



universität
wien

DIPLOMARBEIT

The Influence of Leukemia Inhibitory Factor on Cardiomyogenesis

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Verfasser:	Marc Wiedner
Matrikel-Nummer:	0001993
Studienrichtung:	Molekulare Biologie
Betreuer:	Ao. Univ. Prof. Dr. Georg Weitzer

Wien, am 20.05.2008

Contents

Abstract	4
Zusammenfassung	5
1 Introduction.....	6
1.1 Cardiovascular disease and stem cell therapies	6
1.2 Heart development	7
1.3 Stem cells	10
1.3.1 Embryonic stem cells	11
1.3.2 Embryoid bodies	12
1.3.3 Somatic stem cells	14
1.4 Leukemia Inhibitory Factor	15
1.4.1 Functions and molecular mechanisms	16
1.4.2 Gene structure and isoforms	18
2 Materials	21
2.1 Chemicals for molecular biology	21
2.2 Chemicals for cell culture	22
2.3 Enzymes	23
2.4 Cell lines	23
2.5 Antibodies	24
2.6 PCR primers	24
2.7 Expression vectors	25
2.8 Kits	25
2.9 Other Materials	25
2.10 Inhibitors	26
3 Methods	27
3.1 Cell culture	27
3.1.1 Culture of mammalian cells	27
3.1.1.1 Media and solutions for mammalian cell culture	27
3.1.1.2 Coating of cell culture plates	30
3.1.1.3 Culture of SNL76/7 fibroblasts	30
3.1.1.3.1 Thawing of SNL76/7 fibroblasts	30
3.1.1.3.2 Continuous culture of SNL76/7 fibroblasts	31
3.1.1.3.3 Splitting of SNL76/6 fibroblasts	31

3.1.1.3.4	Production of feeder cells.....	31
3.1.1.3.5	Freezing of SNL76/7 fibroblasts	32
3.1.1.4	Culture of embryonic stem cells.....	33
3.1.1.4.1	Thawing embryonic stem cells	33
3.1.1.4.2	Maintenance of embryonic stem cells.....	33
3.1.1.4.3	Freezing of embryonic stem cells	33
3.1.1.5	Culture of cardioblast-like stem cells	34
3.1.1.5.1	Subcloning of cardioblast-like stem cells	34
3.1.1.5.2	Growth curve of cardioblast-like stem cells.....	34
3.1.1.6	Generation of embryoid bodies	35
3.1.1.7	Isolation of primary cardiomyocytes from neonatal mice	36
3.1.2	Cell culture of insect cells.....	37
3.1.2.1	Media and solutions for insect cell culture	37
3.1.2.2	Sf9 cell culture.....	37
3.1.2.2.1	Thawing and adaptation of SF9 cells.....	37
3.1.2.2.2	Storage of SF9 cells	38
3.1.2.2.3	Infection of SF9 cells	38
3.1.2.2.4	Viral plaque assay	38
3.2	Protein purification from SF9 cells.....	39
3.3	SDS polyacrylamide gel.....	42
3.4	Coomassie staining.....	43
3.5	Silver staining	43
3.6	Western Blot analysis.....	44
3.7	2-D gel electrophoresis.....	46
3.8	Glycoprotein detection	48
3.8.1	Periodic acid-Schiff stain.....	48
3.8.2	Enzymatic deglycosylation with PNGase F.....	49
3.9	Isolation of RNA with Quigen RNeasy Mini Kit	49
3.10	Reverse Transcription of mRNA into cDNA	50
3.11	Semi-quantitative RT-PCR.....	51
3.12	Agarose gels for separation of PCR products.....	53
3.13	Karyotyping	53
3.14	Alkaline phosphatase assay	54
4	Results	56

4.1	Characterization of M-LIF and D-LIF.....	56
4.1.1	Expression of M-LIF and D-LIF	56
4.1.2	Purification of M-LIF and D-LIF	57
4.1.3	Western blot analysis of M-LIF and D-LIF	59
4.1.4	Determination of glycosylation	62
4.1.5	Sequencing of M-LIF and D-LIF	63
4.2	The influence of M-LIF and D-LIF on embryonic stem cells	66
4.2.1	Influence on self-renewal capacity	66
4.2.2	Influence on embryoid body development.....	69
4.3	The influence of M-LIF and D-LIF on primary cardiomyocytes	70
4.3.1	Influence on morphology and contractility/proliferation.....	71
4.3.2	Influence on gene expression	72
4.4	The influence of M-LIF and D-LIF on cardioblast-like stem cells.....	74
4.4.1	Influence on proliferation.....	74
4.4.2	Influence on differentiation.....	75
4.5	Characterization of cardioblast-like stem cells	77
4.5.1	Growth rate of cardioblast-like stem cells	77
4.5.2	Karyotyping of cardioblast-like stem cells	77
4.5.3	Single-cell cloning of cardioblast-like stem cells.....	79
5	Discussion.....	81
5.1	Production and purification of M-LIF and D-LIF.....	81
5.2	M-LIF and D-LIF promote self-renewal in embryonic stem cells.....	82
5.3	M-LIF and D-LIF retard mesoderm formation in embryoid bodies	83
5.4	Glycosylated M-LIF and D-LIF promote contractility and proliferation of primary cardiomyocytes	83
5.5	The influence of M-LIF and D-LIF on gene expression of primary cardiomyocytes...	84
5.6	M-LIF and D-LIF promote proliferation and differentiation of cardioblast-like stem cells	85
5.7	CBLSCs represent an autonomous stem cell line of somatic origin	86
5.8	Conclusion	87
6	References	88
	Acknowledgement	94
	Curriculum vitae	95

Abstract

Heart failure represents one of the leading causes of death in western civilization and is primarily the result of previous myocardial infarction. The fact that cardiac tissue is unable to regenerate after infarction led to the general belief that the heart of mammalian species is a terminally differentiated organ. Recently, however, evidence has emerged that the adult heart contains myocardial cells possessing stem cell-like properties which may have the potential to repair damaged tissue. The identification of factors which could activate these quiescent cardiac stem cells and cause them to differentiate into functional cardiomyocytes would be an important step towards finding new therapies for the treatment of heart diseases.

One such factor has been proposed to be Leukemia Inhibitory Factor (LIF). LIF is a polyfunctional glycoprotein, showing a wide range of biological activities that include growth promotion and cell differentiation of various types of target cells. In previous studies, specifically the matrix-associated isoform of LIF (M-LIF) has been shown to positively influence cardiomyogenesis, in contrast to its diffusible counterpart (D-LIF).

The aim of this diploma thesis was to investigate the potential role of LIF in cardiac differentiation, analyzing differences between the effect of M-LIF and D-LIF on embryonic stem cells, embryoid bodies, cardiac progenitor cells and cardiomyocytes, as well as the impact of glycosylation on biological activity. The conducted experiments reveal that both glycosylated M-LIF and D-LIF, respectively, can be utilized as factors to trigger expansion of cardiac stem cells and induce differentiation into functional heart cells in vitro which may ultimately prove useful for cell therapy of the diseased heart.

Zusammenfassung

Herzversagen stellt weltweit eine der häufigsten Todesursachen dar und ist hauptsächlich die Folge eines vorangegangenen Herzinfarkts. Die Tatsache, dass Herzgewebe nicht imstande ist, sich nach einem Infarkt zu regenerieren, führte zum allgemeinen Glauben, das Herz von Säugetieren sei ein ausdifferenziertes Organ. Kürzlich, jedoch, hat man im erwachsenen Herzen Zellen entdeckt, welche Stammzell-ähnlichen Charakter besitzen und die Fähigkeit haben könnten, geschädigtes Gewebe zu reparieren. Die Identifizierung von Faktoren, welche diese ruhenden Herzstammzellen aktivieren und zur Differenzierung zu funktionellen Herzzellen veranlassen können, wäre ein wichtiger Schritt in Richtung neuer Therapien von Herzkrankheiten.

Ein Faktor, der für diese Tätigkeit in Frage kommt, ist der Leukämie Inhibitions Faktor (LIF). LIF ist ein polyfunktionelles Glykoprotein, welches mannigfaltige biologische Aktivitäten aufweist, wie unter anderem Wachstumsförderung und Differenzierung verschiedenster Zielzellen. In vorangegangenen Studien wurde gezeigt, dass speziell die Matrix-assoziierte Isoform (M-LIF) sich positiv auf die Kardiomyogenese auswirkt, im Unterschied zu ihrem diffundierbaren Gegenstück (D-LIF).

Ziel dieser Diplomarbeit war es, die mögliche Rolle von LIF in Bezug auf Herzzellentwicklung zu untersuchen, wobei Unterschiede zwischen dem Effekt von M-LIF und D-LIF auf embryonale Stammzellen, „Embryoid bodies“, Herzvorläuferzellen und Kardiomyozyten analysiert wurden, sowie der Einfluss des Glykosylierungsgrades auf die biologische Aktivität. In den hier durchgeführten Experimenten konnte gezeigt werden, dass sowohl glykosyliertes M-LIF, als auch D-LIF, als Faktoren für die Expansion und gezielte Differenzierung von Herzvorläuferzellen eingesetzt werden können und sich damit in weiterer Folge als nützlich für die Zelltherapie von Herzkrankheiten erweisen könnten.

1 Introduction

This diploma thesis focuses on the potential role of Leukemia Inhibitory Factor (LIF) in cardiac differentiation, investigating differences between the effect of its matrix-associated and diffusible isoforms (M-LIF and D-LIF, respectively) on embryonic stem cells, embryoid bodies, cardiac progenitor cells and cardiomyocytes, as well as the impact of glycosylation on biological activity. Based on previous data, it was anticipated that treatment with recombinantly produced M-LIF would lead to upregulation of cardiac markers in cardiomyocytes and contribute to expansion and directed differentiation of cardiac progenitor cells in vitro which may ultimately prove useful for cell therapy of the diseased heart.

1.1 Cardiovascular disease and stem cell therapies

Cardiovascular diseases and resulting heart failure represent one of the leading causes of death worldwide killing 17 million people every year (WHO, 2008). In western countries heart failure is primarily the result of previous myocardial infarction (Mahon et al., 1999). Existing therapy is extremely limited focusing on treatment of already established disease predominantly with the use of pharmacological agents, aiming to primarily inhibit the neurohormonal axis that results in excessive cardiac activation through angiotensin- or norepinephrine-dependent pathways (Itescu et al., 2003). For end-stage heart failure patients, the only options remaining to date are heart transplantation which is limited due to the extreme scarcity of donor organs (Mahon et al., 1999; Hognes et al., 1991) and implantation of expensive left ventricular assist devices, not proven for long-term use and associated with significant complications (McCarthy et al., 1991; Frazier et al., 1992; Oz et al., 1997). Therefore, the development of approaches that prevent heart failure rather than treat an already established disease is in dire need.

One such approach is stem cell therapy, involving the clinical application of cultured stem cells. The identification of hematopoietic stem cells in mice heralded the use of this type of therapy (Till and McCulloch, 1961) and has led to the use of bone marrow derived stem cells for the treatment of a broad range of diseases, including breast cancer, leukemia, inflammatory bowel disease and osteogenesis imperfecta in humans (Kochar, 2004). Although success of this treatment is variable (Thomas, 1999), embryonic and somatic stem cells offer great hope for reversing the symptoms of many diseases and conditions such as Parkinson's disease by replacing dopamine-producing cells in the brain, Type I diabetes mellitus by implanting insulin-

producing cells, and heart failure by repairing damaged heart muscle with cardiac muscle cells upon myocardial infarction. The following stem cell characteristics make them good candidates for cell-based therapies (Barker et al., 2003):

- Potential to be harvested from patients
- High capacity of cell proliferation in culture to obtain large numbers of cells from a limited source
- Ease of manipulation to replace existing non-functional genes via gene transfer methods
- Ability to migrate to host's target tissues
- Ability to integrate into host tissue and interact with surrounding tissue.

Recently, human embryonic stem cell-derived cardiomyocytes isolated from so-called embryoid bodies (EBs) were transplanted into the porcine heart after induced atrioventricular block and could be shown to successfully function and integrate with host cells (Kehat et al., 2004). Somatic stem cells have also been used in cell therapy for the heart. Cardiac stem cells have reportedly been isolated from adult human hearts, but thus far have been shown to contribute only poorly to the regeneration of cardiac tissue (Beltrami et al., 2003; Urbanek et al., 2005). The identification of factors which could activate these quiescent cardiac stem cells in vitro and cause them to differentiate into functional cardiomyocytes would be an important step towards finding new therapies for the treatment of heart failure. Candidates for such factors include SPARC, BMP2, and M-LIF, which have all been found to enhance the expression of cardiac markers and promote cardiac differentiation in EBs (Sary et al., 2005; Behfar, 2002; Pastener, 2006).

1.2 Heart development

Cardiogenesis is a spatially and temporally controlled event, involving the differentiation of multiple heart lineages which results in the coordinated formation of the distinct tissue components of the heart, including the four specialized chambers, diverse structures of the conduction system, the endocardium, the heart valves, the coronary arterial tree, and the outflow tract (Harvey, 2002; Brand, 2003). In this regard, three major sources of heart cell precursors have been identified in the embryo: the cardiogenic mesoderm, the cardiac neural crest and the proepicardial organ (Laugwitz et al., 2008).

The first source to arise is the cardiogenic mesoderm, which resembles a horseshoe-like structure appearing on day 7.5 in murine embryogenesis. This structure consists of two populations of cardiac precursor cells that contribute to different parts of the heart. The earliest population of

cardiac progenitors, referred to as the first heart field, originates in the anterior splanchnic mesoderm, gives rise to the cardiac crescent, later to the linear heart tube, and ultimately contributes to parts of the atrial chambers and the left ventricular region. The second cardiogenic region, known as the second heart field, lies anterior and dorsal to the linear heart tube and is derived from the pharyngeal mesoderm medial to the cardiac crescent. Cells from this second heart lineage are added to the developing heart tube and give rise to the outflow tract, the right ventricular region and the main parts of the atrial tissue (Laugwitz et al., 2008).

The second major source of cardiac progenitors, the cardiac neural crest, migrates into the heart after looping of the heart tube on day 8.5 and gives rise to the vascular smooth muscle of the aortic arch, ductus arteriosus and the great vessels; additionally, neural crest contributes to essential components of the cardiac autonomic nervous system (Kirby et al., 1983; Epstein and Buck, 2000). At the same time cells of the third source of heart cell precursors, the proepicardial organ, produce the mesenchyme portion of the developing heart and the majority of epicardial cells (Dettman et al., 1998; Moore et al., 1999; Manner et al., 2001). Coronary vasculature might be partially derived from mesodermal cells in the proepicardium, although the contribution of the proepicardium to the endothelial lineage is still controversial (Poelmann et al., 2002). Figure 1.1 shows a schematic summary of the processes outlined above.

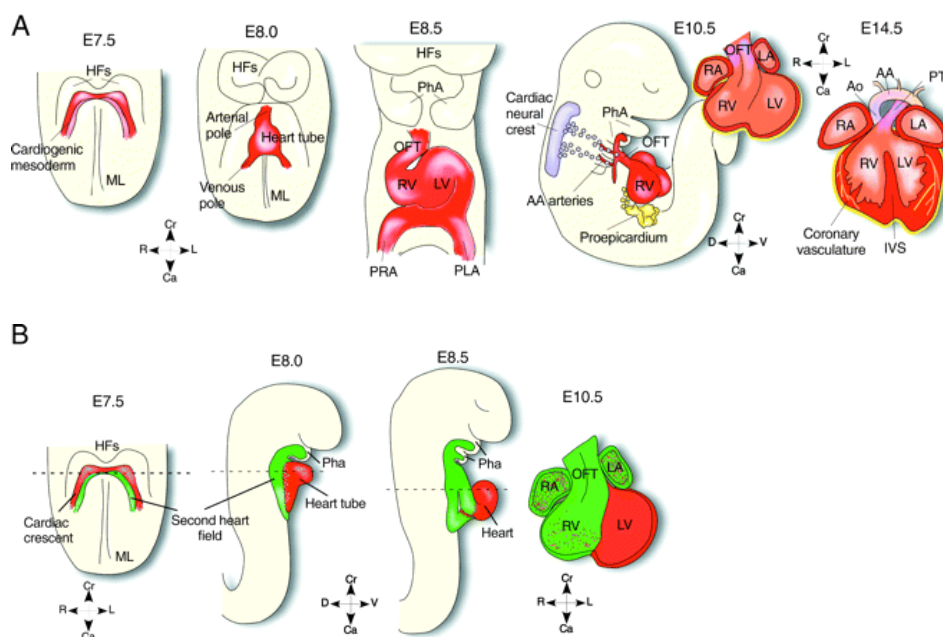


FIGURE 1.1. Murine heart development (Laugwitz et al., 2008). *A*, Contribution of the three populations of embryonic heart progenitors to the developing heart: Cardiogenic mesoderm (red), cardiac neural crest (purple), proepicardial organ (yellow). *B*, First (red) and second (green) heart fields and their contributions to the developing heart. AA, aortic arch; Ao, Aorta; HF, head fold; IVS, interventricular septum; ML, midline; OFT, outflow tract; PhA, pharyngeal arches; PLA, primitive left atrium; PRA, primitive right atrium; PT, pulmonary trunk; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle.

Mesoderm initially becomes instructed to adopt a cardiac fate in response to signals from adjacent tissues (Harvey, 2002; Srivastava and Olsen, 2000). Growth factors of the TGF β family, such as BMP-2 (bone morphogenetic protein) and FGF-4 (fibroblast growth factor) induce commitment of mesodermal cells to the cardiogenic lineage and promote the proliferation of specified cells, respectively (Barron et al., 2000; Lough et al., 1996; Schlange et al., 2000). Other candidates known to influence cardiomyogenesis are members of the Wnt family. Wnt1, Wnt3a, and Wnt8c have an inhibiting effect, whereas Wnt11, expressed in precardiac mesoderm, induces heart formation in the posterior mesoderm (Eisenberg and Eisenberg, 1999). Another candidate able to induce heart development is IGF-2 (insulin-like growth factor 2) which has been shown to lead to upregulation of the T-box protein brachyury in embryoid bodies when added in recombinant form (Morali et al., 2000). Brachyury – along with MESP – is amongst the earliest transcription factors known to direct unspecified cells into the mesoderm lineage (Kitajima et al., 2000; Wilson and Beddington, 1997). LIM-domain-containing transcription factor islet1 (ISL1) is involved in the differentiation of second heart field cells (Cai et al., 2003), whereas the homeodomain-containing transcription factor NKX2.5 is a marker of both heart fields (Buckingham et al., 2005). NKX2.5 activates the target gene eHand, a basic-loop-helix (bHLH) transcription factor which, together with dHAND, is necessary for the looping of the heart tube (Harvey, 1996; Sucov, 1998). Once heart cells are terminally differentiated, markers include NKX2.5, GATA4, MHC α and Tropomyosin α in the case of cardiac muscle cells, NKX2.5 and HF-1b in the case of conduction cells, and HOXB5 in the case of endothelial cells. Figure 1.2 depicts a brief overview of cardiac lineage differentiation with the various transcription factors involved.

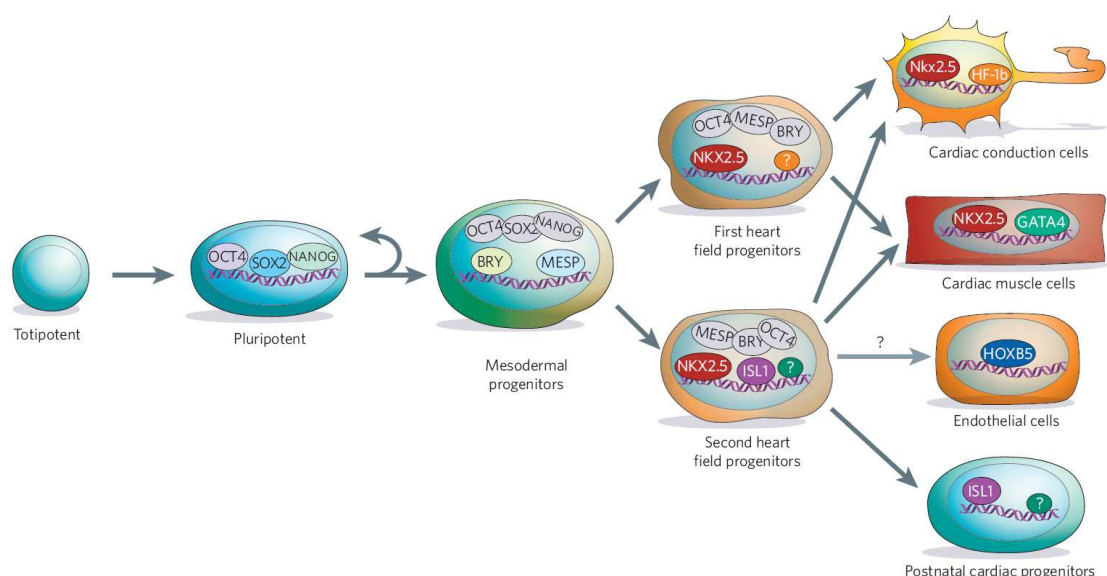


FIGURE 1.2. Differentiation of embryonic stem cells into the cardiac lineage (Srivastava and Ivey, 2006).

Interestingly, remnant second heart field cells may not only be able to differentiate into many cell types, but also persist in the postnatal heart (Laugwitz et al., 2005). The pool of potential cardiac progenitor cells might be involved in continual maintenance of the heart by differentiating into several types of cardiac cells, including muscle, conduction and vascular cells, although the precise lineage potential of distinct subtypes remains to be determined (Srivastava and Ivey, 2006).

1.3 Stem cells

Stem cells are primal undifferentiated cells (self-renewing cells) which have the ability to proliferate and differentiate into cell types of various tissues in vitro and in vivo. Molecular cues provided by their cellular environment or niche activate transcription factors which in turn switch specific genetic programs on or off in a very controlled manner (Ateghang, 2006). Execution of the correct genetic program and therefore differentiation into specific cell types depends crucially on the availability of the right combination and sequence of cues (O'Shea, 2004).

All stem cells are characterized by three inherent abilities. First, they have the capacity of self-renewal. This means they are capable of dividing indefinitely, producing offspring that has the identical potential and nature to that of the mother cell. Second, they are characterized by clonality, i.e. the ability to survive as a single cell and proliferate. And third, they have potency of differentiation, meaning they can differentiate into different types of somatic cells in the presence of certain lineage committing factors. Based on their developmental potential, stem cells can be categorized as follows:

- **Totipotent cells;** these cells have the potential to give rise to all cells found in the adult body and any cell of the extra-embryonic tissue, such as the placenta. The only totipotent cells known to date are fertilized eggs and blastomeres until the morula stage.
- **Pluripotent stem cells;** these cells can differentiate into any cell found in the adult body, but - unlike totipotent cells - cannot contribute to the formation of the extra-embryonic tissue. Three types of pluripotent stem cells exist (Donovan and Gearhart, 2001):
 - o Embryonic stem cells (ESCs): These cells are isolated from the inner cell mass of the pre-implantation embryo.
 - o Embryonic germ cells (EGCs): EGCs are derived from primordial germ cells (PGCs) isolated from the embryonic gonad.

- Embryonic carcinoma cells (ECCs): These cells are derived from PGCs in the embryonic gonad but usually are detected as components of testicular tumors in the adult.
- **Multipotent stem cells;** these cells can only differentiate into a limited number of cell types. They are found in the tissue of the adult organism and are thought to be present in niches within most organs where they eventually replace dead or damaged cells (Ohlstein et al., 2004).

1.3.1 Embryonic stem cells

Embryonic stem cells (ESCs) are derived from pre-implantation embryos. Specifically, mouse ESCs are isolated from the inner cell mass (ICM) of the blastocyst at day 3.5 of murine development (Evans and Kaufman, 1981; Martin, 1981). The blastocyst is made up of three structures: the trophoblast, which is the layer that surrounds the blastocyst and develops into the embryonic membranes and placenta; the blastocoel, which is the hollow cavity inside the blastocyst; and the ICM, which is a group of cells at one end of the blastocoel that gives rise to the embryonic disk of the embryo and, later, the fetus. In order to generate an embryonic stem cell line, cells of the ICM are removed and replated on fresh culture plates (Figure 1.3). After several rounds of re-plating they can be cultured on mitotically inactivated fibroblasts or on feeder cell-free medium containing certain growth factors, such as LIF (Williams et al., 1988; Carpenter et al., 2003). In culture, they can then be propagated as a homogeneous, uncommitted cell population for an almost unlimited period of time without losing their pluripotency and their stable karyotype (Prelle et al., 2002). In the absence of feeder cells or LIF, ESCs begin to spontaneously differentiate in vitro, giving rise to cells of all three germ layers: ectoderm, mesoderm and endoderm (Guan et al., 1999; Amit et al., 2000). Both the pattern and the efficiency of differentiation are affected by parameters such as ESC density and media components (Ateghang, 2006). Directed differentiation into ectodermal, mesodermal, and endodermal cells, respectively, has been shown to be inducible by the addition of certain growth factors (Jiang et al., 2002; Muguruma et al., 2003; Romagnani et al., 2005; Schwartz et al., 2002; Beltrami et al., 2008).

In vitro differentiation provides a basis for detailed studies of developmental mechanisms as well as for the generation of specific cell types for tissue engineering and regenerative medicine

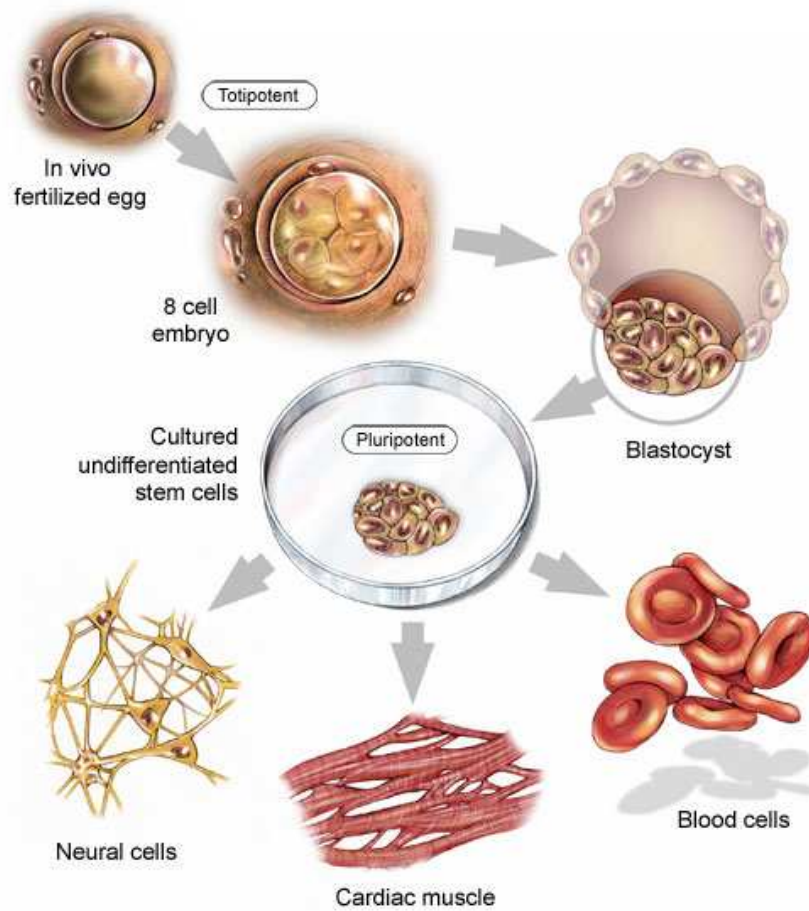


FIGURE 1.3. Embryonic stem cell culture (Kochar, 2004). Embryonic stem cells are isolated from the inner cell mass of the blastocyst and can differentiate into cells of all three germinal layers.

applications. However, reproducibly isolating pure differentiated cell types remains a difficult challenge and will be a major focus of stem cell research for years to come.

1.3.2 Embryoid bodies

Embryoid bodies (EBs) are three-dimensional embryonic stem cell aggregates that show development which is reminiscent of early eutherian embryogenesis (Weitzer, 2006; Figure 1.4). One day after aggregation in hanging drop cultures, the irregular surface smoothens to morphologically resemble the process of morula compaction. Between days 3 and 5, primitive endoderm forms, which secretes components to induce the formation of a basement membrane (Aumailley et al., 2000; Gao et al., 2004; Li et al., 2001; Murray and Edgar 2001; Smyth et al., 1999). At the inner side of the primitive endoderm, ESCs develop into primitive ectoderm that

reorganizes into a columnar epithelium (Ikeda et al., 1999; Smyth et al., 1999) surrounding a central cavity, analogous to the amniotic cavity of the pre-implantation embryo. At day 4.5, when blastocysts implant into the uterine wall, EBs are placed on a collagen-coated surface which substitutes for the Reichert's membrane in the embryo. Primitive endoderm cells then give rise to visceral endoderm and parietal endoderm. Primitive ectoderm develops into embryonic endoderm, definitive ectoderm and mesoderm, the latter of which generates rhythmically beating cardiomyocytes that appear between days 7 and 8 of in vitro differentiation (Doetschman et al., 1985).

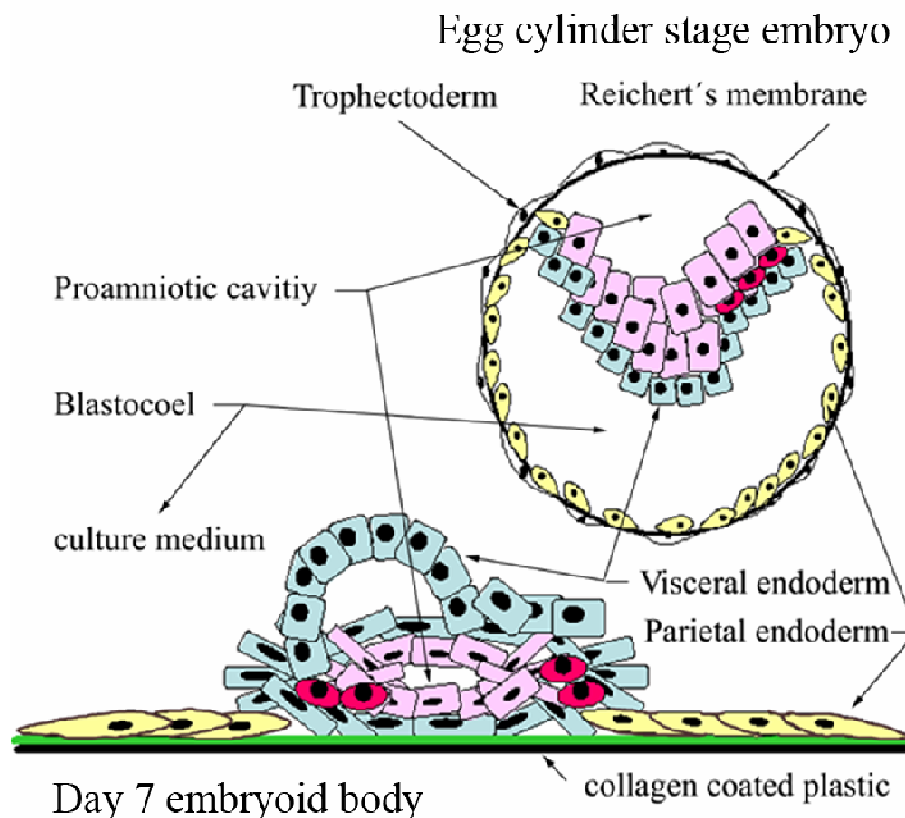


FIGURE 1.4. Schematic comparison of cells and structures between an embryoid body on day 7 and an early egg cylinder mouse stage embryo (Weitzer, 2006).

Thus, EBs serve as a reliable model for in vitro embryogenesis, which may significantly contribute to the direct differentiation of embryonic and somatic stem cells towards a specific cell type useful for therapeutic applications and at the same time reduce animal experiments required for tests in the medical and pharmacological field.

1.3.3 Somatic stem cells

Somatic stem cells (SSC), also referred to as adult stem cells or progenitor cells, are undifferentiated self-renewing cells that are found in specific compartments of specialized tissue. These compartments are referred to as “niches” and provide a microenvironment in which stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing (Ohlstein et al., 2004). So far, SSCs have been isolated from a variety of organs including bone marrow, blood, skeletal muscle, brain, skin, heart, pancreas and liver (Prelle et al., 2001; Beltrami et al., 2007). Their primary functions are to maintain the steady state functioning of a cell (homeostasis) and to replace cells lost due to injury or disease (Holtzer, 1978). While it is questionable if all types of SSCs can be properly expanded in vitro without losing developmental potential, it has been shown that at least oligodendrocyte precursor cells are able to be grown indefinitely in culture (Tang et al., 2001).

SSCs have long been believed to be more restricted in their developmental potential than ESCs and irreversibly committed to specific lineages of differentiation. For instance, it is assumed that SSCs residing in specific niches in the heart are limited to producing solely the cell types needed for this organ, namely cardiac muscle cells for contractility, cardiac conduction cells for coordinated electrical activity and endothelial cells for vessel formation (Figure 1.5).

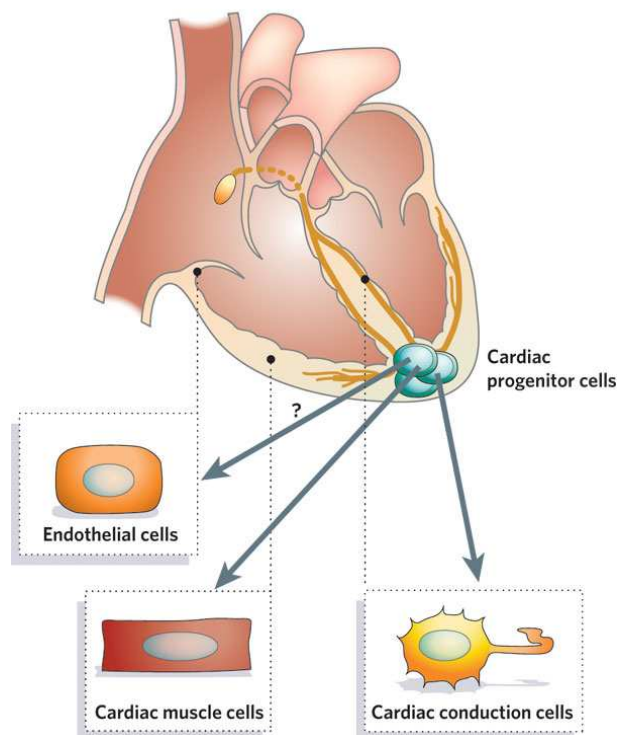


FIGURE 1.5. Cardiac progenitor cells have the potential to produce at least three cell types (Srivastava and Ivey, 2006).

Recently, however, it has been proposed that SSCs could be far less limited in potency than previously assumed, having the ability to differentiate into unrelated cells and even cross lineage boundaries (Krause et al., 2002; Poulson et al., 2002; Beltrami et al., 2007), a phenomenon known as “transdifferentiation” (Anderson et al., 2001) or “stem cell plasticity” (Krause et al., 2002). This plasticity has been observed not only under experimental conditions, but also in patients who have received bone marrow transplants (Forbes et al., 2002).

The utilization of SSCs for medical research and cell therapy represents a practical alternative to that of ESCs, which is much desired due to ethical concerns. Furthermore, immunogenic rejection would be averted, since SSCs could be harvested from the very patient in need of therapy. These advantages explain the increased popularity that somatic stem cell research is receiving in laboratories and the general public alike.

1.4 Leukemia Inhibitory Factor

Leukemia Inhibitory Factor (LIF) is a glycoprotein belonging to the IL-6 class cytokines. It exhibits four α -helices (Figure 1.6) and has a molecular weight (MW) of approximately 20 kD in its unglycosylated form (Hinds et al., 1997). Mature LIF has three disulfide bonds and is heavily N-glycosylated containing six putative N-glycosylation sites in the case of LIF isolated from buffalo rat liver cells (rLIF). In its fully glycosylated form, rLIF has been reported to exhibit a

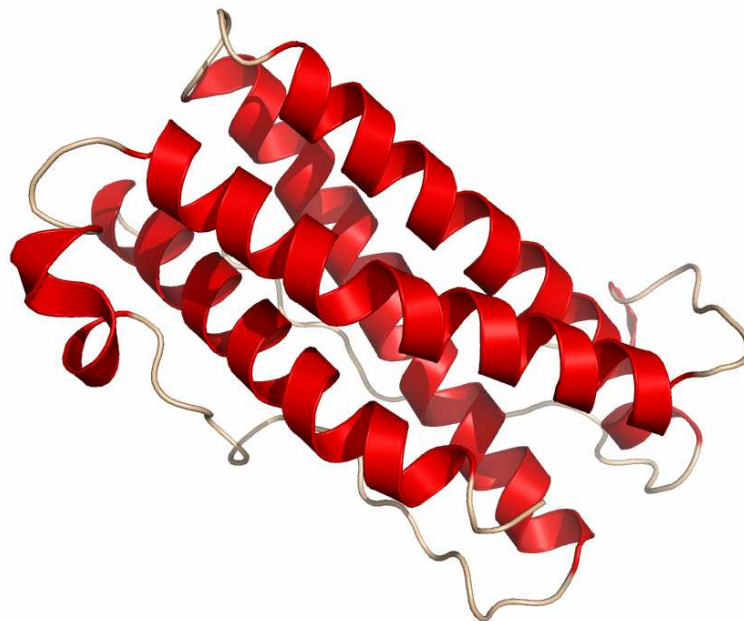


FIGURE 1.6. Crystal structure of Leukemia Inhibitory Factor as published in the Protein Data Bank (PDB: 1LKI). (Robinson et al., 1994)

MW of 43 kD (Sasai et al., 1998). Thus far, LIF has been isolated from humans, mice, rats, sheep and pigs, showing 74 to 92% similarities in terms of amino acid sequence (Willson et al., 1992).

1.4.1 Functions and molecular mechanisms

LIF was first discovered in the year 1987 and originally termed ES cell differentiation inhibiting activity (DIA), due to its ability to actively suppress stem cell differentiation (Smith and Hooper, 1987). It was named “leukemia inhibitory factor”, because it was identified as a factor able to suppress leukemia in mice by inducing macrophage maturation and terminating self-renewal of the undifferentiated highly clonogenic murine myeloid leukemia, M1 (Gearing et al., 1987; Hilton et al., 1988). Now, over twenty years later, neither of these names has been found to do this cytokine justice, since many more biological functions have become apparent. For instance, LIF has been found to induce differentiation of mesenchymal cells into kidney cells (Barasch et al., 1999; Plisov et al., 2001; Yang et al., 2002). Neuronal functions have been identified, such as enhancing survival of sensory and motor neurons (Murphy et al., 1991), causing in vitro proliferation of multipotential human neural progenitor cells (Carpenter et al., 1999), preventing oligodendrocyte death in animal models of multiple sclerosis (Butzkueven, 2002), and enhancing migration of inflammatory macrophages to damaged neuronal tissue (Sugiura, 2000). This proinflammatory property is manifested by LIF’s ability to induce acute phase protein synthesis affecting cell recruitment into the area of damage or inflammation (Chodorowska et al., 2004). LIF has been reported to have multiple effects on endocrine organs or their target tissues, suppressing the proliferation in vitro of breast epithelial cells and breast cancer cells (Grant et al., 2001), inhibiting the production of prolactin and growth hormone (Tomida et al., 2001), and reducing testosterone synthesis by Leydig cells (Mauduit et al., 2001), but, bafflingly, also enhancing the proliferation of primordial germ cells and spermatocyte differentiation (Piquet-Pellorce et al., 2000; Sariola et al., 2001). In vitro studies have shown that LIF can both stimulate (Dazai et al., 2000) and inhibit bone formation (Reid et al., 1990). In regard to muscle action, LIF stimulates the proliferation of muscle satellite cells (Spangenburg et al., 2002) and can ameliorate muscle fiber degeneration in vivo in mdx mice lacking dystrophin (Austin et al., 2000). Furthermore, it is a hypertrophic agent for cardiac muscle (Murata et al., 1999) and has been found to reduce apoptosis in cardiomyocytes (Negoro et al., 2001).

On the basis of this enormous body of work, it becomes clear that LIF acts on many, perhaps all, tissues and is a highly polyfunctional cytokine exerting potentially “adverse” functions in many cases. A partial explanation for this phenomenon can be found by looking into the intracellular signaling events triggered by LIF (Figure 1.7). LIF binds to the signal-transducing receptor component gp130, which is common to all IL-6 family cytokines (Taupin et al., 1998), and to the low-affinity LIF receptor subunit gp190, which is specific for LIF. Following heterodimerization of the receptors, activated Jaks phosphorylate five Tyrosines located on the intracellular domain of gp130. Four of these Phospho-tyrosines (P-Y 126, 173, 265 and 275) can associate with and phosphorylate STAT3, leading to dimerization and translocation to the nucleus, where genes for self-renewal and proliferation are activated. One Phospho-tyrosine (Y 118-P), however, can associate with SHP-2, which leads to the activation of the Ras pathway and translocation of ERK 1/2 to the nucleus, thereby activating genes required for differentiation (Cavaleri and Schöler, 2004).

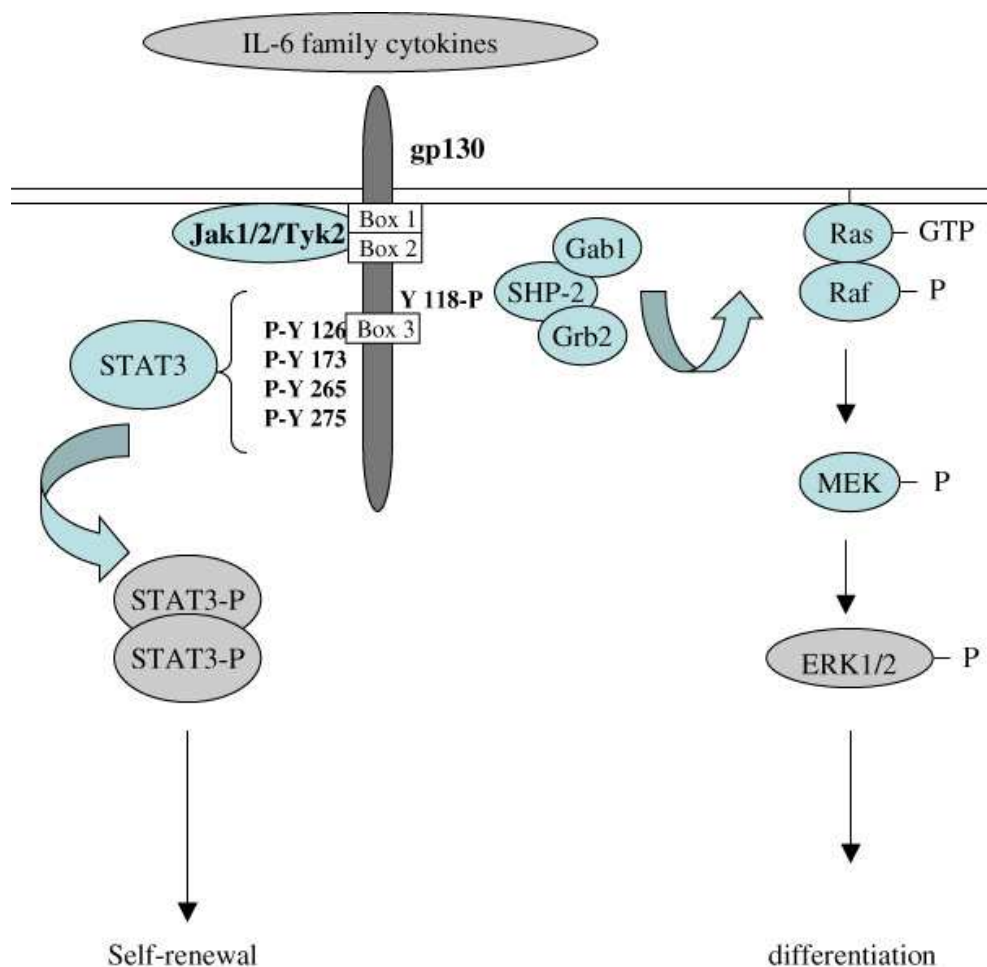


FIGURE 1.7. Signal pathways induced by the IL-6 cytokine family. (Cavaleri and Schöler, 2004)

As previously mentioned, the receptor subunit gp130 is shared by many other cytokines, which - at least in part - explains the finding that LIF seems to be replaceable for almost all discovered functions by one cytokine or another. LIF^{-/-} mice could be produced in expected Mendelian ratios and appeared to develop into healthy young adults (Metcalf, 2003). One dramatic abnormality, however, was that LIF^{-/-} females were absolutely unable to become pregnant (Stewart et al., 1992; Escary et al., 1993). Hence, the only irreplaceable function of LIF seems to be implantation of the blastocyst into the uterine wall.

Since LIF has so many different functions in such a wide array of tissues, regulating mechanisms must exist to modulate LIF action and prevent unwanted action in other tissues. Thus far, two such mechanisms have been identified: First, circulating soluble LIF receptors exist that block the action of any LIF in the circulation, thereby restricting action to a particular site of active LIF production (Layton et al., 1992). And second, negative regulators known as suppressors of cytokine signaling (SOCS) are produced (Starr et al., 1997). These proteins represent highly potent modulators of LIF signaling, with different SOCS proteins most likely acting on different cell types. By way of these mechanisms, LIF action can be regulated both within and between tissues.

1.4.2 Gene structure and isoforms

The gene coding for LIF is widely conserved among eutherian mammals and comprised of three exons. Three alternative sequences exist for exon 1, giving rise to three different isoforms of LIF upon transcription which are generated by alternative splicing (Figure 1.8): diffusible LIF (D-LIF), matrix-associated LIF (M-LIF) and truncated LIF (T-LIF) (Voyle et al., 1999). D-LIF and M-LIF are secreted, whereas T-LIF remains in the cell. RNase protection studies have shown that these transcripts are independently regulated and are therefore likely to have distinct biological functions (Haines et al., 1999).

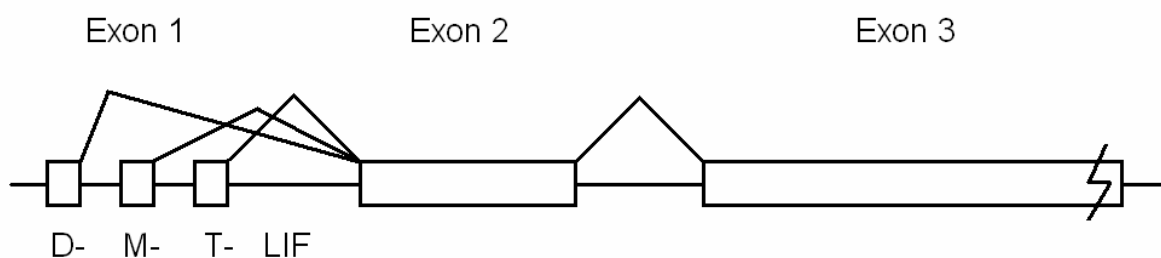


FIGURE 1.8. Schematic representation of exon splicing in the LIF gene.

T-LIF is reported to have a MW of 17 kD, being considerably lighter than its counterparts. Unlike the first exon of murine M-LIF and D-LIF, respectively, exon 1 of T-LIF contains no ATG start codon. Translation is therefore initiated at the ATG codon located in exon 2, giving rise to a truncated version of LIF lacking a large stretch of amino-terminal amino acids found in murine M-LIF and D-LIF which contains a signal secretion sequence. In the mouse, the sequences for D-LIF and M-LIF differ from one other only in the very first amino-terminal amino acids, with D-LIF being merely three amino acids longer than M-LIF (Figure 1.9). D-LIF and M-LIF have calculated masses of 22.29 kD and 22.16 kD, respectively, including a putative hydrophobic leader peptide of 24 amino acids (Gearing et al., 1988) which has been proposed to be cleaved off in the mature versions, giving rise to proteins of approximately 20 kD in weight (Rathjen et al., 1990; Haines et al., 1999).

D-LIF (diffusable):	MK VLAAG IVPLLLL...
M-LIF (matrix-associated):	MRC R IVPLLLL...

FIGURE 1.9. Amino-terminal sequences of murine D-LIF and M-LIF.

The minor difference in sequence and possible cleavage of the putative leader peptide in both isoforms suggest that M-LIF and D-LIF may exert no difference in function. However, findings of previous studies indicate otherwise. Overexpression of M-LIF but not the D-LIF transcript during early mouse embryogenesis has been shown to result in gastrulation defects (Conquet et al., 1992). Furthermore, overexpression of M-LIF but not D-LIF has been found to rescue cardiomyogenesis in *lif*^{-/-} embryoid bodies, rescue and promote smooth muscle cell development in *lif*^{-/-} embryoid bodies, and promote the proliferation of erythrocytes (Weitzer et al., unpublished data). And in yet another study, increased proliferation and longevity of cardiomyocytes have been observed when grown on M-LIF overexpressing parietal endoderm (PE), but not when grown on D-LIF overexpressing PE (Pasteiner, 2006). Taken together, these results indicate a difference between the function of M-LIF and D-LIF in addition to their difference in localization.

The aim of this diploma thesis was to examine the role of LIF in cardiomyogenesis, investigating differences between the effect of M-LIF and D-LIF on embryonic stem cells, embryoid bodies, cardiac progenitor cells and cardiomyocytes, as well as the impact of glycosylation on biological

activity. Based on the above mentioned data, it was anticipated that treatment with recombinantly produced M-LIF would lead to upregulation of cardiac markers in cardiomyocytes and contribute to expansion and directed differentiation of cardiac progenitor cells in vitro.

2 Materials

2.1 Chemicals for molecular biology

Acetic acid	Merck, D
Acrylamide	BioRad, USA
Agarose, Biozyme LE	Biozyme, D
Aprotinin	Sigma, USA
BCIP=5'Brom-4'Chlor-3'Indoylphosphat	Diagnostic Chemicals Limited, D
β-Mercaptoethanol	Loba Feinchemie, A
Bis N,N'-Methylenbisacrylamide	BioRad, USA
Bromphenolblue	Sigma, USA
BSA	Roth, D
Coomassie Brilliant Blue R250	Merck, D
Coumaric acid	Sigma, USA
Dabco	Sigma, USA
dNTPs	MBI Fermentas, Lithuania
Dimethylformamid	Fluka, CH
Dimethylsulfoxid (DMSO)	Acros, B
Dithiothreitol (DTT)	Acros, B
EDTA	Acros, B
Ethanol	Merck, D
Ethidiumbromide	Fluka, CH
Formaldehyde	Merck, D
Fuchsin (Pararosalinin)	Sigma, USA
Giemsa stain	Sigma, USA
Glycerin	Merck, D
Hydrochloric acid (HCl)	Acros, B
Imidazole	Sigma, USA
Iodoacetamide	Merck, D
Leupeptin	Sigma, USA
Methanol	Merck, D
MgCl ₂	MBI Fermentas, Lithuania

Mineral oil	Sigma, USA
Ni-NTA agarose beads	Quiagen, D
Nitrotetrazolium Blue Chloride (NBT)	Fluka, CH
PCR-buffer without MgCl ₂	MBI Fermentas, Lithuania
Pepstatin A	Sigma, USA
Periodic acid	Schuchardt, D
Phenylmethanesulfonylfluoride (PMSF)	Flucka, CH
Ponceau-S	Sigma, USA
Reverse Transcriptase buffer	Invitrogen, USA
SDS	BioRad, USA
Silver nitrate	Merck, D
Sodiumbicarbonate	Sigma, USA
Sodiumchloride	Salinen Austria, A
Sodiumhydrogencarbonate	LifeTechnologies, USA
Sodiumhydrogenphosphate	Roth, D
Sodiumhydroxide	Merck, D
Sodiumthiosulfate	Merck, D
Trichloroacetic acid	Merck D
Tris Base	LifeTechnologies, USA
Triton X100	Sigma, USA
Tween-20	Sigma, USA
Urea	Acros, B

2.2 Chemicals for cell culture

Amphotericin B	Invitrogen, USA
β-Mercaptoethanol	Loba, A
D-Glucose	Acros, B
DMEM powder	LifeTechnologies, USA
DMSO (Dimethylsulfoxide)	Sigma, USA
Fetal Bovine Serum (FBS)	HyClone, USA
Fetal Bovine Serum (FBS)	Gibco, USA
Fetal Bovine Serum (FBS)	Sigma, USA

Gelatine	Difco, USA
Geniticine (G418)	Life Tech, SCO
Glycine	Applichem, D
L-(+)-Glutamin	Acros, B
Mitomycin C	Acros, B
Penicillin G Potassiumsalt	Merck, D
Penicillin / Streptomycin	GibcoBRL
Potassium chloride	Sigma, USA
Recombinant mLIF	Chemicon International
Sf-900 SFM medium	GibcoBRL
Sodiumhydrogencarbonate	LifeTechnologies, USA
Streptomycin	Sigma, USA

2.3 Enzymes

Collagenase	Gibco, USA
DNase I, RNase free	Roche, D
Pancreatin	Worthington, USA
PNGase F	NewEnglandBiolabs, USA
RNaseOUT [®]	LifeTechnologies, USA
Superscript [®] II RNase Reverse Transcriptase	LifeTechnologies, USA
Taq DNA Polymerase	MBI Fermentas, Lithuania
Trypsin	LifeTechnologies, USA

2.4 Cell lines

SNL76/7-Fibroblasts:

These mouse fibroblasts were established by Allan Bradley. They are based on STO fibroblasts which were stably transfected with a neo-resistance expression vector and a LIF expression vector (McMahon and Bradley, 1990).

Embryonic stem cells:

AB2.2 wild type isolated by Allan Bradley from the mouse strain 129Sv (Soriano et al., 1991).

Cardioblast-like stem cells:

The initial cell line was established by Wolfgang Weber. It was isolated from the heart tissue of neonatal HDAC1 +/- mice (neoR) and innitally co-cultured with AB2.2 embryonic stem cells in order to aid in survival. HDAC1 +/- mice possess a neomycin-resistance-gene, whereas AB2.2 ESC do not. Thus, stem cells derived from the heart tissue of these mice can subsequently be selected for by adding G418 (Weber, 2006).

During the course of this study, 11 subclones of this cell line were successfully established.

Primary Cardiomyocytes:

These cells were isolated from the heart tissue of day 1-2 neonatal BalbC mice.

SF9 insect cells:

The SF9 cell line was derived from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*.

2.5 Antibodies

Table 2. 1 Antibodies used during this diploma thesis.

Antibody	Company / supplier
Anti mLif	R&D systems
Anti 6xHIS	Rockland

2.6 PCR primers

Table 2. 2 Primers used during the diploma thesis. All were ordered at VBC Genomics, Vienna.

Primer	Sequence 5' - 3'	Number of nucleotides	Tm in °C	Cycles	Fragment size
<i>Anf fwd</i>	cgggtgtccaacacagatctg	20	50.9	55	~200
<i>Anf rev</i>	tctctcagagggtgggttgac	20	48.5	55	~200
<i>Bcl XI fwd</i>	tggagtaaactgggggtcgcatcg	24	61	64	280

<i>Bcl XI rev</i>	agccaccgtcatgcccgtagg	22	59,1	64	280
<i>GAPDH fwd</i>	cgt ctt cac cac cat gga ga	20	53	27-30	300
<i>GAPDH rev</i>	cgg cca tca cgc cac agt tt	20	60,3	27-30	300
<i>Islet-1 fwd</i>	atg gga gac atg ggc gat cc	20	58,1	38	~360
<i>Islet-1 rev</i>	cgc agg gcg aat tcg tct cc	20	61,4	38	~360
<i>MEF-2C fwd</i>	ggc cat ggt aca ccg agt aca acg agc	27	66	32	400
<i>MEF-2C rev</i>	ggg gat ccc tgt gtt acc tgc act tgg	27	66,8	32	400
<i>MHCα fwd</i>	gga aga gtg agc ggc gca tca agg	24	66,5	30	300
<i>MHCα rev</i>	ctg ctg gag agg tta ttc ctc g	22	54,7	30	300
<i>Nkx2.5 fwd</i>	tct ccg atc cat ccc act tta ttg	24	58	27-35	222
<i>Nkx2.5 rev</i>	ttg cgt tac gca ctc act tta atg	24	56,2	27-35	222
<i>α-Tropomyosin fwd</i>	caagcggaggctgataagaagg	22	55	30	300
<i>α-Tropomyosin rev</i>	tgccctctcactctcatctgc	22	55	30	300

2.7 Expression vectors

Vectors coding for d-lif and m-lif cDNA were kindly provided by Waltraud Pasteiner (Pasteiner, 2006).

2.8 Kits

RNeasy® Mini Kit

Qiagen, D

QIAshredder® Homogenizer

Qiagen, D

2.9 Other Materials

Amicon Ultra centrifugal filter devices

Millipore, USA

Cover slips

Lactan, A

Slides

La Fontaine, D

Hyperfilm

Amersham Biosciences, UK

Immobiline Dry Strip pH 3-10

Amersham Biosciences, UK

DNA ladder (gene ruler, 100bp, 1kb)

MBI Fermentas, Lithuania

Page Ruler™ Protein Ladder

MBI Fermentas, Lithuania

Regenerated cellulose tubular membrane

Roth, D

Sterile disposable scalpels

Swann Morton, UK

2.10 Inhibitors

Aprotinin

Sigma, USA

Leupeptin

Sigma, USA

Pepstatin A

Sigma, USA

Geneticine (G418)

Life Tech, SCO

3 Methods

3.1 Cell culture

All cell culture work is performed under sterile conditions.

Washing glass bottles for media and solutions:

Glass bottles are treated separately in order to avoid contact with detergents. Therefore, the empty glass bottles are filled with water and a small amount of hypochloride and left for several minutes. Then the bottles are flushed five times with tap water, and filled with MilliQ water (endotoxin-free, specially treated). After 1 day bottles are dried and autoclaved at 120°C, 1.4 bar for 20 to 30 minutes.

Washing glass pipettes:

Used pipettes are collected in vessels filled with water and hypochloride. The pipettes are washed with tap water for at least three hours. The vessels themselves are rinsed and refilled with tap water and hypochloride for disinfection.

After washing, the pipettes are left over night in a vessel filled with MilliQ water and then dried at 80°C, plugged with cotton and baked in pipette boxes for eight hours at 180°C. The pipettes are reused exclusively for cell culture work.

3.1.1 Culture of mammalian cells

3.1.1.1 Media and solutions for mammalian cell culture

10xPBS (Phosphate buffered saline)

NaCl	80 g
KCl	2 g
Na ₂ HPO ₄ ·7H ₂ O	10.72 g
KH ₂ PO ₄	2 g

Dissolve salts in 800 ml of MilliQ water and titrate to a pH-value of 7.2 with a saturated Na₂HPO₄·7H₂O-solution. Fill to 1 l with MilliQ water. Sterile filtration (Nalgene Filter, Nalgene

Membrane; 0.22 μ m pore width). Store the stock solution at room temperature in the cell culture room.

1xPBS

Autoclave 450 ml of MilliQ water in a 500 ml cell culture bottle. Fill the bottle to 500 ml with 50 ml 10xPBS in the hood.

100xGPS (Glutamine-Penicillin-Streptomycin)

NaCl	80 g
Penicillin	1.5 g
Streptomycin	2.5 g
L-(+)-Glutamine	14.6 g

Dissolve substances in 500 ml MilliQ water, sterile filtration, aliquot 40 ml in 50 ml Falcons. Store at -20°C in the cell culture refrigerator, store at 4°C after thawing.

100x β -Mercaptoethanol

1xPBS	200 ml
β -Mercaptoethanol	144 μ l

Mix solution, sterile filtration and aliquot in 50 ml Falcons. Store at -20°C in the cell culture refrigerator, store at 4°C after thawing.

Trypsin

NaCl	3.5 g
D-Glucose	0.5 g
Na ₂ HPO ₄ ·7H ₂ O	0.09 g
KCl	0.185 g
KH ₂ PO ₄	0.12 g
EDTA	0.2 g
Trypsin	1.25 g
Tris Base	1.5 g

Dissolve the chemicals in MilliQ water, titrate to pH 7.6 with concentrated HCl, fill to 500 ml with MilliQ water, sterilize by filtration, and aliquot 40 ml in 50 ml Falcons. Store at -20°C in the cell culture refrigerator, store at 4°C after thawing.

1% gelatine stock solution

Solve 100 g of gelatine (Difco) in 1 l MilliQ water. Sterilize by filtration. Store at room temperature in the cell culture room.

0.1% gelatine solution

Autoclave 450 ml of MilliQ water in a cell culture bottle. Add 50 ml of the 1% stock solution in the hood and mix.

Dulbecco's Modified Eagles Medium (DMEM)

To prepare DMEM a 5 l Erlenmayer is filled with 4.5 l of MilliQ water. Exactly one half of a DMEM package (Gibco) is added under stirring. Upon dissolution of the powder 18.5 g of sodiumbicarbonate are added. The Erlenmayer is filled to 5 l and the medium is filtered through sterile filters into autoclaved bottles. 4 ml of medium per bottle are extracted to perform a test for contaminations by incubating the medium at 37°C over night. The medium is analyzed for contaminations under the microscope the next day.

Nutrition media for cell culture

M10Gi: for fibroblasts:

DMEM

10% Fetal bovine serum from Gibco

1x GPS

M15Hi: for ESCs and CBLSCs:

15% = 30 ml Fetal bovine serum from HyClone

1% = 2 ml GPS

1% = 2 ml β -Mercaptoethanol

to 200 ml with DMEM

M15Si: for EBs and parietal endoderm:

15% = 60 ml Fetal bovine serum from Sigma

1% = 4 ml GPS

1% = 4 ml β -Mercaptoethanol

to 400 ml with DMEM

M4Si: for pCMCs:

4% = 16 ml Fetal bovine serum from Sigma

1% = 4 ml GPS

1% = 4 ml β -Mercaptoethanol

to 400 ml with DMEM

Freezing medium:

60% DMEM

20% FBS (Gibco for fibroblasts, HyClone for ES cells, Sigma for parietal endoderm)

20% DMSO

Note: Always add DMEM first; when mixed use it immediately afterwards.

3.1.1.2 Coating of cell culture plates

Cell culture plates have to be gelatinized to serve as a matrix for the cells to adhere.

Apply at least 2 ml of 0.1% gelatine solution per 6 well and 6 cm plate, or 3 ml per 10 cm, leave gelatine on plates for two hours at room temperature, then suck off the solution completely with a Pasteur pipette. Use the cell culture plates directly after gelatinization.

3.1.1.3 Culture of SNL76/7 fibroblasts

Stem cells can only grow on feeder cells. Our feeder cells are produced from SNL 76/7 fibroblasts, which secrete the stem cell renewal factor LIF. The cells have to be mitotically inactivated to serve as feeder cells.

3.1.1.3.1 Thawing of SNL76/7 fibroblasts

The cells are kept in cryotubes at -180°C in liquid nitrogen. Before thawing a vessel with water of 37°C , a sterile 15 ml or 50 ml Falcon tube, M10 Gi, and a 1 ml Gilson pipette have to be prepared. Take the cryotube out of liquid nitrogen (wear safety goggles!), thaw the cells in the water bath quickly until a small ice core is still left. Rinse in 70% alcohol, dry, and put it into the hood. Flame the cryotube and transfer the cells with the 1000 μl Gilson pipette in the prepared Falcon tube. Add 10 ml of M10Gi medium slowly drop by drop with a 10 ml pipette. If done too

quickly the cells undergo an osmotic shock and most of the cells will burst. Flame the Falcon tube, close it, and centrifuge at 1000 rpm, room temperature for 7 min. (Heraeus, Biofuge; „swing-out-buckets“). Carefully suck off medium with a Pasteur pipette. Minimize the risk to suck off the cell pellet by slanting the Falcon tube. Resuspend the cells in 4 ml of fresh M10Gi. Transfer cell suspension to a 10 cm cell culture plate (which doesn't have to be gelatinized). Distribute cells equally by random movements (never in circles, this would concentrate all cell in the middle) and incubate at 37°C. Over night the fibroblasts adhere to the plate, and are confluent after a few days, meaning that the whole surface of the plate is covered with cells.

3.1.1.3.2 Continuous culture of SNL76/7 fibroblasts

The fibroblasts can be cultured on ungelatinized 10 cm cell culture plates, and have to be fed when the medium changes its color to yellow. When cells are confluent, they have to be split 1:5.

3.1.1.3.3 Splitting of SNL76/6 fibroblasts

The old medium is sucked off; cells are washed once with 5 ml of 1xPBS, PBS is sucked off. Add 1 ml trypsin with a 1000 µl Gilson pipette. Incubate the cells for 5 min at 37°C. Control the trypsinization status of the cells in the microscope (10x magnification). Cells should be loose and round because all cell contacts are destroyed. If there are still cells adhering to the plate, incubation time has to be extended. Care must be taken not to trypsinize for too long, since this may damage the cells. Add at least 4 ml of fresh M10Gi, resuspend by up- and down pipeting for several times. Addition of medium inactivates trypsin as soon as a ratio of 1 part trypsin to 2 parts medium is reached. The cells are distributed onto new 10 cm plates. For example, if you wish to split the cells 1:3 you have to transfer a third of your volume (1.6 ml in this case) onto a new plate. Splitting 1:8 should be the maximum, a ratio of 1:5 is best. Fill up the plates to 8 ml with M10Gi. Incubate the cells at 37°C until confluence is reached. With each splitting the passage number is elevated by +1.

3.1.1.3.4 Production of feeder cells

Confluent plates of SNL76/7 cells are mitotically inactivated by adding 80 µl of Mitomycin C to 4 ml culture medium for 3-4 hours. After carefully washing the cells with 1x PBS to remove remaining Mitomycin C, cells are trypsinized for 5-10 min, resuspended in 10 ml of culture

medium, centrifuged for 7 minutes at 1000 rpm, the supernatant is sucked off and the cell pellet is resuspended again in 10 ml of fresh M10Gi culture medium. The cell numbers are determined with the Coulter counter. Feeders in a final concentration of $1.5 - 1.75 \times 10^5$ cells/ml are plated onto gelatinized tissue culture wells – 1 ml in each well of a 24 well plate. The feeder cells are incubated at 37°C. The quality of the feeder cells should be examined on the next day: cells must cover the entire surface of the well or plate evenly. Feeder cells are able to support ES cells for about 2 weeks. If cells are not used immediately, the medium should be changed once it turns yellow or after each week in culture.

Table 3. 1 Volumes of feeder cell suspension for different well sizes.

Plate size (cm) or number of wells	Cell suspension (ml)
10	12
6	4
6-well	2
24-well	1
96-well	0.1

3.1.1.3.5 Freezing of SNL76/7 fibroblasts

Suck off medium with a Pasteur pipette and wash the cells one time with 5 ml of 1xPBS. Cover the cells with 0.7 ml of trypsin and incubate for 5 min at 37°C. Resuspend the loose cells in 2 ml of M10Gi and transfer into a 15 ml or 50 ml Falcon tube. Add 2.7 ml of 2x freezing medium with Gibco serum drop after drop, while permanently swirling the cell suspension in the tube. Transfer 1.8 ml of the cell solution with a 1000 µl Gilson pipette into each of the three cryotubes. Label the tubes with name of the cell line, passage, initials, amount of cells and date of freezing. Close cryotubes; transfer them into tightly closed polystyrene boxes for about 24h at –80°C. Then freeze into liquid nitrogen. Sign in the stored cells in your lab book and the stock registration map.

3.1.1.4 Culture of embryonic stem cells

To maintain ES cells in an undifferentiated state, they have to be grown on a feeder cell layer that produces LIF, or on gelatinized tissue culture plates with LIF enriched medium. Our feeder cells originate from SNL 76/7 fibroblasts, which are able to produce the stem cell renewal factor LIF.

3.1.1.4.1 Thawing embryonic stem cells

ES cells should be thawed quickly at 37°C, but 10 ml of fresh M15Hi medium are added slowly to prevent damage of ES cells by osmotic shock. Cell suspension is then centrifuged at 1000 rpm for 7 minutes. Supernatant is sucked off and ESCs are resuspended in 1 ml of M15Hi medium taken from the pre-fed feeder layers. Plate ES cell suspension on feeder cell layer, which has been pre-fed with M15Hi medium for at least two hours. ES cells will attach within 24 hours – examine growth of ES cells the next day.

3.1.1.4.2 Maintenance of embryonic stem cells

ES cells are grown at 37°C, 5% CO₂, with M15Hi culture medium, which has to be changed every day. Upon confluence of ES cells in a 24-well plate, they have to be pre-fed with 2 ml of fresh M15Hi medium 2 hours before splitting and split by trypsinization with 200 µl of trypsin for 20 min. Stop trypsinization by adding 1 ml of the M15Hi medium of the pre-fed feeder well. Cells are divided 1:3 onto pre-fed feeder cells (again two hours before splitting, the feeder cell medium is removed and feeder cells are pre-fed with M15Hi).

3.1.1.4.3 Freezing of embryonic stem cells

ES cells are trypsinized with 200 µl of trypsin for 20 min, then 800 µl of M15Hi medium is added. The cells are resuspended and transferred into a 14 ml falcon tube. To ensure retrieval of all ES cells, the well is washed with 800 µl of medium again and transferred into the falcon tube. Freezing medium is added in a 1:1 ratio very slowly, ES cells are aliquoted in cryotubes (1.8 ml per cryotube) and frozen at -80°C (slowly) for at least 24 hours, and then stored in liquid nitrogen.

Freezing medium:

1 ml:

0.6 ml DMEM

0.2 ml Serum (the same serum as the cells were cultured with)

3.2 ml DMSO

3.1.1.5 Culture of cardioblast-like stem cells

Cardioblast-like stem cells are thawed, maintained and frozen in the same manner as embryonic stem cells (see 3.1.1.4).

3.1.1.5.1 Subcloning of cardioblast-like stem cells

200 cells are seeded onto a 6-cm dish containing a feeder cell layer. 48 hours thereafter, the medium is exchanged and 40 µl of G418 (180 µg/ml) are added to the medium on a daily basis, thereby selecting for cells conferring neomycine resistance and eliminating any potential bystander cells.

When colonies have reached an appreciable size, they are isolated with a Gilson pipette and transferred to a well of a 96-well plate containing 30 µl of trypsin. After 3 -10 minutes they are resuspended with 80 µl of M15Hi taken from a well of a 96-well plate containing feeder cell layers, which had been pre-fed 2 hours beforehand. The cell suspension is then transferred onto the well containing the feeder cells from which the medium had been taken. After expansion of the colony for a few days the cells are transferred to 24-well plates containing feeder cell layers. This is by definition the first passage of the newly generated subclone.

3.1.1.5.2 Growth curve of cardioblast-like stem cells

50 000 cells/well are seeded onto 6 wells of a 24-well plate containing feeder cell layers. Every 48 hours following seeding, the cells of one well are trypsinized for 15-20 minutes and counted with the Coulter counter. This is repeated until all wells have been counted. The cell numbers are then plotted against the number of hours and a growth curve is established.

The growth rate is calculated using the following formula:

$$\text{Doubling time (h)} = \ln 2 \times \Delta t(\text{h}) / \ln n(t_2) - \ln n(t_1)^*$$

n...number of cells

*Values for t_1 and t_2 must lie within the linear portion of the graph which represents the exponential phase of the growth curve.

3.1.1.6 Generation of embryoid bodies

For the generation of EBs, ES cells are split 1:2 one day prior to making of EBs and trypsinized by adding 200 μ l of trypsin for 20 minutes. After resuspension in 800 μ l of M15Si, cell numbers are counted using the Coulter counter with a program measuring cell sizes between 10 and 20 μ m. Dilute the appropriate volume (you need 2 ml/10cm-plate) to a final cell number of 40 000 cells/ml. 20 μ l drops are put on the lid of a bacterial grade Petri dish, with water filled in the bottom. Drops are made with the 500 μ l Eppendorf pipette which is adjusted to 25x20 μ l. The cells in the hanging drops aggregate and form EBs, which are incubated at 37°C. On day 4.5 after generation of EBs, they have to be plated onto gelatinized 10 cm tissue culture plates with 8 ml of fresh M15Si culture medium. Importantly, EBs are dispersed equally on the plate by slightly shaking plates in two directions.

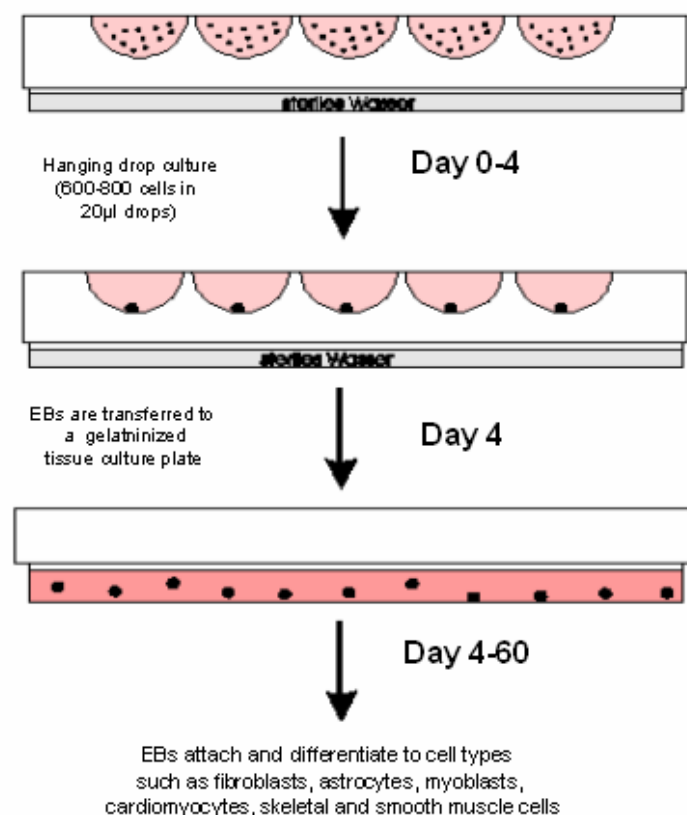


Fig. 3. 1 Generation of embryoid bodies (Stary, 2006).

In general, culture medium of EBs is changed every 3rd day, leaving an aliquot of old medium to supply EBs with their own produced growth factors. For EB culture in M15Si medium, on day 7 8 ml of fresh M15Si medium and 3 ml of old medium, on day 13, 16, 19 and 21, 10 ml of fresh and 4 ml of old medium, on day 25 and every 3 days later (if EB medium turns yellow shorten feeding interval), 12 ml of fresh and 5 ml of old medium are used for feeding EBs. For experiments with low serum the feeding protocol is changed as follows: Serum of EBs in 6 well plates or 24 well plates is completely removed on day 5, 6 or 7 and replaced by 4 ml (for 6 wells) serum free medium (M0), M0,2% Si, M1% Si, M4% Si or M10Si. Medium is changed every 3rd day, thus replacing half of the old medium by fresh medium.

3.1.1.7 Isolation of primary cardiomyocytes from neonatal mice

Primary cardiomyocytes were isolated from d1 and d2 wt mice (BalbC), respectively:

- Prepare hearts from neonatal mice and place in 1x PBS.
- Cut hearts into very small pieces, spin 2 min at 1000 rpm, remove supernatant.
- Add 1 ml of enzyme mixture (Pancreatin/Collagenase), digest 15 min at 37°C, shaking.
- Spin 2 min at 1000 rpm, remove supernatant, add 1 ml of fresh Pancreatin/Collagenase mixture.
- Digest at 37°C, shaking 5-15 min, until a homogenous appearance is achieved.
- spin 2 min at 1000 rpm, remove supernatant and resuspend in M4Si culture medium.
- Pre-adsorb cardiac fibroblasts on gelatinized tissue culture plates for 2-3 h, transfer supernatant containing enriched cardiomyocyte population onto new gelatinized tissue culture plates.
- Cultivate cardiomyocytes at 37°C, 5% CO₂.
- Renew culture medium after 24 h to remove cell debris.
- Change medium every 3-4 days.

Pancreatin/Collagenase enzyme mix:

0.4 ml Pancreatin (Gibco)

5 mg Collagenase (Worthington)

9.6 ml 1x PBS

3.1.2 Cell culture of insect cells

3.1.2.1 Media and solutions for insect cell culture

SF9 thawing and adaptation medium:

Same as culture medium, only with 20-5% FCS.

SF9 culture medium:

Sf-900 medium

2% FBS

1% P/S

Infection medium:

Sf-900 medium

2% FBS

SF9 freezing medium:

20% FBS

20% DMSO

60% Sf-900 medium

Baculovirus freezing medium:

Add 4% FBS to CM containing viral particles

3.1.2.2 Sf9 cell culture

The SF9 cell line was derived from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*. The SF9 cell line is highly susceptible to infection with *Autographa California* nuclear polyhedrosis virus (AcNPV baculovirus), and can be used with all baculovirus expression vectors. Sf9 cells are commonly used to isolate and propagate recombinant baculoviral stocks and to produce recombinant proteins.

3.1.2.2.1 Thawing and adaptation of SF9 cells

SF9 cells are kept in liquid nitrogen for storage and stock purposes.

The cells are quickly thawed and resuspended in SF9 II medium supplemented with 20% serum (Gibco), centrifugated at 1000 rpm for 5 minutes. The resulting pellet is resuspended in 5 ml of 20% serum containing medium again, and the cells are transferred to a culture flask (xs small). The cells are kept in sealed flasks at 28°C.

Once the culture has reached confluency, cells are gently scraped off the surface, collected and centrifugated at 1000 rpm for 5 minutes. The pellet is then resuspended in medium containing

15% serum, and the cells are seeded on a small culture flask. This procedure is continued with serum content decreasing by 5% every time the cells are split. When the serum content is below 5% usually 2 % serum are used for maintenance of cells.

3.1.2.2.2 Storage of SF9 cells

For storage, SF9 cells are collected and resuspended in medium containing 20% serum, equal amounts of freezing medium (containing 20% serum and 20% DMSO) are added drop by drop, and the cells are stored in cryo tubes at -80°C for 24 hours. After that they can be transferred to liquid nitrogen.

3.1.2.2.3 Infection of SF9 cells

For expression of the desired isoforms of LIF, the cells are seeded at a correct cell number required for infection (4×10^7 per large flask in 20 ml medium) and the virus is added according to the desired MOI. Infection is achieved after 96 hours, upon which the cells are visibly bloated and cell lysis sets in.

3.1.2.2.4 Viral plaque assay

Materials:

30 ml of exponential culture of SF9 cells at 5×10^5 cells/ml

6-well plates

1 bottle of 4% agarose gel

1 bottle of Sf-900 medium

0.5 ml baculovirus supernatant

1. under sterile conditions dispense 2 ml of cell suspension per well.
2. Allow cells to settle to bottom of plate and incubate, covered, at RT for 1 hour.
3. Following 1 hour incubation of the plates at RT, observe monolayers under the inverted microscope to confirm cell attachment and 50% confluence.
4. Produce an eight-log serial dilution of the harvested viral supernatant by sequentially diluting 0.5 ml of the previous dilution in 4.5 ml of Sf-9 medium in 15 ml Falcon tubes.

You should conclude with 8 tubes containing each of a 10^{-1} to 10^{-8} dilution of the original virus stock.

5. Move the six well plates and the tubes of diluted virus to the hood. Label the plates, in columns of two, " 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} ".
6. Sequentially remove the supernatant from each well, discard, and immediately replace with 1 ml of the respective virus dilution to each duplicate well. Incubate for 1 h at RT.
7. Melt the agarose solution in a microwave and let cool until handwarm. Quickly dispense 30 ml of Sf-900 medium and 10 ml of the 4% agarose gel to an empty bottle and mix gently.
8. Following this second 1 hour incubation, return the bottle of diluted agarose and the 6-well plates to the hood.
9. Melt the agarose/Sf9 medium solution and let cool until handwarm. Sequentially (from high to low dilution) remove the virus from the wells and replace with 2 ml of the diluted agarose. Work quickly to avoid desiccation of the monolayer.
10. Allow gel to harden for 10-20 minutes before moving.
11. Incubate at 27°C under humidified conditions (e.g. in a sealed plastic bag containing wet paper towels) for 4 to 10 days.
12. Recombinant virus produces milky/gray plaques of slight contrast visible without staining or other detection methods.
13. Monitor plates daily until the number of plaques counted does not change for two consecutive days.
14. To determine the titer of the inoculum employed, an optimal range to count is 3 to 20 plaques per well of a 6-well plate. The titer (pfu/ml) may be calculated with the following formula:

$$\text{Pfu/ml (of original stock)} = 1/\text{dilution factor} \times \text{number of plaques} \times 1/(\text{ml of inoculum/plate})$$

A good high titer virus stock should lie in the range of 1 to 5×10^8 Pfu/ml.

3.2 Protein purification from SF9 cells

After infection, the cells are collected by centrifugation for 5 minutes at 1000 rpm. The supernatant is removed, 5 ml of 1xPBS are added, and the cells are collected by centrifugation

for 3 minutes at 1000 rpm. The 1xPBS is discarded and the cells are frozen at -80°C until further use.

The cell pellet is thawed on ice and resuspended in freshly prepared lysis buffer. This is followed by incubation on ice for 15 minutes. The cell lysate is then mechanically disrupted in a Douncer (on ice), after which it is incubated on ice for 10 minutes. The lysate is then cleared by centrifugation at 14000 rpm, 4°C for 20 minutes. Thereafter, 10 µl of the supernatant are taken for analysis and the rest is distributed equally amongst four 2 ml Eppendorf tubes containing 200 µl of Ni-NTA agarose beads (Quiagen) each, which have been equilibrated in lysis buffer. The beads are then rotated at 4°C for 2.5 hours.

After loading, the beads are pelleted at 4°C, 14000 rpm for 5 minutes.

The beads are washed three times with 500 µl of freshly prepared wash buffer by inverting the tube several times and subsequent pelleting of the beads at 4°C, 14000 rpm for 5 minutes.

Elution fractions E1 and E2 are obtained by adding 200 µl of freshly prepared elution buffer I, followed by shaking at 800 rpm for 10 minutes and subsequent pelleting of the beads at 4°C, 14000 rpm for 5 minutes, after which the supernatant is collected.

Elution fractions E3 and E4 are obtained by adding 200 µl of freshly prepared elution buffer II, followed by shaking at 800 rpm for 10 minutes and subsequent pelleting of the beads at 4°C, 14000 rpm for 5 minutes, after which the supernatant is collected.

The purified protein is stored at 4°C and analyzed on an SDS polyacrylamide gel.

Lysis buffer

50 mM NaH₂PO₄
300 mM NaCl
10 mM Imidazole
adjust pH to 8 using 1 M NaOH
add NP-40 at a dilution of 1:100 directly before use

Wash buffer

50 mM NaH₂PO₄
300 mM NaCl
10 mM Imidazole
adjust pH to 8 using 1 M NaOH

Elution buffer I

50 mM NaH₂PO₄
300 mM NaCl
50 mM Imidazole
adjust pH to 8 using 1 M NaOH

Elution buffer II

50 mM NaH₂PO₄
300 mM NaCl
250 mM Imidazole
adjust pH to 8 using 1 M NaOH

All reagents used in protein purification contain protease inhibitors Aprotinin, Leupeptin, and Pepstatin A at a dilution of 1:1000.

If the protein is to be used for activity assays in cell culture, the sample must be freed from salts and imidazole prior to use. This is achieved by dialysing against a semi-permeable membrane as follows:

Preparation of dialysis membrane:

1. 15 cm of a regenerated cellulose tubular membrane (Roth) are rinsed with distilled water.
2. The membrane is placed into a 50 ml Falcon tube containing 50 ml of Solution 1 and heated in a waterbath at 60°C for 3 h (this removes any traces of heavy metal ions).
3. The membrane is rinsed again with distilled water.

Preparation of Eppendorf tubes:

4. Holes are burnt into the lids of Eppendorf tubes with a heated Pasteur pipette.
5. 400 µl of sample are added per tube.

Dialysis :

6. Pieces of the membrane (~2 cm) are cut off and clamped underneath the lids of the prepared Eppendorf tubes, thereby covering the holes. The rest of the membrane is stored in 40% Ethanol.
7. The tubes are centrifuged upside down at 1000 rpm for 1 minute to test for leakage.
8. If no leakage is visible, the tubes are placed upside down into a beaker containing 1xPBS at a concentration of 1:1000 in relation to the sample amount. Otherwise, repeat step 7.
9. The beaker is left over night at 4°C, stirring.

Solution 1 :

1 mM EDTA

2% Sodiumhydrogencarbonate

After dialysis, concentration and purity of the protein are measured with a Nanodrop device.

3.3 SDS polyacrylamide gel

Isolated proteins are treated with 3x Sample buffer and separated on a denaturing SDS-polyacrylamide gel.

3x Sample buffer:

3 ml Glycerin

0.9 g SDS

3.75 ml of Solution 7

1.75 ml ddH₂O

6 mg Bromphenolblue

- aliquot and store at -20°C
- add 150 µl β-Mercaptoethanol for 850 µl sample buffer aliquot before use

SDS polyacrylamide gel

Table 3. 2 Reagents for one mini protean gel (Biorad).

solution	15%	12%	9%	6%	stack
9	5 ml	4 ml	3 ml	2 ml	250 µl
7	2.5 ml	2.5 ml	2.5 ml	2.5 ml	-
8	-	-	-	-	600 µl
H ₂ O	2.45 ml	3.45 ml	4.45 ml	5.45 ml	1.59 ml
TEMED	5 µl	5 µl	5 µl	5 µl	5 µl
10% APS	50 µl	50 µl	50 µl	50 µl	25 µl

Solution 7:

1.5 M Tris/Cl pH 8.8 (18.17 g Tris)

0.4% SDS (4 ml 10% SDS)

H₂O to 100 ml

Adjust pH to 8.8

Solution 8:

0.5 M Tris/Cl pH 6.8 (6.06 g Tris)

0.4% SDS (4 ml 10% SDS)

H₂O to 100 ml

Adjust pH to 6.8

Solution 9:

30% Acrylamide (30 g Acrylamide)

0.8% Bis N,N'-Methylenbisacrylamide (0.8 g Bis N,N'-Methylenbisacrylamide)

H₂O to 100 ml

After polymerization of the gel, proteins and a size marker (BioRad low range or high range ladder) are run in a BioRad Mini Protean Gel chamber in 1x running buffer at 25 mA for 1-2 hours, depending on the percentage of the gel.

10x Running buffer:

30 g Tris

144 g Glycine

10 g SDS

H₂O to 1l

3.4 Coomassie staining

Most SDS polyacrylamide gels in this study were analyzed by staining with Coomassie brilliant blue for 30 minutes, followed by incubation with destaining solution over night.

Preparative gels for Mass Spectrometry analysis must be stained with fresh Coomassie staining solution in a glass container. Gel pieces are excised using sterile scalpels (Swann Morton) in a laminar flow hood.

Coomassie brilliant blue

45% (v/v) Methanol

10% (v/v) Acetic acid

0.4% (w/v) Coomassie Brilliant Blue

Destain

4.5% (v/v) Methanol

10% (v/v) Acetic acid

3.5 Silver staining

In some cases, SDS polyacrylamide gels were silver stained for increased signal sensitivity:

First, the gel is fixed for 20 minutes with fixer solution. Then the gel is washed for 10 minutes with washing solution and incubated in MilliQ water over night. On the following day, the gel is sensitized for 1 minute with Sensitizing solution, washed twice with MilliQ water for 1 minute each and incubated with Silver solution for 20 minutes at 4°C. The gel is then washed twice with MilliQ water for 1 minute each and developed for 5-10 minutes in Developing solution. Color development is terminated with Stop solution, which is added three times for one minute each. All steps are carried out on a shaker in the hood.

Fixer solution

50 ml Methanol
5 ml Acetic acid
45 ml MilliQ water

Washing solution

50 ml Methanol
50 ml MilliQ water

Developing solution

2 g Sodium carbonate
40 µl 35% formaldehyde
to 100 ml with MilliQ water

Sensitizing solution

0.02 g Sodium thiosulfate
to 100 ml with MilliQ water

Silver solution

0.1 g Silver nitrate
to 100 ml with MilliQ water

Stop solution

5 ml Acetic Acid
to 100 ml with MilliQ water

3.6 Western Blot analysis

Proteins separated by a SDS-Polyacrylamide gel are transferred to a nitrocellulose membrane (Schleicher and Schuell) using a BioRad semi-dry transfer cell. The blot consists of (from the bottom upwards): 6 layers of Whatman paper the same size as the gel, one nitrocellulose membrane the same size as the gel, the gel, and 6 layers of Whatman paper the same size as the gel. All components are soaked with blotting buffer.

Air bubbles are removed by applying pressure with a Falcon tube. Blotting is carried out at 16 V for 1 hour.

10x Blotting buffer:

0.48 M Tris

0.4 M Glycine

ddH₂O

Adjust pH to 9.1

After blotting the protein is stained with Ponceau-S for 5 minutes and rinsed with water to assess transfer and to visualize the protein ladder which can then be marked with a pen.

Ponceau-S

100 mg Ponceau-S

100 ml 1% glacial acetic acid

Antibody incubation and development of the blot:

After blotting, the membrane is blocked with blocking solution for 1 hour. This is followed by three wash steps with 1xTBS/0.1% Tween for 5 minutes each and incubation over night with primary antibody solution. On the following day, the membrane is washed three times with 1xTBS/0.1% Tween for 5 minutes each and incubated with the secondary antibody solution for 1 hour. The membrane is then washed three times with 1xTBS/0.1% Tween for 5 minutes each.

- Detection depends on used secondary antibody:
 - o for an AP conjugated antibody the blot is incubated with AP buffer for 5 minutes
 - o the blot is incubated with 10 ml of AP buffer with 33 µl of NBT (added first) and 66 µl of BCIP (add second)
 - o the blot is incubated in the dark until bands become visible (5-60 minutes), the reaction is stopped by removing AP buffer and washing with water

 - o for HRP conjugated antibody, 1 ml of ECL solution is mixed with 3 µl of H₂O₂ and the blot is incubated with this solution for 1 minute
 - o detection by exposure to a film for 5-60 minutes (Hyperfilm, Amersham)
 - o the film is developed in a AGFA curix60 machine

Blocking solution:

5% dry milk powder (low fat)

dissolve in 1xTBS/0.1% Tween

Primary antibody solution

5% BSA

dissolve in 1xTBS/0.1% Tween

desired amount of primary antibody

Secondary antibody solution

5% dry milk powder (low fat)

dissolve in 1xTBS/0.1% Tween

desired amount of secondary antibody

NBT

75 mg NBT

700 μ l Dimethylformamid

300 μ l H₂O

AP buffer:

100 mM Tris/Cl PH 9.5

100 mM NaCl

50 mM MgCl₂

BCIP

50 mg BCIP

1 ml Dimethylformamid

ECL stock solution:

dilute 20 ml 1MTris in 200 ml H₂O

add 0.5 ml p-Coumaric acid

add 1 ml Luminol

Table 3. 2 Antibodies used for Western blotting.

Primary antibody	Secondary antibody
α LIF 1:500	anti goat AP 1:10 000
α 6xHIS 1:15 000	anti rabbit HRP 1:30 000

3.7 2-D gel electrophoresis

For 2-D-gel electrophoreses 50 μ g of protein* are solved in 250 μ l of Sample buffer and rotated for at least 30 minutes at room temperature, after which the protein sample is loaded on the Immobiline Dry Strip pH 3-10 (Amersham) as follows: the sample has to be carefully dropped in the IPGphor ceramic strip holder (13 cm, Amersham) and the strip placed on the sample with the positive direction on the pointed end. After that the strip has to be covered with 800 μ l of mineral oil.

*using cell CM makes serum starvation necessary to avoid albumin spots which often overlay other spots.

Isoelectric focusing

Isoelectric focusing is performed on IPGphor (Amersham) under following conditions:

Table 3. 3 Program for isoelectric focusing.

Time	Voltage
10 hours, 20°C rehydration	-
30 min	200 V
45 min	500 V
2 h	gradient from 500 V – 3500 V
6 h	3500 V
4 h	100 V

After isoelectric focusing of the sample the gel strip is washed for 15 min with Equilibration buffer 1, and 15 min with Equilibration buffer 2. Then place the strip on a SDS-page gel and cover it with agarose sealing solution. The gel strip replaces the stack gel. Gel run: 200, V 3.5 h. When the gel run is finished, the gel can be used for Coomassie staining (see 3.4), Silver staining (see 3.5) or Western blot analysis (see 3.6).

Sample buffer (Sprenger et al.; 2004):

7 M Urea
2 M Tris-HCl
4 % Triton X-100
2 v/v% Carrier Ampholyte pH 3-10
20 mM Tris-base
55 mM DTT
0.003 % Bromphenolblau
store in 250 µl aliquots at -20°C

Equilibration buffer 1 & 2:

50 mM Tris/HCl pH 8.8
6 M Urea
30 % Glycerin
2 % SDS
0.002 % Bromphenolblau
buffer 1: 6 ml + 150 µg Iodoacetamid
buffer 2: 10 ml + 100 µg DTT
Iodoacetamid and DTT always have to be added immediately before use

Agarose sealing solution:

0.5 v/v% Agarose

0.002 % Bomphenolblau

solved in 1x Running buffer (see SDS polyacrylamide gel)

3.8 Glycoprotein detection

3.8.1 Periodic acid-Schiff stain

Periodic acid-Schiff (PAS) is a staining method mostly used in histology and pathology. This method is primarily used to identify glycogen in tissues. The reaction of periodic acid selectively oxidizes the glucose residues, creates aldehydes that react with the Schiff's reagent and creates a purple magenta color. A suitable basic stain is often used as a counterstain.

Following SDS polyacrylamide gel electrophoresis, samples can be stained directly for the presence of carbohydrates (Van-Seuningen and Davril, 1992):

Table 3. 5 Periodic acid-Schiff stain procedure.

Reagent	Incubation time
20% Trichloroacetic acid	5 min
ddH ₂ O	5 min
0.7% periodic acid + 5% acetic acid	10 min
ddH ₂ O	2 min
ddH ₂ O	2 min
Schiff's reagent	10 min
5% Potassium disulfite + 5% acetic acid	5 min

Schiff's reagent recipe:

1. Dissolve 5 g of basic fuchsin in 900 ml of boiling distilled water.
2. Cool to approximately 50°C and slowly add 100 ml of 1 N HCl.
3. Cool to approximately 25°C and dissolve 10 g of K₂S₂O₅.
4. Shake for 3 minutes and incubate in the dark at room temperature for 24 hours.
5. Add 5 grams of fine activated charcoal and shake for 3 minutes.
6. Filter solution (should be clear).
7. Store at 4°C in a foil covered bottle.

3.8.2 Enzymatic deglycosylation with PNGase F

Peptide: N-Glycosidase F, also known as PNGase F, is an amidase purified from *Flavobacterium meningosepticum* which cleaves between the innermost GlcNAc and asparagines residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins.



This allows for an indirect detection of N-linked carbohydrates on any given protein.

Typical reaction conditions are as follows:

1. Combine 1-20 μg of glycoprotein, 1 μl of 10x Glycoprotein Denaturing Buffer and H_2O (if necessary) to make a 10 μl total reaction volume.
2. Denature glycoprotein by heating reaction at 100°C for 10 minutes.
3. Make a total reaction volume of 20 μl by adding 2 μl of 10x G7 Reaction Buffer, 2 μl of 10% NP-40, H_2O and 1-2 μl of PNGase F.
4. Incubate reaction at 37°C for 3 hours.

After deglycosylation, the protein is run on a SDS polyacrylamide gel, stained with Coomassie, and its molecular weight is compared to that of the undigested form. RNase B is used as a control substrate. This protein has an apparent MW of 17 kD containing one glycan chain of high mannose type. The apparent MW is reduced to 15 kD upon digestion with PNGase F.

3.9 Isolation of RNA with Qiagen RNeasy Mini Kit

For cell isolation the medium is discarded and the cells are washed with 5 ml of ice cold 1xPBS. Then the cells are covered with 1 ml of ice cold 1xPBS. All the following procedures are performed on ice. With a cell scraper go once along the edge and then scrape the cells from top to bottom. With a Gilson pipette the total volume is transferred to a 1.5 ml Eppendorf tube. Then the tubes are centrifuged at 4°C and the supernatant is discarded leaving only the pellet. The pellets are stored at -80°C until further use or RNA isolation is started immediately.

- Pellet is resuspended in 600 μ l RLT buffer and 6 μ l of β -mercaptoethanol, the lysate is transferred into a QIA shredder column, which is positioned on a 2 ml tube.
- Centrifuge for 2 min at 13000 to 14000 rpm.
- Then 600 μ l of 70% EtOH are added and then suspend very well.
- The lysate is transferred onto an RNAeasy column including a 2 ml collection tube.
- Centrifuge the RNAeasy column for 15 sec at 13000 rpm – this is the critical step where binding of the RNA takes place.
- The flow-through is discarded.
- First, the column is washed with 700 μ l of RW1 and then 2x with RPE buffer, inbetween that columns need to be centrifuged for 15 sec, except after the 2nd washing step, here centrifugation needs to take place for 2 min.
- RNAeasy column gets placed on top of a new tube.
- 30 μ l of RNase free water are pipetted directly on the membrane .
- centrifuge at 13000 rpm for 1 minute.

Afterwards, a DNase digestion is performed in order to remove DNA:

30 μ l sample	
+ 3.75 μ l DNase (RNase free!)	
+ 3.75 μ l buffer	incubate the mix for 30 min at 37°C
+ 3.75 μ l stop solution	this mix is incubated for 10min at 65°C

Centrifuge the whole mix for a few seconds to spin down the condensed water. Measure the RNA concentration with a Nanodrop device. RNA can be stored at -80°C, but it is recommended to test for remaining genomic DNA by PCR with GAPDH primers prior to reverse transcription into cDNA.

3.10 Reverse Transcription of mRNA into cDNA

For reverse transcription of mRNA into cDNA, oligo-d(T)s are used as primers, since they bind to the poly-A tail of mRNA. The enzyme that drives the reaction is an RNA dependent DNA polymerase.

- 7.5 µg RNA, dissolved in 29.5 µl water, are mixed with 1 µl oligo-d(T) and incubated at 70°C for 10 min.
- then 3 min incubation on ice, after that centrifugation of the tubes at RT for 30 sec and 13000 rpm.
- the next step is to add 1.8 µl of RT-Mix.
- Incubation of the mix at 42°C for 2 min.
- Addition of 200 U (=1µl) Superscript Reverse Transcriptase.
- incubation at 42°C for 50 min.
- incubation at 70°C for 15 min (the enzyme is deactivated).
- after deactivation the mix is put on ice for 5 min, followed by centrifugation at RT – 13000 rpm for 2 min.
- the cDNA is stored at -20°C.

1x RT-Mix

10 µl	5x Reverse Transcriptase buffer
5 µl	0.1 M DDT (Dithiothreitol)
1.5 µl	RNaseOUT (inhibits RNases, add immediately before use)
2 µl	10 mM dNTPs

3.11 Semi-quantitative RT-PCR

PCR amplifies the DNA sequences enclosed by two primers, which results in a double stranded DNA fragment of a certain length that can be detected on an agarose gel. By reverse transcription (RT)-PCR gene expression levels of distinct genes can be analyzed. Therefore specific primers are used to amplify cDNA that has been reverse transcribed from mRNA.

Table 3. 6 Contents of one PCR mixture (50µl).

PCR water (aqua dest.)	38.75 µl
10x PCR-buffer w/o Mg ²⁺	5 µl
25mM MgCl ₂	3 µl
10mM dNTPs	1 µl

Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Taq DNA-Polymerase	0.25 µl
cDNA	1 µl

For PCR with one primer set for more than 2 samples a master mix (containing everything but cDNA) is prepared first, then aliquoted to 49 µl each, cDNA added and one drop of mineral oil for PCR with Biometra Trio Thermoblock. For PCR with heated lid apparatus no oil is needed.

Table 3. 7 Standard PCR Program.

	Temperature	Time
0	94°C	60 sec
1	94°C	45 sec
2	Annealing temperature	45 sec
3	72°C	60 sec
4	72°C	300 sec
5	4°C	infinitely

Step 1-3 is repeated for 27-40 cycles

The cycle number for each primer pair was determined so that no saturated signal was obtained.

Annealing temperature

The annealing temperature is based on the T_m value of the oligonucleotide, at which half of the DNA molecules are single stranded. A T_m of -7°C is normally used as annealing temperature, since higher temperatures inhibit binding of the primer, and lower temperatures favor unspecific binding. The optimal annealing temperature for each primer is usually determined empirically.

The formula for calculating T_m : $2 \times (A+T) + 4 \times (G+C) = T_m$ in °C

A more precise formula which also takes into account the salt concentration:

$$T_m = 81,5 + 16,6(\log_{10}[J^+]) + 0,41(\%G+C) - (600/l) - 0,63(\%FA)$$

[J+]...concentration of monovalent Kations

l...length of oligonucleotide

FA...Formamid

Table 3. 8 Annealing temperatures for primers used in this study.

Primer	Annealing Temperature (°C)
Anf	55
BCI X1	64
GAPDH	55
Islet-1	60
Mef-2C	66
MHC α	60
Nkx2.5	58
α -Tropomyosin	55

3.12 Agarose gels for separation of PCR products

DNA molecules with a length of 0.1 to 60 kb can be separated on agarose gels. The agarose percentage of the gel depends on the fragment sizes, for PCR fragments of 200-1200 bp, 1.5-2% agarose gels were used. Gels were prepared and run as described in a previous diploma thesis (Stary, 2001). As a size marker, a 100 bp DNA ladder (gene ruler, Fermentas) was used. DNA on gels was stained with ethidium bromide (15 μ l for 150 ml ddH₂O) for 20 min and photographed under UV-light exposure.

3.13 Karyotyping

Prepare cells

1. Harvest the cells by trypsinization and/or pipetting as required and transfer to 15-ml tubes.
2. Centrifuge cell suspension 5 minutes at 1000 rpm, room temperature.

3. Remove supernatant, resuspend pellet in remaining supernatant (<0.5 ml) by vortexing the tube and adding dropwise 4 ml of pre-warmed (37°C) 75 mM hypotonic KCl buffer.
4. Incubate 6 minutes at room temperature.

Fix cells

5. Centrifuge cells 5 minutes at 1000 rpm, room temperature, remove the hypotonic KCl solution, and add 4 ml of fresh 3:1 methanol/glacial acetic acid fixative with a Pasteur pipet.
6. Centrifuge the tubes again as in step 5, discard supernatant, and resuspend the cells in 3 ml fresh 3:1 methanol/glacial acetic acid fixative. Repeat this step once or twice until a suitable cell pellet is obtained.
7. Add 0.5 ml fresh 3:1 methanol/glacial acetic acid fixative and incubate 3 hours at 4°C.

Prepare slides

8. Clean glass microscope slides with 95% ethanol. Wipe slides with dry lint-free paper towels.
9. Resuspend the cells in a small amount of fresh 3:1 methanol/glacial acetic acid fixative until suspension looks slightly turbid ($\sim 5 \times 10^6$ cells/ml). Drop 3 to 4 drops evenly on a slide covered with a thin film of distilled water. After the slide is completely dry, examine with a phase-contrast microscope to check cell density and spread of chromosomes.

If the cell density is too high, add a few more drops of fixative to the cell suspension. If the cell density is low, centrifuge the suspension and resuspend the pellet in a smaller amount of fixative.

10. With a Pasteur pipet, let two drops of cell suspension fall onto each cleaned, wet slide. Mark the slide with a diamond pen or pencil. Air dry slide(s) over night in the fume hood.
11. Stain with Giemsa solution for 2 minutes and rinse with distilled water.

3.14 Alkaline phosphatase assay

Since embryonic stem cells are known to exhibit increased expression of alkaline phosphatase (Resnick et al., 1992), this enzyme can be used as a marker for undifferentiated cells.

1. The cells are washed 2x with 1xPBS for 5 minutes each.

2. The cells are pre-incubated with AP buffer for 5 minutes.
3. AP buffer is sucked off and 1.5 ml of AP coloring solution are added to each well.
4. Incubation in the dark for 15 minutes.
5. Stop reaction with distilled water and count blue colonies.

4 Results

The main tasks of my diploma thesis were, first, to successfully express and purify matrix-associated and diffusible Leukemia Inhibitory Factor (M-LIF and D-LIF, respectively) in their glycosylated forms, and second, to test the influence of these cytokines on various types of cells, encompassing murine embryonic stem cells (ESCs), primary cardiomyocytes (pCMCs) and cardioblast-like stem cells (CBLSCs). Since little is known about the latter cell line, which was only recently established and is believed to represent a line of somatic stem cells isolated from neonatal mouse hearts, an additional task was to begin thorough characterization of this cell line.

4.1 Characterization of M-LIF and D-LIF

After expression and purification of M-LIF and D-LIF, Westernblot analysis was conducted to confirm identity. For further characterization, the degree of glycosylation was enzymatically determined and mass spectrometry analysis was performed.

4.1.1 Expression of M-LIF and D-LIF

The two LIF isoforms of interest were expressed in Sf9 cells via the baculovirus system. Insect cells were chosen, due to their ability to generate posttranslational modifications, such as phosphorylation, myristylation, palmitylation, and glycosylation, to name just a few. Since LIF is known to be heavily glycosylated (Sasai et al., 1998), these modifications could potentially play a key role in activity and/or function of this cytokine.

Sf9 cells were transiently transfected with baculoviruses containing expression vectors for M-LIF and D-LIF, respectively. These vectors were constructed and verified by Waltraud Pasteiner during the course of her doctoral thesis (Pasteiner, 2006). Both viruses were shown to be equally infectious exhibiting a titer of 2.2×10^5 plaque forming units (pfu)/ml, as derived from the viral plaque assay conducted in this study. A multiplicity of infection (MOI) of 0.01 pfu/cell was used for each infection. Generally, one cycle of infection lasted 96 hours, upon which cells were washed, pelleted, and immediately frozen at -80°C . Figure 4.1 shows microphotographs of infected and uninfected cells, taken just before harvesting.

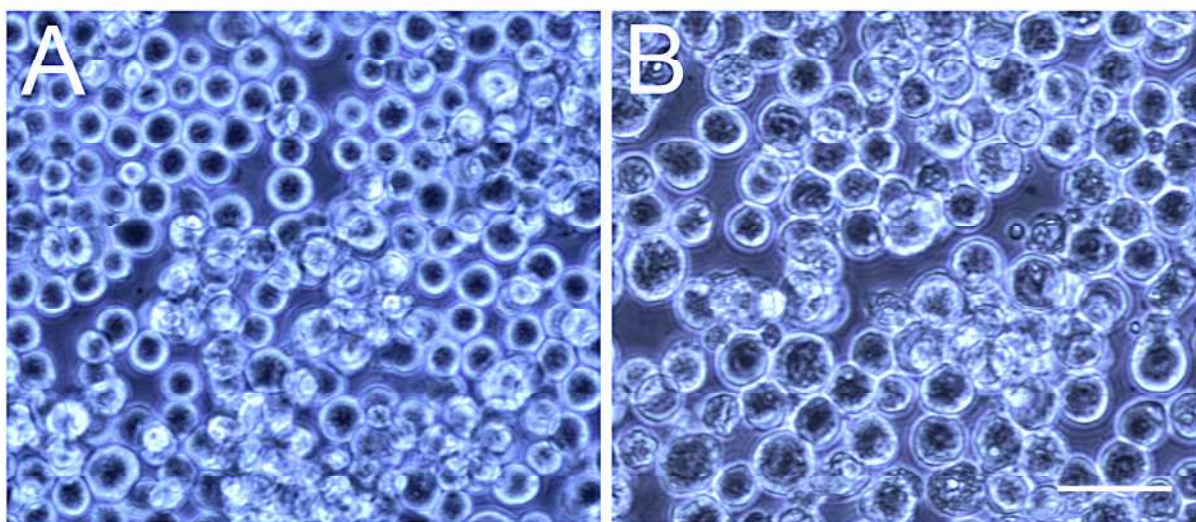


FIGURE 4.1. Morphology of Sf9 cells. A, Sf9 cells 4 days after seeding. B, Sf9 cells 4 days after seeding and infection with baculoviruses. The cells are visibly bloated and granular, as opposed to the uninfected cells in A. Scale bar = 20 μm .

4.1.2 Purification of M-LIF and D-LIF

Since the expression vectors for M-LIF and D-LIF, respectively, contain a carboxy-terminal stretch coding for six consecutive histidines, immobilized-metal affinity chromatography (IMAC) using the chelating ligand nitrilotriacetic acid (NTA) charged with Ni^{2+} and coupled to agarose beads was chosen for purification. NTA occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHIS tag (Figure 4.2). LIF was purified from cell pellets using the batch system and competitively eluted

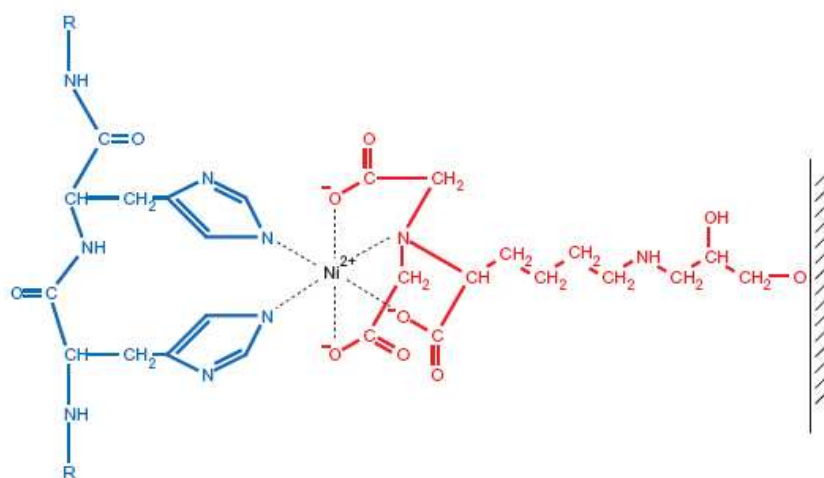


FIGURE 4.2. Interaction between neighboring residues in the 6xHIS tag and Ni-NTA matrix (The QIAexpressionist, 2003).

with two different concentrations of imidazole. Four elution fractions were collected, the first two of which were obtained with 50 mM of imidazole, the second two with 250 mM of imidazole. Since elution steps 3 and 4 were carried out under more stringent conditions than elution steps 1 and 2, the ratio of LIF to background protein was considered to be more favourable in elution fractions 3 and 4. Therefore, these fractions were used for subsequent activity assays after pooling and dialysis. Figure 4.3 shows a typical elution profile of M-LIF which was in no case visibly distinguishable from that of D-LIF.

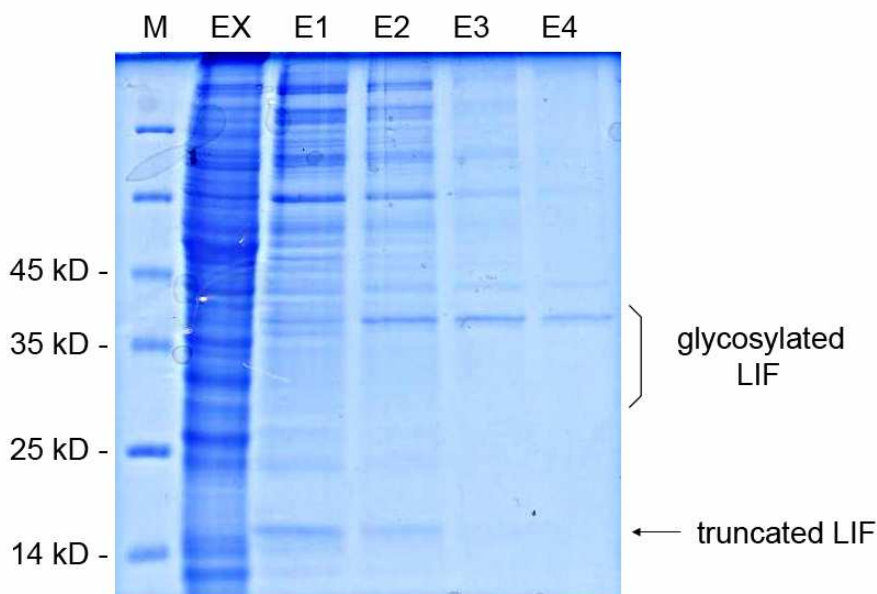


FIGURE 4.3. Purification of M-LIF by Ni^{++} -affinity chromatography. Elution fractions 1-4 (E1-4) and the initial protein extract (EX) of Sf9 cells infected with baculoviruses containing the expression vector for M-LIF are shown on a Coomassie stained SDS polyacrylamide gel. An unglycosylated, truncated form of M-LIF is visible at a MW of approximately 19 kD. Glycosylated forms are less visible, but have been clearly detected at a MW ranging from 30-40 kD in subsequent Western blot analysis (see Figure 4.5). The band seen at approximately 37 kD does not represent a form of LIF, since it was also observed in control extracts (see Figure 4.4).

Samples to be used for activity analyses were freed from salts and imidazole via dialysis across a semi-permeable membrane, and protein concentration was calculated. Photometric analysis was used to determine total protein content, of which 2% were estimated to be LIF. This estimation is based on mass spectrometry analyses of previous purifications conducted in our laboratory.

From Coomassie stained SDS polyacrylamide gels we concluded that LIF was expressed only at low levels. For this reason, samples of M-LIF, D-LIF, and a control fraction were run on a SDS polyacrylamide gel which was silver stained in order to enhance signal sensitivity (Figure 4.4). On such gels, purified LIF could be identified very clearly. As expected, glycosylated forms

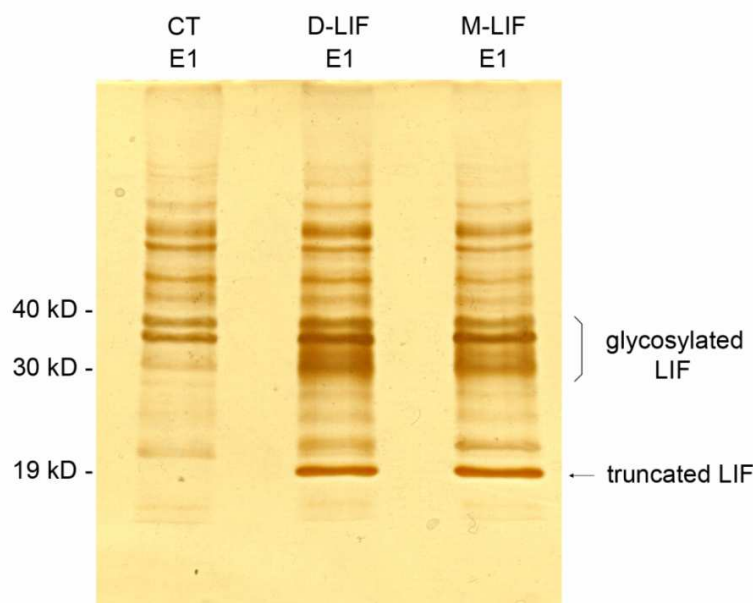


FIGURE 4.4. Silver stain analysis of purified M-LIF and D-LIF. Elution fraction 1 of Sf9 cells infected with baculoviruses containing the expression vector for M-LIF (M-LIF E1), Sf9 cells infected with baculoviruses containing the expression vector for D-LIF (D-LIF E1), and uninfected Sf9 cells (CT E1) are shown on a silver stained SDS polyacrylamide gel. A truncated form of LIF at a MW of 19 kD can clearly be seen in D-LIF E1 and M-LIF E1, as well as glycosylated forms between 30 and 40 kD. These bands do not appear in CT E1.

ranging from a molecular weight (MW) of 30 to 40 kD can be seen, but interestingly also what appears to be an unglycosylated form at 19 kD. Since full-length, unglycosylated LIF containing a HIS-tag must in theory be heavier than 20 kD, this form can only be a truncated version of LIF.

4.1.3 Western blot analysis of M-LIF and D-LIF

To confirm the presence of LIF in the purified samples, Western blots were performed with α LIF antibodies and α 6xHIS antibodies, respectively. Samples of several different purification runs were analyzed, revealing that LIF expression and purification had been successful in every case, but apparently highly variable from bout to bout, judging from differences in band intensities on Western blots. Samples that were obtained from one particularly favorable passage of expression and purification are shown in Figure 4.5, after Western blot analysis with α LIF and α 6xHIS antibodies, respectively. As already observed after silver staining, several bands can be seen at a MW between 30 and 40 kD and one distinct band at 19 kD.

Additionally, Western blot analyses were conducted with samples taken from culture medium that had been kept after infection. Fairly high amounts of LIF could be detected with α LIF

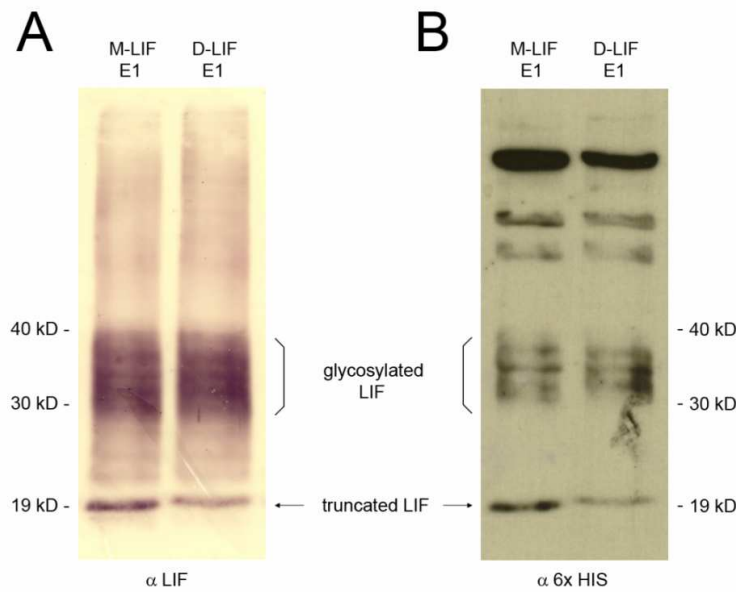


FIGURE 4.5. Identification of M-LIF and D-LIF isoforms by Western blot analysis. *A*, Western Blot of elution fraction 1 (E1) of purified M-Lif and D-Lif, respectively, with α LIF antibodies. Glycosylated forms are visible in the range of 30-40 kD as well as a truncated, unglycosylated form at approximately 19 kD. *B*, WesternBlot with α 6x HIS antibodies. Again, glycosylated and truncated forms of LIF can be seen. Bands >40 kD are unspecific, since they were also detected in the control fraction (not shown).

antibodies in the unconcentrated and unpurified sample, with D-LIF showing consistently higher levels than M-LIF (Figure 4.6). A densiometric analysis was performed, revealing a significantly

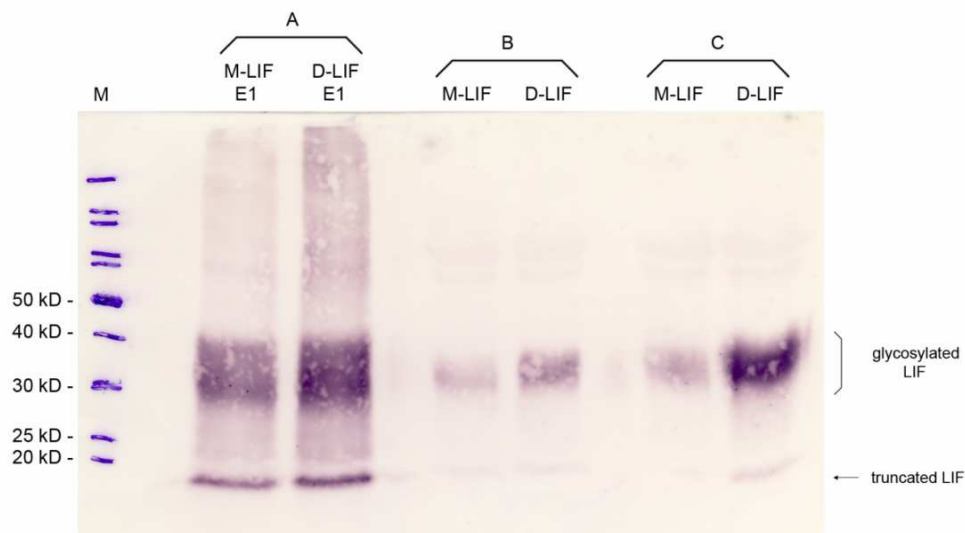


FIGURE 4.6. Sf9 cells produce similar amounts of M-LIF and D-LIF, but secrete much more D-LIF. *A*, Elution fraction 1 (E1) of M-LIF and D-LIF purified from Sf9 cells. Levels for intracellular M-LIF and D-LIF are comparable. *B+C*, Analysis of M-LIF and D-LIF levels, respectively, in the culture medium of infected Sf9 cells taken from two separate rounds of infection. In both cases, more D-LIF than M-LIF could be detected, suggesting a difference in secretion of the two isoforms.

higher amount of D-LIF in all analyzed samples (Figure 4.7). Since M-LIF and D-LIF were proven to be obtainable in equal amounts when isolated from cell lysates (Figure 4.6, A), we may conclude that M-LIF and D-LIF are secreted to a different degree, with D-LIF being the more favorably secreted isoform.

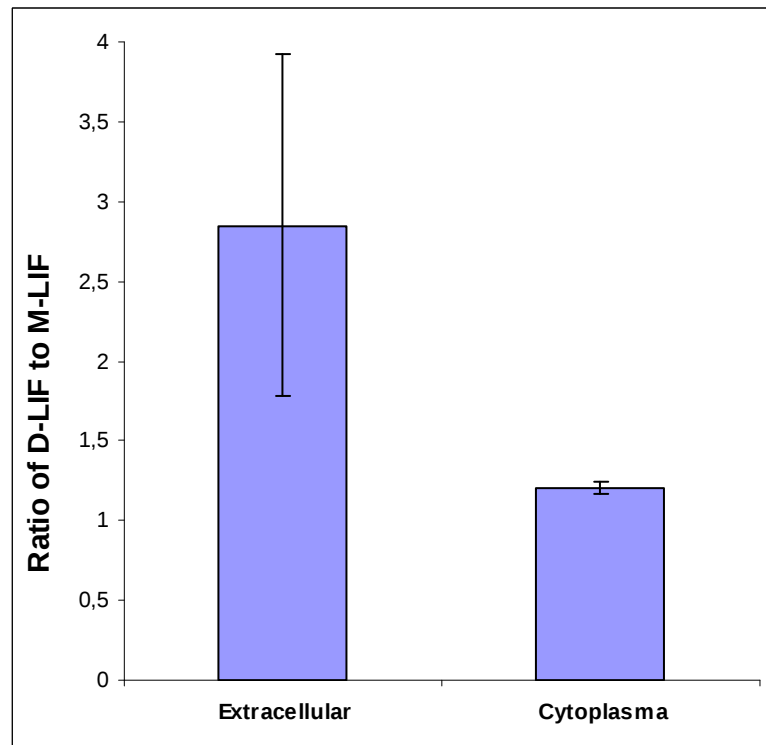


FIGURE 4.7. SF9 cells secrete 3-times more LIF than M-LIF. Expression of d-lif and m-lif cDNA under the control of the same promoter resulted in an equal amount of M-LIF and D-LIF protein in the SF9 cell lysates (cytoplasm) but D-LIF secretion into the medium (extracellular) was significantly increased. Protein concentration was determined in cell lysates from equal numbers of SF9 cells and from equal volumes of culture medium, respectively, by Western blot analysis with α LIF antibodies and densitometric analysis of NBT/BCIP stained nitrocellulose membranes with the program photoshop CS2. Error bars indicate standard deviation $\sigma_{x(n-1)}$; N=3

Moreover, these data reveal that both isoforms of LIF were highly present in infected culture medium. If biological function is proven to be identical to that of intracellularly derived LIF, using culture medium to obtain and purify LIF would be advisable for future experiments, due to the far greater yields thereby achievable.

4.1.4 Determination of glycosylation

The next step was to confirm glycosylation of both LIF isoforms. A Western blot was performed in which M-LIF and D-LIF were compared to commercial LIF that had been produced in *E.coli* and therefore had to be naturally devoid of glycosyl moieties.

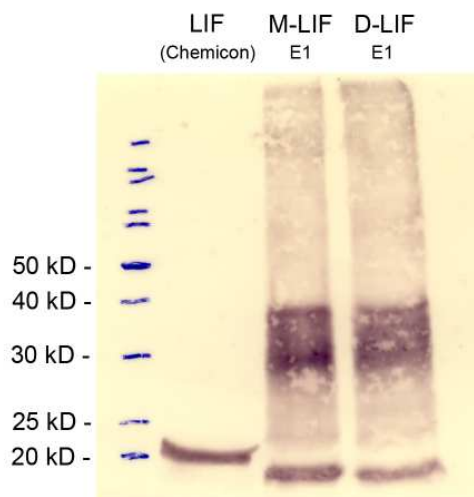


FIGURE 4.8. LIF produced in this study is posttranslationally modified, as opposed to purchased LIF. Western Blot of purchased LIF (Chemicon) and elution fraction 1 (E1) of purified M-Lif and D-Lif, respectively, with α LIF antibodies. Bands in the range of 30-40 do not appear in the lane containing purchased LIF.

In Figure 4.8, we see that commercial LIF lacks the bands ranging from 30 - 40 kD which are present in M-LIF and D-LIF. This finding confirms the presence of posttranslational modifications in M-LIF and D-LIF and a lack thereof in purchased LIF.

To investigate if these modifications were glycosidic in nature, enzymatic deglycosylation of the purified samples and subsequent Western blot analysis with α LIF antibodies were performed. As can be seen in Figure 4.9, the bands at 30 - 40 kD were successfully reduced to a single band at approximately 23 kD upon treatment with the deglycosidase PNGase F. This confirms the existence of several glycosylated subtypes of LIF in the purified samples. Since the MW of one complex N-linked glycosyl moiety was calculated to be 2.86 kD and deglycosylated LIF has a MW of 23 kD, the presence of six glycosyl moieties on M-LIF and D-LIF, respectively, can be assumed, correlating with previously published data (Sasai et al., 1998).

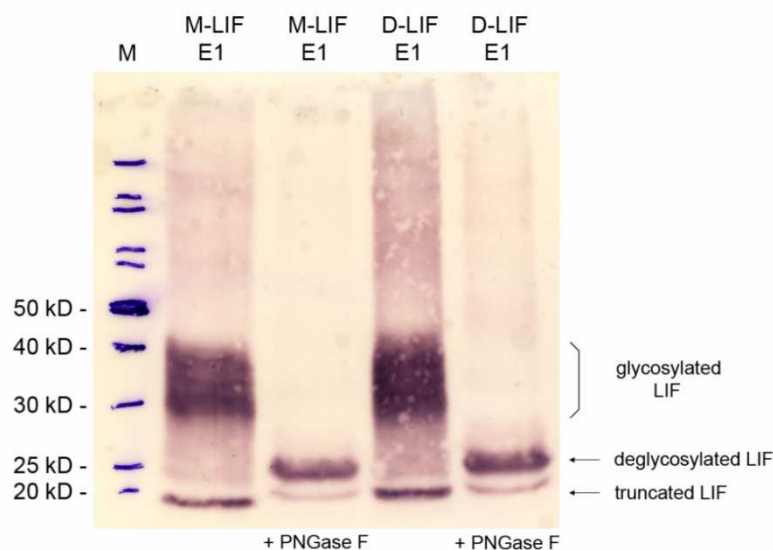


FIGURE 4.9. Glycosidase treatment of M-LIF and D-LIF. Elution fraction 1 (E1) of purified M-LIF and D-LIF, respectively, were treated with PNGase F and analyzed by Western blotting. The glycosylated forms of LIF (30-40 kD) were successfully reduced to a single band at a MW of approximately 23 kD.

4.1.5 Sequencing of M-LIF and D-LIF

The final step in the characterization process was to analyze the sequences of the amino-termini of both purified isoforms of LIF. Since M-LIF and D-LIF differ from one another by only three amino acids in length (corresponding to a MW of 0.38 kD) a difference in molecular weight between the two isoforms cannot be determined by visualization on a SDS polyacrylamide gel. Furthermore, it stands to question if the complete amino-termini are even present in the mature forms of M-LIF and D-LIF, since it has been previously reported that the complete leader sequences upstream of amino acid 24 could not be detected by sequence analysis (Rathjen et al., 1990). Therefore, samples of M-LIF and D-LIF were submitted several times to the VBC mass spectrometry unit for thorough analysis of the protein sequences.

The first samples submitted were the truncated forms of M-LIF and D-LIF at a MW of approximately 19 kD. As can be derived from Figure 4.9, the samples were doubtlessly identified as LIF. However, no peptides upstream of amino acid H39 in the case of D-LIF or H36 in the case of M-LIF (hereinafter referred to as H39/36), and downstream of K193 or K190, respectively (hereinafter referred to as K193/190), could be detected. Since the presence of carboxy-terminal 6xHIS tags had already been confirmed by Western Blot analysis (Figure 4.5 B), it is obvious that amino acids downstream of K193/190 must be present. This, in effect, leaves only the possibility of this particular form of LIF being truncated at the amino-terminus,

D-LIF	
1	MKVLAAGIVP LLLLVLHWKH GAGSPLPITP VNATCAIR HP CHGNLMNQIK
51	NQLAQLNGSA NALFISYYTA QGEPFPNNVE KLCAPNMTDF PSFHGNGTEK
101	TKLVELYRMV AYLSASLTNI TRDQKVLNPT AVSLQVKLNA TIDVMRGLLS
151	NVLCRLCNKY RVGHVDVPPV PDHSDKEAFQ RKKLGCQLLG TYKQ VISVVV
201	QAF
M-LIF	
1	MRCRIVPLLL LVLHWKHGAG SPLPITPVNA TCAIR HPCHG NLMNQIKNQL
51	AQLNGSANAL FISYYTAQGE PFPNNVEKLC APNMTDFPSF HGNGTEKTKL
101	VELYRMVAYL SASLTNITRD QKVLNPTAVS LQVKLNATID VMRGLLSNVL
151	CRLCNKYRVG HVDVPPVPDH SDKEAFQRKK LGCQLLGTYK QVISVVVQAF

FIGURE 4.9. Identification of truncated M-LIF/ D-LIF by mass spectrometry analysis after digestion with Trypsin. The submitted proteins were successfully identified as LIF (detected amino acids are shown in red). Amino acids upstream of H39/36 were not found.

which is in accordance with the finding that the missing amino acids amount to a molecular weight of approximately 4 kD.

After this analysis, samples of deglycosylated LIF with a MW of approximately 23 kD were submitted for examination. First, LysC was used in the fragmentation process, as opposed to Trypsin, which had been used in the former analysis. LysC was chosen, as it would theoretically generate larger, hence, more easily detectable fragments than Trypsin, due to the fact that LysC recognizes fewer cleavage sites within the first 40 amino acids. Again, the samples were undeniably identified as LIF, although fewer peptides could be determined this time around (Figure 4.10). It was possible, though, to identify three amino acids directly upstream of H39/36 in the case of D-LIF, and 6 amino acids directly upstream of H39/36 in the case of M-LIF. Thus, it was proven that amino-acids upstream of H39/36 were present in this form.

Finally, a third mass spectrometry analysis was performed. This time, the deglycosylated 23 kD form was digested with Subtilisin prior to analysis. Subtilisin is a serine endopeptidase which hydrolyses proteins with broad specificity for peptide bonds. Figure 4.11 shows that the carboxy-terminus was almost completely identified in this analysis. As for the amino-terminus, the first amino acid detected this time was S24 for D-LIF and S21 for M-LIF, respectively (hereinafter referred to as S24/21).

At face value, the results obtained from these analyses suggest that the truncated form of LIF present in our samples begins with amino acid H39/36 and “full-length” LIF with amino acid

D-LIF				
1	MKVLAAGIVP	LLLLVLHWKH	GAGSPLPITP	VNATCAIRHP CHGNLMNQIK
51	NQLAQLNGSA	NALFISYYTA	QGEPPNNVE	KLCAPNMTDF PSFHGNGTEK
101	TKLVELYRMV	AYLSASLTNI	TRDQKVLNPT	AVSLQVKLNA TIDVMRGLLS
151	NVLCRLCNKY	RVGHVDVPPV	PDHSDKEAFQ	RKKLGCQLLG TYKQVISVVV
201	QAF			
M-LIF				
1	MRCRIVPLLL	LVLHWKHGAG	SPLPITPVNA	TCAIRHPCHG NLMNQIKNQL
51	AQLNGSANAL	FISYYTAQGE	PPNNVEKLC	APNMTDFPSF HGNGTEKTKL
101	VELYRMVAYL	SASLTNITRD	QKVLNPTAVS	LQVKLNATID VMRGLLSNVL
151	CRLCNKYRVG	HVDVPPVPDH	SDKEAFQRKK	LGCQLLGTYK QVISVVVQAF

FIGURE 4.10. Identification of M-LIF/ D-LIF by mass spectrometry analysis after digestion with LysC. The submitted proteins were successfully identified as LIF (detected amino acids are shown in red). Three amino acids upstream of H39/36 were found in the case of D-LIF, six in the case of M-LIF.

D-LIF				
1	MKVLAAGIVP	LLLLVLHWKH	GAGSPLPITP	VNATCAIRHP CHGNLMNQIK
51	NQLAQLNGSA	NALFISYYTA	QGEPPNNVE	KLCAPNMTDF PSFHGNGTEK
101	TKLVELYRMV	AYLSASLTNI	TRDQKVLNPT	AVSLQVKLNA TIDVMRGLLS
151	NVLCRLCNKY	RVGHVDVPPV	PDHSDKEAFQ	RKKLGCQLLG TYKQVISVVV
201	QAF			
M-LIF				
1	MRCRIVPLLL	LVLHWKHGAG	SPLPITPVNA	TCAIRHPCHG NLMNQIKNQL
51	AQLNGSANAL	FISYYTAQGE	PPNNVEKLC	APNMTDFPSF HGNGTEKTKL
101	VELYRMVAYL	SASLTNITRD	QKVLNPTAVS	LQVKLNATID VMRGLLSNVL
151	CRLCNKYRVG	HVDVPPVPDH	SDKEAFQRKK	LGCQLLGTYK QVISVVVQAF

FIGURE 4.11. Identification of M-LIF/ D-LIF by mass spectrometry analysis after digestion with Subtilisin. The submitted proteins were successfully identified as LIF (detected amino acids are shown in red). The first amino acid detected was S24 or S21, respectively.

S24/21, meaning that preceding peptides must in some way have been cleaved off. In an attempt to verify this hypothesis, the apparent amino-termini were bioinformatically analyzed for enzyme restriction sites using the Expert Protein Analysis System (<http://expasy.org>). Results show that there is indeed a cleavage site for several enzymes directly upstream of H39/36, however, no enzymes could be shown to cleave directly upstream of S24/21 (Figure 4.12).

A

```

          CNBr Ch lo
Ch lo Pn1.3 Pn2 ProtK Therm|
          Therm|
          Ch lo |||
          NTCB Pro | |||
ArgC Clost Tryps | |||
          ProtK| | | |||
ProtK Therm| | | |||
          Therm| | | |||
NTCB ProtK| | | |||
          ProtK| | | |||
          Therm| | | |||
ProtK| | | | | |||
Therm| | | | | |||
ProtK| | | | | |||

```

|||||
TPV NATCAIRHPCHGNLMNQ
-----+-----+

B

```

          ProtK
          ProtK|
          Therm|
          ProtK|
          ProtK | |||
          Therm| | |||
          Ch lo| | |||
          LysC Tryps| | |||
BNPS Ch hi Ch lo Iodo LysN Pn1.3 ProtK| | |||
          Pn1.3| | |||
          Ch lo Pn1.3 Pn2 ProtK| | |||
          Pn1.3 Pn2 ProtK Therm| | |||
          Ch lo Pn1.3 Pn2 ProtK Therm| | |||
          Ch lo Pn1.3 Pn2 ProtK Therm| | |||
          Ch lo Pn1.3 Pn2 ProtK Therm| | |||
          Ch lo Pn1.3 Pn2 ProtK Therm| | |||

```

|||||
LLLLVLHWKHGAGSPLPITP
-----+-----+

FIGURE 4.12. Restriction sites upstream and downstream of H39/36 and S24/21. Amino acid sequences of LIF in the region of H39/36 and S24/21, respectively, were analyzed with EXPASY’s “peptide cutter” program for restriction sites with all available enzymes. *A*, Three enzymes were found to cut directly upstream of H39/36. *B*, No enzyme was found to cleave directly upstream of S24/21.

4.2 The influence of M-LIF and D-LIF on embryonic stem cells

Leukemia Inhibitory Factor is best known for its positive effect on the self-renewal capacity of embryonic stem cells (ESCs) (Smith et al., 1988; Williams et al., 1988). Therefore, ESCs were the first cells chosen in this study to analyze biological function and activity of recombinant M-LIF and D-LIF.

4.2.1 Influence on self-renewal capacity

A typical property of LIF is the maintenance of self-renewal, i.e. the ability to keep stem cells in a proliferating, undifferentiated state. In the absence of LIF or LIF-producing feeder cells, ESCs begin to spontaneously differentiate in vitro after several days. Therefore, the first point of interest was to test purified recombinant M-LIF and D-LIF in terms of their functionality concerning ESC-self-renewal. This was done by conducting an Alkaline Phosphatase assay (AP assay), in which M-LIF and D-LIF, respectively, were added at various concentrations to ESCs

for 4 days. Recombinant, unglycosylated LIF (Chemicon International), which had been produced in *E.coli*, was used as a control, as well as extract of uninfected Sf9 cells. As a third control, cells were left untreated. Commercial LIF was added at concentrations ranging from 0.5 to 5 ng/ml. Since the calculated concentrations for M-LIF and D-LIF relied on estimation (see 4.1.2) and were therefore potentially imprecise, these proteins were added at considerably higher concentrations and a broader range of 10 to 100 ng/ml.

ESCs were plated in 6-well plates at a very low density (5000 ESC per well) in order to avoid support by cell-cell contact. On day 5, cell colonies were analyzed for Alkaline Phosphatase activity, after which coloration and morphology were observed. A classification of degree of differentiation was established, ranging from undifferentiated to differentiated (Figure 4.13).

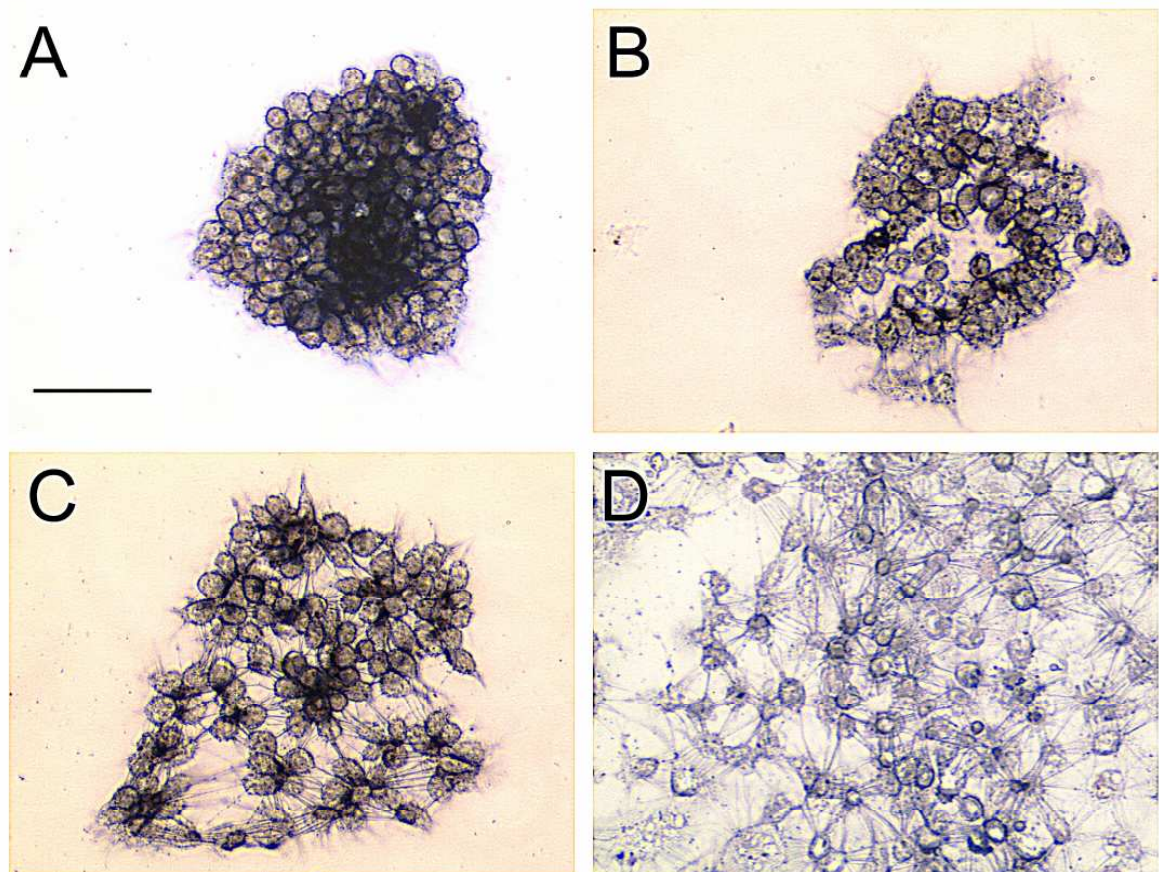


FIGURE 4.13. Colonies of embryonic stem cells (ESCs) evaluated in AP Assay. A, Undifferentiated. B, Slightly differentiated. C, Nearly all differentiated. D, Differentiated. Scale bar = 20µm.

Alkaline Phosphatase is known to be expressed in undifferentiated ESCs at a high level and is therefore often used as a stem cell marker (Resnick et al., 1992). Thus, undifferentiated colonies

appear dark blue after reaction with NBT/BCIP and are morphologically dense, small and round in shape (Figure 4.13 A). Cells in differentiated states show increasingly less coloration and density, with a higher number of protrusions (Figure 4.13 B,C,D).

After evaluation of each well, results of untreated cells and cells treated with LIF stood in stark contrast to one another (Figure 4.14). Almost half of all colonies counted in wells containing

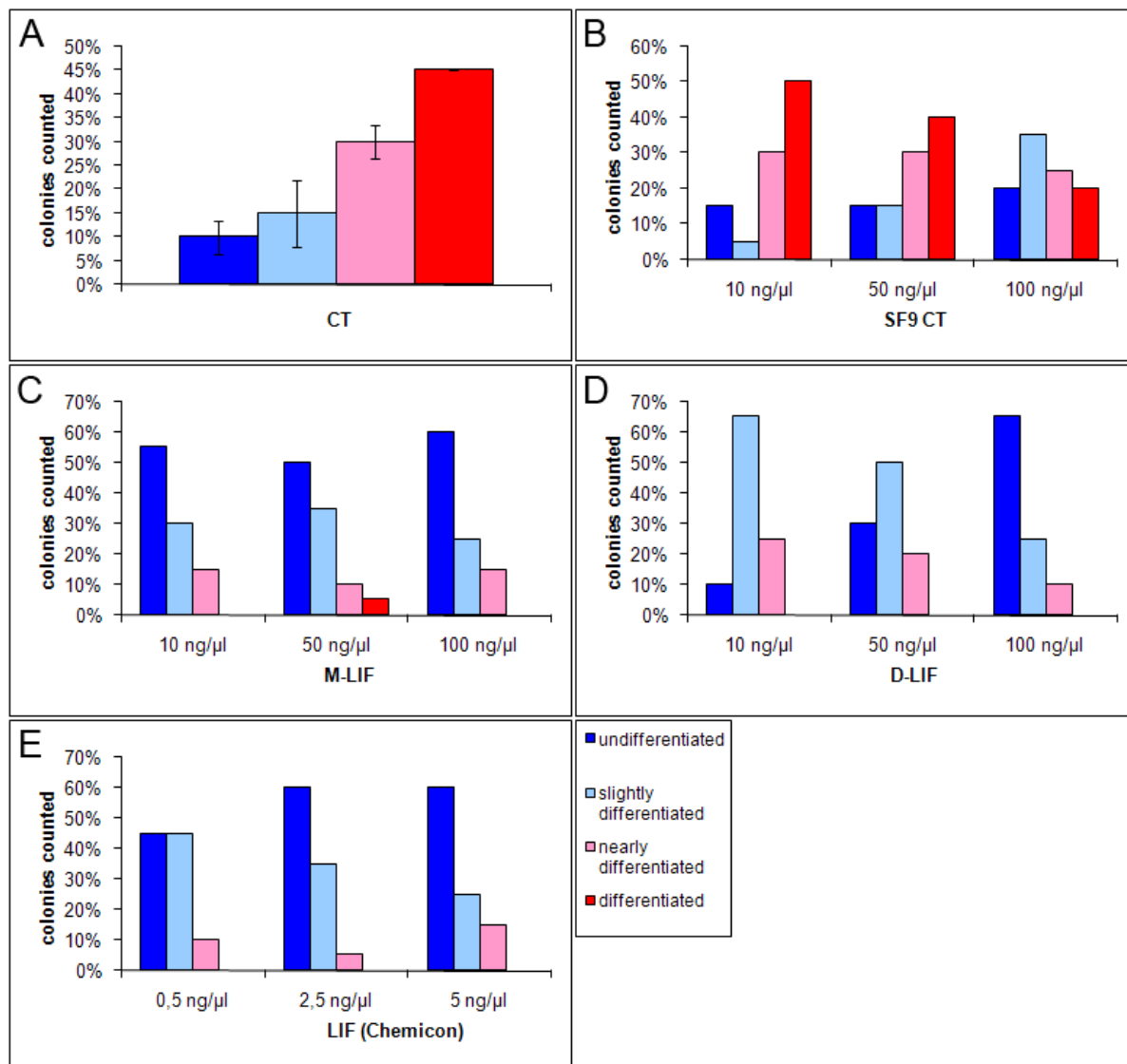


FIGURE 4.14. Analysis of the self renewal capacity of embryonic stem cells (ESCs) in the presence of LIF. A, Nearly half of all colonies counted in wells containing untreated cells appeared to be fully differentiated with only 10% showing complete undifferentiation. Error bars indicate standard deviation $\sigma_{x(n-1)}$; $N=2$ B, Cells treated with the Sf9 extract (SF9 CT) showed a slightly higher degree of undifferentiation as compared to untreated cells, which was dependant on the concentration. C+D+E, More than half of the colonies counted in wells containing cells treated with purified M-LIF, purified D-LIF and commercial LIF (Chemicon), respectively, showed complete undifferentiation at their highest concentration. Furthermore, no fully undifferentiated colonies were present in these wells.

untreated cells appeared to be fully differentiated with only 10% showing complete undifferentiation (Figure 4.14 A). Colonies treated with LIF, however, showed no signs of terminal differentiation and more than half of the analyzed colonies remained completely undifferentiated at the highest concentration (Figure 4.14 C,D,E). Interestingly, purified extract of uninfected Sf9 cells also had a slightly renewing effect when added to the culture medium of ESCs. However, the effect was far weaker than that of LIF, reaching a total percentage of undifferentiation of only 20% at its highest concentration, with an equal amount of terminally differentiated colonies visible (Figure 4.14 B).

Since 100 ng/ml of M-LIF and D-LIF, respectively, displayed an equivalent degree of biological activity to 10 ng/ml of commercial LIF, these concentrations were used in all subsequent experiments.

4.2.2 Influence on embryoid body development

To test the effect of M-LIF and D-LIF, respectively, on cardiomyogenesis in embryoid bodies (EBs), the two cytokines were added at two distinct time points during early EB development – day 5 and day 7, respectively. Controls were EBs treated with unglycosylated LIF (Chemicon International) or extract of uninfected Sf9 cells, respectively, and untreated EBs. EBs were observed from day 7 to 11 and the percentage of EBs containing beating cells was determined.

As can be seen in Figure 4.15, M-LIF and D-LIF inhibited cardiomyocyte development to a slight degree when added on day 5, and to a more significant when added on day 7. In both cases, the inhibiting effect of M-LIF was marginally greater than that of D-LIF. Surprisingly, unglycosylated LIF did not exhibit this effect, having little or no effect when added on day 5 and even a slightly positive effect, as compared to untreated EBs, when added on day 7. The Sf9 cell extract showed no noteworthy effect when added on day 5, but a slight retarding effect when added on day 7, which however lasted only until day 9.

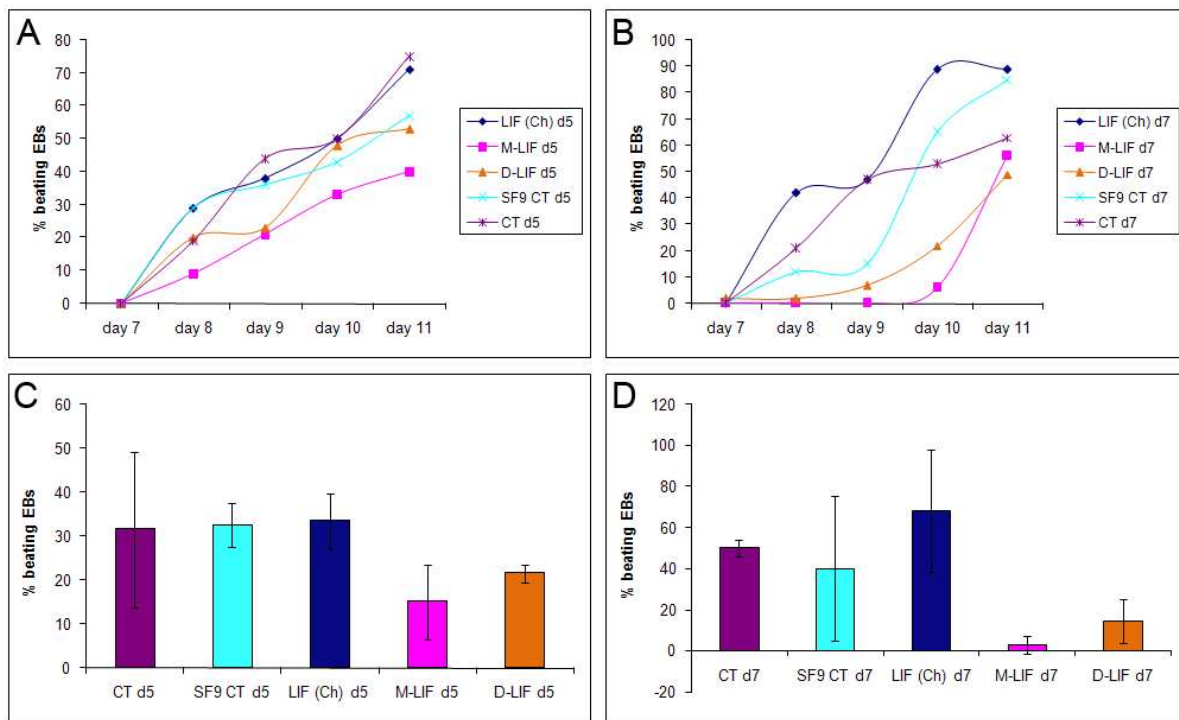


FIGURE 4.15. Embryoid body (EB) development in the presence of LIF. Embryoid bodies were generated and treated with 10 ng/ μ l of LIF (Chemicon), 100 ng/ μ l of M-LIF, 100 ng/ μ l of D-LIF, 100 ng/ μ l of Sf9 extract (SF9 CT) or left untreated (CT), respectively. EBs with beating cardiomyocytes were counted from day 7-11. *A*, M-LIF and D-LIF slightly retard EB development when administered on day 5. *B*, M-LIF and D-LIF drastically retard EB development when administered on day 7. *C*, Mean values calculated from data sampled from day 8/9 of *A*. *D*, Mean values calculated from data sampled from day 9/10 of *B*. Error bars indicate standard deviation $\sigma_{x(n-1)}$; $N=2$

4.3 The influence of M-LIF and D-LIF on primary cardiomyocytes

Increased proliferation and longevity of primary cardiomyocytes (pCMCs) has previously been demonstrated when grown on parietal endoderm (PE) overexpressing M-LIF (Pasteiner, 2006). This beneficial effect could not be observed when grown on PE overexpressing D-LIF. To answer the question of whether this phenomenon could be reproduced by administering LIF in recombinant form, experiments were performed, in which primary cardiomyocytes were treated with recombinant M-LIF and D-LIF, respectively, that had been expressed and purified in this study.

4.3.1 Influence on morphology and contractility/proliferation

Primary cardiomyocytes were isolated from d1-2 neonatal wild-type mice and cultivated on gelatinated 10-cm plates. After attachment of cells (one day after seeding), they were treated daily with M-LIF and D-LIF, respectively. Controls were treated with purchased LIF, Sf9 extract or no agent, respectively. Areas containing beating cells were marked and observed over the course of 7-10 days. Morphology was inspected, as well as contractile ability and proliferation, by counting the number of beating cells within a marked area.

In the first of these experiments, the agents were added to the cells at an interval of 48 hours (Figure 4.16, A). This may explain the periodically fluctuating amount of beating cells

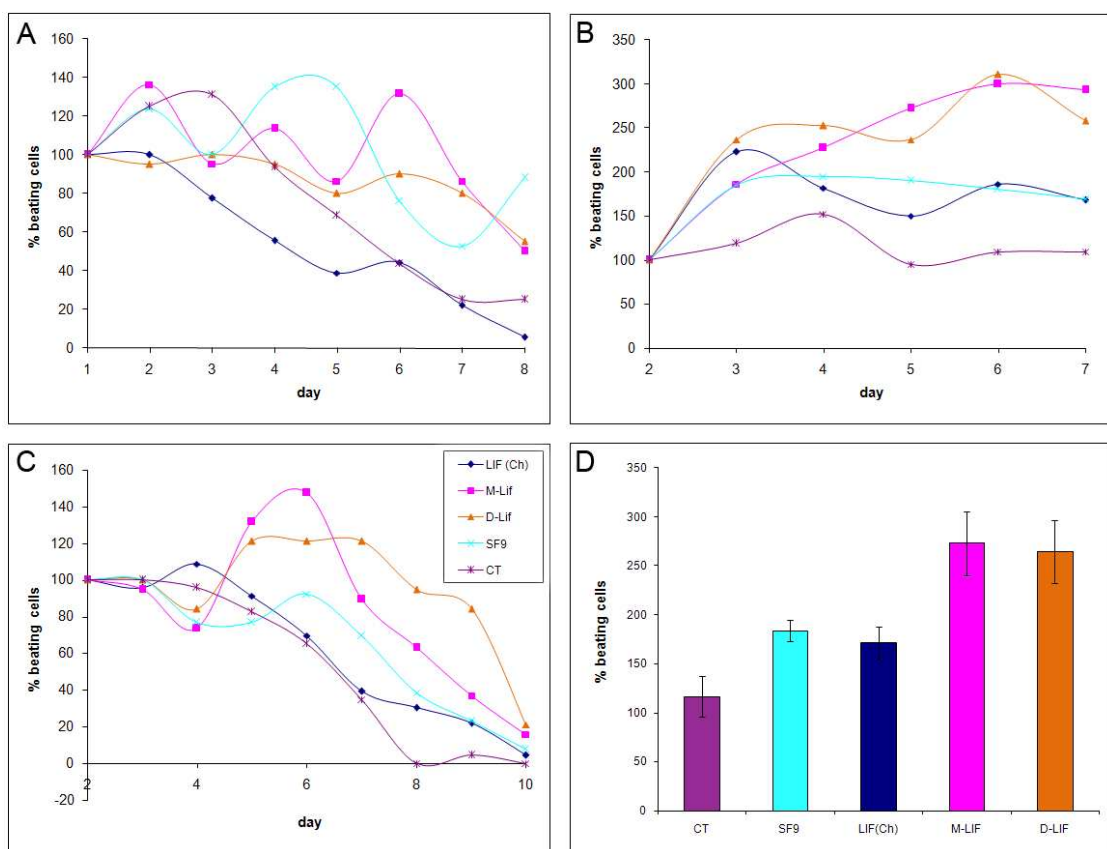


FIGURE 4.16. The influence of LIF on contractile ability / proliferation of primary cardiomyocytes (pCMCs). Primary cardiomyocytes were isolated and LIF was added every second day (A) or every day (B,C), respectively, at a concentration of 100 ng/μl (M-LIF, D-LIF) or 10 ng/μl (LIF(Chemicon)), respectively. Areas containing beating cells were analyzed for contractile activity. A, A fluctuating activity profile can be seen for M-LIF and D-LIF treated cells which can be ascribed to the time-points at which LIF was added to the medium. M-LIF and D-LIF seem to slightly promote longevity of beating cells. B, To a very similar degree, M-LIF and D-LIF apparently promote contractile activity when added on a daily basis. C, Beginning with day 5, M-LIF and D-LIF seem to promote activity. D, Mean values calculated from data sampled from day 4-7 of B. Error bars indicate standard deviation $\sigma_{x(n-1)}$; N=4

encountered. Between day 1 and 5 neither M-LIF, nor D-LIF, displayed any significant effect on the number of beating cells, when compared to untreated cells. On day 6, however, a stark contrast between M-LIF and D-LIF treated cells, respectively, and control cells became visible, the highest number of beating cells being registered in wells treated with M-LIF. By day 8, beating cells had dropped to 25% of the initial number in untreated wells, whereas approximately 50% of cells initially showing contractility were still beating in M-LIF and D-LIF treated wells, respectively. It is interesting to note, that purchased LIF did not display any increase in the number of contracting cells, even displaying a consistently lower number than untreated wells throughout the first 5 days.

In the second experiment (Figure 4.16, B), the agents were added to the wells every day following attachment, in hopes to avoid obtaining fluctuating activity profiles, such as in the first experiment. This time, more even graphs were obtained, with curves for M-LIF and D-LIF treated wells rising steadily from day 2-7. On the final day of observation, the number of beating cells in M-LIF treated wells had risen to nearly 300%, representing an increase by roughly 200%, as opposed to an increase by only 10% in untreated wells. D-LIF had a similar effect, achieving an increase of almost 160%. Both unglycosylated LIF and Sf9 extract showed a minor beneficial effect on contractility/proliferation, achieving an increase by approximately 60% on day 7.

In the third experiment of this nature (Figure 4.16, C), M-LIF and D-LIF again showed a promoting effect beginning on day 5. On day 6, more than twice the amount of beating cells was counted in wells treated with M-LIF, compared to wells containing untreated cells. A substantial increase in terms of longevity could however not be shown, since nearly all cells had stopped beating in each of the wells by day 10.

In regard to morphology, a certain degree of hypertrophy could be detected for cells in all wells (Figure 4.17). The addition of LIF did not appear to promote hypertrophy by a significant degree in these experiments.

4.3.2 Influence on gene expression

To investigate the effect of M-LIF and D-LIF, respectively, on the expression of cardiospecific genes in pCMCs, semi-quantitative RT-PCR was performed (Figure 4.18). pCMCs were treated with LIF and controls as previously described, and mRNA was isolated on days 6 and 10, respectively. mRNA was reverse transcribed to cDNA and used as a template for PCR reactions

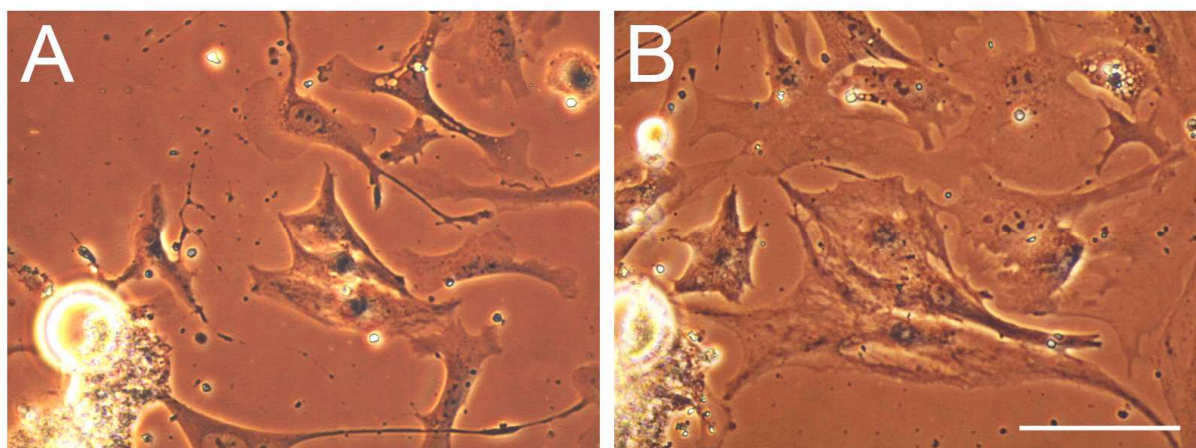


FIGURE 4.17. Isolated primary cardiomyocytes (pCMCs). A, A colony of pCMCs one day after isolation. B, The same colony of pCMCs 6 days after isolation. A certain degree of hypertrophy is apparent. Scale bar = 50 μ m.

with various pairs of primers for a number of different cardiac marker genes (ANF, MHC α , BCL-XL, NKX 2.5, MEF 2C, Tropomyosin α). The well-known housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control.

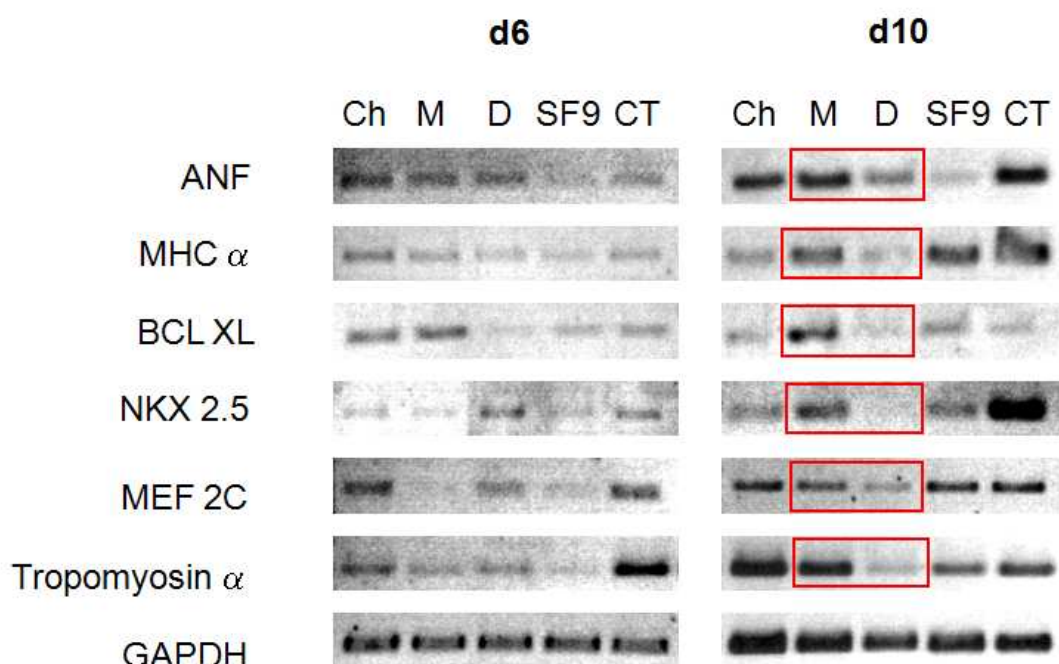


FIGURE 4.18. Analysis of the expression of various cardiac marker genes in the presence of LIF by RT-PCR. mRNA of pCMCs, which had been treated daily with 10 ng/ μ l of LIF (Chemicon, Ch), 100 ng/ μ l of M-LIF (M), 100 ng/ μ l of D-LIF (D), 100 ng/ μ l of Sf9 extract (SF9) or left untreated (CT), respectively, was isolated on day 6 and day 10 after plating, respectively, transcribed to cDNA and analyzed for the presence of various cardiac markers. On day 10, M-LIF treated pCMCs expressed consistently higher levels of mRNA for all tested marker genes than D-LIF treated cells (red boxes). cDNA was normalized by leveling the GAPDH PCR product.

On day 6, ANF expression was upregulated in cells treated with M-LIF, D-LIF and commercial LIF, respectively. MHC α expression was similar for all cells, with slight upregulation in cells treated with purchased LIF. In the case of the antiapoptotic gene BCL-XL, a striking difference could be observed between M-LIF and D-LIF treated cells, with upregulation apparently caused by M-LIF and downregulation by D-LIF. This scenario seems to be reversed in terms of NKX 2.5 and MEF 2C expression, with higher levels of mRNA in the presence of D-LIF. The late cardiospecific marker Tropomyosin α is apparently down-regulated in all treated cells.

On day 10, a striking difference between M-LIF and D-LIF treated cells could be observed for the expression of all analyzed marker genes, with notable downregulation in the presence of D-LIF. mRNA levels in the presence of M-LIF were consistently higher, but only exceeded control levels in the case of BCL-XL and Tropomyosin α , respectively. Treatment with purchased LIF showed apparent downregulation regarding expression of MHC α , BCL-XL and NKX 2.5.

4.4 The influence of M-LIF and D-LIF on cardioblast-like stem cells

4.4.1 Influence on proliferation

To ascertain, whether M-LIF and D-LIF have the same effect on cardioblast-like stem cells (CBLSCs) as on ESCs regarding proliferation, CBLSCs were plated onto gelatinated 24-wells plates at a density corresponding to 1/10 of confluence ($\sim 3 \times 10^5$ cells/well) and treated with M-LIF, D-LIF, and Sf9 control extract, respectively, or left untreated. On day 5, cells were trypsinized and cell numbers were measured (Figure 4.19). As expected, treatment with either form of LIF led to a drastic increase in cell number, showing a 10-fold increase in the presence of M-LIF and an 11-fold increase in the presence of D-LIF, respectively, when compared to the initial values. This is approximately twice as much as the increase found in untreated cells (~ 5 -fold). As already identified for ESCs in the AP-assay (see 4.2.1), the Sf9 control extract also demonstrated a certain degree of biological activity, generating an 8-fold increase in cell number within 5 days.

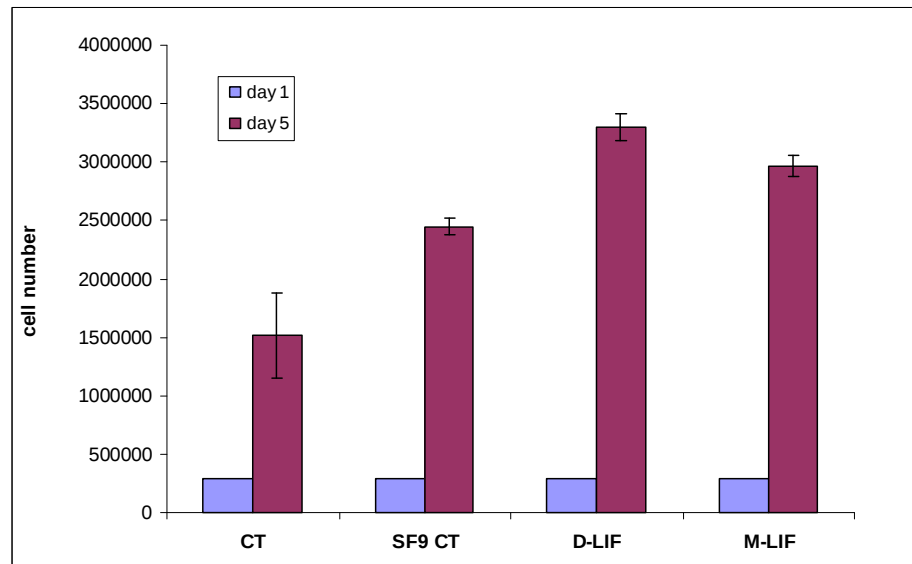


FIGURE 4.19. Proliferation of cardioblast-like stem cells (CBLSCs) in the presence of LIF. 3×10^5 cells were seeded per well and treated with 100 ng/ μ l of M-LIF, 100 ng/ μ l of D-LIF, 100 ng/ μ l of SF9 extract (SF9 CT) or left untreated (CT), respectively, for 4 days. On day 5 they were counted with a Coulter Counter. Treatment with either LIF isoform led to the generation of approximately twice as many cells compared to untreated cells (CT), with D-LIF showing a slightly higher effect than M-LIF. Error bars indicate standard deviation $\sigma_{x(n-1)}$; $N=2$

4.4.2 Influence on differentiation

Following this experiment, an in vitro differentiation assay was conducted. CBLSCs from the former experiment that had been treated for four days with M-LIF, D-LIF, and Sf9 control extract, respectively, or left untreated were seeded onto gelatinated 6-well plates at densities of 1:50, 1:100 and 1:500, respectively, and incubated at 37°C. 12 days after plating, cells were observed in a microscope.

Surprisingly, a marked difference in development had taken place between treated and untreated cells (Figure 4.20). First, treated cells had apparently expanded at a higher rate than control cells, since wells containing treated cells at a dilution of 1:500 showed higher density and discoloration of growth medium, implying higher nutritious demands most likely caused by the increased cell number. At a dilution of 1:100, confluence had been reached in wells containing cells treated with M-LIF, D-LIF, and Sf9 control extract, respectively, as opposed to wells containing untreated cells. Morphologically, cells in the control wells seemed to be for the most part undifferentiated or semi-differentiated, judging from their small size and round shape. Furthermore, cells seemed to be growing on top of one another. This stood in strong contrast to the morphology of LIF treated cells, which were bigger, flatter and often triangular in shape,

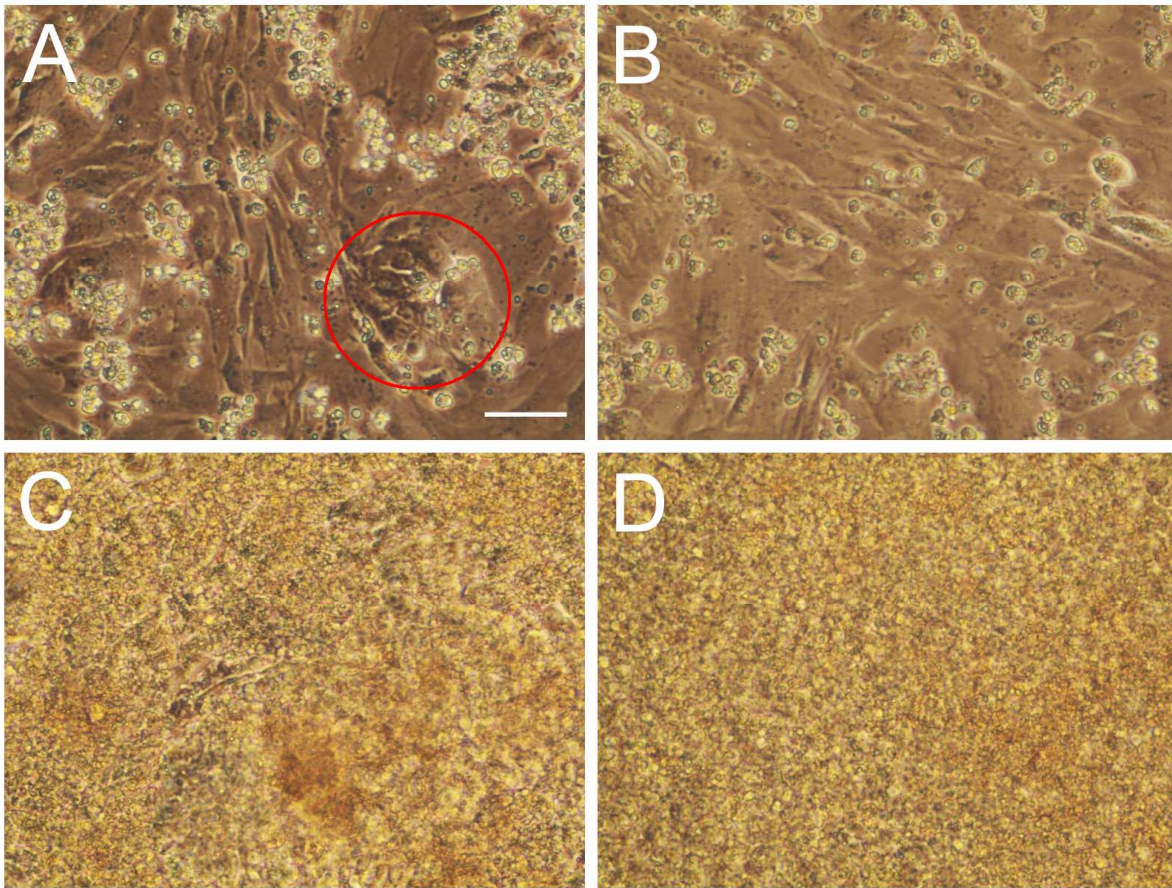


FIGURE 4.20. In vitro differentiation of Cardioblast-like stem cells (CBLSCs). *A*, CBLSCs initially treated with M-LIF. On day 12 after replating, a monolayer of what appeared to be cardiomyocytes was observed with a large number of beating aggregates. One such aggregate is circled in red. *B*, CBLSC initially treated with D-LIF. As was the case for cells initially treated with M-LIF, a monolayer of apparent cardiomyocytes exhibiting contractile ability was observed. *C*, CBLSCs initially treated with Sf9 extract. Most cells in this well seemed to be undifferentiated. A few beating areas were detected, which contracted at a significantly higher frequency than the cells in *A* and *B*. *D*, Untreated cells. In this case, almost all cells appeared to remain undifferentiated, none of which exhibited contractile ability. Scale bar = 50 μm .

quite similar to pCMCs. Since some of these cells were also beating rhythmically, there remains no doubt that heart cells had accrued, induced either directly or indirectly by the addition of M-LIF and D-LIF, respectively. This fact became even clearer upon observation of cells that had been plated at a dilution of 1:50. In wells containing cells treated with either M-LIF or D-LIF, a huge number of beating cells could be detected, the number far exceeding that counted in the 1:100 dilution. These beating cells appeared in clusters (Figure 4.20 *A*) and contracted at a frequency of approximately 50 beats per minute (bpm). Beating cells were also observed in wells that had been treated with the Sf9 control extract. However, two important differences to the

former case must be taken into account: First, these cells had not formed a monolayer and were small, round and tightly packed (Figure 4.20 C). Furthermore, beating areas of cells contracted at a considerably quicker pace, averaging approximately 80 bpm.

Control cells that had never been treated with any of the aforementioned substances displayed absolutely no contractile ability. Small and round in shape, the majority of these cells seemed to have remained undifferentiated (Figure 4.20 D). It must be noted that such cells were also found in wells of cells treated with LIF. However, they seemed to be for the most part dead, as they were not attached to the plate or cell monolayer.

4.5 Characterization of cardioblast-like stem cells

Since little is known regarding the exact nature of cardioblast-like stem cells, it would be of great interest to meticulously characterize these cells in order to decipher their inherent capabilities and make out which and how many of these characteristics differ from those typical for embryonic stem cells. This characterisation process was started in the later stages of this study.

4.5.1 Growth rate of cardioblast-like stem cells

The first step undertaken in terms of characterization was the establishment of a growth curve and calculation of the doubling time (DT) (Figure 4.21). An initial cell number of 5×10^4 cells was seeded per well, and cell numbers were counted every 48 hours. In order to determine the doubling time of CBLSCs, time-points 3 and 4 which lie within the linear portion of the graph (representing the exponential phase of the growth curve) were used. According to this calculation, a DT of 29.9 hours was determined. Doubling times for ESCs have been reported to lie within the range of 22 and 24 hours, thus, the growth rate of CBLSCs seems to be slower than that of ESCs.

4.5.2 Karyotyping of cardioblast-like stem cells

To exclude the possibility of CBLSCs constituting cells generated by fusion of heart cells and ESCs, karyotyping was performed (Figure 4.22). In the case of a possible fusion event, polyploid chromosome spreads would have to be detectable in CBLSCs. After analysis, all cells were found to be diploid in nature, as none of the analyzed chromosome spreads (N=73) displayed

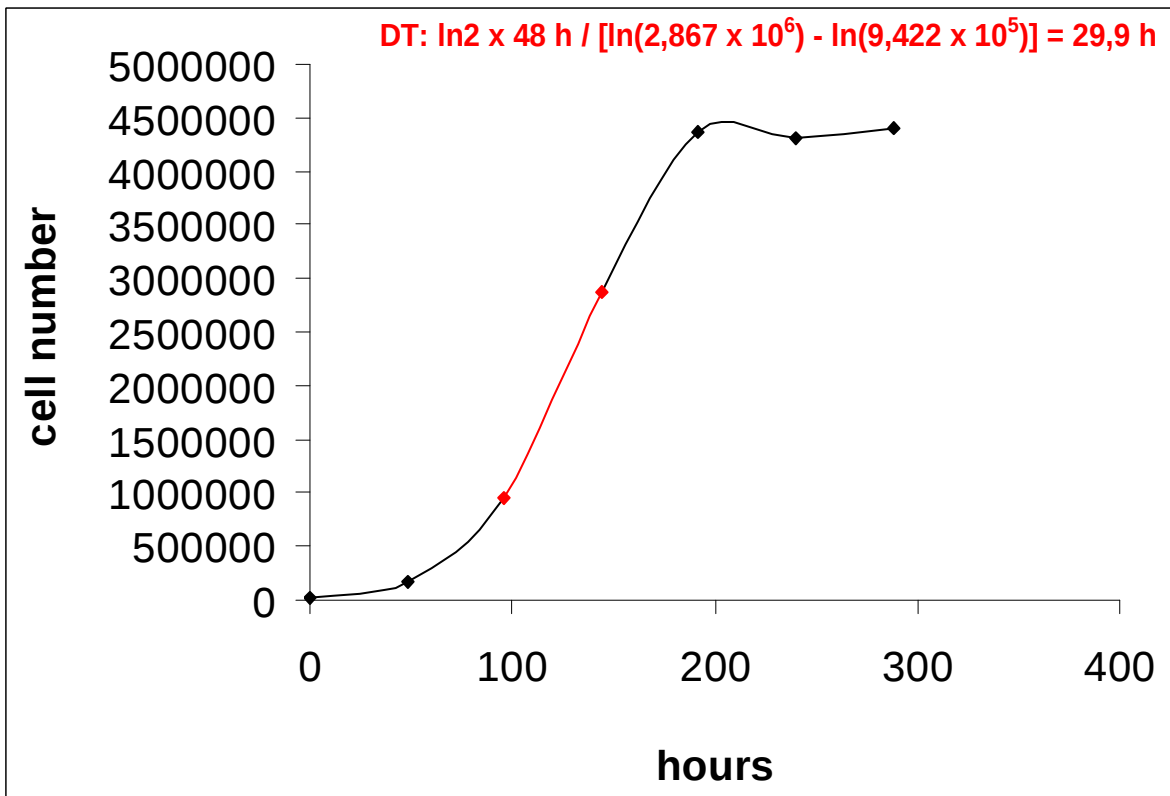


FIGURE 4.21. Growth curve for cardioblast-like stem cells (CBLSCs). 50 000 cells were seeded on 6 wells each of a 24-well plate containing feeder layers. One well was sacrificed every 48 hours and cells were counted with a Coulter counter.

more than the 40 chromosomes naturally occurring in mouse cells. This affirms the notion of CBLSCs as an autonomous, somatic stem cell line.

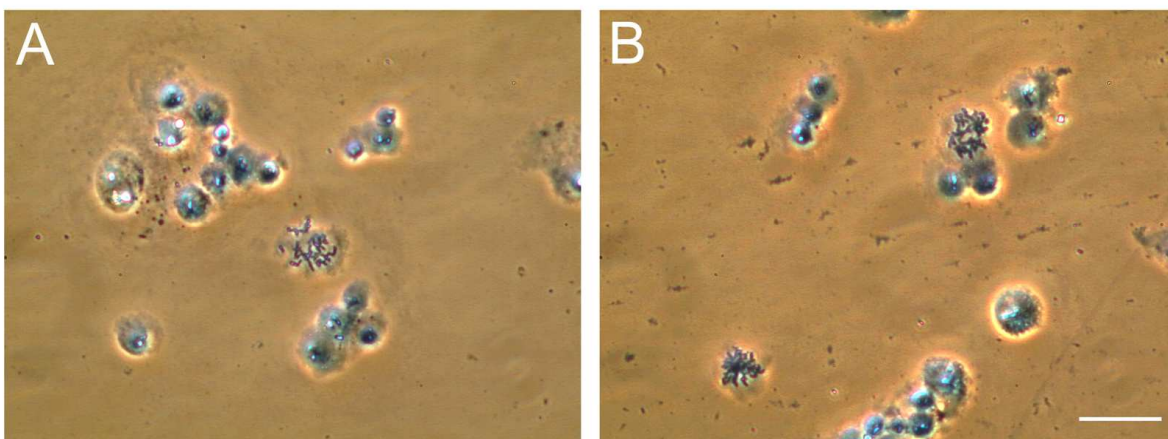


FIGURE 4.22. Karyotyping for cardioblast-like stem cells (CBLSCs). Polyploidy was never encountered, ruling out the possibility of CBLSCs being a hybrid cell line, possibly generated by fusion of cardiac stem cells and embryonic stem cells, the latter of which were used as bystander cells during the isolation process. Scale bar = 50 μm .

4.5.3 Single-cell cloning of cardioblast-like stem cells

Due to the fact that CBLSCs had been isolated in co-culture with ESCs as described in a previous study (Weber, 2006), the possibility of CBLSCs constituting a mixed culture of somatic and embryonic stem cells exists. Therefore, cells were subcloned and selected for with G418. Since CBLSCs had been isolated from the heart tissue of HDAC1 +/- mice possessing a neomycin-resistance-gene, any ESCs should be eliminated by administering the neomycine analogue G418.

In order to achieve this, approximately 200 cells were seeded onto a 6-cm plate containing a feeder cell layer and treated daily with 180 μ g/ml of G418, beginning 48 hours after attachment. One week after seeding, microphotographs were taken (Figure 4.23 A). When compared to a colony of ESCs (Figure 4.23 B) several morphologic differences become evident: CBLSCs appear larger than ESCs. Colonies of CBLSCs are more rounded-up and lack the sharp circumference displayed by colonies of ESCs. Moreover, CBLSCs seem to be less attached to feeder cells and to each another, also exhibiting less overgrowth as opposed to ESCs. These striking morphologic differences strongly suggest an independent identity of CBLSCs and aid in verifying their authenticity.

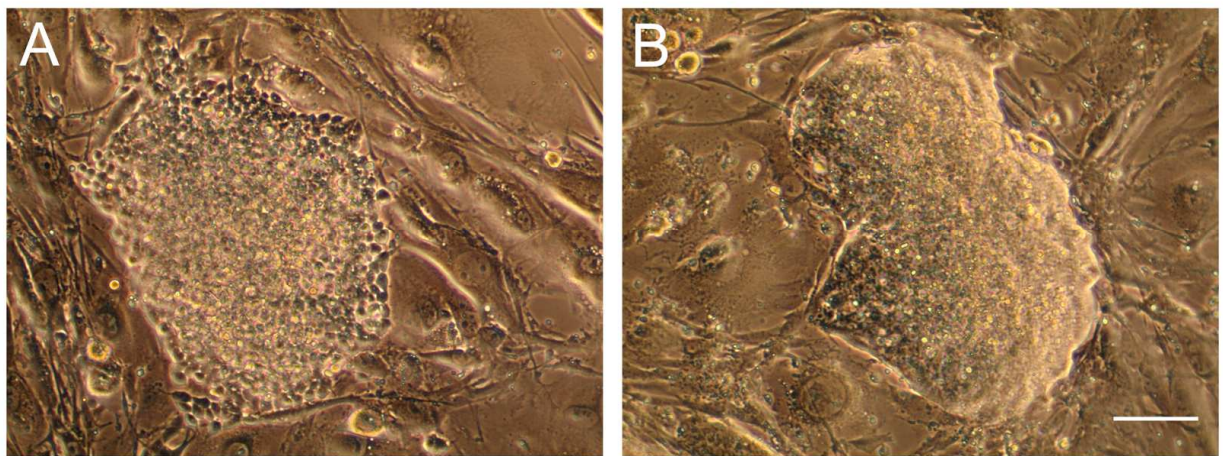


FIGURE 4.23. Morphology of Cardioblast-like stem cells (CBLSCs). A, A colony of CBLSCs, selected for with G418. The morphology is clearly distinct from a colony of ESC-line AB2.2 (B), exhibiting a more rounded-up shape and no sharp circumference. The cells appear to be less well attached to feeder cell layers and to each other. Scale bar = 50 μ m.

10 days after seeding, 12 colonies were picked and replated on a 96-well plate. Of these, 11 could successfully be expanded on feeder cell layers (termed “A3”, “B3”, “C3”, “D3”, “E3”, “F3”, “G3”, “H3”, “A5”, “B5” and “D5”). Currently, the properties of clones G3, H3 and B5 are

under investigation. In regard to growth rate, there seems to be no significant difference amongst each other or between them and ESCs of the line AB2.2. The in vitro differentiation experiment has successfully been reproduced for each clone, with M-LIF and D-LIF treated cells showing beating cells after approximately two weeks. Analysis of gene expression has revealed that the cardiospecific genes NKX 2.5, MEF 2C, and Tropomyosin α are highly upregulated in these cells. Aggregates have been produced and cultured analogously to embryoid bodies. These “cardiac bodies” form beating cardiomyocytes, although with a significant time-lag in respect to embryoid bodies. Interestingly, they have also been shown to generate a large amount of smooth muscle cells, demonstrating that CBLSCs are capable of producing mesodermal cells other than cardiomyocytes.

5 Discussion

Cardiovascular disease and resulting heart failure represent one of the most frequent causes of death in western civilization. Upon heart failure, functional cardiomyocytes are lost, which cannot be fully replaced by the recently described cardiac stem cells (Beltrami et al., 2003; Urbanek et al., 2005). The identification of factors, which could activate these quiescent cardiac stem cells and cause them to differentiate into functional cardiomyocytes would be an important step towards finding new therapies for the treatment of heart diseases.

Previously, it has been suggested that M-LIF may induce increased proliferation and longevity of cardiac progenitor cells. Here, we provide evidence that not only M-LIF, but D-LIF as well, can be utilized as factors to trigger expansion of cardiac stem cells and indirectly induce differentiation into functional heart cells *in vitro*.

5.1 Production and purification of M-LIF and D-LIF

D-LIF and M-LIF were produced in insect cells using the baculovirus system in order to obtain these cytokines in their postrationally modified forms. LIF is known to be heavily modified by glycosylation (Sasai et al., 1998), which could very likely influence biological activity and/or function of this protein. Since vectors encoding D-LIF and M-LIF, respectively, contained coding sequences for carboxy-terminal 6xHIS-tags, Ni⁺⁺-affinity chromatography was used for purification. Western blot and deglycosylation analyses proved that LIF had been successfully expressed and purified in their glycosylated forms ranging from a MW of 30 – 40 kD, which could be reduced to a single band at approximately 23 kD upon deglycosylation. An unglycosylated form of LIF at a MW of approximately 19 kD was also found. Since D-LIF and M-LIF have calculated masses of 22.29 kD and 22.16 kD, respectively, and 6 histidines amount to a MW of 0.84 kD, the 23-kD form was assumed to be LIF in its full-length version.

To investigate the amino-termini of D-LIF and M-LIF, mass spectrometry analysis was performed with several enzymes, revealing that amino acids upstream of H39/36 could not be detected in the 19 kD-form, and amino acids upstream of S24/21 could not be detected in the 23 kD-form. This suggests that the entire leader sequence upstream of amino acid 24/21 is removed in the mature version of both isoforms of LIF, as has been proposed in a previous study (Rathjen, 1990).

However, several findings cast doubt on this conclusion. First, no cleavage site for enzymes could be identified directly upstream of S24/21, raising the question as to how these amino acids

should actually be removed by posttranslational means. Second, the carboxy-termini could not be completely detected in any of the performed mass spectrometry analyses although they were most certainly present, due to the fact that the HIS-tags adjacent to them could be detected via Western blot analysis. Hence, since amino acids were incompletely detected at the carboxy-terminus, the same could apply to amino acids at the amino-terminus. Third, if mass spectrometry results for the amino-termini of both analyzed forms are to be believed, LIF should only be 1.21 kD heavier than the truncated variant of LIF, as this is the number calculated for the stretch of amino acids ranging from S24/21 to H39/36. However, SDS-PAGE analysis revealed a far greater difference in weight of approximately 4 kD, a number that coincides almost perfectly with the MW of the combined first 38/35 amino acids present in full-length LIF, which was calculated to be 3.97 kD in the case of D-LIF, and 3.85 kD in the case of M-LIF. And last but not least, if the leader sequence is indeed missing, then why was LIF found at 23 kD? In the absence of the first 23/20 amino acids LIF containing a HIS tag should have a combined mass of no more than 20.7 kD.

These considerations lead us to believe that the full-length amino-termini may very well be present, in spite of them not being detected by mass spectrometry.

5.2 M-LIF and D-LIF promote self-renewal in embryonic stem cells

Biological activity of recombinantly produced M-LIF and D-LIF was first tested on embryonic stem cells (ESCs). Results obtained from an alkaline phosphatase assay (AP assay) revealed that both M-LIF and D-LIF have the ability to keep ESCs in an undifferentiated state. Glycosylation apparently has no effect in terms of self-renewal capacity, since recombinant LIF produced in *E.coli* had the same effect as the glycosylated versions expressed in Sf9 cells. ESCs in the absence of LIF showed a high degree of differentiation. A small amount of undifferentiated cell colonies could also be observed in this case, which can be explained by the fact that ESCs have the ability to support each other in terms of self-renewal when in close contact. Interestingly, protein extract isolated from uninfected Sf9 cells promoted self-renewal of ESCs to a slight degree, when compared to ESCs that had been left untreated. This can be ascribed to interleukins of insect cell origin that had not been completely removed in the purification process. The effect was however low, when compared to that induced by purified extract containing M-LIF and D-LIF, respectively.

The AP assay conducted in this study proved that LIF had been successfully expressed and purified, since biological activity could be detected to a comparable degree to that of LIF produced in E.coli.

5.3 M-LIF and D-LIF retard mesoderm formation in embryoid bodies

After biological activity of M-LIF and D-LIF had been confirmed, their effect was tested on ESC derived embryoid bodies (EBs). An EB is an in vitro model proposed to recapitulate early embryogenesis. In EBs, mesoderm formation takes place between 2 and 5 days after aggregation (Leahy et al., 1999) and cardiomyocytes begin to beat rhythmically between 6 and 7 days after aggregation (Weitzer et al., 1995). In the presence of glycosylated M-LIF and D-LIF, mesoderm formation was shown to be retarded. When added on day 7, appearance of rhythmically beating cardiomyocytes was delayed by 2 days in the case of D-LIF and by 3 days in the case of M-LIF, respectively. The amount of beating cells was consistently lower in the presence of glycosylated LIF through days 7 to 11, with M-LIF being marginally more inhibiting than D-LIF.

Surprisingly, treatment with unglycosylated LIF showed different results. Inhibition of mesoderm formation could be observed neither when added on day 5, nor on day 7, after aggregation. Quite the contrary, unglycosylated LIF even seemed to promote mesoderm formation when added on day 7, with 89% of EBs containing beating cells on day 10 compared to only 53% in untreated cells. These findings are similar to previously published data in which unglycosylated LIF was shown to positively influence cardiomyocyte formation in EBs when administered after day 4 (Bader et al., 2000). Thus, glycosylation appears to have an impact on differentiation, but not on self-renewal (see 5.2).

5.4 Glycosylated M-LIF and D-LIF promote contractility and proliferation of primary cardiomyocytes

It has been reported that primary cardiomyocytes (pCMCs) show increased proliferation and longevity when grown on *lif*^{-/-}*m-lif*^{ect} parietal endoderm (PE), but not when grown on *lif*^{-/-}*d-lif*^{ect} PE (Pasteiner, 2006). This suggests that M-LIF significantly promotes proliferation and longevity, as opposed to D-LIF. To affirm this conclusion, several experiments were

performed in which pCMCs were treated with recombinantly produced M-LIF and D-LIF, respectively, in the absence of feeder cells. The data of these experiments reveal a certain degree of increased proliferation and contractility, however, no appreciable difference between the effect of M-LIF and D-LIF could be found. Furthermore, longevity did not seem to be markedly increased in the presence of M-LIF and D-LIF, respectively. These findings can in part be explained by the lack of feeder cells, which certainly produce a large number of growth and survival factors aside from LIF that serve to aid in proliferation and longevity. The reason why M-LIF unexpectedly did not show more beneficial results than D-LIF is less clear. Possibly, M-LIF indirectly stimulated pCMCs in the previously mentioned study by triggering the release of other factors from the feeder cells.

A difference could, however, again be found between the effect of glycosylated and unglycosylated LIF, with the latter not stimulating proliferation and contractility in two experiments and only slightly in one of the three performed experiments. This does correlate with previous findings (Pasteiner, 2006). As observed in the case of EB formation, glycosylation seems to play a role in biological activity of LIF.

5.5 The influence of M-LIF and D-LIF on gene expression of primary cardiomyocytes

To investigate changes caused by M-LIF and D-LIF, respectively, on the level of gene expression in pCMCs, the mRNA of several cardio-specific marker genes was analyzed by semi-quantitative RT-PCR. pCMCs treated with D-LIF exhibited decreased gene expression for all analyzed cardiac marker genes after 10 days, when compared to cells treated with M-LIF. The anti-apoptotic gene BCL-XL was found to be marginally upregulated on day 10 as well as day 6 in cells treated with M-LIF. This finding is in concordance with results obtained from M-LIF and D-LIF overexpressing PE, respectively (Pasteiner, 2006). Upregulation could, however, not be demonstrated for any of the other tested marker genes.

5.6 M-LIF and D-LIF promote proliferation and differentiation of cardioblast-like stem cells

M-LIF and D-LIF both induced a significant degree of proliferation in cardioblast-like stem cells (CBLSCs). Since LIF is known to have a positive influence regarding proliferation of ESCs, these findings were expected. What is more intriguing is LIF's influence on differentiation. Approximately 12 days after treatment for 4 days with M-LIF and D-LIF, respectively, and subsequent replating at a dilution of 1:50 (approximately 6×10^4 cells per 6-well plate well), a monolayer of fully differentiated cells could be observed. Since a large number of rhythmically beating aggregates was observed it is clear that heart cells had formed. Wells containing untreated cells, on the other hand, seemed to have remained for the most part undifferentiated, as most cells were small and round and no contractility was observed.

The above findings indicate that LIF directly leads to a surge in proliferation and indirectly leads to differentiation upon removal. These seemingly adverse functions may be explained by the previously described pathways (Cavaleri et Schöler, 2004), one stimulating proliferation via phosphorylation of STAT3 and the other leading to the expression of differentiation-inducing genes via the SHP-2/Ras pathway. How LIF can stimulate differentiation indirectly, however, is unclear. Perhaps the signal for differentiation is outweighed at first by the signal for proliferation induced by LIF and only becomes active after LIF is removed, since this signal may be longer-lasting. Another possibility could be that the formation of differentiated heart cells is merely dependant on the number of seeded cells which was naturally higher for cells that had been treated with LIF. Initial density was indeed found to have an impact on differentiation, since fewer beating cells were observed in wells containing cells that had been seeded at a dilution of 1:100 (approximately 3×10^4 cells) after LIF treatment. This, however, would not explain the morphological differences found between untreated cells and cells treated with LIF. In any case, both M-LIF and D-LIF have been found to ultimately serve as cues for the production of beating heart cells from undifferentiated CBLSCs in as little as 16 days.

Interestingly, beating cells had also formed in wells treated with the control extract of uninfected Sf9 cells. However, these cells had not formed a monolayer and were small, round and tightly packed, quite similar to the appearance of cardiomyocytes found in EBs. Furthermore, these cells contracted at a considerably quicker pace than LIF treated cells, averaging approximately 80 bpm, as opposed to 50 bpm. Again, there is a parallel to EBs, since cardiomyocytes in wild-type EBs are known to contract at a mean frequency of 80 bpm (Hofner et al., 2007).

Perhaps these parallels indicate that CBLSCs treated with the control extract did not differentiate to the same degree as those treated with LIF, giving rise to cardiomyocytes of an earlier developmental stage. Cells initially treated with LIF, on the other hand, seemed to have completely differentiated into viable heart cells.

5.7 CBLSCs represent an autonomous stem cell line of somatic origin

The CBLSCs used in the former experiments had been isolated from neonatal mouse hearts in co-culture with ESCs (Weber, 2006). These cells have a morphology quite similar to that of cardioblasts and exhibit stem cell-like behaviour, hence the name “cardioblast-like stem cells”. They are larger than ESCs and appear to attach less well to feeder cell layers and to one another. Since knowledge of the exact nature of these cells is still very incomplete, several experiments were performed in order to further characterize them.

First, the growth rate of CBLSCs was analyzed. After generation of a growth curve during the period of 12 days, a doubling time (DT) of 29.9 h was calculated. Doubling times for ESCs have been reported to lie within the range of 22 and 24 hours, thus, the growth rate of CBLSCs seems to be markedly slower than that of ESCs. Second, karyotyping was performed. Since CBLSCs had been isolated in co-culture with ESCs, it seems conceivable that they could have been generated by a fusion event. After observation of chromosome spreads, cells were found to be diploid, ruling out the possibility of CBLSCs constituting cells generated by fusion of heart cells and ESCs. Third, single-cell cloning was conducted. The most critical parameter in the definition of stem cells is their clonality which is the ability of single undifferentiated cells to self-renew, proliferate, and differentiate to produce mature progeny cells (Wagers and Weissman, 2004). Therefore, CBLSCs must be subclonable at a single cell level, in order to be justifiably considered as stem cells. Here, eleven colonies of CBLSCs were successfully subcloned under selection of G418, in effect ruling out the possibility of CBLSCs constituting a mixed culture of somatic and embryonic stem cells. These test results, in addition to the observations made regarding morphology, affirm the notion of CBLSCs representing an autonomous, somatic stem cell line.

In recent developments, the subclones have been shown to exhibit similar growth rates. In vitro differentiation has been successfully reproduced and cardiospecific marker genes, such as NKX 2.5, MEF 2C and Tropomyosin α have been shown to be highly upregulated in differentiated

cells. Furthermore, aggregates analogous to embryoid bodies (coined “cardiac bodies”) have been produced, leading to the formation not only of cardiomyocytes, but a large number of smooth muscle cells, as well. This indicates that CBLSCs are capable of producing mesodermal cells besides cardiomyocytes and perhaps even cells of distinct lineages, similar to the findings published by other groups (Krause et al., 2002; Poulsom et al., 2002; Beltrami et al., 2007).

5.8 Conclusion

In this study, glycosylated recombinant M-LIF and D-LIF, respectively, have been found to, first, cause drastic proliferation of cardiac progenitor cells, and second, indirectly serve as cues for differentiation into heart cells in vitro. No significant differences concerning these abilities could be observed between either isoform when administered in recombinant form. Perhaps the short amino-terminal peptide MRCRIV present in M-LIF merely serves as a sorting signal that is capable of targeting polypeptides to the extracellular matrix, as has been previously suggested (Rathjen et al., 1990). A difference between glycosylated and unglycosylated LIF regarding biological activity was observed, but only regarding differentiation.

The above findings imply that recombinant glycosylated M-LIF and D-LIF, respectively, can be utilized as factors to trigger expansion of cardiac stem cells and induce differentiation into functional heart cells in vitro. Since expansion and self-renewal of cardiac progenitor cells seem to be the primary function of LIF, other factors such as Bmp2 - a member of the TGF- β growth factor family which has been shown to actively induce differentiation of heart cells (Behfar et al., 2002) – could perhaps be used in combination with either M-LIF or D-LIF to generate vast in vitro cultures of heart cells quickly and efficiently, which may ultimately be used for cell therapy of the diseased heart.

6 References

- Amit, M. and J. Itskovitz-Eldor (2002). "Derivation and spontaneous differentiation of human embryonic stem cells." J Anat **200**(Pt 3): 225-32.
- Anderson, D.J., F.H. Gage, and I.L. Weissman (2001). "Can stem cells cross lineage Boundaries"? Nat. Med. **7**:393–395.
- Ateghang, B. (2006). "Regulation of cardiotrophin-1 expression during mouse embryonic stem cell differentiation by hypoxia and reactive oxygen species." Justus-Liebig University, Giessen.
- Aumailley, M., M. Pesch, et al. (2000). "Altered synthesis of laminin 1 and absence of basement membrane component deposition in (beta)1 integrin-deficient embryoid bodies." J Cell Sci **113 Pt 2**: 259-68.
- Austin, L., J. J. Bower, et al. (2000). "Leukemia inhibitory factor ameliorates muscle fiber degeneration in the mdx mouse." Muscle Nerve **23**(11): 1700-5.
- Bader, A., H. Al-Dubai, et al. (2000). "Leukemia inhibitory factor modulates cardiogenesis in embryoid bodies in opposite fashions." Circ Res **86**(7): 787-94.
- Barasch, J., J. Yang, et al. (1999). "Mesenchymal to epithelial conversion in rat metanephros is induced by LIF." Cell **99**(4): 377-86.
- Barker, R. A., M. Jain, et al. (2003). "Stem cells and neurological disease." J Neurol Neurosurg Psychiatry **74**(5): 553-7.
- Barron, M., M. Gao, et al. (2000). "Requirement for BMP and FGF signaling during cardiogenic induction in non-precardiac mesoderm is specific, transient, and cooperative." Dev Dyn **218**(2): 383-93.
- Behfar, A., L. V. Zingman, et al. (2002). "Stem cell differentiation requires a paracrine pathway in the heart." Faseb J **16**(12): 1558-66.
- Beltrami, A. P., L. Barlucchi, et al. (2003). "Adult cardiac stem cells are multipotent and support myocardial regeneration." Cell **114**(6): 763-76.
- Beltrami, A. P., D. Cesselli, et al. (2007). "Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow)." Blood **110**(9): 3438-46.
- Boiani, M. and H. R. Scholer (2005). "Regulatory networks in embryo-derived pluripotent stem cells." Nat Rev Mol Cell Biol **6**(11): 872-84.
- Brand, T. (2003). "Heart development: molecular insights into cardiac specification and early morphogenesis." Dev. Biol. **258**: 1-19
- Buckingham, M., S. Meilhac, et al. (2005). "Building the mammalian heart from two sources of myocardial cells." Nat Rev Genet **6**(11): 826-35.
- Butzkueven, H., J. G. Zhang, et al. (2002). "LIF receptor signaling limits immune-mediated demyelination by enhancing oligodendrocyte survival." Nat Med **8**(6): 613-9.
- Cai, C. L., X. Liang, et al. (2003). "Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart." Dev Cell **5**(6): 877-89.
- Carpenter, M. K., X. Cui, et al. (1999). "In vitro expansion of a multipotent population of human neural progenitor cells." Exp Neurol **158**(2): 265-78.
- Carpenter, M. K., E. Rosler, et al. (2003). "Characterization and differentiation of human embryonic stem cells." Cloning Stem Cells **5**(1): 79-88.
- Cavaleri F. and H. Schöler. 2004. Handbook of Stem Cells, Volume 1.
- Chodorowska, G., A. Glowacka, et al. (2004). "Leukemia inhibitory factor (LIF) and its biological activity." Ann Univ Mariae Curie Skłodowska [Med] **59**(2): 189-93.
- Conquet, F., N. Peyrieras, et al. (1992). "Inhibited gastrulation in mouse embryos overexpressing the leukemia inhibitory factor." Proc Natl Acad Sci U S A **89**(17): 8195-9.

- Dazai, S., S. Akita, et al. (2000). "Leukemia inhibitory factor enhances bone formation in calvarial bone defect." *J Craniofac Surg* **11**(6): 513-20.
- Dettman, R. W., W. Denetclaw, Jr., et al. (1998). "Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart." *Dev Biol* **193**(2): 169-81.
- Doetschman, T. C., H. Eistetter, et al. (1985). "The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium." *J Embryol Exp Morphol* **87**: 27-45.
- Donovan, P. J. and J. Gearhart (2001). "The end of the beginning for pluripotent stem cells." *Nature* **414**(6859): 92-7.
- Eisenberg, C. A. and L. M. Eisenberg (1999). "WNT11 promotes cardiac tissue formation of early mesoderm." *Dev Dyn* **216**(1): 45-58.
- Epstein, J. A. and C. A. Buck (2000). "Transcriptional regulation of cardiac development: implications for congenital heart disease and DiGeorge syndrome." *Pediatr Res* **48**(6): 717-24.
- Escary, J. L., J. Perreau, et al. (1993). "Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation." *Nature* **363**(6427): 361-4.
- Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." *Nature* **292**(5819): 154-6.
- ExPASy Proteomics Server. <http://expasy.org>
- Forbes, S. J., P. Vig, et al. (2002). "Adult stem cell plasticity: new pathways of tissue regeneration become visible." *Clin Sci (Lond)* **103**(4): 355-69.
- Frazier, O. H., E. A. Rose, et al. (1992). "Multicenter clinical evaluation of the HeartMate 1000 IP left ventricular assist device." *Ann Thorac Surg* **53**(6): 1080-90.
- Gao, F., H. Y. Shi, et al. (2004). "Maspin plays an essential role in early embryonic development." *Development* **131**(7): 1479-89.
- Gearing, D. P., N. M. Gough, et al. (1987). "Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF)." *Embo J* **6**(13): 3995-4002.
- Gearing, D. P., J. A. King, et al. (1988). "Complete sequence of murine myeloid leukaemia inhibitory factor (LIF)." *Nucleic Acids Res* **16**(20): 9857.
- Grant, S. L., A. M. Douglas, et al. (2001). "Oncostatin M and leukemia inhibitory factor regulate the growth of normal human breast epithelial cells." *Growth Factors* **19**(3): 153-62.
- Guan K., J. Rohwedel and A. Wobus (1999). "Embryonic stem cell differentiation models: cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation in vitro." *Cytotech* **30**: 211-216.
- Haines, B. P., R. B. Voyle, et al. (1999). "Complex conserved organization of the mammalian leukemia inhibitory factor gene: regulated expression of intracellular and extracellular cytokines." *J Immunol* **162**(8): 4637-46.
- Harvey, R. P. (2002). "Patterning the vertebrate heart." *Nat Rev Genet* **3**(7): 544-56.
- Hilton, D. J., N. A. Nicola, et al. (1988). "Resolution and purification of three distinct factors produced by Krebs ascites cells which have differentiation-inducing activity on murine myeloid leukemic cell lines." *J Biol Chem* **263**(19): 9238-43.
- Hinds, M. G., T. Maurer, et al. (1997). "Resonance assignments, secondary structure and topology of leukaemia inhibitory factor in solution." *J Biomol NMR* **9**(2): 113-26.
- Hofner, M., A. Holtrig, et al. (2007). "Desmin stimulates differentiation of cardiomyocytes and up-regulation of brachyury and nkx2.5." *Differentiation* **75**(7): 605-15.
- Hognes, JR. 1991. "The artificial heart: prototypes, policies and patients." *National Academy, Washington*.
- Holtzer H. (1978). "Cell lineages, stem cells and the 'quantal' cell cycle concept. In Lord

- BI, Potten CS and Cole RJ (eds), Stem Cells and Tissue Homeostasis." Cambridge University Press, Cambridge, NY, pp. 1–28.
- Ikeda, W., H. Nakanishi, et al. (1999). "Afadin: A key molecule essential for structural organization of cell-cell junctions of polarized epithelia during embryogenesis." J Cell Biol **146**(5): 1117-32.
- Itescu, S., M. D. Schuster, et al. (2003). "New directions in strategies using cell therapy for heart disease." J Mol Med **81**(5): 288-96.
- Jiang, X., P. Cui, et al. (2002). "[Study on the directed inducing process of cartilage cells differentiated from human marrow mesenchymal stem cells]." Zhonghua Er Bi Yan Hou Ke Za Zhi **37**(2): 137-9.
- Kehat, I., L. Khimovich, et al. (2004). "Electromechanical integration of cardiomyocytes derived from human embryonic stem cells." Nat Biotechnol **22**(10): 1282-9.
- Kirby, M. L., T. F. Gale, et al. (1983). "Neural crest cells contribute to normal aorticopulmonary septation." Science **220**(4601): 1059-61.
- Kitajima, S., A. Takagi, et al. (2000). "MesP1 and MesP2 are essential for the development of cardiac mesoderm." Development **127**(15): 3215-26.
- Kochar, P.G. (2004). What are stem cells? <http://www.csa.com>
- Krause, D. S. (2002). "Plasticity of marrow-derived stem cells." Gene Ther **9**(11): 754-8.
- Laugwitz, K. L., A. Moretti, et al. (2005). "Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages." Nature **433**(7026): 647-53.
- Laugwitz, K. L., A. Moretti, et al. (2008). "Islet1 cardiovascular progenitors: a single source for heart lineages?" Development **135**(2): 193-205.
- Layton, M. J., B. A. Cross, et al. (1992). "A major binding protein for leukemia inhibitory factor in normal mouse serum: identification as a soluble form of the cellular receptor." Proc Natl Acad Sci U S A **89**(18): 8616-20.
- Leahy, A., J. W. Xiong, et al. (1999). "Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation." J Exp Zool **284**(1): 67-81.
- Li, X., Y. Chen, et al. (2001). "Fibroblast growth factor signaling and basement membrane assembly are connected during epithelial morphogenesis of the embryoid body." J Cell Biol **153**(4): 811-22.
- Lough, J., M. Barron, et al. (1996). "Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precordial embryonic mesoderm." Dev Biol **178**(1): 198-202.
- Mahon, N. G., C. O'Rourke, et al. (1999). "Hospital mortality of acute myocardial infarction in the thrombolytic era." Heart **81**(5): 478-82.
- Manner, J., J. M. Perez-Pomares, et al. (2001). "The origin, formation and developmental significance of the epicardium: a review." Cells Tissues Organs **169**(2): 89-103.
- Martin, G. R. (1981). "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells." Proc Natl Acad Sci U S A **78**(12): 7634-8.
- Mauduit, C., I. Goddard, et al. (2001). "Leukemia inhibitory factor antagonizes gonadotropin induced-testosterone synthesis in cultured porcine leydig cells: sites of action." Endocrinology **142**(6): 2509-20.
- McCarthy, P. M., P. M. Portner, et al. (1991). "Clinical experience with the Novacor ventricular assist system. Bridge to transplantation and the transition to permanent application." J Thorac Cardiovasc Surg **102**(4): 578-86; discussion 586-7.
- McMahon, A. P. and A. Bradley (1990). "The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain." Cell **62**(6): 1073-85.

- Metcalf, D. (2003). "The unsolved enigmas of leukemia inhibitory factor." Stem Cells **21**(1): 5-14.
- Moore, A. W., L. McInnes, et al. (1999). "YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis." Development **126**(9): 1845-57.
- Morali, O. G., A. Jouneau, et al. (2000). "IGF-II promotes mesoderm formation." Dev Biol **227**(1): 133-45.
- Muguruma, Y., M. Reyes, et al. (2003). "In vivo and in vitro differentiation of myocytes from human bone marrow-derived multipotent progenitor cells." Exp Hematol **31**(12): 1323-30.
- Murata, M., K. Fukuda, et al. (1999). "Leukemia inhibitory factor, a potent cardiac hypertrophic cytokine, enhances L-type Ca²⁺ current and [Ca²⁺]_i transient in cardiomyocytes." J Mol Cell Cardiol **31**(1): 237-45.
- Murphy, M., K. Reid, et al. (1991). "Generation of sensory neurons is stimulated by leukemia inhibitory factor." Proc Natl Acad Sci U S A **88**(8): 3498-501.
- Murray, P. and D. Edgar (2001). "Regulation of the differentiation and behaviour of extra-embryonic endodermal cells by basement membranes." J Cell Sci **114**(Pt 5): 931-9.
- Negoro, S., H. Oh, et al. (2001). "Glycoprotein 130 regulates cardiac myocyte survival in doxorubicin-induced apoptosis through phosphatidylinositol 3-kinase/Akt phosphorylation and Bcl-xL/caspase-3 interaction." Circulation **103**(4): 555-61.
- Ohlstein, B., T. Kai, et al. (2004). "The stem cell niche: theme and variations." Curr Opin Cell Biol **16**(6): 693-9.
- O'Shea, K. S. (2004). "Self-renewal vs. differentiation of mouse embryonic stem cells." Biol Reprod **71**(6): 1755-65.
- Oz, M. C., M. Argenziano, et al. (1997). "Bridge experience with long-term implantable left ventricular assist devices. Are they an alternative to transplantation?" Circulation **95**(7): 1844-52.
- Pasteiner, W. (2006). "On the synergistic influence of M-LIF and BMP-2 on proliferation and longevity of primary cardiomyocytes." University of Vienna.
- Piquet-Pellorce, C., I. Dorval-Coiffec, et al. (2000). "Leukemia inhibitory factor expression and regulation within the testis." Endocrinology **141**(3): 1136-41.
- Plisov, S. Y., K. Yoshino, et al. (2001). "TGF beta 2, LIF and FGF2 cooperate to induce nephrogenesis." Development **128**(7): 1045-57.
- Poelmann, R. E., H. Lie-Venema, et al. (2002). "The role of the epicardium and neural crest as extracardiac contributors to coronary vascular development." Tex Heart Inst J **29**(4): 255-61.
- Poulsom, R., M. R. Alison, et al. (2002). "Adult stem cell plasticity." J Pathol **197**(4): 441-56.
- Prelle, K., N. Zink, et al. (2002). "Pluripotent stem cells--model of embryonic development, tool for gene targeting, and basis of cell therapy." Anat Histol Embryol **31**(3): 169-86.
- Rathjen, P. D., S. Toth, et al. (1990). "Differentiation inhibiting activity is produced in matrix-associated and diffusible forms that are generated by alternate promoter usage." Cell **62**(6): 1105-14.
- Reid, L. R., C. Lowe, et al. (1990). "Leukemia inhibitory factor: a novel bone-active cytokine." Endocrinology **126**(3): 1416-20.
- Resnick, J. L., L. S. Bixler, et al. (1992). "Long-term proliferation of mouse primordial germ cells in culture." Nature **359**(6395): 550-1.
- Robinson, R. C., L. M. Grey, et al. (1994). "The crystal structure and biological function of leukemia inhibitory factor: implications for receptor binding." Cell **77**(7): 1101-16.

- Romagnani, P., F. Annunziato, et al. (2005). "CD14+CD34^{low} cells with stem cell phenotypic and functional features are the major source of circulating endothelial progenitors." *Circ Res* **97**(4): 314-22.
- Sariola, H. (2001). "The neurotrophic factors in non-neuronal tissues." *Cell Mol Life Sci* **58**(8): 1061-6.
- Sasai, K., J. Aikawa, et al. (1998). "Functions of the N-glycans of rat leukemia inhibitory factor expressed in Chinese hamster ovary cells." *J Biochem* **124**(5): 999-1003.
- Schlange, T., B. Andree, et al. (2000). "BMP2 is required for early heart development during a distinct time period." *Mech Dev* **91**(1-2): 259-70.
- Schwartz, R. E., M. Reyes, et al. (2002). "Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells." *J Clin Invest* **109**(10): 1291-302.
- Smith, A. G., J. K. Heath, et al. (1988). "Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides." *Nature* **336**(6200): 688-90.
- Smith, A. G. and M. L. Hooper (1987). "Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells." *Dev Biol* **121**(1): 1-9.
- Smyth, N., H. S. Vatansever, et al. (1999). "Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation." *J Cell Biol* **144**(1): 151-60.
- Soriano, P., G. Friedrich, et al. (1991). "Promoter interactions in retrovirus vectors introduced into fibroblasts and embryonic stem cells." *J Virol* **65**(5): 2314-9.
- Spangenburg, E. E. and F. W. Booth (2002). "Multiple signaling pathways mediate LIF-induced skeletal muscle satellite cell proliferation." *Am J Physiol Cell Physiol* **283**(1): C204-11.
- Srivastava, D. and E. N. Olsen (2000). "A genetic blueprint for cardiac development." *Nature* **407**: 221-226.
- Srivastava, D. and K. N. Ivey (2006). "Potential of stem-cell-based therapies for heart disease." *Nature* **441**(7097): 1097-9.
- Sterr, R., U. Novak, et al. (1997). "Distinct roles for leukemia inhibitory factor receptor alpha-chain and gp130 in cell type-specific signal transduction." *J Biol Chem* **272**(32): 19982-6.
- Strydom, M., W. Pasternak, et al. (2005). "Parietal endoderm secreted SPARC promotes early cardiomyogenesis in vitro." *Exp Cell Res* **310**(2): 331-43.
- Strydom, M. (2006). Parietal endoderm secreted factors promote early cardiomyogenesis in embryoid bodies. *University of Vienna*.
- Stewart, C. L., P. Kaspar, et al. (1992). "Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor." *Nature* **359**(6390): 76-9.
- Sugiura, S., R. Lahav, et al. (2000). "Leukaemia inhibitory factor is required for normal inflammatory responses to injury in the peripheral and central nervous systems in vivo and is chemotactic for macrophages in vitro." *Eur J Neurosci* **12**(2): 457-66.
- Tang, D. G., Y. M. Tokumoto, et al. (2001). "Lack of replicative senescence in cultured rat oligodendrocyte precursor cells." *Science* **291**(5505): 868-71.
- Taupin, J. L., V. Pitard, et al. (1998). "Leukemia inhibitory factor: part of a large ingathering family." *Int Rev Immunol* **16**(3-4): 397-426.
- Thomas, E. D. (1999). "A history of haemopoietic cell transplantation." *Br J Haematol* **105**(2): 330-9.
- Till J.E. and E.A. McCulloch (1961). "A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells." *Radiat. Res.* **14**, 2213-222.
- Tomida, M., U. Yoshida, et al. (2001). "Leukaemia inhibitory factor and interleukin 6 inhibit secretion of prolactin and growth hormone by rat pituitary MtT/SM cells." *Cytokine* **14**(4): 202-7.

- Urbanek, K., D. Torella, et al. (2005). "Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure." Proc Natl Acad Sci U S A **102**(24): 8692-7.
- Van-Seuningen, I. and M. Davril (1992). "A rapid periodic acid-Schiff staining procedure for the detection of glycoproteins using the PhastSystem." Electrophoresis **13**(1-2): 97-9.
- Voyle, R. B., B. P. Haines, et al. (1999). "Human germ cell tumor cell lines express novel leukemia inhibitory factor transcripts encoding differentially localized proteins." Exp Cell Res **249**(2): 199-211.
- Wagers, A. J. and I. L. Weissman (2004). "Plasticity of adult stem cells." Cell **116**(5): 639-48.
- Weber, W. (2006). "Cardioblast-like stem cells from murine heart." University of Vienna.
- Weitzer, G. (2006). "Embryonic stem cell-derived embryoid bodies: an in vitro model of eutherian pregastrulation development and early gastrulation." Handb Exp Pharmacol(174): 21-51.
- Weitzer, G., D. J. Milner, et al. (1995). "Cytoskeletal control of myogenesis: a desmin null mutation blocks the myogenic pathway during embryonic stem cell differentiation." Dev Biol **172**(2): 422-39.
- Williams, R. L., D. J. Hilton, et al. (1988). "Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells." Nature **336**(6200): 684-7.
- Willson, T. A., D. Metcalf, et al. (1992). "Cross-species comparison of the sequence of the leukaemia inhibitory factor gene and its protein." Eur J Biochem **204**(1): 21-30.
- Wilson, V. and R. Beddington (1997). "Expression of T protein in the primitive streak is necessary and sufficient for posterior mesoderm movement and somite differentiation." Dev Biol **192**(1): 45-58.
- World Health Organisation. 2008. http://www.who.int/cardiovascular_diseases/en/
- Yang, J., A. Blum, et al. (2002). "An epithelial precursor is regulated by the ureteric bud and by the renal stroma." Dev Biol **246**(2): 296-310.

Acknowledgement

First and foremost, I would like to thank my family for supporting me throughout the entire course of my studies.

Sincere thanks to Dr. Georg Weitzer for giving me the opportunity to work in his group, for his continuous support, and for his comments which have improved the quality of this thesis. Special thanks to Dr. Martina Stary for being patient in her tutoring and helping me through rough times. Many thanks to my colleagues Matthias and Christiane for their support.

Thanks also to Martina and Karina, who contributed to the data presented in this study under my supervision.

Curriculum vitae

Personal details

Name:	Marc Wiedner
Date of birth:	09.05.1982
Nationality:	Austrian
Marital status:	unmarried
Address:	Praterstrasse 38/19, 1020 Wien

Education

1986 – 1988:	Preschool at Riverside Elementary School, FLA, USA
1988 – 1993:	Elementary School at Deutsche Schule London, UK
1993 – 2000:	Grammar School at De la Salle Schule Strebersdorf, AUT
2000 – 2003:	University of Vienna, AUT – Medicine
2002 – 2008:	University of Vienna, AUT – Molecular Biology
10/2006 – 12/2006:	Internship at the Institute of Molecular Pathology (IMP), AUT
05/2007 – 05/2008:	Diploma thesis at the Department of Medical Biochemistry, University of Vienna, AUT