Andreas PROKESCH

Large Scale Gene Expression Profiling during Adipogenesis of Murine Bone Marrow Stromal Cells

Master Thesis

Conducted at the [1]Institute for Biomedical Engineering, University of Technology, Graz, Austria and the National Institutes Of Health, Bethesda, United States of America

Supervisor[1] : Ao. Univ.-Prof. Dipl.-Ing., Dr. techn. Zlatko Trajanoski
Head of Institute[1] : Univ.-Prof. Dipl.-Ing., Dr. techn. Gert Pfurtscheller

Graz, June 2002

for my fiancé

ABSTRACT

Obesity is an increasing health problem for many western countries, manifested in a heterogeneous group of associated disorders. Obesity itself is defined as a disorder of energy balance, where energy intake exceeds energy expenditure. Understanding the molecular mechanisms and environmental cues which drive a somatic stem cell to a preadipocyte and further to a mature adipocyte may enable us to fight obesity on a molecular level by designing effective drug treatments to reduce adipose tissue mass with small or no side effects for the patients. The microarray technology is a powerful means to generate a gene expression profile, which allows to simultaneously observe the activity of thousands of genes in one experiment.

The objective was to establish a cell culture model system to study the transcriptional mechanisms which are involved in adipogenesis in vitro. A cell line of murine bone marrow stromal (BMS) cells has been proven to be capable of undergoing adipogenesis upon proper induction. A supplementation of growth medium with dexamethasone and indomethacin massively induced adipogenesis. This was evidenced by oil red O staining.

BMS cells are known to have somatic stem cell character, being able to differentiate into osteocytes, chondrocytes, adipocytes, haematopoiesis-supporting cells and even non-mesodermal cells. The established cell line was investigated for its in vivo properties via an in vivo transplantation assay. The results show that these cells are able to form bone, adipocytes and a complete haematopoietic microenvironment.

Further, the reciprocity of the cells was tested, by means of re-differentiating a mature adipocytic culture into a culture showing an osteogenic phenotype.

Finally, microarray assays were performed in order to delineate transcriptional regulatory networks.

Keywords :

adipocyte, microarray, murine bone marrow stromal cells, obesity, stem cells

KURZFASSUNG

Adipositas ist ein immer größer werdendes Gesundheitsproblem in den westlichen Ländern. Dies manifestiert sich in einer heterogenen Gruppe von Krankheiten, welche mit der Fettsucht in Verbindung stehen. Definiert wird Adipositas selbst als eine Krankheit, in der die Aufnahme von Energie dessen Abbau übersteigt. Das Verständnis der molekularen Mechanismen und die Kenntnis der Umwelteinflüsse, welche eine somatische Stammzelle zu Preadipozyten und weiter zu ausgereiften Adipozyten differenzieren, könnte ermöglichen, Adipositas auf der molekularen Ebene zu bekämpfen. Das Design von neuen, effektiveren Medikamenten könnte helfen die Menge an adipösen Gewebe zu verringern und dies mit weniger oder keinen Nebeneffekten für die Patienten. Die Microarray-Technologie ist ein wertvolles Mittel Genexpressionsprofile zu erstellen, welche es erlauben Tausende von Genen gleichzeitig in einem Experiment zu untersuchen.

Ziel dieser Diplomarbeit war es, ein Zellkultur-Modellsystem zum Studium der transkriptioneller Mechanismen der Adipogenese in vitro zu etablieren. Es wurde bewiesen, dass eine Maus-Knochenmark-Stroma Zelllinie unter Zugabe der entsprechenden Agenzien zur Adipogenese fähig ist. Durch Hinzugabe von Dexamethason und Indomethacin zum Wachstumsmedium wurde eine massive Adipogenese eingeleitet, welche mittels oil red O-Färbung bewiesen wurde.

Knochenmark-Stroma Zellen haben Stammzellcharakter. Sie können sich zu Osteozyten, Chondrocyten und Adipozyten entwickeln und zu Zellen, welche die Hämatopoese unterstützen, sowie zu nicht-mesodermalen Zellen. Die verwendete Zelllinie wurde auf ihre in vivo Eigenschaften hin mittels einem in vivo Transplantationsassay untersucht. Die Ergebnisse zeigen, dass diese Zelllinie fähig ist sich zu Knochen, Fettzellen und einer kompletten, blutbildenden Umgebung zu differenzieren.

Weiters wurde die Reziprozität der Zelllinie untersucht. Dazu wurde eine ausgereifte Adipozyten-Kultur zu einer Kultur mit osteogenischem Phenotyp redifferenziert.

Schließlich wurden Microarray-Experimente zur Rekonstruktion transkriptioneller Netzwerke durchgeführt.

Schlüsselwörter:
Adipositas, Adipocyten, Microarray, Maus-Knochenmark-Stroma Zellen, Stammzellen

CONTENTS

TABLE OF FIGURES

ABBRIEVIATIONS

BMI	Body mass index
BMS	Bone marrow stromal
C/EBP	CCAAT/enhancer binding protein
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyltransferase
CCD	Charged coupled device
cDNA	Complementary DNA
CREB	cAMP response element binding factor
dA	Deoxyadenosine
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DPBS	Dulbecco´s phosphate buffered saline
dT	Deoxythymidine
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediamine tetraacetic acid
EST	Expressed sequence tag
FBS	Fetal bovine serum
IBMX	Isobutyl methylxanthine
MEM	Minimal essential medium
mRNA	Messenger RNA
NaOH	Sodium hydroxide
PPAR	Peroxisome proliferator-activated receptor
RNA	Ribonucleic acid
RT PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SSC	Salt sodium citrate
tiff	Tagged image file format
tRNA	Transfer RNA
WAT	White adipose tissue

1 INTRODUCTION

1.1 Obesity, adipocytes and the adipose tissue

Obesity is defined as a state of increased adipose tissue mass, of sufficient extent to produce adverse health consequences. It is fundamentally a disorder of energy balance, where energy intake exceeds energy expenditure [1]. For each individual, increased adipose tissue mass is the result of a combination of genetic, metabolic, behavioural, environmental, cultural and socio-economic influences. A threshold to define the obese state was introduced by an expert panel, convened by the National Institute of Health (NIH) in 1998 [2]. The Body Mass Index (BMI) was utilised to classify obese and non-obese conditions. The BMI is calculated as weight in kilograms divided by the square of the height in meters (Fig. 1-1).

$$\mathrm{BMI} = \frac{\mathrm{Weight[kilograms]}}{\mathrm{Height[meters]^2}}$$

Fig. 1-1 Calculation of the Body Mass Index

The NIH expert panel proposed adults with a BMI above 30 kg/m² to be considered as obese in a clinical sense, whereas the BMI range between 25 kg/m² and 29.9 kg/m² was suggested to identify overweight. The BMI classification has some limitations, because of its tendency to overestimate body fat mass in very muscular individuals and to underestimate body fat mass in elderly people who have lost muscle mass. Despite this fact, worldwide many scientific and medical organisations endorsed this BMI system for identifying obese and overweight adults. An age- and sex-specific BMI was introduced to define overweight in children and adolescents aged from two to 20 years [3].

However, overweight and obesity is one of the most burdensome health issue in western societies and the burden appears to grow steadily [4,5]. This burden manifests itself in increased mortality and morbidity, in health care costs, in restrained individual well-being and in social stigmatisation. According to a recently published report 34 percent of adults are overweight and 27 percent are obese in the United States of America [4,5]. According to another study 45% are overweight and 11% obese in Germany [6]. Epidemiological studies show that obese individuals (BMI≥30 kg/m²) have a 50 to 100 percent increased risk of premature death from all causes compared to overweight individuals [2]. For example, in the United States of America an estimated 300.000 deaths a year may be attributable to obesity [7].

Morbidity from obesity may be as great as from poverty, smoking or problem drinking. Overweight and obesity was shown to be associated with increased risk for several diseases as listed in Tab. 1-1 [8,9]. For example, type 2 diabetes mellitus is strongly linked to obesity and overweight, with about 80% of the affected individuals being overweight [10].

Overweight and obesity are known risk factors for:	
• diabetes • heart disease • stroke • hypertension • gallbladder disease	• osteoarthritis (degeneration of cartilage and bone of joints) • sleep apnea and other breathing problems • some forms of cancer (uterine, breast, colorectal kidney, and gallbladder).
Obesity is associated with:	
• high blood cholesterol • complications of pregnancy • menstrual irregularities • hirsutism (presence of excess body and facial hair)	• stress incontinence (urine leakage caused by weak pelvic-floor muscles) • psychological disorders such as depression • increased surgical risk.

Tab. 1-1 Health risks associated with obesity and overweight

(Adapted from www.niddk.nih.gov/health/nutrit/pubs/statobes.htm [8])

The increasing prevalence of obesity and overweight is associated with a substantial economic impact. Costs accrue from preventive, diagnostic and treatment

services. The loss of wages by people unable to work due to obesity-related diseases can be considered as ecological factor, too. The economic cost of obesity in the United States was about $117 billion in the year 2000. In Europe the costs attributable to obesity correspond to 2-8% of total health care expenditure [5].

Congenital lipodistrophy is a hereditary disease which is characterised by the absence of fat depots and insulin-resistant diabetes mellitus [11,12]. Thus, in both obesity and lipodistrophy, the incorrect regulation of adipose tissue leads to disease states.

Usually, fat deposits form at sites rich in loose connective tissue, such as subcutaneous layers, around the heart, kidneys and other internal organs[13]. White adipose tissue (WAT) is body's major site of energy storage. The energy is stored in the form of triglycerides inside adipocytes (fat cells) and is mobilised in times of energy deprivation by lipolysis, when triglycerides are broken down into free fatty acids and glycerol [14]. Free fatty acids are then capable to enter the body circulation and can deliver energy by being oxidised in nearly every organ in the body.

Due to a vast number of studies on obesity and adipose tissue the historical view of the adipocytes as mere passive store is disappearing. Fat tissue is now recognised as an endocrine, paracrine and autocrine organ, secreting hormones and cytokines in order to regulate energy homeostasis, lipolysis, lipid synthesis and storage as well as development and growth of itself. Hence, a modulation of adipocyte differentiation can have a profound effect at extra-adipose sites [1].

1.2 Differentiation of adipocytes

Differentiation of adipocytes (adipogenesis) is a process which occurs throughout the lifetime of an organism. Although an adipocyte reacts to energy excess by increasing in size upon fat storage (adipocyte hypertrophy), there is a physical limit to how large this cell can get. On the other hand, fat is stored as long as energy intake exceeds the nutritional requirements. These facts argue in favour of de novo emergence of adipocytes (adipocyte hyperplasia).

Fig. 1-2 displays the process of adipogenesis starting from a pluripotent mesoderm stem cell (adipocytes are suggested to be of mesodermal origin). A lot of data exists about the complex transcriptional cascade that regulates differentiation from preadipocytes to mature adipocytes, although this data is currently just partly understood [15]. Early events supporting determination of primitive mesenchymal stem cells to preadipocytes remain completely unknown [1,16].

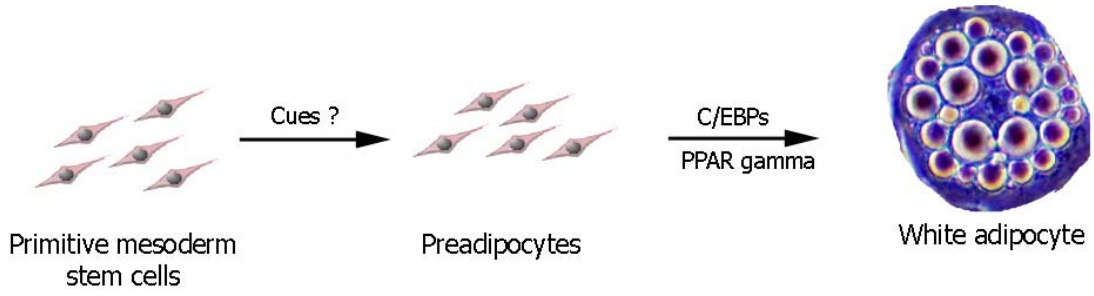

Fig. 1-2 Differentiation of stem cells to mature adipocytes; While the differentiation from preadipocytes to adipocytes is partly understood, early events guiding stem cells to preadipocytes remain unknown

Since the development of an immortal preadipocyte cell line in the 1970s adipocytes differentiation has been a well studied area [1]. Preadipocyte cell lines have been shown to mimic in vivo properties rather authentically, presenting a normal phenotype and behaviour upon transplantation into mice [17].

Preadipocytes are defined by their morphological similarity to fibroblasts and by their commitment to the adipocyte lineage. Currently there is no widely accepted,

clear molecular marker for preadipocytes. If plated into a culture vessel preadipocytes adhere to the bottom of the vessel and grow in a monolayer (like fibroblasts do). They undergo expansion in the form of several cell doublings if the required nutrients are provided. After the cells have covered the whole surface and contact each other, i.e. are confluent, they fall into the state of growth arrest, which is followed by differentiation into a mature adipose phenotype if the proper chemical inducers are provided. Several genetic markers are known during the phases of differentiation. For example, lipoprotein lipase (LPL) appears early [18], together with CCAAT/enhancer binding protein (C/EBP) β and C/EBP δ [19], followed by appearance of the central transcriptional regulators of adipogenesis peroxisome proliferator-activated receptor (PPAR) γ and C/EBP α. These early markers are then followed by expression of most of the genes that characterise the adipocyte phenotype, along with massive fat accumulation [20].

Although many studies revealed mechanisms and their players during the differentiation from a preadipocyte to a mature adipocyte, many questions remain to be answered. For instance, PPARs are transcription factors whose transcriptional targets and their functions are not entirely known [15].

Understanding the regulatory molecular mechanisms of differentiation of adipocytes may enable us to fight obesity on a molecular level by designing effective drug treatments to reduce adipose cell mass, with small or no side effects for the patients.

1.3 Functional genomics and microarrays

The gene expression of a cell is highly specific for the cell type, for the environmental stimuli and for the momentary needs of the cell. Once a gene is transcribed from its DNA template into a messenger RNA (mRNA) molecule, it is then translated into a protein in the cytoplasm of the cell. The concerted regulation of gene expression is a gradual process which is highly controlled by a cascade of molecules, like hormones, transcription factors and coregulators. Understanding these regulatory mechanisms of the transcriptional cascades is the aim of the research area called functional genomics.

Microarrays are an effective means to monitor thousands of expressed genes in a single experiment. Microarrays facilitate the extensive information about DNA sequences available through the achievements of sequencing projects like the Human Genome Project. Annotated known and putative genes are listed in databases and available through cDNA (complementary DNA) libraries. The microarray procedure takes advantage of the highly specific binding of complementary nucleic acid strains (hybridisation), where one strain is immobilised on a surface and the other one is a labelled probe. There are different kinds of microarray technologies: Nylon membrane arrays, radioactive probe arrays, oligonucleotide gene chips and cDNA microarrays. The latter will be briefly described below.

A glass microscope slide is coated with a poly-L-lysine coat, which provides a hydrophobic and positively charged surface [21]. Clones selected from cDNA libraries are amplified with polymerase chain reaction (PCR) using universal primers. The amplicons are then purified and spotted onto the glass surface with the help of a printing robot (spotter). The spotter operates a printhead containing microspotting pins, which hold approximately 1 µl volume in the capillary tube of their tip. DNA is a negatively charged molecule and therefore sticks to the positive poly-L-lysine surface upon contact of the pin. The hydrophobic coat disables the spotted volume to spread and, thereby, allows a deposition of a small volume (approximately 5 nl) to a

precisely defined location on the array. After all cDNAs have been spotted the slides have to be postprocessed. This includes:

- Rehydration; ensures that the cDNA molecules are evenly distributed over each spot;
- Ultraviolet crosslinking; enhances the linkage of cDNA to the surface via ultraviolet irradiation;
- Blocking; chemically converts the positively charged poly-L-lysine surface into a negatively charged, which prevents unspecific binding of the probes;
- Denaturation; resolves possible secondary structures and therefore enables a higher hybridisation efficiency with the labelled probe.

After postprocessing the slides are ready for hybridisation with the labelled probe.

The probes for cDNA microarrays are fluorescently labelled representations of cellular mRNA pools. Typically, total RNA (about 100 µg) is isolated from cells or tissue samples as a snap shot of the momentarily transcribed message. High purity of the RNA is a crucial requirement for a sufficient hybridisation performance because cellular residues can mediate non-specific binding of fluorescently labelled cDNA [22]. The total RNA is than converted into cDNA via oligo-dT primed reverse transcription. Fluorescent labelled deoxy-ribosides (most often dUTP) are incorporated during this step. As illustrated in Fig. 1-3, a microarray experiment employs two fluorescent dyes. Excellent results were obtained using Cy3 and Cy5 dyes because they incorporate efficiently during reverse transcription, they have high photostability and their emission spectra are widely separated. Cy3 fluoresces at a wavelength of 532 nm, which is equivalent to green colour. Cy5 fluoresces at 635 nm, which is equivalent to red colour. One dye labels the RNA pool which is subject to survey and the other dye labels RNA from a basal condition. This enables an analysis of the transcriptional message of cells after a special treatment or in a certain condition (most often a disease condition), compared to a normal condition. Of course, the definition of a normal condition is not an easy one in a biological context [23], but this comparison enables an internal control by delivering an expression ratio rather than a single fluorescence intensity value which could vary due to the frequency of thymine in the mRNA if labelled dUTP is used.

However, following the reverse transcription the labelled cDNA probe has to be purified. Template RNA is therefore degraded by adding NaOH. The sample is filtered to get rid of the small molecules which had to be added for reverse transcription (nucleosides, primer molecules, enzymes). Now the Cy3 and the Cy5 sample are combined and the mixture is ready to be hybridised to the microarray slide. Therefore the mixture is pipetted between the slide surface and a cover glass slip. The slide is sealed in a special hybridisation chamber and submerged in a 65 °C water bath for about 16 hours. Then unbound fluorescent cDNA is removed with a high stringent wash procedure before the slide is scanned by a microarray reader (scanner). This device contains a photomultiplier, which detects the emission of the two dyes, if excited with two separate lasers (one exciting at 532 nm, the other one at 635 nm). This yields to two distinct tiff-format images each providing grey values associated with one dye. Customised computer software assigns a colour to each picture and generates a composite picture (Fig. 1-3), leading to a colour coding for over-regulated and under-regulated genes (highly green or red spots). Genes which are not differentially expressed in the two RNA pools yield a yellow spot.

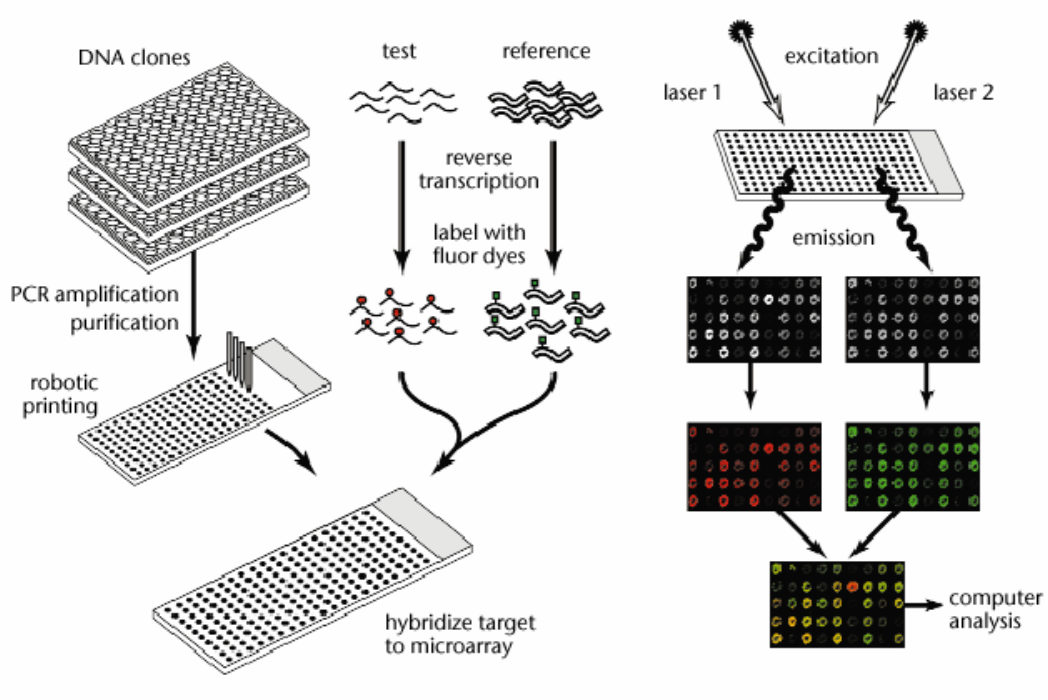

Fig. 1-3 cDNA microarray procedure
(picture adapted from reference [22])

Image analysis software first identifies the shape of the spots, calculates their intensities, determines the local background intensities and calculates the intensity ratio of each spot considering the local background. The software then cross-links information about the spotted clones with all calculated values. This information is then accessible through a excel spread sheet.

Time series experiments are often conducted with microarrays observing the alteration of gene expression over a certain time interval. Thus providing valuable information about the timely regulation of genes, following a specific treatment or exposure to changed conditions. A collection of tissue samples can be compared in the same manner. Prior to comparison the experimental data has to be normalised to take differences into account which arise from uneven amount of applied probes, varying efficiency of probe labelling and fluor excitation and emission [24].

Software algorithms are needed to cope with the vast amount of data accumulating in a time series experiment . Clustering analysis is a powerful tool which partitions genes or biological samples into well-separated and homogenous groups based on their statistical behaviours [25]. Clustering analysis enables a clear visualisation of similar regulated genes (i.e. a gene expression profile) or related tissue samples. Many software packages for microarray data analysis are freely available on the internet.

Microarray data is usually verified in order to deal with any variance emerging in the experiments. Therefore, a subset of genes identified as differentially expressed on the array is confirmed by conventional laboratory methods like northern blot analysis or RT PCR.

Generally, microarray experiments should contain significant repetition and overlapping data to enhance the reliability of the results.

1.4 Bone marrow stromal cells

Bone marrow is historically seen as the major site for haematopoiesis in an organism. The bone marrow microenvironment, consisting of a heterogeneous population of cells, was considered to have merely haematopoiesis supporting properties. In 1974 Friedenstein et al isolated fibroblast-like cells from the bone marrow by their adherence to the plastic surface of a culture vessel [26]. Haematopoietic cells do not adhere to plastic, which enabled an easy separation of these two cell populations. Many names were given to these non-haematopoietic bone marrow cells. Some of them are: mesenchymal cells (pointing out their mesodermal origin) [27], mechanocytes [28], marrow fibroblasts (referring to their fibroblastic morphology) [29], reticular endothelial cells. The term bone marrow stromal (BMS) cells became widely accepted. The word stroma descends from the greek word for mattress (στρομα), indicating that precursors of blood cells rest directly upon surfaces provided by stromal cells.

However, these cells were characterised and investigated extensively since the discovery of Friedenstein et al. Subsequent findings over the years suggest that the BMS cells have somatic stem cell character. Thus, they are capable of developing into more specialised cells of different phenotypes. In vivo different cell types are to be found in the bone marrow :

- Haematopoietic cells and mature blood cells
- Haematopoietic supporting cells
- Osteoblasts (precursors of bone cells)
- Chondrocytes (cells that form cartilage)
- Adipocytes
- Pericytes (involved in exchange of blood between capillaries and tissue)
- Endothelial cells (cells lining the blood vessels)
- Smooth muscle cells

Pericytes, endothelial and smooth muscle cells are located within the blood vessel walls and are not present in the extravascular space of the bone marrow. They are not part of the stroma in a strictly anatomical sense [30].

Chondrocytes, adipocytes, osteoblasts, haematopoietic supporting cells and smooth muscle cells can be derived from the adherent cell layer of BMS cells if cultured in vitro. Whether all this cell types have a common precursor is not known to date. Therefore, BMS cells are considered as a heterogeneous cell population with stem cell character, rather than as a single stem cell [31].

Chondrocytes and osteoblasts are believed to be responsible for bone formation during development and to contribute to bone fraction repair [32]. Several investigators have proposed that chondrocytes and osteoblasts may share a common precursor of osteochondrogenic cells [27].

The function of adipocytes in the bone marrow is subject of permanently discussion. Several hypotheses have been generated [33]:

- Marrow adipocytes may serve a passive role, simply occupying space no longer needed for haematopoiesis;
- Marrow adipocytes are involved in the organisms lipid metabolism similar to adipocytes of the white adipose tissue;
- Marrow adipocytes serve as emergency energy reservoir for haematopoiesis in times of blood loss and for osteogenesis if a bone fraction occurs;
- Marrow adipocytes are steadily and actively supporting haematopoiesis and osteogenesis all through lifetime.

These hypotheses are not mutually exclusive and all of them may prove true. The fraction of adipocytes increases with aging. In newborns the marrow contains few adipocytes and is therefore designated as "red" or erythropoietic marrow. In adolescents more adipocytes appear in the limbs, while in adults fat occupies up to 90 % of the marrow cavity ("yellow" marrow). Experiments have shown that haematopoietic stress like anemia or hypoxia promotes the loss of marrow adipocytes [30,34,35], whereas hypertransfusion of blood into animals turns the marrow to a "yellow", adipocyte-rich phenotype [36].

Further, an increase of adipocytes in the bone marrow on the expense of osteogenic precursors was observed in osteoporosis patients [32]. These observations brought the reciprocal relation between adipocytes and osteoblasts in the spot light. Commitment and differentiation were not usually thought of as a reversible processes. They were rather considered as unidirectional and terminal. BMS cells that were differentiated to mature adipocytes in vitro could de-differentiate to a more proliferative stage and subsequently differentiate to the osteogenic pathway in vitro and even formed bone after transplantation in vivo [37]. Understanding this process in detail and revealing the pivotal players may provide new treatment strategies for bone-related diseases like osteoporosis.

A host of recent studies showed even more surprising potentials of BMS cells. Adult somatic stem cells were considered as committed to differentiate into cell types of their tissue of origin. Viewed developmentally, adult stem cells were thought to develop just within their germ layers (either endoderm, mesoderm or ectoderm). However, a number of recent findings have challenged this notion. Murine [38] and human [40] BMS cells transplanted into ischaemic brains of animals adopted the fate of neural cells (which are of ectodermal origin) and improved functional performance of the animals. Bone marrow cells were shown to contribute to neovascularisation in brains of ischaemic mice, migrating to the site of injury [40]. Hepatocytes [31] and skeletal muscle cells [41] were as well derived from bone marrow. Potential therapeutical applications induced by these findings remain to be determined.

All these findings suggest that the heterogeneous cell population of the bone marrow, named BMS cells, possess a pluripotent somatic stem cell character. They may serve as an alternative to embryonic stem cells, which are ethically questionable. An additional argument in favour of these cells is their straight-forwardness in harvesting them. They can be obtained through a simple bone marrow aspirate. Moreover, they can be easily separated from haematopoietic cells due to their adherence to culture plastic and expand extensively in vitro. Techniques were developed for transient and stable transfection of these cells in vitro [31]. These fact make BMS cells a perfect candidate for cell and gene therapy, as well as for tissue engineering applications [31,32,42].

2 OBJECTIVES

The long term goal of this project is to delineate transcriptional pathways, both during differentiation from stem cells into preadipocytes and from preadipocytes into mature adipocytes.

For large scale gene expression profiling advantage will be taken of the promising microarray technology. A fat-specific customised microarray ("Adipochip") is available, containing about 19 000 genes, many of which are involved in the adipogenic pathway. Genesis clustering software [43] will be utilised to point out genes and groups of genes that are regulated together, hence are active in the same pathway.

The highly interdisciplinary nature of this project demands collaboration between several research institutes. The Institute of Biomedical Engineering, Graz University of Technology cooperates with The Institute of Genomic Research (TIGR), Rockville, MD/USA and the National Institutes of Health (NIH), Bethesda, MD/USA. The Adipochip was assembled and produced at TIGR in 2001. The practical work for this master thesis was entirely performed in the laboratories of the NIH. It was aimed to establish a valuable cell culture model for adipogenesis in BMS cells and to gain expertise and routine with the method of RNA extraction and the microarray assay.

These tasks were grouped in two aims:

2.1 Specific aim #1:

Material, laboratory space and training was provided by the National Institutes of Mental Health (NIMH) under supervision of Dr. Philip W. Gold (Clinical Neuro-endocrinological branch).

Specific aim #1 was to:

- familiarise with aseptic cell culture techniques, including methods like cryo-preservation, viability counting of cells and expanding them to higher cell numbers;
- practise the process of RNA extraction from cell cultures and to conduct associated RNA quality controls;
- design a microarray assay experiment;
- establish the protocols for the microarray assay and to reach a certain level of consistency;
- produce reliable and reproducible data with these protocols;
- conduct image analysis with the provided software;

2.2 Specific aim #2:

All the work concerning specific aim #2 was performed in the laboratories of Dr. Pamela G. Robey at the National Institute of Dental and Craniofacial Research (NIDCR). The objective was to:

- establish a BMS cell culture model that is capable to undergo adipogenesis;
- find the proper combination and concentration of inducers causing the culture to develop mature adipocytes;
- apply the RNA extraction protocol (see specific aim #1) and consider modifications to cope with adipocytes, which usually decrease the purity of isolated RNA;
- verify the pluripotency of the cells by differentiating them into certain lineages in vitro and in vivo;
- extract RNA at eight consecutive time points upon adipogenic induction;

The analysis of the time series during the process of adipogenesis and the interpretation of this data will be conducted at the Institute for Biomedical Engineering in Graz and is not within the framework of this master thesis.

3 METHODS / SPECIFIC AIM #1:

To practise the method for RNA isolation and the procedure for a microarray assay the cell line U937 was chosen. U937 was derived from a patient's lymphoma and was established and characterised in 1976 by Christer Sundstroem and Kenneth Nilsson [44]. This cell line was chosen because it is an easy to handle, high proliferative suspension culture. Further, people in the NIMH laboratories had a lot of experience in working with this cell line and therefore served as an extensive information source. Dr. Sam Listwak kindly provided this cell line which had been frozen in liquid nitrogen at –196 °C for about 7 years.

3.1 Cell culture

Cells were maintained in the following culture medium:
RPMI 1640 medium (ATCC), 10% Fetal Bovine Serum (GibcoBRL), 100 U/ml penicilin and 100 µg/ml streptomycin sulfate (Biofluids). Penicilin and streptomycin sulfate are standard antibiotics for cell cultures.

After thawing the cells at room temperature 1ml of cell suspension (approximately $5*10^5$ cells) was diluted with 50 ml of culture medium. This high dilution factor is necessary to inhibit the toxic effects of dimethylsulfoxid (DMSO), which has to be added to the cells prior to freezing them in order to prevent crystallisation and dehydration of the cell cytoplasm. Cells were then seeded in a culture flask with 175 cm² culturing surface (T 175) and incubated at 37°C in an atmosphere of 100% humidity and 5% CO_2.

A 100 µl aliquot of the cell suspension was mixed to 100 µl trypan blue (GobcoBRL) to count the cells and to assess their viability. This solution was pipetted onto a haematocytometer and counted under the microscope. Trypan blue is a vital dye. Its reactivity is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all cells

which exclude the dye are viable. A "healthy" culture shows a viability around 90%. The viability is calculated by dividing the absolute number of viable cells through the total number of counted cells. To calculate the absolute number of cells one has to take the dilution into account and multiply the count with 10^4, corresponding to the volume that is enclosed under the cover glass slip of the haematocytometer. This leads to the number of cells per milli litre.

As the viability of the cells decreased after reaching a cell number around $8*10^5$ cells/ml –which is a total of 40 million cells in a 50 ml suspension-, cells were passaged every five to six days at a 1:5 dilution (i.e. 10 ml of cell suspension and 40 ml of prewarmed culture medium).

3.2 Preparations

In order to achieve a well distinguishable, significant signal on the microarray slide one needs both, a defined basal condition and an effective treatment. The U937 cell line is known to have an extensive differential expression response to steroids; in particular to glucocoticoids [45]. Dexamethasone is a synthetic glucocorticoid, which is used in vivo as a cortisol surrogate in a vast number of therapeutic approaches.
Fetal bovine serum (FBS) contains lots of steroids among other components (vitamins, growth factors, amino acids, anorganic salts and so on). To create a defined basal condition one needs to remove all steroidal components from the serum. A very simple but reliable method was employed to achieve this. The method of dextran treated charcoal stripping of sera. Charcoal is known to absorb molecular substances like steroids. It was pretreated with dextran in order to stick to a filter membrane after the absorption process. The flow through was filtered again with a 0.22 µm vacuum filter (for sterilisation purposes) after the absorption. The exact protocol of the striping procedure is attached as appendix A.

Afterwards the levels of glucocorticoids were detected with a chemi-luminescence assay. A fully automated chemiluminiscence system from Nichols

Advantage was available. Among other steroids cortisol was undetectable which is equal to a concentration lower than 0.2 µg/dl.

3.3 Treatment

U937 cells are known to respond to dexamethasone at a concentration of 10^{-7} M. Further there is data that after a period of four hours glucocorticoid receptors respond to dexamethasone by showing maximal mRNA level elevation or in some cases maximal mRNA level decrease [46]. Therefore four hours after adding dexamethasone the cells were transferred to a RNase-free polypropylene centrifuge tube and spun down for five minutes at 1 000 rpm and 4 °C in a Sorvall Legend RT cooled table centrifuge with swing rotor. The supernatant was aspirated off. The cell pellet was then washed with 50 ml Dulbecco´s phosphate buffered saline (DPBS) by resuspending the pellet in DPBS, shaking vigorously, spinning it again and aspirating off the supernatant. This step is necessary to remove culture medium residues.

3.4 RNA extraction

The protocol for total RNA isolation from a cell culture (with some alterations applicable to tissue samples, as well) combines the advantages from a procedure recommended from the TRIzol Reagent (Life Technologies/GibcoBRL) manual and the Qiagen RNeasy isolation kit. TRIzol is a reagent that is phenol and guanidine isothiocyanate based which maintains the integrity of RNA, while disrupting the cells and dissolving cell components. The Buffer RLT supplied with the Qiagen kit was found to be not as efficient in homogenizing cells as TRIzol reagent.

A detailed description of the protocol is given in appendix B. The main steps will be briefly described below.

After washing the cells with DBPS (as described in chapter 3.3) the cell pellet was resuspended in 10 ml of TRIzol. The TRIzol manual suggests to use 1 ml TRIzol

reagent per $10*10^6$ cells. The cell count yielded around $35*10^6$ cells in 50 ml of cell suspension, which was the highest cell number prior to a viability decrease. However, 10 ml of TRIzol were used because the homogenisation effect increased with the amount of TRIzol deployed. To further support a disruption of the cells the tube was shaken vigorously. Then chloroform was added, the tube was shaken for 15 seconds and then allowed to rest on the counter for ten minutes. The separation process caused by the interaction of phenol and chloroform started during this time. To enhance the separation the cells were spun at 8 000 rpm at 4 °C for 30 min. After that three clearly distinguishable phases were visible. The phase that contained the DNA at the bottom of the tube; The interphase, which is the organic phase; The upper, clear phase, which contained the RNA. The latter was pipetted off very carefully to not contaminate the solution with proteins from the interphase. To the resulting volume absolute ethanol was added to attain a final volume of 150 %. This ethanol concentration is required for the Qiagen RNeasy kit. The ethanol had to be added drop wise and while mixing with a vortex device to prevent precipitation according to a high local ethanol concentration.

The next steps were conducted according to the Qiagen RNeasy handbook. These steps employ a filter system to decrease the DNA and protein contamination. Every step from the handbook was carried out twice to enhance the purification.

After RNA precipitation with 75 % ethanol and 3M sodium acetate the RNA pellet was washed in 75 % ethanol and resuspended in 100 µl RNase free water.

3.4.1 Measurement of RNA concentration

To measure the concentration of RNA one has to determine the light absorption of a diluted probe at a wavelength of 260 nm. Therefore the spectrophotometer Beckman Du 530 was used. An extinction of one would be equivalent to 40 µg/ml. To calculate the final concentration the dilution has to be adapted to obtain a reading between 0.5 and one. Between these values the reading is in a linear range.

The labelling protocol for the microarray assay requires a RNA concentration of 7µg/ul. If necessary the RNA was concentrated, employing Millipore microcon YM-

30, being a filter system the size of a usual 1.5 ml Eppendorf tube, with a membrane cut-off of 60 nucleotides for single-stranded and 50 nucleotides for double-stranded molecules.

3.4.2 RNA quality

For a microarray assay it is crucial to test both the RNA quality and its integrity, ensuring that they both meet the standard before starting the microarray protocol with this RNA. Cellular proteins, lipids and carbohydrates can mediate significant non-specific binding of fluorescently labelled cDNA to slide surface [22]. Further, degradation of mRNA due to handling (RNases) and exposure to room temperature may change the transcriptional message from the cells.

The ratio of the extinction at 260 nm and at 280 nm wavelength should be between 1.6 and 2.0 for quality RNA.

3.4.3 RNA integrity

Agarose gel electrophoresis was utilised to test the integrity of the RNA molecules (that is no RNA degradation has occurred during or after the isolation process). Between 5 and 10 µg of total RNA were pipetted in one lane of a 1.2 % agarose gel. Staining with ethidium bromide allowed to make the RNA bands visible in an ultra violet light gel reader (BioRad GelDoc 100). For a detailed protocol see appendix C.

Following criteria should be met:

- Two clearly distinct bands should be visible. The upper (5 kb) 28S ribosomal RNA band and the lower (1.9 kb) 18S rRNA band;
- The intensity of the upper 28S band should double the intensity of the lower 18S band;
- No low molecular material should be found on the bottom of the gel;
- No high molecular DNA should appear at the top of the gel;

Fig. 3-1 shows an example of a high quality RNA meeting all of the above criteria.

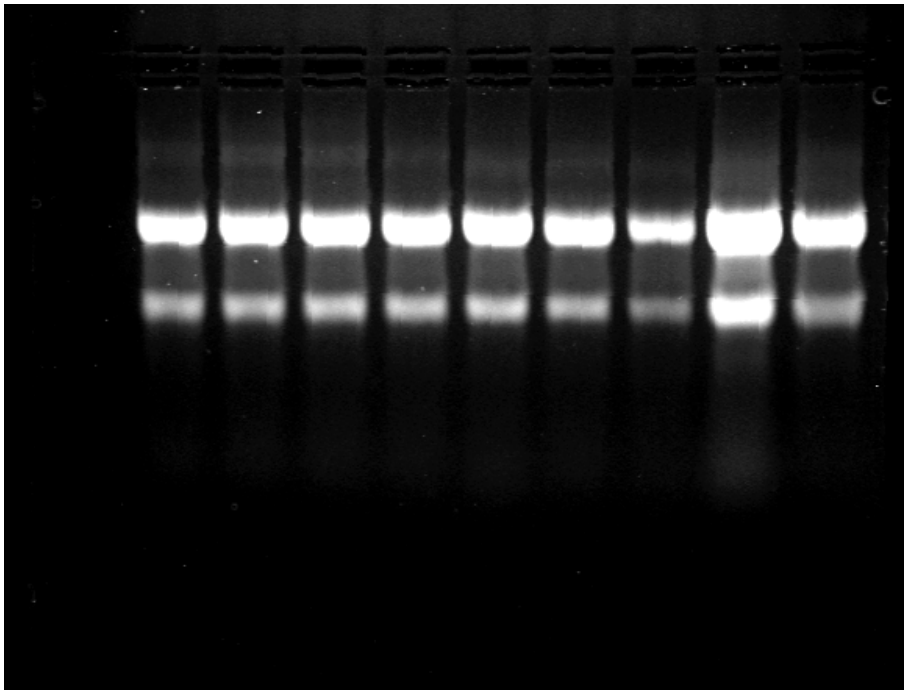

Fig. 3-1 Electrophoreses gel with 5 µg total RNA per lane;

3.5 Microarray assay

The slides were obtained from the microarray core facility for the institutes NHGRI, NIMH and NINDS (URL: http://www.nhgri.nih.gov/UACORE/). The human standard chip contains over 13 000 human sequence verified clones. 9 445 are functionally known or characterised genes, 2 817 are genes whose open reading frame and protein sequence are known, 165 genes are expresses sequence tasks (ESTs) with different level of similarities to known genes. The average clone size is 1 kb and the majority of the clones represent the 3´end of the genes.

A step by step protocol is provided in appendix D. In the following sections, the steps will be explained briefly.

3.5.1 Label procedure

The core of this step is to convert the mRNA into cDNA (complementary DNA) with the help of the enzyme reverse transcriptase and to label the probe with a

fluorescent dye at the same time. The sample treated with dexamethasone was labelled with Cy3 and the basal condition sample (non-treated) with Cy5. The enzyme needs a primer to start the transcription from the RNA template.
The 3´ ends of mRNA molecules consist of a sequence of multiple adenosin residues (50 to 200) and is called the poly A tail. An oligo-dT primer takes advantage of this fact by binding to that poly A tail due to the binding affinity between adenosin and deoxythymidine (dT). The dyes (Cy3 or Cy5) are tagged to deoxyuridine triphosphate (dUTP), which is incorporated into the cDNA during reverse transcription.

The starting amount of total RNA for this protocol is usually between 50 and 200 µg per labelling reaction. With a cell line one is not restricted in the amount of starting material. Therefore it is advisable to go for at least 100 µg per labelling reaction in order to obtain an adequate (i.e. from background noise distinguishable) fluorescence signal. The required starting volume for the annealing of the primer to the target should be 15 µl. This leads to a concentration of at least 7 µg/µl to apply 105 µg per labelling reaction to the slide.

After the reverse transcription, which takes place at 48 °C in a water bath or in a thermocycler, the solution contains a fluorescently labelled cDNA and RNA residues.

3.5.2 Probe purification

To prevent competitive binding between non-labelled RNA and labelled cDNA to the immobilised spots on the slide surface, RNA was degraded by hydrolisation with sodium hydroxide (NaOH) after stopping the labelling reaction with ethylenediamine tetraacetic acid (EDTA). Tris-HCl was added immediately to the reaction to neutralise it (the flours, Cy5 in particular, are sensitive to high pH values). The probes were then purified with Microcon YM-30 spin columns, retaining high molecular cDNA in the membrane and rinsing through small molecules (degraded RNA, loose Cy3 and Cy5-dUTPs, dNTPs). Cy3 and Cy5-labelled probes were then combined and concentrated (again with Microcon YM-30) to a final volume of 37 µl.

To reduce non-specific binding human cot-1 DNA (blocks repetitive DNA sequences) and yeast tRNA (prevents general background haze) was added prior to the concentration step.

3.5.3 Probe hybridisation

Poly dA was added to the Cy3/Cy5 probe mix, to block all poly dT tails so they cannot bind unspecifically to multi adenin sequences on the slide. After denaturing the reaction at 100 °C the proper conditions for hybridisation were created. This is: Adding salt sodium citrate (SSC), 50x Denhardt´s and sodium dodecyl sulfate (SDS) and spinning this reaction ten minutes at 13 000 rpm to pellet any impurity and fluorescent aggregation.

The hybridisation chamber was cleaned with compressed air and three 5 ul spots of 3x SSC were placed on the bottom of the chamber. This circumvents a dehydration in the chamber during the hybridisation at 64 °C.

The hybridisation mix was pipetted from the opposite side of the pellet and a volume of 40 µl was applied to the centre of a 24 mm x 50 mm glass coverslip. The inverted microarray slide was then slowly moved down, until it got in contact with the probe. Upon contact the probe spread evenly over the slide surface. This procedure needed a lot of dexterity and precision to avoid producing bubbles between coverslip and slide and to not introduce dust particles which could interfere with the fluorescence signal.

The slide was then carefully placed in the hybridisation chamber, which was sealed and submerged in a water bath at 64 °C for 16 to 20 hours.

3.5.4 Slide washing

A high stringent wash was performed to get rid off unbound fluorescent cDNA. Here it is crucial to minimise the SDS carry-over between the wash solutions because SDS can interfere with slide imaging. After the third wash slides were dried by spinning 2 minutes at 2000 rpm at room temperature.

3.5.5 Scanning

The hybridised slides were scanned with a prototype device from the company Agilent Technologies, which was provided by the core facility. This device leads to a picture in tiff-format which includes the fluorescence information of both channels. The green channel corresponds to the Cy3 dye (wavelength = 532 nm) and the red channel corresponds to the Cy5 dye (wavelength = 635 nm).

3.6 Image analysis

The objective of the microarray image analysis is to extract sample intensities and intensity ratios of both channels, at each printed cDNA location in a given microarray scan, and then cross-link printed clone information to the calculated ratios, so that further data analysis can be conducted. The utilised software tools were developed at the NHGRI. The tool suit is a collection of IPLab extensions for Macintosh computers. For instance, there are tools for importing the tiff-formatted file and to support the user while aligning the grid to the spots. The core of this suite is DeArray, which is the central processing tool and does most of the image processing tasks including: target segmentation, background intensity estimation and probe intensity extraction. Further a target locator is available which reports target information, refines statistics (such as histogramms and scatter plots) and performs some image enhancement tasks.

As result one receives an Excel spread sheet showing all crucial spot information like: gene description, location on the slide, intensity ratios, accession number of the gene, etc.

4 RESULTS / SPECIFIC AIM #1:

In order to reduce variability due to handling there is a need to gain routine with the protocols. After extensive practising a certain level of consistency and reproducibility was reached, both in isolating RNA and in conducting the microarray assay.

4.1 RNA isolation

The ratio of the extinction at 260 nm and 280 nm was always above 1.6, which is an indicator for a pure RNA solution. The 1.2 % agarose gels met all the criteria, as shown in picture 3-1. This was a solid basis to start the microarray assay with this RNA.

4.2 Microarray assay

Several problems had to be dealt with regarding the microarray assay protocol. The following artefacts occurred on the first few slides:

- Unhybridised areas. This was due to the appearance of air bubbles when applying the probes to the slides.
- Scratches in the hybridised areas. During the first washing step the coverslip lost contact on one corner while sticking to the other one. This caused the corner, that came off later, to scratch the slide surface.
- A raised background haze was visible alongside the slide. This was due to a contamination of the washing solution from slides washed beforehand.

Fig. 4-1 shows examples of the problems encountered. All of these artefacts embodied information loss to some degree. In particular, the background haze reduces the signal-to-noise ratio, which could overstrain the spot recognition algorithm from the image analysis software.

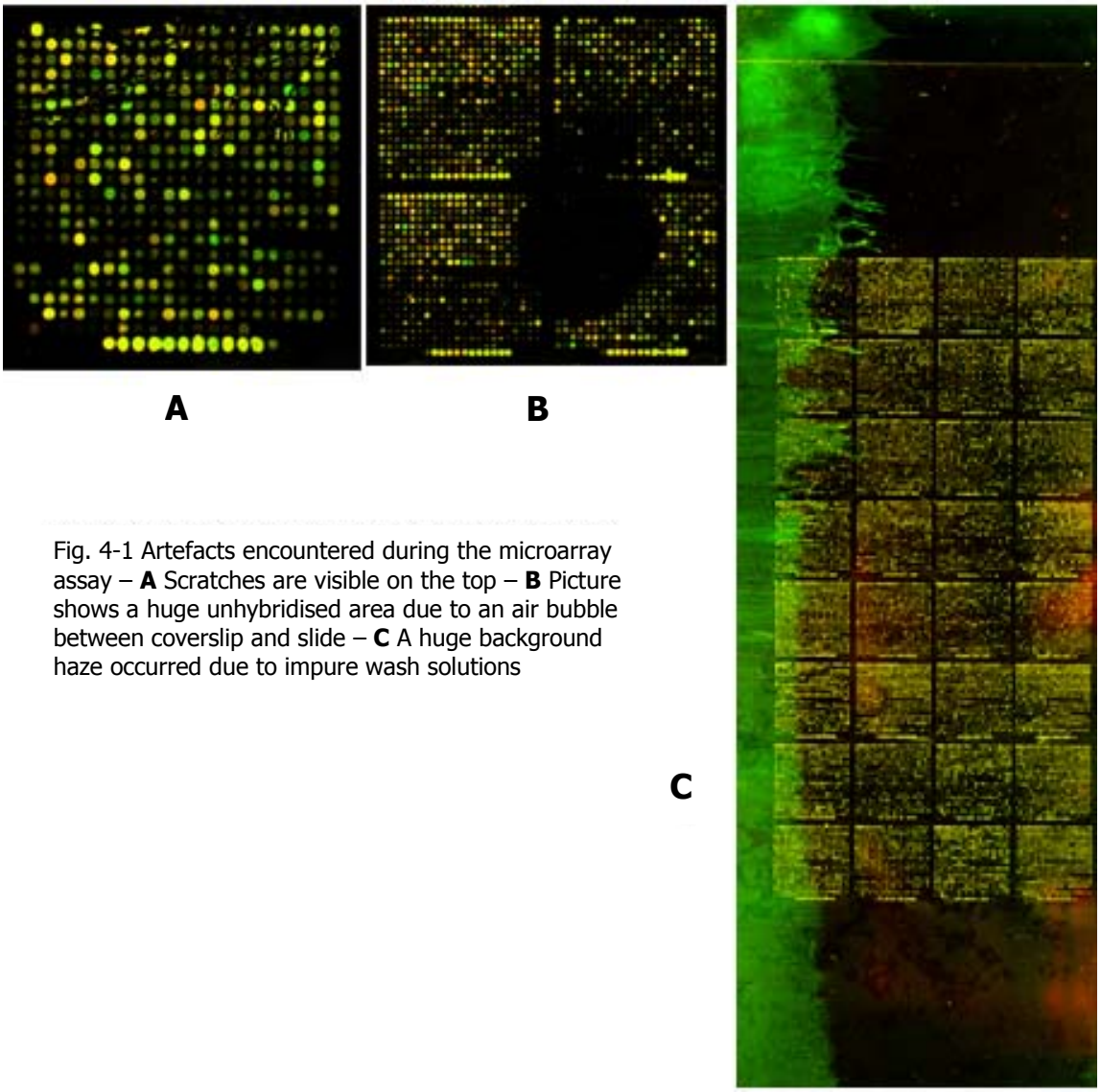

Fig. 4-1 Artefacts encountered during the microarray assay – **A** Scratches are visible on the top – **B** Picture shows a huge unhybridised area due to an air bubble between coverslip and slide – **C** A huge background haze occurred due to impure wash solutions

To prevent bubbles (and therewith unhybridised areas) the probe needs to be pipetted "hunch-shaped" onto the coverslip. This ensures that no air will be enclosed upon contact with the slide. This was accomplished through pipetting more volume onto the middle of the coverslip than towards the ends.

If the volume of the probe, applied to the coverslip, was less than 40 µl scratches were more likely to occur. This may be due to uneven drying under the glass surface while hybridising in the water bath. Hence the coverslip fell off unevenly. Additionally the slide was tilted to minimise the contact of the coverslip while falling off during the first washing step.

The reason for the background haze, shown in Fig. 4-1 C, was the impurity of the washing solutions. This was due to multiple usage of the washes leaving lots of labelled cDNA from previous washes behind, which in turn led to unspecific binding on the slide surface. Therefore it is crucial to prepare a fresh washing solution before each washing procedure.

After coping with and eliminating these sources of artefacts high quality slide scans were obtained. Clear and saturated hybridisation was accomplished yielding to intense fluorescence signal as seen in the detail in Fig. 4-2.

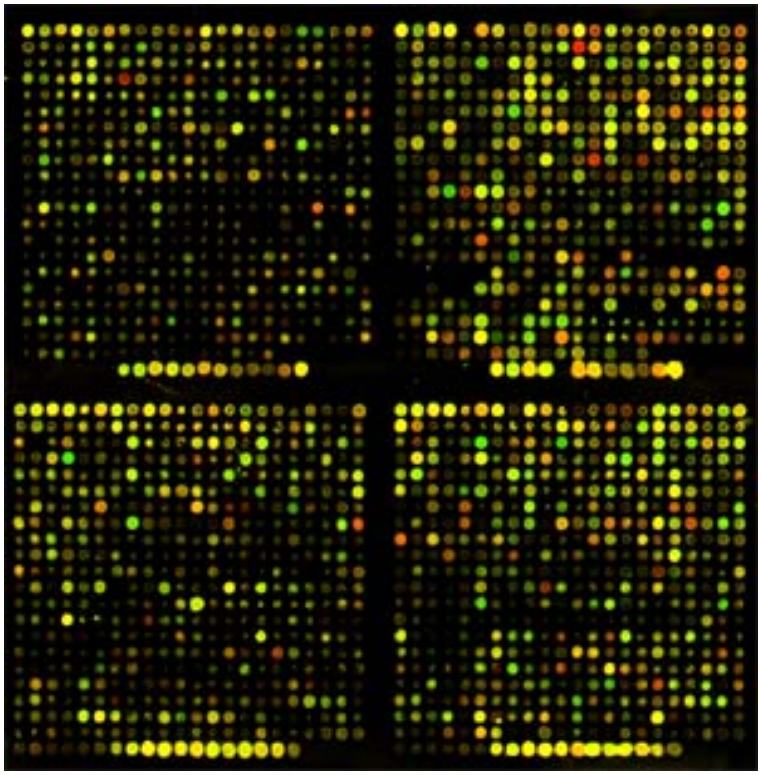

Fig. 4-2 Example of an immaculate fluorescence signal of a microarray assay

Further data analysis was not conducted so far within this thesis. This data and its results could be subject for additional scrutiny.

5 METHODS / SPECIFIC AIM #2:

A valuable in vitro cell model for adipogenesis is usually defined by the ability of the cells to respond to adipogenic inducers by means of accumulating fat vacuoles in their cytoplasm.

In order to develop such a model a cell strain of mouse bone marrow stromal (BMS) cells was provided in the laboratories of Dr. Pamela G. Robey. These cells were harvested from transgenic mice [47]. The transgene is a construct of a reporter gene (chloramphenicol acetyltransferase (CAT)) whose expression is driven by a type I collagen promoter (Col1a1). This promoter is known to be activated in osteogenic tissue in the bone marrow environment. CAT is a commonly employed and easy assayable reporter gene, which, by nature, expresses an enzyme exclusively in prokaryotes. It can be used either for promoter analysis experiments or to verify that transplanted cells are still of donor origin after recovering the cells from the recipient organism (if any CAT activity is detected in these cells it would be a proof that they stem from the donor).

The provided cells appeared to be spontaneously immortalised. Despite their high number of passages (18) these cells displayed an uncommonly high proliferation rate, by doubling every 24 hours. Usually BMS cells do not exceed the eighth passage. According to the widely accepted theory of Leonard Hayflick [48,49] cells cultured ex vivo are usually not capable of exceeding a certain number of doublings, the so-called hayflick limit or crisis. Hence, cells escaping this limit are said to be able to proliferate indefinitely and can be called a cell line. Such cells are likely to have properties of cancer cells. It is essential to test whether these cells were able to maintain their in vivo and in vitro characteristics or not. In the case of BMS cells these characteristics are, among others (see chapter 1.4), the ability to differentiate towards adipocytes, osteocytes and chondrocytes. For this reason cells were assayed not just for their in vitro adipogenic commitment, but also for their ability to differentiate to an osteogenic phenotype in vitro and in vivo. The latter was achieved

by in vivo transplantation in mice, which is the gold standard for defining the differentiation potential of BMS cells.

5.1 Cell culture

Cells were kindly provided by Dr. Sergei A. Kuznetsov. For culture expansion cells at the 16^{th} passage were maintained in growth medium containing a mixture of α Minimal Essential Medium (αMEM; Life Technologies), 20 % fetal bovine serum (FBS; Equitech-Bio), 10^{-8} M dexamethasone (Sigma-Aldritch), 10^{-4} M L-ascorbic acid phosphate (Wako), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate (Biofluids). This growth medium is a standard for culturing BMS cells in the NIDCR laboratories. L-ascorbic acid phosphate serves as a stable source for vitamin C (ascorbic acid). Added directly to the culture medium, ascorbic acid would not be steadily available to the cells, because it is unstable in aqueous solutions under physiological conditions [50,51].

The cells were incubated at 37° C in an atmosphere of 100% humidity and 5% CO_2. Complete medium replacement was performed one day after plating and twice a week thereafter. The cells reached confluency (they occupied the whole culture vessel surface) after three days, if $1.5*10^6$ cells were seeded in a 75 cm^2 tissue culture flask (T75), which is equivalent to a doubling period of less than 24 hours. Then the cells were released from the bottom of the culture vessel by Trypsin-EDTA (Life Technologies) and plated in new flasks for further expansion which is called passaging. At the confluent state each T75 contained about $22*10^6$ cells, as determined by counting with trypan blue (as described in chapter 3).

5.2 Adipogenic induction

Many cocktails for inducing BMS cells to adipogenesis are to be found in the literature. Predominant supplements to the growth medium are 0.1 µM dexamethasone, 50 µM indomethacin (Calbiochem) and 500 µM 3-isobutyl-1-

methylxanthine (IBMX;Sigma). This cocktail was successfully used in a multitude of experiments with BMS cells [52-55] . Other adipogenic inducers had been proposed, like insulin, biotin and ascorbic acid phosphate [52,53,56,57,58 and so forth].
However, three different cocktails were tested with these cell line, with the following supplements to the growth medium (see also Tab. 5-1):

1) 0.1 µM dexamethasone, 50 µM indomethacin and 500 µM IBMX; without ascorbic acid phosphate
2) 0.1 µM dexamethasone and 50 µM indomethacin; without ascorbic acid phosphate
3) 0.1 µM dexamethasone, 50 µM indomethacin and 100 µM ascorbic acid phosphate

Cells were plated in 6-well plates ($2*10^5$ cells per well). Each cocktail was applied to three wells. Additionally, cells were maintained in wells with plane growth medium to serve as a negative staining control.

Growth medium	αMEM, 20% FBS, 10^{-8} M dexamethasone, 10^{-4} M L-ascorbic acid phosphate, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate
Adipogenic cocktail 1)	αMEM, 20% FBS, 10^{-7} M dexamethasone, 50 µM indomethacin, 500 µM IBMX, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate
Adipogenic cocktail 2)	αMEM, 20% FBS, 10^{-7} M dexamethasone, 50 µM indomethacin, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate
Adipogenic cocktail 3)	αMEM, 20% FBS, 10^{-7} M dexamethasone, 10^{-4} M L-ascorbic acid phosphate, 50 µM indomethacin, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate
Osteogenic cocktail	αMEM, 20% FBS, 10^{-2} M β-glycerophosphate , 10^{-8} M dexamethasone, 10^{-4} M L-ascorbic acid phosphate, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate

Tab. 5-1 Overview of utilised media

5.3 Adipocyte histochemistry

Oil red O (ICN) staining was employed to proof the accumulation of lipid vacuoles. Oil Red O is a fat soluble dye. The fat solubility allows a migration through the cell membrane (which is a lipid double layer) and an accumulation in the fat droplets of adipocytes. Hence, adipocytes are recognisable by microscopy.

After aspirating off the medium, cells were rinsed three times with distilled water and fixed with 10% formalin (in phosphate buffered saline) for at least 30 minutes. Oil red O working solution (diluted and filtered) was applied for one hour and the stained cells were washed three times with distilled water. After that, cells were counterstained with light green (ICN). Light green enhances the contrast of the picture by staining the somata of the cells but not their fat droplets. For microscopy images an Axioplan 2 (Zeiss) microscope, equipped with a DMC-1 CCD-device (Polaroid) to acquire digital images, was used.

5.4 Osteogenic induction

The reciprocal relationship between adipocytes and osteocytes is a well known feature of BMS cells [31,33,37,59]. This reversibility can convert a mature adipocyte into an osteocyte under specific circumstances and vice versa. To test, whether the cells maintained this reversibility after spontaneous immortalisation, the media of cultures of mature adipocytes were replaced with a commonly used osteogenic cocktail. Therefore 10^{-2} M β-glycerophosphate was added to the growth medium (see Tab. 5-1), since it is known as a osteogenic inducer together with dexamethasone and ascorbic acid [60,61]. The cells were cultured in the osteogenic medium for five days.

5.5 Osteogenic histochemistry

The ability of matrix mineralisation (an early step in osteogenesis) upon osteogenic induction was assessed by alizarin red staining. Alizarin red is an indicator of calcium phosphate appearing when osteoblasts mineralise. Therefore, cells were rinsed with phosphate buffered saline and fixed for one hour with 10% formalin. After fixed cultures were rinsed with distilled water, they were stained for five minutes with 1% alizarin red in 2% ethanol to reveal mineralisation. Additional oil red O staining was performed on the alizarin red stained cultures to visualise adipocytes.

Cultures maintained in adipogenic cocktail were stained as negative control (also with alizarin red and oil red O).

5.6 In vivo transplantation assay

In vivo transplantation provides the opportunity to determine true osteogenic capacity as identified by the formation of histologically identifiable bone [62]. The in vivo transplantation assay was mainly performed by collaborators in Dr. Pamela G. Robey´s laboratories. Dr. Sergei A. Kuznetsov performed the cross sections and the histochemistry of the cross sections. Dr. Arabella Leet performed the surgical part. The results of this assay have been included in this thesis with their permission. This assay is a crucial proof, whether these cells maintained their in vivo properties upon heterotopic transplantation into immunocompromised mice, or not. If transplanted to extramedullary sites, BMS cells usually start to proliferate and differentiate until they form a complete bone nodule with a cavity containing a haematopoietic microenvironment and haematopoietic cells, which proves their stem cell character [31,42,62-64].

A brief description of the protocol is given in the following:

Cells were grown to confluency and released from the bottom of the culture vessel with Trypsin/EDTA. $2*10^6$ cells were collected by means of centrifugation. 50 mm^3 Gelfoam sponges were prepared as transplantation vehicles. The cells were absorbed into the sponges.

Three anesthetised, immunocompromised mice were used as recipient for subcutaneous transplants; another three received a intracranial graft. For the latter a mid-longitudinal incision of about one centimetre in length was made on the dorsal surface of the cranium and the skin and periosteum was separated. A cranial defect (5 millimetre diameter) was drilled carefully without injuring the dura mater. A transplant was placed into the cranial defect and the skin was sutured. The subcutaneous transplants were placed into subcutaneous pockets, formed in the back to the left and right of the spinal chord. Each pocket received one transplant, with up to two transplants per mouse. The incisions were closed with surgical staples.

Ten weeks later the transplants were retrieved from the mice, fixed, decalcified, dehydrated, embedded in paraffin and sectioned in 5 µm slices. The sections were then deparaffinised and hydrated before they were stained with hematoxylin and eosin. Histochemical pictures were taken with a Zeiss Axioplan 2 light microscope equipped with a CCD device.

5.7 RNA extraction

After an effective adipogenesis inducing cocktail was established, the experiment for the microarray time series was designed. Eight time points were chosen through a period of 24 days after starting to incubate in adipogenic cocktail. One 75 cm^2 culture flask yielded about 100 to 200 µg of total RNA, which is a sufficient amount for a microarray assay. Hence, $1.5*10^6$ cells were plated in each of

eight flask. Cells were cultured in growth medium until confluency and then medium was replaced by the adipogenic cocktail.

Extracting RNA from adipocyte-rich cultures could lead to cutbacks regarding the RNA yield and quality. The high content of triglycerids stored in the vacuoles of the cell cytoplasm can cause technical problems like clogging of membranes, floating to the surface of solutions during the separation step and solidifying on ice. Many suggestions for modulations of RNA isolation protocols for adipocyte-rich cultures are to be found in the literature [65]. These include additional steps (after adding TRIzol) to disrupt the vacuoles and separate the lipids from the solution. Those steps can be: mixing vigorously with a vortex device, incubating in a water bath, multiple passing of the homogenate through a needle and centrifuging the homogenate, to name just a few.

However, it is known that even leaving the homogenate at room temperature for a while could lead to a change in the gene expression profile, because RNA is a very unstable molecule, hence it degrades easily.

Therefore the protocol described in chapter 3 and listed in appendix B was followed thoroughly. The increased volume of TRIzol reagent used in this protocol seemed to be sufficient in order to disrupt lipid vacuoles and no further steps had to be taken.

Additionally RNA from undifferentiated cultures was isolated to serve as basal condition for the microarray assay.

6 RESULTS / SPECIFIC AIM #2

6.1 Cell culture

Due to their high proliferation rate these cells tended to lift from the bottom of the culture vessels and started to form a tissue. Adherence to the plastic is crucial for BMS cells in order to maintain their viability and in vitro properties. Therefore, it was essential to handle these cells very carefully. Reducing the vacuum when aspirating off media, not touching the cell surface with pipette tips and refilling just warm media very slowly, helped to increase mechanical and temperature stress on the cells and therefore prevented their detachment.

Other measures to cope with the effect of detachment are to reduce the concentration of fetal bovine serum (FBS). However, FBS provides the cells with many nutrients and growth factors. A reduction of its concentration slowed down the differentiation process significantly. Hence, it was decided to not decrease the concentration of the serum.

6.2 Adipogenic induction and histochemistry

Cells were cultured in the three different adipogenic cocktails (as listed in Tab. 5-1) for 21 days. Media were refreshed twice a week. Fig. 6-1 shows histochemical stains with oil red O and light green (as described in chapter 5).

Cocktail 1) with IBMX seemed to inhibit adipogenesis at these cells, although it is reported as adipogenesis inducer in other studies [1,56,57]. Fig. 6-1 A shows that no adipocytes emerged in these cultures, as in negative control cultures maintained in growth medium (histochemical pictures not shown).

Cocktail 2) led to massive accumulation of fat droplets, as evidenced in Fig. 6-1 B. An objective lens with a magnification of 40x - which is a total of 400x together

with the magnification of the eyepiece (as opposed to 100x in Fig. 6-1) - was employed for Fig 6-2, which displays the fat droplets in greater detail.

No significant change in the magnitude of adipogenesis was to be found with cocktail 3), which included ascorbic acid phosphate (Fig. 6-1 C). These cultures, though, began to lift from the bottom of the culture vessel as mentioned above.

These results favour cocktail 2) as an effective means to induce adipogenesis in these BMS cell line.

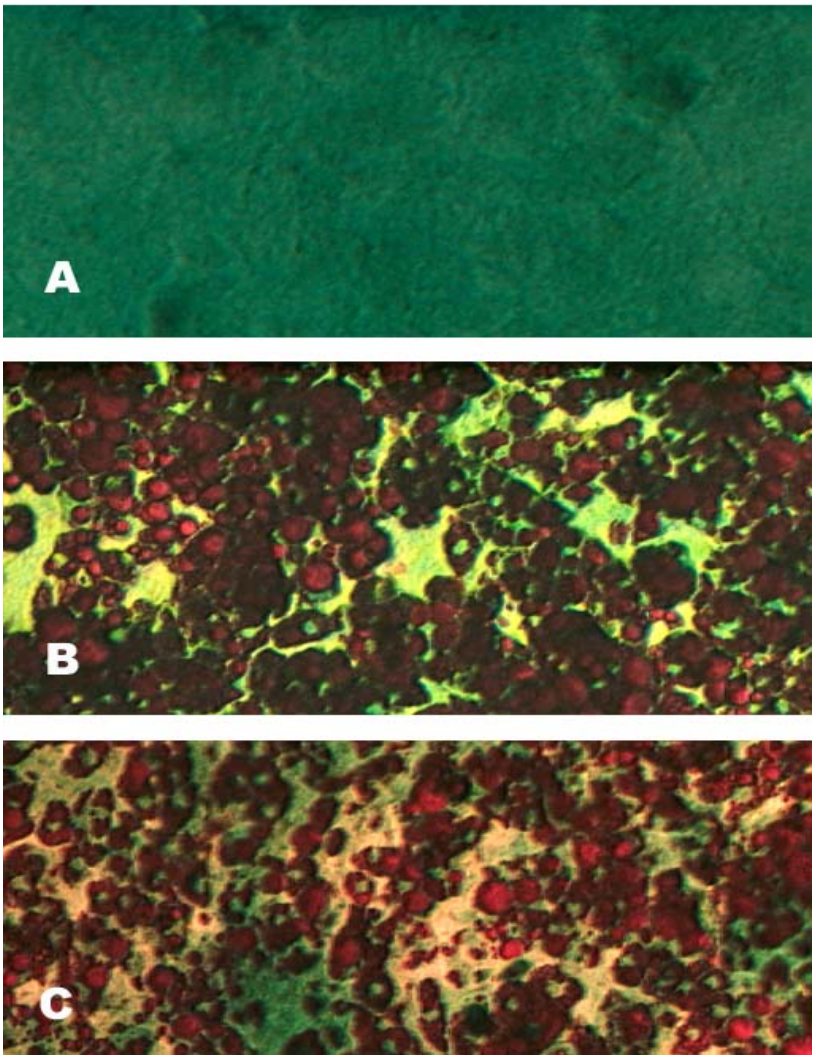

Fig. 6-1 Histochemical staining of three bone marrow stromal cell cultures; Cultures were stained with Oil red O and counterstained with light green after being cultured in three different media with the following adipogenesis-specific inducers (magnification : 100x) – **A** 0.1 µM dexamethasone, 50 µM indomethacin and 500 µM IBMX – **B** 0.1 µM dexamethasone and 50 µM indomethacin – **C** 0.1 µM dexamethasone, 50 µM indomethacin and 100 µM ascorbic acid phosphate

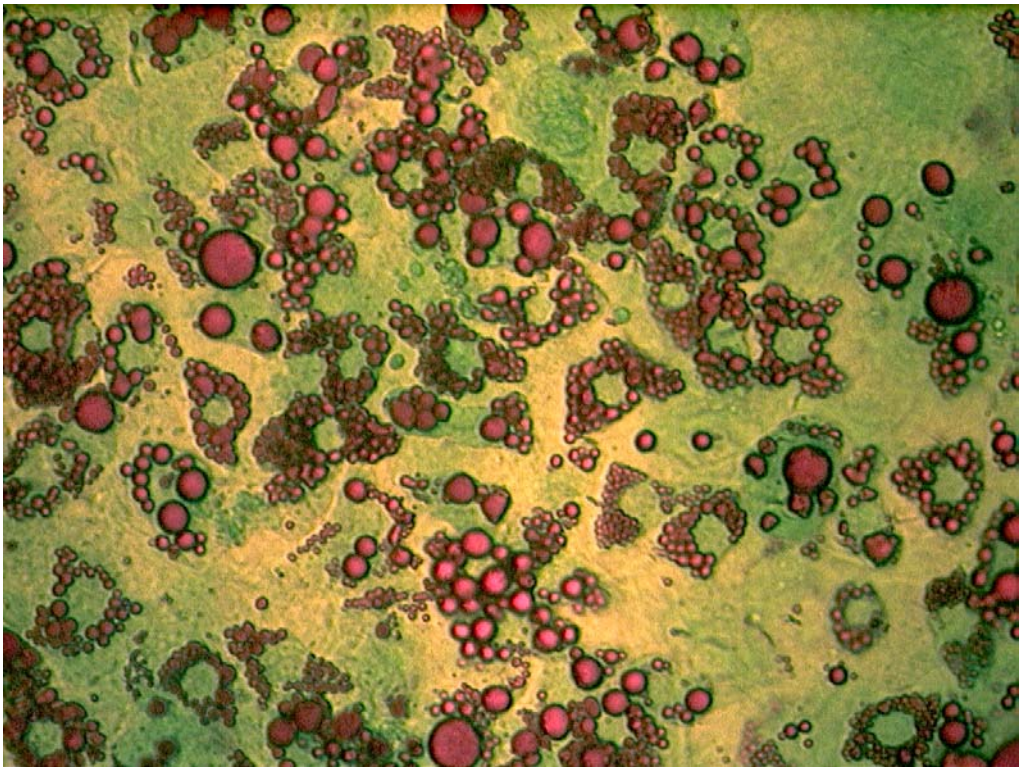

Fig. 6-2 Histochemical staining of an adipocytic cell culture; Oil red O staining and light green counterstaining was performed; This culture was induced to adipogenesis with 0.1 µM dexamethasone and 50 µM indomethacin (magnification : 400x)

6.3 Osteogenic induction and histochemistry

In Fig. 6-3 the presence of calcium phosphate is evidenced by alizarin red staining. Fig. 6-3 A shows a culture maintained in adipogenic cocktail as negative control. In Fig. 6-3 B alizarin red veils are visible which indicates massive mineralisation of the extracellular matrix. Adipocytes are still present in these cultures (dark red cells).

The macroscopic picture (Fig. 6-3 C), too, demonstrates the difference of cultures maintained either in adipogenic or, for five days, in osteogenic conditions (both cultures were stained with alizarin red and oil red O). Further, this picture demonstrates that adipocytes formed colonies. This is a known feature of adipocytes which may be explained by the fact that adipocytes are paracrine cells. This would ensure that were one fat cell develops, others will follow [66].

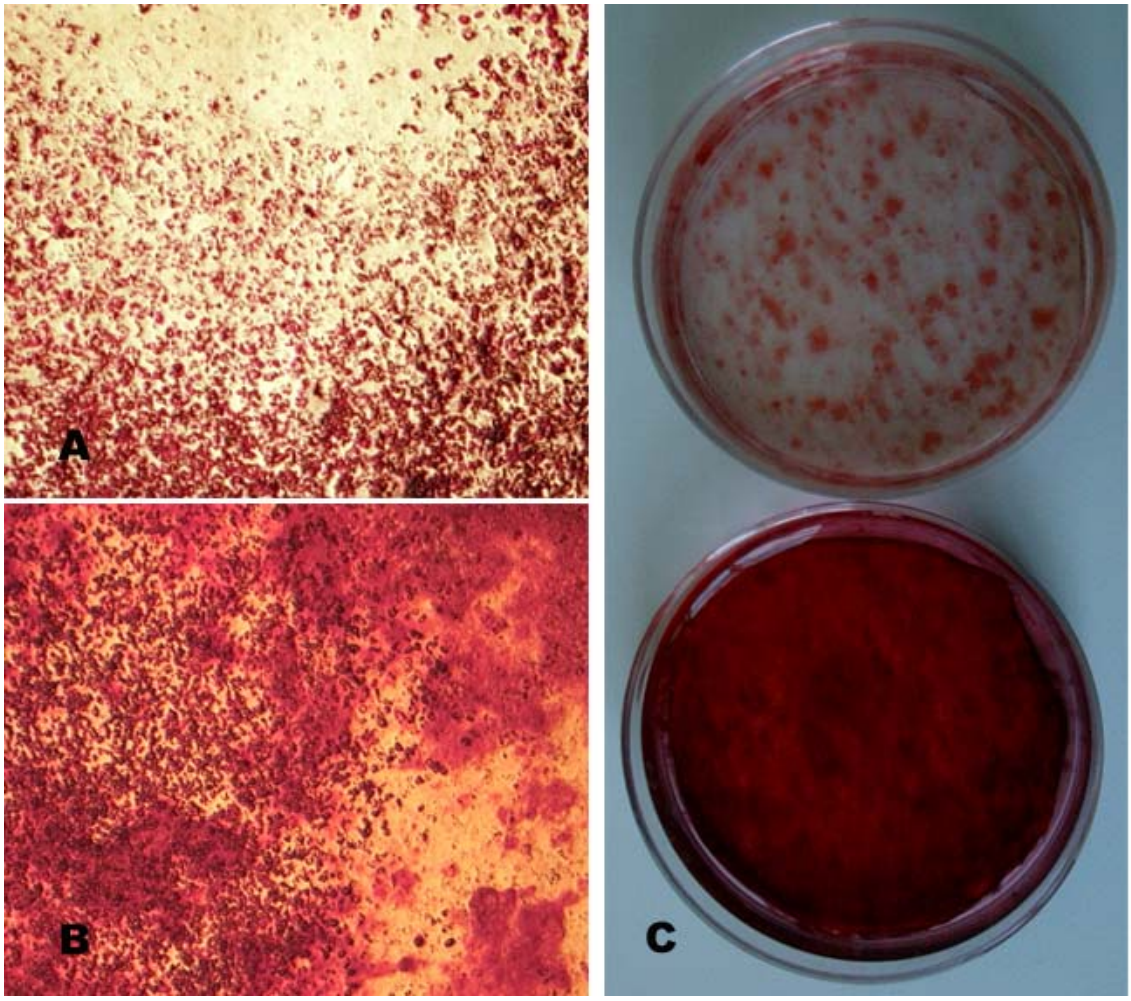

Fig. 6-3 Alizarin red and oil red O staining of bone marrow stromal cells; – **A** Cells cultured in adipogenic conditions (magnification: 25x) – **B** Culture of mature adipocytes changed to an osteogenic medium for 5 days (magnification:25x) – **C** Macroscopic picture of **A** (top) and **B** (bottom)

6.4 In vitro transplantation assay

Ten weeks after transplantation the BMS cells exhibited extensive bone formation, providing cavities for haematopoietic cells. Further osteoblasts (lining the bone), fibroblastic tissue and adipocytes could be easily distinguished at the histological sections (Fig 6-4). Fig. 6-4 A shows a stained section of a subcutaneous transplant, while Fig. 6-4 B shows the newly formed bone from the intracranial transplant and its merging to calvarial bone of recipient origin (at the bottom of the picture).

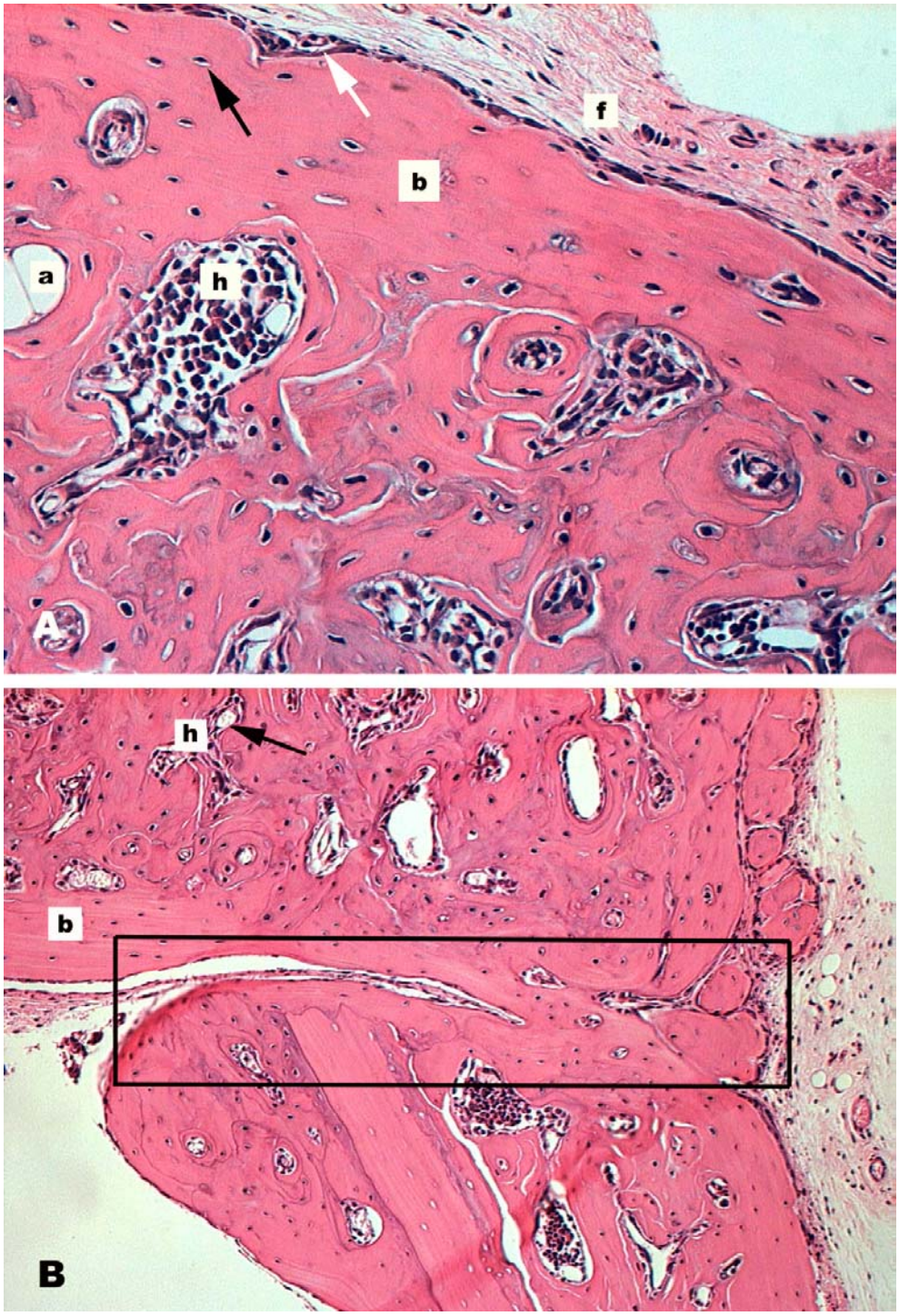

Fig. 6-4 Histological sections of transplanted bone marrow stromal cells; Transplants were sectioned and stained with hematoxylin and eosin - **A** After 10 weeks in vivo the subcutaneous transplants exhibited adipocytes (a), bone (b), haematopoietic tissue (h) and fibroblastic tissue (f); The white arrow designates osteoblasts (lining the bone); The black arrow designates a single osteocyte; - **B** Intracranial transplant merging clavarial bone of the host organism (black framed box); Black arrow designates an adipocyte; Haematopoietic area (h), bone (b) comprised of osteocytes and bone lining tissue(osteoblasts and fibroblasts) is observeable;

6.5 RNA extraction

As described in chapter 3, RNA quality was tested by means of calculating the ratio of extinctions at two different wavelengths (260 nm/280 nm) of a diluted RNA sample. RNA integrity was verified via electrophoresis on an agarose gel.

Fig. 6-5 shows that the RNA extracted at all eight time points is of immaculate integrity.

Spectrophotometer readings ranged from 1.67 to 1.93, which is within the range for high quality RNA (1.6 – 2.0).

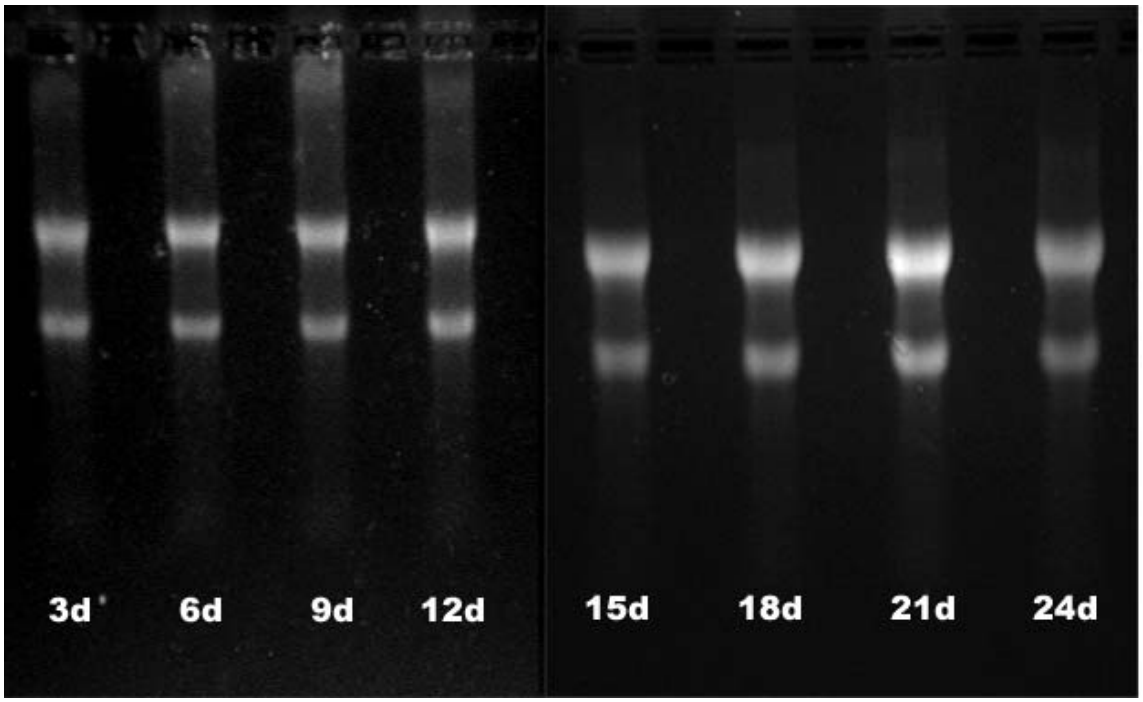

Fig. 6-5 Electrophoresis gel of a RNA extraction time series; Bone marrow stromal cells were induced to adipogenesis at day zero

7 DISCUSSION

A cell line of murine bone marrow stromal (BMS) cells has been proven to be capable to undergo adipogenesis upon proper induction. Therefore, it serves as an in vitro model to study the transcriptional mechanisms which are involved in adipogenesis. Three adipogenic cocktails (see Tab. 5-1) were selected from the literature to survey their efficiency to drive the cells towards the adipocyte pathway. Cocktail 1) with 500 µM IBMX showed no adipocytes and seemed to inhibit adipogenesis. Contradictory notions about the function of IBMX in a culture system are to be found in the literature. On the one hand IBMX is said to inhibit phosphodiesterase, which elevates the intracellular cyclic adenosinemonophosphate (cAMP) level. This, in turn, is claimed to enhance differentiation towards adipocytes [1,56,57]. On the other hand, findings of other research groups suggested an inhibitory role of cAMP regarding adipogenesis [67], which would be consistent with the findings in this study. In preadipocytic cell cultures (3T3-L1) it was recently shown that cAMP initiates adipogenesis via the transcription factor cAMP response element binding factor(CREB) [68]. All these reports indicate that the effect of cAMP on adipogenic differentiation is currently not entirely understood. However, a supplementation of growth medium with dexamethasone and indomethacin massively induced adipogenesis. This was evidenced by in vitro oil red O staining.

Furthermore, the BMS cell line was able to maintain its reversibility, which was shown by re-differentiating a culture that contained mature adipocytes into an osteogenic phenotype. This was evidenced by alizarin red staining of calcium phosphate deposits as demonstrated in other studies [37,59,69].

The in vivo properties of these cells were shown with an in vivo transplantation assay in mice. After a period of ten weeks the transplants had developed bone nodules. The histological sections demonstrated a bone environment with all its constituents like osteocytes, osteoblasts and adipocytes. Haematopoietic tissue was found in bone cavities. These results conform with other studies [62,63,70,71].

A time series containing eight consecutive time points, starting with three days after adipogenic induction, was produced by applying an evaluated RNA isolation protocol. The RNA is of immaculate purity as tested with spectrophotometry (260 nm/280 nm extinction ratio) and with electrophoreses (agarose gels).

A number of assays could be conducted in future to support the data of this thesis. For example, alkaline phosphatase and osteocalcin are specific markers for osteoblasts and proper assay kits are readily available [52,53,72]. The proof of the presence of adipogenic markers will be provided along with the data that will emerge from the microarray assay employing the adipogenesis-specific array.

Moreover, the cell line presents an intriguing model for stem cell differentiation. Investigations with commercially available microarrays along with conventional laboratory techniques could help to delineate their molecular signature and its change to environmental cues. This could make the process of stem cell differentiation more transparent.

Sufficient results were reached with the microarray protocol, as can be seen in Fig. 4-2. A low background and a high fluorescence signal intensity were yielded. The obvious weak point of the protocol is the amount of starting material (100 µg of total RNA). A number of labs recently developed and established protocols, which employ a slightly different technique as described in appendix D [73,74]. Less than 10 µg of total RNA is needed for these new protocols. They take advantage of the indirect labelling of aminoallyl groups. These aminoallyl groups are linked to the nucleotides, which are used for first strand cDNA synthesis (reverse transcription). In a separate step after purification the dyes (Cy3 or Cy5) are incorporated to the aminoallyl-labelled cDNA. This step ensures a more efficiently and evenly incorporation of the dyes leading to a lower required starting amount. For future purposes the application of these protocols should be taken into consideration.

In conclusion, this model system can serve as a valuable means to study the molecular transcriptional mechanisms of the differentiation from somatic stem cells to preadipocytes and further to mature adipocytes. The microarray technology is a powerful assay to observe the activity of thousands of genes at a time. Hence, a gene expression profile will be generated, providing information about the timely and interactive regulation of adipogenesis-related genes. As there is a great deal of similarity between mice and man regarding adipogenesis on a genetic level, the data may allow to be extrapolated to the human metabolism. This may enable us to elucidate the development of obesity from a molecular viewpoint, which may in turn lead to more efficient drug treatment for obese patients.

ACKNOWLEDGMENTS

First of all I want to thank my supervisor Zlatko Trajanoski for his support and for providing the great opportunity to study and work at the National Institutes of Health.

Further, I want to express my gratitude to Pam Robey and Philip Gold, the principal investigators of the laboratories I was honoured to stay. It was a pleasure to learn from Sam Listwak and discuss various problems with him. Martiz Peterson invaluably supported me in every administrative matter. I also want to thank Sergei Kuznetsov for sharing his extensive knowledge about bone marrow stromal cell culturing with me and Xiao-Dong Chen and Marian Young for their help.

The love and encouragement of my fiancé Manuela Wedenig is my daily impetus. I want to thank her for sharing her life with me.

The support of my family and their faith in me was a crucial companion during my study. I want to express my sincere gratitude.

REFERENCE LIST

[1] Rosen ED, Spiegelman BM. Molecular regulation of adipogenesis. Annu.Rev.Cell Dev.Biol. 2000;16:145-71.

[2] National Institute of Health (NIH). Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults--The Evidence Report. National Institutes of Health. Obes Res 1998;6:51S-209S.

[3] National Center for Health Statistics (NCHS), CDC. CDC Growth Charts: United States. http://www.cdc.gov/growthcharts/ . 2002.
Ref Type: Internet Communication

[4] Deitel M. The Surgeon-General's call to action to prevent an increase in overweight and obesity. Released Dec. 13, 2001. Obes Surg. 2002;12:3-4.

[5] Satcher, D. The Surgeon General's Call To Action To Prevent and Decrease Overweight and Obesity. http://www.surgeongeneral.gov/topics/obesity/ . 2001.
Ref Type: Internet Communication

[6] Heseker H, Schmid A. Epidemiologie der Adipositas. Ther.Umsch. 2000;57:478-81.

[7] Allison DB, Fontaine KR, Manson JE, Stevens J, VanItallie TB. Annual deaths attributable to obesity in the United States. JAMA 1999;282:1530-8.

[8] National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Statistics related to obesity and overweight. http://www.niddk.nih.gov/health/nutrit/pubs/statobes.htm . 2000.
Ref Type: Internet Communication

[9] Calle EE, Thun MJ, Petrelli JM, Rodriguez C, Heath CW, Jr. Body-mass index and mortality in a prospective cohort of U.S. adults. N.Engl.J.Med. 1999;341:1097-105.

[10] Nadler ST, Attie AD. Please pass the chips: genomic insights into obesity and diabetes. J.Nutr. 2001;131:2078-81.

[11] Shackleton S, Lloyd DJ, Jackson SN et al. LMNA, encoding lamin A/C, is mutated in partial lipodystrophy. Nat.Genet. 2000;24:153-6.

[12] Speckman RA, Garg A, Du F et al. Mutational and haplotype analyses of families with familial partial lipodystrophy (Dunnigan variety) reveal recurrent missense mutations in the globular C-terminal domain of lamin A/C. Am.J.Hum.Genet. 2000;66:1192-8.

[13] Netter FH. Atlas of Human Anatomy. NJ: Ciba-Geigy, 1997.

[14] Arner P. Techniques for the measurement of white adipose tissue metabolism: a practical guide. Int.J.Obes Relat Metab Disord. 1995;19:435-42.

[15] Mandrup S, Lane MD. Regulating adipogenesis. J.Biol.Chem. 1997;272:5367-70.

[16] Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. Physiol Rev. 1998;78:783-809.

[17] Green H, Kehinde O. Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. J.Cell Physiol 1979;101:169-71.

[18] MacDougald OA, Lane MD. Transcriptional regulation of gene expression during adipocyte differentiation. Annu.Rev.Biochem. 1995;64:345-73.

[19] Darlington GJ, Ross SE, MacDougald OA. The role of C/EBP genes in adipocyte differentiation. J.Biol.Chem. 1998;273:30057-60.

[20] Spiegelman BM, Choy L, Hotamisligil GS, Graves RA, Tontonoz P. Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes. J.Biol.Chem. 1993;268:6823-6.

[21] Eisen MB, Brown PO. DNA arrays for analysis of gene expression. Methods Enzymol. 1999;303:179-205.

[22] Duggan DJ, Bittner M, Chen Y, Meltzer P, Trent JM. Expression profiling using cDNA microarrays. Nat.Genet. 1999;21:10-4.

[23] King HC, Sinha AA. Gene expression profile analysis by DNA microarrays: promise and pitfalls. JAMA 2001;286:2280-8.

[24] Yang YH, Dudoit S, Luu P et al. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 2002;30:e15.

[25] Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc.Natl.Acad.Sci.U.S.A 1998;95:14863-8.

[26] Friedenstein AJ, Deriglasova UF, Kulagina NN et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp.Hematol. 1974;2:83-92.

[27] Dominici M, Hofmann TJ, Horwitz EM. Bone marrow mesenchymal cells: biological properties and clinical applications. J.Biol.Regul.Homeost.Agents 2001;15:28-37.

[28] Friedenstein AJ. Precursor cells of mechanocytes. Int.Rev.Cytol. 1976;47:327-59.

[29] Friedenstein AJ. Marrow stromal fibroblasts. Calcif.Tissue Int. 1995;56 Suppl 1:S17.

[30] Bianco P, Riminucci M. The bone marrow stroma in vivo : ontogeny, structure, cellular composition and changes in disease. In: Beresford JN, Owen M.E., eds. Marrow stromal cell culture. Cambridge: Cambridge University Press 1998:10-25.

[31] Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 2001;19:180-92.

[32] Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71-4.

[33] Gimble JM, Robinson CE, Wu X, Kelly KA. The function of adipocytes in the bone marrow stroma: an update. Bone 1996;19:421-8.

[34] Tavassoli M. Marrow adipose cells and hemopoiesis: an interpretative review. Exp.Hematol. 1984;12:139-46.

[35] Tavassoli M, Maniatis A, Crosby WH. Induction of sustained hemopoiesis in fatty marrow. Blood 1974;43:33-8.

[36] Brookoff D, Weiss L. Adipocyte development and the loss of erythropoietic capacity in the bone marrow of mice after sustained hypertransfusion. Blood 1982;60:1337-44.

[37] Bennett JH, Joyner CJ, Triffitt JT, Owen ME. Adipocytic cells cultured from marrow have osteogenic potential. J.Cell Sci. 1991;99 (Pt 1):131-9.

[38] Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc.Natl.Acad.Sci.U.S.A 1999;96:10711-6.

[39] Zhao LR, Duan WM, Reyes M, Keene CD, Verfaillie CM, Low WC. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Exp.Neurol. 2002;174:11-20.

[40] Hess DC, Hill WD, Martin-Studdard A, Carroll J, Brailer J, Carothers J. Bone marrow as a source of endothelial cells and NeuN-expressing cells After stroke. Stroke 2002;33:1362-8.

[41] Ferrari G, Cusella-De Angelis G, Coletta M et al. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998;279:1528-30.

[42] Bianco P, Robey PG. Stem cells in tissue engineering. Nature 2001;414:118-21.

[43] Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. Bioinformatics. 2002;18:207-8.

[44] Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int.J.Cancer 1976;17:565-77.

[45] Franchimont D, Martens H, Hagelstein MT et al. Tumor necrosis factor alpha decreases, and interleukin-10 increases, the sensitivity of human monocytes to dexamethasone: potential regulation of the glucocorticoid receptor. J.Clin.Endocrinol.Metab 1999;84:2834-9.

[46] Archer TK, Lee HL, Cordingley MG et al. Differential steroid hormone induction of transcription from the mouse mammary tumor virus promoter. Mol.Endocrinol. 1994;8:568-76.

[47] Krebsbach PH, Harrison JR, Lichtler AC, Woody CO, Rowe DW, Kream BE. Transgenic expression of COL1A1-chloramphenicol acetyltransferase fusion genes in bone: differential utilization of promoter elements in vivo and in cultured cells. Mol.Cell Biol. 1993;13:5168-74.

[48] Shay JW, Wright WE. Hayflick, his limit, and cellular ageing. Nat.Rev.Mol.Cell Biol. 2000;1:72-6.

[49] Hayflick L. A brief history of the mortality and immortality of cultured cells. Keio J.Med. 1998;47:174-82.

[50] Chepda T, Cadau M, Girin P, Frey J, Chamson A. Monitoring of ascorbate at a constant rate in cell culture: effect on cell growth. In Vitro Cell Dev.Biol.Anim 2001;37:26-30.

[51] Goldenberg H, Landertshamer H, Laggner H. Functions of vitamin C as a mediator of transmembrane electron transport in blood cells and related cell culture models. Antioxid.Redox.Signal. 2000;2:189-96.

[52] Gimble JM, Morgan C, Kelly K et al. Bone morphogenetic proteins inhibit adipocyte differentiation by bone marrow stromal cells. J.Cell Biochem. 1995;58:393-402.

[53] Thompson DL, Lum KD, Nygaard SC et al. The derivation and characterization of stromal cell lines from the bone marrow of p53-/- mice: new insights into osteoblast and adipocyte differentiation. J.Bone Miner.Res. 1998;13:195-204.

[54] Kelly KA, Gimble JM. 1,25-Dihydroxy vitamin D3 inhibits adipocyte differentiation and gene expression in murine bone marrow stromal cell clones and primary cultures. Endocrinology 1998;139:2622-8.

[55] Dorheim MA, Sullivan M, Dandapani V et al. Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. J.Cell Physiol 1993;154:317-28.

[56] Gimble JM, Dorheim MA, Cheng Q et al. Adipogenesis in a murine bone marrow stromal cell line capable of supporting B lineage lymphocyte growth and proliferation: biochemical and molecular characterization. Eur.J.Immunol. 1990;20:379-87.

[57] Gimble JM, Youkhana K, Hua X et al. Adipogenesis in a myeloid supporting bone marrow stromal cell line. J.Cell Biochem. 1992;50:73-82.

[58] McAveney KM, Gimble JM, Yu-Lee L. Prolactin receptor expression during adipocyte differentiation of bone marrow stroma. Endocrinology 1996;137:5723-6.

[59] Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. J.Cell Sci. 1992;102 (Pt 2):341-51.

[60] Chen TL, Bates RL. Recombinant human transforming growth factor beta 1 modulates bone remodeling in a mineralizing bone organ culture. J.Bone Miner.Res 1993;8:423-34.

[61] Leboy PS, Beresford JN, Devlin C, Owen ME. Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J.Cell Physiol 1991;146:370-8.

[62] Krebsbach PH, Kuznetsov SA, Satomura K, Emmons RV, Rowe DW, Robey PG. Bone formation in vivo: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation 1997;63:1059-69.

[63] Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating skeletal stem cells. J.Cell Biol. 2001;153:1133-40.

[64] Tavassoli M, Crosby WH. Transplantation of marrow to extramedullary sites. Science 1968;161:54-6.

[65] Janke J, Engeli S, Gorzelniak K, Sharma AM. Extraction of total RNA from adipocytes. Horm.Metab Res 2001;33:213-5.

[66] Shillabeer G, Forden JM, Lau DC. Induction of preadipocyte differentiation by mature fat cells in the rat. J.Clin.Invest 1989;84:381-7.

[67] Bianco P, Kuznetsov SA, Riminucci M, Fisher LW, Spiegel AM, Robey PG. Reproduction of human fibrous dysplasia of bone in immunocompromised mice by transplanted mosaics of normal and Gsalpha-mutated skeletal progenitor cells. J.Clin.Invest 1998;101:1737-44.

[68] Reusch JE, Colton LA, Klemm DJ. CREB activation induces adipogenesis in 3T3-L1 cells. Mol.Cell Biol. 2000;20:1008-20.

[69] Halvorsen YD, Franklin D, Bond AL et al. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng 2001;7:729-41.

[70] Satomura K, Krebsbach P, Bianco P, Gehron RP. Osteogenic imprinting upstream of marrow stromal cell differentiation. J.Cell Biochem. 2000;78:391-403.

[71] Krebsbach PH, Kuznetsov SA, Bianco P, Robey PG. Bone marrow stromal cells: characterization and clinical application. Crit Rev.Oral Biol.Med. 1999;10:165-81.

[72] Beresford JN, Joyner CJ, Devlin C, Triffitt JT. The effects of dexamethasone and 1,25-dihydroxyvitamin D3 on osteogenic differentiation of human marrow stromal cells in vitro. Arch.Oral Biol. 1994;39:941-7.

[73] Yu J, Othman MI, Farjo R et al. Evaluation and optimization of procedures for target labeling and hybridization of cDNA microarrays. Mol.Vis. 2002;8:130-7.

[74] Schroeder BG, Peterson LM, Fleischmann RD. Improved quantitation and reproducibility in Mycobacterium tuberculosis DNA microarrays. J.Mol.Microbiol.Biotechnol. 2002;4:123-6.

APPENDIX A - CHARCOAL STRIPPING OF FETAL BOVINE SERUM

Amount specifications for two bottles of fetal bovine serum (FBS; each 500 ml)
Dextran coated charcoal (DCC) should be 1% in final solution

- thaw FBS bottles in water bath (37 °C)
- dissolve 1 g of dextran completely in 100ml TRIS Buffer
- add 10 g of charcoal and dissolve completely
- spin at 3000 rpm for 10 min
- dump off supernatant
- resuspend with 100 ml TRIS Buffer
- repeat the last three steps three times without resuspending at the last step
- add both bottles of 500 ml FBS to the coal pellet (leading to approximately 1% DCC)
- stir for 30 min with a magnetic stirrer
- spin at 12000 rpm for 20 min to pellet the DCC
- dump supernatant through funnel with 3 mm filter paper
- filter again through 0.22μm filter

APPENDIX B - RNA EXTRACTION PROTOCOL

- pellet cells via centrifugation at 1500 rpm for 5 min
- aspirate off the supernatant
- add 10 ml TRIzol to pellet (approximately 1 ml TRIzol per $3*10^5$ cells)
- shake to homogenise
- add 2 ml chloroform per tube
- shake for 15 sec
- let it stand for 10 min (phases should start to separate)
- centrifuge for 15 min at 4 °C and 8000 rpm
- transfer JUST the transparent, clear, upper liquid phase in 50 ml tubes, recording the volume (should be about 4 ml)
- add 0.53 volumes of 100% ethanol drop by drop to all tubes while shaking gently with a vortex device (the RNA would precipitate at a high local concentration of ethanol)
- following steps demand the RNeasy Midi kit from Qiagen
- transfer solution to Qiagen spin column
- centrifuge at 3600 rpm for 3 min
- pour the flow-through back onto the top of the column and centrifuge again (this will increase the yield by 50 %)
- discard the flow-through (RNA is attached to the matrix of the spin column)
- add 4 ml RW1 solution from RNeasy kit to the column (this reduces the protein contamination)
- centrifuge 5 min at 3600 rpm
- repeat last two steps after discarding the filtered liquid
- perform another twofold washing step with ethanol-containing RPE buffer from the RNeasy kit
- the second spin has to be 10 min, because the matrix need ethanol-free

- exchange tubes with new ones
- add 250 µl DEPC water
- let stand for 1 min
- centrifuge for 5 min at 3600 rpm
- don't pour away filtered liquid this time (contains RNA)
- add another 250 µl DEPC water
- centrifuge for 10 min at 3600 rpm
- transfer all (400 to 500 µl) of the solution into a 1.5 ml Eppendorf tube
- add 1 ml of 75% ethanol
- add 40 µl of 3 M sodium acetate (pH 6)
- let it sit for 15 min in freezer (-70 °C)
- centrifuge at 12000xg at 4 °C for at least 20 min to pellet RNA
- aspirate off supernatant
- wash pellet with 500 µl 75% ethanol
- air-dry the pellet for about 20 min
- resuspend RNA in DEPC water (estimate the volume to yield a concentration of 7 µg/µl)
- measure RNA concentration
- concentrate RNA with Microcon YM-30 spin column if needed
- RNA can be stored at –70 °C

APPENDIX C - RNA GEL PREPARATION PROTOCOL

1.2 % agarose gel (dimensions 10x7x0.7 cm)

- add 1.2 g agarose powder to a RNase-free beaker
- add 72ml DEPC water
- heat in the microwave for 30 sec to dissolve agarose powder
- cool solution to room temperature
- add 10 ml 10x MOPS buffer
- add 18 ml formaldehyde solution 12.3 M
- pour into RNA gel chamber
- gel hardens in about 30 min

meanwhile prepare sample:

- add 11 µl RNA (should contain 5 to 10 µg RNA) to a 1.5 ml Eppendorf tube
- add 5 µl 10x MOPS buffer
- add 9 µl formaldehyde solution 12.3 M
- add 25 µl formamid
- incubate in waterbath for 15 min at 55 °C
- add 5 µl RNA gel stained loading solution (10x)
- 1 µl ethidium bromide
- mix gently with vortex device

if gel is hardened:

- carefully remove comb and gel pouring aids from gel
- fill chamber with 1x MOPS (= gel running buffer) until gel is covered
- load sample onto gel (~50 µl / lane)
- apply 70 to 100 V for 1 hour (recommended : 5-10 V / cm)
- destain gel over night in container filled with deionised water, placed on a shaker

APPENDIX D – MICROARRAY ASSAY PROTOCOL

Labelling procedure:

Starting concentration: 7µg/µl for both dyes, 15 µl starting volume → 105 µg total RNA

- prepare on ice :
- add to RNase free PCR tube :
    - 15 µl RNA
    - 2 µl anchor oligo-dT (2 µg/µl)
- mix and incubate at 70 °C for 5 min and cool to 48°C (use thermocycler or heating block)
- while incubating prepare following reagents at room temperature :
    - 8 µl 5x first strand buffer
    - 4 µl 10x low dT dNTP mix
    - 4 µl fluro dUTP (1 mM) i.e. Cy3 or Cy5
    - 4 µl 0.1 M DTT
    - 2 µl SSII reverse transcriptase
- mix and incubate at 48 °C for 60 min

Probe purification : (wear powder-free gloves!)

- add 5 µl of 500 mM EDTA (pH 8.0) to each labelling reaction at room temperature
- mix extensively
- add 10 µl 1 N NaOH and mix well
- incubate at 65 °C for 20 min to hydrolise RNA and cool to room temperature

- add 25 µl 1M Tris-HCl (pH 7.5) to neutralise NaOH
- probes are now ready to be purified and concentrated
- add 200 µl TE buffer to labelled probes
- add this and another 200 µl to Microcon YM-30 spin column
- centrifuge at 13000 rpm for 7 min
- recover each probe by inverting filter into new tube and centrifuging 5 min at 13000 rpm
- if pale pink colour is left on filter, add another 30 µl water and repeat the above step
- combine Cy3 and Cy5 probes and measure volume
- add another 400 µl (200 to probe, 200 to filter) of water to a new Microcon YM-30 column (end volume including 15 µl CoT-1 and 15 µl tRNA should not exceed 500 µl)
- add 15 µl CoT-1 Human DNA (1 µg/µl) and 15 µl of Yeast tRNA (0.4µg/µl) to same column
- mix with pipette in filter
- centrifuge about 8 min 30 sec at 13000 rpm
- final volume should be 37 µl (repeat filtering if volume is too big after recovery)
- recover probe by inverting filter into new tube and centrifuging 5 min at 13000 rpm

Hybridisation :

- add 2 µl of poly dA (1µg/µl) to 37 µl Cy3/Cy5 sample
- mix with pipette and transfer to PCR tube
- denature at 99 °C for 2 min and cool to room temperature
- add:
    - 8 µl 20x SSC
    - 2 µl 50X Denhardt´s
    - 1 µl 10% SDS

- mix gently with pipette
- transfer to 1.5 ml Eppendorf tube
- spin 10 min at 13000 rpm to get rid of any impurity
- clean hybridisation chamber with air pressure
- add 5 µl 3x SSC to three sites in the chamber
- add rubber bands to the edges of the slide
- pipette of the probe from the opposite side of the pelleted impurities (40 µl)
- apply probe to cover slip
- contact slide with cover slip (parallel and centred, make sure that the spotted side points down)
- place in hybridisation chamber and seal chamber with clips
- Hybridise in water bath (or humidified chamber 65 °C) for 16 to 24 hours

High stringent wash : (procedure takes place at room temperature)

- take chamber from water bath and dry with vacuum, especially on edges
- remove clips
- transfer to slide rack
- submerge slide rack under the surface of wash solution #1 (0.1% SDS + 0.5x SSC) until the cover slip falls off and leave it for 2 min
- transfer slide rack directly in wash solution #2 (0.01% SDS + 0.5x SSC) for 2 min
- transfer slide rack directly in wash solution #3 (0.06x SSC) for 2 min
- transfer slide rack immediately to centrifuge and spin for 2 min at 2000 rpm
- slides are ready to be scanned

Preparation of wash solutions :

- Wash solution #1 (0.5x SSC / 0.1% SDS):
    - 25 ml 20x SSC + 965 ml DEPC water
    - filter with 0.5 µm filter device
    - add 10 ml 10% SDS
    - mix well
- Wash solution #2 (0.5x SSC / 0.01% SDS) :
    - 25 ml 20x SSC + 974 ml DEPC water
    - filter with 0.5 µm filter device
    - add 1 ml 10% SDS
    - mix well
- Wash solution #3 (0.06x SSC)
    - 3ml 20x SSC + 997 ml DEPC water
    - filter with 0.5 µm filter device

Store all wash solutions at room temperature