Stroma-leukaemic cell interactions: Analysis of stroma environment-induced effect on human acute myeloid leukaemic cells

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1. SUMMARY

In spite of the progress made in deciphering regulatory networks of cancer cells on the molecular level, the interaction of tumour cells with their stroma has not been adequately analyzed.

Earlier, we have addressed the hypothesis that the murine embryonic microenvironment can induce the differentiation of human tumour cells. To examine such interactions, human leukaemic AML cells were injected into pre-implantation murine blastocysts at embryonic day 3.5 of gestation. Analysis of developing mice revealed the presence of human AML cells in chimaeric embryos and adults and the appearance of haematopoietic differentiation markers on progeny of injected human AML cells. This finding strengthens the notion that the embryonic microenvironment is capable of regulating the proliferation and differentiation of leukaemic AML cells.

Based on these results, I embarked to analyse the consequences of stromal environment-induced changes in human AML cells upon *in vitro* coculture with selected haematopoietic stromal cell lines in terms of changes in differentiation and proliferation properties of AML cells. For this purpose, established human AML cell lines were cocultured on a variety of mitotically inactivated stromal cell lines derived from different murine embryonic/foetal haematopoietic sites such as yolk sac, aorta-gonad-mesonephros (AGM) region and foetal liver. To score for coculture-induced changes, I compared the morphology, histo-chemical properties, immunophenotype, proliferation rate, and gene expression profile in cocultured and non-cocultured AML cells.

Results show that, upon coculture of Kasumi-1 cells- a cell line established from a FAB class M2 patient - with AGM-derived DAS 104-4, but not with other stromal cell lines, Kasumi-1 AML cells exibit decreased proliferation and colony formation capabilities and acquire differentiated morphologies. Along this line, coculturing of Kasumi-1 cells resulted in the up-regulation of the myelo-monocytic lineage cell surface markers CD11b and CD14. Coculture also resulted in increase in lysosomal marker CD68, a hallmark of myeloid differentiation. Interestingly, apart from cell lines, coculture on DAS 104-4 stroma was also efficient in inducing myeloid differentiation of patient derived primary M2-AML cells. Moreover, cocultivation of KG-1 cell line on DAS 104-4 showed activation of β-globin transcription and up-regulation of Glycophorin A on its surface, which indicate DAS 104-4 coculture-induced erythroid differentiation of KG-1 cells.

Analysis of the proliferation rate of Kasumi-1 cells using the CFSE retention assay revealed that upon cocultivation on DAS 104-4, but not on NIH 3T3 cells, there is a decrease both in the proliferation rate and in the frequency of colony forming cells in clonogenic methyl cellulose cultures. Cell cycle analysis revealed the coculture-induced accumulation of G_1-G_0 stage cells.

Gene-expression analysis by quantitative RT-PCR revealed a substantial decrease in the amount of AML1 and AML1-ETO fusion transcripts in parallel with an increase in p16, p21, C/EBPα and PU.1 transcription levels. Interestingly, AML1-ETO transcription down-regulation of AML cells needs direct contact with DAS 104-4 cells.

Knocking down AML1-ETO expression by siRNA strategy led to reduction in proliferation and depletion of colony forming cells in Kasumi1 cell population. siRNA-mediated AML1-ETO knock-down Kasumi-1 cells showed increased susceptibility to stroma-induced myeloid differentiation. However, on its own, AML1-ETO down-regulation was not sufficient to induce myeloid differentiation. This indicates that AML1-ETO down-regulation may have an active

role on the coculture-induced effect but in addition to AML1-ETO down-regulation, further stimuli are required for the coculture-induced myeloid differentiation in the AML cells.

In summary, in the present study I established and characterised a coculture-based *in vitro* system, which is capable of reducing the proliferation while inducing differentiation of human AML cells. The concept emerging from the studies indicates that the stroma environment can affect leukaemic cell proliferation and differentiation in contact-dependent and CD44 activation-independent manner. Furthermore, this study emphasizes the role of AML1-ETO in AML and indicates that AML1-ETO down-regulation is involved in the stroma-induced differentiation of Kasumi-1 cells. The result described here encourages further investigation into the mechanistic details of molecular and cellular interactions between the leukaemic cells and their stroma, which in turn may lead to the identification of new paradigms for a knowledge-based control and reprogramming of leukaemic cells.

2. ZUSAMMENFASSUNG

Neuere Erkenntnisse in der Tumorforschung belegen, dass die Mikroumgebung neben anderen Faktoren eine bedeutende Komponente im komplexen regulatorischen Netzwerk von Tumorzellen darstellt. Doch obwohl in den letzten Jahren große Fortschritte hinsichtlich des molekularen Verständnisses der Tumorzell-Regulation gemacht wurden, wurden die Interaktionen zwischen Tumor- und Stromazellen bislang nur unzureichend analysiert.

In früheren Untersuchungen stellten wir die Hypothese auf, dass die murine embryonale Mikroumgebung humane Tumorzellen zur Differenzierung anregen kann. Um diese Wechselwirkungen zu untersuchen, wurden humane AML-Zellen in murine Blastozysten injiziert und diese in scheinträchtige Ammentiere implantiert. Humane Zellen konnten sowohl in den sich entwickelnden Embryonen als auch in daraus hervorgegangenen adulten Tieren nachgewiesen werden. Außerdem exprimierten die Nachkommen der injizierten AML-Zellen hämatopoetische Differenzierungsmarker. Diese Beobachtungen unterstützen die Hypothese, dass die embryonale Mikroumgebung in der Lage ist, die Proliferation und Differenzierung humaner Leukämiezellen zu beeinflussen.

Aufbauend auf diesen Ergebnissen habe ich damit begonnen, die Stroma-vermittelten Veränderungen in humanen AML-Zellen hinsichtlich ihres Proliferations- und Differenzierungsverhaltens in einem *in vitro* Kokultur-System zu untersuchen. Hierzu wurden etablierte humane AML-Zelllinien mit verschiedenen murinen Stromazelllinien kokultiviert, die embryonalen hämatopoetisch aktiven Geweben (Dottersack, Aorta-Gonaden-Mesonephros-Region, foetale Leber) entstammen. Anschließend wurden verschiedene Parameter, wie Morphology, Histochemie, Immunphänotyp, Proliferationsrate und Expression bestimmter Gene, kokultivierter und nicht kokultivierter AML-Zellen verglichen.

Die Ergebnisse zeigen für Kasumi-1 Zellen, eine etablierte humane Leukämie-Zelllinie vom AML FAB-Typ M2, dass die Kokultur mit DAS 104-4, einer murinen Sromazelllinie, die der AGM-Region entstammt, eine Reduzierung der Proliferations- und Koloniebildungs-Fähigkeit hervorruft und sich die Morphologie der AML-Zellen in Richtung eines differenzierteren Zelltyps ändert.

Übereinstimmend damit können nach Kokultur die myelo-monozytären Differenzierungsmarker CD11b und CD14 auf der Oberfläche der Kasumi-1-Zellen nachgewiesen werden. Die Kokultur führte ebenfalls zu einer Zunahme des lysosomalen Markers CD68, der ebenfalls eine myeloide Differenzierung kennzeichnet. Bemerkenswert ist, dass DAS 104-4 Stromazellen in der Lage sind, myeloide Differenzierung auch in primären M2-AML-Zellen aus einem leukämischen Patienten zu induzieren. Außerdem wurde in KG-1 AML Zellen nach Kokultur mit DAS 104-4 eine Aktivierung der □-Globin-Transkription und eine verstärkte Glycophorin-A-Expression beobachtet, was auf eine Differenzierung der KG-1-Zellen in Richtung erythroide Linie hindeutet.
Untersuchungen zur Proliferationsfähigkeit von Kasumi-1-Zellen mittels CFSE-Retentions-Messungen ergaben, dass nach Kokultur mit DAS 104-4 - nicht aber mit NIH 3T3-Kontrollzellen - die Zellteilungsrate vermindert ist. Gleiches gilt für die Koloniebildungs-Kapazität in Methylzellulose-Kulturen. Zellzyklus-Analysen zeigen eine kokulturinduzierte Akkumulation der AML-Zellen im G_1-G_0 Stadium.

Genexpressionsanalysen mit Hilfe quantitativer RT-PCR verweisen auf eine deutlich herabgesetzte Transkription von AML1 und dem AML1-ETO-Fusionsgen, verbunden mit einem Anstieg der p16-, p21-, C/EBP□ und PU.1-Transkription. Interessanterweise ist die Abnahme von AML1-ETO Transkripten abhängig vom direkten Zellkontakt zwischen AML- und DAS 104-4-Zellen.

Wird die AML1-ETO-Expression nach Einsatz spezifischer siRNA herunter reguliert, führt dies zu einer verminderten Proliferation und zur Depletion koloniebildender Zellen innerhalb der Kasumi-1-Population. Außerdem bewirkt der siRNA-vermittelte *knockdown* von AML1-ETO eine höhere Empfänglichkeit der Kasumi-1-Zellen für die Stroma-induzierte myeloide Differenzierung. Die Verringerung von AML1-ETO Transkripten allein hat allerdings keinen differenzierenden Effekt. Diese Beobachtungen sprechen dafür, dass AML1-ETO zwar aktiv an der kokultur-vermittelten Reaktion beteiligt ist, dass aber zusätzliche Stimuli nötig sind, um myeloide Differenzierung in den AML-Zellen auszulösen.

Zusammenfassend lässt sich feststellen, dass in der vorliegenden Arbeit ein Kokultur-basiertes *in vitro* System entwickelt und charakterisiert wurde, das in der Lage ist, die Proliferationsfähigkeit von humanen AML-Zellen zu senken und ihre Differenzierung in die myeloide Linie zu induzieren. Aus den dargestellten Ergebnissen lässt sich schließen, dass das umgebende embryonale Stroma die Proliferation und Differenzierung leukämischer Zellen beeinflussen kann. Die zugrunde liegenden Mechanismen sind abhängig vom direkten Kontakt zwischen Stroma- und AML-Zellen. Eine CD44-Aktivierung konnte nicht beobachtet werden.

Weiterhin liefert die vorliegende Arbeit Hinweise darauf, dass die Verminderung der AML1-ETO-Transkription ein bedeutendes, jedoch nicht das allein auslösende, Ereignis der stroma-induzierten Differenzierung von Kasumi-1-Zellen darstellt. Die hier beschriebenen Resultate regen zu weiterführenden Untersuchungen an, die Aufschluss über zelluläre und molekulare Details der Interaktionen zwischen Leukämischen und Stromazellen geben sollen. Neue Erkenntnisse über die beteiligten Mechanismen könnten den Ansatz bieten, der es erlaubt, leukämische Zellen aktiv zu kontrollieren und zu reprogrammieren.

3 INTRODUCTION

3.1 Acute myeloid leukaemia – The disease

Acute Myeloid Leukaemia (AML) is a clonal myeloproliferative disease, characterized by an uncontrolled proliferation and block in differentiation of myeloid committed blood cells in the bone marrow (BM) (Jinnai, 1995). The diagnosis of AML is made when over 30% of myeloid blast cells are observed in the BM of the patient. The excessive amount of myeloid blast cells in the bone marrow competes and interferes with the normal haematopoiesis. This results in anemia, granulocytopenia and thrombocytopenia leading to symptoms such as fatigue, dizziness, bleeding and infections. AML occurs as a result of genetic changes often evident as cytogenetic changes, which can be detected in the majority of patient samples. The type of cytogenetic abnormality is considered the most powerful independent risk factor for outcome and the various genetic changes can be classified in a favourable, intermediate or poor risk groups (Grimwade *et al.*, 1998).

AML can also be classified according to its morphology using the French-American-British (FAB) classification (Table 1). Recently, a more clinical relevant classification has been proposed by the World Health Organization (WHO) (Table 2). This classification stratifies hematologic malignancies according to a combination of morphology, immunophenotype, genetic features and clinical syndromes (Harris *et al.*, 1999). According to the WHO classification, the diagnosis of AML is made when over 20% instead of 30% blast cells are present in the Bone marrow.

The incidence of AML is 2.4/100,000 people per year in the United States and it occurs mainly in elderly patients with a median age of onset of 63-65 years (Leith *et al.*, 1995). AML patients under 50 years of age, treated with curative intent chemotherapy, have complete remission (CR) rates that average 70% with a median relapse free survival (RFS) of nearly 2 years (Lowenberg *et al.*, 1999). In contrast, in the elderly AML patients, CR rates only average 50% with a median RFS in these patients of only 9 to 12 months (Bishop *et al.*, 1996; Mayer *et al.*, 1994; Rowe *et al.*, 1995; Taylor *et al.*, 1995). The poorer outcome of AML in the elderly patients may result from adverse cytogenetics, expression of multi-drug resistance proteins, reduced patient tolerance to chemotherapy or a combination of these factors (Leith *et al.*, 1995). However, despite age or biological differences, most patients eventually relapse and die of the disease.

Table 1. **FAB classification of AML depending on the maturation status of AML blast (Casasnovas *et al.*, 1998; Harris *et al.*, 1999)**

French American British (FAB) Classification	Morphology
M0	Acute Myeloblastic Leukaemia with minimal differentiation
M1	Acute Myeloblastic Leukaemia without maturation
M2	Acute Myeloblastic Leukaemia with maturation
M3	Acute Promyelocytic Leukaemia
M4	Acute Myelomonocytic Leukaemia
M4eo	Myelomonocytic Leukaemia with abnormal eosinophils
M5a	Acute Monoblastic Leukaemia
M5b	Acute Monocytic Leukaemia
M6	Acute Erythroleukaemia
M7	Acute Megakaryocytic leukaemia

Table 2. **WHO classification of AML cells, depending on morphology, immuno-phenotype, genetic features and clinical syndromes (Harris *et al.*, 1999)**

WHO classification	Definition
I	AML with recurrent cytogenetic translocations
II	AML with myelodyplasia-related features
III	Therapy-related AML and MDS
IV	AML not otherwise categorized

3.2 Acute Myeloid *leukaemia*: The biology

AML is characterized by an uncontrolled proliferation and accumulation of myeloid blast cells in the bone marrow, blood and other organs of patients. It is thought that the disease is sustained by a small subset of cells capable of extensive self-renewal and proliferation, which gives rise to the more mature myeloid blast cells (Blair *et al.*, 1998; Bonnet and Dick, 1997; Sutherland *et al.*, 1996). These cells, uniquely capable of sustaining the disease, are called AML stem cells.

The haematopoietic system is a complex and tightly regulated and hierarchically structured cell system, in which pluripotent stem cells undergo proliferation and differentiation to

produce mature blood cells of the various lineages while maintaining a compartment of uncommitted cells. As mentioned above, AML occurs as a result of genetic changes. Such changes occur in a primitive haematopoietic cell type that gives rise to the malignant clone. Despite the lack of mature cells derived from the leukaemic clone in the majority of AML patients, there is considerable heterogeneity among the leukaemic blasts even within individual patients, particularly with respect to their capacity to proliferate and/or self-renew (Blair *et al.*, 1998; Bonnet and Dick, 1997; Sutherland *et al.*, 1996). This suggests that subsequent to the transformation and prior to the maturation arrest some differentiation of AML blasts occurs, which creates a hierarchy of AML progenitors just as seen in normal haematopoiesis (Figure 1).

Over the years, several *in vitro* assays have been developed to detect normal haematopoietic progenitors. Lineage committed progenitor cells can be detected *in vitro* by the colony forming cell assay (CFU assay), in which haematopoietic cells are plated in semi-solid media complemented with growth factors (GF) (Eaves CJ, 1999; Sutherland H.J., 1991). More primitive progenitors can be assayed in the long-term culture assay (LTC) (Hogge *et al.*, 1996). In this assay, cells are cultured for 5 to 8 weeks on murine stromal feeders producing human GFs and after 5 to 8 weeks, the cells are harvested and plated in semi-solid media upon which the LTC-initiating cell (LTC-IC) content can be determined. It is thought that the most primitive pluripotent stem cells can only be detected *in vivo* by multi-lineage engraftment in the non-obese diabetic severe combined immunodeficient (NOD/SCID) mice.

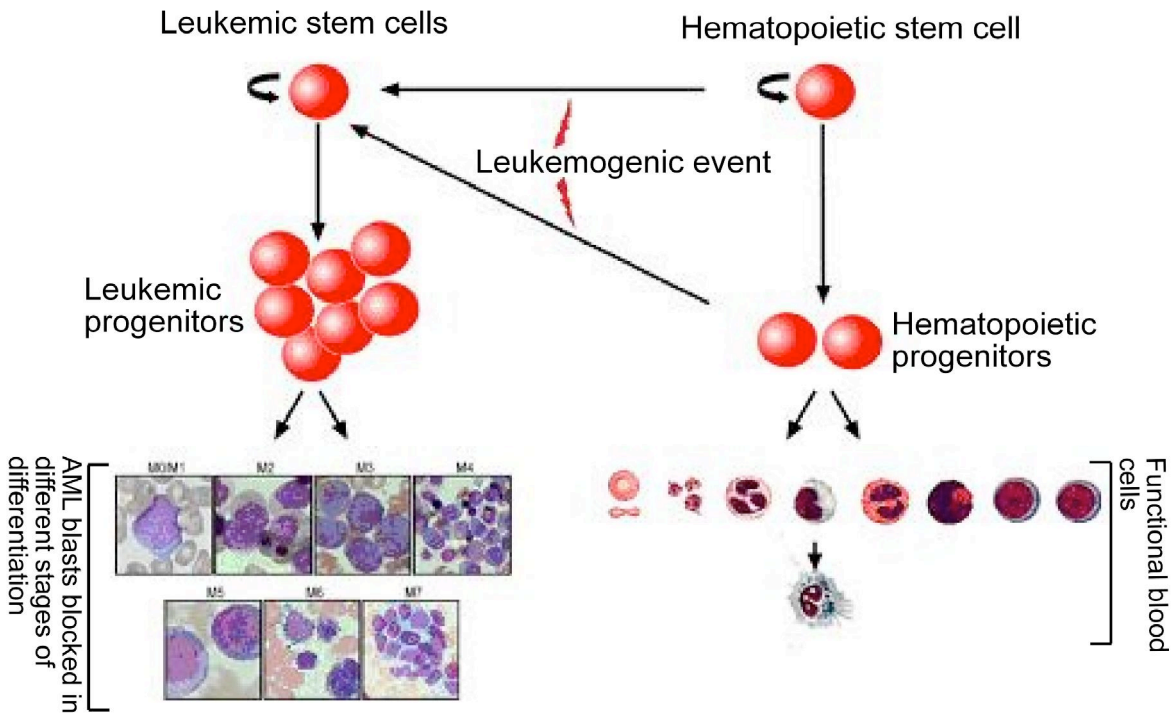

Figure 1. Presently accepted leukaemic stem cell model, which states that AML is a stem cell disease and can arise from normal haematopoietic stem cells or progenitor cells. These leukaemic stem cells give rise to leukaemic progenitors, which in turn lead to AML blast cells and depending on cytogenetics, they get blocked in different stages of differentiation (Bonnet and Dick, 1997; Dick, 2005; Dick and Lapidot, 2005; Hope *et al.*, 2003; Hope *et al.*, 2004). Figure is adapted from Bonnet *et al.*, modified by Michael Dürr and eventually modified by me.

3.2.1 Acute myeloid *leukaemia* and translocation t(8;21)

AML is a clonal disorder resulting from an acquired somatic mutation in haematopoietic progenitor cells and it is characterized by an accumulation of immature myeloid cells in the bone marrow, which are arrested in different stages of maturation. Frequently, in AML, the acquired mutation is the consequence of a balanced reciprocal chromosomal translocation, which disrupts genes residing in the breakpoint regions of the translocation by generating fusion genes (Look, 1997; Lowenberg *et al.*, 1999). The genes located at these breakpoints often encode transcription factors, which are master regulators of haematopoietic cell differentiation, apoptosis or proliferation (Tenen *et al.*, 1997).

Despite the heterogeneity of AML, the identification of recurring chromosomal rearrangements and the resulting molecular abnormalities have identified distinct subgroups of patients with predictable clinical features and therapeutic responses (Downing, 1999). The most frequent cytogenetic abnormalities in AML, accounting for approximately 40% of all AMLs, are the translocations t(8;21), t(15;17), inv(16) and t(9;11), encoding for the AML1-ETO, PML-RARα, CBFβ-SMMHC and MLL-AF9 fusion proteins, respectively (Look, 1997).

AML1-ETO is associated with approximately 40% of AML cases with the M2 subtype according to the French-American-British (FAB) classification and represents one of the most frequent chromosomal translocation in AML (18-20%) (Look, 1997). t(8;21) is a balanced translocation between chromosomes 8 and 21 resulting at the molecular level in the fusion of the AML1 gene normally located on chromosome 21q22 with the ETO (Eight-Twenty-One) gene on chromosome 8q22 (Erickson *et al.*, 1992; Nucifora *et al.*, 1993). The wild type AML1 gene encodes for the CBFβ2 protein, which is a physiological component of the core binding factor (CBF) family (Chang *et al.*, 1993). AML1 appears to function as a transcriptional organizer necessary for the development of definitive haematopoietic stem cells (Carey, 1998). In fact, CBF null mice die *in utero* in the absence of terminal haematopoietic differentiation (Okuda *et al.*, 1998; Speck *et al.*, 1999; Wang *et al.*, 1996). Transcriptional function of AML1 is mediated through the core enhancer DNA sequence present in promoters and enhancers of a large number of haematopoietic specific genes (Mayer *et al.*, 1994). AML1 binds this sequence through the runt homology domain and its DNA binding affinity is increased by heterodimerization with CBFβ (Ogawa, 1993). Finally, AML1 has been shown to co-operate with other transcription factors in activating a set of haematopoietic specific genes (Petrovick *et al.*, 1998). ETO was unknown prior to its identification as the fusion partner of AML1 in t(8;21) (Erickson *et al.*, 1992; Miyoshi *et al.*, 1993; Nisson *et al.*, 1992). ETO is expressed as a nuclear phosphoprotein in brain and in $CD34^+$ haematopoietic cells (Erickson *et al.*, 1996). It contains four evolutionarily conserved regions, the so-called nervy homology regions (NHR) 1-4, which have been shown to make several contacts with co-repressors and histone deacetylases (Amann *et al.*, 2001). Although ETO is a nuclear zinc-finger containing protein and thus might function as a transcriptional regulator, there is no experimental evidence to suggest that it can directly bind to DNA. In the translocation t(8;21), the DNA binding domain (runt domain) of AML1 is fused to nearly the complete ETO gene yielding a protein of 752 amino acids (Erickson *et al.*, 1992; Miyoshi *et al.*, 1991; Nisson *et al.*, 1992). Several important regions of AML1 are lost, like the C-terminal transactivation domain, interaction sites for Groucho/TLE family of transcriptional co-repressors and sin3 co-repressors, the nuclear targeting signal and a MAP kinase phosphorylation site (Licht, 2001). AML1-ETO sequesters the co-activator CBFβ from wild type AML1 by binding CBFβ through the retained runt domain more efficiently (Tanaka *et al.*, 1998). Thus, AML1-ETO can bind to AML1 target genes instead of wild type AML1 (Meyers *et al.*, 1993), and dominantly represses transcription of AML1 target genes by permanently tethering a repressor complex to AML1 responsive promoters through ETO (Gelmetti *et al.*, 1998; Lutterbach *et al.*, 1998; Wang *et al.*, 1998). The importance of disruption of wild type AML1 by AML1-ETO is shown by a similar phenotype in AML1 knock-out and AML1-ETO knock-in studies (Okuda *et al.*, 1998; Yergeau *et al.*, 1997). AML1-ETO blocks the

transactivation of wild type AML1 target genes important for myeloid differentiation, like the GM-CSF, c-fos (Harada *et al.*, 2001; Hwang *et al.*, 1999) or the TGFβ signalling pathway (Jakubowiak *et al.*, 2000). Furthermore, AML1-ETO disrupts the protein-protein interactions of AML1 with important myeloid transcription factors like C/EBPα (Pabst *et al.*, 2001; Westendorf *et al.*, 1998) or MEF-2 (Mao *et al.*, 1999) and also represses transactivation through those transcription factors. Therefore, AML1-ETO might be responsible for the differentiation arrest in t(8;21). Along with that, several cellular and murine model systems have documented the ability of AML1-ETO to inhibit myeloid differentiation (Ahn *et al.*, 1998; Burel *et al.*, 2001; Kitabayashi *et al.*, 1998; Kohzaki *et al.*, 1999; Tanaka *et al.*, 1995).

It has recently been shown that AML1-ETO might also be responsible for the gain-of-function properties characterizing leukemogenesis. Expression of AML1-ETO in primary human $CD34^+$ stem cells, the target cell affected in AML, results in a proliferative and survival advantage of leukaemic cells, which promotes their expansion (Mulloy *et al.*, 2002). Introduction of the AML1-ETO cDNA into the AML1 locus by homologous recombination leads to embryonic lethality in heterozygous animals (Ohneda *et al.*, 1998; Yergeau *et al.*, 1997). Culturing yolk sac cells of these mice, however, yields dysplastic monocytic colonies (Yergeau *et al.*, 1997). In a similar AML1-ETO knock-in mouse study, aberrant myelomonocytic colonies are derived from foetal liver (Ohneda *et al.*, 1998).

In summary, despite the fact that AML1-ETO alone has not been shown to cause leukaemia (Rhoades *et al.*, 2000; Yuan *et al.*, 2001), these studies indicate that AML1-ETO encourages uncontrolled cell growth and might predispose to leukaemia.

3.3 Cell cycle regulatory proteins and their role in cell proliferation and cell cycle arrest

It is a universal process by which cells divide and participate to the growth and development of organisms. G_1 phase is tightly regulated to coordinate normal cell division with cell growth, whereas replication of DNA during S phase is precisely ordered to prevent inadequate events that will lead to genomic instability and cancer. The cell cycle machinery, as such, is a highly coordinated process in which cyclins, cyclin-dependent kinases (CDKs) and their inhibitors (CDKIs) are differentially regulated (Gitig and Koff, 2000; Koepp *et al.*, 1999; Matsushime *et al.*, 1990; Muller *et al.*, 1993; Sherr and Roberts, 2004). Each cyclin can associate with one or more of the CDK family and successive ways of cyclin/CDKs

complexes drive cells through the cell cycle, acting in G_1 to initiate S phase and in G_2 to initiate mitosis. While levels of CDKs remain constant through the cell cycle, expression of cyclins varies following periodic transcriptional or posttranscriptional regulations, so that each cyclin has a unique pattern of expression during the cell cycle. Since cyclin abundance is rate limiting, the different CDKs get activated upon binding to their specific cyclin partner provided that these subunits are available. During the G_1 phase of the cell cycle, two classes of cyclins get successively activated: D type cyclins (cyclins D1, D2 and D3) (Steinman, 2002) and cyclin E (cyclins E1 and E2) (Koepp *et al.*, 1999). These cyclins associate with their respective partners, CDK4 or CDK6 for cyclin D and CDK2 for cyclin E to induce their kinase activities (Bates *et al.*, 1994; Meyerson and Harlow, 1994). Activated CDK4/CDK6 and CDK2 are required for phosphorylation of the retino-blastoma protein (pRb), an event that leads to the release of Rb-E2F repressor complex and hence, to the induction of E2F-dependent genes and cell cycle progression (Bates *et al.*, 1994). Cyclins D and CDK4/6 are responsible for the first phosphorylation of pRb, while cyclin E/CDK2 operates on both the second pRb phosphorylation and the control of S phase entry. The activity of G_1 cyclin-CDK complexes is regulated, at least in part, by CDKIs, among which two members, p21 and p16 play specific roles. Evidence is accumulating that CDKIs are targets of extracellular and intracellular signals that regulate cell growth and differentiation (Harper *et al.*, 1993; Nead *et al.*, 1998; Nishitani *et al.*, 1999; Steinman *et al.*, 1994). The p21 inhibitor is known to be triggered by anti-proliferative and differentiation signals and as a mediator of p53-induced cell cycle arrest after DNA damage (Steinman *et al.*, 1998).

3.4 Response of AML cells to differentiation-inducing agents

AML is a disease that is characterized by a block in the normal process of myeloid differentiation, thereby leading to the accumulation of immature cells termed blasts (Lowenberg *et al.*, 1999; Tenen *et al.*, 1997). The abnormal maturation in AML cells could result from disruption of the function of transcription factors, cytokine receptors and the cell cycle. In other words, leukaemic transformation might involve abnormalities of the transcription factors that normally regulate myeloid development in a stepwise fashion.

AML treatment is based on intensive chemotherapy administered based on: a) induction treatment that aims to bring the patient into complete hematological remission, and b) treatment in remission that aims to eradicate residual disease and prevent AML relapse

(Lowenberg *et al.*, 1999). Treatment in remission with intensive chemotherapy alone or in combination with stem cell transplantation is associated with a relatively high mortality (Bruserud *et al.*, 2000; Lowenberg *et al.*, 1999). The use of a less aggressive therapy is therefore, highly desirable. One potential approach might be the induction of differentiation of leukaemic blasts turning them into non-dividing end cells.

In an experimental setting, AML cells can be induced to differentiate with a variety of agents. In 1978, Sachs and co-workers demonstrated that mouse cells undergo differentiation in the presence of IL6 (Sachs, 1978). Later it was reported that the vitamin A metabolite, retinoic acid (RA), could induce differentiation in the betty-60 cell line (Breitman *et al.*, 1980), with the effect mediated through the retinoic acid receptor (RAR) (Collins *et al.*, 1990). The application of differentiation therapy with All Trans Retinoic Acid (ATRA) is now regarded as the choice for treatment of M3-AML (He *et al.*, 1999; Kogan and Bishop, 1999). There are currently a large number of prototypes and second-generation agents that are capable of inducing differentiation in either myeloid or lymphoid cell lines (Hozumi, 1998; Tallman, 1996), many of which have been used in clinical situations, albeit in only few cases, often this is in combination with other factors (Table 3a & 3b).

Table 3a. **Differentiation inducers of AML**

Direction of differentiation	Soluble mediators	Detection of differentiation in native AML blasts
Neutrophil	SCF or IL-3	Induction of CD15 expression and promyelocyte-myelocyte morphology in $CD34^+$ AML-M1/M2 blasts (Matsushima *et al.*, 2001).
	IL-3, G-CSF or GM-CSF	Differentiation into myelocyte- and metamyelocyte-like leukaemic cells with disappearance of CD34 and HLA-DR expression for a subset of patients (Ikeda *et al.*, 1993).
	SCF	Enhanced differentiation when G-CSF was combined with retinoic acid decreased colony formation in clonogenic assay; these effects were caused by single agents and/or by combinations of mediators.(Ferrero *et al.*, 1992; Santini *et al.*, 1991)
	IFN- , Vit-D3, or Retinoic acid	Increased proportions of mature granulocytes for some patients (Greenberg *et al.*, 1990).
Eosinophil	IL-5	Induction of either pure or mixed leukaemic eosinophilic colonies, no correlation with FAB classification IL-5(Ema *et al.*, 1990; Motoji *et al.*, 1990; Motoji *et al.*, 1995).
Basophil	SCF	Differentiation into cells with segmented nuclei and basophilic/metachromatic granules for a small minority of patients (Matsushima *et al.*, 2001).
	IFN-γ, TNF-α	Increased membrane expression of the monocyte marker CD14 in subsets of patients; effects were caused by single agents or combinations of mediators (Howell *et al.*, 1990).
Monocyte	Vit-D3, or retinoic acid TNF-α	Induction of monocytic morphology with increased phagocytic capacity and expression of CD11b and CD14 (Masciulli *et al.*, 1995).
	IL-3, GM-CSF, G-CSF, or M-CSF	Induction of a macrophage-like morphology and expression of CD13, CD14, and HLA-class II in a minority of patients (Salem *et al.*, 1989).
	SCF, Leukaemia inhibitory factor	Monocyte differentiation in the M1 AML cell line (Brasel *et al.*, 1995).
Megakaryocyte	Thrombopoietin + IL-3 or SCF	Increased expression of platelet-specific antigens in the M-O7e AML cell line (Briggs *et al.*, 2001).
Erythroid	Erythropoietin	Further erythroid differentiation for patients with erythroleukaemia (Salem *et al.*, 1989).

Table 3b. **Candidate drugs for differentiation induction (Tallman, 1996)**

Cytotoxic drugs	Cytarabine, daunorubicin, 6-thioguanine
	Combinations of cytosine arabinoside thioguanine plus retinoic acid
	Hexamethylene or dimethylformamide
Altered Histone Acetylation	Histone deacetylase inhibitors
	Butyrates
	Monosaccharide butyrate derivatives
Methylprednisolone	High-Dose Methylprednisolone
Metal Chelators	Dithizone
ATRA and Vitamin D3 Analogs	ATRA and Vitamin D3 Analogs

3.4.1 Targeting AML with antibodies

Going further ahead in differentiation induction, antibody-based therapy for acute leukaemia has evolved as a possible means of decreasing both relapse rates and mortality (Ruffner and Matthews, 2000). Over the past 25 years, monoclonal antibodies have been evaluated as anti-leukaemic therapy both in unmodified forms and as immuno-conjugates labelled with either radioactive or cytotoxic moieties. For example, anti-GM-CSF antibody (Bouabdallah *et al.*, 1998), humanized anti-CD33 antibody (HuM-195) (Caron *et al.*, 1998) or ^{131}I-labelled anti-CD33 (p67) (Sievers *et al.*, 1999) and anti-CD45 antibody (Sievers, 2000). Most monoclonal antibody targeting approaches have been directed against normal haematopoietic cell surface antigens that are also expressed by leukaemic blast cells (Sievers, 2000).

Recently, it has been shown that the ligation of the CD44 surface antigen by specific anti-CD44 monoclonal antibodies or with its natural ligand, hyaluronan, can induce myeloid differentiation in AML M1 to AML M5 subtypes (Charrad *et al.*, 1999). It was reported previously that one can induce differentiation upon CD44 ligation with the anti-CD44 antibody, A3D8, in the AML cell lines HL60, U937, THp-1, KG-1a and NB4 (Charrad *et al.*, 1999; Zada *et al.*, 2003). Furthermore, it was shown that *in vivo* administration of CD44 activating antibody to leukaemia progenitor-transplanted NOD-SCID mouse, showed significantly less leukaemic repopulation (Jin *et al.*, 2006). This shows a new development for targeted differentiation therapy in AML.

3.5 Tumour cells and microenvironment background

Based on observations from early last century that some cancer types are prevalent in certain families, researchers began to look for genetic alterations that might underlie cancer pathogenesis. Over the following decades, enormous advances were made in identifying the molecular determinants of tumourigenesis. Tumour development became recognized as a multi-step process during which cancer cells accumulate multiple and consecutive genetic alterations (Mueller and Fusenig, 2004). These cancer-, cell-, and genome-centred models have led to the identification and characterization of many oncogenes and tumour-suppressor genes, which in turn yielded detailed information on the molecular basis of tumour development. However, they have largely ignored the heterogeneous and structurally complex nature of the tissue, or "organ", called the tumour. The English surgeon Stephen Paget back in 1889, compared tumour cells with the seed of plants, in that they are both "carried in all directions; but they can only live and grow if they fall on congenial soil". Similarly, he argued that metastatic cells must thrive only where conditions are in some way favourable. The molecular determinants of the "seed" are still much better understood than those of the "soil". It is only recently that tumour progression has been recognized as the product of an evolving crosstalk between different cell types within the tumour and its surrounding supporting tissue or tumour microenvironment. Over the past decade, it has become increasingly apparent that the complex interplay between different cell types and the microenvironment is critical for maintaining normal balanced tissue homeostasis. As a consequence, in tumour tissue, the microenvironment will also profoundly influence many steps of tumour development and progression.

In vivo, the tumour is a complex ecosystem comprising the genetically altered neoplastic cells and the tumour stroma, a framework of different connective tissue cells with extracellular matrix (ECM) and the embedded vasculature. Although changes in the cellularity and ECM composition of the tumour stroma had been previously noticed (Wills, 1967), this connective tissue component of epithelial malignancies was considered mainly as a passive supporting and nourishing system for the cancer cells. Concomitantly with the increasing interest in tumour angiogenesis and the recognition of its essential role for tumour progression and as a new target for tumour therapy, a new view of the significance of the stromal compartment in tumour biology has emerged. With the recognition of the fact that tumours are composed of many cell types and cannot exist in isolation, increasing experimental evidence emphasized the importance of the microenvironment for tumour development and progression. These data indicate that the genetic alterations in the tumour cells themselves are not sufficient to generate a malignant tumour but that a permissive

stromal environment is needed as well. Comparison of the connective tissue of normal organs, which is able to maintain normal tissue homeostasis, with the tumour stroma revealed a disrupted balance in the epithelial-stromal interactions in both composition of the ECM and the functional state of the stromal cells (Coussens *et al.*, 2001; Tlsty, 2001; van den Hooff, 1988). These alterations seem, indeed, to be crucial for tumour growth, invasion, and metastasis (Fidler, 1990; Morikawa *et al.*, 1988).

Recent *in vitro* studies indicate that both cell composition and functional abnormalities of the haematopoietic microenvironment are present in a proportion of patients with myeloid leukaemia, both chronic myeloid leukaemia (CML) and acute myeloid leukaemia. Cell composition abnormalities have been primarily observed in a subset of patients with AML; these abnormalities include reduced numbers of fibroblast progenitors and, in some cases, reduced numbers of macrophages and adipocytes. In terms of function, it has been shown that the marrow stromal cells, from a significant number of both CML and AML patients possess a deficient haematopoietic supportive capacity *in vitro*. However, it is not clear whether the abnormalities in the haematopoietic microenvironment are secondary to myeloid leukaemia or if they precede clinical CML/AML.

Complex interactions between interspecies stroma and cancer have been reported in the context of human AML cell lines. Upon cocultivation, murine stromal MS-5 cells prevent serum deprivation-induced apoptosis and inhibit ara-C-induced apoptosis in HL-60 cells. This effect was shown to be due to murine stroma-induced upregulation of BCL2 expression in the human AML cell line (Konopleva *et al.*, 2002).

3.5.1 Reprogramming of tumour in embryonic environment

Brinster *et al.,* (1976) reported that testicular teratocarcinoma cells transplanted into blastocysts can survive and contribute to the resulting embryos without leading to carcinogenesis (Brinster, 1974). Illmensee *et al.,* (1976), further showed loss of malignant phenotype and germline transmission of blastocyst injected with teratocarcinoma cells (Illmensee and Mintz, 1976). The control of tumour formation is also highlighted by the observation that human mestastatic melanoma cells engrafted into the chick neural crest region acquire neural-crest-cell-like morphologies and populate into host peripheral structures (Kulesa *et al.*, 2006). In addition, work form our lab showed that the murine embryonic microenvironment can induce the differentiation of human AML cells following blastocysts injection. Microinjection of human KG-1 myeloid leukaemia cells and primary

human acute myeloid leukaemia cells led to the generation of chimaeric embryos and adults without signs of tumour formation. Furthermore, analysis of chimaeric E12.5 embryos revealed that the progeny of human KG-1 cells activated erythroid-specific human globin and glycophorin A (CD235a) expression (Durr *et al.*, 2003). In summary, the available data suggest that the embryonic microenvironment can reprogram human leukaemia cells to differentiate.

3.6 Sites of embryonic and adult haematopoiesis and haematopoietic stroma

The different cell lines used in this study are derived from different haematopoietic regions of embryos and adult mice. Therefore, the origin of the haematopoietic system is briefly discussed. Blood development in vertebrates occurs in two phases: The embryonic ("primitive") phase and the definitive ("adult") phase (Davidson and Zon, 2004) (Figure 2). These phases differ in the sites of blood cell production, also in the morphology of the produced cells and in the types of expressed genes. During the embryonic phase of haematopoiesis, the embryo is transiently provided with primitive blood cell types from the extra-embryonic yolk sac. During the definitive phase of haematopoiesis, the embryo is supplied with blood cells that originate from the intra-embryonic aorta-gonad-mesonephros (AGM) region. These definitive-type hematopoetic stem cells (HSCs) last for the entire lifetime. At each stage of development, different subsets of mature haematopoietic cells are generated. Definitive erythroid cells are small and enucleated in adult organisms, while primitive erythroid cells produced during embryonic yolk sac haematopoiesis are large and nucleated. Specific types of B cells and T cells are produced by progenitors in the foetal liver (Bonifer *et al.*, 1998; Dzierzak *et al.*, 1998). There are differences between embryonic/foetal and adult-type macrophages (Faust *et al.*, 1997). The first extra-embryonic sites of haematopoiesis, where primitive erythrocytes are generated in E7.5 embryos, are blood islands of the yolk sac. Blood islands of the yolk sac are in close associations with endothelial and haematopoietic cells, which led to the proposal of a common progenitor cell, for both cell types, the haemangioblast (Choi, 2002). At the beginning of E7, granulocyte-macrophage progenitors and at E8.5, T- and B-lymphoid progenitors can be detected in the yolk sac (Cumano *et al.*, 1993; Liu and Auerbach, 1991). The cells from intra-embryonic para-aortic splanchno-pleura seed the blood system of the embryo and the yolk sac at E8.5. Only short-term repopulating haematopoietic progenitors are generated in the yolk sac. At mid-E9 colony forming unit-spleen cells (CFU-S) are first detected in the AGM region and shortly after, at E10.5, HSCs that can repopulate the entire haematopoietic system are produced in the AGM region (Muller *et al.*, 1994). The first CFU-S progenitors appear in the

dorsal aorta gonads-mesonephros (AGM) region at E9 (Medvinsky and Dzierzak, 1996) and the first long-term repopulating haematopoietic cells are detected at late E10 (Medvinsky and Dzierzak, 1996; Muller *et al.*, 1994). Thus, the first definitive HSCs are produced in the AGM.

The rudiment of the liver is formed at E9 from the evagination of the gut into *septum transfersum*. The liver does not generate its own haematopoietic cells but it is seeded from the yolk sac and AGM region at this stage of development. At E9, the liver contains erythroblasts and at E10, it starts to produce differentiated erythroid cells (Dzierzak and Medvinsky, 1995). At mid-E9, myeloid progenitors appear, at E10, T-cell progenitors and at E10-11, granulocyte-macrophage and B-lineage cells appear (Velardi and Cooper, 1984). At late E10-early E11 HSCs and CFU-S progenitors are detected in the developed foetal liver (Medvinsky and Dzierzak, 1996).

Haematopoietic progenitors and stem cells in the adult mouse are found in bone marrow and spleen. The differentiated cells move quickly through the circulation to the other tissues and organs. Growth factors induce the efficient mobilization of haematopoietic precursors and stem cells into the blood (Bodine, 1995). Colonization of bone marrow with HSCs is believed to take place around E16 of gestation (Ogawa *et al.*, 1988). According to Cumano *et al.* (1996), at E15, prior to bone formation, haematopoietic progenitors have already accumulated in bone marrow, but active B lymphopoiesis does not appear before E17(Cumano *et al.*, 1996). With this background information we decided to use murine embryonic haematopoietic stromal cell lines derived from yolk sac such as yolk sac endodermal (YSE) and yolk sac mesodermal (YSM) (Yoder *et al.*, 1995) AGM-derived DAS 104-4 and DAS 104-8 (Ohneda *et al.*, 1998) and AFT 024 (Moore *et al.*, 1997) derived from foetal liver. Moreover, we took NIH 3T3 as a non-haematopoietic cell line. The specific characteristics of these cell lines play an important role in their capacity to contribute to the organs of developing embryos. These characteristics are described in detail below.

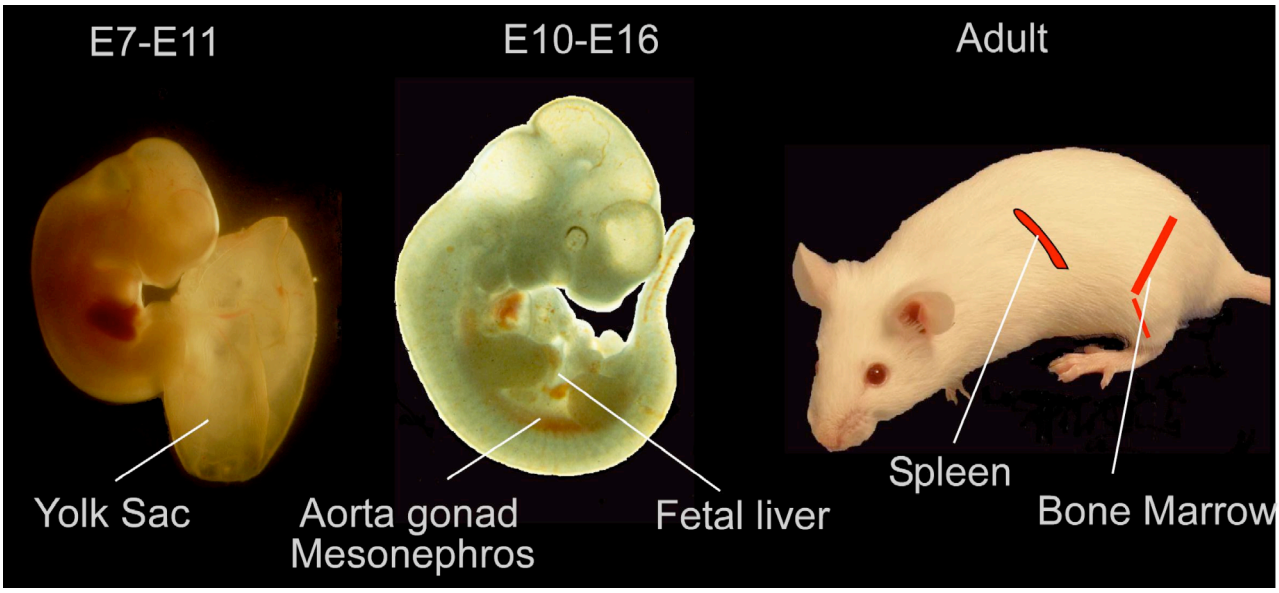

Figure 2. Development of the haematopoietic system in yolk sac, AGM and bone marrow leading to the generation of primitive and definitive haematopoiesis in mouse. In this study, many well described stroma cell lines derived from specific haematopoietic regions were used (See chapter 3.6.1-3).

3.6.1 Dorsal aorta stromal (DAS) 104-4 and DAS 104-8 cell lines

These two cell lines are of endothelial origin. They were isolated from the murine AGM region as supportive cell lines for haematopoietic progenitors and HSCs (Ohneda *et al.*, 1998). To obtain endothelial cell lines, the AGM region of E11 murine embryo was isolated by dissection and cells were transformed using polyoma virus middle T- expressing retroviruses. This oncogene transforms endothelial cells and maintains them in an endothelial-like state (Bautch *et al.*, 1987; Williams *et al.*, 1989) Two morphologically similar $CD34^+$ adherent cell lines DAS 104-4 and DAS 104-8 were selected.

Analysis revealed that DAS 104-8 efficiently induced foetal liver HSCs to differentiate down erythroid, myeloid and B-lymphoid pathways, but did not mediate self-renewal of pluripotent cells. The DAS 104-4 cell line was inefficient at the induction of haematopoietic differentiation, but also provoked expansion of early haematopoietic progenitor cells and was proficient at maintaining foetal liver-derived HSCs (Ohneda *et al.*, 1998). The morphology of these cell lines shows typical flattened, endothelial-like structure. They express a number of endothelial markers: CD34, FLT1, FLK1 (both are VEGF receptors), vWF and CD31 (platelet endothelial cell adhesion marker- PECAM). These cells produce the endothelial growth factor, VEGF, which may allow autocrine-mediated proliferation of these cell lines upon binding to the FLT1 and FLK1. The formation of capillary-like structures, when these cells are grown in Matrigel also supports the fact that they are endothelial cells (Ohneda *et al.*, 1998). Furthermore, DAS

104-4 was shown to be efficient in maintaining haematopoietic stem cells whereas DAS 104-8 promotes differentiation of haematopoietic stem cells (Ohneda *et al.*, 1998).

3.6.2 Yolk sac endodermal (YSE) and Yolk sac mesodermal (YSM) cell lines

These cell lines were established from the visceral endoderm and mesoderm of the yolk sac to better define the role of the murine yolk sac in differentiation and proliferation of the haematopoietic cells (Yoder *et al.*, 1994). Yolk sacs from E9.5 murine embryos were isolated and separated endoderm and mesoderm layers were cultured. Adherent cells were infected with a recombinant retrovirus encoding SV40 large T antigen and permanent lines from yolk sac endoderm and mesoderm were established. The morphologies of the cell lines were similar to freshly isolated visceral cells. They showed polygonal morphology with formation of tight cell clusters. None of these cell lines were contact inhibited, and none showed anchorage-independent growth in soft agar. Polarized morphology, tight junctions, cell surface apical microvilli, coated pits and cytoplasmic coated vesicles were identified in these cells, which is characteristic of endocytically active yolk sac endoderm cells. Markers expressed on these cells are the extracellular matrix proteins - fibronectin, collagen IV, laminin, Endo-B cytokeratin, zonulla occludens-1 and GATA4. They do not express macrophage cell surface molecules Mac-1, F4/80, vimentin intermediate filaments, H513E3 marker for stromal endothelial cells and one of the major secreted proteins in visceral yolk sac - α fetoprotein. Both YSE and YSM cell lines supported expansion of the BM cells depleted of committed progenitors by 5-FU treatment. However, YSE was proven to be more efficient in such maintenance activity than YSM (Yoder *et al.*, 1995). This suggests that these cell lines recapitulate microenvironmental influences that exist in normal yolk sac (Yoder *et al.*, 1994). Moreover, these cell lines significantly stimulate the proliferation of adult murine bone marrow high proliferative potential colony forming cells (HPP-CFC) in coculture (Yoder *et al.*, 1995).

3.6.3 The foetal liver AFT 024 cell line

Cell line AFT 024 was derived from the foetal liver of a 14 to 14.5 day gestation mouse embryo (Moore *et al.*, 1997). This cell line is immortalized with temperature sensitive SV40 T antigen. They represent cells obtained from the microenvironment of the liver that support purified mouse and human $CD34^+$ $CD38^-$ haematopoietic stem/progenitor cells. Delta-like (Dlk1)/preadipocyte factor-1 (pref-1) is preferentially expressed in AFT 024 cells and

functions as a positive stem cell regulator (Moore *et al.*, 1997). This cell line is widely used as stroma for *ex vivo* expansion of both human and murine haematopoietic precursor cells.

3.7 Experimental strategy

The microenvironment, in which a cell resides, may determine its fate. Here, I analyzed the consequences of stroma environment-induced changes in human AML cells upon *in vitro* coculture with murine embryo-derived haematopoietic stromal cells (Figure 3).

In order to answer the question whether the stromal cells from mouse embryonic haematopoietic sites can reprogram human AML cells, the AML cells were cocultivated with stromal cells derived from murine foetal yolk sac, foetal liver or AGM region and any change in terms of immunophenotype was measured by immunostaining for myeloid specific markers. To determine whether the coculture-mediated differentiation of AML cells is accompanied by any coculture-induced change in cell cycle status and proliferation properties, the cell cycle status was analyzed and CFSE retention assay was done to analyze cell proliferation rate. Further, I addressed the question whether the coculture with stroma has any effect on colony forming cell pool of AML cells. By direct cocultivation of AML cells on feeder cells followed by re-seeding cocultured AML cells on semi-solid medium and scoring for the arising colonies, the effect of coculture on CFCs population was analyzed. To understand if the coculture-induced differentiation and change in proliferation need cell-cell contact between the feeder cells and AML cells, AML cells and feeder cells were indirectly cocultivated separated by membrane barrier and were analyzed for the coculture-induced changes. Furthermore, to look for molecular details and possible clue of the process responsible for the coculture-induced effect, I analyzed coculture-induced change in the expression profile of crucial myeloid transcription factors like RUNX1, PU.1 and C/EBPα by real time quantitative RT-PCR. Transcript levels of AML1-ETO oncogene and cell cycle regulator were also analyzed by real time RT-PCR. To understand the role of AML1-ETO transcript level down-regulation in coculture mediated effect on Kasumi-1 cells, AML1-ETO expression was knocked down using siRNA. Finally, to understand if the down-regulation of AML1-ETO have any role in coculture induced differentiation, I have further analyzed the effect of AML1-ETO knock down on colony formation efficiency, cell proliferation and AML cell differentiation together with the coculture.

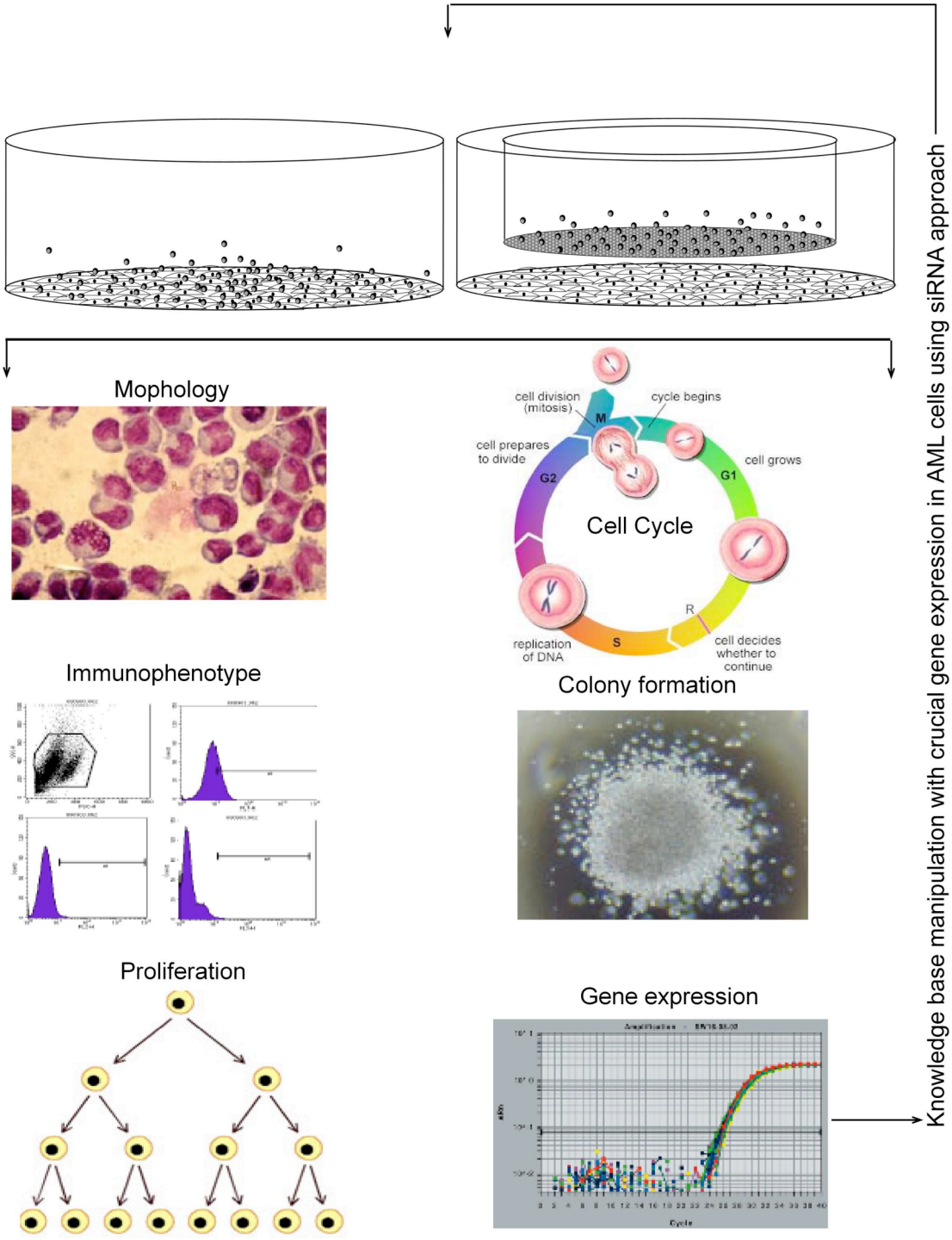

Figure 3. Experimental layout of the project, which includes direct and indirect cocultivation of human AML cell lines on murine stromal cells. Coculture-induced changes were measured in terms of morphology, immunochemistry, immunophenotype, proliferation properties, cell cycle status, fraction of colony forming cells in the population, and transcription status of some crucial genes. Furthermore, information-based manipulation of AML1-ETO gene expression by siRNA method was used to decipher the possible molecular players involved in stroma-induced reprogramming of human AML cells.

4 RESULTS

4.1 Myeloid marker expression following coculture of human AML cell line, Kasumi-1, on murine stromal cell lines

Based on the previous studies in our laboratory, where AML cells were subjected to murine embryonic microenvironment by injecting into the day 3.5 murine blastocysts, and analysis of resulting embryos showed low level Chimaerism. However engrafted donor AML cells revealed no signs of leukaemia in mice derived from the recipient blastocyst. Furthermore, analyses of the donor AML cells in the embryo showed characteristics of erythroid differentiation (Durr *et al.*, 2003). These findings lead to the hypothesis that the microenvironment of embryonic haematopoietic sites can affect and alter the nature of the AML cells and in turn it is possible that microenvironmental cues from the stromal cells derived from active embryonic haematopoietic sites can affect differentiation and proliferation properties of AML cells. Therefore, AML cell lines were cocultivated on characterized stromal cell lines originating from active embryonic haematopoietic sites such as yolk sac, foetal liver and the AGM region. Following coculture, the stroma coculture-induced changes in the AML cells were analyzed. Figure 4 shows effect of coculture on Kasumi-1 cells (Asou *et al.*, 1991), a cell line derived from an AML1-ETO positive AML-M2 patient. Kasumi-1 cells were cocultured by seeding onto adherent and irradiated murine AGM region-derived DAS 104-4 cells (Ohneda *et al.*, 1998). After 6 days of direct coculture, cocultured Kasumi-1 cells were washed off the stromal cell layer, cytospun and examined for the appearance of differentiation markers by morphological and immunocytochemical analyses. Firstly, the morphology was analyzed by Pappenheim staining. Pappenheim staining gives the morphological details. The cytoplasmic granularity and the general morphology indicated myeloid maturation of the cocultured Kasumi-1 cells. In addition, staining for cytoplasmic CD68, which represents a lysosomal marker and was earlier reported to be up-regulated during monocytic differentiation, was used. Cells were stained with anti-CD68 antibody that reacts with a monocyte/macrophage-associated antigen (110Kd) to evaluate for myeloid differentiation (Greiner *et al.*, 1997). As shown in Figure 4, cocultured Kasumi-1 cells acquired the morphology of monocytoid cells with increase in cytoplasmic vacuoles and granularity. Furthermore, upon cocultivation, Kasumi-1 cells showed cytoplasmic CD68 immunoreactivity, approximately 30% of cells showed positive staining for cytoplasmic CD68. This result indicates that upon coculturing on AGM-derived DAS 104-4 cells, Kasumi-1 cells acquire differentiated morphology and cytoplasmic CD68.

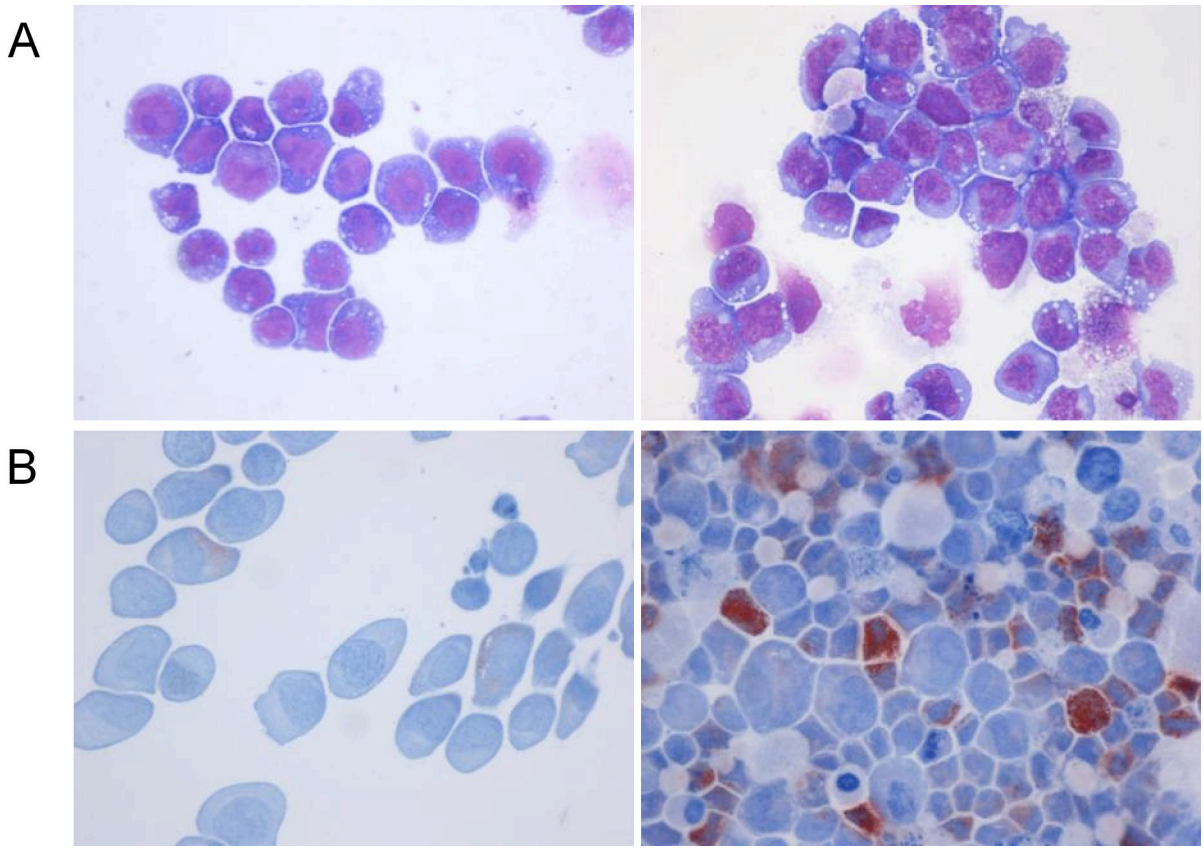

Figure 4. Coculture-induced myeloid/monocytoid differentiation of Kasumi-1 cells. Representative sample stained by (A) Pappenheim or (B) Immunostaining for CD68 (Brown). Left panel shows non-cocultured Kasumi-1 cells whereas right panel shows Kasumi-1 cells cocultured on DAS 104-4 for 6 days. Cells were harvested and cytospun on the objective slide. After air drying, the slides were analyzed by Prof. A. Greiner by Pappenheim and immunostaining for CD68. Magnifications: 400X.

4.2 DAS 104-4 coculture-induced acquisition of myeloid differentiation markers of Kasumi-1 cells

Further, the coculture-induced effects on Immunophenotype of Kasumi-1 cells was analyzed. As shown in chapter 2.1, DAS 104-4 coculture-induced up-regulation of myeloid morphologies and immunochemistry in Kasumi-1 cells, I wanted to see if this result is consistent with up-regulation of myeloid immunophenotype i.e., CD11b and CD14 (Heidenreich *et al.*, 2003). Moreover, it would be interesting to see if stroma-induced differentiation promoting activity is stroma cell-specific. Therefore, I cocultivated Kasumi-1 cells on DAS 104-4; murine foetal liver derived stromal cells, AFT 024 and mouse embryonic fibroblast derived cell line, NIH 3T3 as non-haematopoietic feeder. In parallel, the Kasumi-1

cells were treated with differentiation-inducing agent, all–*trans* retinoic acid (ATRA) as a positive control for myeloid differentiation (Manfredini *et al.*, 1999).

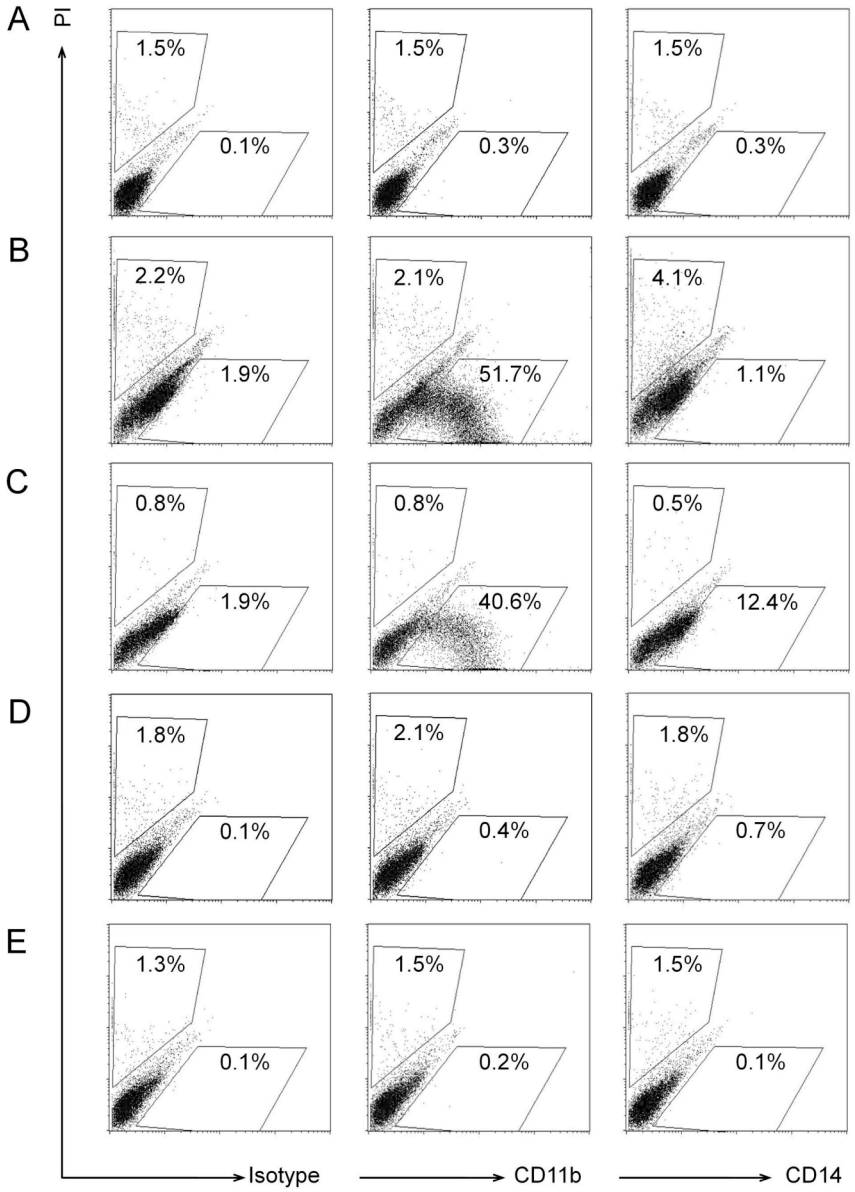

Figure 5. Coculture-induced changes in immunophenotype of Kasumi-1 cells. Representative flowcytometric analyses for CD11b, CD14 and propidium iodide stainings (A) of non-cocultured Kasumi-1 cells, (B) of Kasumi-1 cells following ATRA treatment and of Kasumi-1 cells cocultured for 3 days on (C) DAS 104-4, (D) NIH 3T3 or (E) AFT 024 cells. AML cells were stained with isotype control antibody, with human-specific CD11b or CD14 antibodies in combination with propidium iodide. Live gate on FSC/SSC profiles, enriched in living AML cells and depleted in stromal cells, was used to acquire the readings. Percentages of PI^+ dead cells and of $CD11b^+$ or $CD14^+$ cells are indicated. Representative samples are shown ($n \geq 3$).

After 3 days of direct coculture, cocultured Kasumi-1 cells were washed off the stromal cell layer and harvested cells were examined for staining with either antibody isotype control to exclude any non-specific staining or anti-CD11b or anti-CD14 antibody to look for myeloid

differentiation. And to see if the staining signal is coming from the living cells, cells were double stained for propidium iodide (PI). As seen in Figure 5 and as described earlier (Manfredini *et al.*, 1999), treatment with the differentiating agent all-*trans* retinoic acid (ATRA) induces CD11b up-regulation of Kasumi-1 cells indicative of myeloid differentiation. Furthermore, result shows that Kasumi-1 cells without coculture or cocultured on AFT 024 or NIH 3T3 cells for 3 days does not acquire either CD11b or CD14 staining. Only upon coculture with DAS 104-4 cells, Kasumi-1 cells acquire CD11b and CD14 staining. Propidium iodide staining of cocultured and non-cocultured cells indicate very low percentage of dead cells in the acquired gate. This result indicates that DAS 104-4 stroma can specifically induce Kasumi-1 cells to acquire myeloid differentiated immunophenotype.

Table 4. **Appearance of myeloid immunophenotypes of cocultured human leukaemic cell lines**

	No cocultivation	YSE	YSM	DAS 104-4	DAS 104-8	AFT 024	NIH 3T3	ATRA
Kasumi-1	-	-	-	+	-	-	-	+
KG-1	+	nd	nd	++	nd	nd	++	++
EOL-1	-	-	nd	-	-	nd	nd	nd
NB-4	-	nd	nd	+	nd	-	-	+
M2-AML	+/-	nd	nd	+	nd	nd	nd	nd

Kasumi-1, KG-1, EOL-1, NB-4 cells and primary M2-AML cells were cocultured for 2-6 days on irradiated YSE, YSM, DAS 104-4, DAS 104-8, AFT 024 or NIH 3T3 cells. Following coculture, non-adherent cells were isolated by gentle washes and cells were analyzed for CD11b or CD14 immunoreactivity by FACS. Samples that showed CD11b or CD14 staining are indicated as (+); samples that did not show CD11b or CD14 staining are labelled as (-); (+/-) stands for low ($\leq$10%) and (++) indicates high ($\geq$60%) CD11b or CD14 expression; nd: not done. As control for the induction of myeloid differentiation, cells were treated with all-*trans* retinoid acid (ATRA). Data shown represent the results of at least 2 individual experiments.

4.3 DAS 104-4 stroma can induce myeloid differentiation of patient-derived primary M2-AML cells

In the present experiment, it was analyzed whether the DAS 104-4 stroma has the ability to induce differentiation of primary M2-AML cells. The M2-AML cells were derived from a 70 year-old patient (kindly provided by Dr. R. Henschler, Frankfurt). Cells were cocultivated on DAS 104-4 stromal cells for 2 days. Cocultured M2-AML cells were washed off the stromal

cell layer and harvested cells were examined for the appearance of differentiation markers by morphological and immunocytochemical analyses. Firstly, cells from cocultures were cytospun and the morphology was analyzed by Pappenheim staining. Cytoplasmic granularity and the nuclear structure indicated myeloid maturation of the cocultured patient-derived primary M2-AML cells. Secondly, staining with anti-CD68 antibody was done to evaluate myeloid differentiation (Greiner *et al.*, 1997). As shown in Figure 6, cocultured Kasumi-1 cells acquired the morphology of monocytoid cells with cytoplasmic CD68 immunoreactivity, approximately 30-40% of primary M2-AML cells, cocultured on DAS 104-4 cells, showed positive staining for cytoplasmic CD68, whereas ≤2% non-cocultured M2-AML cells showed positive staining for cytoplasmic CD68. This result indicates that upon coculturing on AGM-derived DAS 104-4 cells, primary M2-AML cells acquire monocytoid-specific immunochemistry and morphology.

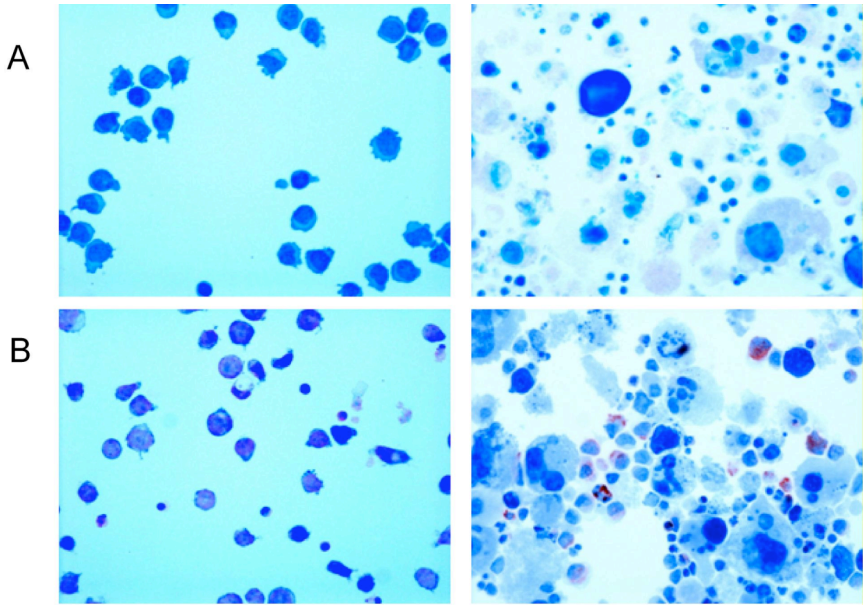

Figure 6. Coculture-induced myeloid/monocytoid differentiation of patient-derived M2-AML cells. Shown are representative samples of (A) Pappenheim staining and of (B) CD68 immunostaining of non–cocultured patient-derived M2-AML cells (left) and of patient-derived M2-AML cells cocultured on irradiated DAS 104-4 cells (right). Non-cocultured or patient-derived M2-AML cells cocultured for 2 days were harvested and cytospun on objective slides. Air-dried slides were sent to Heidelberg to Prof. Axel Griener. Cells were there analyzed by Pappenheim staining or by CD68 (PGM1)-specific immunostaining (brown). Magnifications: 400X.

In addition, the AML cells were harvested after 2 days of cocultivation and double stained with propidium iodide and either isotype control antibody to rule out any non-specific staining or antibodies for myeloid markers CD11b, CD14 or the antibody against c-KIT. c-KIT is known as the receptor for stem cell factor hence, correlating to primitive progenitors/stem cells. Results indicate the fraction single positive for PI only consists of small fraction in all cases (Figure 7). It is interesting to see that the $CD11b^{+}$ PI^{-} cells increased from 4.3% in non-cocultivated primary M2-AML cells to 28.1% upon cocultivation with DAS 104-4 cells.

Similarly, there was also cocultivation-induced increase in the $CD14^+$ PI^- primary M2-AML cells. Moreover, the result with c-KIT staining showed that there is a marginal decrease in c-KIT^+ PI^- population upon cocultivation (Figure 7). In summary, Patient derived primary M2-AML cells shows marked increase in myeloid differentiation markers, CD11b and CD14, in parallel with marginal decrease in primitive cell marker i.e., c-KIT in primary M2-AML cells when they are cocultivated on murine AGM-derived stromal cells, DAS 104-4.

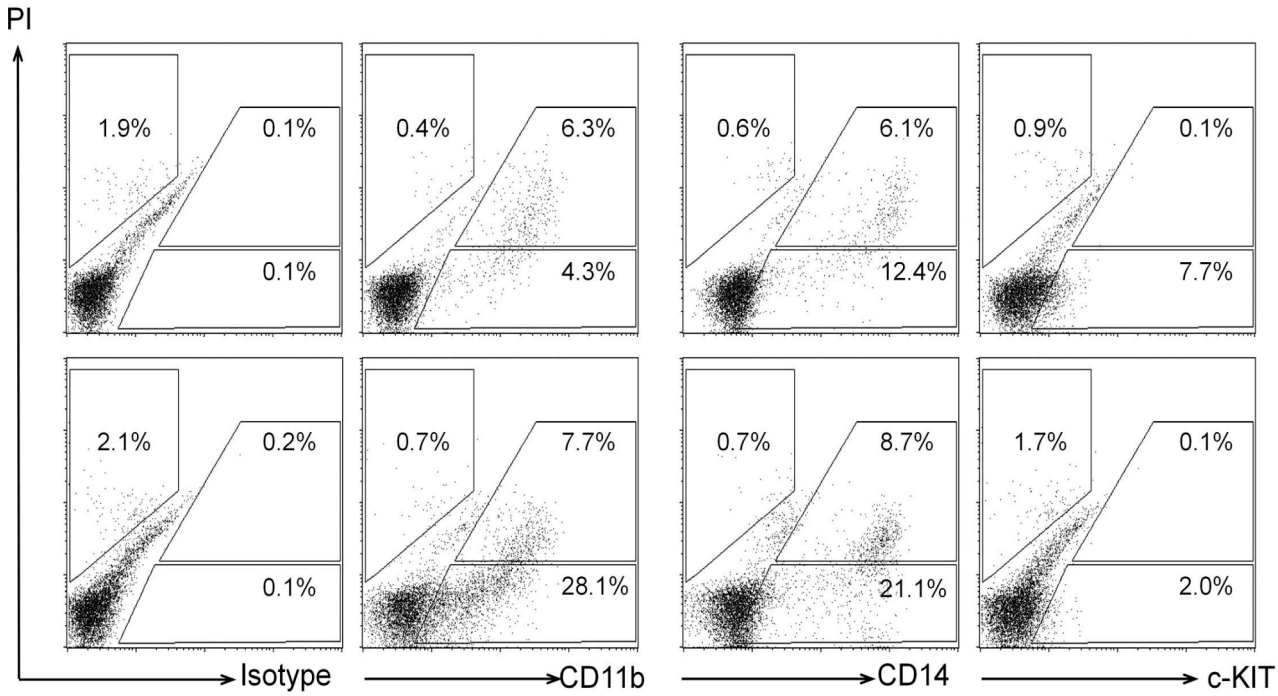

Figure 7. M2-AML cells, derived from 70 year-old, male patient, were thawed and washed twice with culture medium to remove any traces of cryo-preservant. $8x10^5$ cells were seeded onto irradiated DAS 104-4 stromal cells. After two days of coculture, cells were isolated, gently washed and AML cells were stained with isotype control antibody, human-specific antibodies against myeloid monocytic markers, CD11b or CD14 or the progenitor cell marker c-KIT in combination with PI. Live gate on FSC/SSC profiles, enriched in living AML cells and depleted in stromal cells, was used to acquire the readings. Percentages of PI^+ dead cells and of $CD11b^+$ or $CD14^+$ or c-KIT^+ cells are indicated. Representative samples are shown (n=2).

4.4 Effect of cocultivation on KG-1 AML cells

KG-1 is an AML cell line. Previously, chimeric animals derived from blastocysts microinjection of KG-1, showed that upon introduction to the murine microenvironment, AML cells were undergoing change and started expressing elevated level of erythroid markers such as Glycophorin A and elevated level of β-globin expression. In the present study I asked the question, "What are the consequences of cocultivation of KG-1 cells on DAS 104-4

cells?"

KG-1 cells were either grown alone or cocultured on DAS 104-4. After 3 days of direct coculture, KG-1 cells were washed off the stromal cell layer and harvested cells were examined for double staining with PI and either antibody isotype control to omit out any non-specific staining or anti-CD11b antibody to look for myeloid differentiation. Figure 8 shows that the coculture of KG-1 cells on DAS 104-4 leads to increase in CD11b staining (non-cocultured KG-1 cells: 25±15% $CD11b^+$ cells; day 3 of coculture: 74±16% $CD11b^+$ cells). In addition, propidium iodide staining of cocultured and non-cocultured cells indicate very low percentage of dead cells in the acquired gate. In summary, cocultivation on DAS 104-4, leads to increase in myeloid immunophenotype of KG-1 cells.

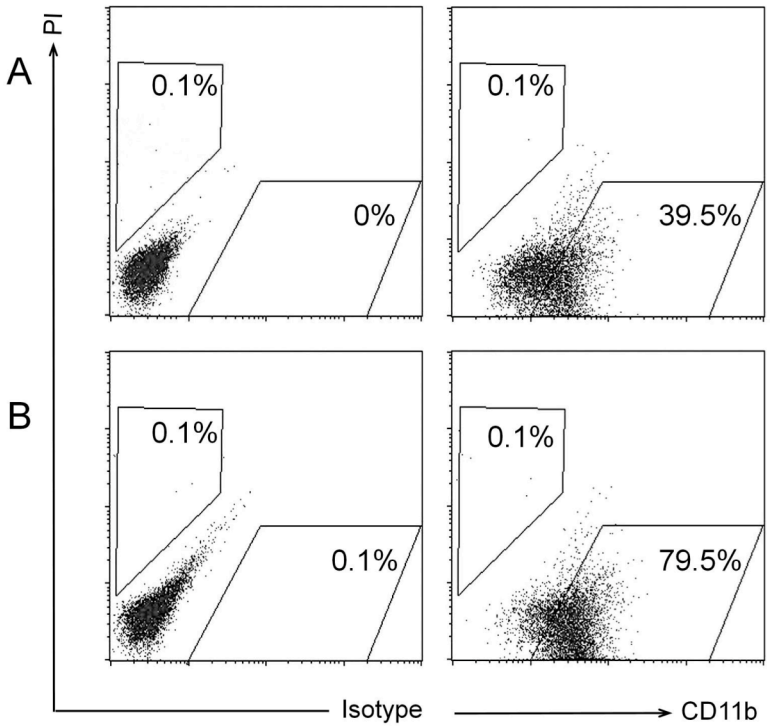

Figure 8. Coculture-induced up-regulation of myeloid immunophenotype of KG-1 cells. Representative flowcytometric analyses of CD11b and PI stainings. (A) Non-cocultured KG-1 cells stained with isotype control and PI (Left) and stained with CD11b (right). (B) KG-1 cells cocultured on DAS 104-4 for 3 days, stained with isotype (left) or CD11b human-specific antibody. AML cells were stained with isotype control antibody, with human-specific CD11b antibody in combination with PI. Live gate on FSC/SSC profiles, enriched in living AML cells and depleted in stromal cells, was used to acquire the readings. Percentages of PI^+ dead cells and of $CD11b^+$ cells are indicated. Representative samples are shown (n=3).

As previously shown, KG-1 cells can be induced to undergo erythroid differentiation by introducing them into the murine embryonic microenvironment (Durr *et al.*, 2003). Here, I

wanted to study the consequences of *in vitro* cocultivation on KG-1 cells in presence of DAS 104-4 and its effect on erythroid markers. KG-1 cells were either grown alone (non-coculture) or cocultured on irradiated DAS 104-4 stromal layer. After coculture for 1, 2 and 3 days, KG-1 cells were harvested, total RNA was extracted and cDNA was made. The transcription levels of β-globin were compared between the non-cocultured and cocultured KG-1 cells by human-specific β-globin RT-PCR.

Furthermore, It will be interesting to see whether there is any change in CD235a (Glycophorin A) expression on the KG-1 cell surface upon cocultivation. KG-1 cells either cultured alone or cocultured on irradiated DAS 104-4 stroma for 3 days. After cocultivation, KG-1 cells were harvested from the stromal layer and double stained with DAPI and anti-CD235a antibody.

RT-PCR result shows transcriptional up-regulation of β-globin gene upon cocultivation of KG-1 on DAS 104-4, starting from 2nd day (Figure 9A). The immunostaining for human-specific Glycophorin A shows that higher level of staining is acquired when KG-1 is cocultivated on DAS 104-4 for 3 days (Figure 9B). This result indicates that KG-1 cells can acquire differentiated features when exposed to DAS 104-4 stroma environment *in vitro*, in a manner similar when they are exposed to embryonic environment *in vivo*.

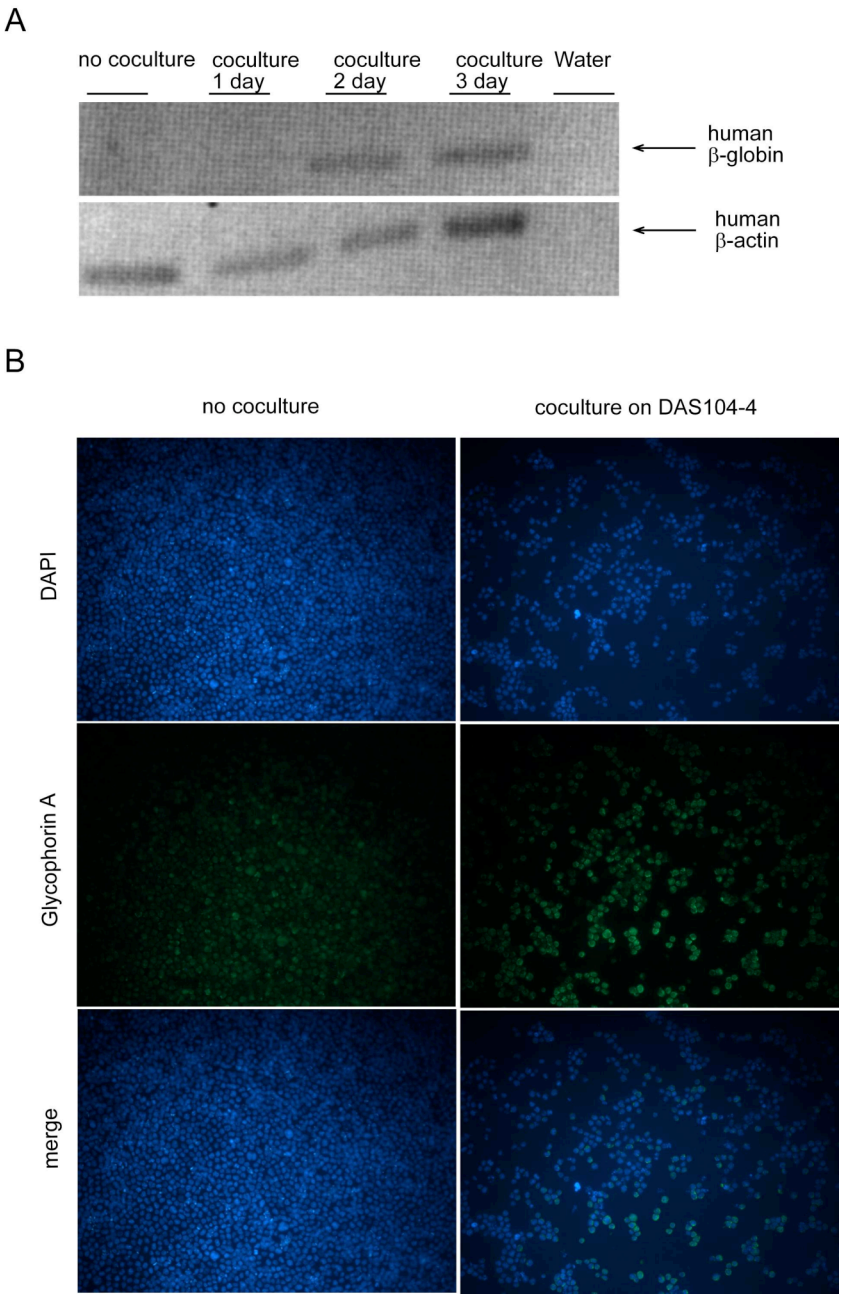

Figure 9. Up-regulation of erythroid features in KG-1 upon DAS 104-4 coculture. (A) β-globin-specific RT-PCR on RNA isolated from KG-1 cells either non-cocultured or cocultured on DAS 104-4 for 1, 2 and 3 days. (B) Immunostaining for Glycophorin A (CD235a), the left column shows non-cocultured KG-1 cells stained with DAPI and Glycophorin A antibody and the right column shows the KG-1 cells, cocultured on DAS 104-4 stromal cells for 3 days, stained with DAPI and Glycophorin A antibody.

4.5 Coculture-induced change in proliferation properties of AML cells

Next, it was analyzed whether stroma-environment can change proliferation properties of human AML cells. I wanted to see, if there is any cocultivation-induced difference in cell cycle stage of Kasumi-1 cells. The results of cell cycle analysis of non-cocultured or cocultured Kasumi-1 cells by PI DNA staining revealed that coculture of Kasumi-1 on DAS 104-4 cells induces accumulation of cells in G_1 phase and a subsequent decrease of cells in S and G_2/M phases (Figure 10).

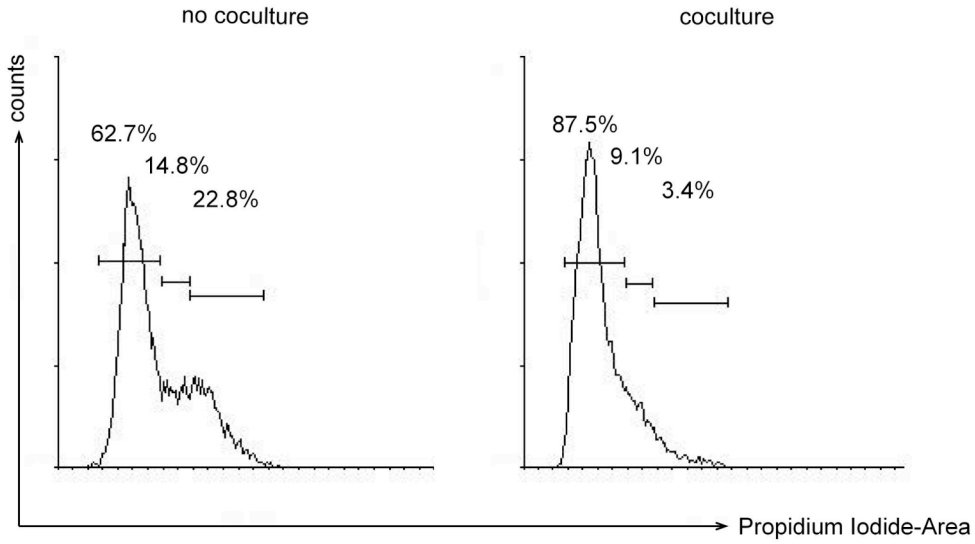

Figure 10. Analysis of cell cycle distribution. Cell cycle analysis by PI staining of non-cocultured (left) and of 2 days cocultured Kasumi-1 cells on DAS 104-4 (right). Percentages of cells in individual stages of the cell cycle are indicated. Showing one representative experiment of 3 individual experiments.

I further asked the question whether the coculture-induced accumulation of Kasumi-1 cells in G_1 phase of cell cycle is also reflected as coculture-induced decrease in proliferation. To analyze the effect of coculture on AML cell proliferation rate, CFSE label retention assay was used (Lyons and Parish, 1994), where loss of CFSE serve as indication of cell proliferation rate. The distribution of cells according to their CFSE fluorescence was analyzed at 2, 4, 6, and 8 days of coculture (Figure 11). CFSE loss was decreased in Kasumi-1 cells cocultured on DAS 104-4 cells compared to the non-cocultured Kasumi-1 cells. There was no difference in CFSE dilution rates between the Kasumi-1 cells cocultivated on NIH 3T3 or without any stromal cells. This observation indicates that cocultivation with DAS 104-4 induces a specific reduction in cell proliferation rate of human AML cells.

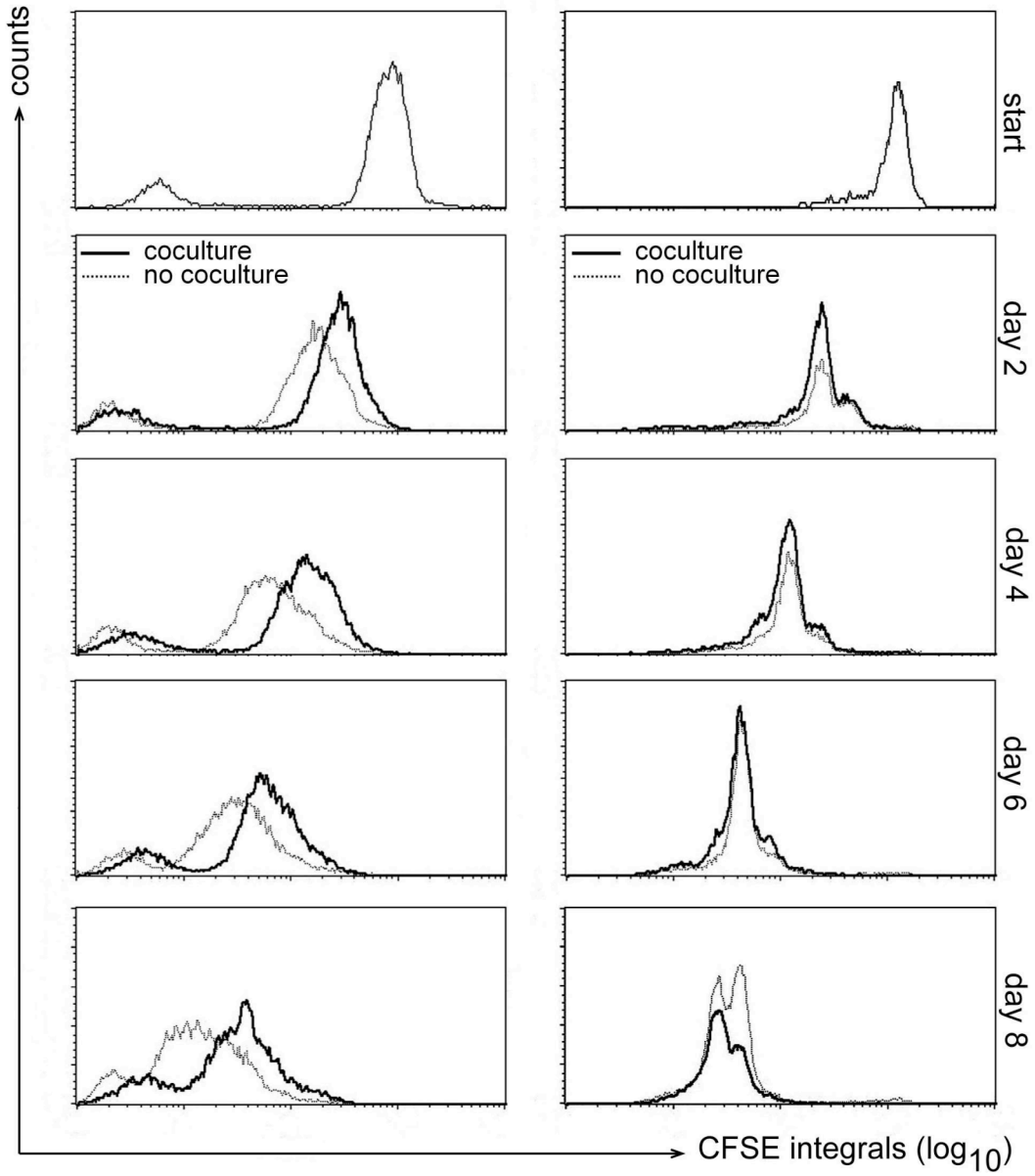

Figure 11. Analysis of proliferation of Kasumi-1 cells. (A) Fluorescence profiles of CFSE-labelled Kasumi-1 cells that were either non-cocultured or cocultured on DAS 104-4 (left panels) or NIH 3T3 (right panels) cells. Following CFSE labelling, Kasumi-1 cells were split into samples that were either cocultured or were kept without stroma cells. Shown are fluorescence profiles of labelled Kasumi-1 cells at the start of the experiment before coculture and after 2, 4, 6 and 8 days of coculture.

4.6 DAS 104-4 induces decrease in total cell number and reduction in primitive colony forming cell pool of cocultured AML cell population

As shown above, DAS 104-4 stroma induces accumulation of Kasumi-1 cells in G_1 stage paralleled by decrease in CFSE dilution rate indicating a coculture-mediated reduction of cell proliferation. I further asked the question whether coculture has any effect on colony forming

cell (CFC) population in a given cell pool of Kasumi-1 cells. To achieve that, Kasumi-1 cells were cultured without stroma or cocultured on either DAS 104-4 or AFT 024 or NIH 3T3 for 4 days. Counting of total number of trypan blue negative cells showed that there was significant decrease in total Kasumi-1 cell number when cocultivated on DAS 104-4 compared to the cells without any cocultivation or cocultivated on AFT 024 or NIH 3T3 stroma (Figure 12). Moreover, there was no significant difference between total number of viable cells when they were not cocultivated or cocultivated on NIH 3T3. Similarly, cocultivation of Kasumi-1 cells on AFT 024 did not show any change in total number of viable cells compared to the non-cocultivated. This result indicates that DAS 104-4 in particular exerts anti-proliferative effect on cocultivated Kasumi-1 cells and feeder derived from foetal liver AFT 024 and NIH 3T3 do not have such activity.

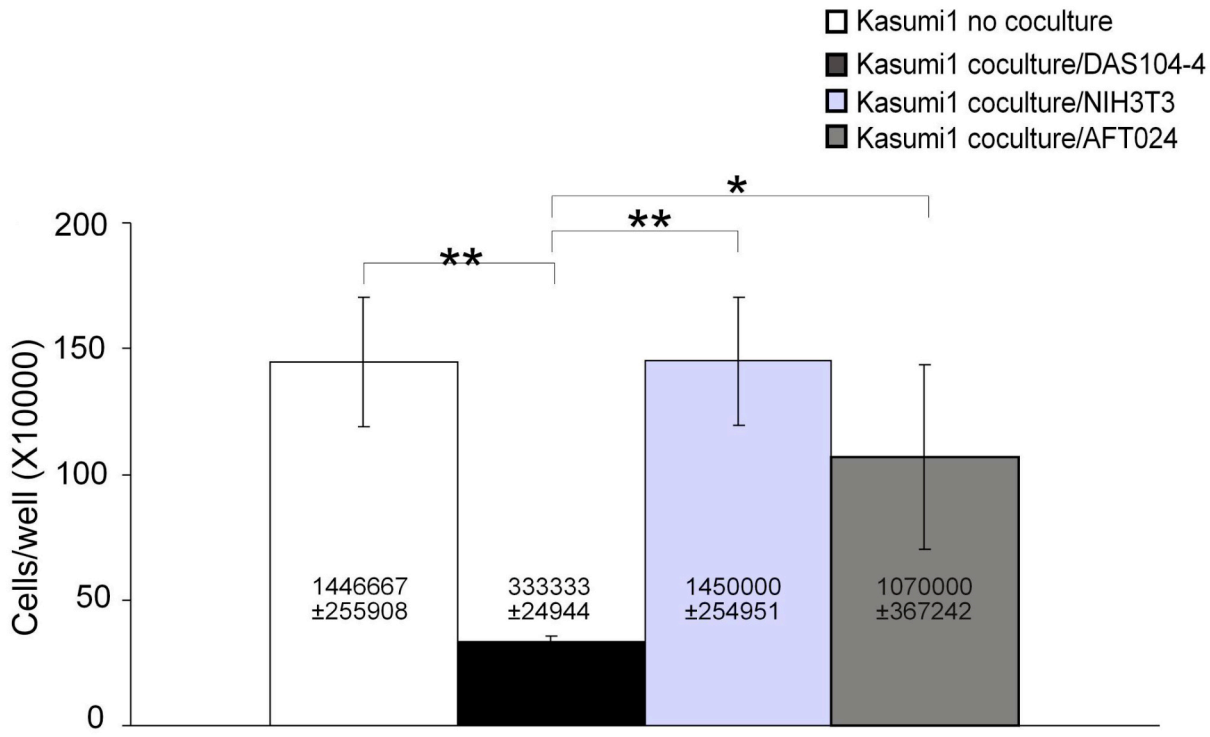

Figure 12. Effect of coculture on the cell number of Kasumi-1 cells. $5x10^5$ Kasumi-1 cells were seeded per well of a 6 well plate in the presence or absence of stromal cells (in triplicate). After 4 days, Kasumi-1 cells were harvested from stromal cells and the total cell number per well was counted by trypan blue staining.

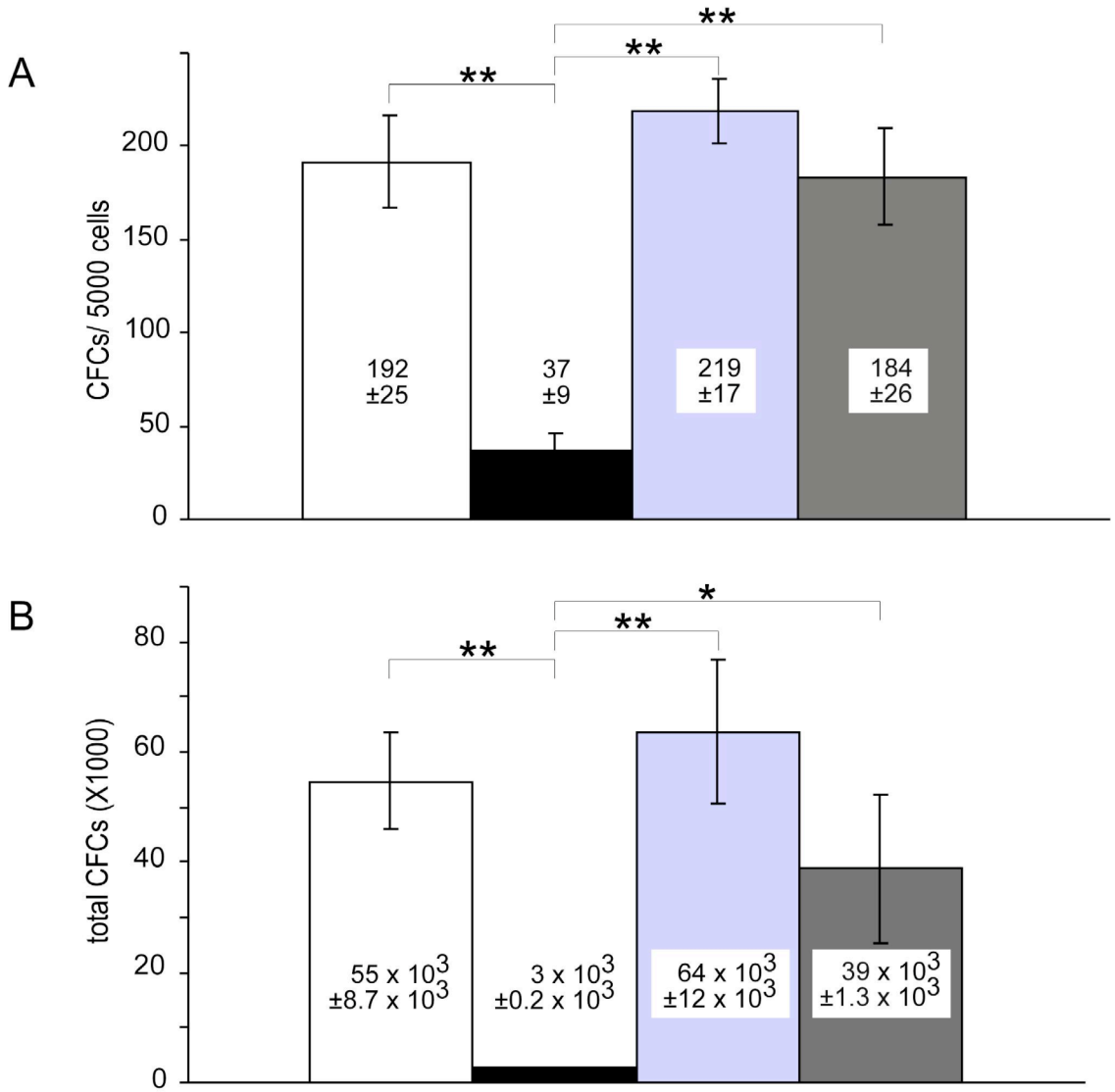

Figure 13. Effect of coculture on the clonogenicity of Kasumi-1 cells. $5x10^5$ Kasumi-1 cells were seeded per well of a 6 well plate in the presence or absence of stromal cells (in triplicate). After 4 days, Kasumi-1 cells were harvested from stromal cells and (A) non-cocultured and cocultured cells were seeded into semi-solid methyl cellulose medium and 14 days after plating colony numbers were counted. (B) Total number of CFCs after 4 days with or without coculture. The total CFC numbers were calculated by multiplication of viable cell numbers/well (taken from Figure 14) with CFCs/5000 cell numbers (from Figure 15A). Cells/well, CFCs/5000 cells and total numbers of CFCs/well are indicated; error bars show standard deviations (n=3). Statistical significance was tested by Student *t*-test. (*) indicates $P<0.05$; (**) stands for $P<0.01$.

In addition, it was analyzed whether cocultivation with DAS 104-4 has any effect on colony forming cell fraction of Kasumi-1 cell population. These CFCs are supposed to be primitive cells, which drive total population. Kasumi-1 cells were either cultured without any feeder cells or cocultivated on either DAS 104-4 or AFT 024 or NIH 3T3 cells for 4 days. Viable cells were counted by their tryphan blue exclusion capability. 5000 viable cells from cultures were

seeded on semi-solid methyl cellulose plates. Colonies were let to grow in 37°C humidified incubator for 14 days. Results showed no significant difference between colony numbers of non-cocultivated Kasumi-1 cells and that of Kasumi-1 cells cocultivated on NIH 3T3 or AFT 024. In contrast, significant decrease in the colony numbers were observed, when they are cocultured on DAS 104-4 in comparison to the Kasumi-1 cells without cocultivation or cocultivated on NIH 3T3 or AFT 024 (Figure 13). Furthermore, I calculated the absolute number of colony forming cells of Kasumi-1 cells, which were either cultured alone or cocultured on DAS 104-4, AFT 024 or NIH 3T3. Results showed that absolute number of colony forming cells decreased to less than 1% of non-cocultured Kasumi-1 cells when they were cocultured on DAS 104-4. Whereas, there was no significant difference between the non-cocultivated Kasumi-1 cells and the Kasumi-1 cells cocultivated on AFT 024 or NIH 3T3. These results indicate that DAS 104-4 microenvironment can specifically and significantly reduce the total colony forming cell fraction of Kasumi-1 cell pool.

4.7 Effects of direct and indirect coculture

I asked the question whether the coculture-induced effect of DAS 104-4 needs direct cell to cell contact between stromal cells and AML cells or it is mediated through some soluble factor(s). In that case I can see the effect even if the stroma and AML cells are placed apart by a permeable transmembrane. In the present experiment, AML cells were either cultured alone or cocultivated directly on feeder cells or indirectly cocultured, separated from feeder layer by 0.4µm pore-size permeable transmembrane.

Kasumi-1 cells were stained with CFSE and then split them to seed either alone or directly on irradiated feeder layer of DAS 104-4 or across the transwell for indirect cocultivation and then looked over window of time for CFSE dilution which in turn related directly to the cell proliferation rate of Kasumi-1 cells. The result (Figure 14) indicated that direct cocultivation has maximum effect on proliferation reduction capability, whereas, Kasumi-1 cells cocultured indirectly on transwell shows lesser degree of proliferation reduction. This study indicates that to exert maximum efficacy on Kasumi-1 cells in terms of cell proliferation inhibition, DAS 104-4 stroma needs direct cell to cell contact with AML cells.

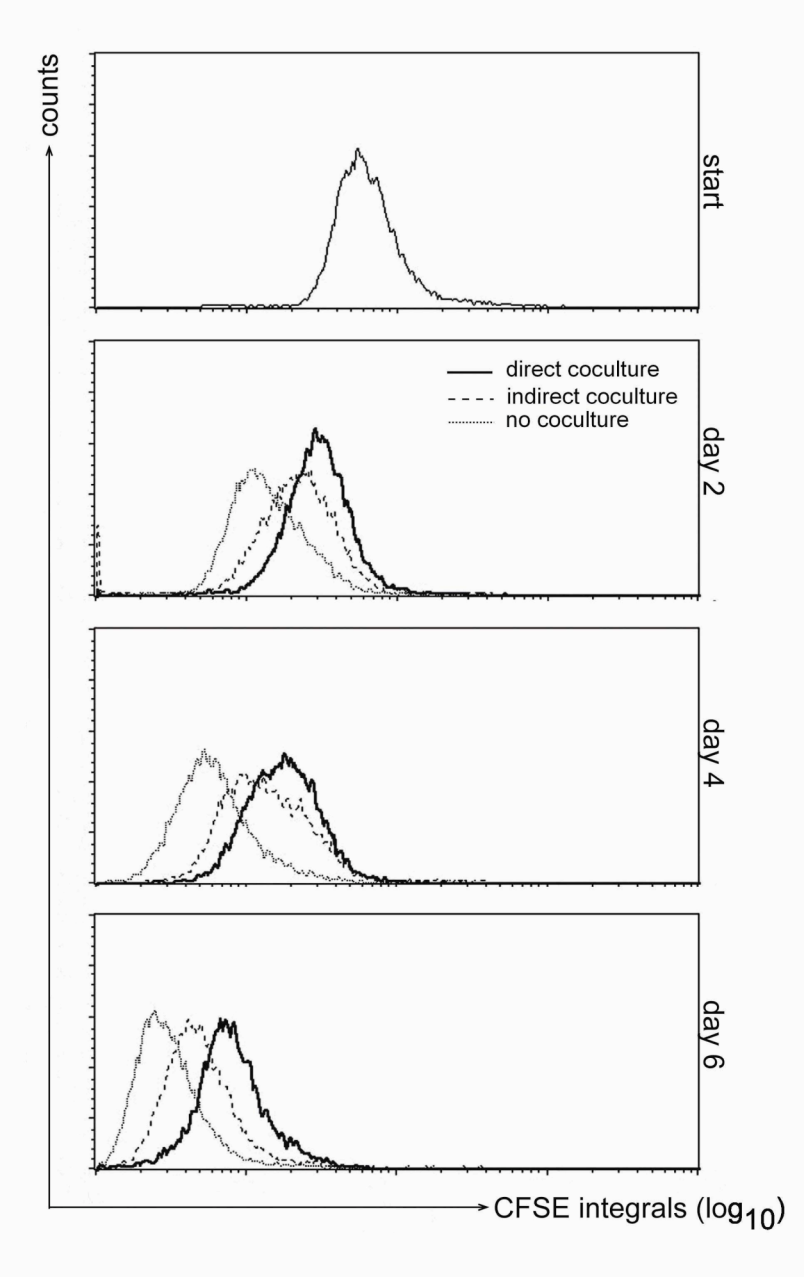

Figure 14. Effect of direct versus indirect coculture on proliferation of AML cells. CFSE-labelled Kasumi-1 cells were either non-cocultured or directly cocultured on DAS 104-4 cells or labelled Kasumi-1 cells and DAS 104-4 cells were seeded in opposite sides of a 0.4µm pore-size polycarbonate membrane (transwell). CFSE fluorescence of Kasumi-1 cells was recorded at 2, 4 or 6 days post start of coculture. A representative experiment is shown (n=3).

Furthermore, I asked the question whether the Kasumi-1 differentiation promotion activity of DAS 104-4 feeder cells indeed need direct cell to cell contact or it can be facilitated through soluble factor when stroma and AML cells are separated by permeable membrane. To address this, Kasumi-1 cells were cocultivated for 3 days either directly on irradiated feeder cells or indirectly when the AML and feeder cells were separated by 0.4µm pore-size

permeable transmembrane and I compared these with the Kasumi-1 cells cultured without any stromal cells for 3 days. AML cells were harvested after 3 days post culture and either stained with isotype control antibody to rule out any non-specific staining or stained with anti-CD11b antibody to indicate myeloid differentiation. Results (Figure 15) indicate that only Kasumi-1 cells cocultured directly on DAS 104-4 showed induction of myeloid differentiation, whereas, the Kasumi-1 cells indirectly cocultured on DAS 104-4 showed no staining for myeloid differentiation as similar to the Kasumi-1 cells cultured without any feeder. From this result it is evident that the direct stroma-AML cell contact is required for coculture-induced myeloid differentiation of Kasumi-1 cells by DAS 104-4 stroma.

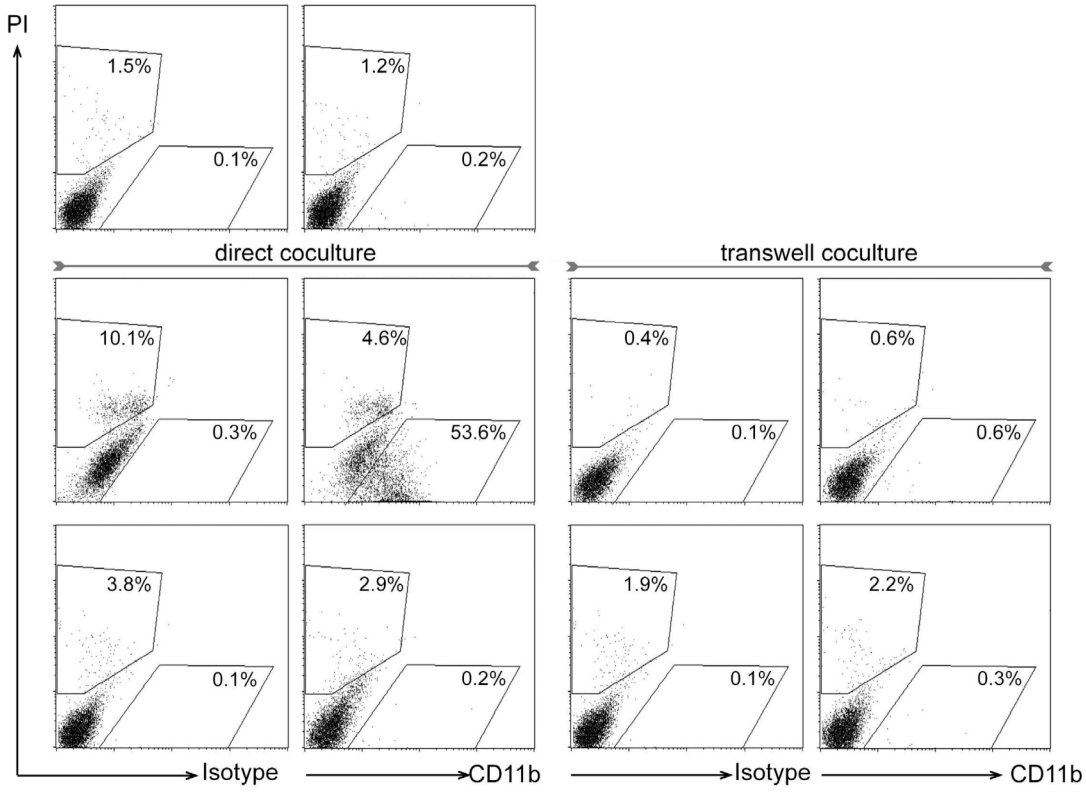

Figure 15. Effect of direct versus indirect coculture on AML differentiation. Upper row shows Kasumi-1 cells cultured for 3 days without stroma. Middle row represents Kasumi-1 cells cocultured on DAS 104-4 for 3 days either directly or indirectly. Lower row shows Kasumi-1 cells cocultured directly or indirectly on NIH 3T3 for 3 days. AML cells were stained with isotype control antibody or with human-specific CD11b antibody in combination with PI. Live gate on FSC/SSC profiles, enriched in living AML cells and depleted in stromal cells, was used to acquire the readings. Percentages of PI^+ dead cells and of $CD11b^+$ cells are indicated. Representative samples are shown (n=3).

4.8 Effect of fixed DAS 104-4 in reduction of proliferation and induction of differentiation of cocultured Kasumi-1 cells

I asked question, whether the fixed DAS 104-4 is sufficient to induce proliferation reduction of AML cells. To answer our question, Kasumi-1 cells were labelled with CFSE and cultured them as either non-cocultured or directly cocultivated Kasumi-1 cells either on the living or on fixed stromal layer of DAS 104-4. Kasumi-1 cells were harvested after 3 days of coculture and CFSE fluorescence retention was measured using flowcytometry. The fluorescence measurement in Figure 16 indicates that fluorescence reduction was maximum in non-cocultured Kasumi-1 cells, this indicates that the Kasumi-1 cell proliferation rate was maximum when they were not cocultured with DAS 104-4. Kasumi-1 cells cocultivated on paraformaldehyde-fixed stroma cells showed lower CFSE loss compared to the non-cocultured Kasumi-1 cells, that indicates that the proliferation rate of Kasumi-1 cells on fixed DAS 104-4 is lesser than that of Kasumi-1 cells grown without stroma. The Kasumi-1 cells cocultured on living (unfixed) DAS 104-4 cells showed maximum CFSE retention, i.e., maximum AML cell proliferation inhibition activity. In summary, living DAS 104-4 stroma can most effectively induce cell proliferation inhibition, than the PFA-fixed DAS 104-4 stroma which in turn shows marginally reduced cell proliferation inhibition than the Kasumi-1 cells grown without any DAS 104-4 stroma. This indicates that living DAS 104-4 stroma is required to induce maximum reduction in proliferation of Kasumi-1 cells.

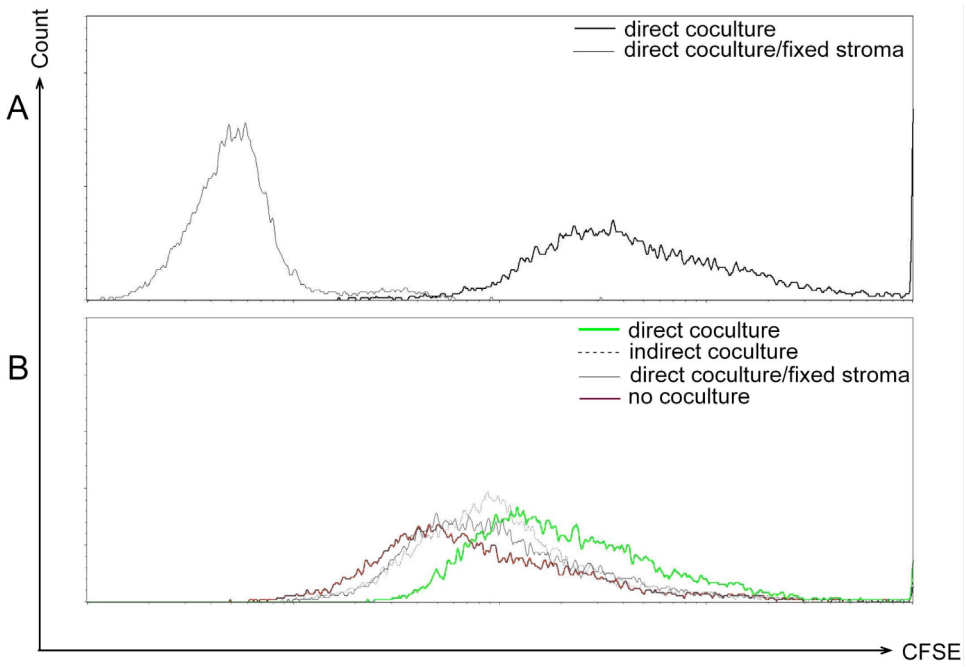

Figure 16. Effect of physiologically active versus fixed DAS 104-4 stroma layer on proliferation of cocultured Kasumi-1 cells. (A) Unlabelled and CFSE-labelled Kasumi-1 cells at the start. (B) CFSE-labelled Kasumi-1 cells were directly cocultured on either living or fixed DAS 104-4, or indirectly cocultured by seeding Kasumi-1 cells and DAS 104-4 cells in opposite sides of a 0.4µm pore-size polycarbonate membrane (transwell). CFSE fluorescence of Kasumi-1 cells was recorded at 3 days post start of coculture. A representative experiment is shown (n=2).

4.8.1 Effect of fixed stroma on differentiation

The question addressed here is whether the fixed DAS 104-4 is sufficient to induce myeloid differentiation in AML cells. To address this question, Kasumi-1 cells were cultured alone or directly cocultured either on the living or on the fixed stromal layer of DAS 104-4. In addition to that, Kasumi-1 cells, indirectly cocultured on living DAS 104-4, were also included in this study. Kasumi-1 cells were harvested after 3 days of coculture and stained with isotype control or myeloid marker CD11b-specific antibody. Figure 17 shows that Kasumi-1 cells cocultured on fixed stromal cells showed no CD11b staining, i.e., showed no sign of myeloid differentiation, similar to the Kasumi-1 cells cultured without stromal cells, whereas, Kasumi-1 cells directly cocultivated on living (unfixed) DAS 104-4 showed positive staining for CD11b. These results indicate that metabolically active DAS 104-4 is required for its myeloid differentiation inducing activity in AML cells.

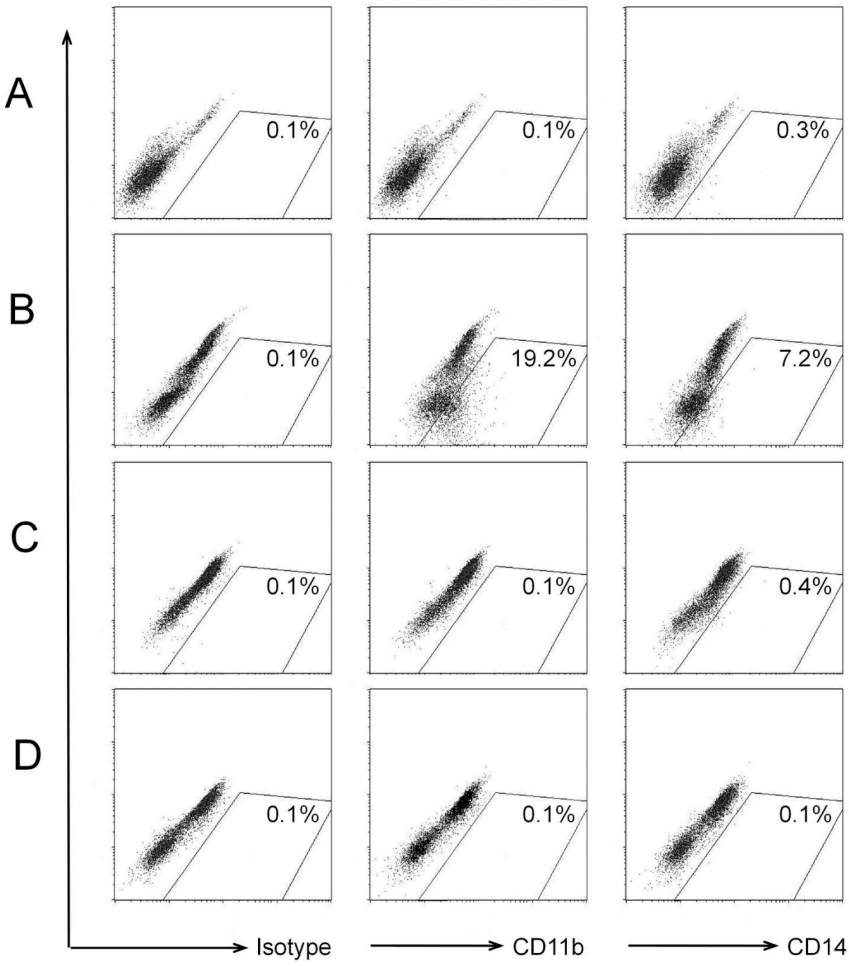

Figure 17. Comparison of direct cocultivation on physiologically active DAS 104-4 stroma versus fixed stroma on AML differentiation inducing capability. Flowcytometric analysis of CD11b, CD14 and PI stainings of (A) non-cocultured Kasumi-1 cells, (B) Kasumi-1 cells cocultured for 3 days directly on living DAS 104-4 cells, (C) Kasumi-1 cells cocultured directly on 2% PFA-fixed DAS 104-4 stromal cells and (D) Kasumi-1 cells cocultured indirectly on DAS 104-4 stromal cells

where AML and stroma were separated by transmembrane with 0.4µm pore size. Following coculture, AML cells were stained with isotype control antibody, with human-specific CD11b or CD14 antibodies in combination with PI. Live gate on FSC/SSC profiles, enriched in living AML cells and depleted in stromal cells, was used to acquire the readings. Percentages of PI^+ dead cells and of $CD11b^+$ or $CD14^+$ cells are indicated. Representative samples are shown (n=2).

4.9 Effect of DAS 104-4 stroma on Kasumi-1 AML1-ETO expression

As AML1-ETO fusion gene is present in 40% of M2-AML and this fusion oncogene is present in Kasumi-1 cells, it was studied whether there is any specific change in AML1-ETO transcription following coculture of Kasumi-1 cells on DAS 104-4.

4.9.1 DAS 104-4 induced reduction in AML1-ETO transcript level of cocultivated Kasumi-1 cells

To look for DAS 104-4 coculture induced molecular consequences in Kasumi-1 cell, I compared the transcript level of AML1-ETO in Kasumi-1 cells cultured alone or cocultured either on DAS 104-4 or NIH 3T3 or AFT 024 for 3 days. Figure 18 shows more than 10-fold decrease in AML1-ETO transcript level of Kasumi-1 cells cocultured on DAS 104-4 cells compared to that of Kasumi-1 cells which are non-cocultivated or cocultivated on either NIH 3T3 or AFT 024.

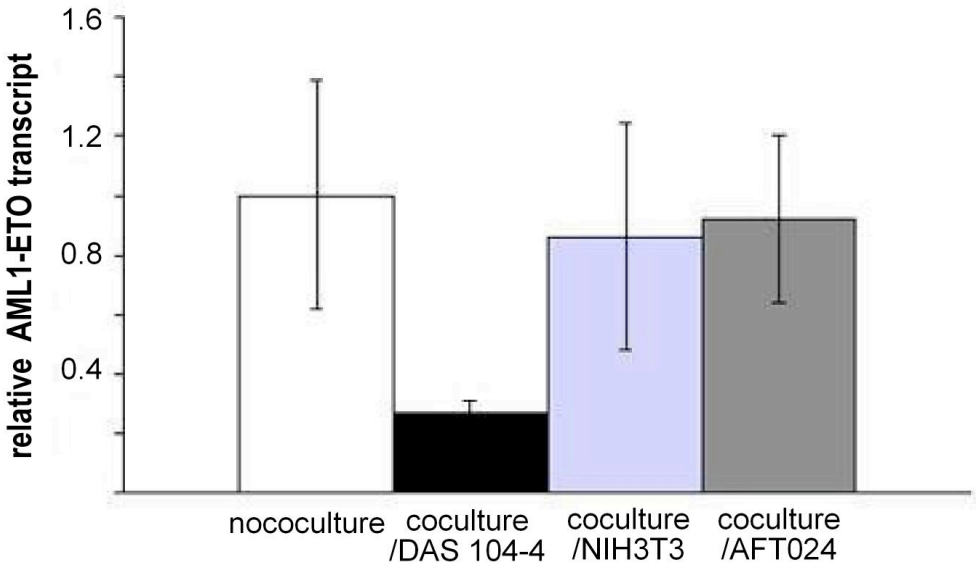

Figure 18. Quantitative AML1-ETO RT-PCR analysis. Total RNA was isolated from non-cocultured Kasumi-1 cells or from direct cocultures of Kasumi-1 cells on DAS 104-4, NIH 3T3 or AFT 024 cells at day 3 of coculture. The relative mRNA levels of AML1-ETO were normalized by β-actin-specific RT-PCR. Gene expression of tested mRNAs in non-cocultured Kasumi-1 cells was set to 1 (n=3). PCR primers are human-specific.

4.9.2 Stroma-AML contact-dependent down-regulation of AML1-ETO transcription

As it was shown in the previous section that there is coculture-induced down-regulation of AML1-ETO transcript level, I further went ahead to ask the question whether the DAS 104-4 stroma-induced AML1-ETO transcriptional down-regulation is contact-dependent. Figure 19 shows that direct cocultivation of Kasumi-1 cells on DAS 104-4 stroma for 3 days leads to significant reduction in AML1-ETO transcription compared to that of non-cocultured Kasumi-1 cells. In contrast, Kasumi-1 cells indirectly cocultured on DAS 104-4 showed no significant change in the AML1-ETO transcript levels compared to non-cocultured Kasumi-1 cells.

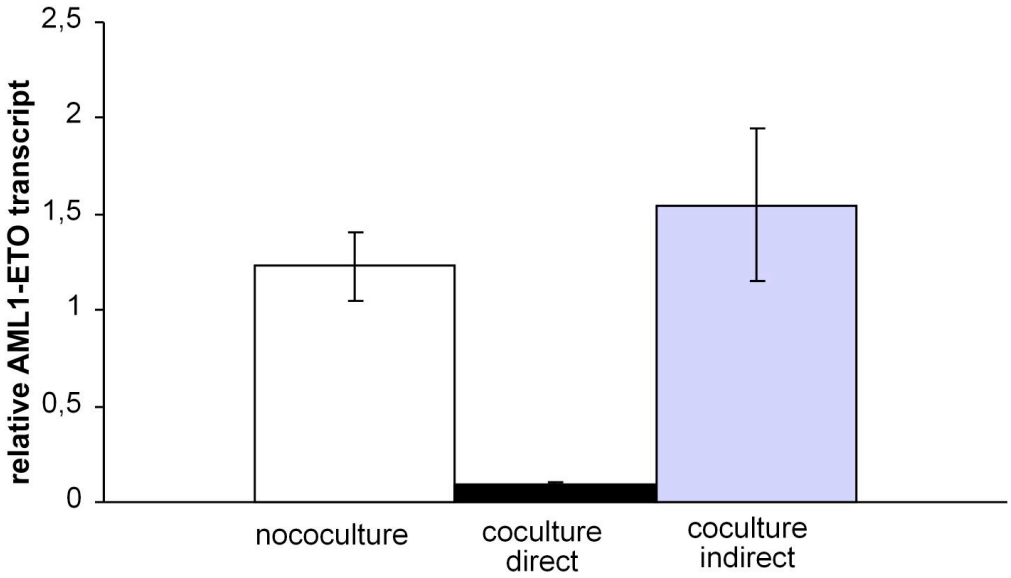

Figure 19. Effect of direct and indirect coculture on AML1-ETO transcript level in Kasumi-1 cells. Total RNA was isolated from non-cocultured Kasumi-1 cells or from Kasumi-1 cells directly or indirectly cocultured on DAS 104-4 for 3 days. The relative mRNA levels of AML1-ETO were normalized by β-actin-specific RT-PCR. Gene expression of tested mRNAs in non-cocultured Kasumi-1 cells was set to 1 (n=3). PCR primers are human-specific.

4.10 CD44 ligation-induced differentiation of AML cells

It has been shown (Charrad *et al.*, 1999) that many AML primary and established cell lines can be induced to differentiate by activation of CD44 receptor by ligation with CD44–ligating

antibody. Here, the possibility of CD44 activation being the mechanism of observed DAS 104-4 cocultivation-induced myeloid differentiation of Kasumi-1 cells was checked.

4.10.1 Expression of CD44 on Kasumi-1, KG-1 and NB-4 cells

I looked for CD44 expression on AML cell lines, Kasumi-1, KG-1 and NB-4. Staining with the CD44–ligating antibody (A3D8) and FACS analyses showed that all three cell lines express CD44 on their surface with similar intensities (Figure 20).

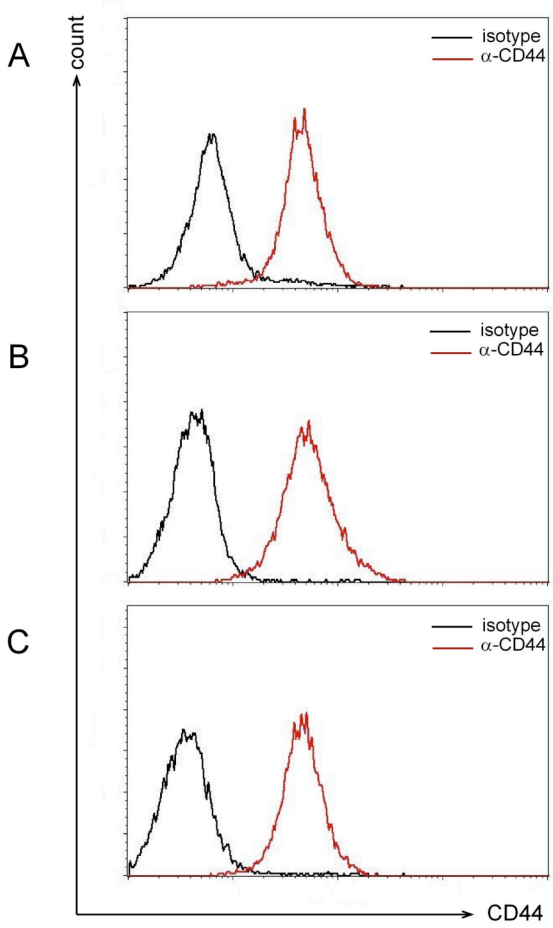

Figure 20. Expression of CD44 on the surface of different AML cell lines. (A) Kasumi-1 (B) KG-1 and (C) NB-4. Cells were stained with mouse anti-human CD44 antibody, clone A3D8, subsequently stained with rabbit anti-mouse secondary antibody coupled with Cy3 chromophore (n=3).

4.10.2 CD44-ligating antibody, A3D8 mediated differentiation of AML cells

As CD44 ligation can induce myeloid differentiation of human AML cells (Charrad *et al.*, 1999), I addressed the question whether CD44 activation is the mean by which cocultivation is mediating its myeloid differentiating effect on AML cells. AML cells, Kasumi-1, KG-1 and

NB-4 were incubated, either with 20µg/ml concentration of CD44 activating antibody, A3D8 or same concentration of IgG1 isotype control. Cells were harvested after 3 days of incubation and were either stained with isotype control to rule out any non-specific staining or stained with antibody against myeloid marker, CD11b. Second staining with PI was done to be sure that the signal is not coming from the dead cells. I observed myeloid differentiation in NB-4 (no CD44 activation: 0.07±0.03% $CD11b^+$ cells; day 3 of CD44 treatment: 27.6±10.3% $CD11b^+$ cells) and KG-1 cells (no CD44 activation: 59.2±6.1% $CD11b^+$ cells, day 3 of CD44 treatment: 92.6±5.7% $CD11b^+$ cells). In contrast, CD44 treatment of Kasumi-1 cells did not induce myeloid differentiation (no CD44 activation: 0.03±0.04% $CD11b^+$ cells, day 3 of CD44 treatment: 0.05±0.03% $CD11b^+$ cells). This result indicates that CD44 activation by itself is not sufficient to induce myeloid differentiation in Kasumi-1 cells (Figure 21).

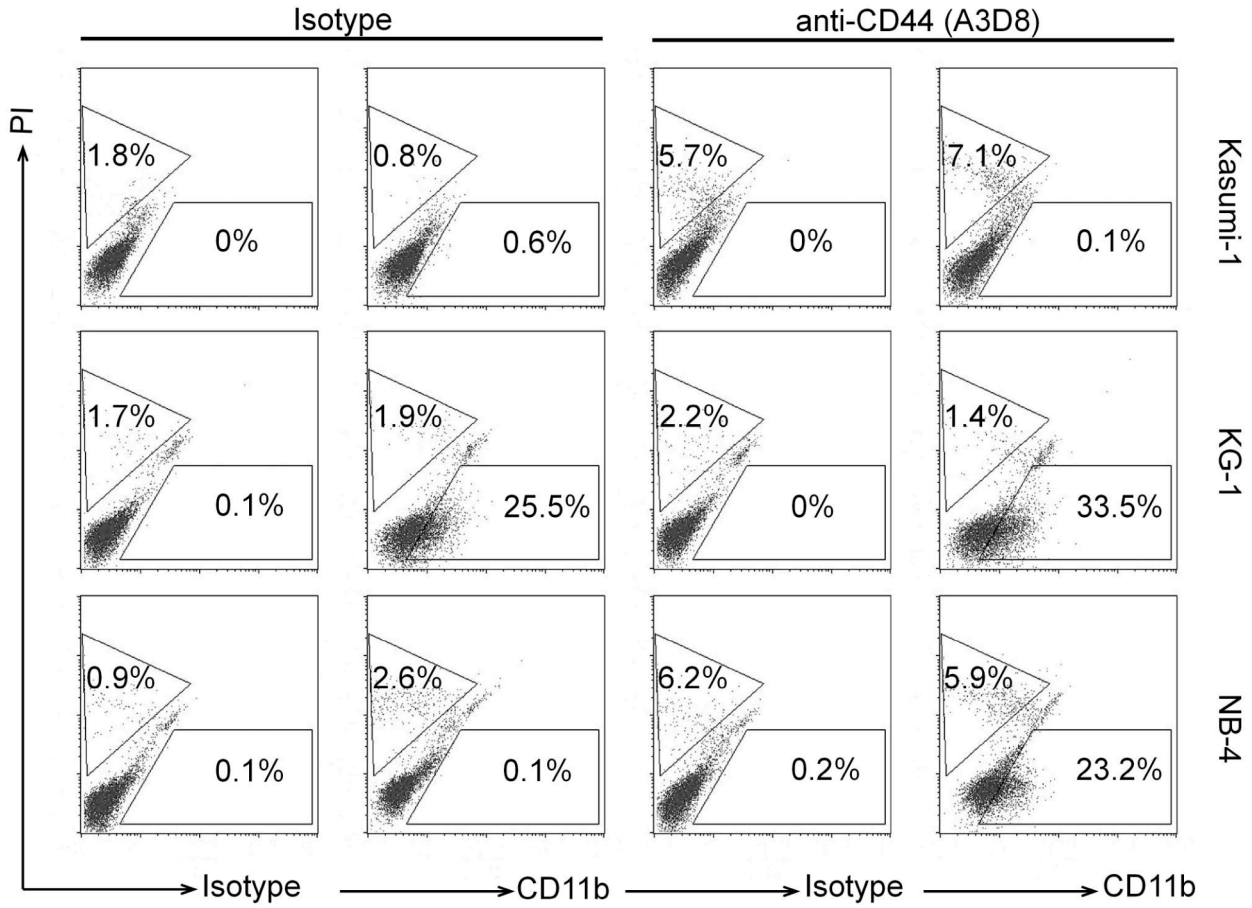

Figure 21. Effect of CD44-ligating (activating) antibody on differentiation of AML cells. Kasumi 1 cells were either incubated with isotype control antibody (20µg/ml) or same concentration of CD44-ligating antibody for 3 days. Upper row shows Kasumi-1 incubated either in isotype control antibody (left two) or incubated with A3D8 (right two). Middle row shows KG-1 incubated either in isotype control antibody (left two) or incubated with A3D8 (right two). Lower row shows NB-4 incubated either in isotype control antibody (left two) or incubated with A3D8 (right two). Flowcytometric analyses of CD11b and PI stainings. AML cells were stained with isotype control antibody or with human-specific CD11b antibody in combination with PI. Live gate on FSC/SSC profiles, enriched in living AML cells and depleted in stromal cells, was used to acquire the readings. Percentages of PI^+ dead cells and $CD11b^+$ cells are indicated. Representative samples are shown (n=3).

4.11 Gene expression analysis of critical mediators of proliferation and myeloid differentiation

Since coculture-mediated reduction in proliferation and induction of myeloid differentiation were observed as described in the previous sections, I wanted to see if these manifestations are accompanied by alteration in expression of differentiation-associated and cell cycle regulator genes.

4.11.1 PU.1

PU.1 transcription factor plays an important role in myeloid differentiation and it was previously shown that mice PU.1 hypomorphs shows myeloproliferative disorders (Mueller *et al.*, 2002), suggesting its important role in myeloid differentiation pathway. Therefore, the transcript level of PU.1 between Kasumi-1 cells cocultured on DAS 104-4 and Kasumi-1 cells cultured without any feeder cells was compared. The result indicates increase in the PU.1 transcript level of cocultured Kasumi-1 cells (Figure 22).

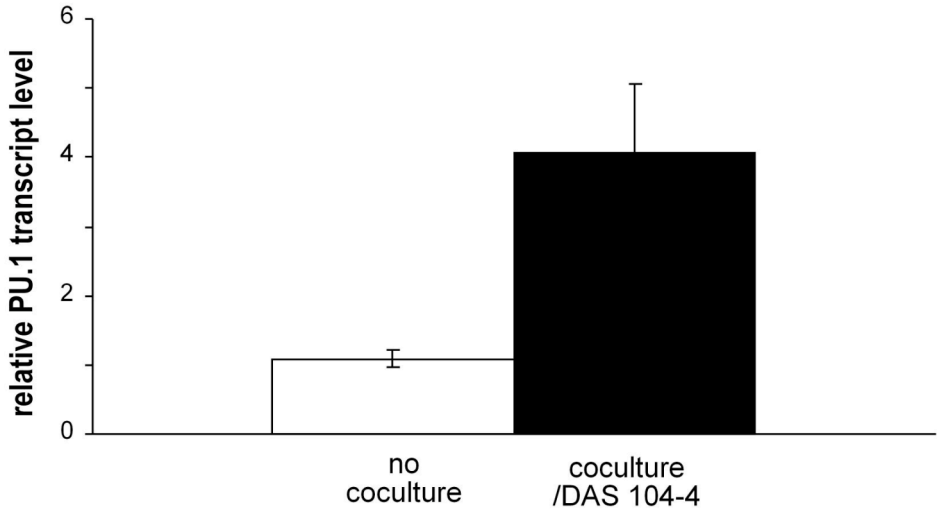

Figure 22. Quantitative RT-PCR analysis for PU.1 transcript level. Total RNA was isolated from non-cocultured Kasumi-1 cells or from direct coculture of Kasumi-1 on DAS 104-4 cells at day 3 of coculture. The relative mRNA levels of PU.1 were normalized by β-actin-specific RT-PCR. Gene expression of tested mRNAs in non-cocultured Kasumi-1 cells was set to 1 (n=3). PCR primers are human-specific.

4.11.2 RUNX1 (AML1)

AML1; also known as runt-related transcription factor 1, or RUNX1. This gene was originally cloned from the breakpoint of the t(8;21) reciprocal chromosome translocation and was later recognized as one of the most frequent targets of leukaemia-associated gene aberrations. Gene-targeting experiments revealed that transcriptionally active AML1 is essential for the establishment of definitive haematopoiesis (Okuda *et al.*, 2001). Moreover, as shown in previous section that the direct DAS 104-4 coculture induces significant decrease in AML1-ETO transcript level in Kasumi-1 cells, Therefore, I asked the question whether there is any difference in transcript level of RUNX1. As the 5' upstream gene region of AML1 is retained in AML1-ETO gene, the transcript level of AML1 between Kasumi-1 cells cocultured on DAS 104-4 and Kasumi-1 cells cultured without any feeder cells was compared. This result indicates substantial decrease in the AML1 transcript level of cocultured Kasumi-1 cells compared to that of non-cocultured Kasumi-1 cells (Figure 23).

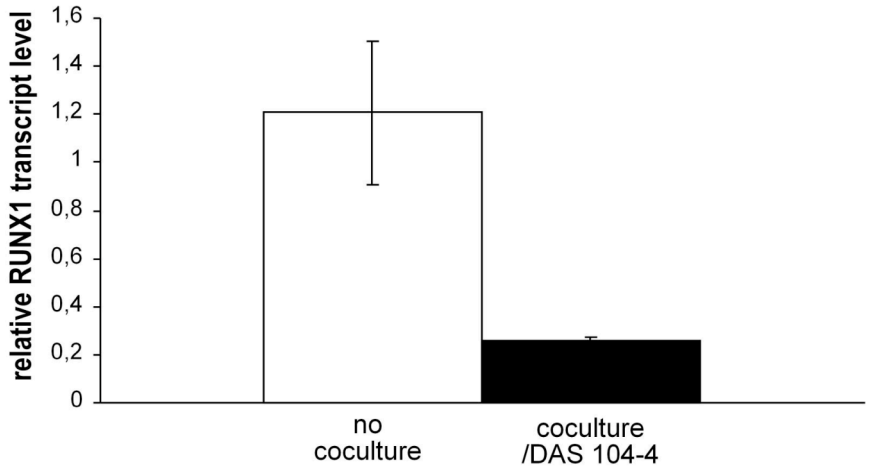

Figure 23. Quantitative RT-PCR analysis for RUNX1 transcript level. Total RNA was isolated from non-cocultured Kasumi-1 cells or from direct coculture of Kasumi-1 on DAS 104-4 cells at day 3 of coculture. The relative mRNA levels of RUNX1 were normalized by β-actin-specific RT-PCR. Gene expression of tested mRNAs in non-cocultured Kasumi-1 cells was set to 1 (n=3). PCR primers are human-specific.

4.11.3 C/EBPα

C/EBPα is one of the myeloid transcription factors, the role of which is lot more implicated in myeloid differentiation (Zhang *et al.*, 1996). It has been shown that the C/EBPα protein directly interacts with CDK2 and CDK4 and arrests cell proliferation by inhibiting these

kinases. A region between amino acids 175 and 187 of C/EBPα was determined to be responsible for direct inhibition of cyclin-dependent kinases and caused growth arrest in cultured cells (Wang *et al.*, 2001). Apart from this C/EBPα can also exert its proliferation inhibitory effect through the p21(WAF) (Timchenko *et al.*, 1996). Therefore, I compared the transcript level of C/EBPα between Kasumi-1 cells cultured without any feeder or cocultured on either DAS 104-4 or AFT 024 or NIH 3T3. The result indicates increase in the C/EBPα transcript level when the Kasumi-1 is cocultured on DAS 104-4 (Figure 24).

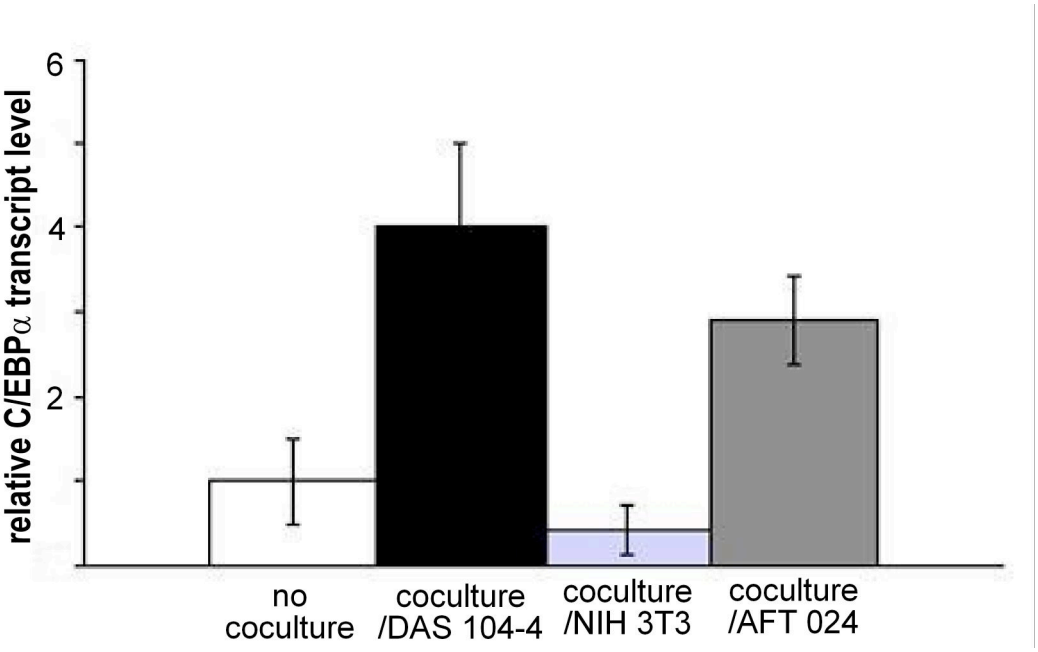

Figure 24. Quantitative RT-PCR analysis of C/EBPα. Total RNA was isolated from non-cocultured Kasumi-1 cells or from direct cocultures of Kasumi-1 cells on DAS 104-4, NIH 3T3 or AFT 024 cells at day 3 of coculture. The relative mRNA levels of C/EBPα were normalized by β-actin-specific RT-PCR. Gene expression of tested mRNAs in non-cocultured Kasumi-1 cells was set to 1 (n=3). PCR primers are human-specific.

4.11.4 p16 (INK4A)

p16 is important cell cycle regulatory factor which inhibits CDK4 and causes G_1 checkpoint arrest. As DAS 104-4 coculture induce reduction of AML cell proliferation accompanied by accumulation of cells in G_1 phase, I compared the transcript level of p16 between Kasumi-1 cells cocultured on either DAS 104-4 or NIH 3T3 or AFT 024 and Kasumi-1 cells cultured without any feeder cells. The result indicates increase in the p16 transcript level of Kasumi-1

cells cocultured on DAS 104-4 stromal cells compared to that of non-cocultivated Kasumi-1 cells. Kasumi-1 cells cocultivated on NIH 3T3 also showed elevated level of p16 transcript, leading us to conclude that p16 transcript level up-regulation is not DAS 104-4 coculture-specific (Figure 25).

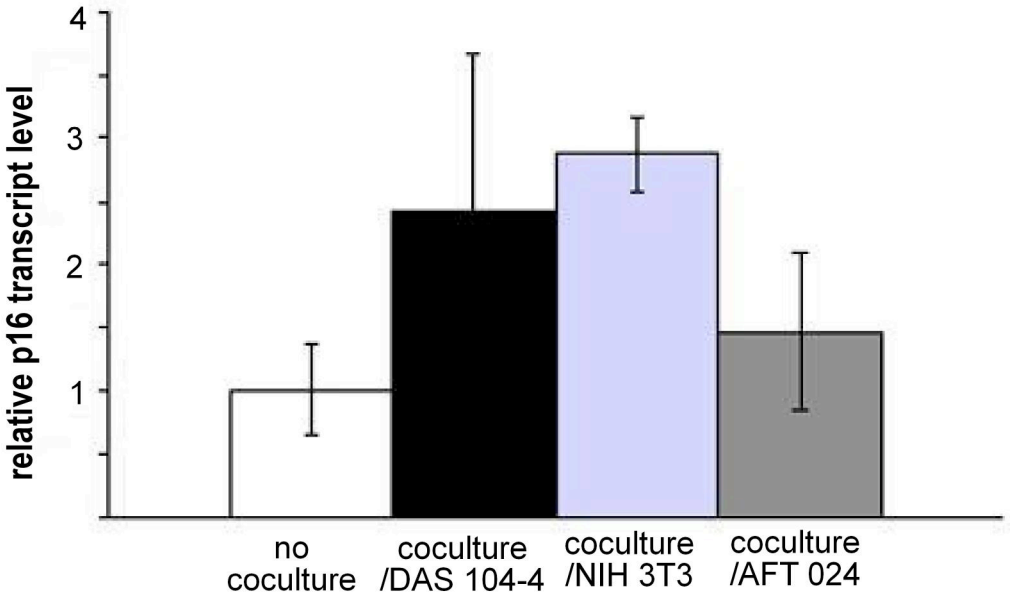

Figure 25. Quantitative RT-PCR analysis of p16. Total RNA was isolated from non-cocultured Kasumi-1 cells or from direct cocultures of Kasumi-1 cells on DAS 104-4, NIH 3T3 or AFT 024 cells at day 3 of coculture. The relative mRNA levels of p16 were normalized by β-actin-specific RT-PCR. Gene expression of tested mRNAs in non-cocultured Kasumi-1 cells was set to 1 (n=3). PCR primers are human-specific.

4.11.5 p21(WAF)

p21 is an important cell cycle regulatory factor, which inhibits cyclin dependent kinase-1,-2,-4 & 6 and causes checkpoint arrest. Apart from being a cell cycle control protein, its role in differentiation is also been previously indicated (Harper *et al.*, 1993). I compared the transcript level of p21 between Kasumi-1 cells cocultured on either DAS 104-4 or NIH 3T3 or AFT 024 and Kasumi-1 cells cultured without any feeder cells. The result indicates considerable increase in the p21 transcript level of Kasumi-1 cells cocultured on DAS 104-4

stromal cells compared to non-cocultured Kasumi-1 cells as well as Kasumi-1 cells cocultured on NIH 3T3 or AFT 024 (Figure 26).

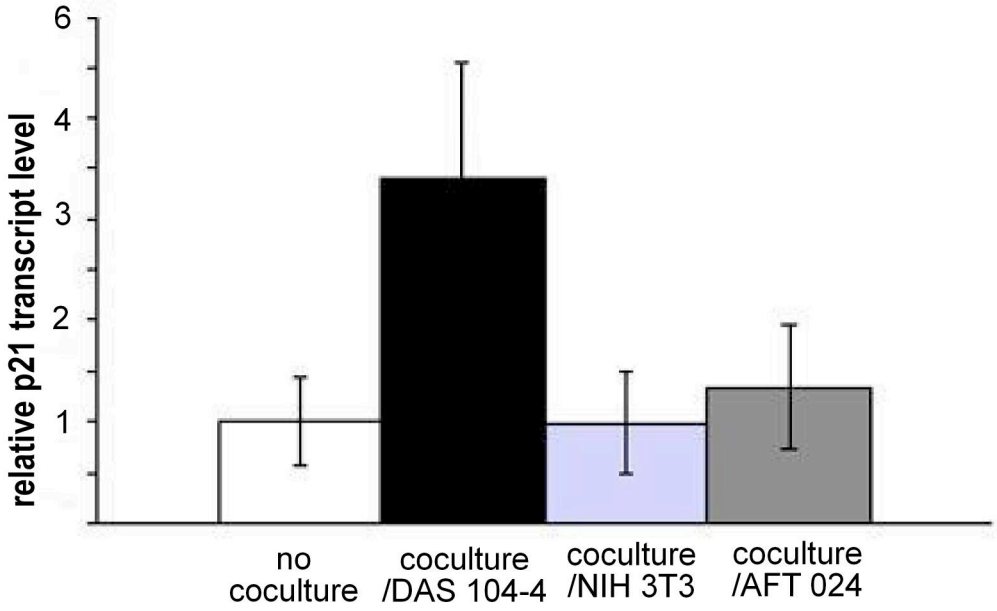

Figure 26. Quantitative RT-PCR analysis of p21. Total RNA was isolated from non-cocultured Kasumi-1 cells or from direct cocultures of Kasumi-1 cells on DAS 104-4, NIH 3T3 or AFT 024 cells at day 3 of coculture. The relative mRNA levels of p21 were normalized by β-actin-specific RT-PCR. Gene expression of tested mRNAs in non-cocultured Kasumi-1 cells was set to 1 (n=3). PCR primers are human-specific.

In summary, quantitative RT-PCR of RNAs from cocultured Kasumi-1 cells with human-specific primers showed that the coculture-induced down-regulation of AML1-ETO is paralleled by an increase in C/EBPα, p16 (INK4A) and p21 (WAF) expression. Therefore, coculture of Kasumi-1 on DAS 104-4 cells results in the decrease of AML1-ETO transcription and in the increase in transcription of regulators associated with differentiation and cell cycle regulation.

4.12 AML1-ETO siRNA decreases proliferation and induces myeloid differentiation of cocultured Kasumi-1 cells

As DAS 104-4 coculture induces down-regulation in the transcript level of AML1-ETO. I further analyzed if the down-regulation itself can promote differentiation, or if AML1-ETO transcription down-regulation act in concert with coculture derived signal. This question was addressed by employing siRNA to specifically down-regulate AML1-ETO expression.

4.12.1 siRNA transfection efficiency

siAM (siRNA against AML1-ETO) sequence were adapted from Heidenreich *et al.* (Heidenreich *et al.*, 2003). To optimize out the best siRNA transfection conditions for Kasumi-1 cells. I titrated between different concentrations of fluorescent-labelled siAM and amount of transfectin^{TM} reagent per transfection. 8X10^5 Kasumi-1 cells were transfected using shown concentrations of FAM labelled siAM and transfectin^{TM} agent. After 24hr post transfection, cells were harvested, washed and analyzed by flowcytometry for the incorporated fluorescent-labelled siRNA. The result shows that the siAM transfection efficacy can be brought to as high as 83% by using 1µg of siRNA and 4µl of transfectin^{TM} reagent per transfection reaction (Figure 27).

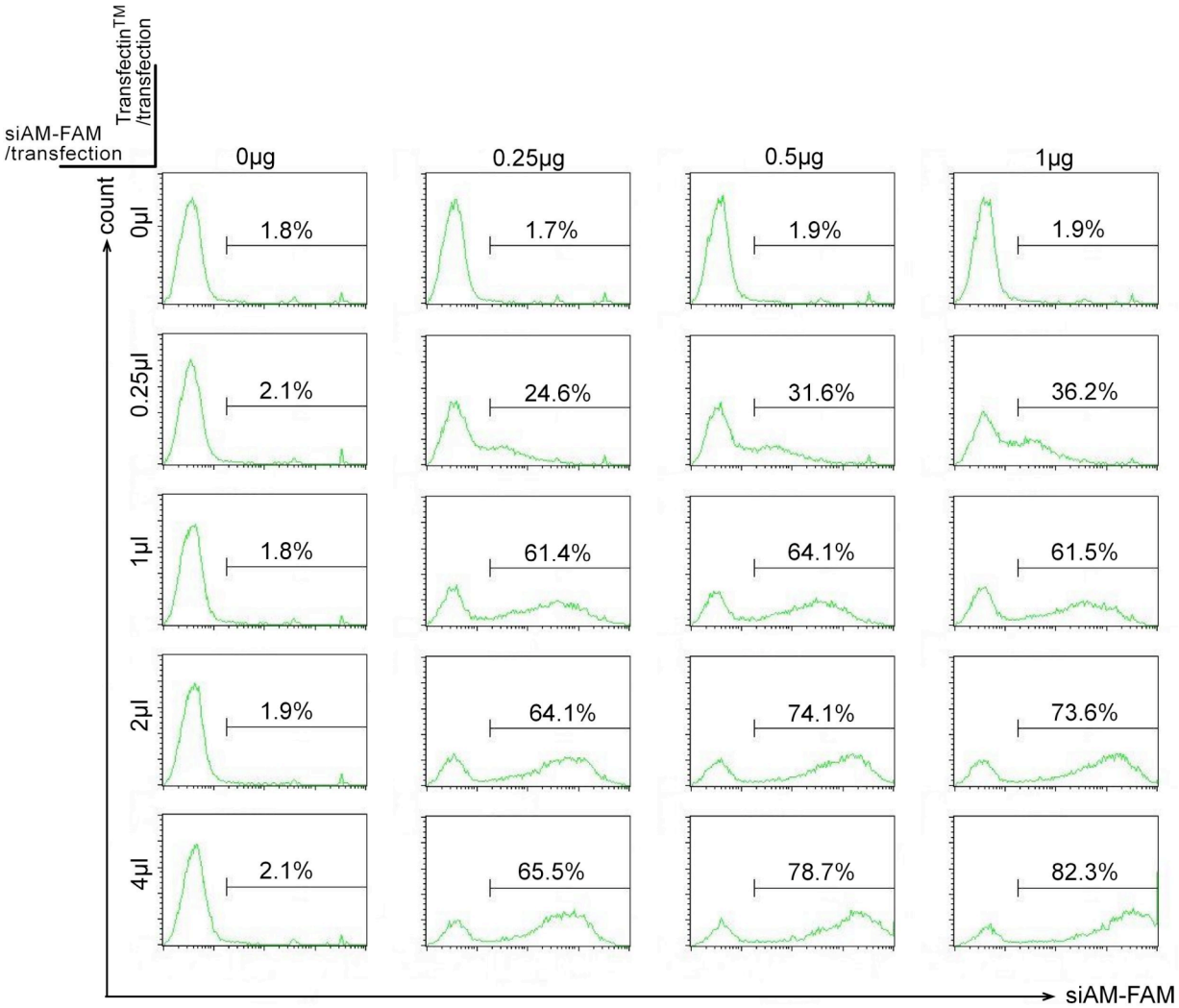

Figure 27. Transfection of FAM labelled siAM. Kasumi-1 cells were transfected with different concentrations of siRNA specific to AML1-ETO (siAM) labelled with fluorescent dye, FAM (different columns). Different amounts of transfection agent, transfectin™, were also tested (different rows). Cells were washed 24hr post transfection and flowcytometric analyses were done to analyze intracellular siAM positive cells. Percentages of siAM$^+$ cells are indicated.

4.12.2 siRNA mediated down-regulation of AML1-ETO expression

Total RNA was isolated 48 hour post-transfection from Kasumi-1 cells either mock transfected (without any siRNA) or transfected either with AML1-ETO specific siRNA (siAM) or control siRNA (siCTL). The quantitative RT-PCR result shows no significant difference in AML1-ETO transcript level between Kasumi-1 cells either transfected with control siRNA or no siRNA. In contrast, Kasumi-1 cells transfected with siAM show significant decrease in AML1–ETO transcript level (Figure 28).

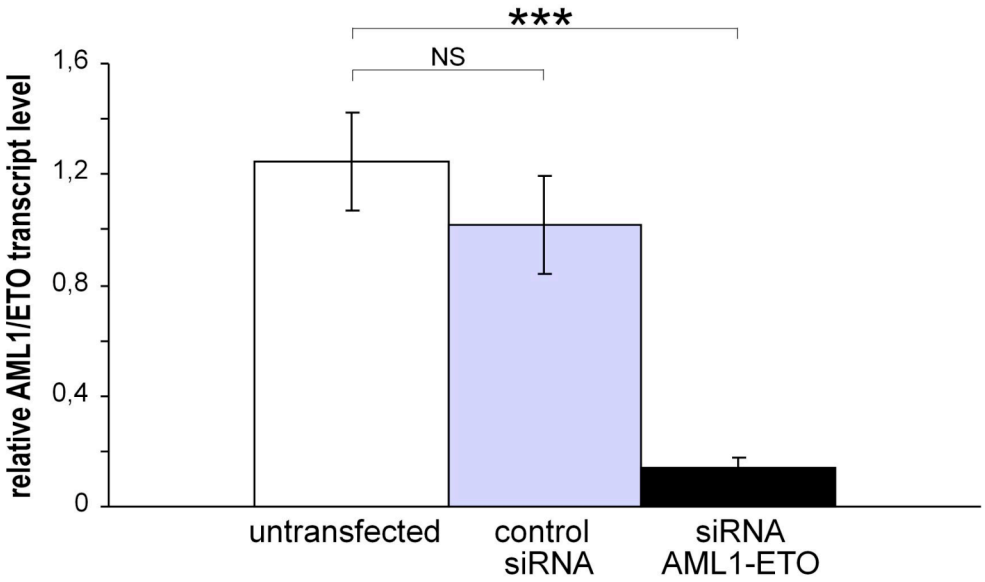

Figure 28. Quantitative RT-PCR analysis of AML1-ETO. Total RNA was isolated 48hr post-transfection of Kasumi-1 cells either without any siRNA or with non-specific siRNA or with siAM. The relative mRNA levels of AML1-ETO were normalized by β-actin-specific RT-PCR. Gene expression of tested mRNAs of untransfected Kasumi-1 cells was set to 1 (n=3). PCR primers are human-specific.

4.12.3 siRNA mediated reduce in colony formation efficiency

Here I addressed the question whether the AML1-ETO knock-down alone has any effect on colony forming cell fraction of Kasumi-1 cell population. 24 hour post-transfection with siAM or siCTL, 5000 Kasumi-1 cells were seeded per methyl cellulose plate and the plates were incubated in humidified 37°C incubator for 14 days. Result shows that Kasumi-1 cells transfected with siAM showed reduced number of colony forming cells in the population pool compared to the cells transfected with siCTL. This result (Figure 29) indicates that knocking down of AML1-ETO gene transcription alone can lead to significant decrease in colony forming cells in the AML cell pool. This result is comparable to the previously reported data (Heidenreich *et al.*, 2003).

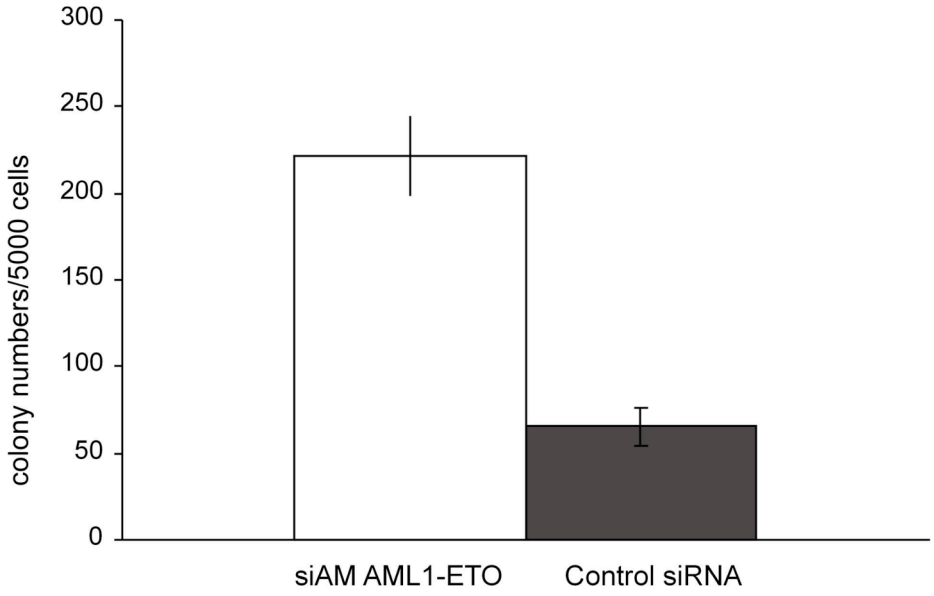

Figure 29. Effects of AML1-ETO knock-down on the clonogenicity of Kasumi-1 cells. $8X10^5$ Kasumi-1 cells were transfected with either control siRNA or siRNA specific to AML1-ETO (siAM). Cells were harvested 48hr post-transfection and seeded on methyl cellulose plates (5000 cells/plate) and 14 days after plating, colony numbers were scored (n=3).

4.12.4 siRNA mediated reduction in cell proliferation

Previously, I have seen coculture-induced down-regulation of AML1-ETO expression in Kasumi-1 cells in parallel with reduction in proliferation. Here I asked the question if AML1-ETO gene expression knock-down alone is sufficient to induce cell proliferation reduction in AML cells. I labelled the cells with CFSE and then split them to transfect with either siAM or siCTL. Subsequently, transfected cells were analyzed on 2, 3 and 4 day for CFSE retention by flowcytometry. The result shows that the Kasumi-1 cells transfected with siAM showed lower rate of CFSE loss compared to the Kasumi-1 cells transfected with siCTL (Figure 30). Therefore, knocking down AML1-ETO expression alone in Kasumi-1 cell leads to lower proliferation rates compared to the Kasumi-1 cells transfected with control siRNA.

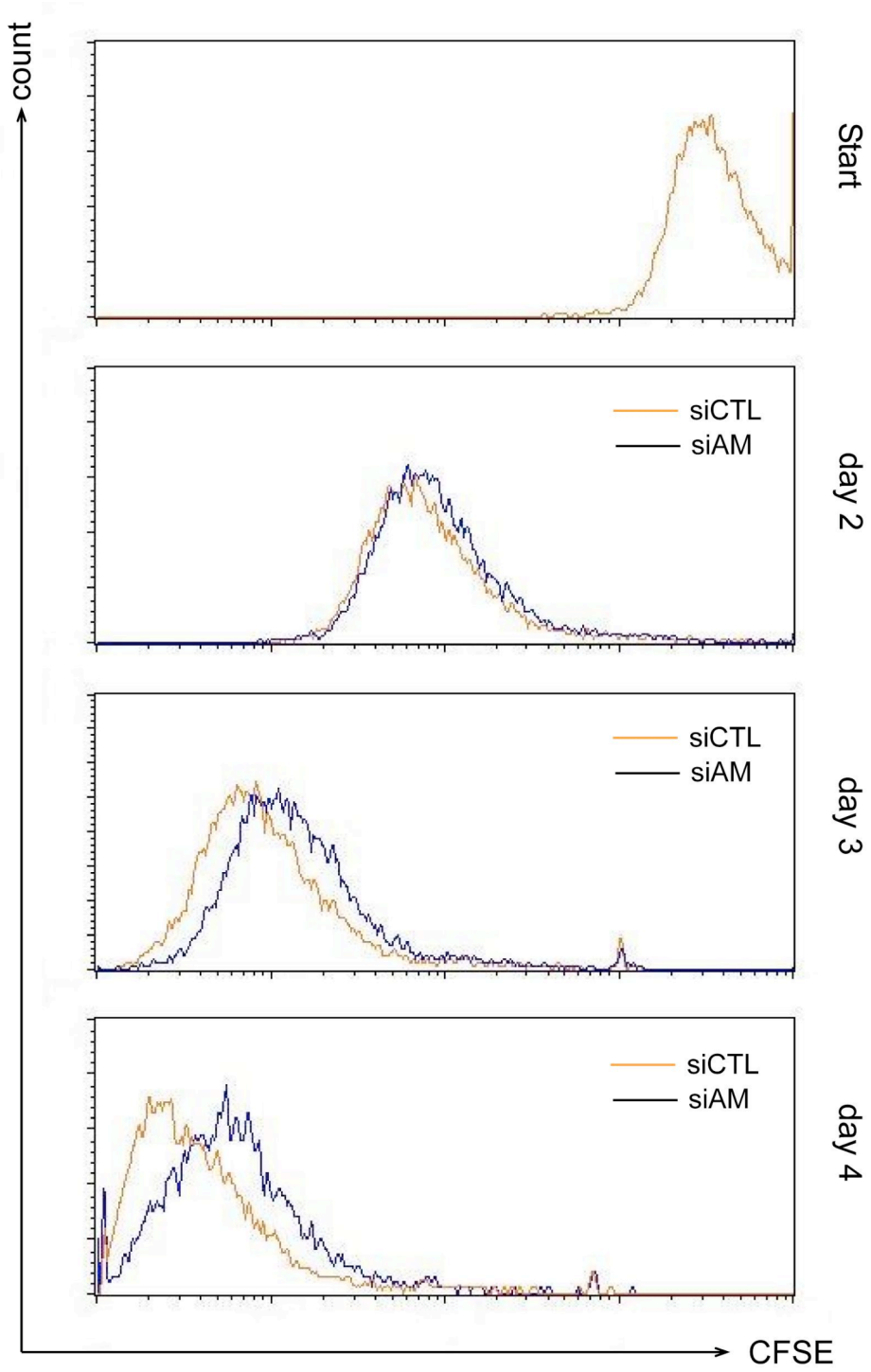

Figure 30. Effect of siRNA mediated knock-down of AML1-ETO transcription on proliferation rate of Kasumi-1 cells. Following CFSE labelling, Kasumi-1 cells were split and transfected with either control siRNA or siAM. Fluorescence profiles of the transfected CFSE-labelled Kasumi-1 cells are shown at the start and after 2, 3 and 4 days of coculture (n=2).

4.12.5 Effect of AML1-ETO knock-down on DAS 104-4 induced differentiation of Kasumi-1 cells

As DAS 104-4 coculture induces down-regulation in AML1-ETO transcript level in parallel with coculture-induced up-regulation in myeloid markers in Kasumi-1 cells, I asked the question if coculture-induced AML1-ETO down-regulation plays any role in DAS 104-4 stroma-induced myeloid differentiation in Kasumi-1 cells or they are just parallel events. Kasumi-1 cells were transfected with either siCTL or siAM. Transfected Kasumi-1 cells were either cultured without stromal cells or cocultured on either NIH 3T3 or DAS 104-4 cells for 3 days. Result shows that non-cocultured Kasumi-1 cells or Kasumi-1 cells cocultured on NIH 3T3, irrespective of either transfected with either siCTL or siAM, did not show any CD11b or CD14 staining. Therefore, it implicates that AML1-ETO transcription down-regulation by itself is not sufficient to induce myeloid differentiation of Kasumi-1 cells. Furthermore, only upon direct coculture on DAS 104-4, siAM transfected Kasumi-1 cells showed higher levels of CD11b and CD14 specific staining compared to the cocultured Kasumi-1 cells transfected with siCTL. These results indicate that AML1-ETO down-regulation alone in Kasumi-1 cells is insufficient to induce myeloid differentiation and it can only work synergistically along with coculture on DAS 104-4 stromal cells (Figure 31).

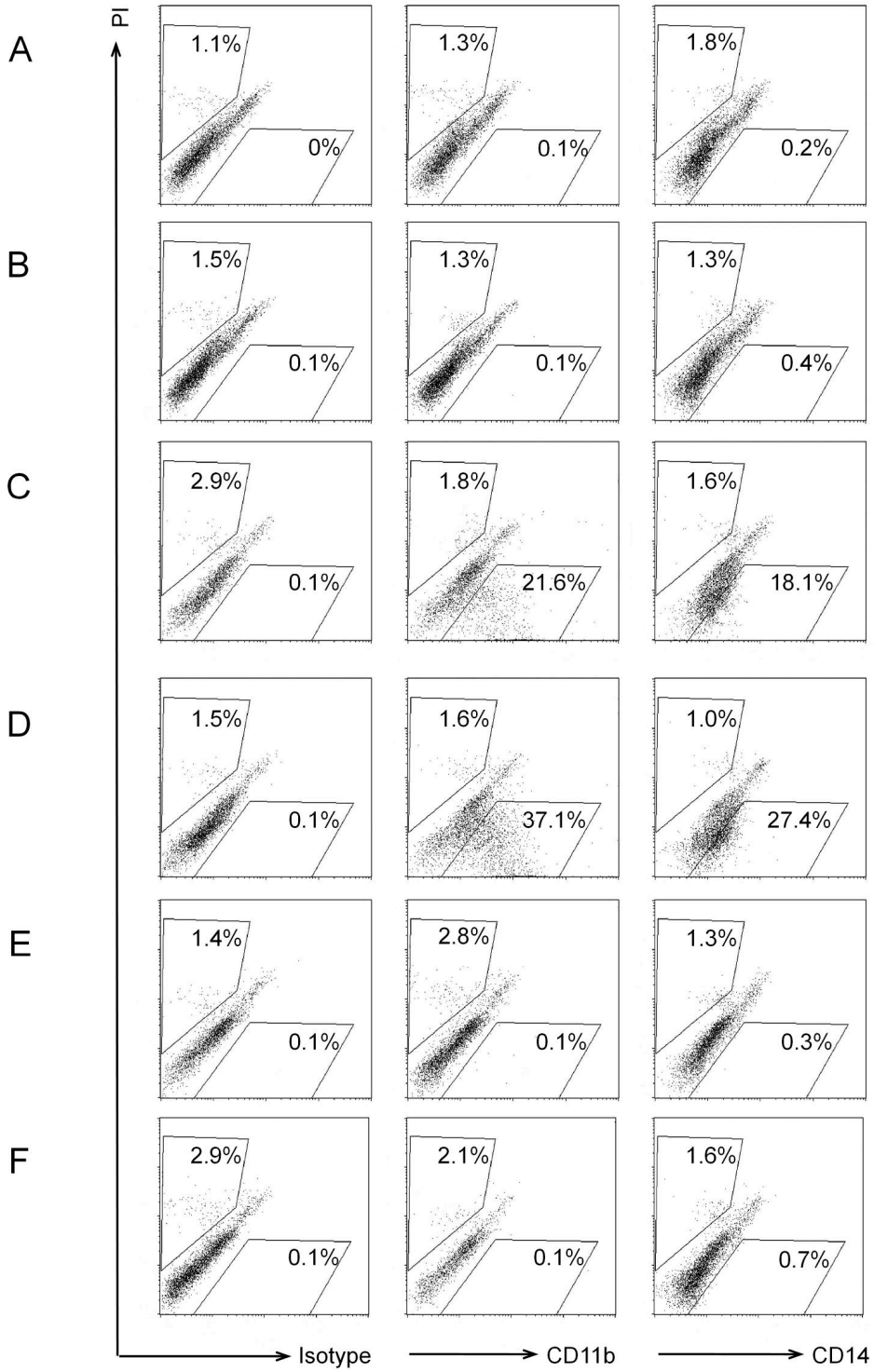

Figure 31. Effects of AML1-ETO siRNA-mediated down-regulation on cocultured Kasumi-1 cells. (A) non-cocultured Kasumi-1 cells transfected with siCTL. (B) non-cocultured Kasumi-1 cells transfected with siAM. (C) Kasumi-1 cells transfected with siCTL, cocultured on DAS 104-4 cells. (D) Kasumi-1 cells transfected with siAM, cocultured on DAS 104-4 cells. (E) Kasumi-1 cells transfected with siCTL, cocultured on NIH 3T3 cells. (F) Kasumi-1 cells transfected with siAM, cocultured on NIH 3T3 cells. Percentages of PI^+ dead cells, of $CD11b^+$ and $CD14^+$ cells are indicated. FACS analyses were done after 3 days of coculture with transfected cells. Live gate on FSC/SSC profiles, enriched in living AML cells and depleted in stromal cells, was used to acquire the readings. A representative experiment is shown (n=3).

5 DISCUSSION

Aim of this study was to analyze the effect of stroma environment on leukaemic cells *in vitro*. It is known that normal haematopoietic cell proliferation and differentiation *in vivo* are tightly regulated both by soluble factors as well as complex network of cell-to-cell contacts provided by the stromal cells in their niche. Moreover, the picture generating from molecular and functional data indicates that molecular crosstalk between normal haematopoietic cells and their niche is orchestrated by large number of molecules like cadherins, integrins, chemokines, cytokine signalling molecules and receptors. It is well documented that stromal cell lines isolated from embryonic or foetal haematopoietic sites maintain or even support HSC activity *ex vivo* (Moore *et al.*, 1997; Ohneda *et al.*, 1998; Oostendorp *et al.*, 2002; Yoder *et al.*, 1995). While some stroma cells support HSC maintenance other stromal cell lines induce haematopoietic differentiation (Ohneda *et al.*, 1998; Yoder *et al.*, 1994). Therefore, haematopoietic stromal cells may play extensive roles in growth, proliferation, survival and maintenance of normal haematopoietic cells.

In contrast to the present belief that the tumor development and progression is a cell autonomous process and solely depends on genetic and epigenetic features of tumor cells, recent published data indicate that the microenvironment plays a profound influence on tumor cells and their malignant features. Moreover, it has been shown that in case of both chronic myeloid leukaemia and AML are associated with change in composition of stromal compartment (Mayani, 1996). This has been suggested in previous studies, in which stromal cells were shown to prevent spontaneous or induced apoptosis in AML cells (Bendall *et al.*, 1998). Coculturing on the bone marrow-derived MS-5 stromal cell line can rescue the PML cell lines NB4 and HL60 from apoptosis by stroma-induced up-regulation of anti-apoptotic BCL2 expression in the stroma supported AML cells (Konopleva *et al.*, 2002). These results indicate that the stroma cells may play an active role in leukemogenesis and they add additional layer to the complex regulatory network controlling tumor development and progression.

Previous work in the laboratory addressed the hypothesis that the murine embryonic microenvironment can induce the differentiation of human tumor cells. To examine such interactions, human leukaemic AML cells were injected into pre-implantation murine blastocysts at embryonic day 3.5 of gestation. Analysis of developing mice revealed the presence of human AML cells in chimeric embryos and adults, albeit at lower frequency, and

the appearance of erythroid differentiation markers on progeny of injected human AML cells. These chimaeric animals grow and survive normally without succumbing to leukaemia (Durr *et al.*, 2003). In contrast, transplantation of leukaemic cells into adult murine recipient eventually leads to proliferation of AML blast cells (Henschler *et al.*, 2005). This finding strengthens the notion that the embryonic microenvironment is capable of regulating the proliferation and differentiation of leukaemic AML cells.

In this study, I have investigated the effect of stroma environment on leukaemic cells *in vitro*. For this purpose I have analyzed whether proliferation and differentiation properties of AML cells are influenced by direct or indirect coculture with haematopoietic stromal cells from embryonic and foetal sources. Results from the coculture experiments, showed that coculture with AGM-derived DAS104-4 cells can induce myeloid differentiation not only in established AML cell lines like Kasumi-1or KG-1, but it can also cause primary AML cells derived from patient to undergo myeloid differentiation. Whereas other tested murine embryo-derived haematopoietic stromal cells like AGM-derived DAS104-8, Yolk sac endoderm-derived YSE, yolk sac mesoderm-derived YSM and a foetal liver-derived AFT024 were ineffective in inducing myeloid differentiation in neither AML cell line or in patient derived M2-AML cells. Furthermore cocultivation with DAS104-4 stroma shows upregulation of erythroid specific markers such as β-globin and Glycophorin A in the cocultivated KG-1 cells. These observation support the previously observed results that KG-1 cells can be reprogrammed by murine blastocysts microenvironment which leads to predominantly erythroid differentiation of KG-1 cells. These observation further implicate the spectrum of stroma environment effect can be context specific i.e., depending on the nature of AML cells.

Further, coculture experiments with direct and transwell coculture of AML cells on DAS104-4 showed that only directly cocultured Kasumi-1 cells showed the immunophenotype of differentiated myeloid cells. Kasumi-1 cells indirectly cocultured with DAS104-4 showed no sign of myeloid differentiation. In indirect coculture, AML cells and stroma cells were separated by 0.4µm transmembrane, by which any cell-cell interaction between stroma and AML cells was avoided, allowing only soluble factors to diffuse through the membrane. Therefore, it can be interpreted that direct cell-to-cell contact is essential for DAS104-4 induced differentiation of Kasumi-1 cells.

Comparing proliferation rate of Kasumi-1 cells either cultured without stromal cells or directly cocultured on DAS104-4 or NIH3T3 showed that upon direct cocultivation on DAS104-4, Kasumi-1 cells shows reduction in proliferation, whereas Kasumi-1 cells cocultured on NIH3T3 show similar rate of proliferation as Kasumi-1 cells cultured without any stromal cells. These results indicate that DAS104-4 stroma environment has specific proliferation reduction activity on Kasumi-1 cells. Similar results were observed when the number of Kasumi-1 cells was counted on the basis of trypan blue exclusion microscopy after they were either simply cultured without stroma or directly cocultured either on DAS104-4 or AFT024 or NIH3T3. Kasumi-1 cell counts cocultured on either NIH3T3 or AFT024 did not show significant variation than the Kasumi-1 cells cultured without any stroma. In contrast, there was significant reduction in the Kasumi-1 cell count compared to non-cocultured control, when they were cocultured on DAS104-4. Further, cell cycle analysis of the cells directly cocultured on DAS104-4 for 3 days showed accumulation of cells in G_1 phase compared to the non-cocultured Kasumi-1 cells. In summary, DAS104-4 environment reduces the proliferation of Kasumi-1 cells. Interestingly, comparison among proliferation rates of Kasumi-1 cells directly cocultivated on DAS104-4 feeder or transwell separated coculture or the coculture experiment when the DAS104-4 stroma layer was fixed with 2% PFA showed that for maximum AML proliferation reduction activity, direct stroma-AML contact is needed. Our observation is analogously consistent with the previous report showing that the normal haematopoietic progenitor cells cultured physically separated from stroma in a transwell plate shows higher proliferation than the progenitor with direct stroma contact (Hurley *et al.*, 1995). Therefore, for most efficient AML proliferation reduction, direct contact with the metabolically active stroma is an important requisite.

Complementing to the AML cell proliferation reduction ability, the direct coculture on DAS104-4 cells also showed reduction of colony forming cells from the population pool of Kasumi-1 cells. In contrast, the Kasumi-1 cells directly cocultured on NIH3T3 or AFT024 showed similar levels of CFCs compared to the Kasumi-1 cells cultured without any stromal cells. This is a very interesting observation as the colony forming cells on semisolid medium are the cells that really drive the population. Therefore, DAS104-4 stroma-induced reduction in Kasumi-1 CFCs indicates towards stroma-induced reduction in tumorigenic potential of Kasumi-1 cells.

To focus more light on molecular consequences and the governing mechanism of coculture-induced effect on AML cells, we compared and quantitated transcript levels of some important myeloid specific transcription factors and cell cycle regulatory genes. Quantitative RT-PCR analysis indicated that there is coculture-induced up-regulation of transcription factors such as CCAAT/enhancer binding protein alpha (C/EBPα) and ETS family transcription factor PU.1. Previously, it has been shown that both of these transcription factors are crucial for myeloid differentiation. The murine hypomorphs for these transcription factors are spontaneously affected with myeloproliferative disorder (Mueller *et al.*, 2002). Moreover, it has been shown that other than working as a transcription factor, C/EBPα can physically interact with crucial cell cycle regulatory kinases like CDK2 and CDK4 and promotes cell cycle check point block. Therefore, we find an indirect correlation that perhaps up-regulation of PU.1 and C/EBPα may be the important event during DAS104-4 coculture-induced reduction in proliferation, G_1 phase accumulation and differentiation of Kasumi-1 cells. The comparative analyses of crucial cell cycle regulatory genes like p16 and p21 show DAS104-4 coculture-induced up-regulation in transcript levels, again this is consistent with the observed coculture-induced reduction in proliferation of Kasumi-1 cells that we have discussed above.

Often AML cells carry chromosomal aberrations like translocations. The t(8:21) type of translocation is one of the prevalent genetic aberration among FAB class M2 – the most predominant class among all AML classes (Lowenberg *et al.*, 1999). The t(8:21) translocation generates the leukaemic AML1-ETO fusion gene which is associated with 10-15% of all *de novo* cases of AML (Heidenreich *et al.*, 2003). This translocation event leads to fusion of the acute myeloid Leukaemia-1 (AML1, RUNX1) transcription factor gene located at chromosome 21 to the eight twenty-one (ETO, MTG8, CBF2T1) and joins the DNA binding part of AML1 to the ETO co-repressor. This leads to conversion of a transcriptional activator of definite haematopoiesis into a constitutive and transdominant transcriptional repressor. The importance of the AML1-ETO is indicated in the observation that AML1-ETO down-regulation in an AML cell line renders it sensitive to the differentiation inducing agents. Pabst *et al.* (2001) demonstrated that the AML1-ETO fusion protein suppresses C/EBPα expression. The myeloid transcription factor C/EBPα is crucial for normal granulopoiesis, and dominant-negative mutations of the C/EBPα gene are found in a significant proportion of patients with myeloblastic subtypes (M1 and M2) of AML. Analysis of AML1-ETO transcript levels in Kasumi-1 cells either cultured alone or cocultured either on DAS104-4 or NIH3T3 or AFT024 reveals down-regulation of AML1-ETO transcript in DAS104-4 cocultured Kasumi-1 cells. Whereas, there is no difference between AML1-ETO transcript levels of non-cocultured

cells or cells cocultivated either on NIH3T3 or AFT024 cells. Further analyses of Kasumi-1 cells, directly or indirectly cocultured on DAS104-4, display stroma contact-dependent decrease in AML1-ETO transcript level, which correlate well with the functional data such as stroma contact-dependent myeloid differentiation of the Kasumi-1 cells.

From the previous studies, it has been shown (Charrad *et al.*, 1999) that many AML primary and established cell lines can be induced to differentiate by activation of CD44 receptor by ligating antibodies. And even *in vivo* administration of CD44 ligating antibodies to the non-obese diabetic-severe combined immuno-deficient (NOD-SCID) mice transplanted with human AML showed marked reduction in leukaemic repopulation. As staining for CD44 showed that all three AML cell lines i.e., Kasumi-1, KG-1 and NB4 show positive staining for CD44 receptor on the surface, further it was interesting to see if the activation of CD44 is just the underlying mechanism for the DAS104-4 environment-induced myeloid differentiation of Kasumi-1 cells. However, my result indicated that DAS104-4 induced differentiation is independent of CD44 activation. Hence, the embryonic stromal environment may provide an alternative means to differentiate human AML cells.

To understand the possible role of AML1-ETO transcription down-regulation in coculture-induced effect on AML cells, transcription from AML1-ETO gene was knocked down and the consequences were analyzed. Upon siRNA mediated knockdown of AML1-ETO gene expression alone, Kasumi-1 cells showed lower rate of proliferation, together with decrease in colony forming cells. However, knocking down the expression of AML1-ETO alone by itself was not found to be sufficient for myeloid differentiation. However, in combination with DAS104-4 cocultivation, siRNA mediated AML1-ETO knock down compared to the cells transfected with control siRNA showed additive effect on coculture mediated Kasumi-1 myeloid differentiation. This indicates that AML1-ETO down-regulation may have an active role on the coculture-induced effect but in addition to AML1-ETO down-regulation further stimuli are required for the coculture-induced myeloid differentiation in the AML cells.

The concept emerging from this study indicates that the stroma environment can affect leukaemic cell proliferation and differentiation. In the present scenario, stroma may elicit atleast two different signals to the responding leukaemic cells: One soluble signal that can diffuse through the transwell membrane and causing reduction in proliferation without having any direct effect on differentiation and second signal that is cell contact-dependent and which can account for both differentiation induction and cell proliferation reduction. The results described here add a new prospective to our understanding of interaction between leukaemic

cells and its environment. Furthermore, this study encourages further investigation into the mechanistic details of molecular and cellular interaction between the leukaemic cells and their stroma, which in turn may lead to the identification of new paradigm for active control and reprogramming of human leukaemic cells.

6. MATERIALS AND METHODS

6.1 Materials

6.1.1 Antibodies

6.1.1.1 Primary antibodies

Specificity	Clone	Isotype	Conjugate	Supplier
β–Actin	I-19	IgG1	HRP	Santa Cruz
CD117 (c-kit)	3H389	IgG1	FITC	United States Biologicals
CD11b	44	IgG1	FITC	Cymbus Biotech
CD14	2D-15C	IgG1	FITC, Biotin	Chemicon
CD15	ZC-18C	IgM	FITC	Chemicon
CD235a	YTH89.1	RAT IgG2b	no conjugate	DPC Biermann
CD38	T16	IgG1	FITC	Immunotech
CD44	A3D8	IgG1	no conjugate	Sigma-Aldrich
CD45	HI30	IgG1, κ	FITC	BD Bioscience
ETO	C-20	IgG1	no conjugate	Santa Cruz Biotech
Mouse CD16/CD32	2.4G2	IgG2a, κ	no conjugate	BD Bioscience/made in lab
Mouse IgG1 isotype control	-	IgG1	FITC	Chemicon

6.1.1.2 Secondary antibodies

Specificity	Host	Conjugate	Supplier
Goat	Cow	HRP	Dianova
Mouse	Goat	Cy3	Chemicon
Rat	Goat	FITC	Dianova

6.1.1.3 Conjugate for antibody

Conjugate	Supplier
Strptavidin-FITC	BD Bioscience

6.1.2 Cell culture related materials

10% BSA in Iscove´s MDM	StemCell Technologies Inc
ATRA	Sigma-Aldrich
β-Mercaptoethanol	Merck
Bovine serum albumin	Boeringer Mannheim
Dulbecco´s MEM	GibcoBRL
Foetal calf serum	Biochrom KG
Ficoll	Sigma-Aldrich
IL-3	Stem Cell Techologies Inc
Iscove´s MDM	GibcoBRL
L-Glutamine	GibcoBRL
MethoCult™ M3134	StemCell Technologies Inc
Non-essential amino acids	GibcoBRL
Penicillin/Streptomycin	GibcoBRL
RPMI 1640	PAA
Sodium pyruvate	GibcoBRL
Streptomycin	Sigma-Aldrich
Trypsin-EDTA (1X)	GibcoBRL

6.1.3 Consumables

3mm-Whatman paper	Scheicher & Schüll
Cell sieve	BD, Falcon
Cover slip	Hartenstein
Disposable gloves	Kimberly-Clark
FACS tubes	Elkay, Greiner
Filter cup	Nalgene
Nitrocellulose membrane	Scheicher & Schüll
Pipette tips	University store
Plastic material for cell culture	Falcon, Greiner, Nunc, Sarstedt
Scalpel	Ratiomed
Sterile filter	Schleicher & Schüll
Super-Frost® Plus slide	Menzel-Gläser
Syringes	Braun, Dispomed
X-ray film	Fuji, Sigma-Aldrich

6.1.4 Chemicals

4´,6-Diamidin-2-phenylindol (DAPI)	Sigma-Aldrich
7-AAD	Sigma-Aldrich
Acetic acid	Applichem
Actinomycin D (AD)	Sigma-Aldrich
Agarose NEEO (Ultra Quality)	Roth AG
Ammonium persulfate (APS)	Sigma-Aldrich
Blocking milk powder	Roth
Boric acid	Applichem

Bovine serum albumin	Boeringer Mannheim
CFSE	Molecular Probes
Dextran T500	Roth
Diff-Quik®	Dade Behring
Dithiotreitol (DTT)	Applichem
DMSO	Sigma-Aldrich
DNA size marker	MBI Fermentas
EDTA	Applichem
Ethanol	Applichem
Ethidium bromide	Merck
Gelatin	Sigma-Aldrich
Glycerol	Applichem
HCl, 37%	Applichem
Hepes	Sigma
Isopropanol	Applichem
Methanol	Sigma-Aldrich
Mounting medium	DAKO corporation
N,N,N´,N´-Tetramethylendiamin (TEMED)	Sigma-Aldrich
Para formaldehyde (PFA)	Applichem
Phenol-Chloroform-Isoamyl alcohol	Applichem
Potassium chloride	Applichem
Protein size marker	MBI Fermentas
Rotiphorese® Gel 30	Roth
SDS	Sigma-Aldrich
Sodium azide	Applichem
Sodium borate	Applichem
Sodium chloride	Applichem
Sodium citrate	Applichem
Sodium hydroxide	Applichem
SYBR green mix	Fynnzyme
Tris	Applichem
Triton® X-100	GibcoBRL
Trypan blue	Sigma-Aldrich
Tween 20	Applichem
Water Ultra pure	Merck
Xylol	Applichem

6.1.5 Instruments

Agarose gel cast	MSZ workshop
Cell freezing container	Nalgene
Centrifuge	Hettich, Eppendorf, Sorvall
CytoSpin	ThermoShandon
Digital camera	Olympus
Digital weighing balance	Sartorius
Film developer machine	Agfa
Flow cytometer	Becton Dickinson

Gamma ray irradiation	Central facility MSZ
Gel and blotting chamber	BioRad
Incubator	Forma Scientific
Inverted microscope	Zeiss
Neubauer chamber	Marienfeld
pH-Meter	Knick
Photometer	Eppendorf
Real Time PCR	Gene Rotor
Sterile bench	Heraeus
T3 Thermocycler	Biometra
Vortexer	Scientific Industries
Water bath	GFL

6.2 Cell culture

6.2.1 AML and stromal cells and their maintenance

Human leukaemic Kasumi-1 (DSMZ, Braunschweig, Germany) (Asou *et al.*, 1991); KG-1 (DSMZ, Braunschweig, Germany) (Koeffler *et al.*, 1981); EOL-1 (Saito *et al.*, 1985) and NB-4 cells (Lanotte *et al.*, 1991) and murine stromal cells AFT 024 (Moore *et al.*, 1997); DAS 104-4 (Ohneda *et al.*, 1998) and DAS 104-8 (Ohneda *et al.*, 1998), YSE and YSM (Yoder *et al.*, 1995) were expanded in RPMI 1640 (PAA) in the presence of 20% FCS, penicillin 100units/ml, streptomycin 100µg/ml and 10mM 2-ME. Cells were cultured in a humidified 37°C incubator with 5% CO_2. Primary M2-AML cells were kindly provided by Prof. Reinhard Henschler (Institute for transfusion medicine and immuno-haematology, University of Frankfurt). Patient-derived primary AML cells are originating from a 70 year-old male patient with FAB M2 AML and derived observing approved institutional procedures for obtaining informed consent according to the declaration of Helsinki (Ethics Committee, Medical Faculty, University of Frankfurt, Germany). The primary AML cells were expanded in the cell culture medium, RPMI 1640 (PAA), in the presence of 20% FCS, penicillin 100units/ml, streptomycin 100µg/ml and 10mM 2-ME.

6.2.2 Passaging of Stromal cell lines

Stromal cell lines proliferate very well and must be passaged every 3-4 days. 70-80% confluence is the best and before adding 1ml of 1X Trypsin-EDTA (Gibco) to dislodge the stromal cells from the plates, stromal monolayer is washed once by 1X PBS. Then 5ml of the medium is added followed by flushing of cells in and out by 5ml pipette 3-4 times to ensure that the cells are not clumped before plating.

6.2.3 Freezing of AML and Stromal cell lines

1. Spin down the cells at 200xg (RCF) for 10 min, RT
2. Resuspend in 90% foetal calf serum and 10% dimethylsulphoxide (DMSO)
3. Freeze $2x10^6$/ml (min - $1x10^6$/ml; max - $5x10^6$/ml), approximately 1.8ml/vial
4. Freeze in a freezing box with isopropanol at -70°C overnight
5. Next day put the vials in liquid nitrogen

6.2.4 Thawing of AML and Stromal cell lines from liquid nitrogen storage

1. Put the vial into a water bath at 37°C for a short time (1min)
2. Pour the cells into a 50ml Falcon tube in the laminar flow hood
3. Dilute slowly by adding 10ml complete medium drop wise over 10min
4. Leave the cells for 10min in the laminar flow hood
5. Spin them down at 200xg, 10min, RT
6. Pour off the supernatant
7. Resuspend the pellet in 5ml complete medium
8. Pour into T25 Falcon culture flask, leave in the incubator (5% CO2, 98% humidity, 37°C) overnight

6.2.5 Removing of dead AML and Stromal cell lines cells

1. Count the living cells the day after thawing in the 5ml culture
2. Pour Histopaque™ in a sterile tube under the hood
3. Drop 5ml of cell suspension over Histopaque™, carefully, do not disturb the interface
4. Spin down at 400xg (RCF), 30min, RT
5. The live cells accumulate at the interface while the dead cells sink to the bottom
6. Collect the cells off the interface (take 5ml of cell suspension plus 1ml interface/Histopaque™) into a 15ml Falcon tube which contains 5ml of medium
7. Spin down again at 200xg (RCF), 10min, RT
8. Pour off the supernatant and resuspend the pellet at $8x10^4$/ml cells in medium

6.3 Coculture

6.3.1 Feeder preparation and direct coculture

Stromal cells are grown to the log phase by splitting them one day before irradiation. For irradiation, cells are trypsinized and counted; 2-5x10^6 cells are suspended in 1 ml of cell culture medium and irradiated at 40Gy (single dose, ^{137}Cs-Gammatron, 0.511MeV, dose rate=1.7Gray/min). After irradiation, cells are plated to confluence. On the next day, 5x10^5 leukaemic cells are seeded directly onto stromal cells and cocultivated for given period of time (Figure 32). Cocultured leukaemic cells are harvested by mild washes with culture medium. The recovery rate of cocultured leukaemic cells was in the range of 60-80% as analyzed by microscopy using the cell size difference between leukaemic and stromal cells as parameter.

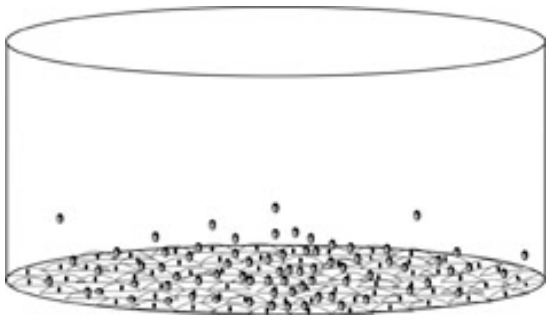

Figure 32. Sketch showing directly cocultured AML cells on irradiated feeder monolayer. 3x10^5 γ-irradiated cells (40Gy) are seeded in each well of 6 well polystyrene tissue culture plate (Greiner bio-one) and incubated for overnight in the incubator (5% CO_2, 98% humidity, 37°C) to make stromal monolayer with ≥80% confluence. On the following day, the old medium is aspirated and 3ml/well fresh medium with 5x10^5 AML cells are seeded directly on the stromal monolayer.

6.3.2 Indirect coculture

Exponentially growing stromal cells are irradiated with 40Gy. After irradiation, cells are plated to confluence. 0.4µm pore size transwell insets (Costar) are used for indirect coculture. Transwell insets are placed gently on the wells with irradiated confluent stroma and the 5x10^5 leukaemic cells are seeded on the transwell (Figure 33). Cocultured leukaemic cells are harvested by mild washing of transmembrane inset with culture medium.

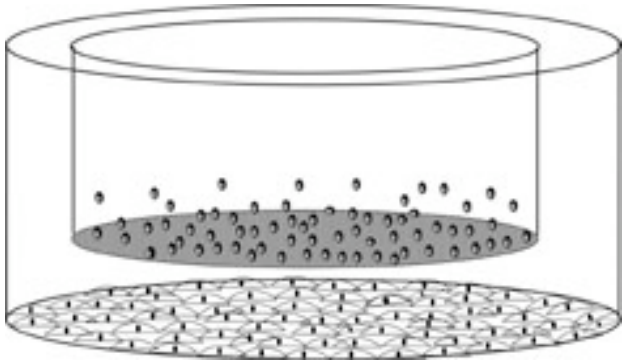

Figure 33. Sketch showing indirect cocultured AML cells on irradiated feeder monolayer. $3x10^5$ γ-irradiated cells are seeded in each well of 6 well tissue culture plate (Greiner bio-one) and incubated overnight in the incubator (5% CO_2, 98% humidity, 37°C) to make stromal monolayer with ≥80% confluence. On the following day, the old medium is aspirated and fresh 1ml/well medium is gently placed over the monolayer. Subsequently, polystyrene transwell insets with 0.4µm pore size polycarbonate membrane (Costar) are placed gently over the well. Finally, $5x10^5$ AML cells suspended in 2ml medium are gently placed on the membrane.

6.3.3 Coculture on fixed stroma

Stroma cells are irradiated with 40Gy and seeded till next morning to form confluent layer. The stromal monolayer is washed twice with 1X PBS before treating with 2% PFA in 1X PBS for 5 minutes. Later, the monolayer is washed 5 times with PBS to remove any trace of fixing solution before seeding $5x10^5$ leukaemic cells for coculture (Figure 34). Cocultured leukaemic cells are harvested by mild washes with culture medium. The recovery rate of cocultured leukaemic cells was in the range of ≥80% as analyzed by microscopy using the cell size difference between leukaemic and stromal cells as parameter.

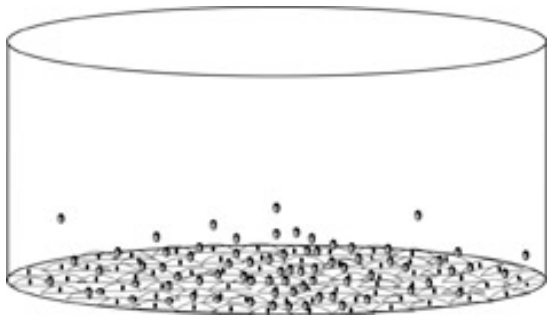

Figure 34. Sketch showing directly cocultured AML cells on irradiated fixed feeder monolayer. $3x10^5$ γ-irradiated cells (40Gy) are seeded in each well of 6 well polystyrene tissue culture plate (Greiner bio-one) and incubated for overnight in the incubator (5% CO_2, 98% humidity, 37°C) to make stromal monolayer with ≥80% confluence. On the following day, the old medium is aspirated followed by overlaying the monolayer with 3ml of fixing solution (2% PFA in 1X PBS). After incubation at room temperature for 10 min, the fixing solution is aspirated followed by five rounds of washing with 1X PBS to remove any trace of fixative agent. Finally, 3ml/well fresh medium with $5x10^5$ AML cells are seeded directly on the fixed stromal monolayer.

6.4 Flowcytometry

For immunophenotyping by flowcytometry, $2x10^5$ cultured cells are washed with FACS buffer (PBS, 0.4% BSA, 0.02% NaN_3, pH 7.4). After blocking of Fc receptors (FcgRII/III) with 2.4G2 antibody, cells are incubated with appropriate antibodies for 30min at 4°C. The following antibodies were used: IgGa isotype-FITC control (Chemicon), CD11b-FITC (Chemicon) and CD14-FITC (Chemicon). To visualize dead cells, 10µg/ml propidium Iodide (PI) (Sigma) is added and 10,000 PI-negative, living cells are acquired on a *FACSCalibur*.

6.5 Immuno-cytochemistry and morphological analysis

Immuno-cytochemistry is performed in the laboratory of Prof. Axel Greiner (Heidelberg) on cytospins, fixed with acetone for 10min at room temperature and air dried (Greiner *et al.*, 1997). Slides are incubated for 20min with 0.03% H_2O_2/PBS to inactivate endogenous peroxidase, followed by a blocking step with 10% horse serum/PBS for 20min at room temperature. The specific antibody (PGM1, DAKO) is diluted in 10% horse serum/PBS at a final concentration of 36µg/ml and added for 30min. For immuno-cytochemical detection, slides are incubated with a peroxidase-coupled rabbit anti-mouse antibody (DAKO) for 30min. The peroxidase was displayed using the Sigma Fast diaminobenzidine peroxidase substrate tablet set (Sigma). For morphological analysis of cocultured cells, $2x10^5$ cells were cytospun. Myeloid differentiation was quantified by Pappenheim staining (Chuang and Li, 1997).

6.6 CFSE staining

CFSE (5- and 6-carboxyfluorescein diacetate succinimidyl ester) is a fluorescein derivative that is cell permeable and non-fluorescent. The cleavage of the acetate groups by intracellular esterase renders the molecule fluorescent, and the attachment of the fluorescein group to all intracellular proteins via aminolysis of the succinimidyl ester ensures that the dye is retained within the cells (Figure 35A). As the cells divide, CFSE is distributed between the daughter cells (Lyons and Parish, 1994). Using flowcytometry, the rate of cell proliferation can be correlated with the rate of fluorescence loss (Figure 35B). For labelling the Kasumi-1 cells with CFSE, 5-$10x10^6$ Kasumi-1 cells are incubated for 8min at RT in 5µM CFSE solution (Molecular Probes). Further dye uptake is prevented and unincorporated CFSE is

sequestered and washed by adding excess RPMI medium/20% FCS. Prior to culturing, traces of unbound CFSE are removed by intensive washes of cells in RPMI medium/20% FCS. After growth for overnight, the cells are seeded for coculture on the following day. After coculture, cells are harvested and CFSE fluorescence is assayed on a *FACSCalibur* (Becton Dickinson).

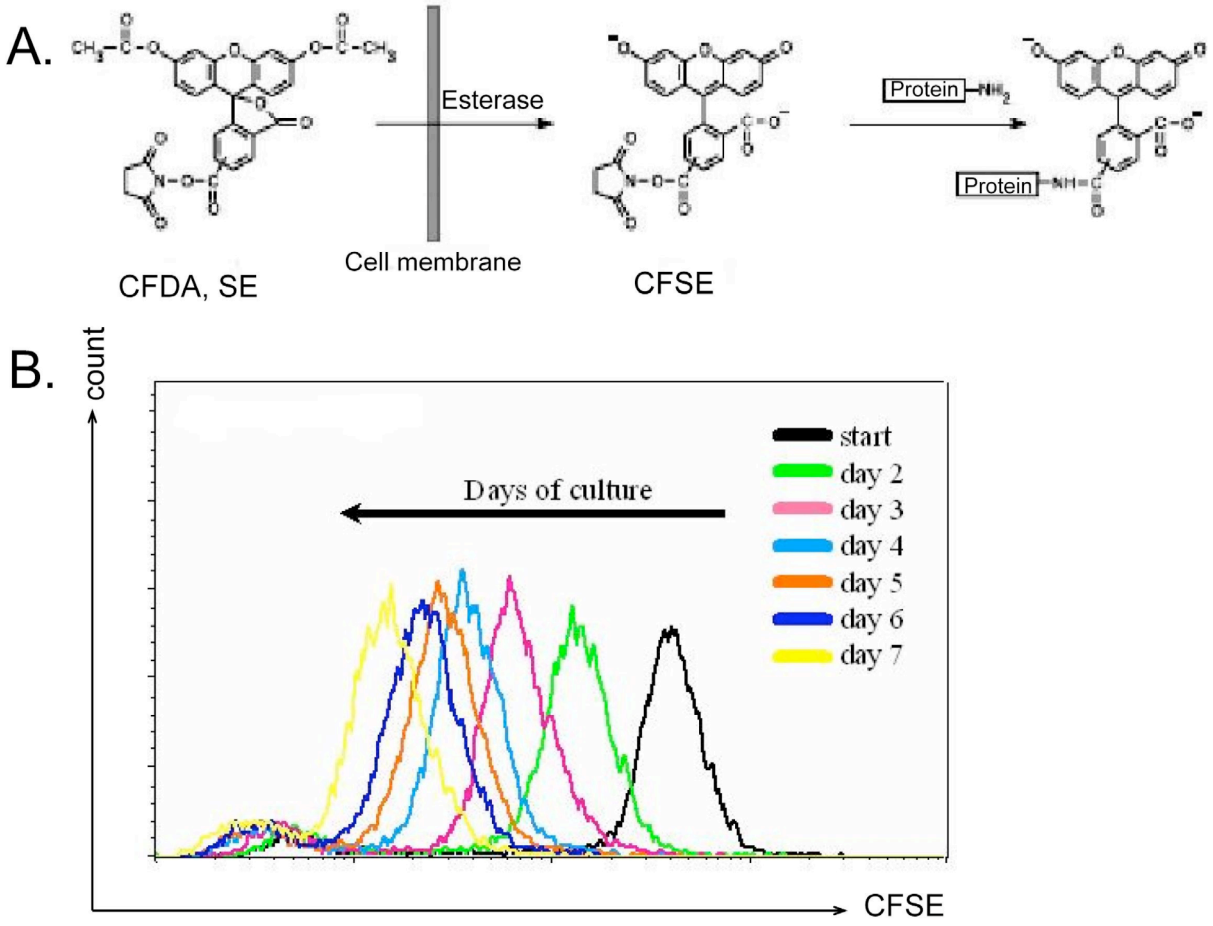

Figure 35. CFSE retention assay. (A) Showing scheme of CFSE incorporation and fluorescent labelling of intracellular proteins. (B) Showing an experiment where Kasumi-1 cells were labelled with CFSE and subsequently allowed to divide in optimum cell culture conditions, flowcytometric analysis of the CFSE labelled cells on subsequent days shows sequential reduction in fluorescence of Kasumi-1 cells.

6.7 Methyl cellulose colony forming assay

This method quantifies the amount of early progenitor cells in the total cell pool. The colony starting cells on semisolid medium are known as colony forming cells (CFCs). The semisolid methyl cellulose medium is based on IMDM medium with following components

- 40% v/v MethoCult™ M3134
- 30% v/v FCS
- 1% w/v BSA
- 100U/ml Penicillin/Streptomycin
- 2mM L-Glutamine
- $1x10^{-4}$M β-Mercaptoethanol

The semisolid medium is made and aliquoted in 15ml conical bottom tubes, special care is taken to avoid introduction of air bubbles. AML cells are counted and $1.5x10^4$ trypan blue-negative AML cells are suspended in 300µl of IMDM medium and added to the aliquoted 2.7ml MC medium, followed by mixing by vortexing to have even cell suspension. In doublet, 1ml MC cell suspension mix is plated carefully, without introducing any bubbles, on 36mm cell culture dishes. The plates are incubated in incubator (37°C, 5% CO_2 and 100% humidity)

6.8 Cell cycle analyses

One million cells are washed with FACS buffer, resuspended and incubated in PI staining solution containing 1.6% sodium citrate (Applichem), 0.04% Triton X-100 (BioChemika) and 0.1% PI (Sigma) and 20µg/ml RNAse (Sigma) for 10min followed by washing and suspending in FACS buffer. PI labelling is assayed with a *FACSCalibur*. The total DNA content is plotted as a function of PI-area. The Cylchred software version 1.0.2, based on algorithms by Watson *et al.* (Watson *et al.*, 1987), is used to determine cell cycle status.

6.9 RNA and DNA isolation

6.9.1 Isolation of DNA

1. Put the tissue or cells in 700µl of lysis buffer with proteinase K
2. Incubate over night (o/n) at 56°C in the rotation incubator
3. Add 7µl of RNAse to degrade RNA
4. Incubate for 30min at 37°C
5. Add an equal volume of Phenol-Chloroform, shake for 5min
6. Centrifuge for 10min at 20000xg (10000rpm)

7. Transfer supernatant to another tube; hydrophilic DNA is in aqueous phase of supernatant, while the proteins are in the interphase
8. Add an equal volume of phenol/chloroform to the water phase, shake for 5 min
9. Centrifuge for 10min at 10000rpm
10. Transfer the supernatant to a new tube
11. Add an equal volume of isopropanol to the water phase, shake
12. Incubate for 20min at -20°C
13. Centrifuge for 10min at 20000xg (14000rpm); DNA will be visible as a white pellet at the bottom of the tube
14. Remove supernatant and add 700µl of 70% ethanol (washing of DNA)
15. Vortex to dissolve the pellet
16. Centrifuge for 20min at 20000xg
17. Remove supernatant and add 700µl of 70% ethanol
18. Centrifuge for 20min at 20000xg
19. Remove ethanol and dry the DNA
20. Dissolve DNA pellet in ddH_2O

Measurement of DNA concentration

The aromatic rings of purine and pyrimidine bases of nucleic acids dissolved in water absorb the light at 260nm wave length. The concentration of DNA and optical density at 260nm (OD_{260}) are in linear relationship so it is possible to calculate the concentration with very simple formula:

C= OD_{260}x 50µg/ml double stranded DNA

C= OD_{260}x 40µg/ml single stranded DNA

C= OD_{260}x 33µg/ml single stranded RNA

Bio Photometer (Eppendorf) is used for measuring of DNA concentration.

6.9.2 RNA isolation and RT-PCR

Wash 1 million cells once in PBS and pellet by centrifuging for 10 minutes at 5000xg (3500rpm).

Resuspend the cell pellet in 700µl of RNA peq gold, mix by gentle pipetting

Add 100µl of chloroform, mix by gentle vortexing

Centrifuge for 10min at 20000xg (14000rpm)

Transfer the aqueous supernatant to fresh tube

Add equal volume of isopropanol to the aqueous phase and mix gently by inverting.

Precipitate nucleic acid at -20°C for 20 minute

Centrifuge for 10min at 20000xg (14000 rpm)
Discard the supernatant and resuspend the pellet in 1ml of 70% Ethanol.
Centrifuge for 10min at 20000xg (14000 rpm)
Discard the supernatant and again resuspend the pellet with 1ml of ethanol for second wash
Centrifuge for 10min at 20000xg (14000 rpm)
Decant the supernatant and suspend the pellet in 20µl DEPC-treated water
Bio Photometer (Eppendorf) is used for quantitation of RNA

6.9.2.1 DNAse treatment

To remove the contaminating genomic DNA from the total RNA, the RNA sample is treated with RNAse free recombinant DNAse by the following method:

Mix 1µg of RNA with 1.3µl of 10X DNAse buffer (Ambion) and add DEPC treated water to 12µl, finally add 1µl (2U) of DNAse (Ambion)
Incubate for 30 minute at 37°C
Stop reaction by adding 1µl of 25mM EDTA, 10min inactivation at 65°C.
Half of the reaction mix containing 0.5µg RNA is used for cDNA synthesis and half for "no reverse transcription control"

6.9.2.2 First strand synthesis

Reverse transcription reaction to synthesize first cDNA strand from DNAse treated total RNA include 0.5µg of RNA in 6.5µl volume, 1µl (200ng) of oligo d-T (16-mer) and 5µl of DEPC treated water, the total mix is heated at 65°C for 5 minutes to resolve all secondary RNA structures. Then 1µl of dNTPs (10mM), 4µl of 5X first strand synthesis buffer, 2µl of 100mM DTT and 0.5µl of M-MLV (Invitrogen) are added to make final 20µl mixture, incubation for 1hr at 37°C.
cDNAs are then stored at -20°C.

6.9.2.3 Polymerase chain reaction (PCR)

The amplification of specific DNA fragments is performed in T3 Thermoblock (Biometra, Goettingen). Total cDNA is used as template. All the oligonucleotides (primers) are synthesized by MWG Biotech.

Human β–Actin PCR

PCR reaction:
5µl 10X PCR buffer
1µl dNTPs (2.5mM each)
200ng sense primer (5´-AGCCTCGCCTTTGCCGA-3´)
200ng antisense primer (5´-CTGGTGCCTGGGGCG-3´)
0.15µl Super Taq Polymerase (5U/µl)

Add ddH_2O up to 50µl

95°C 5min 1 cycle
95°C 30sec
60°C 30sec 36 cycles
72°C 90sec
72°C 2min 1 cycle
Fragment size: 180bp

Primer sequences for PCR are taken from literature (Kreuzer *et al.*, 1999)

AML1-ETO PCR

PCR reaction:
5µl 10X PCR buffer
1µl dNTPs (2.5mM each)
200ng sense primer (5'- AGCTTCACTCTGACCATCAC -3')
200ng antisense primer (5'- TCAGCCTAGATTGCGTCTTC -3')
0.15µl Super Taq Polymerase (5U/µl)

Add ddH_2O up to 50µl

95°C 5min 1 cycle
95°C 60sec
60°C 30sec 35 cycles
72°C 35sec
72°C 2min 1 cycle
Fragment size: 200bp
Primer sequences for PCR are taken from literature (Mrozek *et al.*, 2001)

C/EBPα PCR

PCR reaction:
5µl 10X PCR buffer
1µl dNTPs (2.5mM each)
200ng sense primer (5'-GGGCCAGGTCACATTTGTAAA-3')
200ng antisense primer (5'-AGTAAGTCACCCCCTTAGGGTAAGA-3')
0.15µl Super Taq Polymerase (5U/µl)
200ng DNA

Add ddH_2O up to 50µl

95°C 5min 1 cycle
95°C 10sec
60°C 30sec 32 cycles
72°C 35sec
72°C 2min 1 cycle
Fragment size: 110bp
Primer sequences for PCR are taken from literature (Ishiyama *et al.*, 2003)

β-globin PCR

PCR reaction:
5µl 10X PCR buffer
1µl dNTPs (2.5mM each)

200ng sense primer (5'-GAGAAGTCTGCCGTTACTGCC-3')
200ng antisense primer (5'- CCGAGCACTTTCTTGCCATGA-3')
0.15µl Super Taq Polymerase (5U/µl)

Add ddH_2O up to 50µl

95°C 5min 1 cycle
95°C 30sec
60°C 60sec 35 cycles
72°C 60sec
72°C 2min 1 cycle
Fragment size: 188bp
Primer sequences for PCR for forward primer is same as the coding sequence between 22-47 of the ORF and reverse primer corresponds to 209-189 of the coding sequence.

p14 ARF PCR

PCR reaction:
5µl 10X PCR buffer
1µl dNTPs (2.5mM each)
200ng sense primer (5'- AGCAGCCGCTTCCTAGAAGAC -3')
200ng antisense primer (5'-CACGGGTCGGGTGAGAGT-3')
0.15µl Super Taq Polymerase (5U/µl)

Add ddH_2O up to 50µl

95°C 5min 1 cycle
95°C 30sec
60°C 60sec 40 cycles
72°C 60sec
72°C 2min 1 cycle
Fragment size: 119bp
Primer sequences for PCR are taken from literature (Linggi *et al.*, 2002)

p16 (INK4A)

PCR reaction:

5µl 10x PCR buffer
2µl dNTPs (2.5mM each)
200ng sense primer (5'-tggacctggctgaggagct-3')
200ng antisense primer (5'-gaccttccgcggcatctat-3')
0.15µl Super Taq Polymerase (5U/µl)
add till 50µl ddH2O
95°C 5min 1 cycle
95°C 30 sec
60°C 60 sec 40 cycles
72°C 60 sec
72°C 2min 1 cycle
Fragment size: 108
Primer sequences for PCR are taken from literature (Linggi *et al.*, 2002)

p21 (WAF1) PCR

PCR reaction:
5µl 10X PCR buffer
1µl dNTPs (2.5mM each)
200ng sense primer (5'-GGACAGCAGAGGAAGACCATGT-3')
200ng antisense primer (5'-TGGAGTGGTAGAAATCTGTCATGC-3')
0.15µl Super Taq Polymerase (5U/µl)

Add ddH_2O up to 50µl

95°C 5min 1 cycle
95°C 10sec
60°C 30sec 35 cycles
72°C 35sec
72°C 2min 1 cycle
Fragment size: 150bp
Primer sequences for PCR are taken from literature (Koga *et al.*, 2003)

PU.1 PCR
PCR reaction:

5µl 10X PCR buffer
1µl dNTPs (2.5mM each)
200ng sense primer (5'--3')
200ng antisense primer (5'--3')
0.15µl Super Taq Polymerase (5U/µl)

Add ddH_2O up to 50µl

95°C 5min 1 cycle
95°C 10sec
60°C 30sec 40 cycles
72°C 35sec
72°C 2min 1 cycle
Fragment size: 86bp
Primer sequences for PCR are taken from literature (Walter *et al.*, 2005)

RUNX1 PCR
PCR reaction:
5µl 10X PCR buffer
1µl dNTPs (2.5mM each)
200ng sense primer (5'-CCCACCGTGGTCCTACGAT-3')
200ng antisense primer (5'-CTGGCACGTCCAGGTGAAA-3')
0.15µl Super Taq Polymerase (5U/µl)

Add ddH_2O up to 50µl

95°C 5min 1 cycle
95°C 10sec
60°C 30sec 40 cycles
72°C 35sec
72°C 2min 1 cycle
Fragment size: 96bp
Primer sequences for PCR are taken from literature (Mikhail *et al.*, 2002)

6.9.3 Quantitative RT-PCR

cDNA is amplified using Rotor-Gene RG-300 (Corbett Research) with SYBR Green mix (Fynnzyme). For touch-down PCR, the primer annealing temperature is decreased from 65°C to 60°C (0.5°C/cycle), denaturation is at 95°C and extension at 72°C for 10 cycles, followed by 35 cycles at 60°C annealing temperature. SYBR Green analyses are normalized to human β-actin gene expression. Sequences of primers are human specific and desribed above with individual PCR.

6.9.4 Separation of amplicon fragments on agarose gel

The amplified DNA fragments are separated on 1.5% agarose gel. 6gm of agarose is dissolved in 400ml of TBE-buffer, cooked in microwave and 20µl of 0.5% ethidium bromide solution is added. 10-20µl of PCR product is mixed with 3µl of Bromophenol blue buffer. Electrophoretic separation is dependent on fragment size and is performed in electric field of 5-8V/cm for 30-60 min. Ethidium bromide incorporates between nucleic acids and visualisation is possible at 560nm on UV light. Standard size of markers is compared with separated PCR products.

Markers: pTZ plasmid digested with restriction enzyme HinfI and pSM with Hind III. DNA fragments of pTZ-HinfI have size from 44 to 1202bp, and that of pSM-HindIII between 145 and 3440bp.

6.10 siRNA studies

0.75-8x10^6 Kasumi-1 cells are grown to log phase and are transfected with 120nmol of either AML1-ETO siRNA (MWG) (Heidenreich *et al.*, 2003) or control siRNA (Santa Cruz Biotech) using Tranfectin™ (Biorad). The number of fluorescently stained cells is determined by flowcytometry (FACSCalibur) at 24hr post- transfection using FAM-labelled siRNA against AML1-ETO. Flowcytometry provides quantification of transfection efficiency directly as a percent of positively-fluorescent cells. Sequences of siRNAs are:
AML1-ETO siRNA (5´-$[CCUCGAAAUCGUACUGAGA]_{RNA}$ $[TT]_{DNA}$-3´);
(3´-$[TT]_{DNA}$ $[GGAGCUUUAGCAUGACUCU]_{RNA}$-5´);

FAM-labelled AML1-ETO siRNA (5´-[FAM]-[CCUCGAAAUCGUACUGAGA]$_{RNA}$ [TT]$_{DNA}$-3´); (3´-[TT]$_{DNA}$[GGAGCUUUAGCAUGACUCU]$_{RNA}$-5´)

6.11 Treatment of AML cell lines with CD44-activating antibody

AML cells are seeded in triplicate at a concentration of 2×10^5 cells/ml in RPMI 1640 (PAA) in the presence of 20% FCS, penicillin 100units/ml, streptomycin 100µg/ml and 10mM 2-ME in 96-well tissue culture plates (Nunc) in a total volume of 150µl/well. AML samples are treated for 3 days with CD44-activating monoclonal antibody (A3D8 clone, Sigma) or IgG1 isotype control antibody at a final concentration of 20µg/ml as described (Charrad *et al.*, 1999).

6.12 Statistical analysis

Student t-test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California, USA.

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List of Abbreviations

A	adenine
AGM	aorta-gonad-mesonephros
ALL	acute lymphocytic leukaemia
AML	acute myeloid leukaemia
ATRA	all-trans retinoic acid
BCL-2	B-cell lymphoma-2
BM	bone marrow
BSA	bovine serum albumin
C	bovine serum albumin
CBF	core binding factor
CD	cluster of differentiation
cDNA	complemetary DNA
CFU-S	colony-forming-unit of the spleen
CML	chronic myeloid leukaemia
CMPs	common myeloid preogenitors
IL-3	interleukin 3
CR	complete remission
bp	base pair
DAPI	4', 6-diamidino-2-phenylindol
DAS	dorsal- aorta-stromal
dATP	deoxi-adenosine-triphosphate
dCTP	deoxi-cytosine-triphosphate
dGTP	deoxi-guanosine-triphosphate
DMSO	dimethylsulphoxide
DNA	deoxiribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxi-nucleotide-triphosphates
DSMZ	Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH
dTTP	deoxi-thymidine-triphosphate
dUTP	deoxi-uridine-triphosphate
E	embryonic day
ECM	extracellular matrix
EDTA	ethylendiaminotetraacetic acid
ETO	eight-twenty-one
EtOH	eight-twenty-one
FAB	French-American-British
FACS	fluorescence-activated cell sorter
FCS	foetal calf serum
FITC	fluorescein-5-isothiocyanate
G	guanine
HDAC	histone deacetylase
g	gravitation: 9.81m/s2
kb	kilobases
LIF	leukaemia inhibitory factor
Lin	lineage
LSCs	leukaemic stem cells
LTC	long term culture
M	molar
mAb	monoclonal antibody
MC	methyl cellulose
MDS	myelodysplastic syndrome
min	minutes
ml	mililitre
mM	milimolar
NCCs	neural crest cells
nd	not done
ng	nanogram
nm	nanometer
NOD/SCID	non-obese diabetic/severe combined immunodeficient
ns	not significant
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
PI	propidium iodide
RCF	relative centrifugal force
RFS	relapse free survival
RNase	ribonuclease
Rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription -PCR
SDS	sodium dodecyl sulphate
sec	second
SMMHC	smooth muscle myosin heavy chain
SV40LT	Simian virus 40 large T antigen
T	thymine
t(8;21)	translocation(8;21)
TBE	tris-borate-EDTA
TGFβ	transforming growth factor beta
TWEEN20	polyoxyethylenesorbitan monolaurate
U	units
V	volt
WHO	world health organization
YSE	yolk sac endodermal

G-CSF	granulocyte-colony stimulating factor	YSM	yolk sac mesodermal
GM-CSF	granulocyte/macrophage-colony stimulating factor	µg	microgram
HEPES	N-2-hydroxyethylpiperazin-N'-2-ethansulphonacid	µl	microlitre
HSCs	haematopoietic stem cells	µm	micrometer
IMDM	Iscove's modified Eagle's medium		

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Part of this work is presented in form of poster and oral presentation in international symposiums/workshop and for manuscript preparation as listed below:

POSTER PRESENTATIONS

Pramanik, K., Greiner, A., Müller A.M., Differentiation of leukaemia cells *in vitro*: Validation of stromal environment-induced differentiation of human AML cells Poster: 13th AEK/AIO cancer meeting, Wuerzburg, March 2005

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ORAL PRESENTATION

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