Summer 1998

Leukemia Inhibitory Factor (LIF): Murine Preimplantation Embryo Development, Implantation Rates, and Skeletal Development

Michael Hayes Mitchell
Old Dominion University

LEUKEMIA INHIBITORY FACTOR (LIF):

MURINE PREIMPLANTATION EMBRYO DEVELOPMENT,

IMPLANTATION RATES, AND

SKELETAL DEVELOPMENT

by

Michael Hayes Mitchell
A.S. June 1981, Tidewater Community College
B.S. August 1981, Old Dominion University
M.S. December 1987, Old Dominion University

A Dissertation submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY
August 1998

Approved by:

_____
R. James Swanson (Director)

_____
Keith Carson (Member)

_____
Mark Elliott (Member)

_____
Sergio Oehninger (Member)

ABSTRACT

LEUKEMIA INHIBITORY FACTOR (LIF):
MURINE PRE-IMPLANTATION EMBRYO DEVELOPMENT,
IMPLANTATION RATES AND
SKELETAL DEVELOPMENT.

Michael Hayes Mitchell

Old Dominion University, 1998
Director: Dr. R. James Swanson

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine which demonstrates perplexing physiological effects. It has been demonstrated that LIF is essential for implantation in mice. Little is known relating to the manner by which LIF effects pre-implantation and post-implantation development. The objectives of this project were to determine the effects LIF on pre-implantation development, to determine the effects that it may have on implantation rates, successful pregnancy rates, and resorption rates, and to determine the effects that LIF has on the skeletal development of mice. For the embryo transfer experiments, embryos were exposed to test compounds in the transfer medium only at the time of transcervical embryo transfer.

The results obtained from this project illustrate that murine LIF stimulates the development of pre-implantation mouse embryos to the blastocyst stage. Additionally, human LIF and an anti-murine LIF antibody had and inhibitory effect. Dose dependent effects were demonstrated and murine LIF significantly enhanced development in a dose related manner with the highest developmental indices recorded for the highest concentration studied and significance observed

for all concentrations. For the transcervical embryo transfer experiments, transfer medium supplemented with 5000 U/ml mrLIF almost doubled implantation, pregnancy, and resorption rates whereas the mcab approximately halved these rates when compared to controls. Finally, it was observed that transfer medium supplemented with 5000 U/ml mrLIF or 5000 U/ml of the anti-mrLIF mcab had profound effects on the skeletal development of mouse fetuses. Murine rLIF significantly reduced the overall length of the cartilagenous bone precursor as well as the length of the ossification centers in the humerus and scapula. The mcab reversed this behavior and actually stimulated both parameter significantly.

The clinical ramifications for LIF use in in vitro fertilization clinics as a medium supplement are obvious and have been suggested by clinicians. The results frcm this project show that LIF enhances the implantation of, not only healthy embryos, but those that would not normally survive to term. Additionally, a single expose at the time of embryo transfer significantly retards skeletal development in mice. These results warrant further studies before LIF is used in a clinical setting.

ACKNOWLEDGMENTS

I would like to take this opportunity to thank those who made this degree possible. First, and most importantly are my parents, Paul and Hazel Mitchell. Without their unconditional love and support I would not be at this juncture. I would also like to thank my research committee, especially Dr. R. James Swanson, PhD, and Dr. Sergio Oehninger, MS, MD for their guidance and support. Dr. Swanson has been an inspiration and motivating force in my educational and research endeavors. Additionally, many thanks to my colleagues, friends, and fellow students, especially Rokshana R. Thanadar for her ideas and help in editing this document, and Carlos Villar-Gosalvez and Steven L. Taylor for their comments, advice and assistance in experimental design and interpretation. And to Wayne L. Hynes, thank you for all your guidance and advice concerning the logistics of writing and submitting this document. Many thanks to Gail Stenberg for the answers to so very many questions at the most inopportune times and for the countless and thankless hours of extra time....thank you very very much. I would also like to thank Nancy L. Wade for 25 years of friendship, advice, and her philosophies on life. And thank you Nancy for over three decades of unconditional love and dedication to teaching. Finally, to Jamie S. Heisig for her continued love and support - thank you all.

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

TABLE Page

LIST OF FIGURES

FIGURE Page

NOMENCLATURE

ACRONYM	DEFINITION
ACTH	Adrenocorticotropin hormone
AP-2	Activator protein-2
Body M	Body mass
BRL	Buffalo rat liver cells
BSA	Bovine serum albumin
CDF	Cholinergic differentiation factor
cDNA	Complementary DNA
CMH	Cochran-Mantel-Haenszel statistics
CNDF	Cholinergic neuronal differentiation factor
CNTF	Ciliary neurotrophic factor
C/R	Crown/rump length
CRH	Corticotropin releasing hormone
CSF-1	Colony stimulating factor-1
CT-1	Cardiotrophin-1
CyRE	Cytokine response element
D-factor	Differentiation stimulating factor or differentiation factor
DI	Developmental index
DIA	Differentiation inhibiting factor
DIF	Differentiation inducing factor
DRA	Differentiation retarding factor
DS	Developmental score
EC	Embryonal carcinoma cells
ECM	Extracellular matrix
ED_{50}	Effective dose - dose at which 50% of maximum effect is observed
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ES	Embryonal stem cells
EX	Estimation of ossification in exoccipital bone
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony stimulating factor
GFAP	Glial fibrillar acidic protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
gp	Glycoprotein
Grb	Growth factor receptor binding protein
hCG	Human chorionic gonadotropin
HILDA	Human interleukin for DA cells
HLN	Length of humerus
HLPI	HILDA/LIF production index
HOL	Length of diaphyseal ossification center in humerus

hrLIF ..... Human recombiant LIF
HSFIII..... Hepatocyte stimulating factor III
hsp..... Heat shock protein
ICM ..... Inner cell mass
IFN..... Interferon
IL..... Interleukin
IP ..... Intraperitoneal
kb..... Kilobase
K_d ..... Dissociation constant
kDa ..... Kilodalton
LBP..... LIF-binding protein
LIF..... Leukemia inhibitory factor
LIFR..... LIF receptor
LPS..... Lipopolysaccharide endotoxin
M-CSF ..... Macrophage colony stimulating factor
MANOVA ..... Multiple analysis of variance
MAPK..... Mitogen activated protein kinase
mcab or ab..... monoclonal antibody
MGI-2B ..... Macrophage-granulocyte inducer-2B
MLPLI ..... Lipoprotein lipase inhibitor
MMP-9 ..... Matrix metalloproteinase gelatinase B
MMP ..... Matrix metalloproteinase
mrLIF ..... Murine recombinant LIF
mRNA ..... Messenger RNA
ng..... Nanogram
NGF ..... Nerve growth factor
NK..... Natural killer cells
OAF ..... Osteoclast activating factor
OSM..... Oncostatin M
PA..... Plasminogen activator
PCR ..... Polymerase chain reaction
PDGF..... Platelet derived growth factor
PGC..... Primordial germ cells
PHA ..... Phytohemagglutinin
PL ..... Placental length
pM..... Picomolar
PM ..... Placental mass
PMSG ..... Pregnant mare's serum gonadotropin
POM-C..... Proopiomelanocortin-C
PTH..... Parathyroid hormone
PW..... Placental width
RT-PCR ..... Reverse transcription - polymerase chain reaction
SLN..... Length of scapula
SOS ..... Length of ossification center in scapula
SOS ..... Son of sevenless protein

SP ..... Substance P
STAT..... Signal transducer and activator of transcription
T cell ..... T Lymphocyte
TGFβ..... Transforming growth factor β
TIMP ..... Tissue inhibitors of metalloproteinases
TL..... Tail length
TNFα..... Tumor necrosis factor α
tPA ..... Tissue plasminogen activator
TUN ..... Trophouteronectin
µg..... Microgram
µl..... Microliter
uPA..... Urokinase plasminogen activator
UTPH ..... Placental uterotrophic hormone
VIP ..... Vasointestinal peptide
VSPC ..... Dorsal gap between vertebral pedicles

INTRODUCTION

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine which demonstrates perplexing and varying physiological effects. It is known by no less than ten synonyms. This diverse nomenclature is a reflection of an equally diverse and sometimes paradoxical array of biological activities as illustrated by several reviews (Cornish, Callon, King, Edgar, and Reid, 1993; Heath, 1992; Hilton and Gough, 1991; Kurzrock, Estrov, Wetzler, Gutterman, and Talpaz, 1991; Yamamori, 1991). LIF belongs to a relatively new super-family of cytokines, characterized by their receptors and known as the hematopoietin receptor super-family of cytokines. Various cytokines belong to this super-family including the interleukins (IL's) 2, 3, 4, 6, 7, and 11, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), ciliary neurotrophic factor (CNTF), prolactin, growth hormone, erythropoietin, oncostatin M (OSM), and cardiotrophin-1 (CT-1) (Cosman et al., 1990; Cullinan et al., 1996). This introduction begins with a description of the LIF gene and its protein. After the discussion of the LIF gene and protein, the various physiological properties attributed to LIF including its effects on various reproductive parameters will be reviewed.

Gene and Protein Structure

Partial sequence analysis of murine LIF (Hilton, Nicola, Gough, and Metcalf, 1988) produced from Krebs ascites tumor cells was established by

---

The journal model for this dissertation is *Behavioral Neuroscience*.

Simpson and associates. These experiments led to the partial cloning of the LIF Gene (Gearing et al., 1987; Simpson et al., 1988). The initial sequencing resulted in the identification of 125 of the 179 amino acids comprising the protein. These authors determined that the protein had a molecular mass of approximately 60 kDa and a non-glycosylated form (treated with endoglycosidase F to remove carbohydrate moieties) with a mass of 22 - 24 kDa (Simpson et al., 1988). In 1987 Gearing and associates employed various cell differentiation assays to conclude that two forms of LIF are expressed in the mouse, LIF-A and LIF-B (Gearing et al., 1987). These authors characterized these two peptides as differing in the amount of glycosylation. LIF-A was determined to be the same as differentiation factor and macrophage-granulocyte inducer 2B or MGI-2B (Hilton et al., 1988). Additionally, the murine LIF-A gene was found to produce two different forms of the protein based on solubility. A diffusible or "D" form and a matrix-associated or "M" form were identified. It was determined that the differences in the two resulted from variant expression from the exon I/II boundary and differing promoter regions. Rathjen and others suggested the "D" form had its effect on cells at great distances from its source and provided a wide variety of biological effects. They further postulated that the "M" form was associated with a more specific location and provided a focused effect or may be stored for later use (Rathjen, Toth, Willis, Heath, and Smith, 1990; Rathjen et al., 1990; Rathjen, Nichols, et al., 1990).

Gearing et al., used a yeast expression vector plasmid (YEpsec1) to introduce the gene into the genome of the yeast *Saccharomyces cerevisiae* and partially cloned the cDNA for murine LIF (mLIF) (Gearing et al., 1987). From these experiments, the structures of the LIF gene and the peptide were further described and comparison with other cytokines conducted. It was also demonstrated that LIF was produced by two murine T-lymphocyte-cell lines (LB3 and E9.D4), as well as murine Krebs II ascites tumor cells. The latter also produced other cytokines such as (G-CSF), (GM-CSF), and tumor necrosis factor α (TNFα). Various assays have been employed to examine the biological properties of cytokines. Comparisons were established based on the ability of these proteins to induce differentiation in murine myeloid leukemia cell lines such as M1 and WEHI-3B D^+. Some of these cytokines were able to induce a terminal macrophage-like phenotype in these cells, which terminated their neoplastic/proliferative nature. G-CSF, for example, was able to induce differentiation in both the M1 and WEHI-3B D^+ cells, whereas GM-CSF produced a weak differentiation of the WEHI-3B D^+ cells only. LIF induced a macrophage phenotype in the M1 line without effecting the WEHI-3B D^+ cells or stimulating proliferation in normal granulocyte/macrophage progenitor cells. These authors determined that the cloned mLIF gene produced a transcript of approximately 0.8 to 1.0 kilobases (kb). The mature mLIF peptide was found to consist of 179 amino acids. The un-glycosylated peptide exhibited a molecular mass of approximately 20 kDa, with seven potential N-linked and four potential

O-linked glycosylation sites. The glycosylated form of this peptide was shown be approximately 58 kDa if produced by the Krebs ascites tumor cells as compared to 67-100 kDa if derived from yeast (Gearing et al., 1987). A complete clone of the murine protein was produced the following year. This work resulted in the identification of a leader sequence consisting of 24 amino acids and the mature murine protein consisting of 179 amino acids with a non-glycosylated mass of 19,758 Da (Gearing, King, and Gough, 1988).

The above authors cloned the human LIF (hLIF) gene in 1988, again using the yeast vector, *S. cerevisiae*. They observed 78% homology between the human and murine amino acid sequences. The recombinant human product had similar effects as the murine peptide in inducing differentiation in the M1 cell line and was able to compete with native mLIF for binding with receptors on murine cells. The human gene was found to consist of two exons separated by a single intron of 693 base pairs (Table 1, Gough et al., 1988).

Table 1
Percent Homology Between Murine and Human rLIF (Gough et al., 1988).

	Gene	Protein
Exon 1	88	91
Exon 2	77	74
Mature Protein		78

Using Southern analysis, Sutherland and co-workers determined that the gene for human LIF resided on the long arm of chromosome number 22

(Sutherland et al.,1989). Kola and associates identified chromosome 11 as the location for the murine gene (Kola, Davey, and Gough, 1990).

In 1988 a protein named human interleukin for DA cells (HILDA) was purified and described by Godard and co-workers. This cytokine maintained the growth of an interleukin 3 (IL-3)-sensitive murine cell line known as DA2 and was shown to activate eosinophils. It was determined that this protein had a mass of 38 kDa and was active in concentrations between 10^8 - 10^9 U/mg (Godard et al., 1988).

HILDA was later determined to be identical to D-factor (Lowe et al.,1989). This work described a murine D-factor produced from Ehrlich ascites tumor cells and found it to be almost identical to LIF produced from Krebs II ascites tumor cells. D-factor contained one additional serine residue in the amino terminal end of the protein when compared to LIF. The authors predicted that the mature murine and human proteins contained 180 amino acids each and that both demonstrated a non-glycosylated mass of 19.7 kDa. In addition, a partial clone for the human D-factor gene was produced using COS-1 (transformed simian cells transfected with the human D-factor gene) and *Escherichia coli* as the expression vector. They determined the gene to consist of the two intron-three exon arrangement discussed in more detail below. Comparison of the human LIF and murine D-factor genes demonstrated a single difference between the two. Thymine was shown to be located at position 2203 for the human gene and cytosine at this same location for the murine gene. Protein products from COS-1

cells and *E. coli* were both active in inducing differentiation in various cell cultures. Two other phenotypes used to indicate differentiation in M1 cells were the expression of the Fc receptor and the synthesis of PGE_2, both of which were demonstrated in the above experiment (Lowe et al., 1989). The nucleotide base sequence determined for murine D-factor was found to be the same as that for HILDA as determined by Moreau (Moreau et al., 1988).

Schmelzer et al.,describe the human, recombinant D-factor/LIF protein as being an approximately 45 kDa glycoprotein containing 30% neutral sugars and 12% sialic acid (Schmelzer, Burton, and Tamony, 1990). They determined the activity for the recombinant human protein and reported the ED_{50} to be 0.25 ng/ml (12.7 pM) based on one half of its maximum ability to induce differentication in M1 cells.

These authors also determined that the non-glycosylated form had a mass of approximately 21.5 kDa and demonstrated biological activity as per M1 differentiation with ED_{50} = 0.40 ng/ml (Schmelzer et al., 1990) . This activity compares with 17 pM for native D-factor produced by L929 cells (Tomida, Yomamoto-Yamaguchi, and Hozumi, 1984a), 20 pM for D-factor produced by Ehrlich ascites tumor cells, and 6 pM for LIF-A (Tomida, Yomamoto-Yamaguchi, and Hozumi et al., 1984b).

Stahl and co-workers determined that the nucleotide base sequences for the murine and human LIF genes consisted of 6.0 and 6.3 kb respectively (Stahl et al.,1990). These genes consisted of three exons and two introns, four TATA-

like segments, and two transcriptional start sites. One of the start sites, designated as the major start site, was located 60-64 base pairs upstream (5') of the transcriptional start codon (positions 898 - 902), just downstream (3') from a TATA box located at positions 867 or 869. The second start site, designated as the minor start site, was found to be located 160 bp upstream of the start codon (positions 800 - 801), also next to a TATA-like box. The first exon coded for the first six amino acids of the leader sequence, exon two coded the rest of the leader sequence plus 53 amino acids of the mature protein, and exon three coded for the rest of the mature protein (137 amino acids). The mRNA synthesized from these genes was determined to be approximately 4.1 kb in size. Potential protein binding regions were identified in the promoter (5') flanking region. A transcription activator protein-2 (AP-2)-like binding region was identified in the murine and human gene in addition to an SP-1-like binding region in the murine gene within the cloned sections near the LIF gene. AP1 and SP2 were identified as transcription factors coded for by a group of nuclear-acting proto-oncogenes. This proto-oncogene family included *c-fos*, *c-myc*, *c-myb*, *c-erbA*, p53, and *c-jun*. (Bishop, J. M., 1987). Oncogenes were defined as those which caused or perpetuated the proliferative characteristics of cancer and have been shown to influence development on several levels (Klug and Cummings, 1994; Bishop, J. M., 1987). The polypeptide products from two groups of genes, *fos* and *jun*, were found to interact to stimulate the AP-1 responsive gene (Chiu et al.,1988; Beckmann, Matsumoto, and Wilce, 1997).

AP-1, related to AP-2 and SP-1, was found to consist of the protein products of the *jun* and *fos* genes, as Jun homodimers or Fos/Jun heterodimeric complexes. These dimers were shown to function by binding to palindromic response elements on the DNA, thereby activating the genes. These transcription factor sequences are not well conserved in the human gene (Stahl et al.,1990). Schule et al., investigated potential metabolic effects of AP-1 by demonstrating that the glucocorticoid receptor and AP-1 repressed the other's transcriptional activation (Schule et al., 1990). Recently, the *c-fos* binding component for exon one of the pro-opiomelanocortin (POM-C) gene was shown to be identical to the AP-1 binding site. The *c-fos* proto-oncogene was shown to be stimulated by corticotropin releasing hormone (CRH) which synergized with LIF to stimulate an eight-fold increase in the POM-C gene induction (Bousquet, Ray, and Melmed, 1997).

In 1992, Willson and others cloned the ovine and porcine LIF genes and compared the nucleotide base sequences of these to each other and to the human and murine genes described by Stahl and associates (Willson, Metcalf, and Gough, 1992). It was determined that the coding segments (exons) for the genes were highly conserved, whereas the non-coding segments (introns) were poorly conserved. One notable exception is a highly conserved segment of approximately 150 bases within intron one. The four TATA boxes and two transcriptional start sites identified above were also revealed in the porcine and ovine genes. Additionally, the murine gene contained a poorly conserved

sequence in intron one that coincided with an alternative gene transcript that coded for the matrix or "M" form of the protein as opposed to the diffusible or "D" form. Table 2 provides the sequence homologies for the four species observed (Stahl et al., 1990; Willson et al., 1992). The overall homology observed among all four species was approximately 71%. These authors determined that the ovine and porcine LIF genes consisted of the same structural and encoding arrangement as found in the murine and human genes, i.e. three exons separated by two introns. (Willson et al., 1992).

Table 2
Percent Homologies Among Rat, Human, Ovine, and Porcine LIF (Willson, Metcalf, and Gough, 1992).

Species	Murine	Rat	Human	Ovine	Porcine
Murine		92	79	74	78
Rat			81	75	78
Human				88	87
Ovine					84

The gene and protein for oncostatin M (OSM), a glycoprotein belonging to the same super-family of cytokines as LIF, has also been described (Rose and Bruce, 1991). These authors found striking similarities in the genetic arrangement for LIF, OSM, and interleukin-6 (IL-6), another member of this cytokine super-family. As with LIF, the genetic structure of OSM demonstrated exon one encoding for the beginning of the leader sequence, exon two encoding

the rest of the leader sequence plus the first segment of the mature protein, and exon three coding for the rest of the mature protein. Like the LIF gene, the OSM gene was also located on chromosome 22. These authors determined OSM to be a glycoprotein with a mass of approximately 28 kDa. In addition to sharing similar genetic structures, these cytokines share similarities in a receptor subunit known as gp130 (Gearing et al., 1992; Taga et al., 1989) and all are able to induce the differentiation of a macrophage phenotype in the murine myeloid leukemia cells known as M1 (Rose and Bruce, 1991). These authors also provided an amino acid sequence comparison for OSM, LIF, IL-6, and G-CSF. Significant differences were observed when the genetic structures for these four proteins were compared to the structural arrangement for granulocyte/ macrophage-colony stimulating factor (G/M-CSF), M-CSF, interleukins 1-5, tumor necrosis factor (TNF), Steel factor, and interferon (IFN), significant differences were noted (Rose and Bruce, 1991).

Another member of this cytokine family is cardiotrophin-1 (CT-1) which was observed to induce myocardiocyte hypertrophy. Pennica et al.,described that during heart failure, fetal heart-development genes are reactivated which produce substances such as CT-1 that induce cardiac myocyte hypertrophy. This compensatory reaction results in enlarged myocardiocytes, accumulation of sarcomeric proteins without mitotic division, and general myocardial dilation (Pennica et al., 1995b). Pennica and associates also observed that CT-1 demonstrated many of the biological activities of LIF and bound to a soluble form

of the LIF receptor (Pennica et al., 1995a). These authors suggest that the soluble form of the LIF receptor may be a normal pathway for some of the biological activities of CT-1.

The development of a human LIF-specific, double monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) was successfully completed by Kim et al.,and the methods published in 1992. This technique provided a simple and highly sensitive assay for LIF detection (Kim, Alphonso, Schmelzer, and Lowe, 1992).

Since its discovery several sources have been reported for LIF. LIF was found to be produced by a number of cells and tissues *in situ* and several in vitro cell lines which were found to synthesize high levels of the protein. Appendix i is provided as a partial listing of sources and some of the references for each. Several of these sources are discussed throughout this introduction. Akita and associates reported LIF gene expression in human adult pituitary tissue and fetal pituitary corticotropes, somatotropes, and other cell types. It was demonstrated that, in murine cell cultures, LIF stimulated the pro-opio-melanocortin (POM-C) gene and adrenocorticotropin hormone (ACTH) synthesis (Akita et al., 1995). In 1996 Wang, Ren and Melmed observed the constitutive expression of the LIF gene and the gene for the LIF receptor (LIFR) in mouse hypothalamic and pituitary tissue. These authors demonstrated an increase in LIF production by these tissues when lipopolysaccharide endotoxin (LPS) was administered to mice (Wang, Ren, and Melmed, 1996).

The above discussion demonstrates the diverse nature of LIF related to the structural analysis, description, and sources for the gene and its protein. The following discussion will cover a varied array of physiological properties attributed to this protein which will add to an already diverse and sometimes disparate nomenclature.

Bone

Several groups demonstrated that LIF increased bone turnover, favoring resorption over deposition (Abe, 1988; Allan 1990; Lowe, Cornish, Martin, and Reid, 1991; Malaval, Gupta, and Aubin, 1995; Metcalf and Gearing, 1989a; Reid et al., 1990). It was shown that LIF shared certain physiological properties with osteoclast activating factor (OAF) (Abe et al., 1986). In 1995 Malaval, Gupta, and Aubin concluded that LIF had both anabolic as well as catabolic effects on rat calvaria cell cultures. For these experiments it was reported that LIF inhibited bone nodule formation by rat calvaria cell cultures (Malaval et al., 1995). LIF was observed to stimulate an increase in DNA synthesis as well as bone resorption in bone organ cultures (Lowe et al., 1991). In 1989, Metcalf et al.,described various pathological conditions induced in mice by engrafting them with cells capable of synthesizing high levels of LIF. Among these conditions were cachexia (wasting syndrome in more detail under the Lipid Metabolism section of this introduction) as well as excessive new bone formation, and calcification in heart, skeletal muscle, and other tissues (Metcalf and Gearing, 1989a) and neutrophil leucocytosis, enlarged spleen, and an increase in

numbers of hemopoietic cells in liver and spleen (Metcalf and Gearing, 1989b). Van Beek and associates reported that LIF inhibited growth and mineralization of mouse early fetal metacarpal cultures. They described how LIF inhibited resorption in 17-day old mouse metacarpal cultures with the osteoclast precursors remaining in the periosteum rather than the matrix. For day 18 and 19 they also recorded significant inhibition of resorption (Van Beek et al., 1993).

LIF receptors were identified on osteoblasts, and, as observed for parathyroid hormone (PTH) receptors, they were not found to be expressed by osteoclasts. In light of LIF's predominantly osteoclastic activity it was suggested that LIF stimulates the osteoblasts which, in turn, activate osteoclasts by some cell-to-cell mechanism which remains to be identified (Allan, 1990). Recently, Greenfield and others demonstrated that PTH stimulated osteoblasts to release IL-6 as well as LIF. Both of these cytokines were observed to stimulate osteoclastic activity resulting in bone resorption (Greenfield, Horowitz, and Lavish, 1996). Ishimi and associates determined that IL6 was released by osteoblasts and induced osteoclastic bone resorption (Ishimi et al., 1990). Cornish et al.,determined that the bone resorption due to the effects of LIF was prostaglandin-dependent (Cornish et al., 1993; Abe et al., 1986; Allan, 1990). Additionally, elevated levels of LIF were measured in the synovial fluid of patients with rheumatoid arthritis, but not those with osteoarthritis, suggesting a role in the former pathological condition (Ichikawa, 1970; Ishimi et al., 1992). In the absence of a resorption cycle, LIF was shown to inhibit bone nodule

formation in fetal rat calvaria cultures whereas dexamethasone stimulated such development. This inhibitory effect by LIF was demonstrated with and without dexamethasone added to the medium (Malaval et al., 1995). Dexamethasone was shown to inhibit the release of arachidonic acid, a precursor to prostaglandins (Gilman, Rall, Nies, and Taylor, 1990). Reid et al., detailed an experiment in which indomethacin (a drug known to block the synthesis of prostaglandins by inhibiting cyclooxygenase) (Gilman et al., 1990) inhibited LIF-induced activities such as an increase in calcium release from mouse calvaria cultures and an increase in the number of osteoclasts. This work supported the role of prostaglandins in LIF-mediated mechanisms (Reid et al., 1990). The exact relationships among LIF, prostaglandins, rheumatoid arthritis, and bone turnover remain to be elucidated. LIF and its transcript were identified in osteoblast-like cell cultures suggesting a possible autocrine or paracrine role (Abe et al., 1988; Ishimi et al., 1992). For mice in which the gene for the low-afinity LIF receptor was disrupted, acute osteopenia (reduction in bone formation) was observed. Fetal bone volume was more than three fold lower than controls with a six fold increase in osteoclasts, implicating LIF s having a role in skeletal development (Ware et al., 1995).

Hemopoietic Tissues

In 1979, Hozumi reported a factor or factors produced by the rat sarcoma cell line clone YS-722 which stimulated differentiation in the mouse myeloid leukemia cell line M1 (discussed in more detail below). Differentiation was

represented by a terminal macrophage phenotype that demonstrated phagocytosis, motility, and lysosomal enzyme activities (Hozumi et al., 1979; Ichikawa, 1969; Ichikawa, 1970). Hozumi et al., later described other cytokines with similar effects on the M1 line (Hozumi, 1983). It was later reported by Tomida and others that a differentiation stimulating factor or factors (D-Factor) produced by the murine L929 cell line also induced differentiation of a macrophage phenotype in M1 cells (Tomida et al., 1984a). This D-Factor activity was also found to be produced by mouse Ehrlich ascites tumor cells (Yamamoto-Yamaguchi, Tomida, and Hozumi, 1986; Yamamoto-Yamaguchi et al., 1989) and by an osteoblastic cell line known as the MC3T3-E1 cell line (Shiina-Ishimi, Abe, Tanaka, and Suda, 1986). Lowe et al., cloned the human gene for D-Factor (*vide ante*) and demonstrated similarities between LIF and D-Factor (Lowe et al., 1989). In separate experiments, Takeda et al., found that a differentiation inducing factor (DIF), produced from monocytes and the phytohemagglutinin (PHA)-stimulated T cell line, HUT-102, induced differentiation in the M1 cells. However, this lymphokine exhibited a molecular mass of approximately 17 kDa and Takeda and co-workers suggested that it may be TNF (Takeda et al., 1986). During that same year, Abe and co-workers defined a differentiation inducing factor (DIF) from L929 and Ehrlich ascites tumor cells that induced differentiation in M1 cells and resembled OAF in its ability to stimulate bone resorption (Abe et al., 1986).

Several groups demonstrated LIF's influences on a variety of hemopoietic cell lines. It was not found to produce a proliferative effect on normal hemopoietic progenitor cells (Hilton, Nicola, Waring, and Metcalf, 1991; Metcalf, Hilton, and Nicola, 1988; Willson et al., 1992). A standard assay for measuring LIF's activity sprang from its ability to induce a mature macrophage-like phenotype in the M1 cells (Abe et al., 1989; Gearing et al.,1987; Gough et al., 1988; Hilton et al., 1988; Hilton and Gough, 1991; Hilton, Nicola, and Metcalf, 1988; Lowe et al., 1989; Metcalf et al., 1988; Rose and Bruce, 1991; Tomida et al., 1984a; Tomida et al., 1984b). LIF receptors were identified on M1 cells by Hilton and co-workers (Hilton, Nicola, and Metcalf, 1988). By inducing this mature phenotype, LIF efficiently terminated the neoplasm's "immortal" or proliferative nature, thus the name leukemia inhibitory factor. Hozumi and others published a review article suggesting such induction of differentiation as a possible method of therapy for certain neoplasms (Hozumi, 1983; Maekawa, and Metcalf, 1989; Maekawa, Metcalf, and Gearing, 1990). LIF's effects on M1 cells are shared by IL-6 and G-CSF (Metcalf and Gearing, 1989a).

LIF was found to be identical to HILDA which maintained proliferation in the murine, interleukin-3-dependent, myeloid, leukemia cell line DA2, later renamed DA-1a (Gascan et al., 1989; Gascan et al., 1990; Godard et al., 1988; Moreau et al., 1987; Moreau et al., 1988). Leary et al., determined that LIF was as efficient in this respect as IL-6 or G-CSF (Leary, Wong, Clark, Smith, and Ogawa, 1990).

This action was contrary to LIF's ability to stimulate differentiation and thereby inhibit the continued proliferation in the myeloid leukemia M1 cells. Reported in 1988 was HILDA's ability to activate eosinophils. The same article described HILDA as a 38 kDa glycoprotein produced by T-lymphocytic clones after antigen stimulation (Godard et al., 1988). Anegon and co-workers determined that HILDA/LIF was synthesized by activated monocytes, monocyte-derived macrophages, and myelomonocytic cell lines especially when these cells were stimulated with phorbol ester and 1,25-dihydroxycholicalciferol (vitamin D_3 or VD_3) as well as (PHA) (Anegon et al., 1990). Other laboratories determined that LIF/HILDA stimulated the growth and proliferation of human erythroid and eosinophil precursors in the presence of serum (Verfaillie and McGlave, 1991). It was later discovered that LIF/HILDA production was stimulated by interleukin-1 (IL-1) and inhibited by dexamethasone in endothelial cells derived from human umbilical vein and human bone marrow (Grosset et al., 1995). Additionally, it was determined that LIF was secreted by bovine pituitary follicular cells and this inhibited the growth of adult bovine aortic endothelial cells. Paradoxically, LIF was observed to stimulate cell division in adrenal cortex capillary endothelial cells (Ferrara, Winer, and Henzel, 1992).

Metcalf and co-workers found that LIF alone had no effect on murine megakaryocyte colonies in vitro unless these colonies were first treated with IL-3 (Metcalf, Hilton, and Nicola, 1991). When injected into mice, LIF caused weight loss, an increase in serum calcium concentration, and an increase in the number

of megakaryocytes (precursors and mature) in both the spleen and bone marrow (Metcalf, Nicola, and Gearing, 1990). By observing LIF-deficient mice, Escary et al., concluded that LIF was required for the "support" of hemopoietic stem cells, especially from the spleen and bone marrow. They observed fewer spleen and bone marrow stem cells in LIF deficient mice suggesting that LIF maintained a population of pluripotent cells for these cell lines (Escary, Perreau, Dumenil, Ezine, and Brulet, 1993). Leary and others observed that LIF increased the number of interleukin-3 (IL-3) stimulated cells in vitro, however, it demonstrated no influence without the IL-3 pre-treatment (Leary et al., 1990).

Observing the two neoplastic cell lines, Foss human melanoma and human neuroblastoma, Heymann et al., found that LIF and OSM stimulated an increase in the integrin known as $\alpha v\beta 1$ as well as an increase in tumor cell-fibronectin association. Integrins have been found to provide a role in cell-to-cell or cell-to-matrix association and may be involved in the migratory/metastatic behavior of certain neoplasms. The increase in integrin $\alpha v\beta 1$ was observed to be correlated with an increased association of, Foss human melanoma and the human neuroblastoma cell line, SK-N-SH, with their fibronectin matrix potentially reducing their metastatic capacity by "tightening" their matrix association. Similar results were also observed when treating the medium with TNFα. These authors suggested possible roles for these cytokines in the regulation of tumor cell metastasis (Heymann et al., 1995). In unrelated experiments, it was determined that the extracellular domain of the gp130 transmembrane signal transduction

component of the LIF receptor consisted of fibronectin type III units suggesting further association of LIF withthe extracellular matrix components (Hibi et al., 1990).

Lipid Metabolism

Metcalf and associates demonstrated that, in mice engrafted with LIF-producing cells or mice injected with LIF, LIF inhibited lipoprotein lipase in adipocytes (Metcalf and Gearing, 1989b, Metcalf, 1989; Metcalf et al., 1990). These animals developed a pathologic condition known as cachexia in which a dramatic loss in body weight was observed due to an increase in subcutaneous lipid catabolism and a decrease in fatty acid intake by adipocytes. Several human pathologies such as cancer have been associated with various degrees of "wasting" seen in patients. Mori et al., 1989, demonstrated elevated LIF concentrations in a human melanoma cell line known as SEKI. These cells were derived from a patient suffering a form of melanoma concomitant with marked cachexia. When these cells were injected into tumor-bearing nude mice, marked cachexia developed. These effects were attributed to melanocyte-derived lipoprotein lipase inhibitor (MLPLI) determined to be the same as LIF (Mori, Yamaguchi, and Abe, 1989). Similar pathological conditions were reported when cultures of the adipocyte cell line 3T3-L1 were exposed to TNF (Price, Olivecrona, and Pekala, 1986) or IL1 (Beutler and Cerami, 1985) and both teams found that these cytokines also inhibited lipoprotein lipase synthesis.

In some patients with chronic myelogenous leukemia as well as certain primary malignant and stromal cell cultures, measurable levels of LIF were reported. In these cultures, LIF expression was shown to be augmented by cytokines such as IL-12, IL-1β , TNFα , and transforming growth factor β (TGFβ) (Wetzler et al., 1990).

Hepatic Function

Baumann and others demonstrated that LIF induced the production of acute phase proteins by cultured monocytes, tissue macrophages, keratinocytes, and hepatocytes. These proteins were shown to be produced by the liver in response to stress such as tissue damage or exposure to various toxins (Baumann, Onorato, Gauldie, and Jahreis, 1987; Baumann and Schendel, 1991; Baumann and Wong, 1989; Hilton, 1992). Examples of acute phase proteins are α_1 -anti-trypsin, α_1 -anti-chymotrypsin, α_2 -macroglobulin, fibrinogen, hemopexin, cystein protease inhibitors, and others (Baumann et al., 1987; Hilton, 1992). Baumann and associates concluded that LIF is identical to hepatocyte stimulating factor III (HSFIII) (Baumann and Wong, 1989; Baumann, Jahreis, Sauder, and Koj, 1984) which was previously observed to stimulate acute phase protein synthesis (Baumann, Won, and Jahreis, 1989; Baumann, Hil, Sauder, and Jahreis, 1986). LIF receptors were identified on fetal and adult parenchymal hepatocytes (Hilton, Nicola, and Metcalf, 1991).

Neural Effects

In 1979, ciliary neurotrophic factor (CNTF) a cytokine with similar properties to cholinergic neuronal differentiation factor (CNDF), was identified as a factor from whole chick embryo extracts that supported the survival of chick ciliary ganglionic neurons. CNDF was also known as CDF or cholinergic differentiation factor (Martinou, Martinou, and Kato, 1992). It was determined that intraocular tissues contained relatively high levels of CNTF (Adler, Landa, Manthorpe, and Varon, 1979). CNTF was later purified, cloned, and found to be distinct from nerve growth factor (NGF) and LIF (Stockli et al., 1989; Lin et al., 1979). Rat and rabbit CNTF demonstrated 80% amino acid sequence homology and the genes were shown to have similar structures (Masiakowski et al., 1991). Fukada et al., reported a 45 kDa (22 kDa de-glycosylated form) protein factor produced by newborn rat heart cell cultures that induced adrenergic neurons to acquire a cholinergic phenotype, as evidenced by their synthesis of acetylcholine (Fukada, 1985). Conover illustrated that, like CNDF, CNTF receptors were also expressed on embryonal stem (ES) cells and these proteins were able to maintain pluripotency in ES cells (Conover et al., 1993). Stahl et al., demonstrated that LIF and CNTF both used gp130 and perhaps the β subunit for theLIF receptor (LIFR-β). These authors reported similar signal transduction pathways (Stahl et al., 1993). It was also determined that LIF induced a cholinergic (acetylcholine producing) phenotype in sympathetic (adrenergic) neural progenitor cells as effectively as CNDF and CNTF (Fukada, 1985) and in 1989 Yamamori and co-

workers, determined that LIF and CNDF were identical (Yamamori et al., 1989). CNDF/LIF was observed to induce the expression of acetylcholine and suppress both catecholamine synthesis and noradrenergic function in these cells (Adler et al., 1979; Yamamori et al., 1989; Fukada, 1985; Weber, 1981). In related experiments, Ludlam and Kessler reported that LIF and CNTF regulated the expression of muscarinic receptors on neonatal rat superior cervical ganglion (SCG) neurons (Ludlam and Kessler, 1993). Michikawa, Kikuchi, and Kim examined the effects of LIF on murine spinal cord neuron cell cultures and reported an increase in choline acetyltransferase activity in these cells under the influence of LIF (Michikawa, Kikuchi, and Kim, 1992).

Murphy reported the ability of LIF treated medium to function in concert with NGF in neural cell development. In these experiments, it was established that LIF was necessary for the initial differentiation step in which cells acquired neurofilaments and that further differentiation and survival of these cells relied on NGF (Murphy, Reid, Brown, and Bartlett, 1993). This work illustrated a temporal association between LIF and NGF in regard to neural cell development. Murphy and associates also showed that LIF supported the differentiation of sensory neurons in mouse embryo neural crest and dorsal root ganglion cell cultures (Murphy, Reid, Hilton, and Bartlett, 1991). Freidin observed that LIF increased the production of the neuropeptide, substance P (SP) in neuronal and non-neuronal cultures. SP has been implicated in a number of neural/immunological functions (Freidin and Kessler, 1991). Ludlam and others demonstrated LIF-

mediated IL-1-stimulated SP receptor transcript synthesis in rat neuronal explants and demonstrated possible relationships among LIF, IL-1 , and SP production in immune reaction after axotomy (Ludlam, Chandross, and Kessler, 1995). In related studies, Nakagaito, et al., illustrated that LIF, not epidermal growth factor (EGF), stimulated glial fibrillar acidic protein (GFAP) expression in murine astrocyte progenitor cells obtained from embryonic cerebral hemispheres. GFAP is a specific marker for astrocyte differentiation (Nakagaito, Yoshida, Satoh, and Takeuchi, 1995).

Recently, Li and others discovered that LIF receptor-deficient/mutant mice demonstrated a 35% loss of facial motor neurons, 40% loss of spinal motor neurons, and 50% loss of neurons from the nucleus ambiguous, suggesting possible roles for this cytokine in the normal development of motor, as well as sensory neurons (Li, Sendtner, and Smith, 1995). In related experiments, CDF/LIF was shown to increase survival of day 14 fetal rat motor neurons (Martinou, Martinou, and Kato, 1992).

Renal

Bard and Ross examined the effects of LIF on renal development. They determined that supra-physiological levels of LIF selectively inhibited nephrogenesis without having an effect on the development of the collecting ducts. These experiments illustrated that LIF was able to inhibit the development of the mesenchyme which generates the nephrons while having no effect on the

epithelium that undergoes successive bifurcation to form the collecting ducts (Bard and Ross, 1991).

The multiplicity of physiological effects (the pleiotropic nature of LIF) disscussed thus far was well demonstrated by a series of experiments by Ware and associates which targeted genetic disruption at the low-affinity LIF receptor. In mutant mice, placental development was disrupted thereby obstructing nutritional flow to fetuses, however, fetuses developed to term. An acute reduction in the fetal vascular component of the placenta was observed. In addition, acute osteopenia (reduction in bone formation) was observed. Fetal bone volume was more than three fold lower than controls with a six fold increase in osteoclasts. Central nervous system effects were reflected in reduced astrocytes in both the brain and spinal cord of mutant mice. Hepatic lesions were indicated by abnormally large glycogen stores suggesting possible metabolic disorders. The multiplicity of defects proved to be incompatible with life and the term pups died on the first day post parturition (Ware et al., 1995).

Metabolism

In a series of experiments conducted by Hilton and others, native and recombinant LIF were injected into mice to determine their clearance rates. Both were found to have an initial clearance half-life of six to eight minutes with a longer second clearance phase. The predominant organ for metabolic breakdown was determined to be the kidneys, however, LIF accumulation was

observed in several other organs/tissues (Hilton, Nicola, Waring, and Metcalf, 1991).

Receptors

Concomitant with the diverse biological properties attributed to LIF is an equally diverse distribution of its receptors (appendix ii). Smith and others observed ES cells that possessed 4500 high-affinity (dissociation constant or K_d = 90 pM) LIF receptors per cell (Smith et al., 1988). Yamamoto-Yamaguchi, Tomida, and Hozumi (1986) reported high-affinity binding of D-factor to the M1 cells discussed earlier. This factor was derived from mouse Ehrlich ascites tumor and L929 cells (Yamamoto-Yamaguchi et al., 1986). Hilton and associates studied the binding of ^{125}I-labeled LIF in several normal and cultured cell lines. These authors demonstrated that LIF binding occurred on normal murine macrophages, monocytes and their precursors from the peritoneal cavity, bone marrow, and spleen. Also examined were several cell lines, however, only the M1 murine monocytic leukemic line revealed binding at high levels. Three to five hundred high-affinity (K_d = 100 - 200 pM) receptors per cell were observed. In addition, half of the maximum induction of M1 cells occurred with only five percent of the receptors occupied by ligand. Hilton et al., reported that no binding was observed on neutrophilic or eosinophilic cell lines, mast cells, or erythrocytes (Hilton et al., 1988). These findings were later reconfirmed (Hilton et al., 1991). Cells of macrophage/monocyte lineage illustrated binding to ^{125}I-labeled LIF with approximately 150 receptors per cell. In the same article the

authors describe receptor binding in several cell lines and an especially high level of binding was seen on hepatocytes from fetal and adult hepatic parenchyma with as many as 2000 receptors per cell (Hilton et al., 1991).

Layton and others identified a murine serum protein that binds LIF. This protein had a molecular mass of 90 kDa and displayed a dissociation constant found in the low-affinity range (K_d = 600 pM). These investigators also determined that the protein was a truncated form of the α chain of the cellular LIF receptor. Concentrations as high as 1 μg/ml were measured for this LIF binding protein (LBP) and these levels rose during pregnancy. LBP (later determined to be the soluble form of the LIFR-β) inhibited the activities of LIF in culture and may have a similar role in vivo (Layton et al., 1992). Analysis of a recombinant soluble mouse D-factor/LIF receptor (expressed in COS-7 cells) indicated the receptor to be a 150 kDa protein with a dissociation constant of 12 nM when mouse LIF was bound to the mouse soluble LIF receptor and 0.67 nM when human LIF binds to the mouse soluble receptor. These authors observed that the murine LIF had a mass of 50 kDa (Tomida, 1995). Two forms of the murine LIF receptor, a membrane-bound form and a soluble form, were described by Owczarek et al., as being the product of differential splicing of the same gene locus. The authors found differential expression of the two forms of the LIF receptors. High levels of receptor transcripts were identified in the liver, placenta, and uterus during pregnancy and pseudopregnancy (Owczarek, Layton, Robb, Nicola, and Begley, 1996).

Gearing et al., succeeded in producing a clone for the murine and human LIF receptor and provided data pertaining to its structure (Gearing et al., 1991; Gearing, 1993). Nicola and Metcalf published a mini-review describing binding promiscuity amongst various cytokine receptors with various ligands. This may in part explain the pleiotropic nature for some of these cytokines (Nicola and Metcalf, 1991). It has been demonstrated that, although hLIF binds to both the hLIF receptor (hLIFR) and mLIF receptor (mLIFR), mLIF is only able to bind hLIFR (Layton, Lock, Metcalf, and Nicola 1994; Owczarek et al., 1993).

gp 130

The receptor for IL-6 was described by Taga et al., in 1987. The cytokine was shown to bind to an 80 kDa polypeptide which associated with murine gp130 (Taga et al., 1989). Gearing et al.,found that OSM bound to the high-affinity LIFR but not the low-affinity LIFR. They determined that gp130 was the candidate subunit that induced high-affinity binding properties in the otherwise low-affinity receptor and this same gp130 was responsible for binding of OSM to the high-affinity LIFR. (Gearing et al., 1992). CNTF, which shared several biological properties with LIF, also expressed the gp130 signal transducing receptor subunit (Ip et al., 1992). Additionally, Davis and associates described the CNTF receptor complex as a tripartite system consisting of the CNTF receptor α subunit, the LIF receptor β subunit, and the gp 130 protein (Davis et al., 1993).

Hibi et al., studied the gp130 component and found it to be a 918 amino acid peptide that consisted of a transmembrane domain and an extracellular domain (approximately 200 amino acids) that was composed of six units of fibronectin type III (Hibi et al., 1990). The gp130 subunit had a large intracytoplasmic domain, yet displayed no catalytic activity. However, its activation, due to ligand binding to its receptor, resulted in rapid phosphorylation and activation of a kinase pathway which resulted in gene transcription (Lord et al., 1991; Murakami et al., 1993)

Ernst, Gearing, and Dunn demonstrated another protein associated with the LIF receptor complex. They reported that Hck, a Src-related tyrosine kinase, mediated LIFR signal transduction and physically associated with gp130. With increased Hck production by mutagenic cell, the LIF required to maintain ES cells in their pluripotent state was reduced by approximately 15-fold (Ernst, Gearing, and Dunn, 1944). The exact relationship between Hck and LIF physiology remains to be determined.

The crystalline structure for LIF was described as four alpha helices with long crossover loops between the first two and the last two helices (Robinson et al., 1994). Hudson, Vernallis, and Heath characterized the receptor binding sites of hLIF. They reported that the hLIFR binding sites on hLIF were the amino terminus of the D-helix, carboxyl terminus of the B-helix, and the C-D loop. The A and C helices were reported to be the binding sites for gp130 (Hudson, Vernallis, and Heath, 1996).

Signal Transduction Mechanism

LIF and CNTF induced a tyrosine phosphorylation cascade that involved three proteins known as CLIP1, CLIP2, and CLIP3. Tyrosine phosphorylation of the three proteins was specific for LIF and CNTF and was not observed in several other cytokines tested such as fibroblast growth factor (FGF), nerve growth factor (NGF), platelet derived growth factor (PDGF), EGF, and others. These authors found that LIF as well as CNTF induced the CLIP phosphorylation pathway which was followed by activation of the *tis11* immediate-early response gene. The response reached maximum transcription in 45 minutes and returned to basal levels within 120 minutes. Whereas, the *tis11* gene was activated in the MAH (rat pathoadrenal progenitors immortalized with the v-*myc* oncogene) and EW-1 (Ewing sarcoma) cell lines, the *c-fos* immediate early response gene was activated by these two cytokines in the EW-1 cell line only. Beta fibroblast growth factor (FGF), on the other hand, induced gene activation in MAH cells after stimulation with FGF without activating the *tis11* gene. LIF also induced the phosphorylation of proteins of the same size as CLIP1, CLIP2, and CLIP3 in M1 cells. However, IL-6 induced the phosphorylation of the CLIP2 and CLIP3 and thereby revealed a possible bifurcation in the pathways stimulated by these two cytokines. CNTF did not induce tyrosine phosphorylation in the M1 cell line and CNTF receptors were not demonstrated on these cells. These authors discovered that CLIP1 and CLIP2 possessed extracellular domains that probably

participated in the receptor complex and that the CLIP2 was identical to gp130 (Ip et al., 1992).

Michishita and associates (1991) found that ligand binding to the receptor resulted in the phosphorylation of a 27 kDa heat shock protein (hsp27) with an iso-electric point of 5.6. This activity was measurable within five minutes and reached a maximum within ten minutes.

Although the signaling transduction pathway for LIF has not been fully described, data for the OSM and other related cytokine pathways have accumulated. OSM was determined to be another pleiotropic cytokine related to LIF, IL-6, IL-11, and CNTF. In 1991, Rose et al., exhibited data that showed that OSM bound to a LIF/OSM receptor (Rose and Bruce, 1991). Ligand binding of these cytokines with their receptors involved homo-dimerization of gp130 or hetero-dimerization of gp130 with the LIF receptor (Chauhan et al., 1995). Cytokine binding to these receptors activated a series of tyrosine kinases. OSM activation of gp130 was shown to induce tyrosine phosphorylation of JAK2, however, JAK1 or Tyk2 (members of the tyrosine kinases of the Janus family of protein kinases), were unaffected. In other systems, gp130 was shown to associate with JAK1 as well as JAK2. JAK2 was also activated by prolactin binding to its receptor (Ferrag, Chiarenza, Goffin, and Kelly, 1996). Chauhan and associates demonstrated an interaction between phosphorylated JAK2 and the SH2 domain of growth factor receptor binding protein (Grb2). OSM stimulated mitogen activated protein kinases (or MAP kinases) in a pathway that

depended on the Grb2 proteins. Sos (son of sevenless, a protein first described in *Drosophila* and later mammals) was found located within the JAK2-Grb2 complex. Sos appeared to bind to the SH3 domain of the Grb2 protein. Given that Sos was involved in the activation of Ras genes, this relationship may have indicated a position for Ras genes in the OSM induced pathway cascade. Similarities between this OSM pathway and that for LIF signal transduction remains to be uncovered (Chauhan et al., 1995). Other proteins found to be related to the Ras/MAP kinase activation pathway were mitogen-activated protein kinase kinase (MAPK kinase), and S6 protein kinase (Boulton, Stahl, and Yancopoulos, 1994; Schiemann et al., 1994; Schwarzschild et al.,1994; Yin and Yang, 1994).

EGF, another related cytokine, was found to elicit homo-dimerization and phosphorylation of its membrane receptor (encoded by the *erb* B gene) resulting in a cascade of reactions. Again, SOS complexed with Grb2, was found to be included in the pathway. In addition, this pathway included members of the ras family, Raf 1 (a serine-threonine kinase), MAP kinases, as well as MAPK. The path leading to the nucleus effected transcription factors such as *myc*, *jun*, *Erk-1*, *Erk2* (Marx, 1993a; Marx, 1993b; Stokoe, MacDonald, Cadwallader, Symons, and Hancock, 1994; Wu et al., 1993). Darnell and associates described a signal transduction pathway in which interferon activated the phosphorylation of signal transducers and activators of transcription (STAT) proteins which entered the nucleus to induce transcription (Darnell, Kerr, and Stark, 1994). Symes et al.,

studied a signal transduction pathway for the LIF-stimulated transcription of the vasointestinal peptide (VIP) gene (Symes, Corpus, and Fink, 1995). They demonstrated that LIF and interferon-γ (IFN-γ) stimulated the phosphorylation of members of the Jak-Tyk family of tyrosine kinases, however, their receptors did not demonstrate endogenous kinase activity. Also within the cascade pathways for LIF and INF-γ were found Jak-STAT (signal transducer and activator of transcription) proteins. Once activated by phosphorylation, these STAT proteins (STAT1 from IFN and STAT3 from LIF stimulation) migrated to the nucleus to activate transcription. Yet LIF and IFN exhibit different biological properties. LIF induced an increase in VIP from sympathetic neurons and neuroblastoma cell lines (NBFL) *in vitro* and was reported to be the major physiological activator of VIP after post-ganglionic axotomy of the superior cervical ganglion (Rao, 1993). LIF binding to its receptor induced the STAT proteins to bind to the cytokine response element (CyRE) on the gene. This CyRE was found to be approximately 180 bp long and located about 1330 bp upstream from the start site in a portion of the promoter. It was shown to be activated by LIF, CNTF, OSM, and IL-6. Mutation studies of the STAT site indicates that, although the STAT site was important to the activation of the CyRE, alone it was insufficient to induce maximum transcription. Transcription did occur after targeted mutagenic-deletion of the STAT site. The authors suggest that a region of the CyRE, other than the STAT-binding portion, must have also contributed to this transcription. Possible candidates included the C/EBP-related binding sites which were shown

to bind to a protein complex. Its role in the signal transducing pathways for LIF and IFN remains to be described. LIF also activated other signal transduction pathways. LIF as well as CNTF stimulated the phosphorylation of phospholipase C , phosphoinositol-3-kinase, phosphotyrosine phosphatase, and pp120src substrate (Symes et al., 1995) as well as the Ras pathway discussed earlier.

Reproduction

The earliest reports that linked LIF to reproductive physiology involved media conditioning techniques. It was known that co-culture of cells along with specialized "feeder" cells could effect the differentiation/development of the former. In 1975, Martin and Evans developed a technique of employing a murine fibroblast cell line, known as STO fibroblasts, as an efficient feeder cell system (Martin and Evans, 1975). That same technique was later used to examine the totipotent/pluripotent nature of ES and embryonal carcinoma (EC) cells. EC cells were pleuripotent cells derived from teratocarcinoma cell lines as described by Bradley and associates (Bradley, Evans, Kaufman, and Robertson, 1984; Wobus, Hozhausen, Jakel, and Schoneich, 1984). Evans and Kaufmann, for example, extended the use of STO fibroblasts as feeder cells for the development of a system which provided a source of totipotent cells derived from early embryos. These ES cells were harvested from embryos that were in the blastocyst through early post-implantation stages of development (Evans and Kaufman, 1981; Hooper, Hardy, Handyside, Hunter, and Monk, 1987). Once the embryo developed beyond these stages, differentiation of the cells began and

totipotency was eventually lost. The authors discovered that ovariectomy and hormone treatment of pregnant mice at appropriate times induced the embryos into a condition described as "delayed implantation" in which development/ differentiation slowed and nidation (implantation of the embryo) was postponed. These embryos were then cultured and separated into trophoblast and inner cell mass (ICM) groupings according to the technique of Solten and Knowles (Solter and Knowles, 1975). Those cells from the ICM were then cultured until an "egg cylinder" developed. At this point the cells were co-cultured with mitomycin C-treated inactivated STO fibroblasts. This system maintained the ES cells in their totipotent state which prevented differentiation (Evans and Kaufman, 1981; Nichols, Evans, and Smith, 1990).

In 1981, Martin developed a second technique for observing pluripotent cells. In this system EC's were used. ICM cells from normal embryos were cultured with PSA-1 EC cells and STO fibroblasts. The ICM cells acquired characteristics that were indistinguishable from teratocarcinoma tumor cells, which maintained their pluripotent/totipotent nature (Martin, 1981).

Koopman and Cotton described a polypeptide of approximately 57 kDa that was derived from the STO mouse fibroblast-treated medium. They termed this activity as differentiation retarding factor (DRF). These authors employed the use of pluripotent EC cells (NG2 and F9 lines) and determined that DRF inhibited differentiation in these cells (Koopman and Cotton, 1984). In similar studies, Gearing and associates were able to produce LIF-releasing *E. coli* which

prevented differentiation in ES cells. DRF was determined to be identical to LIF (Gearing et al., 1989).

Another feeder cell system was developed using buffalo rat liver (BRL) cells. This system also prevented differentiation in ES and EC cell lines. In this case, the putative agent was named differentiation inhibiting activity (DIA). The authors determined this to be a 20 - 35 kDa peptide (Smith and Hooper, 1987). Smith et al., later compared DIA with HILDA and LIF and observed structural and functional similarities. In these experiments, DIA was shown to be a glycoprotein with a molecular mass of 43 kDa (20 kDa deglycosylated). It was further demonstrated that DIA prevented ES differentiation at concentrations of 10 ng/ml and removal of DIA induced the ES to differentiate (Smith et al., 1988; Smith, Nichols, Robertson, and Rathjen, 1992).

Another comparison among DIA, feeder cells, and LIF was conducted by Williams and others. These authors determined that differentiation of several ES and EC cell lines was prevented by DIA or feeder cells. One such feeder cell system used the human bladder carcinoma cell line known as 5637, which was shown to produce LIF. During these experiments, the authors reported that DIA and LIF were peptides of approximately 50 kDa and that both were heavily glycosylated. They also observed that both DIA as well as LIF were produced by the 5637 cell line, both peptides were able to maintain totipotency/pluripotency in ES and EC cells, and both promoted proliferation in the murine DA1.1a cell line. The authors also reported that 50% of the maximum effect of LIF on ES cells

was obtained at an activity of 50 - 100 U/ml. Dissociation constants were determined to be approximately 9 x 10^{-9} or 9 pM for the three ES/EC cell lines, EK_{CS}-1, PCCA1, and F9 (Williams et al., 1988).

In 1990, Pease et al.,presented data detailing the maintenance of totipotency in several strains of the MBL ES cell lines with no loss of developmental potential. In these experiments it was shown that LIF effectively replaced feeder cells in this regard (Pease, Braghetta, Gearing, Grail, and Williams, 1990). Pease and associates published another article in which they reported that LIF was substituted for feeder cells in the maintenance of the ES cell line D3 using 1000 U/ml LIF (Pease and Williams, 1990). Similar results were demonstrated using other cytokines in the same family such as CNTF (Conover et al., 1993) and LIF, IL-6, OSM, and CNTF (Piquet-Pellorce, Grey, Moreau, and Heath, 1994) to maintain ES totipotency.

An anatomical description of the granulated cells in the mouse endometrial (metrial) gland was provided by Stewart and Peel in 1981. In this study, the authors described the manner in which these cells were concentrated in the decidua basalis and metrial glands around implantation sites. The authors suggested a possible role for implantation/pregnancy regulation by products from these cells (Stewart and Peel, 1981).

One requirement for implantation was determined to be the "hatching" of the embryo from its surrounding zona pellucida on day 4 - 5 of gestation. The outer layer of cells, the trophoblasts or trophectoderm, were shown to give rise to

components of the placenta and extraembryonic membranes. The inner cell mass (ICM) contributed to the embryo proper and umbilical cord (Nobil, Neill, Greenwald, Markert, and Pfaff, 1994). LIF was found to be associated with the trophoblast cells by Conquet and Brulet in 1990. Transcripts (mRNA) for LIF were assayed in mouse embryonic and extraembryonic tissues. They described LIF transcripts located in extraembryonic ectoderm on day 7.5 (post coitus or p.c.), however none was found in the decidua (maternal tissue). After analyzing the placenta, they determined that transcripts were synthesized on days 9.5, 10.5, and 12.5 p.c., yet no LIF mRNA was detected from the embryo proper. Therefore, transcripts appeared to be synthesized by the fetal contribution of the placenta (from the trophoblasts) and not the maternal contribution to the placenta or embryo proper. Transcripts produced by the blastocysts were detected just before nidation on day 3.5 p.c. (Conquet and Brulet, 1990). The relationship between the trophectoderm and the ICM have not been completely described. These experiments suggested that the murine trophectoderm cells maintained a regulatory role in controlling ICM development *vis-à-vis* LIF production. LIF receptors were located on ICM-like ES cells which suggested to the authors that trophectoderm-derived cells may have had an influence on ICM cells. It has been suggested that for these ES cells, the ICM may regulate the growth and development of the trophectoderm (Conquet and Brulet, 1990; Layton et al., 1992; Robertson, Lavranos, and Seamark, 1990). Other cytokines that are produced by the pre-implantation blastocysts and the placenta include

transforming growth factor (TGF-α, TGF-β), and PDGF (Conquet and Brulet, 1990; Graham, Lysiak, McCrae, and Lala, 1992; Rappolee, Brenner, Schultx, Mark, and Werb, 1988), GM-CSF (Robertson and Seamark, 1992), as well as human placental uterotrophic hormone (UTPH) (Beas and Flores, 1969; Roblero, Beas, Arrau, and Rojas, 1983; Roblero, Beas, and Rojas, 1981; Roblero, Beas, Arrau, and Rojas, 1981).

In a series of experiments investigating which mouse tissues synthesize LIF, Bhatt et al., found that LIF transcript was most abundant in the uterine endometrial glands, particularly on day 4 of pregnancy. This transient synthesis of LIF temporally corresponded to implantation. Similar timing for LIF release was observed in pseudopregnant females and those undergoing delayed implantation (Daniel, 1970) suggesting maternal control of LIF synthesis (Bhatt, Brunet, and Stewart, 1991; Finn and McLaren, 1967). Similar experiments conducted by Shen and associates revealed that peak LIF mRNA levels were observed just after ovulation and relatively high levels were found in the mouse uterus throughout pregnancy and during pseudopregnancy. High levels were also recorded for the period when the blastocyst arrived in the uterine lumen. In addition, these authors reported that LIF partially blocked embryonic body development in vitro by inhibiting the formation of primitive ectoderm (not primitive endoderm). For these experiments, Shen et al.,employed ES (D3 and CCE cell lines) cells co-cultured with mitomycin C-treated embryonic STO fibroblasts (known to produce LIF). Several cell markers were used to

differentiate the effects LIF had on ectodermal vs endodermal differentiation and development (Table 3). This data suggested that LIF inhibited the formation of primitive ectoderm in embryonic bodies that otherwise would have appeared in ES cells (Shen and Leder, 1992).

Table 3
Tissue Markers for the Study of LIF.

Tissue examined	Protein Marker	Effect vs control
Endoderm		
- visceral	α-fetoprotein	little effect
- visceral and parietal	H19	little effect
- parietal	collagen type IV	little effect
	laminin β_1	little effect
mesoderm		
- primitive RBC islands	ξ-globulin	inhibited
- cardiac & skeletal muscleα	cardiac actin	inhibited
- primitive ectoderm & streak	Fgf-5	inhibited
- undifferentiated ES & ICM cells	REX-1	remains high
- ICM & primitive ectoderm	Oct-3	remains high

(in various embryonic tissues in the mouse - Shen and Leder 1992).

In addition to production by placental components, LIF was shown to be synthesized by the embryo. Polymerase chain reaction (PCR) techniques were used by Murray et al., to determine the presence of IL-6 and LIF transcripts in mouse blastocysts on day 3.5 of gestation. They suggested a role for LIF in the growth and development of the trophoblasts as well a ES cells. (Murray, Lee, and Chiu, 1990). Conquet and Brulet also determined that LIF was synthesized by the mouse embryo on day 3.5 of pregnancy by using *in situ* hybridization and

reverse transcription polymerase chain reaction (RT-PCR) techniques (Conquet and Brulet, 1990).

LIF secretion patterns in the rabbit closely paralleled those in mice. Low levels were detected in the endometrial epithelium, myometrium, and endometrial glands of non-estrous and estrous females. High levels were reported on day five of pregnancy and pseudopregnancy and levels declined during days six and seven. No LIF was detected in the serosa and stromal cells during the study. Very little LIF was detected by day 13 (Yang, Le, Chen, and Harper, 1994). During the following year, Yang and associates described the expression of the LIF receptor and gp130 in the rabbit. They found low levels of the receptor in estrous and non-estrous animals with peak levels observed on days five and six of pregnancy and pseudopregnancy. The authors found little difference in the levels found in the mesometrial and antimesometrial regions of the implantation sites. Maximum gp130 levels were observed on day 6 of pregnancy and pseudopregnancy. They did not observe the receptor or gp 130 in the stromal cells (Yang, Le, Chen, Yasukawa, and Harper, 1995).

Continued experimentation by Yang et al., revealed a differential regulation of LIF expression/secretion as reflected by mRNA synthesis. These authors found that, in the rabbit, LIF was upregulated by progesterone alone or in combination with estradiol-17-β. Estradiol-17-β alone had no effect in the rabbit. However, in the mouse, estradiol-17-β alone or in combination with progesterone caused upregulation of LIF, but progesterone alone had no effect. The results

from these experiments demonstrate variable regulation of LIF in different species. (Yang, Chen, Le, and Harper, 1996) Parallel results were observed when studying the effect of steroids on implantation. In the mouse and rat, estradiol-17-β is necessary for implantation if the uterus has been primed by progesterone (Yoshinaga and Adams, 1966). However, in the rabbit, hamster, and pig, estradiol-17-β was not and absolute requirement (Kwun and Emmens, 1974; Orsini and Meyer, 1962; Heap, Flint, and Gadsby, 1981).

Anegon et al., observed peak levels of LIF transcripts in the porcine endometrium on day 11 of the estrous cycle, with maximum protein levels reported on day 12 and 13. Implantation of the blastocyst was recorded around day 14 of pregnancy (Anegon et al., 1994; Perry and Rowlands, 1962).

In the ovine uterus, LIF transcripts were found in peak levels from day 16 to day 20 of pregnancy in both epithelial and stromal cells. LIF protein was found at maximum levels in the luminal epithelium associated with glandular caruncles as well as in trophoblast cells from day 17 blastocysts. These levels were reduced under the influence of estradiol-17-β treatment and further reduction was observed by adding progesterone and estradiol-17-β to the medium (Vogiagis, Fry, Sandeman, and Salamonsen, 1997).

Human studies revealed that increased levels of LIF mRNA synthesis occured in the endometrial tissues and decidua. Low levels were recorded for the first trimester chorionic villi and term placenta. During these experiments, LIF synthesized by the endometrial glands during the menstrual cycle were

examined. A 9.5 fold increase in LIF was observed during the secretory phase as compared to the proliferative phase. In addition it was determined that the epithelial component of the endometrium synthesized a 3.3 fold greater level of LIF than the stromal components (Kojima et al., 1994).

In 1995, Kojima et al.,reported LIF receptor subunit mRNA synthesis in the human endometrium, placenta, and decidua. They determined two components of the receptor complex, the LIF receptor proper (LIF-R) and the previously mentioned glycoprotein, gp130. Their findings reported no detectable LIF-R mRNA in the non-pregnant endometrium, yet transcripts for gp130 were found in all tissues examined. Throughout pregnancy the authors detected no increase in LIF-R while reporting an increase in gp130 after the second trimester. In this same report the authors described the presence of LIF-R and gp130 on BeWo human choriocarcinoma cells in culture. Further, they determined LIF's ability to inhibit forskolin-induced human chorionic gonadotropin β (hCG-β) subunit synthesis by these cells (Kojima et al., 1995).

Charnock-Jones and associates determined that, in the human uterus, LIF transcript production was low or undetectable during the proliferative phase and observed a six fold increase during the mid to late stage of the secretory phase. The authors found that the endometrium synthesized LIF at the time of implantation and that the blastocyst expressed mRNA for LIF receptors (Charnock-Jones, Sharkey, Fenwick, and Smith, 1994).

Delage and associates formulated an HILDA/LIF production index or HLPI. They calculated HLPI by Day 5:Day 1 LIF ratios using human endometrial explant cultures. Enzyme-linked immunosorbent assays were employed to reveal LIF production by the endometrium at every phase of the reproductive cycle in both normal women and those presenting a history of reproductive failures. Although LIF was indeed synthesized in both groups, its levels were significantly lower in the latter (Delage et al., 1995).

Additional human studies were conducted by Chen and associates. They observed LIF synthesis in the human endometrium by both glandular epithelial as well as stromal primary culture cells. Their experiments showed LIF production was low during the follicular and late luteal phase of both tissues examined. Maximum production levels of LIF were recorded during the mid-luteal phase at a time that correlated with implantation. Levels were consistently lower in the stromal tissues as compared to the epithelial tissues for all phases of the reproductive cycle studied (Chen et al., 1995). Cullinan and associates reported LIF expression (not OSM or CNTF) in the human endometrial glands during the secretory/postovulatory period, not the proliferative/preovulatory period. They found the LIFRβ to be expressed during the proliferative and secretory phases in the luminal epithelium only. Both the luminal and glandular epithelium expressed gp130 throughout the cycle (Cullinan et al., 1996). Similar data were reported by Arici and associates who observed peak LIF transcripts in the human endometrium during the mid and late luteal phase. These authors

also found transcripts in decidual tissues from the first trimester of pregnancy. An increase in LIF expression was noted when endometrial stromal cultures were treated with IL-1, TNFα, PDGF, EGF, and TGFβ. Interferon-γ (IFNγ) inhibited this induction (Arici, Engin, Attrar, and Olive, 1995). Vogiagis and associates reported LIF mRNA in the human endometrium during the middle and late secretory phases. They found moderate to high levels of LIF in the stroma throughout the cycle and low levels in the epithelium during the proliferative phase and maximum epithelial levels in the middle and late secretory phases (Vogiagis, Marsh, Fry, and Salamonsen, 1996).

Little variation was observed in the LIF transcript levels produced by the human fallopian tubes throughout the normal menstrual cycle. However, significant increases in tubal LIF transcripts were associated with ectopic pregnancy. Highest levels were observed in the mucosa of the ampulla as compared to the more proximal segments. IL1α, TNFα, TGFβ all increase the expression of LIF in both epithelial and stromal cells. TGFβ increased stromal production by four fold. Epithelial cells produced approximately 13 times greater levels than stromal cells unless stromal cells were treated with TGFβ, TNFα, or IL1α, or a combination of EGF and PDGF (Keltz et al., 1996).

Jokhi et al., demonstrated an increase in specialized LIF-producing leukocytes during implantation in humans. Leukocytes comprised 40% of the cells found in the early decidual stroma and of these, 70-80% were found to belong to the natural killer (NK) cell type. These cells were defined as being able

to recognize and kill many types of tumor cells without damaging normal cells (Paul, 1993). An increase in $CD56^+$ -NK cells was observed during the secretory phase in women, a time congruent with the time of implantation. In addition, $CD56^+$ cells were especially numerous in the decidua basalis. This was recognized as the location for trophoblast migration into the endometrium during implantation. During this process, $CD56^+$ cells were observed in close proximity to the trophoblast cells and a functional relationship was suggested. These authors also suggested a role of $CD56^+$ NK cells as producers of cytokines that effect trophoblast differentiation. They described the ability of decidual $CD56^+$ NK cells to produce LIF, CSF-1, IFN-γ , as well as TNF-α and TNF-β_1. Those $CD56^+$ NK cells found in the peripheral circulation were observed to produce only the latter two cytokines (Jokhi, King, Sharkey, Smith, and Loke, 1994).

LIF was shown to have various biological effects on the same reproductive tissues in which it was made. For cultured mouse placental cells, LIF, OSM, IL-6, and IL-11 were all found to inhibit growth hormone-releasing hormone (GHRH) secretion. The signal transduction component for all four cytokines was gp130. The significance of GHRF's impact on placental biology is unknown, however, it has been demonstrated that it regulates growth hormone expression by somatotropes in the fetal pituitary gland. These cells appear in the embryo at 18 days post-coitus (Yamaguchi et al., 1995). Yamaguchi and associates also examined the effect of LIF on other mouse placental cytokines. These authors

found that LIF, IL-11, and OSM inhibit murine placental lactogen II (mPL-II) from placental cell cultures from days 7, 9, and 12 of pregnancy while stimulating mPL-I release on day 9. Additionally, LIF and OSM were found to reduce the amount of mPL-II mRNA in these cells. All three of these cytokines were shown to increase the number of syncytiotrophoblasts (giant cells) containing mPL-I alone or both mPL-I and II, however, the number of cells containing mPL-II alone decreased. Therefore, it appeared that these cytokines inhibited giant cell differentiation. IL-6, which also utilized the gp130 signal transduction protein, had no effect on mPL-I expression and only inhibited mPL-II release before mid-pregnancy unless soluble IL-6 receptor plus IL-6 were both added to the culture medium. The authors suggested that functional receptors for IL-6 were not expressed in the embryo until after mid-pregnancy . These experiments also demonstrated that the cytokines studied shared a common subunit in their signal transduction systems, yet employed additional, unique components allowing for a variety of different biological properties. Table 4 provides other cytokines and hormones and their effects on mPL levels (Yamaguchi, Taga, Kishimoto, and Miyake, 1995).

Table 4
Other Cytokines and their Effects on Placental Lactogens (Yamaguchi, Taga, Kishimoto, and Miyake, 1995).

Progesterone	decrease in mPL-I
EGF and TGF	increase in mPL-I
EGF + TGF + IL-6 + TNF	decrease in mPL-II

Note. Abbreviations for Table 4: EGF (epidermal growth factor); TGF (transforming growth factor); IL-6 (interleukin 6); TNF (tumor necrosis factor) Note: gp130 is not a component of the TGF receptor)

LIF was shown to have specific effects on proteinases which have been implicated in contributing to the processes by which the embryonic trophoblasts invade the maternal endometrial tissues thereby facilitating nidation. Two classes in particular have been studied in relationship to LIF. These are the plasminogen activators (PAs) and the matrix metalloproteinases (MMPs). PAs are serine proteinases which transform plasminogen into its active form, plasmin, and have been implicated in the process of implantation (Dano et al., 1985; Axelrod, 1985). Although plasmin may directly degrade the extracellular matrix (ECM), its primary action was thought to be involved in the proteolytic cascade that was responsible for activating MMPs. Two major forms of PAs have been isolated in mouse and rat reproductive systems. The first, tissue PAs (tPAs), were observed as products of mouse and rat oocytes and no synthesis was noted past the 2-cell embryo stage. The second major class of PAs, the

urokinase PAs (uPAs), were observed to be produced by the 2-cell rat embryo and the mouse blastocyst. Receptors for uPAs were identified on human trophoblasts, which reflected the proteolytic activity at the leading surface of the invading cells (Huarte and Vassalli, 1985; Zhang, Kidder, Zhang, Khamsi, and Armstrong, 1994; Roldan et al., 1990). In addition to these enzymes, their inhibitors have also been implicated in the regulation of implantation. For example, sows have been shown to demonstrate a particular non-invasive form of implantation and uterine fluid from pregnant females contained inhibitors of PAs which implicated PAs in the invasive type of implantation observed in other mammals (Mullins, Bazer, and Roberts, 1980). The MMPs have been shown to be the rate-limiting enzymes in ECM remodeling (Werb, 1989). Harvey et al., studied the levels of these enzymes during pregnancy in the mouse. They found transcripts for the receptors of tissue inhibitors of metalloproteinases (TIMP) and uPA in the pre and peri-implantation embryo. Both uPA and a related enzyme, matrix metalloproteinase gelatinase B (MMP-9) were reported in the peri-implanting mouse embryo. It was found that MMP-9 was produced by the trophoblast giant cells of the 7.5 day mouse embryo while TIMP-3 was synthesized by the decidua near the implantation site. LIF and EGF were both shown to stimulate uPA and MMP-9 after three days of culture and EGF had no effect by days 5-6 of culture. LIF was shown to reduce the levels of both enzymes. uPA activity was localized to the trophoblasts and the ectoplacental cone (Harvey et al., 1995).

In addition to its effect on uterine and placental cells, LIF was shown to influence primordial germ cells (PGCs). De Felici and Dolci studied the effect of LIF (and other cytokines and growth factors) on mouse PGCs. These authors co-cultured PGCs with an LIF-producing Sertoli cell feeder system known as TM_4. They found that LIF prolonged the proliferation of PGCs and retarded the degree of PGC degeneration (DeFelici and Dolci, 1991). Matsui et al.,also examined the effect LIF exerted on mouse PGCs and observed results similar to those of De Felici and Dolci. They determined that Steel factor and its receptor, the *c-kit* ligand (and its tyrosine kinase receptor) as well as LIF were temporally involved in the normal development of PGCs (Matsui et al., 1991; Williams, De Vries, Namen, Widmer, and Lyman, 1992). This growth factor has been identified in a membrane-bound and soluble form. The membrane-bound form of Steel factor is necessary for normal hematopoiesis (McNiece, Langley, and Zsebo, 1991). Also known as mast cell growth factor (MGF), stem cell factor (SCF), or *kit* ligand, Steel factor demonstrates a complex history similar to that of LIF. It is a pleiotropic growth factor that effects differentiation and development (Williams et al., 1992). Steel factor has been shown to be the ligand for the receptor c-Kit (a proto-oncoprotein) and binding causes tyrosine phosphorylation of many cellular proteins (Sattler et al., 1997). In related work, Cheng et al., observed LIF receptors on the surface of PGCs. Anti-LIFR antibodies abolished PGC survival stimulated by LIF. These authors found that OSM and CNTF also promoted PGC growth in vitro (Cheng et al., 1994). The members of the

hemopoietic family of cytokines were shown to differentially effect PGC development and behavior. OSM and LIF promoted proliferation of migratory PGCs and the viability of colonizing PGCs. IL-11 stimulated migratory PGC growth, yet had no effect on the colonizing cells. IL-6 and ciliary neurotrophic factors had no effect on PGCs (Koshimizu et al., 1996). LIF and CNTF were also determined to prolong the survival of Sertoli cells and gonocytes, in culture, in a temporal and dose dependent manner. During these experiments, IL-6 had no effect on either cell type (De Miguel et al., 1996).

In 1990 Robertson, Lavranos, and Seamark demonstrated similarities between D-factor, DRF, and LIF and examined LIF for possible embryotrophic effects. They exposed 8-cell mouse embryos to 1000 U/ml LIF and observed no effect of LIF on pre-blastocyst embryos and an increased trophoblast outgrowth (expressed in arbitrary units) due to LIF treatment, especially after day 5 of culture (Robertson et al., 1990).

In order to more precisely ascertain the reproductive functions for LIF Stewart et al., conducted a series of elegant experiments in which a mouse strain was produced for which the LIF gene was mutated (Stewart, 1994; Stewart et al., 1992). Functional LIF was not produced by these mice. Several insights into LIF's function were brought to light by these experiments. First, was the discovery that LIF was critical to implantation in mice. Implantation did not occur in the mutant recipient females whether transferred embryos were homozygous for the mutation or wild-type embryos able to produce LIF. Furthermore, it was

found that the mutant males and females were both fertile. They were able to produce viable gametes and normal fertilization took place. If the resultant embryos were transferred to wild-type females, implantation and development progressed normally. However, if the embryos were allowed to remain in the mutant female, implantation failed to occur and the embryos entered into a state indistinguishable from that seen during delayed implantation. Characteristic of such embryos is the cessation of proliferation subsequent to their hatching from the zona pellucida (Daniel, 1970). From these results it was deduced that LIF of endometrial gland origin was essential for implantation (Stewart et al., 1992, Stewart, 1994). This information supported the findings of Bhatt and associates in 1991. They demonstrated that LIF was produced at the time of implantation and its appearance coincided with elevated estrogen levels. A single injection of estrogen was sufficient to induce implantation in "delayed" mouse embryos (Bhatt et al., 1991).

In 1995, Lavranos, Rathjen, and Seamark demonstrated an increase from 62.1% to 85.1% in the number of 8-cell mouse embryos developing to the hatching stage when exposed to 1000 U/ml human LIF in vitro. They also reported an increase in hatching embryos from 7.65% to 33.8%, an increase in trophoblast outgrowth from 0 to 13.5% at 120h post human chorionic gonadotropin (hCG) injection, and an increase in trophoblast outgrowth from 47.0% to 85.1% at 144h post hCG injection. In addition, these authors observed

an increase in embryo survival after transfer of LIF-treated embryos to pseudopregnant females (Lavranos, Rathjen, and Seamark , 1995).

Mitchell and co-workers observed a significant increase in the number of 2-cell mouse embryos that reached the blastocyst stage when cultured in medium supplemented with 1000 U/ml mrLIF. These authors also determined that mrLIF significantly reduced embryo fragmentation/degeneration (Mitchell, Swanson, Hodgen, and Oehninger, 1994).

Kauma and Matt observed an increase in 2-cell mouse embryo from the $B_6C_3F_1$ strain that developed to blastocyst when these embryos were co-cultured in medium containing the LIF-producing Vero cells or embryonic fibroblasts which were shown to produce LIF (Kauma and Matt, 1995).

Fry et al.,completed experiments observing LIF's effect on ovine embryos. For these experiments the authors exposed ovine morulae or early blastocysts to 1000 U/ml LIF and examined the effects on cultured embryos as well as embryo transferred to recipient females (Fry, Batt, Fairclough, and Parr, 1992). They reported an increase in the number of embryos that hatched form the zona pellucida from 16% for cultures without LIF to 64% for LIF treated embryos. Also observed was a decrease in the number of degenerating embryos from 27% for controls to 9% for this treatment group. Pregnancy rates were analyzed and results demonstrated the highest rate of 89% for those ewes that received two embryos soon after embryo collection (no LIF treatment, no prolonged culturing). The lowest rate (16%) was observed for ewes receiving only one embryo after

48 h in a control culture (no LIF treatment). Pregnancy rates of 52% was observed for ewes that received only one embryo soon after embryo collection (no LIF treatment, no prolonged culturing). Fifty percent (50%) pregnancy rates were observed for ewes receiving only one embryo after 48 h in an LIF treated medium. When only one embryo was transferred to recipient ewes, the results clearly demonstrated the deleterious effects of culturing embryos for 48 h in this system which was compensated for by LIF treatment. (Fry, 1992; Fry et al., 1992).

Funston and associates found varying results for bovine embryos treated with LIF under different culture medium conditions. In one, serum free medium, they reported no improvement in the percentage of embryos to reach the blastocyst stage, however, they recorded an increase in the number of cells per blastocyst. In medium supplemented with LIF, no increase in development to blastocyst was recorded (Funston, Nauta, and Seidel, 1997).

LIF was found to have varied effects on human embryos and the data available is conflicting. Using 5, 7.5, 10, and 20 ng/ml recombinant hLIF Jurisicova found no significant difference in human 2-cell to 6-cell embryos that developed to blastocysts as compared to controls (Jurisicova, Ben-Chetrit, Varmuza, and Casper, 1995). Dunglison et al., however, found a significant increase in the number of embryos that developed to the blastocyst stage when cultured in a special medium containing 1000 U/ml recombinant hLIF. Not only did the Jurisicova group find an increase in the number of blastocysts, they

reported an increase in the quality of those blastocysts using a grading scale based on morphology (Dunglison, Barlow, and Sargent, 1996).

Studying chimeric mouse embryos engineered to over express LIF, Conquet, Peyrieras, Tiret, and Brulet reported that over expression of the diffusible (D) form of LIF had little effect on embryo morphology while over expression of the matrix associated (M) form has significant effects. In the latter group the authors observed an atypical production of tissues. In addition, gastrulation was inhibited as exemplified by the fact that embryonic ectoderm failed to differentiate into mesoderm. These conditions were not compatible with life and became lethal by day 9.5 postcoitus (Conquet, Peyrieras, Tiret, and Brulet, 1992).

Nachtigall et al., described another possible role for LIF in the process of implantation. They reported that LIF decreased the production of human chorionic gonadotropin (hCG) and its β subunit mRNA by human trophoblasts. They also observed an significant increase in trophouteronectin (TUN) a type of oncofetal fibronectin which anchors the trophoblasts to the endometrium. No effect was observed on the steroidogenic activity (progesterone production) of the trophoblasts. This work suggested a mechanism by which LIF switched the phenotype of the trophoblasts from the hormone producing syncytiotrophoblasts to the anchoring trophoblast type in much the same way as TGFβ thus bringing about embryo attachment during the process of implantation (Nachtigall et al., 1996; Strickland and Richards, 1992). Conversely, Sawai et al.,reported that LIF

increased levels of hCG produced, in humans, by the trophoblasts & decidua as cornpared to chorionic tissues (Sawai et al., 1995).

Little is known about the effect of LIF on the development of the embryo exposed to LIF as early as the two-cell stage. Information concerning the effect of this peptide on the process by which the embryo "hatches" from the zona pellucida is also lacking. This hatching is a necessary prerequisite to implantation. Also lacking is information comparing LIF's effects on different strains of the same murine species. Additionally, possible dose dependent effects on the early embryo are not available. Finally, no data are available comparing the results of exposing murine embryos to LIF and/or its monoclonal antibodies on embryo transfer, resorption rates, pregnancy rates, and gross morphological anomalies.

The objectives of this project were to clarify various effects that leukemia inhibitory factor had on the mouse embryo and fetus, both pre- and post-implantation. Insight into factors capable of supporting embryo development in vitro and in vivo will lead to understanding better the complex relationships in embryo development and has direct application in the growing field of reproductive medicine. Experiments were divided into three major categories or experimental groups reflecting the specific aims of the project.

Category A experiments examined possible effects of (1) murine recombinant LIF (mrLIF) (2) human recombinant LIF (hrLIF) (3) an anti-human recombinant LIF monoclonal antibody (anti-hrLIF mcab) (4) combinations of the

mrLIF or hrLIF with the mcab - on pre-implantation embryo development from the 2-cell to the blastocyst/hatching stage. Conducted were seven sub-divisions of this category (experiments 1 through 7) in addition to controls. Two-cell mouse embryos were cultured in medium conditioned with murine recombinant LIF (mrLIF), human rLIF (hrLIF), and/or a mouse anti-hLIF monoclonal antibody. A brief description of the seven experiments for category A follows:

Experiment 1. Temporal effect of exposing embryos from females of the CD_1 and B_6CBAF_1 /J strains to mrLIF . (1) Protocol 1: Embryos exposed immediately upon collection at the 2-cell stage or day 1 of embryonic development. (2) Protocol 2: 2-cell embryos allowed to culture in control medium for 24 h then exposed to LIF on day 2 of embryonic development. (3) Protocol 3: 2-cell embryos allowed to culture in control medium for 36 h then exposed to LIF on day 3 of embryonic development. The null hypothesis for the Experiment 1 was - LIF and/or its antibodies will have no significant temporal effect on the number of 2-cell embryos developing to the hatched blastocyst stage.

Experiment 2. The effects of murine recombinant LIF (mrLIF) on 2-cell embryos from B_6CBAF_1 /J females mated with CD_1 males. The null hypothesis for Experiment 2 was: mrLIF will have no significant effect on the number of 2-cell embryos that develop to the hatched blastocyst stage.

Experiment 3. The effects of human recombinant LIF (hrLIF) on embryos from B_6CBAF_1 /J females mated with CD_1 males. The null hypothesis for

Experiment 3 was - hrLIF will have no significant effect on the number of 2-cell embryos that develop to the hatched blastocyst stage.

Experiment 4. The effects of a murine anti-human LIF monoclonal antibody on embryos from B_6CBAF_1 /J females mated with CD_1 males. For Experiment 4, the null hypothesis was - the murine anti-human LIF monoclonal antibody will have no significant effect on the number of 2-cell embryos that develop to the hatched blastocyst stage.

Experiment 5. The effects of mrLIF plus a murine anti-human LIF monoclonal antibody on embryos from females of B_6CBAF_1 /J mice, mated with CD_1 males. The null hypothesis for Experiment 5 was - mrLIF plus a murine anti-human LIF monoclonal antibody will have no significant effect on the number of 2-cell embryos that develop to the hatched blastocyst stage.

Experiment 6. The effects of hrLIF plus a murine-anti human LIF monoclonal antibody on embryos from B_6CBAF_1 /J females mated with CD_1 males. The null hypothesis for Experiment 6 was - hrLIF plus a murine anti-human LIF monoclonal antibody will have no significant effect on the number of 2-cell embryos that develop to the hatched blastocyst stage.

Experiment 7. The effects of various concentrations of mrLIF on embryos from females of the CD_1 strain mated with CD_1 males. For Experiment 7, the null hypothesis was - Varying the concentrations of mrLIF will have no significant effect on the number of 2-cell embryos that develop to the hatched blastocyst stage for CD_1 mice.

Category B experiments were designed to determine the effects of a single exposure to mrLIF, or an anti-mrLIF monoclonal antibody (mcab), at the time of transcervical embryo transfer, on implantation rates, pregnancy rates (as determined by the number of viable pups on day 17), and resorption rates. For all embryo transfers, day zero (0) was considered to be the day that the donor females were examined for the presence of vaginal plugs as an indication of mating. The null hypothesis for this category was - a single exposure of mrLIF or an anti-mrLIF mcab at the time of transcervical embryo transfer will have no significant effect on implantation rates, pregnancy rates, and resorption rates in embryos transferred.

Category C experiments were designed to determine the effects of a single exposure to mrLIF, or an anti-mrLIF monoclonal antibody (mcab), at the time of transcervical embryo transfer, skeletal development in this species. Transcervical embryo transfers were conducted as in category 2 experiments . For category three experiments, the null hypothesis was - a single exposure of mrLIF or an anti-mrLIF mcab at the time of transcervical embryo transfer will have no significant effect on the skeletal development in embryos transferred.

MATERIALS AND METHODS

Experiments were placed into one of three major categories: Category A in which two-cell embryo were exposed to various combinations of mLIF, hLIF, and an anti-human LIF monoclonal antibody; Category B in which transcervical embryo transfer supplementing the transfer medium with mLIF or an anti-murine LIF monoclonal antibody were conducted to determine implantation, pregnancy, and resorption rates; Category C employed fetal clearing and differential staining of developing cartilage and bone for assessment of skeletal development.

Embryos from the mouse, *Mus musculus* were observed during this project. Six to eight week old CD_1 (Charles River Laboratories, Wilmington, Massachusetts) and B_6CBF_1 (The Jackson Laboratory, Bar Harbor, Maine) were given water and food (Agway PROLAB) *ad libitum*. Females from both strains will be mated with CD_1 males (at least 8 weeks of age).

A modified Kreb's medium (mKreb's) supplemented with 4 mg bovine serum albumin (4% BSA)/ml (Sigma, St. Louis) was used for collection and culture of embryos as described by Ackerman (Ackerman, Swanson, Adams, and Wortham, 1983). Medium was prepared filter-sterilized through 0.22 cellulose acetate membrane filters (Corning), dispensed into 25 cm^2 tissue culture flasks (Corning), and refrigerated at 0 - 4° C until utilized. Medium was incubated at 37° C in 5% CO_2 in 100% humidified air for at least 2 h prior to use.

Superovulation was induced by injecting female mice with 5 IU pregnant mare's serum gonadotropin (PMSG - Sigma, St. Louis) IP followed in 48 h by

injecting 5 IU human chorionic gonadotropin (hCG - Sigma, St. Louis) IP. At the time of hCG injection each female was placed with an individually caged, proven fertile male CD_1. The following morning, approximately 16 h after the hCG injection, the female mice were inspected for vaginal plugs. This was considered to be day 0 of pregnancy throughout these experiments. On the morning after inspection for vaginal coagulation plugs (day 1 of pregnancy), the pregnant donor females were killed by cervical dislocation and the abdominal skin will be removed. Under sterile conditions the abdomen was opened and the oviducts removed and placed in culture medium. Using a Zeiss Urban Quadrascope/dissecting microscope the embryos were removed from the oviducts by inserting into the fimbriated end of the oviduct a 30-gauge needle connected to a 1cc tuberculin syringe filled with mKreb's medium applying gentle pressure to expel the embryos.. The 2-cell embryos were flushed from the oviducts into sterile 35 X 10 mm polystyrene culture dishes (Falcon) containing 2 ml of culture medium. Morphologically normal 2-cell embryos were collected and appropriated to the various test/control groups using micropipettes hand-drawn from Pasteur pipettes. Embryos were cultured in 100 µl droplets under mineral oil at 37° C in 5% CO_2 in 100% humidified air.

Category A Experiments

Collected 2-cell embryos were exposed to test compounds in vitro under three temporal protocols. For Protocol 1, fifteen 2-cell embryos were introduced to a 100 µl droplet containing 1000 U/ml of the test compound immediately after

the embryos were collected from the oviducts. Fifteen 2-cell embryos were also introduced to a 100 µl droplet of mKreb's medium as the control. All droplets were covered with mineral oil prior to embryo collection and allowed to equilibrate in the incubator. Embryo development was observed and recorded during the next 6 days. For Protocol 2, fifteen 2-cell embryos were introduced to a 100 µl droplet of mKreb's culture medium and allowed to culture as controls for 24 h at which time they were transferred into a 100 µl droplet of test medium containing 1000 U/ml of the test compound. Fifteen 2-cell embryos were similarly cultured in mKrebs medium as controls. Embryo development was observed and recorded during the next 5 days. For protocol 3 fifteen 2-cell embryos were introduced to a 100 µl droplet of mKreb's culture medium and allowed to develop as controls for 48 h at which time they were transferred to a 100 µl droplet of test medium containing 1000 U/ml of the test compound. Again, fifteen 2-cell embryos were similarly cultured in mKrebs medium as controls. Embryo development was observed and recorded during the next 4 days.

Category A experiments were further divided into Experiments 2-7 according to the test compound used. With the exception of Experiment 8 (varying concentrations of mrLIF), each was conducted using the temporal protocols described above. These experiments were performed using embryos from female B_6CBF_1 mated with CD_1 males.

- Experiment 2 - Test compound: 1000 U/ml murine rLIF using embryos from B_6CBF_1 females mated with CD_1 males.
- Experiment 3 - Test compound: 1000 U/ml human rLIF using embryos from B_6CBF_1 females mated with CD_1 males.

- Experiment 4 - Test compound: 1000 U/ml murine anti-human LIF monoclonal using embryos from B_6CBF_1 females mated with CD_1 males.
- Experiment 5 - Test compound: 1000 U/ml murine rLIF plus 1000 U/ml murine anti-human LIF monoclonal antibody. using embryos from B_6CBF_1 females mated with CD_1 males.
- Experiment 6 - Test compound: 1000 U/ml human rLIF plus 1000 U/ml murine anti-human LIF monoclonal antibody. using embryos from B_6CBF_1 females mated with CD_1 males.
- Experiment 7 - to determine whether a dose dependent effect of LIF on blastocyst development and fragmentation existed. For these experiments embryos from CD_1 females will be mated with CD_1 males. Embryos were exposed to 1000, 2000, 5000, and 10000 U/ml murine recombinant LIF under the protocol 1.

For Category A experiments, murine and human recombinant Leukemia Inhibitory Factor (derived from Chinese hamster ovarian cells) as well as a murine anti-human LIF monoclonal antibody were provided by Genentech, San Francisco, Ca.

Category B Experiments - Transcervical embryo transfer - implantation, pregnancy, and resorption rates

For Categorie B and C experiments, murine recombinant LIF and a goat anti- murine recombinant LIF monoclonal antibody (IgG) was purchased from R&D Systems Inc. Test groups included: (1) 5000 U/ml mrLIF (2) 1000 U/ml anti-mLIF mcab (3) 2500 U/ml anti-mLIF mcab and (4) 5000 U/ml anti-mLIF mcab. Two groups of female mice were prepared, one group as embryo donors (B_6CBF_1), the other as the recipient females (CD_1). Females from both groups received IP injections of 5 IU (0.1 ml) of pregnant mare serum gonadotropin (PMSG - Sigma, St. Louis) followed in 48 hours by IP injection of 5 IU (0.1 ml) of human chorionic gonadotropin (hCG - Sigma, St. Louis). The injection schedule for recipients was one day later than that for donors to allow cultured embryo development to syncrhonize with pseudopregnant uterine development. Donor females were placed with proven fertile CD_1 males. The next morning (approximately 16 hours) donor females were examined for the presence of vaginal coagulation plugs as an indication of mating. The recipient females were placed with vasectomized males in the afternoon of this day immediately following the hCG injection (day 0 of pregnancy) and examined for vaginal plugs the following morning. Embryos from donors were cultured in mKreb's medium for 72 h at 37^{o}C in 5% CO_2 in 100% humidified air. On day 4 of embryonic development, pseudopregnant or recipient females were tranquilized using 0.1 mg/Kg body mass acepromazine maleate (10mg/m, Promace, Henry Schein

Inc., Fort Dodge) . The embryo transfer tubing used was Clay Adams PE 10 tubing (I.D. 0.28 mm; O.D. 0.61 mm, Baxter Scientific) connected to a 30 ga needle fitted to a 500 µl, threaded, Halmilton syringe(Hamilton #1750TP). The PE10 tubing and syringe were filled with mKreb's culture medium. Approximately one cm of air was pulled into the distal tip of the tubing followed by 3 cm (1.85 µl) medium containing fifteen blastocysts from the donor group. Embryos were collected in as little medium as possible (1/4 turn of threaded Hamilton syringe) to reduce the risk of having them wash out of the uterus upon removal of the transfer tubing. An additional 0.5 cm of air was drawn into the transfer tube. The cervical os was visualized with the aid of a glass speculum constructed from a Pasteur pipette. Using a Wild Heerbrugg dissecting microscope, the distal end of the embryo transfer tube was inserted approximately 1.5 - 2 cm into the cervical os and the embryos flushed out of the tube until the proximal air spacer was observed passing the cervical os. The transfer tubing was gently removed and females individually caged and allowed to recover from anesthesia. On day 17 of pregnancy, pregnant females and their fetuses were killed by lethal overdose using IP injection of sodium pentobarbital (64.8 mg/ml, Anpro Pharmaceuticals - Henry Schein Inc.). The abdominal skin of pregnant females was removed and the abdomen opened in order to externalize the uterus. The uterus was then inspected for number of fetuses and/or resorption sites which were then removed. Fetuses were then removed from amniotic membranes and separated from the placentas. The following data were

recorded: number of fetuses; which uterine horn they were collected from; fetal tail length, fetal crown-rump length; fetal mass; placental mass and linear dimensions; number of resorption sites and their dimensions; resorbing embryos were weighed if fetal attributes could be recognized. Implantation rates, pregnancy rates, and resorption rates were determined. The following definitions pertain to data: (1) Implantation rates - includes all implantation sites (those of both viable pups and resorbed fetuses) (2) Pregnancy rates - viable pups which appeared morphologically normal at day 17 (3) Resorption rates - amorphous resorption sites or fetuses demonstrating severe developmental retardation.

Category C Experiments - Whole fetus clearing/staining for determination of Skeletal Development

Fetuses were removed and measured as described for category 3 experiments on day 17 of pregnancy then cleared and differentially stained for cartilage and bone as described by Hanken and Wassersug (Hanken and Wassersug, 1981) in order to evaluate skeletal development. Once removed from uteri and measurements taken, the fetuses were fixed in 10% neutral buffered formalin (4g NaH_2PO_4 + 6.5 g Na_2HPO_4 in 1 L 10% formalin) for at least 24 hrs. After fixation, evisceration was facilitated by using watchmaker forceps. At this time the fetuses were washed in several changes of distilled water to remove most of the formaldehyde. After washing, fetuses were placed in an alcian blue solution (10 mg alcian blue - Sigma Chemical Co. + 70 ml 100% ethanol, + 30 ml glacial acetic acid) for 12-48 hours. Once the cartilage was stained, the dehydration

step ensued by placing the fetuses in a minimum of two absolute alcohol baths for 24 hours each. Next the fetuses were exposed to a series baths consisting of decreasing concentrations of ethanol (i.e. 75% - 2 hrs; 50% - 2 hrs; 25%) each for two hrs followed by two consecutive distilled water baths for one hr each. After re-hydration, fetuses were partially macerated for one or two hrs in a solution containing 1 g of trypsin, 30 ml saturated aqueous sodium borate ($Na_2B_4O_7 \cdot 10H_2O$ Fischer Scientific, Co.), and 70 ml distilled water. Fetuses were left in this solution until the soft tissues become transparent and the embryo proper had a consistency of warm gelatin. Bone was stained by placing fetuses a solution of 0.5% KOH then adding drops of 0.1% alizarin red in water (Sigma Chemical Co.) until the solution turned deep, almost opaque purple. Fetuses remained in this solution for 24 hours. The final step was embryo clearing. Fetuses were placed in a 25% glycerin in 0.5% aqueous KOH solution for 48 hours. To this solution, 1 ml hydrogen peroxide for every 10 ml glycerin was added. Fetuses were then placed in succeeding solutions of increasing glycerin concentrations, 50% glycerin with 0.5% KOH; 75% glycerin with 0.5% KOH, and 100% glycerin each for 24 hours. Specimens were be stored in glycerin to which a few drops of 88% liquid phenol (Mallinckrodt) was added. Fetuses were then examined and photographed using a Wild Heerbrugg dissecting microscope. Number and condition of ribs, humerus length (HLN), length of diaphyseal ossification (bone) in the humerus (HOL), width of dorsal gap between the vertebral pedicles or the vertebral space (VSPC), estimated percentage of

ossification in the exoccipital bone (EX), length of ipsilateral scapula (SLN) measured from vertebral border to glenoid cavity, and length of ossification center in scapular spine (SOS) were recorded. All lengths were measured in millimeters (mm).

Vasectomy

Eight to ten week old CD_1 males were tranquilized by an IP injection of 0.1 mg/Kg body mass acepromazine maleate (10mg/ml, Promace, Fort Dodge - Henry Schein Inc.) and anesthetized using 30 mg/kg body mass sodium pentobarbital (64.8 mg/ml, Anpro Pharmaceuticals - Henry Schein Inc.), IP. This protocol has proven to be very efficacious in our laboratory. Dopram-V (20 mg/ml, Doxapram hydrochloride - Aveco - Henry Schein Inc.) was available if needed as an antidote in the case of any life threatening reactions to the general anesthesia. The scrotum was shaved and swabbed with 100% ethanol. Approximately 0.25 - 0.50 ml 2% lidocaine hydrochloride (Abbott Laboratories - Henry Schein, Inc.) was subcutaneously injected as a local analgesic/anesthetic. A 0.5 cm, mid-line incision, through the skin of the scrotum is was made, taking care not to involve the underlying tunics. The skin was separated from these underlying layers before proceeding. A 0.5 cm incision was made through the tunic approximately 0.25 cm to either side of the midline septum or raphe. The tunic layer was stabilized with self-closing watchmaker forceps. By rotating the testis within the scrotum, the vas defferens could be visualized and externalized without removing the testis which greatly facilitated replacement of tissues. The

vas defferens was ligated approximately 0.05 cm from the cauda epididymis. Another ligature was placed approximately 1.0 cm proximal to the first. The segment of vas deferens between the two ligatures was removed using micro-scissors. Any externalized viscera was replaced into the abdominal cavity. Wound closing was effected by 06 Prolene sutures, for the abdominal wall (tunic) incision with discontinuous sutures spacing them 2-4 mm apart. 06 Prolene sutures were also used to close the skin incision with the same technique. I do not recommend continuous suturing because if the animal removes any segment, the rest will untie rather easily. A surgical staple was also used to provide an excellent deterrence to the animal removing its own sutures. Animals were placed in recovery and observe daily. Approximately one month minimum recovery was observed prior to mating.

Statistics

For Category A experiments, Cochran-Mantel-Haenszel (CMH) statistics were employed to determine potential differences in the means of that data used to determining the developmental indices (ID's) used throughout experiments 1 - 7. As explained below, these indices evaluate the reproductive vitality of the embryos.

For Category B & C experiments, Multiple analysis of variance (MANOVA) was utilized to determine significant differences observed among the various dependent variables with Tukeys studentized range (HSD) test used to

determine in which independent variables the differences existed. All lengths were measured in millimeters (mm) and masses in grams.

Justification of animal use

The species used was *Mus musculus*. This was a basic research project to determine fundamental mechanisms of embryo development once fetuses were exposed to leukemia inhibitory factor or its monoclonal antibody. This species is a large enough animal to perform transcervical and transtubal embryo transfer with ease, collection of fetuses is relatively simple, and the species has been proven to be very proliferative. The latter insures maximum numbers for embryo retrieval with minimum adult animal expenditure. Additionally, this work was a continuation of 20 years of research on embryotoxicity in this laboratory. Therefore a substantial data base was available to compare with new results. The principal investigator was familiar with abided by all parts of the Animal Welfare Act, and the provisions described by the USDA, OPRR, and the University's assurance. No veterinary care of these animals was required.

To ensure minimum discomfort, distress, pain, and injury , mice were anesthetized to stage III, plane III of the surgical level of anesthesia, and the standard techniques for vasectomy for appropriate males as mentioned earlier was employed. Anesthetic, tranquilizer, and analgesic used were: Pentobarbital Sodium (anesthetic), Acepromazine Maleate (tranquilizer), and Lidocaine Hydrochloride (analgesic). Acepromazine maleate (0.1 mg/Kg), a veterinary tranquilizer, was administered intra-peritoneally (IP) on all animals at least one

hour before surgical anesthesia was induced by peritoneal (IP) injection of Na-pentobarbital (30 mg/Kg). Intradermal (ID) and/or subcutaneous (SC) Lidocaine HCl (2% solution) was used at all surface sites prior to incisions. Animals were not allowed to reach consciousness throughout the duration of the experiment until wound closure was completed. Surgical experience for the principal investigator consisted of five years of training (1990 - 1995) under the supervision of Dr. Swanson. Techniques include: Inhalation & general anesthesia, local analgesia and general surgery. Experiences include work with 1) mice, 2) rats, 3) rabbits, and 4) hamsters

When euthanasia was required the drug and/or procedure to be used was (1) cervical dislocation or (2) overdose (OD) with IV injection of Na-Pentobarbital. Both of these procedures are consistent with the recommendations of the American Veterinary Medical Association Panel on Euthanasia for the species concerned (AVMA, 1978), the Guide for the Care and Use of Laboratory Animals (USDHHS-NIH publication No. 86-23), and the Experimental and Surgical Technique in the Rat (Waynforth and Fleckness, 1992).

Results

Category A, Experiment 1 - Temporal Effects

For category 1 experiments a developmental index (DI) was formulated in which embryos in each stage of development were assigned developmental scores. These scores were ranked from -1 to 7 and corresponded to the following developmental stages respectively: -1 for fragmenting or degenerating embryos (f/d); 0 for 2-cell embryos; 1 for 3-4-cell embryos; 2 for 5-8-cell embryos; 3 for morulae (M); 4 for early blastocysts (e); 5 for expanded blastocysts (E); 6 for hatching blastocysts (h); 7 for completely hatched blastocysts H, Table 5). This index was designed to allow a negative impact for f/d embryos and included several developmental stages to reflect the graded response to treatment. This developmental index represents the following formula: The developmental scores (DS's) were multiplied by the number of embryos in that developmental stage for a particular day of development and these numbers were summed. This total was divided by the total number of embryos in that test group to get an average representing the development of the entire group of embryos (Table 5). The DI is represented by the following formula:

$$DI = \frac{\Sigma \text{ (DS x number of embryos in each stage)}}{\text{total embryos in each treatment group}}$$

Developmental indices were statistically analyzed using Cochran-Mantel-Haenszel (CMH) statistics to compare means.

Figures 1, 2, 3, and 4 provide data on experimental sub-category 1, the effect of exposing murine 2-cell embryos to the various test compounds at different times during early embryo development. For protocol 1, 2-cell embryos were exposed to test compounds on the day they were collected from the oviducts. For protocols 2 and 3, embryos were exposed to test compounds after culturing as controls for 24 and 48 hours respectively. Table 6 provides a simplified presentation of sub-category 1 experiments. For this table, ">" and "<" characters were employed to indicate when a statistically significant increase (>) or decrease (<) was recorded between any of the three temporal protocols for developmental days three through six for each of sub-categories one through five. When comparing the effect of exposing preimplantation embryos to the various test compounds under the three different protocols, results varied according to the test compound studied (Table 6, Figures 1 - 4). For sub-category 2 embryos (exposed to mrLIF) and sub-category 5 embryos (exposed to mrLIF plus the mcab), the order of temporal effect from highest developmental index (DI) to lowest follows the pattern: protocol 1, 2, and 3, with protocol 3 having the lowest DI. For sub-category 3 embryos (exposed to hrLIF) as well as sub-category 6 embryos (exposed to hrLIF plus the mcab), protocol hierarchy followed a pattern of protocol 2, 3, and 1 or 2 and 3 compared to 1. When combined, as in the latter pattern (2 and 3 compared to 1), no significant difference was detected between protocols 2 and 3. Embryos exposed to the mcab alone, sub-category 4, protocol 2 demonstrated a higher DI than protocol 1

for developmental days three and four, whereas protocols 2 and 3 had significantly higher DI values for developmental days five and six (Table 6, Figures 1 - 4).

Similar analysis was conducted to determine the effect that mrLIF had on embryos derived from CD_1 females mated with B_6CBAF_1/J males (sub-category 7). For these experiments, no significant differences were observed among the three protocols for days 3, 4, and 5. However, on day 6, protocol 3 embryos were recorded to have a significantly higher developmental index compared to protocol 1 embryos.

Table 5
Developmental Index Categories.

Developmental Score (DS)	Developmental Stage	Number of embryos in Stage
-1	fragmenting or degenerating	n_1
0	2-cell	n_2
1	3-4-cell	n_3
2	5-8-cell	n_4
3	morula	n_5
4	early blastocyst	n_6
5	expanded blastocyst	n_7
6	hatching blastocyst	n_8
7	hatched blastocyst	n_9

$$DI = \frac{\Sigma \text{ (DS x number of embryos in each stage i.e. } n_1 \ldots n_9)}{\text{total embryos in each treatment group}}$$

Table 6
Category A, Experiment 1. Comparison of Developmental Indices for Protocols 1, 2, and 3.

	mrLIF	hrLIF	mcab	mrLIF + ab	hrLIF + ab
Day 3	NS	2 > 1	2 > 1	NS	2 > 1
Day 4	1 & 2 > 3	2 > 3 > 1	2 > 1	1 & 2 >3	2 & 3 > 1
Day 5	1 > 2 > 3	2> 3 > 1	2 & 3 > 1	1 & 2 > 3	2 & 3 > 1
Day 6	1 > 2 > 3	2 & 3 > 1	2 & 3 > 1	1 & 2 > 3	2 & 3 > 1

Note. Increases (>) or decreases (<) in developmental indices indicate statistically significant differences between the protocols.

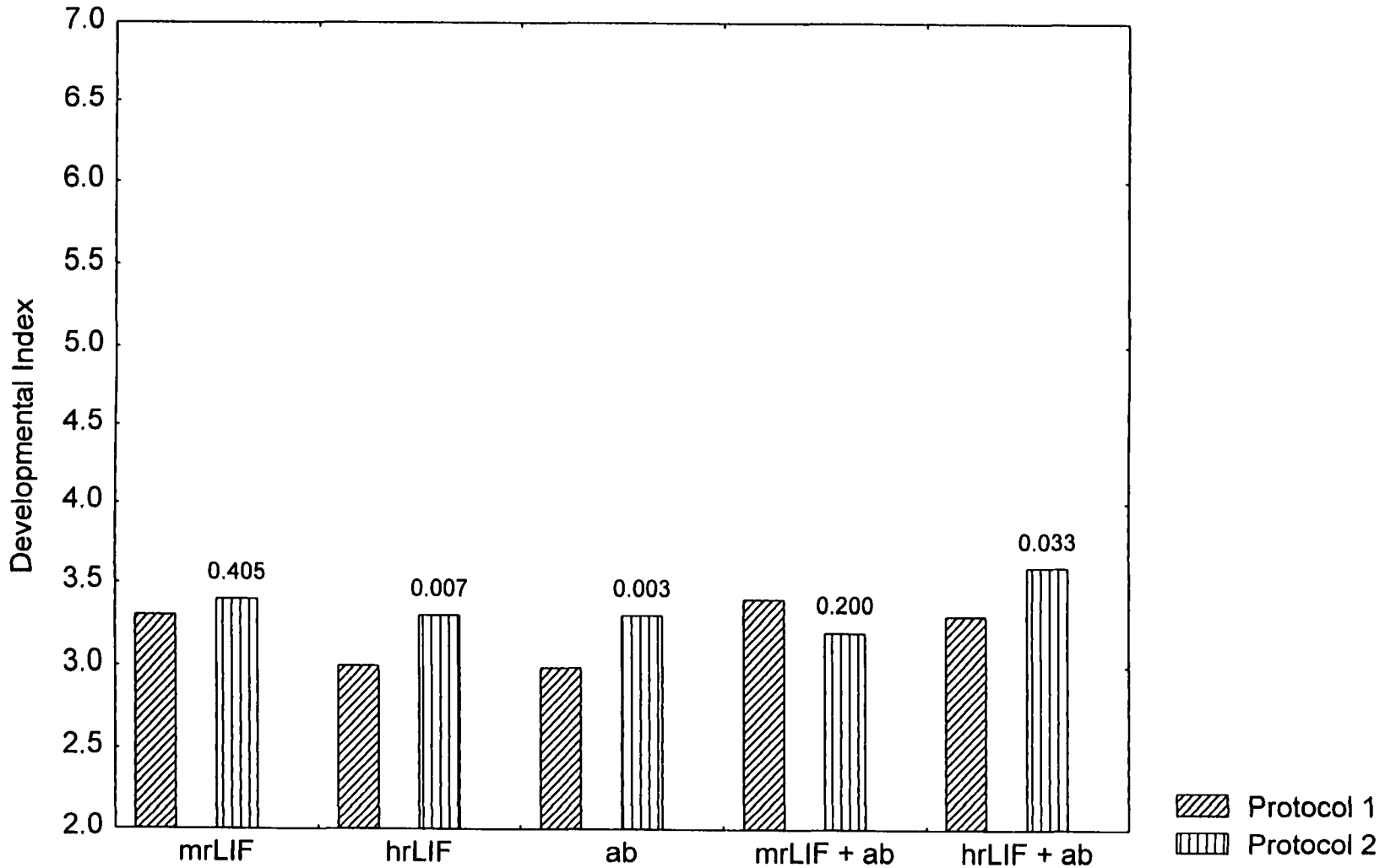


Figure 1. Temporal comparison - Day 3 - Category A, Experiment 1. Protocols 1 and 2. p values located above histograms for each group.

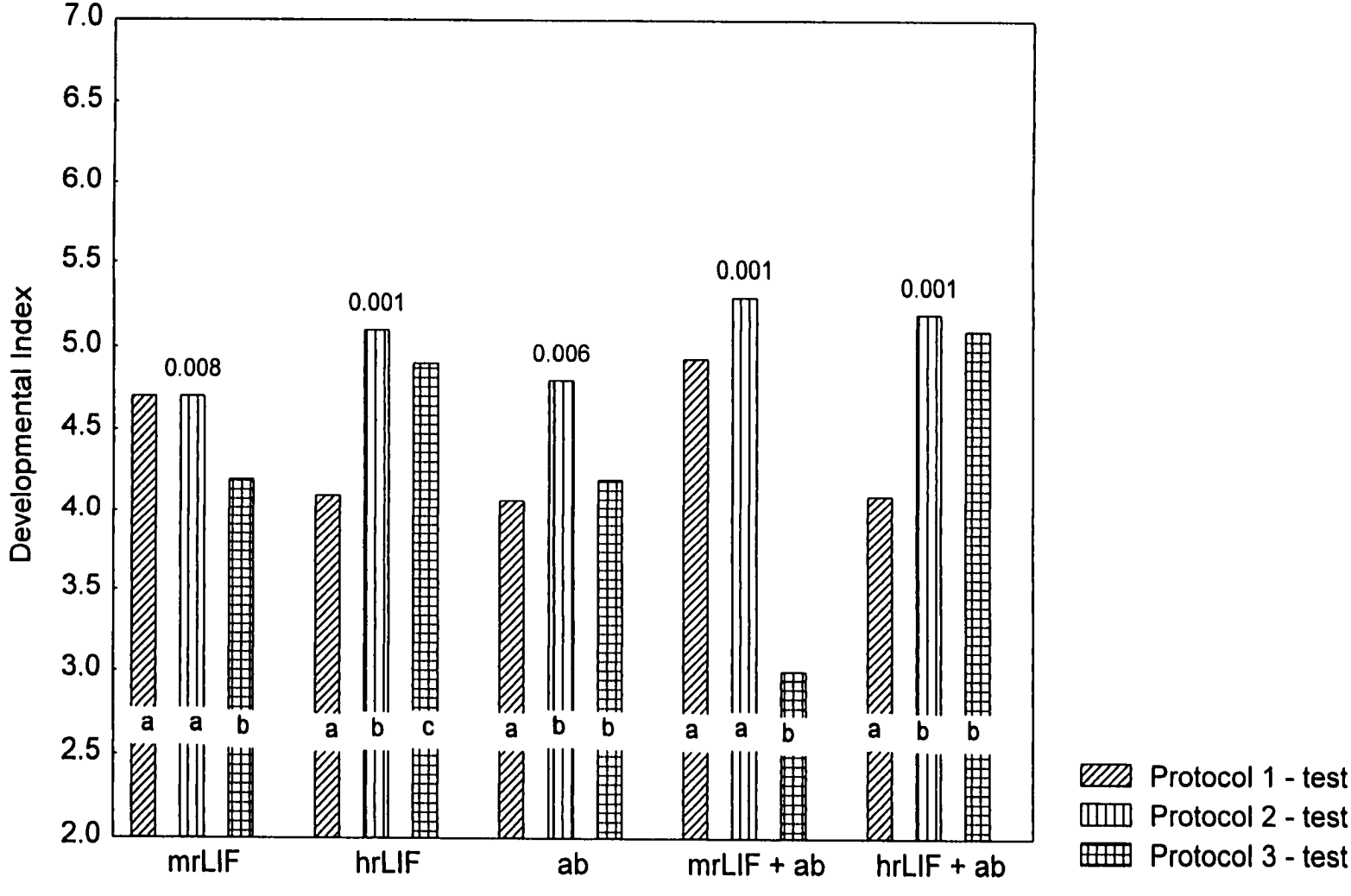


Figure 2. Temporal comparison - Day 4 - Category A, Experiment 1. Protocols 1, 2, and 3. p values located above histograms for each group. Within each group, histograms with the same letter are not significantly different.

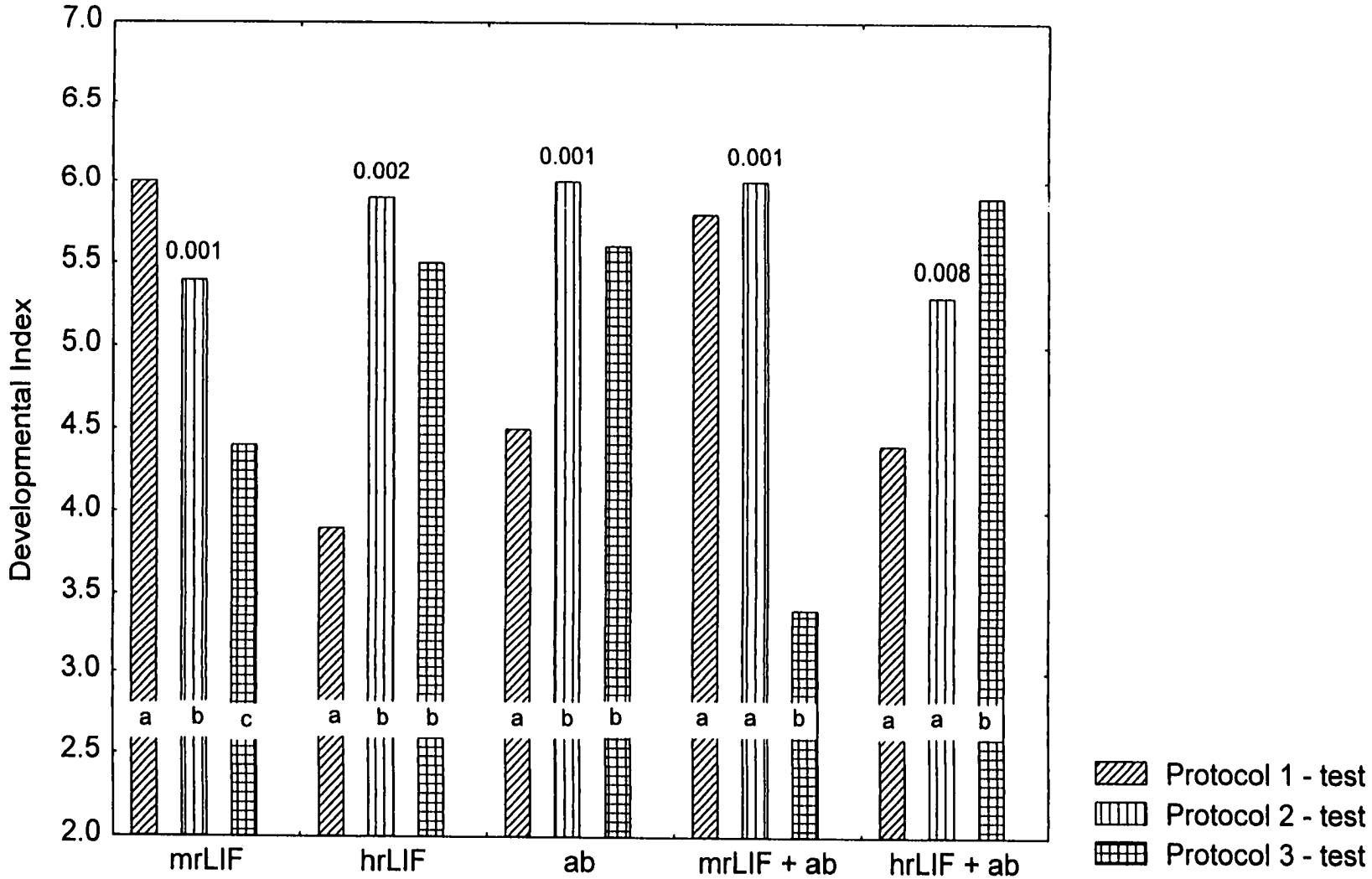


Figure 3. Temporal comparison - Day 5 - Category A, Experiment 1. Protocols 1, 2, and 3. p values located above histograms for each group. Within each group, histograms with the same letter are not significantly different.

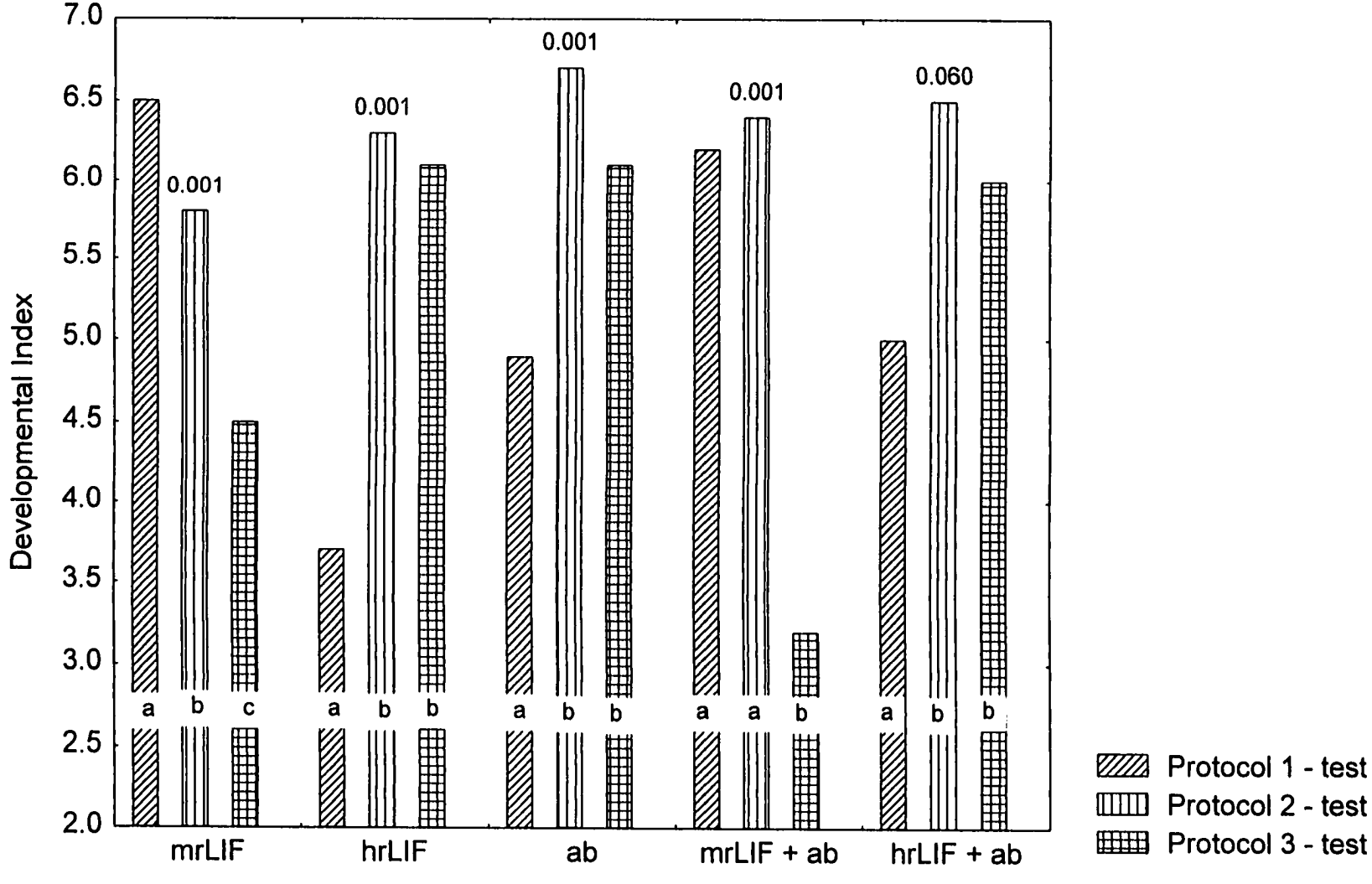


Figure 4. Temporal comparison - Day 6 - Category A, Experiment 1. Protocols 1, 2, and 3. p values located above histograms for each group. Within each group, histograms with the same letter are not significantly different.

Category A, Experiments 2-6 - mrLIF, hrLIF, and mcab

Table 7 provides the magnitude in differences for developmental indices for each test compound compared to its control as well as the direction for that change (i.e. increase or decrease in developmental indices). For these experiments embryos were collected from B_6CBAF_1/J females mated with CD_1 males. Appendix iii provides developmental index categories (DI), data totals, percents for each developmental stage, and developmental indices for individual developmental day for each protocol for Experiments 2-6. Appendix iv shows the raw data collected for these experiments. Figures 5 - 15 provide bar/column graphs for individual developmental day (days 3 - 6) for each protocol for experiments 2-6.

For protocols 1 and 2, 1000 U/ml mrLIF (Experiment 2) enhanced 2-cell embryo development for every developmental day observed when compared to controls (Figures. 5 - 12). For days 3 - 6, mrLIF significantly increased the developmental indices during protocols 1 and 2 except for day 5 of protocol 2. Although an increase was observed on this day, it was not statistically significant ($p = 0.5444$, Table 7). For protocol 3 (Figures 13 - 15), no increase was observed on day 4 for the mrLIF group and a slight decrease in development was recorded for day 5 (Figures. 13 and 14) with a greater decrease for day 6 (Table 7), although neither of these were significant ($p = 0.298$ and 0.124 for days 5 and 6, protocol 3, Table 7). When combined with 1000 U/ml of an anti-hrLIF monoclonal antibody (mcab) (Experiment 5) the stimulatory effect of 1000

U/ml mrLIF was still observed for all developmental days during protocols 1 and 2 (Figures 5 - 15, Table 7), however, none of these were statistically significant. As with the mrLIF alone, the combination of mrLIF and the mcab had an insignificant effect on embryos for protocol 3 (Figures 13 - 15, Table 7). Supplementing medium with mrLIF tends to over-ride the inhibitory effect seen from the mcab alone, but not with statistical significance.

Human rLIF, at a concentration of 1000 U/ml (Experiment 3), had an increasingly inhibitory effect on 2-cell embryo development with each ensuing day for protocol 1 when compared to controls with significance observed on day 4 ($p = 0.033$, Table 7). For protocols 2 and 3, hrLIF had an stimulatory effect on embryonic develop as reflected by developmental indices when compared to controls with statistically significant results recorded for days 3, 4, and 6 during protocol 2 and day 4 for protocol 3. The inhibitory effect of hrLIF was also observed when 1000 U/ml hrLIF was combined with the 1000 U/ml of the mcab (Experiment 6) for each developmental day during protocols 1, 2, and 3. Statistically, this inhibition was significant for all developmental days for protocol 1 and for days 4 and 5 ($p = 0.019$ and 0.001 respectively) for protocol 2 (Figures 5 - 15, Table 7).

The anti-hrLIF mcab alone (1000 U/ml) had very little effect on embryonic development compared to controls for developmental day 3 of protocol 1 (Experiment 4). However, a significant decrease in development was recorded for developmental day 4 and the magnitude of this tendency increased

temporally through day 6 ($p = 0.022$, 0.004, and 0.006 respectively). For protocol 2, an increase in development was observed, for days 4 - 6 with only day 4 being significant. A slight decrease was recorded for all 3 developmental days of protocol 3 with none of these being significant (Table 7).

Table 7
Category A, Experiments 2-6 - Comparison of Test Compounds.

	Protocol 1				
Treatment →	m (n=138)	h (n=105)	ab (n=106)	m + ab (n=30)	h + ab (n=31)
Devel Day 3	+ 0.2	- 0.1	- 0.01	+ 0.4	- 0.4
	p=0.003	p=0.239	p=0.927	p=0.189	p=0.004
Devel Day 4	+ 0.5	- 0.5	- 0.43	+ 0.73	- 1.5
	p=0.003	p=0.033	p=0.022	p=0.246	p=0.001
Devel Day 5	+ 1.4	- 0.8	- 1.3	+ 1.2	- 1.8
	p=0.003	p=0.131	p=0.004	p=0.125	p=0.002
Devel Day 6	+ 1.5	- 1.3	- 1.4	+ 1.5	- 1.4
	p=0.003	p=0.057	p=0.006	p=0.054	p=0.026

	Protocol 2				
Treatment →	m (n=100)	h (n=61)	ab (n=62)	m + ab (n=30)	h + ab (n=31)
Devel Day 3	+ 0.3	+ 0.2	+ 0.3	+ 0.2	- 0.1
	p=0.001	p=0.041	p=0.035	p=0.944	p=0.944
Devel Day 4	+ 0.5	+ 0.5	+ 0.3	+ 1.1	- 0.4
	p=0.011	p=0.001	p=0.082	p=0.049	p=0.019
Devel Day 5	+ 0.8	+ 1.2	+ 0.2	+ 1.4	- 0.9
	p=0.444	p=0.060	p=0.470	p=0.095	p=0.001
Devel Day 6	+ 0.8	+ 1.4	+ 0.4	+ 1.7	+ 0.1
	p=0.003	p=0.046	p=0.196	p=0.051	p=0.683

	Protocol 3				
Treatment →	m (n=95)	h (n=57)	ab (n=59)	m + ab (n=30)	h + ab (n=31)
Devel Day 4	=	+ 0.3	- 0.3	- 0.8	- 0.4
	p=0.965	p=0.037	p=0.807	p=0.140	p=0.056
Devel Day 5	- 0.2	+ 0.8	- 0.2	- 1.2	- 0.3
	p=0.298	p=0.607	p=0.805	p=0.135	p=0.795
Devel Day 6	- 0.5	+ 1.1	- 0.2	- 1.5	- 0.3
	p=0.124	p=0.137	p=0.937	p=0.166	p=0.143

Note. Embryos from B_6CBAF_1/J females mated with CD_1 males. Comparison of developmental indices exposing 2-cell embryos to 1000 U/ml: mrLIF (m), hrLIF (h), an anti-hrLIF monoclonal antibody (ab), and combinations of the ab with m and h. Upper numbers indicate the magnitude of increase (+) or decrease (-) in the developmental index as compared to controls for each treatment. Lower numbers are p values derived from Cochran-Mantel-Haenszel (CMH) statistics.

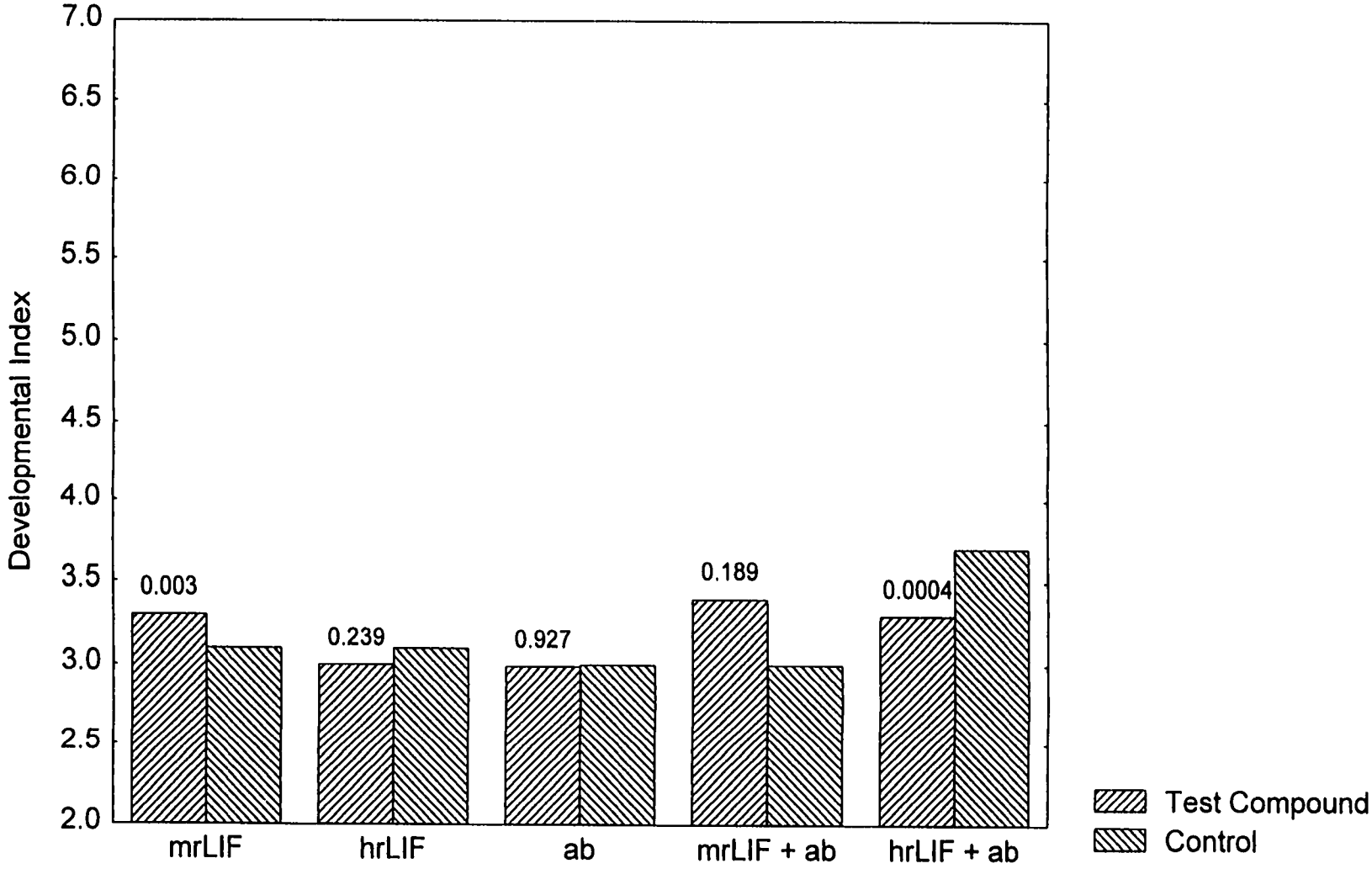


Figure 5. Category A, Experiments 2 - 6, Protocol 1, Day 3.
p values located above histograms for each group.

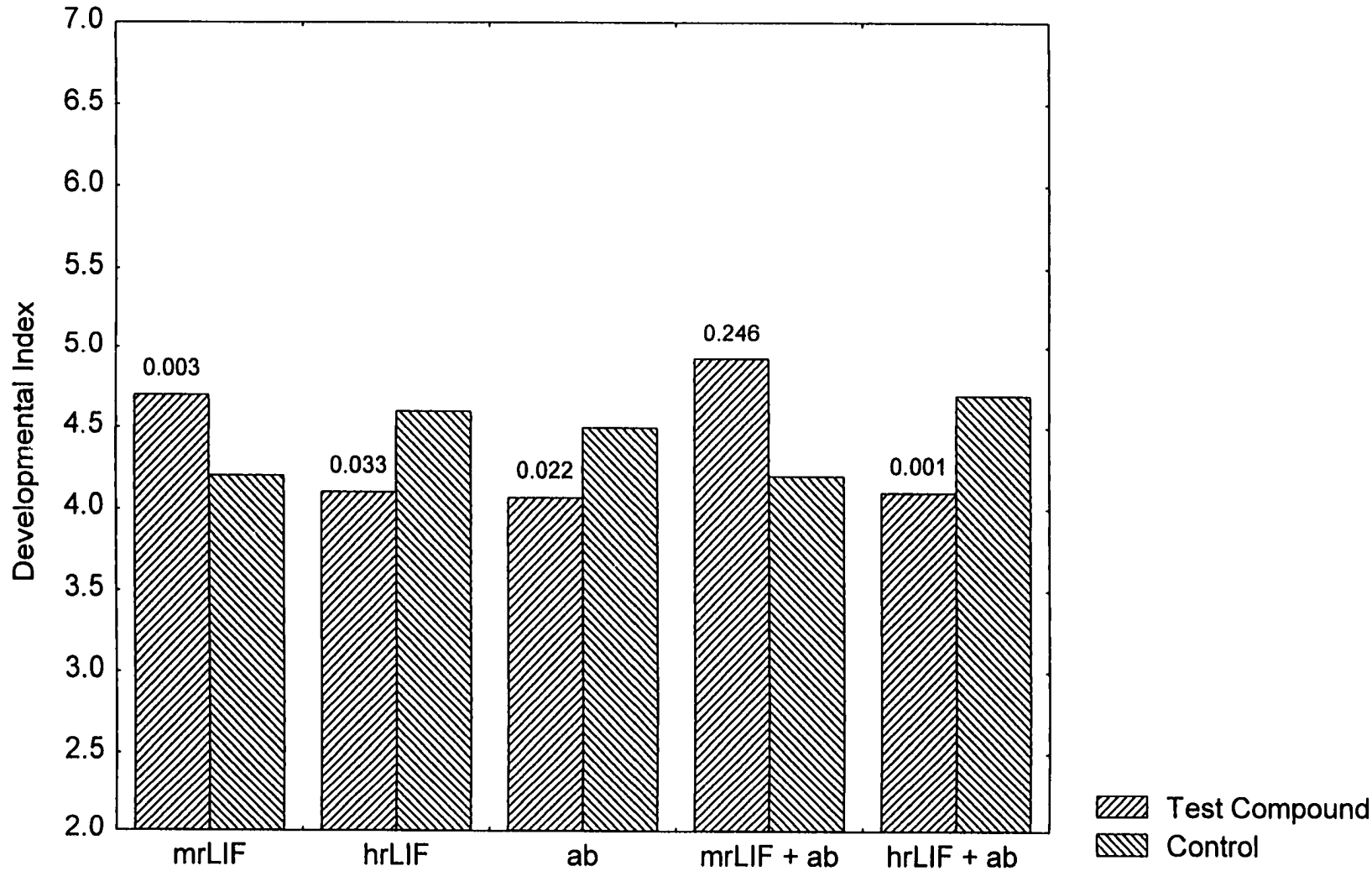


Figure 6. Category A, Experiments 2 - 6, Protocol 1, Day 4.
p values located above histograms for each group.

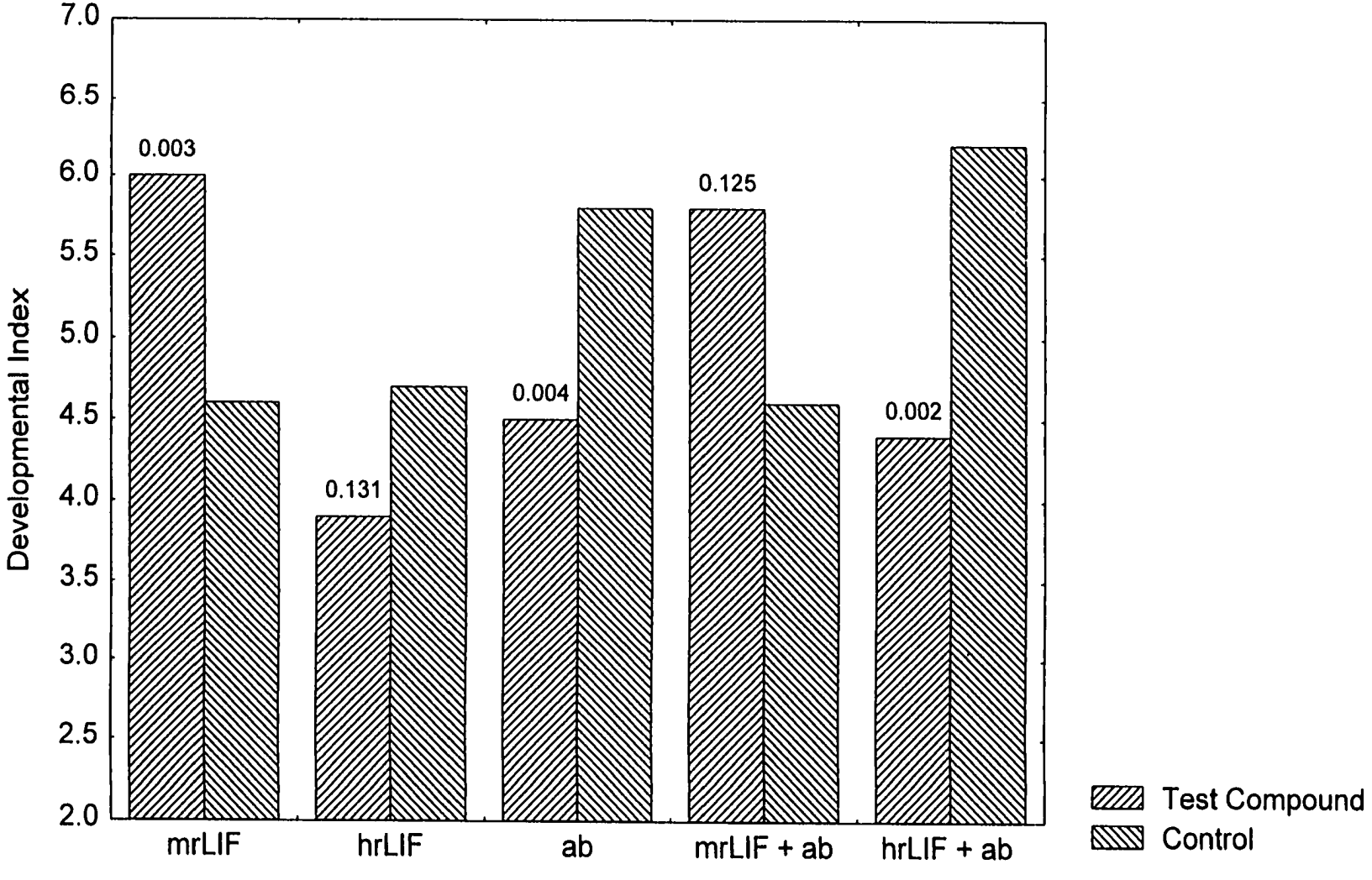


Figure 7. Category A, Experiments 2 - 6, Protocol 1, Day 5.
p values located above histograms for each group.

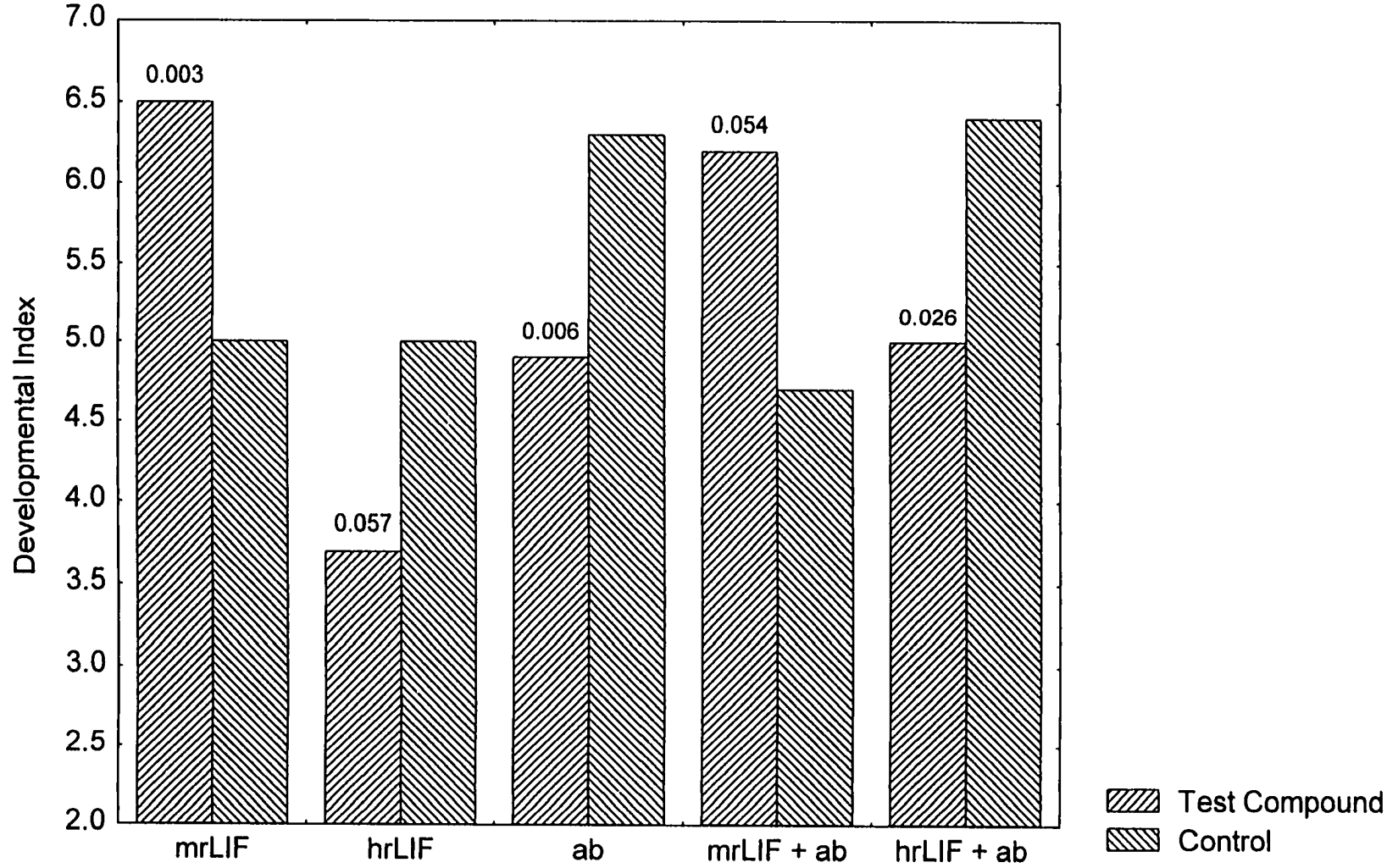


Figure 8. Category A, Experiments 2 - 6, Protocol 1, Day 6.
p values located above histograms for each group.

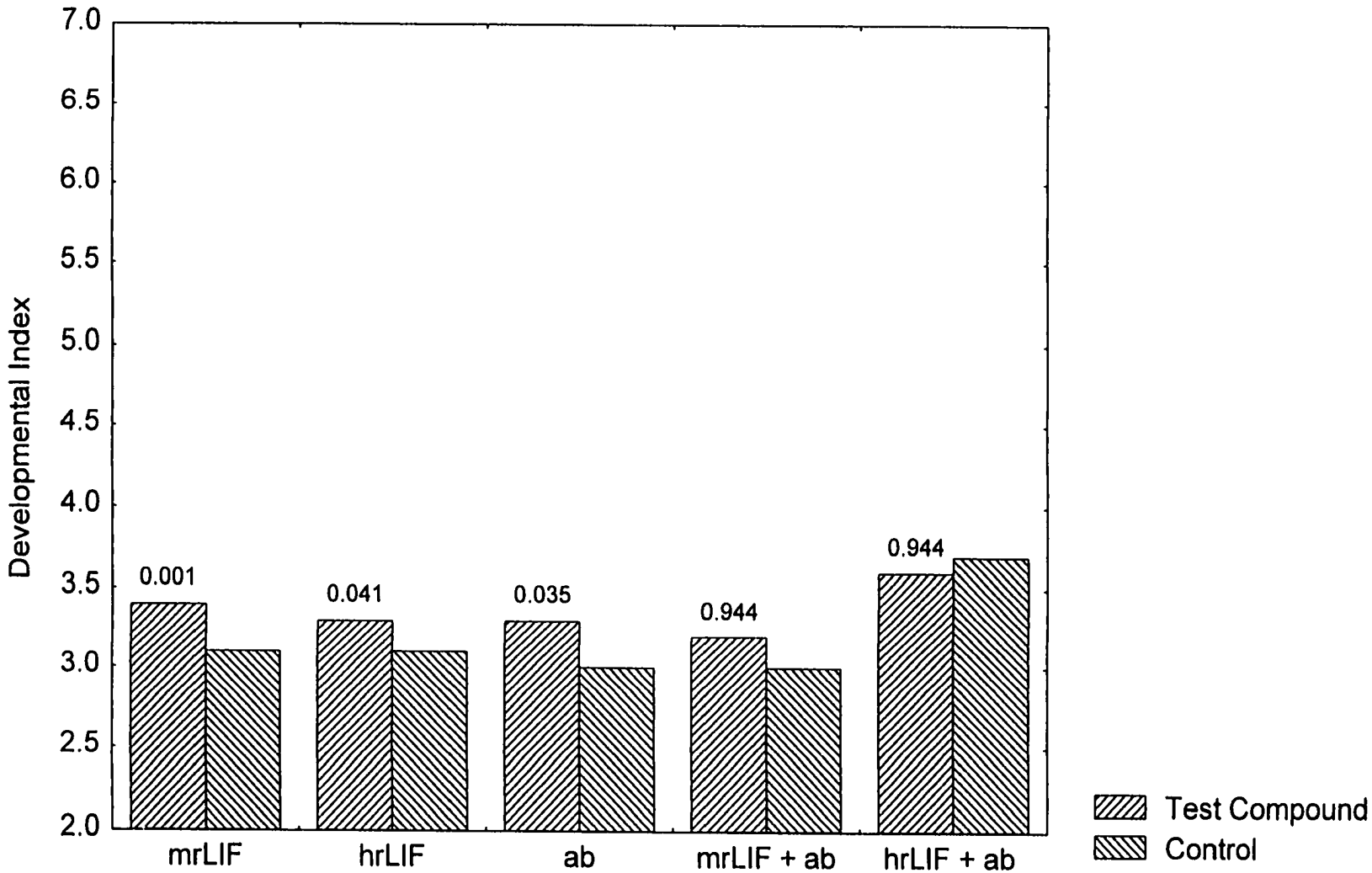


Figure 9. Category A, Experiments - 2 - 6, Protocol 2, Day 3.
p values located above histograms for each group.

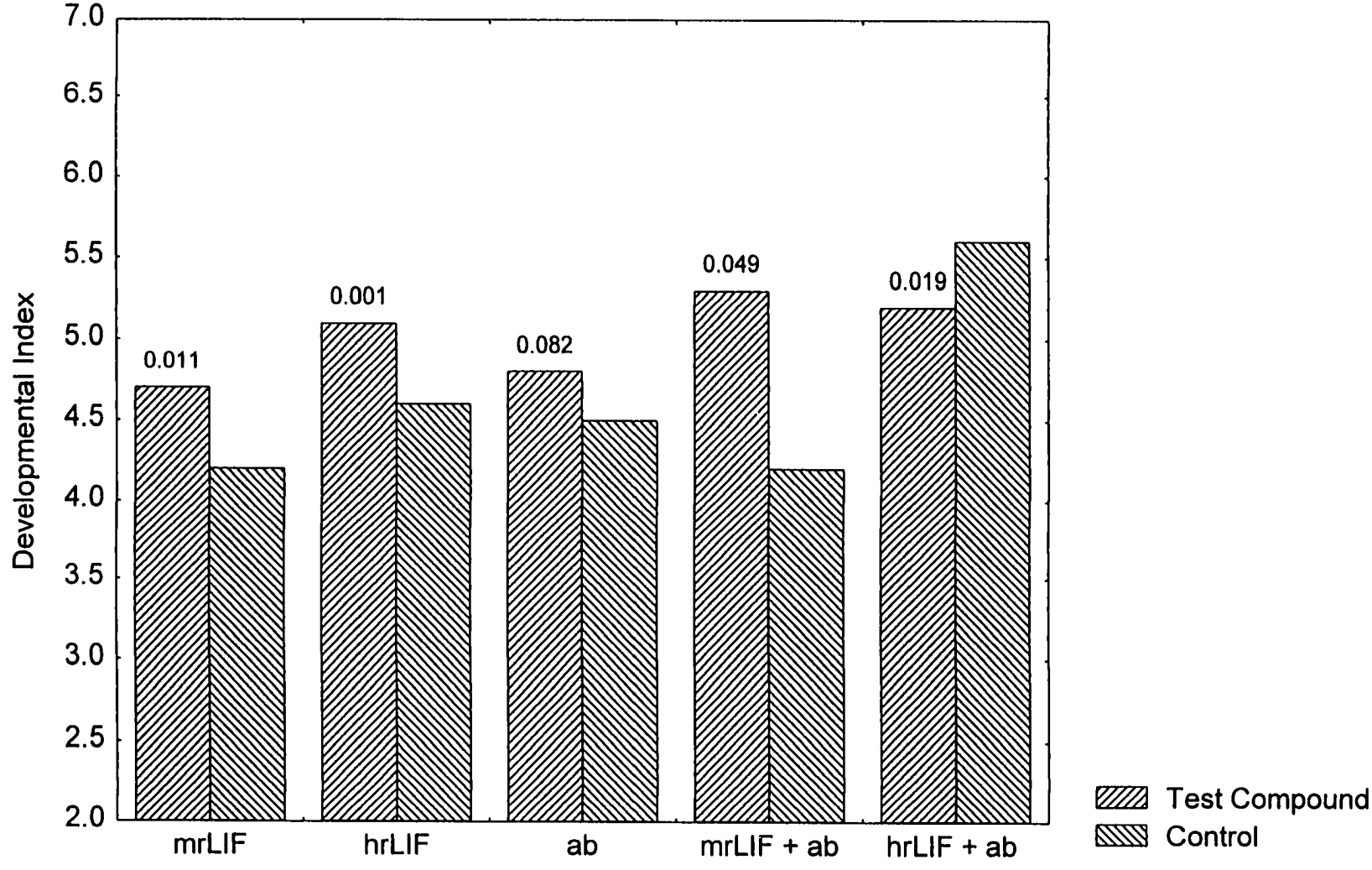


Figure 10. Category A, Experiments 2 - 6, Protocol 2, Day 4.
p values located above histograms for each group.

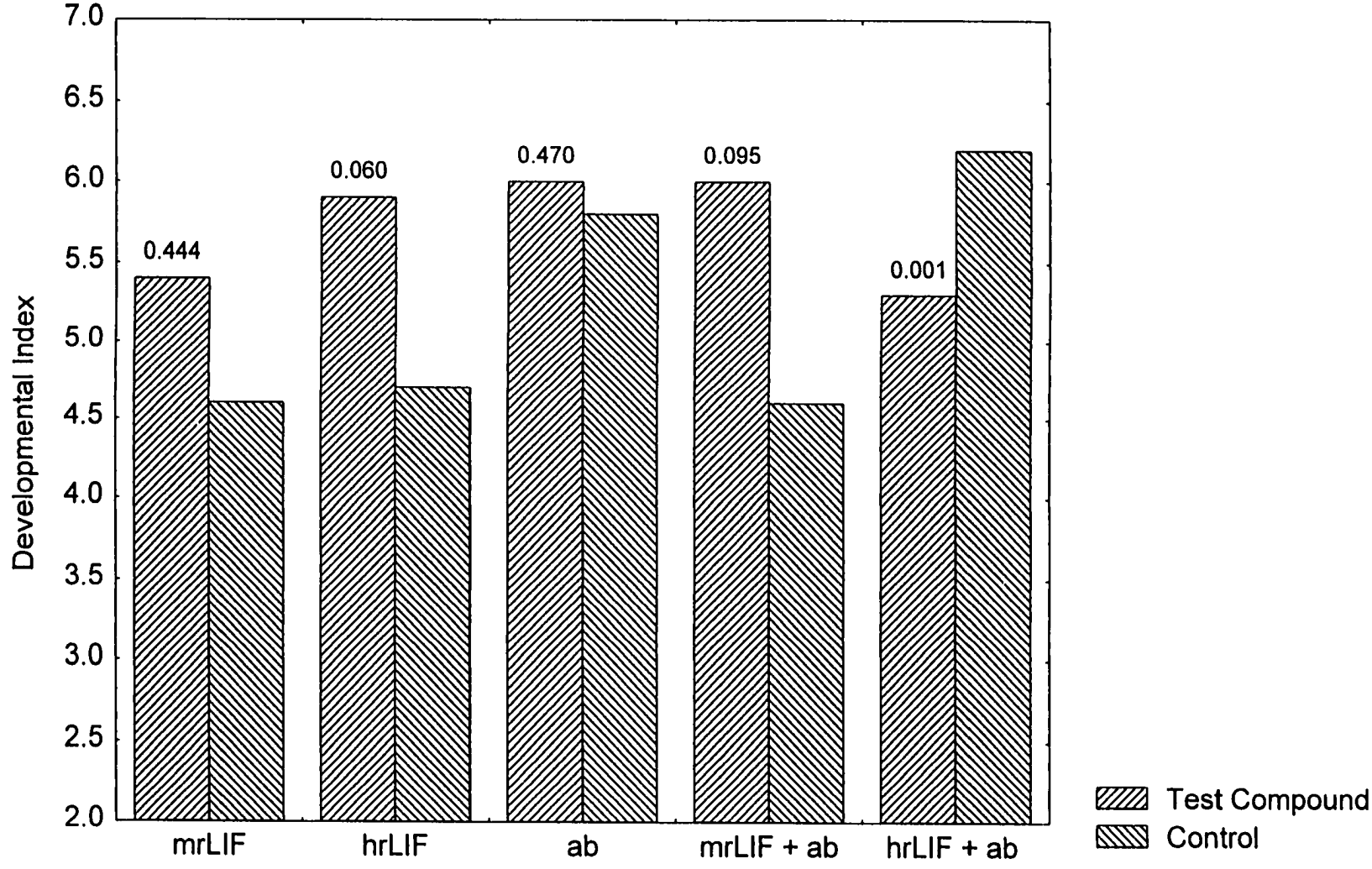


Figure 11. Category A, Experiments 2 - 6, Protocol 2, Day 5.
p values located above histograms for each group.

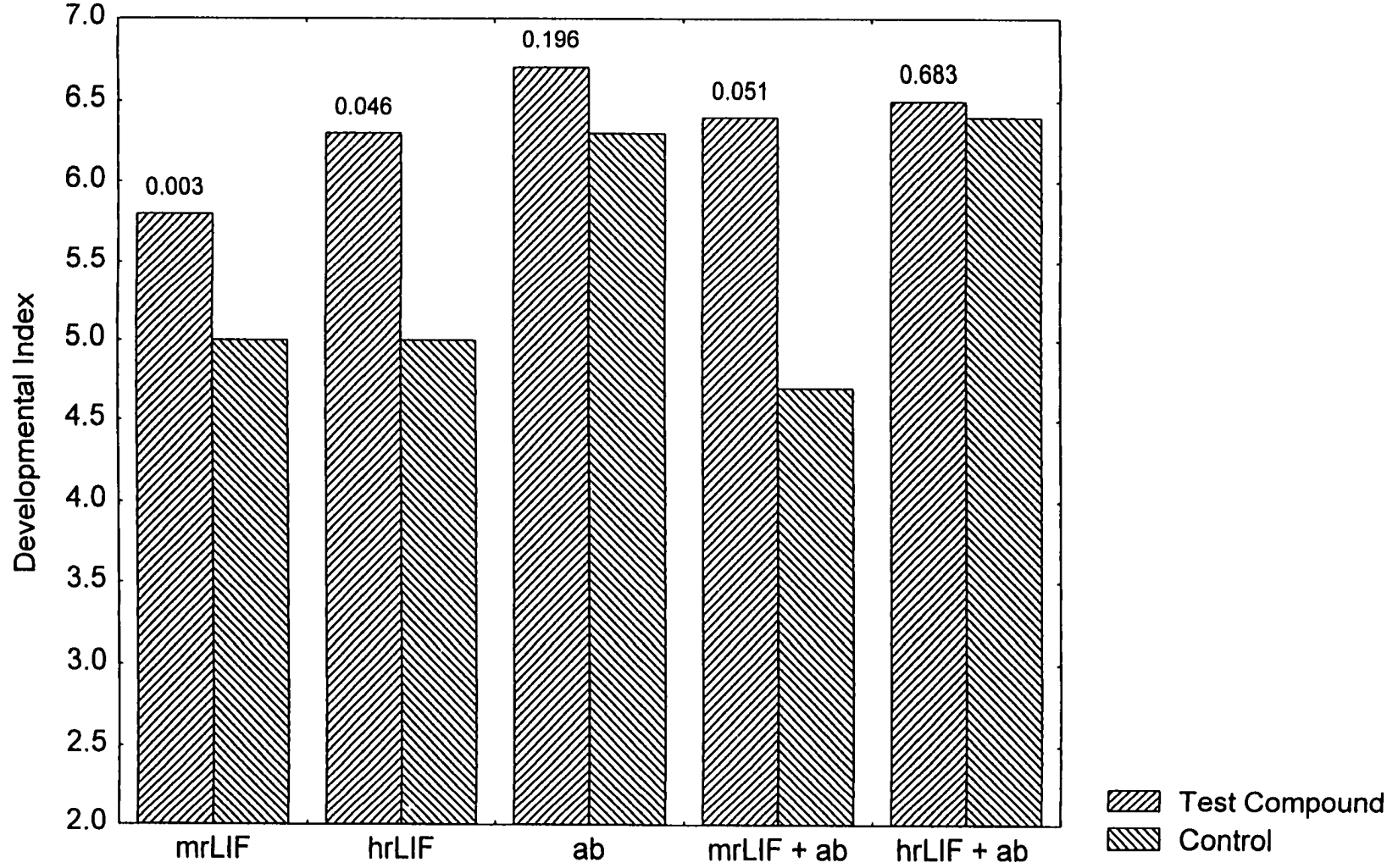


Figure 12. Category A, Experiments 2 - 6, Protocol 2, Day 6.
p values located above histograms for each group.

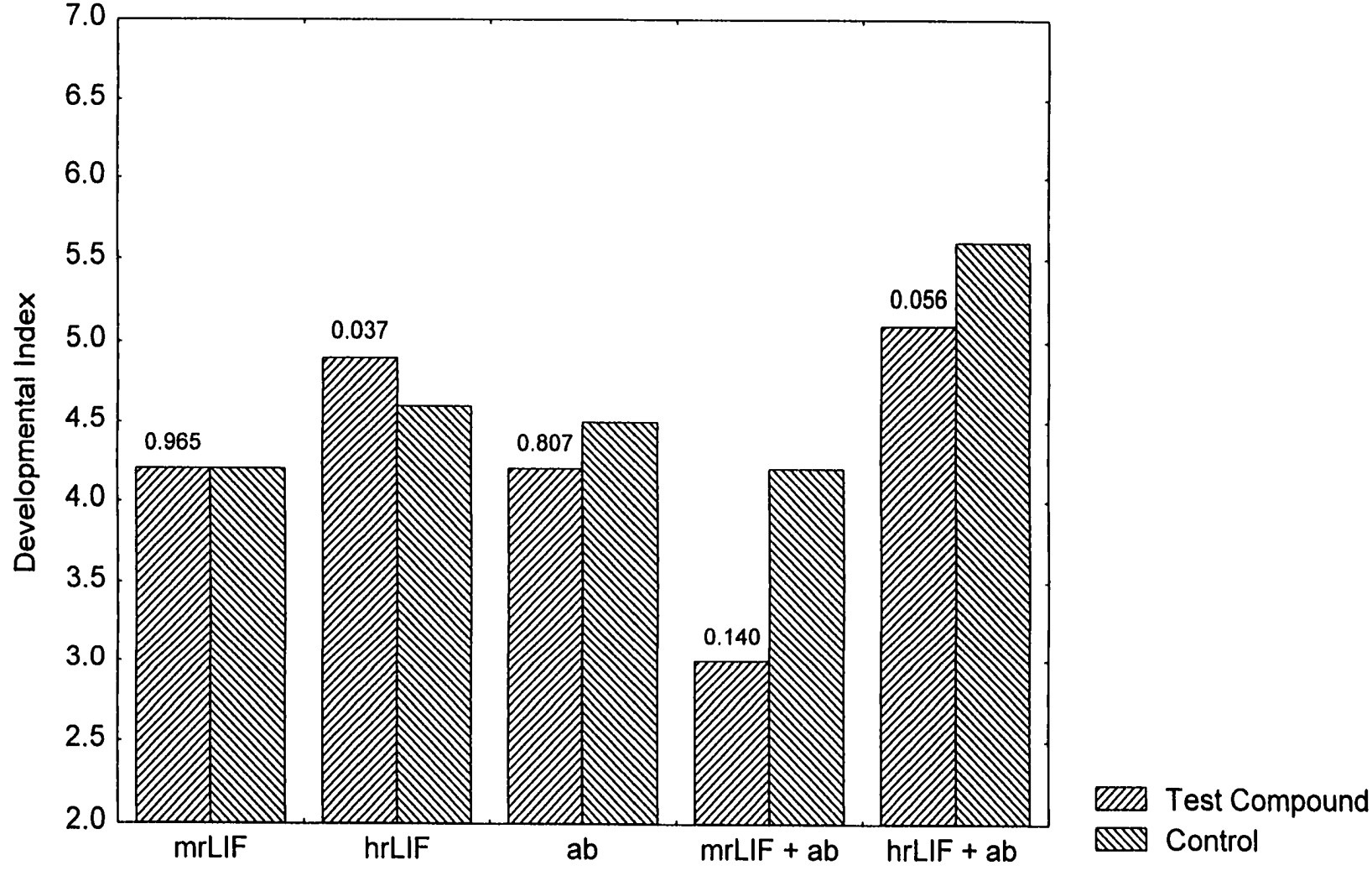


Figure 13. Category A, Experiments 2 - 6, Protocol 3, Day 4.
p values located above histograms for each group.

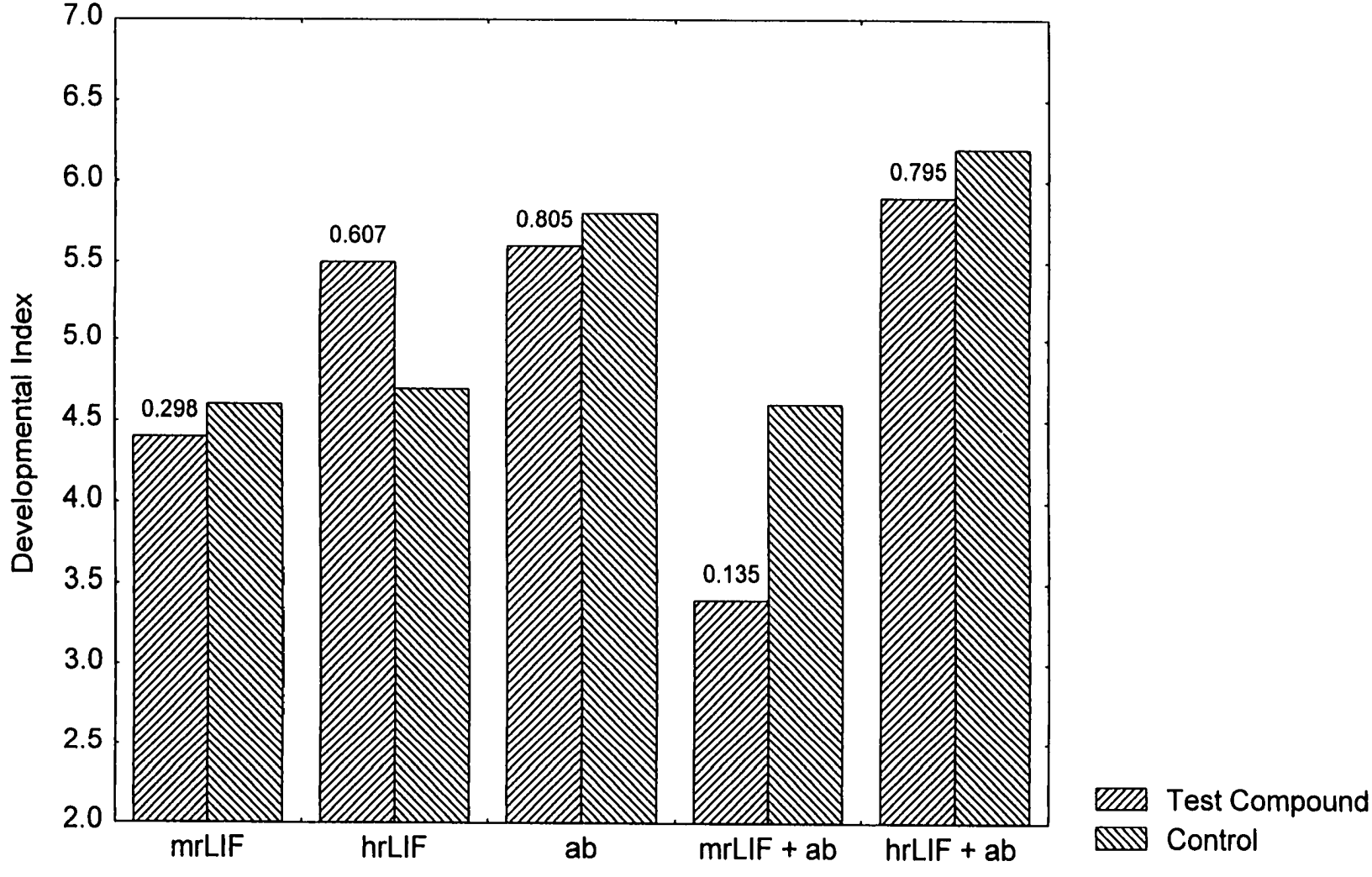


Figure 14. Category A, Experiments 2 - 6, Protocol 3, Day 5.
p values located above histograms for each group.

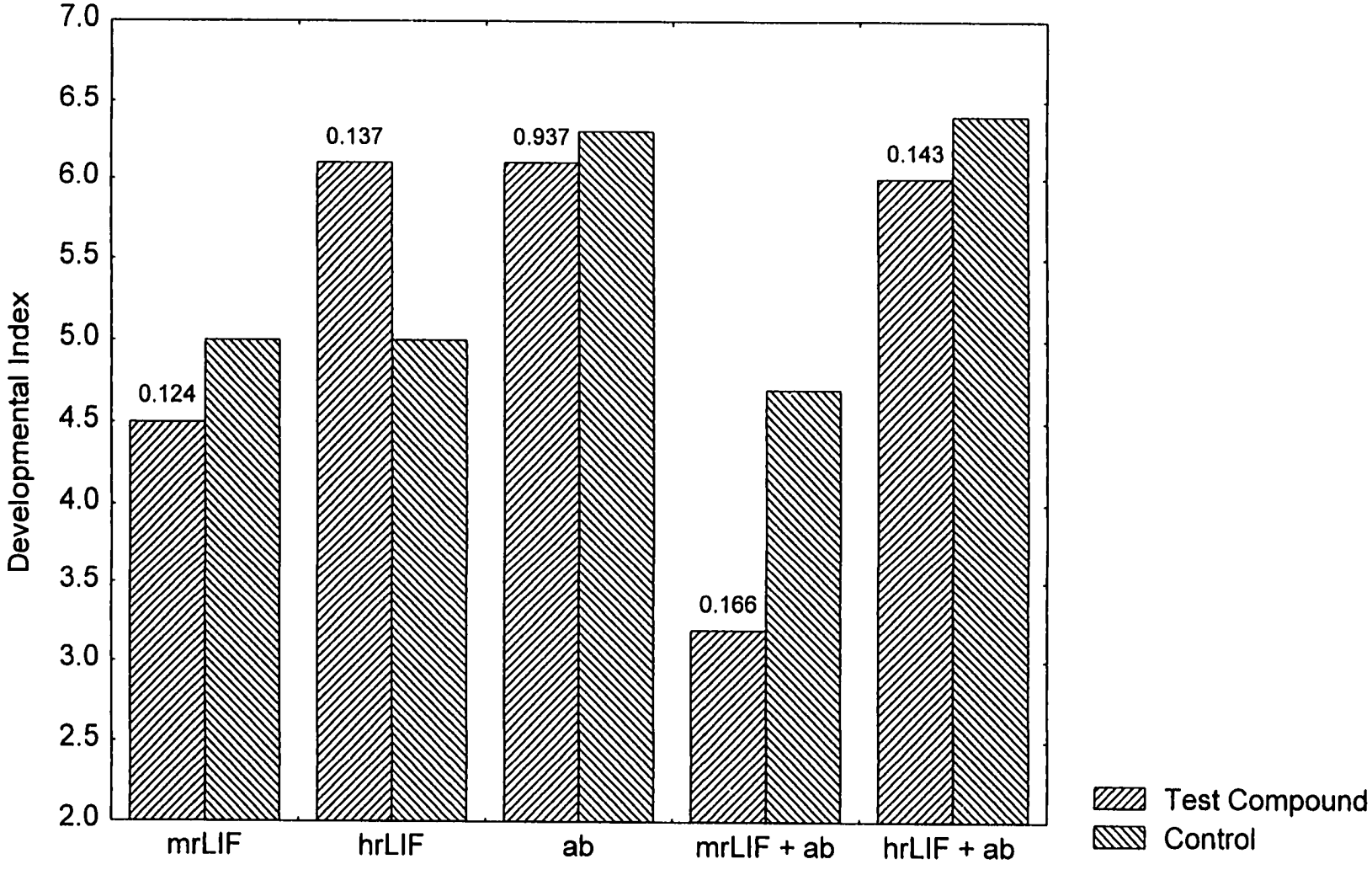


Figure 15. Category A, Experiments 2 - 6, Protocol 3, Day 6.
p values located above histograms for each group.

Category A, Experiment 7 - Dose Dependency Experiments

Experiment 7 investigated dose dependent effects of various concentrations of U/ml mrLIF on 2-cell embryo development. Table 8 provides the magnitude in differences for developmental indices for each test compound compared to its control as well as the direction for that change (i.e. increase or decrease in developmental indices). Figure 16 gives the results of these experiments and Appendix v provides data totals, percents for each developmental stage, and developmental indices for the individual developmental day for each protocol for experiment 7 with Appendix vi providing raw data. For these experiments, protocol 1 only was observed using embryos collected from CD_1 females mated with CD_1 males. When testing 1000, 2000, 5000, and 10,000 U/ml mrLIF, maximum embryo development was observed at the highest dose tested (10,000 U/ml) for each day when compared to controls. Significant increases in development were documented for developmental days 3 and 4 for all four levels of mrLIF. For developmental day 5, significant increases were observed for 1000 U/ml and 10000 U/ml mrLIF groups. For developmental day 6, only the 10000 U/m mrLIF group demonstrated a significant increase in development (Table 8, Figure 16).

Table 8
Category A, Experiments 7 - Dose Dependent Study.

	1000 U/ml (n=60)	2000 U/ml (n=60)	5000 U/ml (n=60)	10000 U/ml (n=60)
Devel Day 3	+ 0.5	+ 0.6	+ 0.7	+ 0.7
	p=0.005	p=0.001	p=0.001	p=0.001
Devel Day 4	+ 1.1	+ 1.2	+ 1.0	+ 1.5
	p=0.001	p=0.001	p=0.001	p=0.001
Devel Day 5	+ 0.7	+ 0.2	+ 0.3	+ 0.9
	p=0.007	p=0.615	p=0.105	p=0.034
Devel Day 6	+ 0.6	+ 0.3	- 0.2	+ 1.3
	p=0.110	p=0.277	p=0.798	p=0.002

Note. Developmental indices for embryos collected from CD_1 females mated with CD_1 males comparing various concentrations of mrLIF to controls in order to examine potential dose dependent responses. Upper numbers indicate the magnitude of increase (+) or decrease (-) in the developmental index. Lower numbers are p values derived from Cochran-Mantel-Haenszel (CMH) statistics.

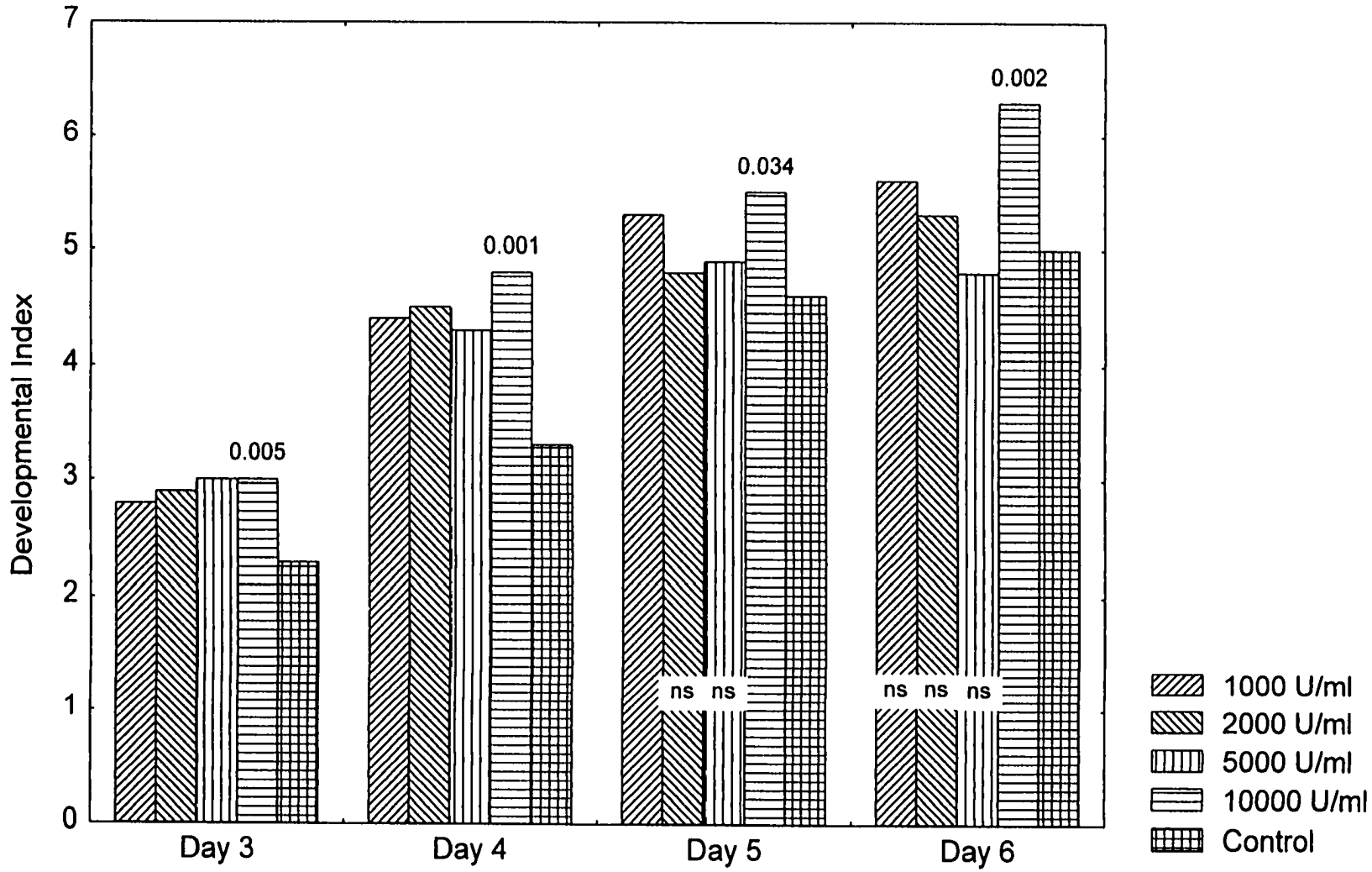


Figure 16. Category A, Experiment 7, Dose Dependent Study, Protocol 1 only. p values located above histograms for each group. * = non-significant.

Category B Experiments - Implantation, Pregnancy, and Resorption Rates

Implantation and Pregnancy rates for embryos derived from B6CBAF1/J, exposed to 5000 U/ml mrLIF, were approximately twice the rates for controls (62.96% and 55.56% compared to 33.33%, p = 0.0002 and 30.00%, p = 0.0001) respectively (Table 9, Figure 17). However, embryos in this group were also observed to have about a two-fold increase in resorption rates (51.85%) when compared to controls (30.00%, p = 0.011). Embryos exposed to the two lower concentrations of an anti-LIF monoclonal antibody (anti-mLIF mcab) reflected results similar to the LIF-treated embryos with and increase in implantation, pregnancy, and resorption rates as compared to controls. Significantly different results were observed when embryos were exposed to 5000 U/ml anti-mLIF mcab. For this group, decreases in implantation rates (15.38%) were reflected in both pregnancy (7.69%) and resorption (11.54%) rates as compared to controls (33.33%, p = 0.002; 30.00%, p = 0.001; and 30.00%, p= 0.011 respectively). No significant difference was observed when comparing females with resorption sites only (failed pregnancies with no viable pups) among the five dependent variables (p = 0.622). Two-by-five contingency tables were employed to investigate possible differences in the various rates (implantation, pregnancy, and resorption).

Multiple analysis of variance was utilized for fetal body mass, crown-rump length, tail length, placental length and width, and placental mass (Table10). The Wilks' lambda indicated significant differences for these dependent

variables (p = 0.0001). The individual ANOVA results of this analysis demonstrated a significant difference in body mass (p = 0.0002) and placental length (p = 0.0078). Tukey's studentized range (HSD) test located the differences in body mass to be greatest between the 5000 U/ml anti-mLIF mcab-treated group and the 5000 U/ml LIF-treated group compared to the 1000 U/ml and 2500 U/ml anti-LIF-treated groups respectively. For placental lengths, the greatest difference was observed between controls and 1000 U/ml anti-mLIF mcab-treated embryos. Raw data for category B experiments is provided in Appendix vii.

Table 9
Category B - Implantation, Pregnancy, and Resorptiojn Rates.

	C(n=30)	LIF(n=27)	10 (n=14)	25 (n=14)	50 (n=26)
Implantation Rates	33.33 (10)	62.96 (17)	50.00 (7)	66.67 (10)	15.38 (4)
Pregnancy Rates	30.00 (9)	55.56 (15)	50.00 (7)	66.67 (10)	7.69 (2)
Resorption Rates	30.00 (9)	51.85 (14)	21.43 (3)	13.33 (2)	11.54 (3)

Note. C= Control; 50 = 5000 U/ml mcab; 25 = 2500 U/ml mcab; 10 = 1000 U/ml mcab
n= # recipients/group
Implantation Rates: including resorption sites as well as fetuses determined to be morphologically normal (apparently viable) at day 17.
Pregnancy Rates: Those fetuses determined to be morphologically normal (apparently viable) at day 17 .

Table10
Catetory B - Fetal Lengths and Masses.

Treatment	Body M	C/R	TL	PL	PW	PM
Controls (n=21)	0.572	17.667	7.000	9.762	9.524	0.174
5000 U/ml LIF (n=43)	0.528	17.559	6.884	9.488	9.326	0.156
1000 U/ml mab (n=15)	0.629	17.333	7.400	8.533	9.000	0.154
2500 U/ml mab (n=19)	0.730	18.210	7.680	8.789	9.789	0.174
5000 U/ml mab (n=7)	0.450	16.570	6.860	9.714	9.000	0.200

Note. Linear parameters and masses for various test groups and controls. Category 2 Experiments: n = number of fetuses in each group. Body M = body mass, C/R = crown-rump length, TL = tail length, PL = placental length, PW = placental width, PM = placental mass.

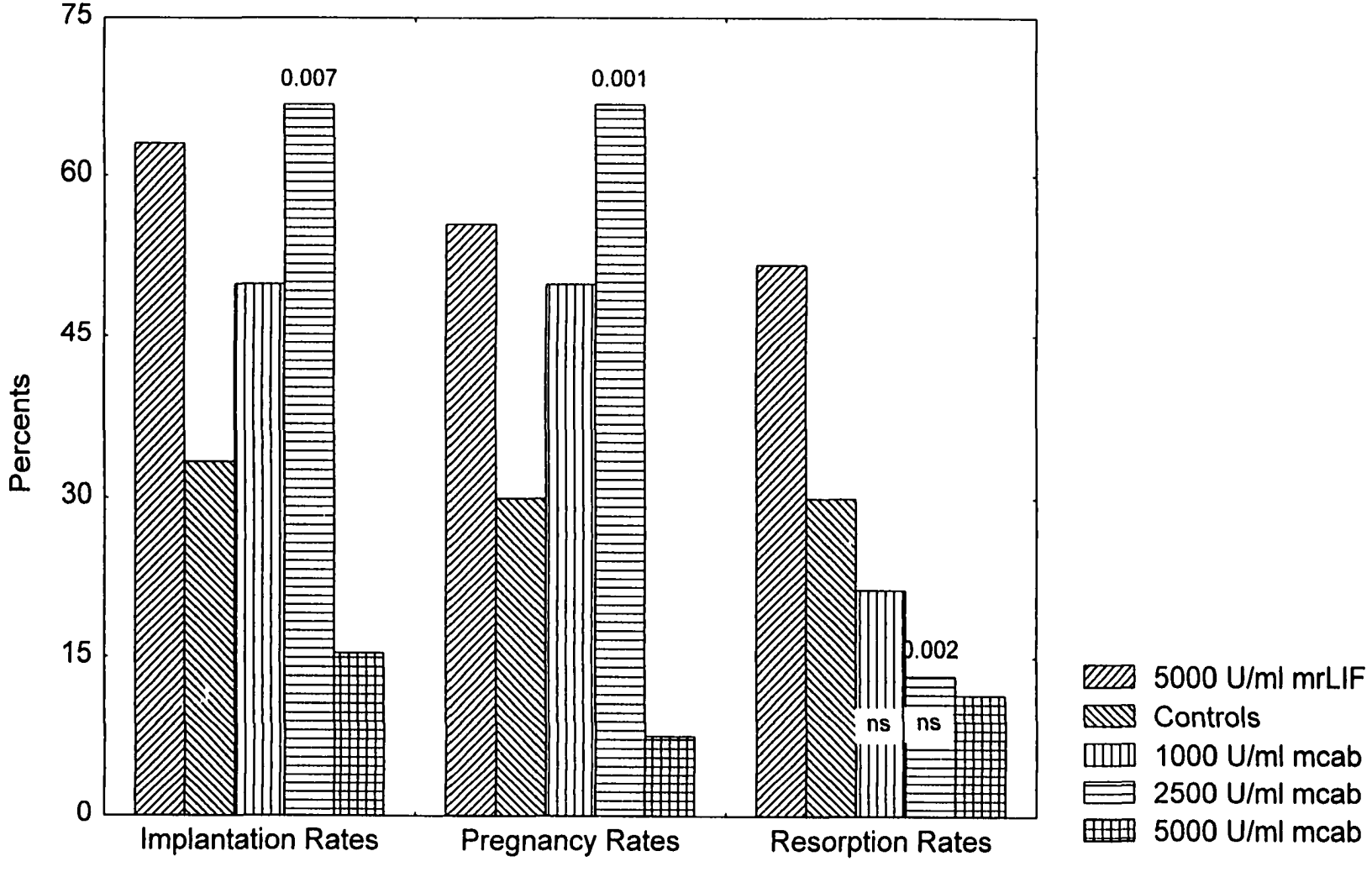


Figure 17. Category B Experiments, Implantation, Pregnancy, & Resorption Rates. p values located above histograms for each group. * = non-significant.

Category C Experiments - Murine Skeletal Development and LIF

Multiple analysis of variance demonstrated significant differences observed among the various dependent variables: humerus length ($p = 0.0027$), length of diaphyseal ossification in the humerus ($p = 0.0001$), width of space between the vertebral pedicles/laminae ($p = 0.0073$), approximate percentage of ossification in the exoccipital bone ($p = 0.0003$), length of ipsilateral scapula ($p = 0.0099$), and length of ossification center in scapula ($p = 0.0001$). Tukeys' studentized range (HSD) test was used to determine between which independent variables the differences existed (Table11, figure 18, raw data for category C experiments provided in Appendix viii). Figure 18 shows significant differences between the 1000 U/ml and 2500 U/ml anti-mLIF mcab groups compared to the 5000 U/ml LIF and controls which were significantly higher compared to 5000 U/ml anti-mLIF mcab group. In the latter group, significantly lower values were recorded for all variables except the space between the vertebral pedicles. Although not significantly different from controls, LIF-treated embryos demonstrated lower values for ossification and overall bone length when compared to the 1000 U/ml and 2500 U/ml anti-mLIF mcan groups with higher values compared to 5000 U/ml anti-mrLIF mcab. Tukey's studentized range (HSD) test demonstrated that these differences were observed for HLN, HOS, SLN, and SOS. For the estimated amount of ossification in the exoccipital bone (EX) a significant difference was observed between the 50 U/ml anti-mLIF mcan group and all the other independent variables. Finally, the greatest amount of difference for the

variable SOS was determined to exist between the 50 U/ml anti-mLIF mcan group and the 25 U/ml anti-mLIF mcan group.

LIF-treated fetuses were not significantly different when compared to controls for overall bone length and length of ossification centers. When controlling for fetal size by using an ossification index, significant differences were observed. This index was formulated by dividing the length of the ossification center in the bone (humerus and scapula) by the total length of the developing bone. Table 12 shows that when using this index, LIF-treated fetuses demonstrate significantly lower values for bone ossification/development when compared to controls ($p = 0.0001$). No significant differences were noted between the controls and the 1000 U/ml anti-mrLIF mcab-treated fetuses or the 2500 U/ml anti-mrLIF mcab-treated fetuses. Additionally, the fetuses treated with the 5000 U/ml anti-mrLIF mcab showed significantly lower values for skeletal development compared to the LIF-treated fetuses.

Table11
Category C - Skeletal Development.

Test Group	HLN	HOS	VSPC	EX	SLN	SOS
50 U/ml mcab (n=9)	2.848+	.587+	.946	46.70+	2.251+	.564+
LIF (n=42)	2.992	.891+	.818	82.75-	2.400	.911
CONTROLS (n=21)	3.058	1.052	.822	88.57-	2.401	.977
10 U/ml mcab (n=16)	3.277-	1.293-	1.027	90.00-	2.660-	1.211
25 U/ml mcab (n=19)	3.412-	1.293-	.960	94.44-	2.759-	1.280-
MANOVA p values	0.0027	0.0001	0.0073	0.0003	0.0099	0.0001

Note. C= Control; 50 = 5000 U/ml mcab; 25 = 2500 U/ml mcab; 10 = 1000 U/ml mcab n= # recipients/group "+" and "-" indicate groups, within columns that are statistically different from each other. HLN = total length of humerus, HOS = length of ossification center of humerus, VSPC = dorsal gap between vertebral pedicles/lamina, EX = estimated percent ossification of exoccipital bone, SLN = total length of scapula, SOS = length of ossification center of scapula.

Table12
Category C - Ossification Index.

Test Group	HO/HL	SO/SL
CONTROLS (n=21)	0.333a	0.403a
LIF (n=42)	0.296b	0.384b
1000 U/ml mcab (n=16)	0.386a	0.446a
2500 U/ml mcab (n=19)	0.386a	0.453a
5000 U/ml mcab (n=9)	0.206c	0.249c
MANOVA p values	0.0001	0.0001

Note. C= Control; n= # recipients/group. HO/HL = length of ossification center in the humerus divided by the total length of the humerus. SO/SL = length of ossification center in the scapula divided by the total length of the scapula. Groups with different letters following the ossification indices are significantly different.

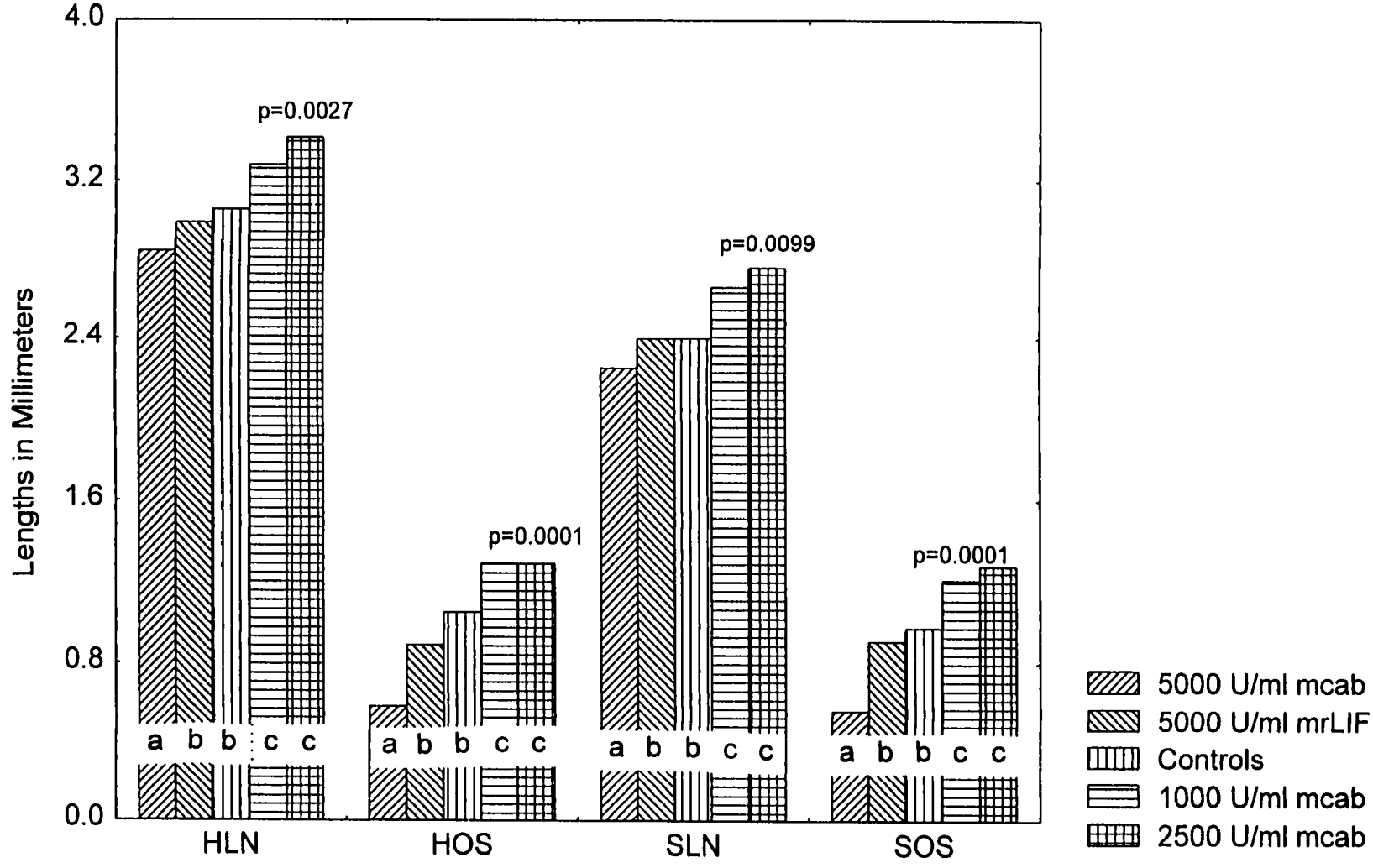


Figure 18. Category C Experiments, Skeletal Development.
Histograms with the same letter are not significantly different.

Discussion

Leukemia inhibitory factor (LIF) is a multifunctional (pleiotroic) cytokine that enhances in vitro murine pre-implantation embryo development with temporal and dose-dependent effects (Mitchell et al., 1994). It has been shown that LIF is essential for implantation in murine species (Stewart, et al., 1992).

This project shows that LIF has a significant effect on pre-implantation embryo development, both in a temporal and dose dependent manner. Additionally, LIF was shown to have a significant effect on implantation, pregnancy (i.e. successful pregnancy), and resorption rates as well as the skeletal development of the mouse fetus.

Category A, experiment 1 demonstrated the effect that LIF exerted on the pre-implantation development of 2-cell murine embryos to the hatched blastocyst stage. Different temporal protocols were shown to be optimal for embryos exposed to different test compounds. Protocol 1 was optimal for embryos exposed to 1000 U/ml mrLIF or a combination of the mrLIF plus the mcab. For mouse embryos exposed to mrLIF, the day 2 protocol demonstrated developmental indices significantly less than those for protocol 1 and significantly higher than those for protocol 3. This data indicates that these embryos will develop best when exposed to recombinant mLIF at the time of 2-cell embryo collection. Additionally, it appears that the longer these embryos culture without being exposed to rmLIF, the lower their developmental potentials will be.

Protocol 2 was optimal for development of embryos exposed to hrLIF, the mcab alone, and a combination of the hrLIF plus the mcab (table 6). Therefore, murine embryos from female B_6CBAF_1/J mice obtain the highest developmental index when exposed to mrLIF immediately after their collection from the fallopian tubes. However, if these same embryos are exposed to hrLIF, they tend to develop further if allowed to culture as controls for 24 hours prior to exposure to hrLIF (protocol 2). This is not surprising considering the fact that hrLIF is a non-native xenobiotic cytokine for the murine species.

Investigated in category A, experiments 2-6, was the effect that 1000 U/ml mrLIF, hrLIF, the anti-hLIF monoclonal antibody alone as well as in combination with the two previous test compounds, had on the development of 2-cell embryos (table 7, figures 5 through 15). Supplementing culture medium with mrLIF (sub-category 2) exerted a stimulatory effect on development for embryos derived from B_6CBAF_1/J female mice, whereas, supplementation with hrLIF exerted an inhibitory effect, significant on day four and six. The exception for the stimulatory effect from mrLIF was noted for protocol 3. Additionally, hrLIF exerted a stimulatory effect during protocol 2 with significance on days 3, 4, and 6 and protocol 3 with significance on day 3. There is evidence of the presence of low affinity and high affinity binding. It is possible that high affinity binding to receptors is acquired by the embryo by day 3 and this high affinity binding is sufficient to bind enough hrLIF to cell membrane receptors to allow some positive effect. No evidence to this effect has been demonstrated. It is possible

that by the third day of development a window for the positive effect of mrLIF-supplemented medium has passed, however, this is purely speculation and requires further investigation. Even though 78% homology has been described between LIF derived from these two species (Gough et al., 1988), it is not surprising that xenobiotics, such as hrLIF in the mouse, do not exert as much of a stimulatory effect as endogenous cytokines, and as in this case, and they produce significant inhibitory effects. The anti-hrLIF monoclonal antibody demonstrated an inhibitory effect for protocol 1, a slightly stimulatory, however, predominantly non-significant effect or protocol 2, and a non-significant inhibition for protocol 3. These results suggest that native mLIF (produced by the test animals) may be associated with the anti-human rLIF mcab to induce a negative effect during the first day of development when compared to controls. In light of the fact that the mcab alone was inclined to exert an inhibitory effect on pre-implantation development, a surprising result was the fact that a stimulatory effect was observed in sub-category 5 (mrLIF plus anti-hrLIF mcab). If binding of this anti-human rLIF mcab to native and recombinant mLIF is weak, and if the stimulatory effect of mrLIF is strong, it is possible that the significant stimulatory effect from the mrLIF is great enough to override the non-significant inhibitory effect seen by the mcab alone. The inhibitory effect of the combined hrLIF plus mcab for each protocol may reflect the antibodies ability to bind to its ligand, the hrLIF, with a high enough affinity to reduce the stimulatory effect of hrLIF during protocol 2 and 3.

LIF transcripts hav been detected in 3.5 post-coital mouse embryo and transcripts for the receptor have been observed in the 4-day-old embryos (Conquet and Brulet, 1990; Murray et al., 1990). Additionally, LIF was shown to be produced by the mouse uterine endometrial glands on day 4 of pregnancy (Bhatt et al., 1991). These authors and others have demonstrated that maximum LIF release from endometrial glands corresponds to implantation (Bhatt et al., 1991; Conquet and Brulet, 1990; Shen and Leder, 1992). The results from these and the present project suggest a physiological role for LIF in the process of implantation. The present study demonstrates definitive embryo trophic effects of mrLIF, hrLIF, the anti-human rLIF mcab and combinations of these on the development of 2-cell mouse embryos.

Category A, experiment 7, investigated possible dose dependent effects for 2-cell embryos, derived from CD_1 females mated with CD_1 males exposed to mrLIF. Compared were concentrations of 1000 U/ml, 2000 U/ml, 5000 U/ml, and 10000 U/ml with controls. For sub-category 7 experiments, only protocol 1 was utilized. For each of developmental days 3 - 6, test groups demonstrated significantly higher developmental indices when compared to controls (table 8, 10, figure 16). Additionally, the highest concentration examined (10000 U/ml) resulted in the highest developmental indices for each day suggesting that supplementing medium with supra-physiological levels of mrLIF enhances embryo development to the blastocyst and hatching/hatched blastocyst stages.

However, as will be discussed below, unfavorable side effects may develop from using such supra-physiological levels.

It has been shown that LIF plays a critical role in murine reproduction. LIF has been shown to be an absolute requirement for implantation in murine species (Stewart, et al., 1992). Although its precise function in humans is unknown, its therapeutic potential in IVF clinics merits investigation. In vitro experiments indicate that 1000 U/ml LIF are required to maintain undifferentiated embryonal stem (ES) cells (Williams et al., 1988). In our lab we have demonstrated that LIF has a pronounced effect on implantation and resorption in the mouse. These experiments demonstrate that transfer medium, supplemented with LIF increases the implantation rate of both healthy or viable embryos as well as those of "poorer quality" that may not otherwise survive to term as demonstrated by almost parallel increases in both successful pregnancy rates and resorption rates (fialed pregnancies). Further studies are needed to determine if LIF will increase the risk of resorption or spontaneous abortion (miscarriage) in primates such as humans.

The lower levels of anti-mLIF mcab actually increase rather than decrease implantation rates (table 9). These lower levels of mcab may not be sufficient to block all LIF-receptor binding. Additionally, lower levels of the mcab may be binding to other, inhibitory proteins, in a non-specific manner. More work needs to be conducted to determine the mechanism of this effect. Significant

reductions in implantation rates are seen when transfer medium is supplemented with 5000 U/ml anti-mrLIF mcab.

LIF has been shown to increase the resorption of bone (Abe et al., 1988; Ishimi et al., 1992; Reid, et al., 1990). High levels have been associated with pathological conditions such as cachexia, tissue calcification, pancreatitis, gonadal and thymus anomalies (Metcalf and Gearing, 1989). Malaval and associates demonstrated the ability of LIF to inhibit bone nodule formation in fetal rat calvaria cell cultures (Malaval, Gupta, and Aubin, 1995). Additionally, LIF was observed to inhibit growth and mineralization of early fetal mouse long bone cultures (Van Beek et al., 1993).

For the present study we investigated the effects of a single exposure of LIF on the skeletal development of mice in vivo. These data show that LIF does not significantly inhibit the development of the overall bone length (cartilagenous template). However, LIF has a significantly inhibitory effect on the ratio of ossification to overall bone length (Table 12). This indicates that supplementing embryo transfer medium with supra-physiological levels of LIF may significantly impair skeletal development. Further investigation is warranted to determine the effect that various concentrations of LIF-supplemented transfer medium may have on mouse as well as human embryos.

In conclusion, mrLIF has a significant positive embryotrphic effect. It increases the number of embryos developing to the hatched blastocyst stage. LIF has been shown to have a pronounced effect on implantation. It increases

the implantation of both healthy embryos as well as those that may not otherwise survive until term. Finally, LIF exerts an inhibitory effect on the process of ossification in developing bone. More studies are warranted before LIF or its monoclonal antibody should be used in the clinical setting.

REFERENCES

Abe, E., Tanaka, H., Ishimi, Y., Miyaura, C., Hayashi, T., Nagasawa, H., Tomida, M., Yamaguchi, Y., Hozumi, M., and Suda, T. (1986). Differentiation-inducing factor purified from conditioned medium of mitogen-treated spleen cell cultures stimulates bone resorption. *Proceedings of the National Academy of Science, USA. 83*, 5958-5962.

Abe, T., Murakami, M., Sato, T., Kajiki, M., Ohno, M., and Kodaira, R. (1989). Macrophage differentiation inducing factor from human monocytic cells is equivalent to murine leukemia inhibitory factor. *Journal of Biological Chemistry. 264*, 8941-8945.

Abe, E. Ishimi, Y., Takahashi, N., Akatsu, T., Ozawa, H., Yamana, H., Yoshiki, S., and Suda, T. (1988). A differentiation-inducing factor produced by the osteoblastic cell line MC3T3-E1 stimulates bone resorption by promoting osteoclast formation. *Journal of Bone and Mineral Research. 3*, 635-645.

Ackerman, S. B., Swanson, R. J., Adams, P. J. and Wortham, J. W. E. (1983). Comparison of strains and culture media used for mouse in vitro fertilization. *Gamete Research. 7*, 103-109.

Adler, R., Landa, K. B., Manthorpe, M,. and Varon, S. (1979). Cholinergic neuronotrophic factors: Intraocular distribution of trophic activity for ciliary neurons. *Science. 204*, 1434-1436.

Akita, S., Webster, J., Ren, S-G., Takino, H., Said, J., Zand, O., and Melmed, S. (1995). Human and murine pituitary expression of leukemia inhibitory factor: Novel intrapituitary regulation of adrenocorticotropin hormone synthesis and secretion. *Journal of Clinical Investigation. 2565*, 1288-1298.

Allan, E. H., Hilton, D. J., Brown, M. A., Evely, R. S., Yumita, S., Metcalf, D., Gough, N. M., Ng, K. W., Nicola, N. A., and Martin, T. J. (1990). Osteoblast display receptors for and responses to leukemia-inhibitory factor. *Journal of Cellular Physiology. 145*, 110-119.

Anegon, I., Moreau, J-F., Godard, A., Jacques, Y., Peyrat, M-A., Hallet, M-M., Wong, G., and Soulillou, J. P. (1990). Production of human interleukin for DA cells (HILDA)/leukemia inhibitory factor(LIF) by activated monocytes. *Cellular Immunology. 130*, 50-65.

Anegon, I., Cyturi, M. C., Godard, A., Moreau, M., Terqui, M., Martinat-Botte, F., and Soulillou, J. P. (1994). Presence of leukemia inhbibitory factor and interleukin-6 in porcine uterine secretions prior to conceptus attachment. *Cytokine. 6*, 493-499.

Arici, A., Engin, O., Attar, E., and Olive, D. L. (1995). Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. *Journal of Clinical Endocrinology. 80*, 1908-1915.

Axelrod, H. R. (1985). Altered trophoblast functions in implantation-defective mouse embryos. *Developmental Biology. 108*, 185-190.

Bard, J. B., and Ross, A. S. A. (1991). LIF, the ES-cell inhibition factor, reversibly blocks nephrogenesis in cultured mouse kidney rudiments. *Journal of Cellular Biology. 113*, 193-198.

Baumann, H., Hill, R. E., Sauder, D. N., and Jahreis, G. P. (1986). Regulation of major acute-phase plasma proteins by hepatocyte-stimulating factors of human squamous carcinoma cells. *Journal of Cellular Biology. 102*, 370-383.

Baumann, H., and Schendel, P. (1991). Interleukin-11 regulates the hepatic expression of the same plasma protein genes as interleukin-6. *Journal of Biological Chemistry. 266*, 20424-20427.

Baumann, H., Won, K-A., and Jahreis, G. P. (1989). Human hepatocyte-stimulating factor-III and interleukin-6 are structurally and immunologically distinct but regulate the production of the same acture phase plasma proteins. *Journal of Biological Chemistry. 264*, 8046-8051.

Baumann, H., Jahreis, G. P., Sauder, D. N., and Koj, A. (1984). Human keratinocytes and monocytes release factors which regulate the synthesis of major acute phase plasma proteins in hepatic cells. *Journal of Biological Chemistry. 259*, 7331-7342.

Baumann, H., Onorato, V., Gauldie, J., and Jahreis, G. P. (1987). Distinct sets of acute phase plasma proteins are stimulated by separate human hepatocyte-stimulating factors and monokines in rat hepatoma cells. *Journal of Biological Chemistry. 262*, 9756-9768.

Baumann, H., and Wong, G. G. (1989). Hepatocyte-stimulating factor III shares structural and functional identity with leukemia-inhibitory factor. *Journal of Immunology. 143*, 1163-1167.

Beas, F., and Flores, H. (1969). Characterization and properties of a new placental protein. *Nature. 221*, 574.

Beckmann, A. M., Matsumoto, I., and Wilce, P. A. (1997). AP-1 and Egr DNA-binding activities are increased in rat brain duirng ethanol withdrawal. *Journal of Neurochemistry. 69*, 306-314.

Berke, G. (1993). The functions and mechanisms of action of cytolytic lymphocytes. In William E. Paul. (Ed.), Fundamental immunology (pp 965-1014). New York. Raven Press.

Beutler, B. A., and Cerami, A. (1985). Recombinant interleukin-1 suppresses lipoprotein lipase activity in 3T3-L1 cells. *Journal of Immunology. 135*, 3969-3971.

Bhatt, H., Brunet, L. J., and Stewart, C. L. (1991). Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proceedings of the National Academy of Science, USA. 88*, 11408-11412.

Bishop, J. M. (1987). The molecular genetics of cancer. *Science. 235*, 305-311.

Boulton, T. G., Stahl, N., and Yancopoulos, G. D. (1994). Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin-6/oncostatin M family of Cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other Cytokines and *Growth Factors. Journal of Biological Chemistry. 269*, 11648-11655

Bousquet, C., Ray, D. W., and Melmed, S. (1997). A common pro-opiomelanocortin-binding element mediates leukemia inhibitory factor and corticotropin-releasing hormone Transcriptional Synergy. *Journal of Biological Chemistry. 272*, 10551-10557.

Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature. 309*, 255-256.

Charnock-Jones, D. S., Sharkey, A. M., Fenwick, P., and Smith, S. K. (1994). Leukaemia inhibitory factor mrna concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *Journal of Reproduction and Fertility. 101*, 421-426.

Chauhan, D., Kharbanda, S. M., Ogata, A., Urashima, M., Frank, D., Malik, N., Kufe, D. W., and Anderson, K. C. (1995). Oncostatin M induces association of Grb2 with Janus kinase JAK2 in multiple myeloma cells. *Journal of Experimental Medicine. 182*, 1801-1806.

Chen, D-B., Hilsenrath, R., Yang, Z.-M., Le, S-P., Kim, R., Chuong, C. J., Poindexter, A. N. III, and Harper, M. J. K. (1995). Leukaemia inhibitory factor in human endometrium during the menstrual cytcle: Cellular origin and action on production of glandular epithelial cell prostaglandin *in vitro*. *Human Reproduction*. *10*, 911-918.

Cheng, L., Gearing, D. P., White, L. S., Compton, D. L., Schooley, K., and Donovan, P. J. (1994). Roll of leukemia inhibitory factor and its receptor in mouse primordial germ cell growth. *Development*. *120*, 3145-3153.

Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988). The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive gene. *Cell*. *54*, 541-552.

Conover, J. C., Ip, N. Y., Poueymirou, W. T., Bates, B., Goldfarb, M. P., DeChiara, T. M., and Yancopoulos, D. (1993). Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. *Development*. *119*, 559-565.

Conquet, F., Peyrieras, N., Tiret, L., and Brulet, P. (1992). Inhibited gastrulation in mouse embryos overexpressing the leukemia inhibitory factor. *Proceedings of the National Academy of Science, USA*. *89*, 8195-8199.

Conquet, F., and Brulet, P. (1990). Developmental expression of myeloid leukemia inhibitory factor gene in preimplantation blastocysts and in extraembryonic tissue of mouse embryos. *Mollecular and Cellular Biology*. *10*, 3801-3805.

Cornish, J., Callon, K., King, A., Edgar, S., and Reid, I. R. (1993). The effect of leukemia inhibitory factor on bone in vivo. *Endocrinology*. *132*, 1359-1366.

Cosman, D., Lyman, S. D., Idzerda, R. L., Beckmann, M. P., Park, L. S., Goodwin, R. G. , and March, C. J. (1991). A new Cytokine receptor superfamily. *Trends in Biochemical Sciences* *15*, 265-270.

Cullinan, E. B., Abbondanzo, S. J., Anderson, P. S., Pollard, J. W., Lessey, B A., and Stewart, C. L. (1996). Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. *Proceedings of the National Academy of Science, USA*. *93,* 3115-3120.

Daniel, J. C. Jr. (1970). Dormant embryos of mammals. *BioScience*. *20*, 411 - 415.

Dano, K., Andreasen, P. A., Grondakl-Hansen, J., Kristensen, P., Nielsen, L. S., and Skriver, L. (1985). Plasminogen activators, tissue degradation, and cancer. *Advances in Cancer Researchearch*. *44*, 139-266.

Darnell, J. E. Jr., Kerr, I. M., and Stark, G. R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*. *264*, 1415-1416.

Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y., and Yancopoulos, G. D. (1993). LIFR-β and gp 130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science*. *260*, 1805-1808.

De Felici, M., and Dolci, S. (1991). Leukemia inhibitory factor sustains the survival of mouse primordial germ cells cultured on TM_4 feeder layers. *Developmental Biology*. *147*, 281-284.

De Miguel, M. P., De Boer-Brouwer, M., Paniagua, R., Van Den Hurk, R., De Rooij, D. G., and Van Dissel-Emiliani, F. M. F. (1996). Leukemia inhibitory factor and ciliary neurotropic factor promote the survival of sertoli cells and gonocytes in a coculture system. *Endocrinology*. *137*, 1885-1893.

Delage, G., Moreau, J -F., Taupin, J.-L., Freitas, S., Hambartsoumian, E., Olivennes, F., Fanchin, R., Letur-Konirsch, H., Frydman, R., and Chaouat, G. (1995). In-vitro endometrial secretion of human interleukin for DA cells/leukaemia inhibitory factor by explant cultures from fertile and infertile women. *Human Reproduction* *10*, 2483-2488.

Dunglison, G. F., Barlow, D. H., and Sargent, I. L. (1996). Leukaemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium. *Human Reproduction*. *11*(1), 191-196.

Ernst, M., D. P. Gearing, and A. R. Dunn. (1994). Functional and biochemical association of Hck with the LIF/IL-6 receptor signal transducing subunit gp130 in embryonic stem cells. *Journal of the European Molecular Biology Organization*. *13*, 1574-1584.

Escary, J-L., Perreau, J., Dumenil, D., Ezine, S., and Brulet, P. (1993). Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. *Nature*. *363*, 361-364.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature*. *292*, 104-106.

Ferrag, F., Chiarenza, A., Goffin, V., and Kelly, P. A. (1996). Convergence of signaling transduced by prolactin (PRL)/Cytokine chimeric receptors on PRL-responsive gene transcription. *Molecular Endocrinology*. *10*, 451-460.

Ferrara, N., Winer, J., and Henzel, W. J. (1992). Pituitary follicular cells secrete an inhibitor of aortic endothelial cell growth: Identification as leukemia Inhibitory Factor. *Proceedings of the National Academy of Science, USA. 89*, 698-702.

Finn, C. A., and McLaren, A. (1967). A study of the early stages of implantation in mice. *Journal of Reproduction and Fertility. 13*, 259-267.

Freidin, M., and Kessler, J. A. (1991). Cytokine regulation of substance P expression in sympathetic neurons. *Proceedings of the National Academy of Science, USA. 88*, 3200-3203.

Fry, R. C., Batt, P. A., Fairclough, R. J., and Parr, R. A. (1992). Human leukemia inhibitory factor improves the viability of cultured ovine embryos. *Biology of Reproduction. 46*, 470-474

Fry, R. C. (1992). The effect of leukaemia inhibitory factor (LIF) on embryogenesis. *Reproduction, Fertility, and Development. 4*, 449-458.

Fukada, K. (1985). Purification and partial characterization of a cholinergic neuronal differentiation factor. *Proceedings of the National Academy of Science, USA. 82*, 8795-8799.

Funston, R. N., Nauta, W. J., and Seidel, G. E., Jr. (1997). Culture of bovine embryos in Buffalo Rat liver cell-conditioned media or with leukemia inhibitory factor. *Journal of Animal Science. 75*, 1332-1336.

Gascan, H., Anegon, I., Praloran, V., Naulet, J., Godard, A., Soulillou, J-P., and Jacques, Y. (1990). Constitutive production of human interleukin for DA cells/leukemia inhibitory factor by human tumor cell lines derived from various tissues. *Journal of Immunology. 144*, 2592-2598.

Gascan, H., Godard, A., Ferenz, C., Naulet, J., Praloran, V., Peyrat, M-A., Hewick, R., Jacques, Y., Moreau, J-F., and Soulillou, J-P. (1989). Characterization and NH_2-terminal amino acid sequence of natural human interleukin for DA cells: Leukemia inhibitory factor. *Journal of Biological Chemistry. 264*, 21509-21515.

Gearing, D. P. (1993). The leukemia inhibitory factor and its receptor. *Advances in Immunology. 53*, 31-58.

Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F., and Cosmann, D. (1992). The il-6 signal transducer, gp130: An oncostatin M receptor and affinity converter for the lif receptor. *Science. 255*, 1434-1437.

Gearing, D. P., Thut, C. J., VandenBos, T., Gimpel, S. D., Delaney, P. B., King, J., Price, V., Cosman, D., and Beckmann, M. P. (1991). Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. *Journal of the European Molecular Biology Organization. 10*, 2839-2848.

Gearing, D. P., Nicola, N. A., Metcalf, D., Foote, S., Willson, T. A., Gough, N. M., and Williams, R. L. (1989). Production of leukemia inhibitory factor in *Escherichia coli* by a novel procedure and its use in maintaining embryonic stem cells in culture. *Biotechnology. 7*, 1157-1161.

Gearing, D. P., King, J. A., and Gough, N. M. (1988). Complete sequence of murine myeloid leukaemia inhibitory factor (LIF). *Nucleic Acids Research. 16*, 9857.

Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A., and Metcalf, D. (1987). Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). *Journal of the European Molecular Biology Organization. 6*, 3995-4002.

Gilman, A. G., Rall, T. W., Nies, A. S., and Taylor, P. (Eds.). (1990). Goodman and Gilman's: The pharmacological basis of therapeutics (8th ed.) (pp 659 & 1443). N.Y. Pergamon Press.

Godard, A., Gascan, H., Naulet, J., Peyrat, M-A., Jacques, Y., Soulillou, J-P., and Mareau, J-F. (1988). Biochemical characterization and purification of HILDA, a human lymphokine active on eosinophils and bone marrow cells. *Blood. 71*, 1618-1623.

Gough, N., M., Gearing, D. P., King, J. A., Willson, T.A., Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988). Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor. *Proceedings of the National Academy of Science, USA. 85*, 2623-2627.

Graham, C., Lysiak, J. J., McCrae, K. R., and Lala, P. K. (1992). Localization of transforming growth factor-β at the human fetal-maternal interface: Role in trophoblast growth and differentiation. *Biology of Reproduction. 46*, 561-572.

Greenfield, E. M., Horowitz, M. C., and Lavish, S. A. (1996). Stimulation by parathyroid hormone of interleukin-6 and leukemia inhibitory factor expression in osteoblasts is an immediate-early gene response induced by cAMP signal transduction. *Journal of Biological Chemistry. 271*, 10984-10989.

Grosset, C., Jazwiec, B., Taupin, J-L., Liu, H., Richard, S., Mahon, F-X., Reiffers, J., Moreau, J-F., and Ripoche, J. (1995). In vitro biosynthesis of leukemia inbhibitory factor/human interleukin for DA cells by human endothelial cells: Differential Regulation by Interleukin-1α and Glucocorticoids. *Blood. 86*, 3763-3770.

Hanken, J., and Wassersug, R. (1981). The visible skeleton. A new double-stain technique reveals the *Nature* of the "hard" tissues. *Functional Photography. July/August*, 22-26.

Harvey, M. B., Leco, K. J., Arcellana-Panlilio, M. Y., Zhang, X., Edwards, D. R., and Schultz, G. A. (1995). Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor. *Development. 121*, 1005-1014.

Heap, R. B., Flint, A. P. F., and Gadsby, J. E. (1981). Embryonic signals and maternal recognition. In S. R. Glasser and D. W. Bullock (Eds.), Cellular and molecular aspects of implantation (pp. 311-326). New York: Plenum Press.

Heath, J. K. (1992). Can there be life without LIF? *Nature. 359*, 17.

Heymann, D., Harb, J., Ringeard, S., Blanchard, F., Lassort, D., Raher, S., and Gadard, A. (1995). Upmodulation of αvβ1 integrin expression on human tumor cells by human interleukin for DA cells/leukemia inhibitory factor and oncostatin M: Correlation with increased cell adhesion on fibronectin. *Journal of Cellular Biology. 58*, 305-314.

Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T., and Kishimoto, T. (1990). Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell. 63*, 1149-1157.

Hilton, D. J., Nicola, N. A., and Metcalf, D. (1991). Distribution and comparison of receptors for leukemia inhibitory factor on murine hemopoietic and hepatic cells. *Journal of Cellular Physiology. 146*, 207-215.

Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988). Specific binding of murine leukemia inhibitory factor to normal and leukemic monocytic cells. *Proceedings of the National Academy of Science, USA. 85*, 5971-5975.

Hilton, D. J., Nicola, N. A., Gough, N. M., and Metcalf, D. (1988). Resolution and purification of three distinct factors produced by krebs ascites cells which have differentiation-inducing activity on murine myeloid leukemic cell lines. *Journal of Biological Chemistry. 263*, 9238-9243.

Hilton, D., J. (1992). LIF: Lots of interesting functions. *Trends in Biochemical Sciences. 17*, 72-76.

Hilton, D. J., Nicola, N. A., Waring, P. M., and Metcalf, D. (1991). Clearance and fate of leukemia-inhibitory factor (LIF) after injection into mice. *Journal of Cellular Physiology. 148*, 430-439.

Hilton, D. , and Gough, N. M. (1991). Leukemia inhibitory factor: a biological perspective. *Journal of Cellular Biochemistry. 46*, 21-26.

Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987). HPRT-deficient (Lesch-Nyyhan) mouse embryos derived from germline colonization by cultured cells. *Nature. 326*, 292-295.

Hozumi, M. (1983) Fundamentals of chemotherapy of myeloid leukemia by induction of leukemia cell differentiation. *Advances in Cancer Research. 38*, 121-169.

Hozumi, M., Umezawa, T., Takenaga, K., Ohno, T., Shikita, M., and Yamane, I. (1979). Characterization of factors stimulating differentiation of mouse myeloid leukemia cells from a Yoshida sarcoma cell line cultured in serum-free medium. *Cancer Research. 39*, 5127-5131.

Huarte, J., Belin, D., and Vassalli, J-D. (1985). Plasminogen activator in mouse and rat oocytes: induction during meiotic maturation. *Cell. 43*, 551-558.

Hudson, K. R., Vernallis, A. B., and Heath, J. K. (1996). Charcterization of the receptor binding sites of human leukmia inhibitory factor and creation of antagonists. *Journal of Biological Chemistry. 271*, 11971-11978.

Ichikawa, Y. (1970). Further studies on the differentiation of a cell line of myeloid leukemia. *Journal of Cellular Physiology. 76*, 175-184.

Ichikawa, Y. (1969). Differentiation of a cell line of myeloid leukemia. *Journal of Cellular Physiology. 74*, 223-234.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., and Yancopoulos, G. D. (1992). CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp 130. *Cell. 69*, 1121-1132

Ishimi, Y., Abe, E., Jin, C. H., Miyaura, C., Hong, M. H., Oshida, M., Kurosawa, H., Yamaguchi, Y., Tomida, M., Hozumi, M., and Suda, T. (1992). Leukemia inhibitory factor/ differentiation-stimulating factor (LIF/D-Factor): Regulation of its production and possible roles in bone metabolism. *Journal of Cellular Physiology. 152*, 71-78.

Ishimi, Y., Miyaura, C., Jin, C. H., Akatsu, T., Abe, E., Nakamura, Y., Yamaguchi, A., Yoshiki, S., Matsuda, T., Hirano, T., Kishimoto, T., and Suda, T. (1990). IL-6 is produced by osteoblasts and induces bone resorption. *Journal of Immunology. 145*, 3297-3303.

Jokhi, P. P., King, A., Sharkey, A. M., Smith, S. K., and Loke, Y. W. (1994). Screening for Cytokine messenger ribonucleic acids in purified human decidual lymphocyte populations by the reverse-transcriptase polymerase chain reaction. *Journal of Immunology. 153*, 4427-4435.

Jurisicova, A., Ben-Chetrit, A., Varmuza, S. L., and Casper, R. F. (1995). Recombinant human leukemia inhibitory factor does not enhance in vitro human blastocyst formation. *Fertility and Sterility. 64*, 999-1002.

Kauma, S. W., and Matt, D. W. (1995). Coculture cells that express leukemia inhibitory factor (LIF) enhance mouse blastocyst development *in Vitro*. *Journal of Assisted Reproduction and Genetics. 12*, 153-156.

Keltz, M. D., Attar, E., Buradagunta, S., Olive, D. L., Kliman, H. J., and Arici, A. (1996). Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in the human fallopian tube. *American Journal of Obstetrics and Gynecology. 175*, 1611-1619.

Kim, K. J., Alphonso, M., Schmelzer , C. H.,and Lowe, D. (1992). Detection of human leukemia inhibitory factor by monoclonal antibody based ELISA. *Journal of Immunology. 156*, 9-17.

Klug, W. S., and Cummings, M. R. (1997). The genetics of cancer. In W. S. Klug and M. R. Cummings, Concepts of genetics (5th ed.) (p 595). New Jersey: Prentice Hall.

Kojima, K., Kanzaki, H., Iwai, M., Hatayama, H., Fujimoto, M., Narukawa, S., Higuchi, T., Kaneko, Y., Mori, T., and Fujita, J. (1995). Expression of leukaemia inhibitory factor (LIF) receptor in human placenta: A possible role for LIF in the growth and differentiation of trophoblasts. *Human Reproduction*. 10, 1907-1911.

Kojima, K., Kanzaki, H., Iwai, M., Hatayama, H., Fujimoto, M., Inoue, T., Horie, K., Nakayama, H., Fujita, J., and Mori, T. (1994). Expression of leukemia inhibitory factor in human endometrium and placenta. *Biology of Reproduction. 50*, 882-887.

Kola, I., A. D., and Gough, N. M. (1990). Localization of the murine leukaemia inhibitory factor gene near the centromere on chromosome 11. *Growth Factors. 2*, 235.

Koopman, P., and Cotton, R. G. H. (1984). A factor produced by feeder cells which inhibits embryonal carcinoma cell differentiation. *Experimental Cell Research. 154*, 233-242.

Koshimizu, U., Taga, T., Watanabe, M., Saito, M., Shirayoshi, Y., Kishimoto, T., and Nakatsuji, N. (1996). Functional requirement of gp130-mediated signaling for growth and survival of mouse primordial germ cells in vitro and derivation of embryonic germ (EG) cells. *Development. 122*, 1235-1242.

Kurzrock, R., Estrov, Z., Wetzler, M., Gutterman, J. U., and Talpaz, M. (1991). LIF: Not just a leukemia inhibitory factor. *Endocrine Research. 12*, 208-217.

Kwun, J. K., and Emmens, C. W. (1974). Hormone requirements for implantation and pregnancy in the ovariectomized rabbit. *Australian Journal of Biological Sciences. 27*, 275-283.

Lavranos, T. C., Rathjen, P. D., and Seamark, R. F. (1995). Trophic effects of myeloid leukaemia inhibitory factor (LIF) on mouse embryos. *Journal of Reproduction and Fertility. 105*, 331-338.

Layton, M. J., Cross, B. A., Metcalf, D., Ward, L. D., Simpson, R. J., and Nicola, N. A. (1992). A major binding protein for leukemia inhibitory factor in normal mouse serum: Identification as a soluble form of the cellular receptor. *Proceedings of the National Academy of Science, USA. 89*, 8616-8620.

Layton, M. J., Lock, P., Metcalf, D., and Nicola, N. A. (1994). Cross-species receptor binding characteristics of human and mouse leukemia inhibitory factor suggests a complex binding interaction. *Journal of Biological Chemistry. 269*, 17048-17055.

Le, P., T., Lazorick, S., Whichard, L. P., Yang, Y-C., Clark, S. C., Haynes, B. F., and Singer, K. H. (1990). Human thymic epithelial cells produce IL-6, granulocyte-monocyte-CSF, and leukemia inhibitory Factor. *Journal of Immunology. 145*, 3310-3315.

Leary, A. G., Wong, G. G., Clark, S. C., Smith, A. G., and Ogawa, M. (1990). Leukemia inhibitory factor differentiation-inhibiting activity/human interleukin for DA cells augments proliferation of human hematopoietic stem cells. *Blood. 75*, 1960-1964.

Li, M., Sendtner, M., and Smith, A. (1995). Essential function of lif receptor in motor neurons. *Nature. 378*, 724-727.

Lin, L-F. H., Mismer, D., Lile, J. D., Armes, L. G., Butler , E. T. III., Vannice, J. L., and Collins, F. (1979). Cholinergic neuronotrophic factor: Intraocular distribution of trophic activity for ciliary neurons. *Science. 246*, 1023-1025.

Lord, K. A., Abdollahi, A., Thomas, S. M., DeMarco, M., Brugge, J. S., Hoffman-Liebermann, B., and Liebermann, D. A. (1991). Leukemia inhibitory factor and interleukin-6 trigger the immediate early response, including tyrosine phosphorylation upon induction of myeloid leukemia differentiation. *Mollecular and Cellular Biology. 11*, 4371-4379.

Lowe, C., Cornish, J., Martin, T. J., and Reid, I. R. (1991) . Effects of leukemia inhibitory factor on bone resorption and DNA synthesis in neonatal mouse calvaria. *Calcified Tissue Research. 49*, 394-397.

Lowe, D. G., Nunes, W., Bombara, M., McCabe, S., Ranges, G. E., Henzel, W., Tomida, M., Yamamoto-Yamaguchi, Y., Hozumi, M., and Goeddel, D. V. (1989). Genomic cloning and heterologous expression of human differentiation-stimulating factor. *DNA. 8*, 351-359.

Ludlam, W. H., Chandross, K. J., and Kessler, J. A.. (1995). LIF-and IL-1 β-mediated increases in substance P receptor mRNA in axotomized, explanted or dissociated sympathetic ganglia. *Brain Research. 685*, 12-20.

Ludlam, W., and Kessler, J. A.. (1993). Leukemia inhibitory factor and ciliary neurotrophic factor regulate expression of muscarinic receptors in cultured sympathetic neurons. *Developmental Biology. 155*, 497-506.

Maekawa, T., Metcalf, D., and Gearing, D. P. (1990). Enhcanced suppression of human myeloid leukemic cell lines by combinations of IL-6, LIF, GM-CSF, and G-CSF. *International Journal of Cancer. 45*, 53-358.

Maekawa, T., and Metcalf, D. (1989). Clonal suppression of HL60 and U937 cells by recombinant human leukemia inhibitory factor in combination with GM-CSF or G-CSF. *Leukemia. 3*, 270-276.

Malaval, L., Gupta, A. K., and Aubin, J. E. (1995). Leukemia inhibitory factor inhibits osteogenic differentiation in rat calvaria cell cultures. *Endocrinology. 136*, 1411-1418.

Marshall, J. S., Gauldie, J., Nielsen, L., and Bienenstock, J. (1993). Leukemia inhibitory factor production by rat mast cells. *European Journal of Immunology. 23*, 2116-2120.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Science, USA. 78*, 7634-7638.

Martin, G. R., and Evans, M. J. (1975). Differentiation of clonal lines of teratocarcinoma cells: Formation of embryoid bodies *in vitro*. *Proceedings of the National Academy of Science, USA. 72*, 1441-1445.

Martinou, J-C., Martinou, I., and Kato, A. C. (1992). Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. *Neuron. 8*, 737-744.

Marx, J. (1993a). Forging a path to the nucleus. *Science. 260*, 1588-1589.

Marx, J. (1993b). Two major signal pathways linked. *Science. 262*, 988-990.

Masiakowski, P., Liu, H., Radziejewski, C., Lottspeich, F., Oberthuer, W., Wong, V., Lindsay, R. M., Furth, M. E., and Panayotatos, N. (1991). Recombinant human and rat ciliary neurotrophic factors. *Journal of Neurochemistry. 57*, 1003-1012.

Matsui, Y., Toksoz, D., Nishikawa, S., Nishikawa, S-I., Williams, D., Zsebo, K., and Hogan, B. L. M. (1991). Effect of steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture. *Nature. 353*, 750-752.

McNiece, I. K., Langley, K. E., and Zsebo, K. M. (1991). The role of recombinant stem cell factor in early B cell development. *Journal of Immunology. 146*, 3785-3790.

Metcalf, D., Hilton, D., and Nicola, N. A. (1991). Leukemia inhibitory factor can potentiate murine megakaryocyte production *in vitro*. *Blood. 77*, 2150-2153.

Metcalf, D., Nicola, N. A., and Gearing, D. P. (1990). Effects of injected leukemia inhibitory factor on hematopoietic and other tissues in mice. *Blood. 76*, 50-56.

Metcalf, D. (1989). Actions and interactions of G-CSF, LIF, and IL-6 on normal and leukemic murine cells. *Leukemia. 3*, 349-355.

Metcalf, D., Hilton, D. J., and Nicola, N. A. (1988). Clonal analysis of the actions of the murine leukemia inhibiory factor on leukemic and normal murine hemopoietic cells. *Leukemia. 2*, 216-221.

Metcalf, D., and Gearing, D. P. (1989b). A myelosclerotic syndrome in mice engrafted with cells producing high levels of leukemia inhibitory factor (LIF). *Leukemia. 3*, 847-852.

Metcalf, D., and Gearing, D. P. (1989a). Fatal syndrome in mice engrafted with cells producing high levels of leukemia inhibitory factor. *Proceedings of the National Academy of Science, USA. 86*, 5948-5952.

Michikawa, M., Kikuchi, S., and Kim, S. U. (1992). Leukemia inhibitory factor (LIF) mediated increase of choline acetyltransferase activity in mouse spinal cord neurons in culture. *Neuroscience Letters. 140*, 75-77.

Michishita, M., Satoh, M., Yamaguchi, M., Hirayoshi, K., Okuma, M., and Nagata, K. (1991). Phosphorylation of the stress protein HSP27 is an early event in murine myelomonocytic leukemic cell differentiation induced by leukemia inhibitory factor/D-factor. *Biochemical and Biophysical Research Communications. 176*, 979-984.

Mitchell, M. H., Swanson, R. J., Hodgen, G. D., and Oehninger, S. (1994). Enhancement of in vitro murine embryo development by recombinant leukemia inhibitory factor. *Journal of the Society of Gynecological Investigations. 1*, 215-219.

Moreau, J-F., Donaldson, D. D., Bennett, F., Witek-Giannotti, J., Clark, S. C., and Wong, G. G. (1988). Leukaemia inhibitory factor is identical to the myeloid growth factor human interleukin for DA cells. *Nature. 336*, 690-692.

Moreau, J-F., Bonneville, M., Godard, A., Gascan, H., Gruart, V., Moore, M. A., and Soulillou, J. P. (1987). Characterization of a factor produced by human t cell clones. *Journal of Immunology. 138*, 3844-3849.

Mori, M., Yamaguchi, K., and Abe, K. (1989). Purification of a lipoprotein lipase-inhibiting protein produced by a melanoma cell line associated with cancer cachexia. *Biochemical and Biophysical Research Communications. 160*, 1085-1092.

Mullins, D. E., Bazer, F. W., and Roberts, R. M. (1980). Secretion of a progesterone-induced inhibitor of plasminogen activator by the porcine uterus. *Cell. 20*, 865-872.

Murakami, M., Hibi, M., Nakagawa, N., Nakagawa, T., Yasukawa, K., Yamanishi, K., Taga, T., Kishimoto, T. (1993). IL-6-induced homodimerization of gp 130 and associated activation of a tyrosine kinase. *Science. 260*, 1808-1810.

Murphy, M., Reid, K., Hilton, D. J., and Bartlett, P. F. (1991). Generation of sensory neurons is stimulated by leukemia inhibitory factor. *Proceedings of the National Academy of Science, USA. 88*, 3498-3501.

Murphy, M., Reid, K., Brown, M. A., and Bartlett, P. F. (1993). Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. *Development. 117*, 1173-1182.

Murray, R., Lee, F., and Chiu, C-P. (1990). The genes for leukemia inhibitory factor and interleukin-6 are expressed in mouse blastocysts prior to the onset of hemopoiesis. *Mollecular and Cellular Biology. 10*, 4953-4956.

Nachtigall, M. J., Kliman, H. J., Feinbert, R. F., Olive, D. L., Enguin, O., and Arici, A. (1996). The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: A potential role in human Implantation. *Journal of Clinical Endocrinology. 81*, 801-806.

Nakagaito, Y., Yoshida, T., Satoh, M., and Takeuchi, M. (1995). Effects of leukemia inhibitory factor on the differentiation of astrocyte progenitor cells from embryonic mouse cerebral hemispheres. *Developmental Brain Research. 87*, 220-223.

National Institutes of Health (US). 1985. Guide for the care and use of laboratory animals. (NIH publication No. 86-23). Bethesda (MD): U. S. Government Printing Office.

Nichols, J., Evans, E. P., and Smith, A. G. (1990). Establishment of germ-line-competent embryonic stem (es) cells using differentiation inhibiting activity. *Development. 110*, 1341-1348.

Nicola, N. A., and Metcalf, D. (1991). Subunit promiscuity among hemopoietic growth factor receptors. *Cell. 67*, 1-4.

Noble, E., Neill, J. D., Greenwald, G. S., Markert, C. L., and Pfaff, D. W.. eds. (1994). in The physiology of reproduction, 2nd ed. Raven Press, N. Y. pp 328-332.

Orsini, M. W., and Meyers, R. K. (1962). Effect of varying doses of progesterone on implantation in the ovariectomized hamster. *Proceedings of the Societcy for Experimental Biology and Medicine. 110*, 713-715.

Owczarek, C. M., Layton, M. J., Metcalf, D., Lock, P., Willson, T. A., Gough, N. M., and Nicola, N. A. (1993). Inter-species chimeras of leukaemia inhibitory factor define a major human receptor-binding determinant. *Journal of the European Molecular Biology Organization. 12*, 3487-3495.

Owezarek, C., Layton, M. J., Robb, L. G., Nicola, N. A., and Begley, C. G. (1996). Molecular basis of the soluble and membrane-bound forms of the murine leukemia inhibitory fafctor receptor α-chain. *Journal of Biological Chemistry. 271*, 5495-5504.

Papaxanthos-Roche, A., Daniel, J-Y., Taupin, J-L., Moreau, J-F., and Mayer, G. (1994). Human interleukin for DA cells or leukemia inhibitory factor is released by vero cells in human embryo coculture. *Fertility and Sterility. 63*, 648-650.

Pease, S. and Williams, R. L. (1990). Formation of germ-line chimeras from embryonic stem cells maintained with recombinant leukemia inhibitory factor. *Experimental Cell Research. 190*, 209-211.

Pease, S., Braghetta, P., Gearing, D., Grail, D., and Williams, R. L. (1990). Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). *Developmental Biology. 141*, 344-352.

Pennica, D., Shaw, K. J., Swanson, T. A., Moore, M. W., Shelton, D. L., Zioncheck, K. A., Rosenthal, A., Tagal, T., Paoni, N. F., and Wood, W. I.. (1995a). cardiotrophin-1. *Journal of Biological Chemistry. 270*, 10915-10922.

Pennica, D., King, K. L., Shaw, K. J., Luis, E., Rullamas, J., Luoh, S-M., Darboone, W. C., Knutzon, D. S., Yen, R., Chien, K. R., Baker, J. B., and Wood, W. I. (1995b). Expression cloning of cardiotrophin 1, a Cytokine that induces cardiac myocyte hypertrophy. *Proceedings of the National Academy of Science, USA. 92*, 1142-1146.

Perry, J. S., and Rowlands, I. W. (1962). Early pregnancy in the pig. *Journal of Reproduction and Fertility. 4*, 175-187.

Piquet-Pellorce, C., Grey, L., Moreau, A., and Heath, J. K. (1994). Are LIF and related Cytokines functionally equivalent. *Experimental Cell Research. 213*, 340-347.

Price, S. R., Olivecrona, T., and Pekala, P. H. (1986). Regulation of lipoprotein lipase synthesis by recombinant tumor necrosis factor - the primary regulatory role of the hormone in 3T3-L1 adipocytes. *Archives of Biochemistry and Biophysics. 251*, 738-746.

Rao, M. S., Sun, Y., Escary, J. L., Perreau, J., Tresser, S., Patterson, P. H., Zigmond, R. E., Brulet, P., and Landis, S. C. (1993). Leukemia inhibitory factor mediates an injury response but not a target-directed developmental transmitter switch in sympathetic neurons. *Neuron. 11*, 1175-1185.

Rappolee, D A., Brenner, C. A., Schultz, R., Mark, D., and Werb, Z. (1988). Developmental expression of PDGF, TGF-α , and TGF-β genes in preimplantation mouse embryos. *Science. 241*, 1823-1825.

Rathjen, P. D, Nichols, J., Toth, S., Edwards, D. R., Heath, J. K., and Smith, A. G. (1990). Developmentally programmed induction of differentiation inhibiting activity and the control of stem cell populations. *Genes and Development. 4*, 308-2318.

Rathjen, P. D., Toth, S., Willis, A., Heath, J. K., and Smith, A. G. (1990). Differentiation inhibiting activity is produced in matrix-associated and diffusible forms that are generated by alternate promoter usage. *Cell. 62*, 1105-1114.

Reid, I. R., Lowe, C., Cornish, J., Skinner, S. J. M., Hilton, D. J., Willson, T. A., Gearing, D. P., and Martin, T. J. (1990). Leukemia inhibitory factor: A novel bone-active Cytokine. *Endocrinology. 126*, 1416-1420.

Robertson, S. A., and Seamark, R. F. (1992). Granulocyte-macrophage colony stimulating factor (GM-CSF): One of a family of epithelial cell-derived Cytokines in the preimplantation uterus. *Reproduction, Fertility, and Development. 4*, 435-448.

Robertson, S. A., Lavranos, T., and Seamark, R. F. (1990). In vitro models of the maternal-fetal interface. In T. G. Wegmann, E. Nesbitt-Brown, & T. J. Gill. (Eds.), The molecular and cellular Immunobiology of the maternal-fetal interface (pp191-206). New York: Oxford Univ. Pres.

Robinson, R. C., Grey, L. M., Staunton, D., Vankelecom, H., Vernallis, A. B., Moreau, J. F., Stuart, D. I., Heath, J. K., and Jones, E. Y. (1994). The crystal structure and biological function of leukemia inhibitory factor: implications for receptor binding. *Cell. 77*, 1101-1116.

Roblero, L, Beas, F., and Arrau, J. (1981). Embryo development under the effects of a new placental protein (UTPH) and its antiserum. *Hormone and Metabolic Research. 13*, 124-125.

Roblero, L., Beas, F., Arrau, J., and Rojas, I. (1981). Uptake and incorporation of uridine-^{3}H, leucine-^{3}H and thymidine-^{3}H by delayed mouse blastocysts under the effect of a human placental protein (UTPH) and its antiserum. *Hormone and Metabolic Research.* 13, 188-89.

Roblero, L., Beas, F., Arrau, J., and Rojas, I. (1983). Induction of implantation by a human uterotrophic placental protein (UTPH) in female mice and rats with delayed implantation. *Hormone and Metabolic Research. 15*, 53-55.

Roldan, A. L., Cubellis, M. V., Masucci, M. T., Behrendt, N., Lund, L. R., Dano, K., Appella, E., and Blasi, F. (1990). Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis. *Journal of the European Molecular Biology Organization. 9*, 467-474.

Rose, T. M., and Bruce, A. G. (1991). Oncostatin M is a member of a Cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor, and interleukin 6. *Proceedings of the National Academy of Science, USA. 88*, 641-8645.

Sattler, M., Salgia, R., Shrikhande, G., Verma, S., Pisick, E., Prasad, K. V. S., and Griffin, J. D. (1997). Steel factor induces tyrosine phosphorylation of CRKL and binding of CRKL to a complex containing c-Kit, phosphatidylinositol 3-kinase, and p120^{CBL}. *Journal of Biological Chemistry. 272*, 10248-10253.

Sawai, K., Matsuzaki, N., Kameda, T., Hashimoto, K., Okada, T., Shimoya, K., Nobunaga, T., Taga, T., Kishimoto, T., and Saji, F. (1995). Leukemia inhibitory factor produced at the fetomaternal interface stimulates chorionic gonadotropin production: its possible implication during pregnancy, including implantation period. *Journal of Clinical Endocrinology. 80*, 1449-1456.

Schiemann, W. P., Graves, L. M., Baumann, H., Morella, K. K., Gearing, D. P., Nielsen, M. D., Krebs, E. G., and Nathanson, N. M. (1995). Phosphorylation of the human leukemia inhibitory factor (LIF) receptor by mitogen-activated protein kinase and the regulation of lif receptor function by heterologous receptor activation. *Proceedings of the National Academy of Science, USA. 92*, 5361-5365.

Schmelzer, C. H., Burton, L. E., and Tamony, C. M. (1990). Purification and partial characterization of recombinant human differentiation-stimulating factor. *Protein Expression and Purification. 1*, 54-62.

Schule, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M., and Evans, R. M. (1990). Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell. 62*, 1217-1226.

Schwarzschild, M. A., Dauer, W. T., Lewis, S. E., Hamill, L. K., Fink, J. S., and Hyman, S. E. (1994). Leukemia inhibitory factor and ciliary neurotrophic factor increase activated Ras in a neuroblastoma cell line and in sympatheic neuron cultures. *Journal of Neurochemistry. 63*, 1246-1254.

Shen, M M., and Leder, P. (1992). Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation *in vitro*. *Proceedings of the National Academy of Science, USA. 89*, 8240-8244.

Shiina-Ishimi, Y., Abe, E., Tanaka, H., and Suda, T. (1986) Synthesis of colony-stimulatingfactor (CSF) and differentiation-inducing factor (D-factor) by osteoblastic cells, clone MC3T3-E1. *Biochemical and Biophysical Research Communications. 134*, 400-406.

Simpson, R. J., Hilton, D. J., Nice, E. C., Rubira, M. R., Metcalf, D., Gearing, D. P., Gough, N. M., and Nicola, N. A. (1988). Structural characterization of a murine myeloid leukaemia inhibitory factor. *European Journal of Biochemistry. 175*, 541-547.

Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M., and Ragers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature. 336*, 688-690.

Smith, A. G., Nichols, J., Robertson, M., and Rathjen, P. D. (1992). Differentiation inhibiting activity (DIA/LIF) and mouse development. *Developmental Biology. 151*, 339-351.

Smith, A. G., and Hooper, M. L. (1987). Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Developmental Biology. 121*, 1-9.

Solter, D., and Knowles, B. B. (1975). Immunosurgery of mouse blastocysts. *Proceedings of the National Academy of Science, USA. 72*(12),5099-5102.

Stahl, N., Davis, S., Wong, V., Taga, T., Kishimoto, T., Ip, N. Y., and Yancopoulos, G. D. (1993). Cross-linking identifies leukemia inhibitory factor-binding protein as a ciliary neurotrophic factor receptor component. *Journal of Biological Chemistry. 268*, 7628-7631.

Stahl, J., Gearing, D. P., Willson, T. A., Brown, M. A., King, J. A., and Gough, N. M. (1990). Structural organization of the genes for murine and human leukemia inhibitory factor. *Journal of Biological Chemistry. 265*, 8833-8841.

Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F., & Abbondanzo, S. J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature. 359*, 76-79

Stewart, I., and Peel, S. (1981). Granulated metrial gland cells in the virgin and early pregnant mouse uterus. *Journal of Anatomy. 133*, 535-541.

Stewart, C. L. (1994). Leukaemia inhibitory factor and the regulation of pre-implantation development of the mammalian embryo. *Molecular Reproduction and Development. 39,* 233-238.

Stockli, K. A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Gotz, R., Lindholm, D., and Thoenen, H. (1989). Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. *Nature. 342*, 920-923.

Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. (1994). Activation of raf as a resut of recruitment to the plasma membrane. *Science. 264*, 1463-1467.

Strickland, S., and Richards, W. G. (1992). Invasion of the trophoblasts. *Cell. 71*, 355-357

Sutherland, G. R., Baker, E., Hyland, V. J., Callen, D. F., Stahl, J., and Gough, N. M. (1989). The gene for human leukemia inhibitory factor (LIF) maps to 22q12. *Leukemia. 3*, 9-13.

Symes, A. J., Corpus, L., and Fink, J. S. (1995). Differences in nuclear signaling by leukemia inhibitory factor and interferon-α: The role of STAT proteins in regulating vasoactive intestinal peptide gene expression. *Journal of Neurochemistry. 65,* 1926-1933.

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., and Kishimoto, T. (1989). interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell. 58*, 573-581.

Takeda, K., Iwamoto, S., Sugimoto, H., Takuma, T., Kawatani, N., Noda, M., Masaki, A., Morise, H., Arimura, H., and Konno, K. (1986). Identity of differentiation inducting factor and tumor necrosis factor. *Nature. 323*, 338-340.

Tomida, M., Yamamoto-Yamaguchi, Y., and Hozumi, M. (1984a). Purification of a factor inducing differentiation of mouse myeloid leukemic m1 cells from conditioned medium of mouse fibroblast L929 cells *Journal of Biological Chemistry. 259*, 10978-10982.

Tomida, M. (1995). Analysis of recombinant soluble mouse D-factor/LIF receptor. *Journal of Biochemistry. 117*, 1228-1231.

Tomida, M., Yamamoto-Yamaguchi, Y., and Hozumi, M. (1984b). Characterization of a factor inducing differentiation of mouse myeloid leukemic cells purified from conditioned medium of mouse ehrlich ascites tumor cells. *Letters of the Federation of European Biochemical Society. 178*, 291-296.

Van Beek, E., Van Der Wee-Pals, L., Van De Ruit, M., Nijweide, P., Papapoulos, S., and Löwik, C. (1993). Leukemia inhibitory factor inhibits osteoclastic resorption, growth, mineralization, and alkaline phosphatase activity in fetal mouse metacarpal bones in culture. *Journal of Bone and Mineral Research. 8*, 191-198.

Verfaillie, C., and McGlave, P. (1991). Leukemia inhibitory factor/human interleukin for DA cells: A growth factor that stimulates the in vitro development of multipotential human hematopoietic progenitors. *Blood. 77*, 263-270.

Vogiagis, D., Fry, R. C., Sandeman, R. M., and Salamonsen, L. A. (1997). Leukaemia inhibitory factor in endometrium during the oestrous cycle, early pregnancy and in ovariectomized steroid-treated ewes. *Journal of Reproduction and Fertility. 109*, 279-288.

Vogiagis, D., Marsh, M. M., Fry, R. C., and Salamonsen, L. A. (1996). Leukaemia inhibitory factor in human endometrium throughout the menstrual cycle. *Journal of Endocrinology. 148*, 95-102.

Wang, Z., Ren, S-G., and Melmed, S. (1996). Hypothalamic and pituitary leukemia inhibitory factor gene expression *in vivo*: A novel endotoxin-inducible neuro-endocrine interface. *Endocrinology. 137*, 2947-2953.

Ware, C. B., Horowitz, M. C., Renshaw, B. R., Hunt, J. S., Liggitt, D., Koblar, S. A., Gliniak, B. C., McKenna, H. J., Papayannopoulou, T., Thoma, B., Cheng, L., Donovan, P. J., Peschon, J. J., Bartlett, P. F., Willis, C. R., Wright, B. D., Carpenter, M. K., Davison, B. L., and Gearing, D. P. (1995). Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural, and metabolic defects and results in perinatal death. *Development. 121*, 1283-1299.

Waynforth, H. B., and Flecknell, P. A. (1992). Experimental and surgical technique in the rat (2nd ed). New York: Academic Press. pp 100-125.

Weber, M. J. (1981). A diffusible factor responsible for the determination of cholinergic functions in cultured sympathetic neurons. *Journal of Biological Chemistry. 256*, 3447-3453.

Werb, Z. (1989). Proteinases and matrix degradation. In W. N. Kelley, E. D. Harris, Jr., S. Ruddy, and C. B. Sledge. W. B. Saunders. (Eds), Texbook of rheumatology (pp. 248-268). Philadelphia, PA..

Wetzler, M., Kurzrock, R., Taylor, K., Spitzer, G., Kantarjian, H., Baiocchi, G., Ku, S., Gutterman, J. U., and Talpaz, M. (1990). Constitutive and induced expression of *Growth Factors* in normal and chronic phase chronic myelogenous leukemia ph^1 bone marrow stroma. *Cancer Research. 50*, 5801-5805.

Williams, D. E., DeVries, P., Namen, A. E., Widmer, M. B., and Lyman, S. D. (1992). The *Steel* factor. *Developmental Biology. 151*, 368-376.

Williams, R. L, Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P, Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature. 336*, 684-687.

Willson, T. A., Metcalf, D., and Gough, N. M. (1992). Cross-species comparison of the sequence of the leukaemia inhibitory factor gene and its protein. *European Journal of Biochemistry. 204*, 21-30.

Wobus, A. M., Holzhausen, H., Jakel, P., and Schoneich, J. (1984). Characterization of a pluripotent stem cell line derived from a mouse embryo. *Experimental Cell Research. 152*, 212-219.

Wu, K., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1993). Inhibition of the EGF-activated MAP kinase signaling pathway by adenosing 3',5'-monophosphate. *Science. 262*, 1065-1069.

Yamaguchi, M., Miki, N., Ono, M., Ohtsuka, C., Demura, H., Kurachi, H., Inoue, M., Endo, H., Taga, T., Kishimoto, T., and Miyake, A. (1995). Inhibition of growth hormone- releasing factor production in mouse placenta by Cytokines using gp 130 as a signal transducer. *Endocrinology*. 136, 1072-1078.

Yamaguchi, M., Taga, T. Kishimoto, T., and Miyake, A. (1995). Cytokine that use gp130 as a signal transducer stimulate mouse placental lactogen-i (mPL-I) but Inhibit mPL-II production in vitro. *Biology of Reproduction. 53*, 399-406.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M-J., and Patterson, P. H. (1989). The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science. 246*, 1412-1416.

Yamamori, T. (1991). Localization of cholinergic differentiation factor/leukemia inhibitory factor mRNA in the rat brain and peripheral tissues. *Proceedings of the National Academy of Science, USA. 88*, 7298-7302.

Yamamoto, Y., Tomida, M., and Hozumi, M. (1980). Production by mouse spleen cells of factors stimulating differentiation of mouse myeloid leukemic cells that differ from the colony-stimulating factor. *Cancer Research. 40*, 4804-4809.

Yamamoto-Yamaguchi, Y., Tomida, M., and Hozumi, M. (1986). Specific binding of a factor inducing differentiation to mouse myeloid leukemic M1 cells. *Experimental Cell Research.* 164, 97-102.

Yamamoto-Yamaguchi, Y., Tomida, M., Hozumi, M., Maurer, H. R., Okabe, T., and Takaku, F. (1989). Combined effects of differentiation-inducing factor and other Cytokines on induction of differentiation of mouse myeloid leukemic cells. *Japanese Journal of Cancer Research. 80*, 115-121.

Yang, Z-M., Le, S-P., Chen, D-B., and Harper, M. J. K. (1994). Temporal and spatial expression of leukemia inhibitory factor in rabbit uterus during early pregnancy. *Molecular Reproduction and Development. 38*, 148-152.

Yang, Z-M., Le, S-P., Chen, D-B., Yasukawa, K., and Harper, M. J. K. (1995). Expression patterns of leukaemia inhibitory factor receptor (LIF) and the gp130 receptor component in rabbit uterus during early pregnancy. *Journal of Reproduction and Fertility. 103*, 249-255.

Yang, Z-M., Chen, D-B., Le, S-P., and Harper, M. J. K. (1996). Differential hormonal regulation of leukemia inhibitory factor (LIF) in rabbit and mouse uterus. *Molecular Reproduction and Development. 43,* 470-476.

Yin, T., and Yang, Y-C. (1994). Mitogen-activated protein kinases and ribosomal S6 protein kinases are involved in signaling pathways shared by interleukin-11, interleukin-6, leukemia inhibitory factor, and oncostatin M in mouse 3T3-L1 cells. *Journal of Biological Chemistry. 269*, 3731-3738.

Yoshinaga, K., and Adams, C. E. (1966). Delayed implantation in the spayed, progesterone-treated adult mouse. *Journal of Reproduction and Fertility. 12,* 593-595.

Zhang, X., Kidder, G. M., Zhang, C., Khamsi, F., and Armstrong, D. T. (1994). Expression of plasminogen activator genes and enzymatic activities in pre-implantation embryos from the rat and mouse. *Journal of Reproduction and Fertility. 101*, 235-240.

APPENDIX i

SOURCES FOR LIF

Sources	References
- *Escherichia coli*	Lowe, et al., 1989
- Yeast (*Saccharomyces cerevisiae*)	Gearing, et al., 1987
	Gough, et al., 1988
- murine (m) endometrial glands	Bhatt et al., 1991
	Hilton, 1992
	Shen and Leder, 1992
	Smith et al., 1992
	Stewart, et al. 1992
- m extraembryonic tissues	Conquet and Brulet, 1990
- m placenta/decidua	Conquet and Brulet, 1990
- m blastocysts	Conquet and Brulet, 1990
- m fibroblasts	Williams, et al., 1988
- m L cells	Robertson, Lavranos, and Seamark, 1991
- m spleen cells	Abe, et al. 1986
	Chen, Platzer, Moore, Mertelsmann, and Welte, 1986
- m T lymphocytes	Hilton, Nicola, and Metcalf, 1991
	Gearing, et al., 1987
- m osteoblastic cell line	Abe, et al., 1988
	Ishimi, et al., 1992
- m primary osteoblast-like cells	Ishimi, et al., 1992
- m L929 cells	Abe, et al., 1986
	Tomida 1984
- m Ehrlich ascites tumor cells	Abe, et al., 1986
	Franchimont, Demoulin, and Valcke, 1988
	Lowe, et al., 1989
- m Krebs II ascites tumor cells	Chen, et al., 1986
	Gearing, et al., 1987
	Hilton, Nicola, Gough, and Metcalf, 1988
	Hilton, Nicola, and Metcalf, 1991
	Lowe, et al., 1989
	Simpson, et al., 1988
- m neutrophils and	Hozumi, et al., 1983
- m peritoneal macrophages	Hozumi, et al., 1983
- Buffalo rat liver (BRL) cells	Moreau, et al., 1988
	Smith, et al., 1988
	Smith and Hooper, 1987
- rat sarcoma cell line	Hozumi, et al., 1979

- rat heart cell culture ..... Fukada, 1985
  Yamamori, et al., 1989
- rat Yoshida sarcoma ..... Hozumi, et al., 1979
- rat osteoblasts ..... Allan, et al., 1990
- rat mast cells ..... Marshal, Gauldie, Nielson, and Biens, 1993
- hamster - COS cells..... Lowe, et al., 1989
  Moreau, et al., 1988
  Schmelzer, Burton, and Tamony, 1990
- various mammalian cell lines..... Lowe, et al., 1989
- rabbit uterus..... Yang, Le, Chen, and Harper, 1994
- bovine pituitary follicular cells ..... Ferrara, Winer, and Henzel, 1992
- monkey kidney epithelium (Vero cells) Papaxanthos-Roche, Jean-Yves, Taupin, Moreau, and Mayer, 1994
- human (h) endometrium, decidua..... Delage, et al., 1995
- h blastocyst..... Charnock-Jones, Sharkey, Fenwick, and Smith, 1994
- h umbilical vein endothelial cells..... Grosset, et al., 1995
- h bone marrow endothelial cells ..... Grosset, et al., 1995
- h thymic epithelial cells..... Le, et al. 1990
- h aloreactive T lymphocyte clones ..... Godard, et al., 1988
  Metcalf, 1988
  Moreau, et al., 1988
- h keratinocytes, SVK-14 ..... Baumann, 1984
  Gascan, et al., 1990
- h mammary epithelium (SV40) ..... Gascan, et al., 1990
- h PHA stimulated Tcell line HUT102 .... Takeda, et al., 1986
  Wetzler, 1991
- h killer cells Jokhi et al, 1994
- h T cell line (C10-MJ2)..... Moreau, et al., 1988
- h H23 cells..... Moreau, et al., 1988
- h PHA stimulated lymphocytes ..... Takeda, et al., 1986
- h peripheral blood lymphocytes..... Takeda, et al., 1986
- h monocytes, macrophages..... Takeda, et al., 1986
- h myelomonocytes..... Anegon, et al., 1990
- h bone marrow stromal cultures ..... Wetzler, et al., 1991
- h $CD56^{+}$ NK cells ..... Jokhi, King, Sharkey, Smith, and Loke, 1994
  Yamaguchi, Kishimoto, and Miyake, 1995
- h melanoma cell line - SEKI..... Mori, Yamaguchi, and Abe, 1989
- h 5637 medium..... Williams, et al., 1988
- h bladder carcinoma cell line - 5637 Gascan, et al., 1990
  Williams, et al., 1988
- h lung adenocarcinoma, NCI-H23 ..... Gascan, et al., 1990

- h epidermoid carcinoma, HLFa.....Gascan, et al., 1990
- h colonadenocarcinoma, SW948.....Gascan, et al., 1990
- h colonadenocarcinoma, HRT18 .....Gascan, et al., 1990
- h pancreatic carcinoma, Mia Paca.....Gascan, et al., 1990
- h amelanotic melanoma, C32.....Gascan, et al., 1990
- h breast adenocarcinoma .....Gascan, et al., 1990
- h squamous carcinoma, COLO-16 .....Baumann, Onorato, Gauldie, and Jahreis, 1987
  Baumann and Wong, 1989
- FDC-P1 cell lines .....Metcalf and Gearing, 1989
- h bone marrow stromal cells.....Wetzler, 1991
- h monocytic leukemia, THP-1 .....Abe, et al., 1989
- h mitogen treated spleen cells.....Abe, et al., 1986
- h STO embryonic fibroblasts .....Evans and Kaufman, 1981
  Koopman and Cotton, 1984
  Rathjen, et al., 1990
  Smith, et al., 1988
- h fallopian tube .....Keltz et al., 1996
- h uterus .....Charnock-Jones et al., 1994
  Chen et al., 1995
  Cullinan et al., 1996
  Delage et al., 1995
  Kojima et al., 1994
  Kojima et al., 1995
  Vogiagis, et al., 1996

APPENDIX ii

RECEPTORS

Normal cell types displaying LIF receptors with receptors per cell (RPC) and dissociation constants (DC) in parentheses (when available) compared to Embryonyl stem cell (ES) and embryonic carcinoma cell (EC) lines.

Cell Type	RPC (DC)
Bone Marrow	
monocyte	155
promonocyte	270
lymphocyte	70
Spleen	
blast	430
monocyte	240
lymphocyte	120
Peritoneal Cavity	
Macrophage	360
Lymphocyte	170
fetal hepatocyte	1500
fetal hepatic blast	140
adult hepatocyte	2000
serum LBP	1 µg/ml
osteoblasts	
mouse placenta	
megakaryocyte	

Cell Type	RPC (DC)
ES and EC Cell Lines	
M1(ES)	300-500 (100-200 pM)
M1	170 (1 x 10^{-0}M)
EKcs-1 (ES)	295 HA (90 pM)
PCC3A1 (EC)	190 HA (90 pM)
F9 (EC)	330 HA (90 pM)
D3 (ES)	
NG2	
PC13	
P19	
CBL63 (ES)	
HD5 (ES)	
CC (ES)	
CP1 (ES)	
Cloned Cell Lines	
COS7	
B9	
MAH (rat adrenals)	
Ewing's sarcoma cells	
SK-N-LO (neuroepithelioma)	
EW-1	

(Allan, et al., 1990; Conquet and Brulet, 1990; Gearing, 1991; Gough, et al., 1988; Hilton, Nicola, and Metcalf, 1988; Hilton, Nicola, and Metcalf, 1991; Ip, et al., 1992; Layton, et al., 1992; Shen and Leder, 1992; Tomida, 1995; Willaiams, et al., 1988; Yamaguchi, et al. 1995; Yamamoto-Yamaguchi, Tomida, and Hazumi, 1986;)

APPENDIX iii

Category A, Experiments 2 - 6

Developmental Indices, Percents, and Totals

Protocol 1

DS	Day3	m	%	Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2				5	3.57	0.0	3	2.86	0.0	2.0	2.00	0.0				1.0	0.96	0.0
1	3-4	1	0.72	1.0	4	2.86	4.0	5	4.76	5.0				4	3.77	4.0	2.0	1.92	2.0
2	5-8				5	3.57	10.0	8	7.62	16.0	6.0	6.00	12.0	8	7.55	16.0	3.0	2.88	6.0
3	M	84	60.87	252.0	90	64.29	270.0	65	61.90	195.0	70.0	70.00	210.0	79	74.53	237.0	75.0	72.12	225.0
4	e	52	37.68	208.0	36	25.71	144.0	24	22.86	96.0	22.0	22.00	88.0	15	14.15	60.0	20.0	19.23	80.0
5	E																		
6	h																		
7	H																		
-1	d	1	0.72	-1.0													3.0	2.88	-3.0
	Tot	138	100	460.0	140	100.00	428.0	105	100.00	312.0	100	100.00	310.0	106	100.00	317.0	104.0	100.00	310.0
Devel. Indices			▶	3.3			3.1			3.0			3.1			2.99			3.0
			p =	0.003					p =	0.239					p =	0.927			

	Day4	m	%	Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2			0.0	2	1.45	0.0	2	2.25	0.0	1.0	1.00	0.0						
1	3-4				2	1.45	2.0					0.00		2	1.89	2.0			
2	5-8				2	1.45	4.0	4	4.49	8.0		0.00		3	2.83	6.0	1.0	0.99	2.0
3	M	1	0.72	3.0	2	1.45	6.0	5	5.62	15.0	2.0	2.00	6.0	5	4.72	15.0	1.0	0.99	3.0
4	e	63	45.65	252.0	58	42.03	232.0	41	46.07	164.0	54.0	54.00	216.0	56	52.83	224.0	49.0	48.51	196.0
5	E	39	28.26	195.0	32	23.19	160.0	21	23.60	105.0	14.0	14.00	70.0	28	26.42	140.0	22.0	21.78	110.0
6	h	33	23.91	198.0	31	22.46	186.0	13	14.61	78.0	24.0	24.00	144.0	8	7.55	48.0	24.0	23.76	144.0
7	H	1	0.72	7.0			0.0				3.0	3.00	21.0						
-1	d	1	0.72	-1.0	9	6.52	-9.0	3	3.37	-3.0	2.0	2.00	-2.0	4	3.77	-4.0	4.0	3.96	-4.0
	Tot	138	100	654.0	138	100.00	581.0	89	100.00	367.0	100	100.00	455.0	106	100.00	431.0	101.0	100.00	451.0
Devel. Indices			▶	4.7			4.2			4.1			4.6			4.07			4.5
			p =	0.003					p =	0.033					p =	0.022			

	Devel Day 5	b6 m	Day 1 %	Proto Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2																		
1	3-4																		
2	5-8																		
3	M																		
4	e	1	1.59	4.0	1	1.61	4.0	5	16.13	20.0	3	10.34	12.0	5	16.67	20	1	3.13	4.0
5	E	18	28.57	90.0	15	24.19	75.0	9	29.03	45.0	3	10.34	15.0	10	33.33	50	9	28.13	45.0
6	h	16	25.40	96.0	18	29.03	108.0	6	19.35	36.0	12	41.38	72.0	8	26.67	48	10	31.25	60.0
7	H	27	42.86	189.0	16	25.81	112.0	4	12.90	28.0	6	20.69	42.0	3	10.00	21	11	34.38	77.0
-1	d	1	1.59	-1.0	12	19.35	-12.0	7	22.58	-7.0	5	17.24	-5.0	4	13.33	-4	1	3.13	-1.0
	Tot	63	100.00	378.0	62	100.00	287.0	31	100.00	122.0	29	100.00	136.0	30	100.00	135	32	100.00	185.0
Devel. Indices			▶	6.0			4.6			3.9			4.7			4.5			5.8
			p =	0.003					p =	0.131					p =	0.004			

	Devel Day 6	b6 m	Day 1 %	Proto Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2																		
1	3-4																		
2	5-8																		
3	M																		
4	e	1	1.59	4.0	1	1.61	4.0	5	16.13	20.0	4	13.79	16.0	4	13.33	16			
5	E	8	12.70	40.0	5	8.06	25.0	7	22.58	35.0				9	30.00	45	7	21.88	35.0
6	h	3	4.76	18.0	6	9.68	36.0				5	17.24	30.0	1	3.33	6	2	6.25	12.0
7	H	50	79.37	350.0	37	59.68	259.0	10	32.26	70.0	15	51.72	105.0	12	40.00	84	22	68.75	154.0
-1	d	1	1.59	-1.0	13	20.97	-13.0	9	29.03	-9.0	5	17.24	-5.0	4	13.33	-4	1	3.13	-1.0
	Tot	63	100.00	411.0	62	100.00	311.0	31	100.00	116.0	29	100.00	146.0	30	100.00	147	32	100.00	200.0
Devel. Indices			▶	6.5			5.0			3.7			5.0			4.9			6.3
			p =	0.003					p =	0.057					p =	0.006			

3-4 = 3-4-cell embryo; 5-8 = 5-8-cell embryo; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocyst; H = completely hatched blastocyst; f/d = fragmenting or degenerating embryos. m = mrLIF; h = hrLIF; ab = anti h-LIF monoclonal antibody; DS = Develpmental Score; p values derived from Cochran-Mantel-Haenszel (CMH) statistics.

APPENDIX iii CONTINUED

Category A, Experiments 2 - 6

Developmental Indices, Percents, and Totals

Protocol 1

DS	Day3	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2				2	6.25	0.0	1	3.23	0.0			
1	3-4				3	9.38	3.0						
2	5-8	1	3.33	2.0									
3	m	16	53.33	48.0	9.0	28.13	27.0	19	61.29	57.0	9	29.03	27.0
4	e	13	43.33	52.0	17.0	53.13	68.0	11	35.48	44.0	22	70.97	88.0
5	E												
6	h												
7	H												
-1	d				1.0	3.13	-1.0						
	Tot	30	100.00	102	32	100.00	97.0	31	100.00	101.0	31	100.00	115.0
Devel. Indices	▶			3.40			3.0			3.3			3.7
			p =	0.189					p =	0.004			

	Day4	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2							1	3.23	0.0			
1	3-4												
2	5-8				1	3.23	2.0						
3	m	1	3.33	3.0	1.0	3.23	3.0	3	9.68	9.0	1	3.23	3.0
4	e	3	10.00	12.0	3.0	9.68	12.0	6	19.35	24.0	1	3.23	4.0
5	E	16	53.33	80.0	7.0	22.58	35.0	17	54.84	85.0	9	29.03	45.0
6	h	9	30.00	54.0	14.0	45.16	84.0	2	6.45	12.0	19	61.29	114.0
7	H										1	3.23	7.0
-1	d	1	3.33	-1.0	5.0	16.13	-5.0	2	6.45	-2.0			
	Tot	30	100.00	148	31	100.00	131.0	31	100.00	128.0	31	100.00	173.0
Devel. Indices	▶			4.93			4.2			4.1			5.6
			p =	0.246					p =	0.001			

	Devel Day 5	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2												
1	3-4												
2	5-8												
3	m												
4	e	2	6.67	8	2	6.25	8.0	2	6.45	8.0	1	3.23	4.0
5	E	4	13.33	20	3	9.38	15.0	12	38.71	60.0	5	16.13	25.0
6	h	6	20.00	36	8	25.00	48.0	2	6.45	12.0	12	38.71	72.0
7	H	16	53.33	112	12	37.50	84.0	9	29.03	63.0	13	41.94	91.0
-1	d	2	6.67	-2	7	21.88	-7.0	6	19.35	-6.0			
	Tot	30	100.00	174	32	100.00	148.0	31	100.00	137.0	31	100.00	192.0
Devel. Indices	▶			5.8			4.6			4.4			6.2
			p =	0.125					p =	0.002			

	Devel Day 6	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2												
1	3-4												
2	5-8												
3	m												
4	e				2	6.25	8.0	2	6.45	8.0			
5	E	3	10.00	15		0.00	0.0	3	9.68	15.0			
6	h	2	6.67	12	3	9.38	18.0	1	3.23	6.0	4	12.90	24.0
7	H	23	76.67	161	19	59.38	133.0	19	61.29	133.0	25	80.65	175.0
-1	d	2	6.67	-2	8	25.00	-8.0	6	19.35	-6.0	2	6.45	-2.0
	Tot	30	100.00	186	32	100.00	151.0	31	100.00	156.0	31	100.00	197.0
Devel. Indices	▶			6.2			4.7			5.0			6.4
			p =	0.054					p =	0.026			

3-4 = 3-4-cell embryo; 5-8 = 5-8-cell embryo; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocyst; H = completely hatched blastocyst; f/d = fragmenting or degenerating embryos. m = mrLIF; h = hrLIF; ab = anti h-LIF monoclonal antibody; DS = Develpmental Score; p values derived from Cochran-Mantel-Haenszel (CMH) statistics.

APPENDIX iii CONTINUED

Category A, Experiments 2 - 6

Developmental Indices, Percents, and Totals

Protocol 2

DS	Day3	m	%	Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2				5	3.57	0.0				2	2.00	0.0				1	0.96	0.0
1	3-4	1	1.09	1.0	4	2.86	4.0										2	1.92	2.0
2	5-8	1	1.09	2.0	5	3.57	10.0	2	3.28	4.0	6	6.00	12.0				3	2.88	6.0
3	m	49	53.26	147.0	90	64.29	270.0	38	62.30	114.0	70	70.00	210.0	40	64.52	120.0	75	72.12	225.0
4	e	41	44.57	164.0	36	25.71	144.0	21	34.43	84.0	22	22.00	88.0	21	33.87	84.0	20	19.23	80.0
5	E																		
6	h																		
7	H																		
-1	d													1	1.61	-1.0	3	2.88	-3.0
	Tot	92	100.00	314.0	140	100.00	428.0	61	100.00	202.0	100	100.00	310.0	62	100.00	203.0	104	100.00	310.0
Devel. Indices			▶	3.4			3.1			3.3			3.1			3.3			3.0
			p =	0.001					p =	0.041					p =	0.035			

	Day4	m	%	Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2				2	1.45	0.0				1	1.00	0.0						
1	3-4				2	1.45	2.0												
2	5-8	1	1.09	2.0	2	1.45	4.0										1	0.99	2.0
3	m	4	4.35	12.0	2	1.45	6.0				2	2.00	6.0	3	4.92	9.0	1	0.99	3.0
4	e	23	25.00	92.0	58	42.03	232.0	12	19.67	48.0	54	54.00	216.0	16	26.23	64.0	49	48.51	196.0
5	E	47	51.09	235.0	32	23.19	160.0	31	50.82	155.0	14	14.00	70.0	24	39.34	120.0	22	21.78	110.0
6	h	16	17.39	96.0	31	22.46	186.0	15	24.59	90.0	24	24.00	144.0	17	27.87	102.0	24	23.76	144.0
7	H							3	4.92	21.0	3	3.00	21.0						
-1	d	1	1.09	-1.0	9	6.52	-9.0				2	2.00	-2.0	1	1.64	-1.0	4	3.96	-4.0
	Tot	92	100.00	436.0	138	100.00	581.0	61	100.00	314.0	100	100.00	455.0	61	100.00	294.0	101	100.00	451.0
Devel. Indices			▶	4.7			4.2			5.1			4.6			4.8			4.5
			p =	0.011					p =	0.001					p =	0.082			

	Day5	m	%	Scr				h	%	Scr				ab	%	Scr			
0	2																		
1	3-4																		
2	5-8																		
3	m																		
4	e	4	6.35	16.0	1	1.61	4.0				3	10.34	12.0	1	3.03	4.0	1	3.13	4.0
5	E	22	34.92	110.0	15	24.19	75.0	10	32.26	50.0	3	10.34	15.0	7	21.21	35.0	9	28.13	45.0
6	h	22	34.92	132.0	18	29.03	108.0	13	41.94	78.0	12	41.38	72.0	9	27.27	54.0	10	31.25	60.0
7	H	12	19.05	84.0	16	25.81	112.0	8	25.81	56.0	6	20.69	42.0	15	45.45	105.0	11	34.38	77.0
-1	d	3	4.76	-3.0	12	19.35	-12.0				5	17.24	-5.0	1	3.03	-1.0	1	3.13	-1.0
	Tot	63	100.00	339.0	62	100.00	287.0	31	100.00	184.0	29	100.00	136.0	33	100.00	197.0	32	100.00	185.0
Devel. Indices			▶	5.4			4.6			5.9			4.7			6.0			5.8
			p =	0.444					p =	0.060					p =	0.470			

	Day6	m	%	Scr				h	%	Scr				ab	%	Scr			
0	2																		
1	3-4																		
2	5-8																		
3	m																		
4	e	4	6.35	16.0	1	1.61	4.0				3	10.34	12.0						
5	E	9	14.29	45.0	5	8.06	25.0	4	12.90	20.0	1			3	9.09	15.0	7	21.88	35.0
6	h	5	7.94	30.0	6	9.68	36.0	5	16.13	30.0	5	17.24	30.0	5	15.15	30.0	2	6.25	12.0
7	H	40	63.49	280.0	37	59.68	259.0	21	67.74	147.0	15	51.72	105.0	25	75.76	175.0	22	68.75	154.0
-1	d	5	7.94	-5.0	13	20.97	-13.0	1	3.23	-1.0	5	17.24	-5.0		0.00	0.0	1	3.13	-1.0
	Tot	63	100.00	366.0	62	100.00	311.0	31	100.00	196.0	29	96.55	142.0	33	100.00	220.0	32	100.00	200.0
Devel. Indices			▶	5.8			5.0			6.3			4.9			6.7			6.3
			p =	0.003					p =	0.046					p =	0.196			

3-4 = 3-4-cell embryo; 5-8 = 5-8-cell embryo; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocyst; H = completely hatched blastocyst; f/d = fragmenting or degenerating embryos. m = mrLIF; h = hrLIF; ab = anti h-LIF monoclonal antibody; DS = Develpmental Score; p values derived from Cochran-Mantel-Haenszel (CMH) statistics.

APPENDIX iii CONTINUED

Category A, Experiments 2 - 6

Developmental Indices, Percents, and Totals

Protocol 2

DS	Day3	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2				2	6.25	0.0		0.00	0.0			
1	3-4				3	9.38	3.0						
2	5-8												
3	m	23	76.67	69.0	9	28.13	27.0	12	38.71	36.0	9	29.03	27.0
4	e	7	23.33	28.0	17	53.13	68.0	19	61.29	76.0	22	70.97	88.0
5	E												
6	h												
7	H												
-1	d				1	3.13	-1.0						
	Tot	30	100.00	97.0	32	100.00	97.0	31	100.00	112.0	31	100.00	115.0
Devel. Indices				3.2			3.0			3.6			3.7
			p =	0.944					p =	0.944			

	Day4	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2												
1	3-4												
2	5-8				1	3.23	2.0						
3	m				1	3.23	3.0				1	3.23	3.0
4	e	2	6.45	8.0	3	9.68	12.0	2	6.45	8.0	1	3.23	4.0
5	E	20	64.52	100.0	7	22.58	35.0	23	74.19	115.0	9	29.03	45.0
6	h	8	25.81	48.0	14	45.16	84.0	5	16.13	30.0	19	61.29	114.0
7	H	1	3.23	7.0				1	3.23	7.0	1	3.23	7.0
-1	d				5	16.13	-5.0						
	Tot	31	100.00	163.0	31	100.00	131.0	31	100.00	160.0	31	100.00	173.0
Devel. Indices				5.3			4.2			5.2			5.6
			p =	0.049					p =	0.019			

	Day5	m+ab	%	Scr				h+ab	%	Scr			
0	2												
1	3-4												
2	5-8												
3	m												
4	e				2	6.25	8.0	1	4.76	4.0	1	3.23	4.0
5	E	8	25.81	40.0	3	9.38	15.0	14	66.67	70.0	5	16.13	25.0
6	h	14	45.16	84.0	8	25.00	48.0	5	23.81	30.0	12	38.71	72.0
7	H	9	29.03	63.0	12	37.50	84.0	1	4.76	7.0	13	41.94	91.0
-1	d				7	21.88	-7.0						
	Tot	31	100.00	187.0	32	100.00	148.0	21	100.00	111.0	31	100.00	192.0
Devel. Indices				6.0			4.6			5.3			6.2
			p =	0.095					p =	0.001			

	Day6	m+ab	%	Scr				h+ab	%	Scr			
0	2												
1	3-4												
2	5-8												
3	m												
4	e				2	6.25	8.0	1	3.23	4.0			
5	E	7	22.58	35.0				4	12.90	20.0			
6	h	4	12.90	24.0	3	9.38	18.0	4	12.90	24.0	4	12.90	24.0
7	H	20	64.52	140.0	19	59.38	133.0	22	70.97	154.0	25	80.65	175.0
-1	d				8	25.00	-8.0				2	6.45	-2.0
	Tot	31	100.00	199.0	32	100.00	151.0	31	100.00	202.0	31	100.00	197.0
Devel. Indices				6.4			4.7			6.5			6.4
			p =	0.051					p =	0.683			

3-4 = 3-4-cell embryo; 5-8 = 5-8-cell embryo; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocyst; H = completely hatched blastocyst; f/d = fragmenting or degenerating embryos. m = mrLIF; h = hrLIF; ab = anti h-LIF monoclonal antibody; DS = Develpmental Score; p values derived from Cochran-Mantel-Haenszel (CMH) statistics.

APPENDIX iii CONTINUED

Category A, Experiments - 2-6

Developmental Indices, Percents, and Totals

Protocol 3

DS	Day3	m	%	Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2																		
1	3-4																		
2	5-8																		
3	m																		
4	e																		
5	E																		
6	h																		
7	H																		
-1	d																		
	Tot																		
	Day4	m	%	Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2	4	4.21	0.0	2	1.45	0.0	2	3.51	0.0	1	1.00	0.0						
1	3-4	3	3.16	3.0	2	1.45	2.0					0.00							
2	5-8	2	2.11	4.0	2	1.45	4.0										1	0.99	2.0
3	m	5	5.26	15.0	2	1.45	6.0	1	1.75	3.0	2	2.00	6.0	4	6.78	12.0	1	0.99	3.0
4	e	23	24.21	92.0	58	42.03	232.0	15	26.32	60.0	54	54.00	216.0	5	8.47	20.0	49	48.51	196.0
5	E	29	30.53	145.0	32	23.19	160.0	20	35.09	100.0	14	14.00	70.0	27	45.76	135.0	22	21.78	110.0
6	h	21	22.11	126.0	31	22.46	186.0	15	26.32	90.0	24	24.00	144.0	15	25.42	90.0	24	23.76	144.0
7	H	3	3.16	21.0				4	7.02	28.0	3	3.00	21.0						
-1	d	5	5.26	-5.0	9	6.52	-9.0				2	2.00	-2.0	8	13.56	-8.0	4	3.96	-4.0
	Tot	95	100.00	401.0	138	100.00	581.0	57	100.00	281.0	100	100.00	455.0	59	100.00	249.0	101	100.00	451.0
Devel. Indices	▶			4.2			4.2			4.9			4.6			4.2			4.5
			p =	0.965					p =	0.037					p =	0.807			
	Devel	b6	Day 3	Proto															
	Day 5	m	%	Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2																		
1	3-4																		
2	5-8																		
3	m																		
4	e	7	10.45	28.0	1	1.61	4.0	2	6.67	8.0	3	10.34	12.0				1	3.13	4.0
5	E	21	31.34	105.0	15	24.19	75.0	15	50.00	75.0	3	10.34	15.0	11	35.48	55.0	9	28.13	45.0
6	h	13	19.40	78.0	18	29.03	108.0	8	26.67	48.0	12	41.38	72.0	6	19.35	36.0	10	31.25	60.0
7	H	14	20.90	98.0	16	25.81	112.0	5	16.67	35.0	6	20.69	42.0	12	38.71	84.0	11	34.38	77.0
-1	d	12	17.91	-12.0	12	19.35	-12.0				5	17.24	-5.0	2	6.45	-2.0	1	3.13	-1.0
	Tot	67	100.00	297.0	62	100.00	287.0	30	100.00	166.0	29	100.00	136.0	31	100.00	173.0	32	100.00	185.0
Devel. Indices	▶			4.4			4.6			5.5			4.7			5.6			5.8
			p =	0.298					p =	0.607					p =	0.805			
	Devel	b6	Day 3	Proto															
	Day 6	m	%	Scr	c	%	Scr	h	%	Scr	c	%	Scr	ab	%	Scr	c	%	Scr
0	2																		
1	3-4																		
2	5-8																		
3	m																		
4	e	3	4.69	12.0	1	1.61	4.0				4	13.79	16.0						
5	E	15	23.44	75.0	5	8.06	25.0	9	30.00	45.0				5	16.13	25.0	7	21.88	35.0
6	h	3	4.69	18.0	6	9.68	36.0				5	17.24	30.0	3	9.68	18.0	2	6.25	12.0
7	H	28	43.75	196.0	37	59.68	259.0	20	66.67	140.0	15	51.72	105.0	21	67.74	147.0	22	68.75	154.0
-1	d	15	23.44	-15.0	13	20.97	-13.0	1	3.33	-1.0	5	17.24	-5.0	2	6.45	-2.0	1	3.13	-1.0
	Tot	64	100.00	286.0	62	100.00	311.0	30	100.00	184.0	29	100.00	146.0	31	100.00	188.0	32	100.00	200.0
Devel. Indices	▶			4.5			5.0			6.1			5.0			6.1			6.3
			p =	0.124					p =	0.137					p =	0.937			

3-4 = 3-4-cell embryo; 5-8 = 5-8-cell embryo; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocyst; H = completely hatched blastocyst; f/d = fragmenting or degenerating embryos. m = mrLIF; h = hrLIF; ab = anti h-LIF monoclonal antibody; DS = Develpmental Score; p values derived from Cochran-Mantel-Haenszel (CMH) statistics.

APPENDIX iii CONTINUED

Category A, Experiments - 2-6

Developmental Indices, Percents, and Totals

Protocol 3

DS	Day3	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2												
1	3-4												
2	5-8												
3	m												
4	e												
5	E												
6	h												
7	H												
-1	d												
	Tot												
	Day4	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2	5	16.67	0.0				1	3.23	0.0			
1	3-4	4	13.33	4.0									
2	5-8				1	3.23	2.0						
3	m	1	3.33	3.0	1	3.23	3.0				1	3.23	3.0
4	e	1	3.33	4.0	3	9.68	12.0	1	3.23	4.0	1	3.23	4.0
5	E	14	46.67	70.0	7	22.58	35.0	19	61.29	95.0	9	29.03	45.0
6	h	2	6.67	12.0	14	45.16	84.0	10	32.26	60.0	19	61.29	114.0
7	H	1	3.33								1	3.23	7.0
-1	d	2	6.67	-2.0	5	16.13	-5.0						
	Tot	30	100.00	91.0	31	100.00	131.0	31	100.00	159.0	31	100.00	173.0
Devel. Indices	▶			3.0			4.2			5.1			5.6
			p =	0.140					p =	0.056			
	Devel												
	Day 5	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2												
1	3-4												
2	5-8												
3	m												
4	e	2	6.67	8.0	2	6.25	8.0				1	3.23	4.0
5	E	7	23.33	35.0	3	9.38	15.0	5	16.13	25.0	5	16.13	25.0
6	h				8	25.00	48.0	9	29.03	54.0	12	38.71	72.0
7	H	10	33.33	70.0	12	37.50	84.0	15	48.39	105.0	13	41.94	91.0
-1	d	11	36.67	-11.0	7	21.88	-7.0	2	6.45	-2.0			
	Tot	30	100.00	102.0	32	100.00	148.0	31	100.00	182.0	31	100.00	192.0
Devel. Indices	▶			3.4			4.6			5.9			6.2
			p =	0.135					p =	0.795			
	Devel												
	Day 6	m+ab	%	Scr	c	%	Scr	h+ab	%	Scr	c	%	Scr
0	2												
1	3-4												
2	5-8												
3	m												
4	e	2	6.67		2	6.25	8.0						
5	E	2	6.67	10.0									
6	h				3	9.38	18.0	11	35.48	66.0	4	12.90	24.0
7	H	14	46.67	98.0	19	59.38	133.0	18	58.06	126.0	25	80.65	175.0
-1	d	12	40.00	-12.0	8	25.00	-8.0	2	6.45	-2.0	2	6.45	-2.0
	Tot	30	100.00	96.0	32	100.00	151.0	31	100.00	190.0	31	100.00	197.0
Devel. Indices	▶			3.2			4.7			6.1			6.4
			p =	0.133					p =	0.143			

3-4 = 3-4-cell embryo; 5-8 = 5-8-cell embryo; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocyst; H = completely hatched blastocyst; f/d = fragmenting or degenerating embryos. m = mrLIF; h = hrLIF; ab = anti h-LIF monoclonal antibody; DS = Develpmental Score; p values derived from Cochran-Mantel-Haenszel (CMH) statistics.

APPENDIX iv

Category A, Experiments 2 - 6

Day2

Raw Data

Treatment =		m			c			h			c			ab			c			m+ab			c			h+ab			c		
Protocol =	Series	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2-Cell	2&3										4	1					4	1													
	4																														
	5&6	8			1		1	1			1	2	2					3													
	9				4		9	2			1							1				2			7	1					
	10																														1
	12B				1																										
Tot 2		8	0	0	6	0	10	3	0	0	2	6	3	0	0	0	0	4	5	0	0	0	2	0	7	1	0	0	0	0	1
3-4-Cell	2&3	1			1		5	11			3	26	8	3			3	26	8				4	4	6						
	4	12			9			5			9			7			9														
	5&6	7			8	1	5	8			12	10	13	14			3	14	9												
	9				10	5	7	14			13	2	7	14			4		10	1						7			3	5	1
	10				1	1		1					6	4				1		2						4					
	12A	3			1																										
	12B	9			4	4	9																								
Tot 3-4		32	0	0	34	11	26	39	0	0	37	38	34	42	0	0	19	41	27	3	0	0	4	4	6	11	0	0	3	5	1
5-8-Cell	2&3	9			8	12	6	4			6		21	11			6		21												
	4	29			35			29			35			33			35														
	5&6				6	14	10	6			2	4		1			3		2												
	9	9				9	2				1	7	8	1			12	11	5	13			8	11	2	7			9	7	12
	10	5			12	15	13	12			10	16	10	9			14	13	15	9			14	10	8	7			11	16	12
	12A	10			12	14	16																								
	12B	7			10	10	6																								
Tot 5-8		69	0	0	83	74	53	51	0	0	54	27	39	55	0	0	70	24	43	22	0	0	22	21	10	14	0	0	20	23	24
M	2&3	5			6	3	4							1																	
	4	5			2			10			2			5			2														
	9	6				1						6						4		1			1	6	7	1			4	3	3
	10	9			3		2	2			5			2			1	3		4			2			4			4		2
	12A	3			3	2																									
	12B				1	2	1																								
Tot M		28	0	0	15	8	7	12	0	0	7	6	0	8	0	0	3	7	0	5	0	0	3	6	7	5	0	0	8	3	5
f/d	5&6																1														
	9																						1								
	10	2															1	1													
Tot f/d		2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	1	0	0	0	0	0	0	0	0

m = mrLIF; h = hrLIF; ab = anti-hrLIF monoclonal antibody; c = controls; all concentrations = 1000 U/ml; No embryos developed beyond the morulae (m) stage on day 2 of development. M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocysts; H completely hatched blastocysts; f/d = fragmenting or degenerating embryos. Developmental stages without data points for experiments were omited.

APPENDIX iv CONTINUED

Category A, Experiments 2 - 6

Day 3

Raw Data

Treatment =		m			c			h			c			ab			c			m+ab			c			h+ab			c		
Protocol =	Serie	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2-Cell	4				1						1						1														
	5&6				1		1	1					3						3												
	9				3		5	2			1												2		5	1					
	10																														1
Tot 2		0	0	0	5	0	6	3	0	0	2	0	3	0	0	0	1	0	3	0	0	0	2	0	5		0	0	0	0	1
3-4-Cell	2&3							2					1	1					1												
	5&6	1	1					1					2				1		2												
	9				4		8							2									3		6						
	10							2						1			1														
Tot 3-4		1	1	0	4	0	8	5	0	0	0	0	3	4	0	0	2	0	3	0	0	0	3	0	6	0	0	0	0	0	0
5-8-Cell	2&3							2			2			1			2														
	5&6								2				1	5			1														
	9				3		2	6			4								1						1						
	10				2									2						1											
	12B		1																												
Tot 5-8		0	1	0	5	0	2	8	2	0	6	0	1	8	0	0	3	0	1	1	0	0	0	0	1	0	0	0	0	0	0
M	2&3	3	4		3		8	9	15		7		27	10	11		7		27												
	4	39			39			28			39			38			39														
	5&6	14	13		14		14	13	13		15		9	10	14		15		9												
	9	5	11		4		1	8	5		7		14	13	7		12		14	7	14		8		2	11	10		8		7
	10	5	6		8		4	7	5		2		9	8	8		2		5	9	9		1		2	8	2		1		3
	12A	5	9		10		12																								
	12B	13	6		12		13																								
Tot M		84	49	0	90	0	52	65	38	0	70	0	59	79	40	0	75	0	55	16	23	0	9	0	4	19	12	0	9	0	10
e	2&3	12	11		12		7	2					2	3	4				2												
	4	6			6			16			6			8			6														
	5&6																														
	9	10	4						10		3		1		8		4			8	1		2		13	4	5		8		9
	10	10	10		8		11	6	11		13		6	4	9		10		10	5	7		15			7	14		14		11
	12A	11	7		6		4																								
	12B	3	9		4		3																								
Tot e		52	41	0	36	0	25	24	21	0	22	0	9	15	21	0	20	0	12	13	8	0	17	0	13	11	19	0	22	0	20
f/d	9						2												1				1		1						
	10	1													1		3														
	12A																														
	12B																														
Tot f/d		1	0	0	0	0	2	0	0	0	0	0	0	0	1	0	3	0	1	0	0	0	1	0	1	0	0	0	0	0	0

m = mrLIF; h = hrLIF; ab = anti-hrLIF monoclonal antibody; c = controls; all concentrations = 1000 U/ml; No embryos developed beyond the early blastocyst stage on day 3 of development. M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocysts; H completely hatched blastocysts; f/d = fragmenting or degenerating embryos. Developmental stages without data points for experiments were omited.

APPENDIX iv CONTINUED

Category A, Experiments 2 - 6

Day 4

Raw Data

Treatment =		m				c		h				c		ab				c		m+ab				c		h+ab				c	
Protocol =	Series	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2-Cell	2&3									1																					
	4									1																					
	9			4	2			2			1											5				1					
	10																											1			
Tot 2		0	0	4	2	0	0	2	0	2	1	0	0	0	0	0	0	0	0	0	0	5	0	0	0		0	1	0	0	0
3-4-Cell	9			3	2									1								4									
	10													1																	
Tot 3-4		0	0	3	2	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
5-8-Cell	2&3							1																							
	9			2	2			2														1									
	10							1						3			1														
	12B		1																												
Tot 5-8		0	1	2	2	0	0	4	0	0	0	0	0	3	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
M	2&3							1			1				2			1													
	5&6			1	1			2						5	1	4															
	9		1	1							1									1		1	1			3			1		
	10		1					2		1																					
	12A	1	1	1																											
	12B		1	2	1																										
Tot M		1	4	5	2	0	0	5	0	1	2	0	0	5	3	4	1	0	0	1	0	1	1	0	0	3	0	0	1	0	0
e	2&3	6	7	3	9																										
	4	31			34			20			34			40			34														
	5&6	15	13	15	13			13	12	10	14			10	13	5	15														
	9		2					7		3	6			6	2					3	2		3			1	2	1	1		
	10	1		1				1		2					1							1				5					
	12A	4	1	1	1																										
	12B	6		3	1																										
Tot e		63	23	23	58	0	0	41	12	15	54	0	0	56	16	5	49	0	0	3	2	1	3	0	0	6	2	1	1	0	0
E	2&3							8	11	5	6			12	9	8	6														
	9	9	12	2	1			5	10	5	7			7	6	11	11			6	9	3	3			7	10	9	5		
	10	11	12	5	7			8	10	10	1			9	9	8	5			10	11	11	4			10	13	10	4		
	12A	10	11	13	12																										
	12B	9	12	9	12																										
Tot E		39	47	29	32	0	0	21	31	20	14	0	0	28	24	27	22	0	0	16	20	14	7	0	0	17	23	19	9	0	0
h	2&3	8	8	12	6			2	4	9				2	3	6															
	4	13			10			8			10			4			10														
	9	6			1				5	4					7	2	5			5	4		2			2	3	6	9		
	10	4	3	6	9			3	6	2	14			2	7	7	9			4	4	2	12				2	4	10		
	12A	1	3	1	3																										
	12B	1	2	2	2																										
Tot h		33	16	21	31	0	0	13	15	15	24	0	0	8	17	15	24	0	0	9	8	2	14	0	0	2	5	10	19	0	0
H	2&3	1									2																				
	5&6								3	4	1																				
	10			3																	1	1					1		1		
Tot H		1	0	3	0	0	0	0	3	4	3	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	0	0
f/d	2&3							3						1		1															
	4	1			2						2			2			2														
	5&6		1		1											5	2														
	9			4	6									1		2						2	5			2					
	10			1											1					1											
Tot f/d		1	1	5	9	0	0	3	0	0	2	0	0	4	1	8	4	0	0	1	0	2	5	0	0	2	0	0	0	0	0

m = mrLIF; h = hrLIF; ab = anti-hrLIF monoclonal antibody; c = controls; all concentrations = 1000 U/ml; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocysts; H completely hatched blastocysts; f/d = fragmenting or degenerating embryos. Developmental stages without data points for experiments were omited.

APPENDIX iv CONTINUED

Category A, Experiments 2 - 6

Day 5

Raw Data

Treatment=		m			c			h			c			ab			c			m+ab			c			h+ab			c		
Protocol =	Serie	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
e	9		1	4	1			5			2			4						1				2					1			1	
	10									2	1			1	1		1			1				2					2			
	12A		3	3																											
	12B	1																													
Tot e		1	4	7	1	0	0	5	0	2	3	0	0	5	1	0	1	0	0	2	0	2	2	0	0	2	1	0	1	0	0
E	9		6	3				5	5	7	1			5	3	9	6			1	2	4	3			7	8	1	3		
	10	2	8	2	1			4	5	8	2			5	4	2	3			3	6	3				5	6	4	2		
	12A	9	3	5	8																										
	12B	7	5	11	6																										
Tot E		18	22	21	15	0	0	9	10	15	3	0	0	10	7	11	9	0	0	4	8	7	3	0	0	12	14	5	5	0	0
h	9	2	5		2				7	4	4			4	5	1	5			2	8		1			1	3	4	7		
	10	4	6	5	7			6	6	4	8			4	4	5	5			4	6		7			1	2	5	5		
	12A	5	7	5	2																										
	12B	5	4	3	7																										
Tot h		16	22	13	18	0	0	6	13	8	12	0	0	8	9	6	10	0	0	6	14	0	8	0	0	2	5	9	12	0	0
H	9	13	2					1	3	4	2				7	4	5			10	5		3			4	3	10	5		
	10	9	1	8	8			3	5	1	4			3	8	8	6			6	4	10	9			5	8	5	8		
	12A	2	3	4	6																										
	12B	3	6	2	2																										
Tot H		27	12	14	16	0	0	4	8	5	6	0	0	3	15	12	11	0	0	16	9	10	12	0	0	9	11	15	13	0	0
f/d	9		1	11	11			5			5			2		2				1		11	7			4		1			
	10	1	1	1				2						2	1		1			1						2		1			
	12A																														
	12B		1		1																										
Tot f/d		1	3	12	12	0	0	7	0	0	5	0	0	4	1	2	1	0	0	2	0	11	7	0	0	6	0	2	0	0	0

m = mrLIF; h = hrLIF; ab = anti-hrLIF monoclonal antibody; c = controls; all concentrations = 1000 U/ml; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocysts; H completely hatched blastocysts; f/d = fragmenting or degenerating embryos. Developmental stages without data points for experiments were omited.

APPENDIX iv CONTINUED

Category A, Experiments 2 - 6

Day 6

Raw Data

Tratment =		m				c		h				c		ab				c		m+ab				c		h+ab				c	
Protocol =	Serie	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
e	9		2	2				6	1		4			4									2			1			1			
	10				1																	2				2	1				
	12A		2	1																											
	12B	1																													
Tot e		1	4	3	1	0	0	6	1	0	4	0	0	4	0	0	0	0	0	0	0	2	2	0	0	3	1	1	0	0	0
E	9		3					3	1	4				5	1	4	5			1	3	2				1	4				
	10	1	2	2				3	2	5				4	2	1	2			2	4					1		3			
	12A	4	3	5	2																										
	12B	3	1	8	3																										
Tot E		8	9	15	5	0	0	6	3	9	0	0	0	9	3	5	7	0	0	3	7	2	0	0	0	2	4	3	0	0	0
h	9	1	1					2						2						1	2						1	3	3		
	10	1	2	1	1			3		5				1	3	3	2			1	2		3			1	3	4	1		
	12A	1	1	2	2																										
	12B		1		3																										
Tot h		3	5	3	6	0	0	0	5	0	5	0	0	1	5	3	2	0	0	2	4	0	3	0	0	1	4	7	4	0	0
H	9	14	8		2			1	11	11	5			4	12	10	11			12	10	1	6			10	10	11	11		
	10	14	10	12	14			9	10	9	10			8	13	11	11			11	10	13	13			9	12	7	14		
	12A	11	10	8	12																										
	12B	11	12	8	9																										
Tot H		50	40	28	37	0	0	10	21	20	15	0	0	12	25	21	22	0	0	23	20	14	19	0	0	19	22	18	25	0	0
f/d	9		1	14	12			6			5			2		2				1			12	8			4			1	2	
	10		2	1				3	1	1				2			1			1							2			1		
	12A																														
	12B	1	2		1																										
Tot f/d		1	5	15	13	0	0	9	1	1	5	0	0	4	0	2	1	0	0	2	0	12	8	0	0	6	0	2	2	0	0

m = mrLIF; h = hrLIF; ab = anti-hrLIF monoclonal antibody; c = controls; all concentrations = 1000 U/ml; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocysts; H completely hatched blastocysts; f/d = fragmenting or degenerating embryos. Developmental stages without data points for experiments were omited.

APPENDIX iv CONTINUED

Category A, Experiments 2 - 6

Day 7

Raw Data

Treatment =		m			c			h			c			ab			c			m+ab			c			h+ab			c		
Protocol =	Serie	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
e	9		4												1	2											1				
	10							2	3					2			1				1		1			2		2			
	12A		2	1																											
	12B	1																													
Tot e		1	6	1	0	0	0	2	3	0	0	0	0	2	1	2	1	0	0	0	1	0	1	0	0	2	1	2	0	0	0
E	9		1											1	1	1					1						2				
	10	2		2				3		5				1	2	1				1	1					1		3			
	12A	4	3	5	2																										
	12B	3	1	8	3																										
Tot E		9	5	15	5	0	0	3	0	5	0	0	0	2	3	2	0	0	0	1	2	0	0	0	0	1	2	3	0	0	0
h	9	1							1						3						1						1				
	10		1	1	1				1		6			1	2	2	2			1	1	1	2				1		1			
	12A	1	1	2	2																										
	12B		1		3																										
Tot h		2	3	3	6	0	0	0	2	0	6	0	0	1	5	2	2	0	0	1	2	1	2	0	0	0	2	0	1	0	0
H	9	14	9					1	13	11				3	9	10	12				13						11				
	10	13	12	13	15			8	11	9	10			8	13	11	13			12	12	13	13			11	15	9	14		
	12A	11	10	8	12																										
	12B	11	12	8	9																										
Tot H		49	43	29	36	0	0	9	24	20	10	0	0	11	22	21	25	0	0	12	25	13	13	0	0	11	26	9	14	0	0
f/d	9		1	16	14			15	1	4	14			11	1	3	4			15		15	16			16		16	16		
	10	1	3					2	1	1				3	1	1				1	1	1				1		1			
	12A																														
	12B	1	2		1																										
Tot f/d		2	6	16	15	0	0	17	2	5	14	0	0	14	2	4	4	0	0	16	1	16	16	0	0	17	0	17	16	0	0

m = mrLIF; h = hrLIF; ab = anti-hrLIF monoclonal antibody; c = controls; all concentrations = 1000 U/ml; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocysts; H completely hatched blastocysts; f/d = fragmenting or degenerating embryos. Developmental stages without data points for experiments were omited.

APPENDIX v

Category A, Experiments 7
Developmental Indices, Percents, and Totals
Protocol 1 Only

	Day 3	1000			2000			5000			10000					
DI	Trt =	mrLIF	%	Scr	mrLIF	%		mrLIF	%	Scr	mrLIF	%	Scr	c	%	Scr
0	2				1	1.67	0.0									
1	3-4	5	8.33	5.0	3	5.00	3.0	5	8.33	5.0				10	15.38	10.0
2	5-8	3	5.00	6.0	1	1.67	2.0	2	3.33	4.0	4	6.67	8.0	11	16.92	22.0
3	M	49	81.67	147.0	49	81.67	147.0	44	73.33	132.0	50	83.33	150.0	34.0	52.31	102.0
4	e	3	5.00	12.0	6	10.00	24.0	9	15.00	36.0	6	10.00	24.0	5.0	7.69	20.0
-1	d													5.0	7.69	-5.0
	Tot	60	100.00	170.0	60	100.00	176.0	60	100.00	177.0	60	100.00	182.0	65	100.00	149.0
Devel. Indices			▶	2.8			2.9			3.0			3.0			2.3
			p =	0.005		p =	0.001		p =	0.001		p =	0.001			
	Day 4															
			%	Scr		%	Scr		%	Scr		%	Scr		%	Scr
0	2															
1	3-4													7	11.67	7.0
2	5-8	1	1.67	2.0										10	16.67	20.0
3	M	5	8.33	15.0	4	6.67	12.0	7	11.67	21.0	4	6.67	12.0	12.0	20.00	36.0
4	e	13	21.67	52.0	22	36.67	88.0	20	33.33	80.0	19	31.67	76.0	19.0	31.67	76.0
5	E	29	48.33	145.0	24	40.00	120.0	18	30.00	90.0	25	41.67	125.0	12.0	20.00	60.0
6	h	8	13.33	48.0	5	8.33	30.0	12	20.00	72.0	12	20.00	72.0			
7	H	1	1.67	7.0	3	5.00	21.0									
-1	d	3	5.00	-3.0	2	3.33	-2.0	3	5.00	-3.0						
	Tot	60	100.00	266.0	60	100.00	269.0	60	100.00	260.0	60	100.00	285.0	60	100.00	199.0
Devel. Indices			▶	4.4			4.5			4.3			4.8			3.3
			p =	0.001		p =	0.001		p =	0.001		p =	0.001			
	Day 5															
			%	Scr		%	Scr		%	Scr		%	Scr		%	Scr
4	e	8	13.33	32.0	13	21.67	52.0	5	8.33	20.0	10	16.67	40.0	11	18.33	44.0
5	E	21	35.00	105.0	14	23.33	70.0	11	18.33	55.0	17	28.33	85.0	19	31.67	95.0
6	h	12	20.00	72.0	16	26.67	96.0	17	28.33	102.0	18	30.00	108.0	11	18.33	66.0
7	H	16	26.67	112.0	11	18.33	77.0	18	30.00	126.0	14	23.33	98.0	11	18.33	77.0
-1	d	3	5.00	-3.0	6	10.00	-6.0	9	15.00	-9.0	1	1.67	-1.0	8	13.33	-8.0
	Tot	60	100.00	318.0	60	100.00	289.0	60	100.00	294.0	60	100.00	330.0	60	100.00	274.0
Devel. Indices			▶	5.3			4.8			4.9			5.5			4.6
			p =	0.077		p =	0.615		p =	0.105		p =	0.034			
	Day 6															
			%	Scr		%	Scr		%	Scr		%	Scr		%	Scr
4	e	4	6.67	16.0	6	10.00	24.0	3	5.00	12.0	3	5.00	12.0	3	5.00	12.0
5	E	9	15.00	45.0	4	6.67	20.0	5	8.33	25.0	10	16.67	50.0	14	23.33	70.0
6	h	4	6.67	24.0	3	5.00	18.0	24	40.00	144.0	5	8.33	30.0	10	16.67	60.0
7	H	37	61.67	259.0	38	63.33	266.0	17	28.33	119.0	41	68.33	287.0	24	40.00	168.0
-1	d	6	10.00	-6.0	9	15.00	-9.0	11	18.33	-11.0	1	1.67	-1.0	9	15.00	-9.0
	Tot	60	100.00	338.0	60	100.00	319.0	60	100.00	289.0	60	100.00	378.0	60	100.00	301.0
Devel. Indices			▶	5.6			5.3			4.8			6.3			5.0
			p =	0.110		p =	0.277		p =	0.798		p =	0.002			

3-4 = 3-4-cell embryo; 5-8 = 5-8-cell embryo; M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocyst; H = completely hatched blastocyst; f/d = fragmenting or degenerating embryos. m = mrLIF; p values derived from Cochran-Mantel-Haenszel (CMH) statistics. Developmental stages without data points for experiments were omitted.

APPENDIX vi

Category A, Experiment 7

Raw Data

Devel Day 2	Trt =	1	2	5	10	c
	Exp					
2-Cell	1	2	2	1		1
	2			1	1	3
	3		1	3		1
	4		1		1	
3-4 Cell	1	10	9	12	12	12
	2	12	15	12	12	8
	3	14	13	10	15	11
	4	13	13	13	15	13
5-8 Cell	1	3	3	2	3	3
	2	3	1	2	2	4
	3	1	1	2	1	3
	4	2	1	2		2
M	1	1				
	2					
	3					
	4					
e	1					
	2					
	3					
	4					
E	1					
	2					
	3					
	4					
h	1					
	2					
	3					
	4					
H	1					
	2					
	3					
	4					
d	1					
	2					
	3					
	4					
Tot		61	60	60	62	61

Devel Day 3		1	2	5	10	c
	Exp					
2-Cell	1		1			
	2					
	3					
	4		1			
3-4 Cell	1					2
	2	2	3	2		4
	3	1		3		3
	4	2			1	1
5-8 Cell	1	1			3	3
	2	1				2
	3			1		3
	4	1	1	1	1	3
M	1	14	14	15	11	9
	2	12	9	10	13	9
	3	12	14	7	14	8
	4	11	12	12	13	8
e	1		1		1	1
	2		3	3	2	
	3	2	1	4	2	1
	4	1	1	2	1	3
E	1					
	2					
	3					
	4					
h	1					
	2					
	3					
	4					
H	1					
	2					
	3					
	4					
d	1					
	2					
	3					
	4					
Tot		60	61	60	62	60

Devel Day 4		1	2	5	10
	Exp				
2-Cell	1				
	2				
	3				
	4				
3-4 Cell	1				
	2				
	3				
	4				
5-8 Cell	1	1			
	2				
	3				
	4				
M	1	3	1	4	
	2		1	2	3
	3			1	1
	4	2	2		
e	1	1	2	2	1
	2	6	6	6	3
	3		5	3	2
	4	3	4	3	8
E	1	4	3	1	3
	2	6	7	5	5
	3	12	7	4	9
	4	7	7	8	8
h	1	1	1	2	5
	2	3		2	4
	3	3	3	5	3
	4	1	1	3	
H	1		1		
	2		1		
	3				
	4	1	1		
d	1	2	2		
	2		1		
	3			2	
	4	1		1	
Tot		57	56	54	55

M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocyst;
H = completely hatched blastocyst; f/d = fragmenting or degenerating embryos; m = mrLIF;
c = control. 1 = 1000; 2 = 2000; 5 = 5000; 10 = 10000; all values in U/ml mrLIF; c = control

APPENDIX vi CONTINUED

Category A, Experiment 7

Raw Data

Devel Day 5		1	2	5	10	c
	Exp					
2-Cell	1					
	2					
	3					
	4					
3-4 Cell	1					
	2					
	3					
	4					
5-8 Cell	1					
	2					
	3					
	4					
M	1					
	2					
	3					
	4					
e	1	4	5	2	2	3
	2	3	3	1	3	5
	3		2	1	1	
	4	1	3	1	4	3
E	1	6	2	2	42	4
	2	6	7	2	5	4
	3	5	3	3	3	7
	4	4	2	4	5	5
h	1	2	5	4	5	2
	2	1	1	5	4	4
	3	6	5	4	7	3
	4	3	5	4	2	2
H	1	2	2	3	4	1
	2	5	3	5	2	2
	3	4	3	5	4	5
	4	5	3	5	4	2
d	1	1	1	4		5
	2		1	2	1	
	3		2	2		
	4	2	2	1		3
Tot		60	60	60	98	60

Devel Day 6		1	2	5	10	c
	Exp					
2-Cell	1					
	2					
	3					
	4					
3-4 Cell	1					
	2					
	3					
	4					
5-8 Cell	1					
	2					
	3					
	4					
M	1					
	2					
	3					
	4					
e	1	3	5			2
	2	1		1	2	1
	3			3	1	
	4		1			
E	1	1	2	2	4	4
	2	4	1	1	3	6
	3	2	1			2
	4	2		2	4	2
h	1				2	2
	2	1	2			2
	3	2	1		2	3
	4	1		2	1	3
H	1	13	5	12	14	2
	2	9	10	11	9	6
	3	10	11	11	12	9
	4	10	12	10	11	7
d	1	3	3	6		5
	2		2	2	1	
	3	1	2	2		1
	4	2	2	1		3
Tot		65	60	66	66	60

Devel Day 7		1	2	5	10	c
	Exp					
2-Cell	1					
	2					
	3					
	4					
3-4 Cell	1					
	2					
	3					
	4					
5-8 Cell	1					
	2					
	3					
	4					
M	1					
	2					
	3					
	4					
e	1	1	2			3
	2			1	2	
	3					
	4		1	2	3	
E	1		2		1	1
	2	2				1
	3		1			1
	4	1				1
h	1				1	1
	2	1	1			2
	3					1
	4	1				1
H	1	10	8	9	13	3
	2	11	13	12	12	10
	3	14	13	11	15	12
	4	10	13	12	12	7
d	1	4	3	6		7
	2	1	1	2	1	2
	3	1	1	3		1
	4	3	1	1		6
Tot		60	60	59	60	60

M = morulae; e = early blastocyst; E = expanded blastocyst; h = hatching blastocyst;
H = completely hatched blastocyst; f/d = fragmenting or degenerating embryos; m = mrLIF;
c = control. 1 = 1000; 2 = 2000; 5 = 5000; 10 = 10000; all values in U/ml mrLIF; c = control

APPENDIX vii

CATEGORY B EXPERIMENTS
IMPLANTATION, PREGNANCY, AND RESORPTION RATES
RAW DATA

	Controls											P	P	P			R
Exp	Recip	Trt	Conc	# Fem	#PG Fem	#Em	Age	Hrn	Body M	CR	TL	L	W	M	# R	Hrn	Di
6	M5	1	5000	0													
6	M6			0													
7	M1	1	5000	0													
8	M1	1	5000	0													
8	M2			2	2	1	17	R	0.34	15	4	11	9	0.2	1	R	3
						2	17	R	0.35	17	5	13	8	0.2			
8	M3			0													
9	M1	1	5000	0													
9	M2			1	1	1	17	L	0.7	20	8	10	10	0.2	3	L	4
																L	3
																L	6
9	M3			2	2	1	17	L	0.63	20	8	11	9	0.2	4	L	2
						2	17	L	0.73	20	8	10	8	0.2		L	3
																L	3
																L	4
10	M1	1	5000	0													
10	M2			2	2	1	17	R	0.72	21	8	11	10	0.3	6	L	3
						2	17	R	0.62	19	8	10	10	0.2		L	3
																L	3
																L	3
																R	3
																R	3
11	M1	1	5000	0													
12	M1	1	5000	0													
	M2			0													
	M3			0													
13	M1	1	5000	0													
	M2			0													
	M3			4	4	1	17	L	0.76	20	8	9	9	0.1			
						2			0.48	18	7	9	9	0.1			
						3			0.69	21	8	10	10	0.2			
						4			0.49	18	7	10	9	0.1			
14	M4	1	5000	0													
	M5			0											3	R	2
																R	6
																R	4
	M6			1	1	1	17	L	0.87	20	9	9	9	0.1	3	L	7
																L	2
																L	4
15	M1	1	5000	0													
16	M1	1	5000	0													
	M2			3	3	1	17	R	0.39	16	6	9	9	0.2	1	R	5
						2	17	R	0.45	16	6	9	9	0.1			
						3	17	R	0.53	18	7	9	10	0.2			
17	M1	1	5000	0													
	M2			0													
	M3			0													

Fem = # females included in experiment; # PG Fem = # females with fetuses; # em = numerical assignment to embryos within same female; age = fetal age in days of development; Hrn = right (R) or left (L) uterine horn w/ fetuses; Body M = body mass of fetuses; CR = Crown-rump length; TL = tail length; PL = placental length; PW = placental width; PM = placental mass. TOT # R = total # resorptions; NPG R = # resorptions in females with no viable pups; PGR = # resorptions in females with viable pups; R Di = diameter of resorptions. (all lengths in millimeters, all masses in grams)

APPENDIX vii CONTINUED

CATEGORY B EXPERIMENTS

IMPLANTATION, PREGNANCY, AND RESORPTION RATES

RAW DATA

	LIF											P	P	P			R
Exp	Recip	Trt	Conc	# Fem	#PG Fem	#Em	Age	Hrn	Body M	CR	TL	L	W	M	# R	Hrn	Di
6	M3	2	5000	0											2	L	3
6	M3	2	5000	0												L	3
7	M2	2	5000	0											1	R	8
8	M6	2	5000	1	1	1	17	R	0.47	18	6	13	10	0.27	4	L	3
																R	3
9	M4	2	5000	0												R	3
9	M5	2	5000	0												R	3
9	M6	2	5000	0													
9	M7	2	5000	6	6	1	17	L	0.37	17	5	13	10	0.36	2	L	3
						2	17	L	0.42	17	6					L	3
						3	17	L	0.37	15	6	9	8	0.14		L	4
						4	17	L	0.41	15	6	8	8	0.15			
						5	17	L	0.46	18	7	10	8	0.18			
						6	17	L	0.33	14	5	10	9	0.17			
10	M3	2	5000	1	1	1	17	L	0.44	17	7	11	10	0.18			
10	M4	2	5000	1	1	1	17	R	0.38	16	6	10	8	0.15	2	L	2
																R	2
10	M5	2	5000	1	1	1	17	R	0.53	19	7	9	9	0.18	3	R	4
																R	3
																R	1
11	M2	2	5000	3	3	1	17	L	0.69	19	8	8	8	0.10	4	L	3
						2	17	L	0.54	18	9	10	10	0.15		L	3
						3	17	L	0.63	20	9	9	8	0.11		L	3
																L	5
11	M3	2	5000	3	3	1	17	L	0.66	21	9	8	7	0.13			
						2	17	L	0.60	18	8	10	10	0.20			
						3	17	L	0.76	22	9	11	11	0.21			
12	M5	2	5000	2	2	1	17	L	0.53	19	7	9	9	0.23			
						2	17		0.60	19	8	11	11	0.24			
13	M4	2	5000	2	2	1	17	R	0.72	21	9	11	11	0.17			
						2	17	R	0.72	21	9	9	9	0.14			
	M5	2	5000	3	3	1	17	L	0.69	20	9	10	10	0.18	1	L	2
						2	17	L	0.58	19	7	9	9	0.18			
						3	17	L	0.74	21	8	11	11	0.17			
14	M2	2	5000	2	2	1	17	R	0.28	13	5	10	10	0.14	2	R	4
						2	17	R	0.29	14	4	10	12	0.20		R	4
	M3	2	5000	4	4	1	17	R	0.66	18	8	9	9	0.13	1	R	2
						2	17	R	0.65	20	7	9	9	0.16			
						3	17	R	0.51	18	6	10	11	0.21			
						4	17	R	0.63	19	8	9	9	0.14			
16	M3	2	5000	5	5	1	17	R	0.47	16	6	8	9	0.16	2	R	6
						2	17	R	0.52	17	6	9	9	0.16		R	2
						3	17	R	0.50	16	7	9	9	0.18			
						4	17	R	0.55	17	6	8	9	0.16			
						5	17	R	0.53	17	8	9	8	0.20			
	M4	2	5000	4	4	1	17	L	0.58	17	6	9	11	0.18	2	L	2
						2	17	L	0.49	16	7	8	11	0.17		L	2
						3	17	L	0.57	18	7	8	10	0.18			
						4	17	L	0.57	17	7	9	9	0.14			
17	M4	2	5000	5	5	1	17	L	0.48	17	5	8	8	0.10	1	L	2
						2	17	L	0.42	14	4	9	9	0.14			
						3	17	L	0.53	17	7	8	8	0.14			
						4	17	L	0.44	15	6	8	8	0.15			
						5	17	L	0.38	15	6	9	9	0.13			

Fem = # females included in experiment; # PG Fem = # females with fetuses; # em = numerical assignment to embryos within same female; age = fetal age in days of development; Hrn = right (R) or left (L) uterine horn w/ fetuses; Body M = body mass of fetuses; CR = Crown-rump length; TL = tail length; PL = placental length; PW = placental width; PM = placental mass. TOT # R = total # resorptions; NPG R = # resorptions in females with no viable pups; PGR = # resorptions in females with viable pups; R Di = diameter of resorptions. (all lengths in millimeters, all masses in grams)

APPENDIX vii CONTINUED

CATEGORY B EXPERIMENTS

IMPLANTATION, PREGNANCY, AND RESORPTION RATES

RAW DATA

Exp	AB Recip	Conc	# Fem	#PG Fem	#Em	Age	Hm	Bod M	CR	TL	P L	P W	P M	# R	Hm	R DI
6	M1	5000	0													
6	M2	5000	0											1	L	5
7	M3	5000	0													
8	M7	5000	0													
8	M8	5000	0													
9	M9	5000	0													
9	M10	5000	0													
9	M11	5000	0													
10	M6	5000	0													
10	M7	5000	0													
10	M8	5000	0													
11	M4	5000	0													
12	M6	5000	0													
	M7	5000	0													
	M8	5000	0													
13	M6	5000	4		1	17	R	0.40	16	6	8	8	0.13			
					2	17	R	0.50	18	7	10	10	0.21			
					3	17	R	0.34	16	7	9	8	0.16			
					4	17	R	0.38	16	7	9	8	0.11			
	M7	5000	0											1	L	2
14	M7	5000	0													
	M8		0													
	M9		0													
15	M3	5000	0													
16	M5	5000	0													
	M6		0													
17	M7	5000	0													
	M8		3		1	17	L	0.54	17	7	9	10	0.17	2	L	2
					2	17	L	0.49	17	7	13	9	0.32		L	2
					3	17	L	0.47	16	7						
	M9		0													

Fem = # females included in experiment; # PG Fem = # females with fetuses; # em = numerical assignment to embryos within same female; age = fetal age in days of development; Hm = right (R) or left (L) uterine horn w/ fetuses; Body M = body mass of fetuses; CR = Crown-rump length; TL = tail length; PL = placental length; PW = placental width; PM = placental mass. TOT # R = total # resorptions; NPG R = # resorptions in females with no viable pups; PGR = # resorptions in females with viable pups; R Di = diameter of resorptions. (all lengths in millimeters, all masses in grams)

APPENDIX viii

CATEGORY C EXPERIMENTS
SKELETAL DEVELOPMENT AND LIF
RAW DATA

ET	Grp	II		Clear				at L13	% OS			
Exp	M#	Trt	Em#	H-Ln	H-Os	HO/L	Ribs	V Spc	Excocc	S-Ln	S-Os	SO/L
8	2	C	a	2.25	0.50	0.22	13	1.25	50	1.75	0.38	0.21
8	2	C	b	2.44	0.69	0.28	.	1.00	20	1.75	0.50	0.29
8	6	L	a	3.00	1.13	0.38	13	0.63	100	2.50	1.00	0.40
9	2	C	a	3.75	1.50	0.40	13	0.50	100	2.88	1.50	0.52
9	3	C	a	3.13	1.13	0.36	13	0.50	100	2.75	1.38	0.50
9	3	C	b	3.50	1.13	0.32	13	0.50	100	2.75	1.25	0.45
9	7	L	a	2.75	0.75	0.27	13	0.75	100	2.25	0.75	0.33
9	7	L	b	2.63	0.75	0.29	13	0.63	100	1.63	0.75	0.46
9	7	L	c	3.00	0.75	0.25	13	1.00	100	2.58	0.63	0.24
9	7	L	d	2.50	0.38	0.15	13	1.25	100	1.88	0.63	0.34
9	7	L	e	2.25	0.63	0.28	13	1.13	100	1.63	0.75	0.46
9	7	L	f	2.75	0.81	0.29	13	0.88	100	2.25	0.75	0.33
10	2	C	a	3.13	1.00	0.32	13	0.63	100	2.63	1.13	0.43
10	2	C	b	3.25	1.25	0.38	13	0.63	100	2.13	1.38	0.65
10	3	L	a	2.75	0.75	0.27	13	1.00	100	2.13	0.75	0.35
10	4	L	a	3.00	0.88	0.29	13	0.75	100	2.25	0.88	0.39
10	5	L	a	3.25	1.00	0.31	13	0.88	100	2.25	1.00	0.44
11	2	L	a	2.63	1.25	0.48	13	1.00	.	3.00	1.50	0.50
11	2	L	b	2.50	1.00	0.40	13	0.38	70	2.50	1.00	0.40
11	2	L	c	3.50	1.13	0.32	13	0.88	.	1.75	1.00	0.57
11	3	L	a	3.00	1.00	0.33	13	0.63	100	2.50	1.13	0.45
11	3	L	b	3.50	1.25	0.36	13	0.63	100	2.75	1.25	0.45
11	3	L	c	3.00	1.00	0.33	13	0.75	100	2.25	1.00	0.44
12	5	L	a	3.50	1.00	0.29	13	0.75	100	2.50	1.00	0.40
12	5	L	b	3.13	0.88	0.28	13	0.75	100	3.28	1.00	0.30
13	3	C	a	3.00	1.00	0.33	13	1.25	100	2.25	1.13	0.50
13	3	C	b	3.00	1.38	0.46	13	0.50	100	2.63	1.13	0.43
13	3	C	c	3.25	0.75	0.23	13	0.75	100	2.50	1.00	0.40
13	3	C	d	3.38	1.50	0.44	13	0.50	100	2.75	1.00	0.36
13	4	L	a	3.00	1.00	0.33	13	1.25	100	2.75	1.25	0.45
13	4	L	b	3.25	1.00	0.31	13	0.75	100	2.75	1.25	0.45
13	5	L	a	3.50	1.63	0.47	13	0.88	100	3.00	1.50	0.50
13	5	L	b	3.25	1.25	0.38	13	1.00	100	2.63	1.25	0.48
13	6	A	a	2.50	0.50	0.20	13	1.00	100	2.13	0.50	0.23
13	6	A	b	2.25	0.38	0.17	13	1.50	20	1.75	0.38	0.21
13	6	A	c	3.00	0.75	0.25	13	0.88	80	2.25	0.88	0.39
13	6	A	d	2.50	0.63	0.25	13	1.00	70	2.00	0.56	0.28
14	3	L	a	3.25	1.00	0.31	13	0.50	100	3.00	1.00	0.33
14	3	L	c	3.38	1.00	0.30	13	0.50	100	2.63	1.13	0.43
14	3	L	d	3.25	1.00	0.31	13	0.75	100	2.50	1.00	0.40
14	6	C	a	4.00	1.88	0.47	13	1.25	100	3.13	1.75	0.56
16	2	C	a	3.00	1.00	0.33	13	0.75	100	2.50	1.00	0.40
16	2	C	b	2.75	0.75	0.27	13	1.00	100	2.25	0.88	0.39
16	2	C	c	2.75	0.63	0.23	13	0.75	30	2.13	0.63	0.29
16	3	L	a	2.88	0.75	0.26	13	1.00	100	2.00	1.00	0.50
16	3	L	b	3.00	1.25	0.42	13	0.75	100	2.50	1.25	0.50
16	3	L	d	3.00	0.88	0.29	13	0.63	100	2.50	1.00	0.40
16	3	L	e	2.75	1.00	0.36	13	0.88	80	2.25	0.75	0.33
16	4	L	a	3.00	0.75	0.25	13	1.00	70	2.50	1.00	0.40
16	4	L	b	2.88	0.50	0.17	13	0.88	30	2.25	0.38	0.17
16	4	L	c	3.25	1.00	0.31	13	0.75	90	2.50	1.00	0.40
16	4	L	d	3.13	0.88	0.28	13	1.00	30	2.50	0.88	0.35
17	4	L	a	3.00	0.75	0.25	13	1.00	20	2.25	0.63	0.28
17	4	L	c	2.88	0.75	0.26	13	0.88	30	2.25	0.63	0.28
17	4	L	d	2.75	0.50	0.18	13	1.00	40	2.00	0.38	0.19
17	4	L	e	2.75	0.63	0.23	13	0.88	40	2.13	0.63	0.29
17	8	A	a	3.13	0.63	0.20	13	0.88	50	2.38	0.63	0.26
17	8	A	b	3.00	0.63	0.21	14	0.75	30	2.50	0.50	0.20
17	8	A	c	3.00	0.63	0.21	13	0.75	40	2.25	0.50	0.22
18	2	L	a	3.13	0.75	0.24	13	0.75	10	2.25	0.50	0.22

H-Ln = total length of humerus
H-Os = length of diaphyseal ossification center in humerus
HO/L = index of H-Os/H-Ln
Ribs = number of ribes
V Spc = dorsal space between vertebral pedicles
Exocc = estimated percent of ossification in exoccipital bone
S-Ln = total length of scapula
S-Os = length of ossification center in scapula
SO/L = index of S-Os/S-In

VITAE

MICHAEL HAYES MITCHELL

REPRODUCTIVE ENDOCRINOLOGY/PHYSIOLOGY GROSS HUMAN ANATOMY

OLD DOMINION UNIVERSITY, DEPARTMENT OF BIOLOGICAL SCIENCES AND
THE JONES INSTITUTE FOR REPRODUCTIVE MEDICINE EASTERN VIRGINIA MEDICAL SCHOOL NORFOLK, VIRGINIA, USA.

MILITARY EXPERIENCE: U. S. Navy, 1970 - 1976

EDUCATION:

6/81	AS	Tidewater Community College, Virginia Beach, VA, Science
8/81	BS	Old Dominion University, Norfolk, VA, Biology
12/86	MS	Old Dominion University, Norfolk, VA, Biology/Mammalian Reproduction
08/98	PhD	Old Dominion University, Norfolk, VA, Sciences/Cellular Endocrinology & Reproductive Biology

EMPLOYMENT/APPOINTMENTS:

1991 - 1998	**LECTURER/INSTRUCTOR/GRADUATE FACULTY/ TEACHING ASSISTANT/ RESEARCH ASSISTANT**, Department of Biological Sciences, Old Dominion University, Norfolk, Virginia
1989 - 1991	**ADJUNCT INSTRUCTOR**, Department of Math and Natural Sciences, Tidewater Community College, Virginia Beach, Virginia
1983 - 1990	**TEACHER**, High School
1989 - 1991	**DIRECTOR/COUNSELOR** , Sea Camps Summer Marine Program, ODU
1977 - 1979	**FIRST MATE "A" - OCEANS RESEARCH**, The Virginia Institute of Marine Science, Gloucester, Virginia

TEACHING EXPERIENCE:
Undergraduate Clases: **Biol 141/142**: Human Anatomy and Physiology with concurrent Lab (TCC); **Biol 101/102**: General Biology; **Chem 111**: General Chemistry Lab(TCC); **Biol 116**: General Biology Lab (ODU); **Biol 250/251**: Human Anatomy and Physiology with concurrent Lab (ODU); **Biol 108/109**: Life Sciences with concurrent Lab (ODU); **Biol 332** - Genetics (ODU)
Undergraduate/Graduate Courses (ODU): **Biol 489/589**: Survey of Human Anatomy Lab; **Biol 484/584** Advanced Human Anatomy Lecture; **Biol 484/584** Advanced Human Anatomy Lab; **Biol 490/590**: Advanced Human Physiology Lecture; **Biol 480/580**: Advanced Human Physiology Lab; **Biol 789/889**: Gross Anatomy Lab; **Biol 493/593**: Human Neurophysiology

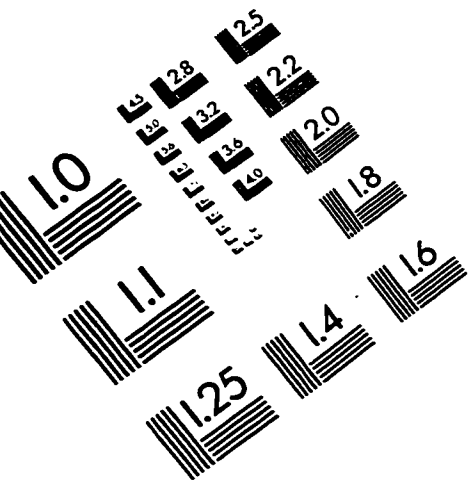
PUBLICATIONS
PAPERS (in referreed journals)

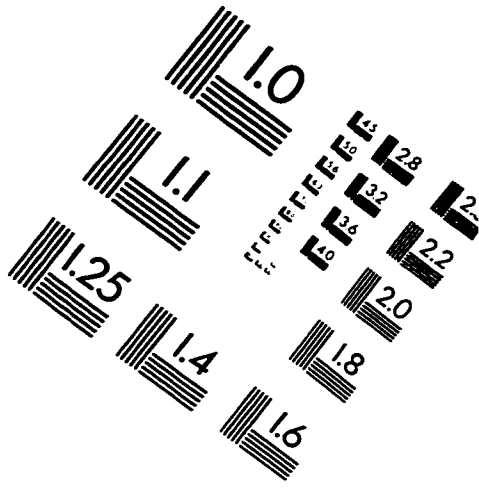
Michael H. Mitchell, MS, Robert J. Swanson, PhD, Gary D. Hodgen, PhD, and Sergio Oehninger, MD. Enhancement of In Vitro murine Embryo Development by Recombinant Leukemia Inhibitory Factor. *The Journal of the Society for Gynecologic Investigations,* 1994, 1(3):215-219.

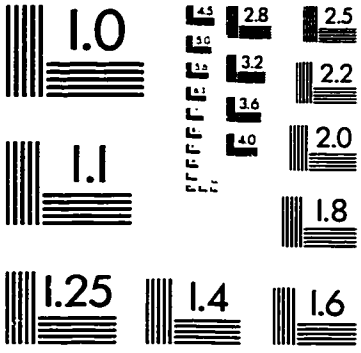
Rose, R. K. and M. H. Mitchell. Reproduction in the Hispid Cotton Rat, *Sigmodon Hispidus* Say and Ord (Rodentia: Muridae), in Southeastern Virginia. *Brimleyana - The Journal of the North Carolina State Museum of Natural Sciences*, 1990, 16:43-59.

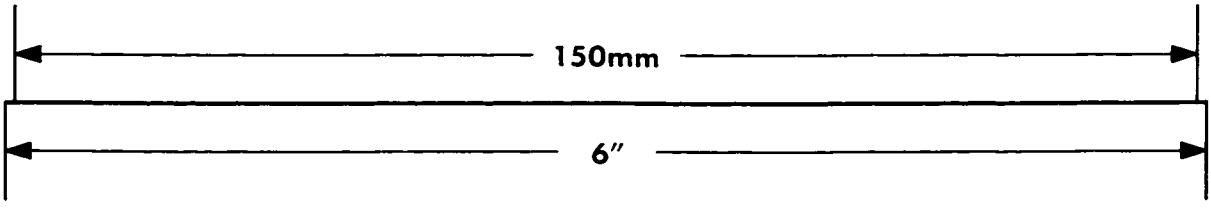
ABSTRACTS: Mitchell M., Swanson RJ, Jin Kim K, Oehninger. Enhancement of in vitro murine embryo development (blastocyst formation and hatching) by recombinant leukaemia inhibitory factor (rLIF). *Society for Gynecologic Investigation: Fortieth Annual Meeting, Scientific Program Abstracts*, #S60:98, 1993.

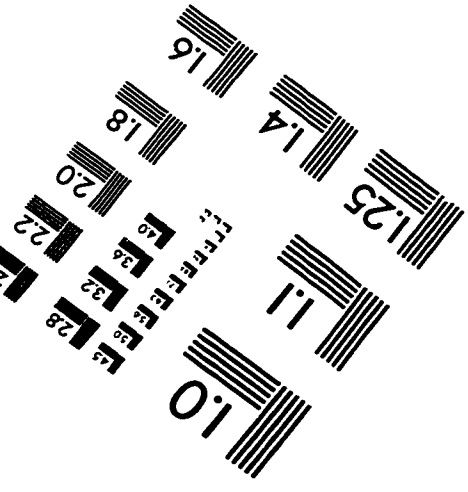
IMAGE EVALUATION TEST TARGET (QA-3)











APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989