MICROARRAY ANALYSIS REVEALS PATHWAYS AND BIOLOGICAL PROCESSES IN MYELOMA CELL LINE L363 WHICH ARE INFLUENCED BY MICROENVIRONMENT

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Abstract: Multiple myeloma, also called Kahler disease or myelomatosis is a debilitating and incurable malignancy characterized by proliferation of malignant plasma cells and an increased production of monoclonal paraproteins. In multiple myeloma, the abnormal proliferation of plasma cells within the bone marrow interferes with the production of blood cells therefor leading to anemia, leukopenia and thrombocytopenia. Another characteristic of disease is the activation of osteoclasts which leads to osteolytic lesions accompanied by fractures, bone pain, hypercalcemia and renal failure.

Since a key factor in the pathology of multiple myeloma is represented by the interaction between bone marrow stroma and plasma cells we have designed an *in vitro* experiment where L363 myeloma cells have been grown together with bone marrow stromal cells. The negative control was represented by L363 cells cultured alone. Following coculture, RNA from L363 plasma cells was extracted, revers-transcribed and analyzed by microarray techniques to identify biological processes and pathways which are affected by differentially expressed genes. Among these biological processes we mention regulation of cell cycle, apoptosis, and STAT genes activation.

INTRODUCTION

Multiple myeloma (MM) is cancer of plasma cells which manifests through abnormal elevated levels of monoclonal antibodies called paraproteins and accumulation of malignant plasma cells in the bone marrow. Collections of malignant plasma cell in bones lead to bone destruction and interfere with normal production of blood cells. Because plasma cells are responsible with antibody synthesis, MM patients suffer from immunodeficiency their malignant plasma cells being unable to produce normal antibodies.

Research studies on MM reveals that bone marrow microenvironment is an important player in survival and proliferation of malignant plasma cells and offers protection against therapeutic drugs (Feng *et al.* 2010; Hao *et al.* 2011; Hideshima *et al.* 2002; Nefedova *et al.* 2003; Podar *et al.* 2009; Zlei *et al.* 2007).

Current study was focused on understanding which pathways and biological processes in L363 MM cell line are affected by the presence of bone marrow stromal cells (BMSCs).

To achieve our goal we have elaborated an *in vitro* experiment where L363 MM cells have been cultivated together with BMSCs. Confluent layers of BMSCs were generated first before proceeding to real experiment. Next, L363 cells grown separately where placed in co-culture with BMSCs layers obtained previously.

During co-culture L363 have separated into two groups because some plasma cells became adherent to stroma.

Following co-culture, RNA was extracted from both adherent and non-adherent fractions of L363 cells and was used next for microarray. We have used Gene Set Enrichment Analysis tool from Ariadne Genomics Pathway Studio software to investigate and identify groups of genes significantly changed which takes part in similar biological processes.

Results suggest that most these genes are connected to apoptosis, cell cycle regulation, cell adhesion, transcription, STAT pathways, chromatin remodeling.

MATERIAL AND METHODS

BMSCs where harvested from myeloma patients and healthy volunteers and seeded with Myelocult H5100 media (StemCell Technologies, Vancouver, British Columbia, Canada) in 75 cm^2 flasks (Nunc, Denmark) at 37°C in a 5% CO_2 humidified atmosphere. A volume of 25 ml media per flask was used and it was changed every 3-4 days. With this setup BMSCs cultures have reached confluency within 5 weeks.

The myeloma cell line L363: cells were cultured in RPMI 1640 media supplemented with 10% fetal calf serum (FCS; Gibco) and incubated at 37°C and 5% CO_2. Media was changed every 2-3 days.

L363 cells prior labeled with 10μM CFSE where co-cultured with confluent layers of BMSCs. Some L363 cells have become adherent to BMSCs while the rest have remained in suspension. The non-adherent fraction of MM cells was collected after 72hrs of co-culture and at the same time point BMSCs with the adherent fraction of L363 cells were

enzymatically detached with a solution containing trypsin and EDTA. Cells were sorted later on to a purity of 90-95% with a MoFlo™ High-Performance Cell Sorter (Dako-Cytomation) based on the CFSE expression in MM cells. Following sorting procedure cells where centrifuged at 300 G and pellets were stored at -70°C.

RNA was extracted from frozen pellets with Qiagen RNeasy extraction kits and Biotin-labeled target cRNA was obtained using Ambion Message Amp II-Enhanced kit. cRNA was hybridized for 16h at 45°C on Affymetrix HG-U133 Plus 2.0 arrays and arrays were scanned using GCOS software.

For data analysis we used the Ariadne Pathway Studio software version 6 where data was imported as Affymetrix files. Find differentially expressed genes tool from the software was applied which briefly it was a two paired correlated T-test with FDR correction Benjamini-Hocheberg (Benjamini *et al.* 1995). The correlated groups were adherent L363 versus control L363 and non-adherent versus control. The algorithm is based on one way ANOVA test to calculate p-value and expression differences. To find group of genes that share similar biological functions or regulation and pathways that these genes have in common we used Gene Set Enrichment Analysis (GSEA) (Mootha *et al.* 2003; Subramanian *et al.* 2005) function of Pathway Studio software with Mann-Whitney U Test as enrichment algorithm with p-value cut-off set to 0.05.

RESULTS AND DISCUSSIONS

During co-culture, L363 cells have separated into two fractions: one fraction remained in suspension, non-adherent to BMSCs and the second fraction strongly adherent to stromal cells (Fig.1).

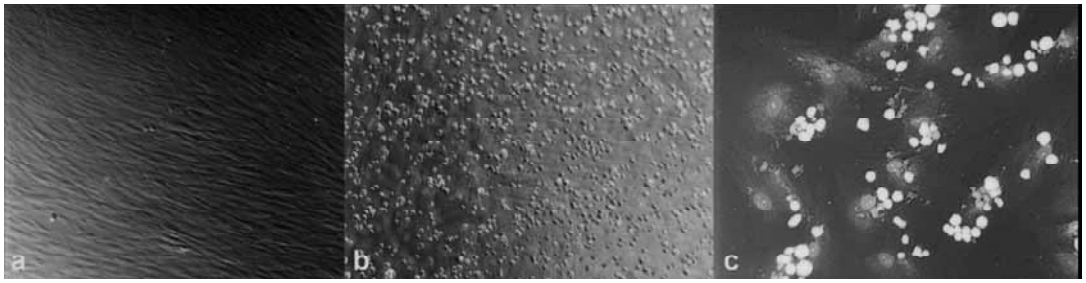

Figure 1. *a)Confluent layers of bone marrow stromal cells in culture (objective x10, phase contrast). b)and c) Co-culture of L363 MM cells with bone marrow stromal cells: b) The entire population of L363 and BMSCs (objective x10, phase contrast). c) BMSCs and adherent population of L363 (objective x40, Dia Panoptic staining)*

The GSEA analysis of these two populations of L363 gave slightly different results (Tables 1 and 2).

For adherent group we could note several important groups of genes involved in: regulation of apoptosis, transcription, cell cycle regulation, cell proliferation, cell-matrix adhesion, Stat pathways and many others, the full list is shown in Table 1.

Table 1. *GSEA analysis for adherent fraction of L363.Groups of genes and pathways are sorted according to p-value.*

Name	Type	p-value	Gene Set Category
protein transport	Group	5.49456e-012	biological_process
Actin Cytoskeleton Regulation	Pathway	7.97844e-011	Ariadne Pathways
regulation of transcription from RNA polymerase II promoter	Group	1.90138e-010	biological_process
transcription	Group	2.47822e-010	biological_process
regulation of transcription, DNA-dependent	Group	1.286e-008	biological_process
EndothelinRb -> AP-1/CREB/ELK-SRF signaling	Pathway	3.42193e-008	Ariadne Pathways
Cell Cycle Regulation	Pathway	4.37057e-008	Ariadne Pathways
protein amino acid phosphorylation	Group	6.13805e-008	biological_process
Focal Adhesion Regulation	Pathway	7.38964e-008	Ariadne Pathways
EndothelinRa -> AP-1/CREB signaling	Pathway	1.4404e-007	Ariadne Pathways
Hedgehog Pathway	Pathway	1.59695e-007	Ariadne Pathways
ubiquitin cycle	Group	1.88078e-007	biological_process
heart development	Group	3.11742e-007	biological_process
apoptosis	Group	4.7738e-007	biological_process
response to DNA damage stimulus	Group	5.29973e-007	biological_process
EDG3/5 -> AP-1/ELK-SRF signaling	Pathway	1.05171e-006	Ariadne Pathways
cell proliferation	Group	1.08143e-006	biological_process
ubiquitin-dependent protein catabolic process	Group	1.55949e-006	biological_process
EphrinR -> actin signaling	Pathway	1.70039e-006	Ariadne Pathways
cell cycle	Group	2.96537e-006	biological_process
B Cell Activation	Pathway	3.51272e-006	Ariadne Pathways
AdrenergicRa -> STAT3 signaling	Pathway	4.86739e-006	Ariadne Pathways
T Cell Activation	Pathway	5.67735e-006	Ariadne Pathways
ICAM1 -> AP-1/CREB/ELK-SRF signaling	Pathway	5.86233e-006	Ariadne Pathways
liver development	Group	6.01069e-006	biological_process
GFR -> AP-1/CREB/CREBBP/ELK-SRF/MYC signaling	Pathway	7.69736e-006	Ariadne Pathways
ProstaglandinIR -> ATF1/ELK-SRF/CREB signaling	Pathway	8.19262e-006	Ariadne Pathways
AngiotensinR -> CREB/ELK-SRF/TP53 signaling	Pathway	8.50676e-006	Ariadne Pathways
regulation of cell cycle	Group	8.90809e-006	biological_process
collagen fibril organization	Group	9.23608e-006	biological_process
protein ubiquitination	Group	9.58185e-006	biological_process
cell adhesion	Group	9.62985e-006	biological_process
VIPR -> CREB/CEBP signaling	Pathway	1.13924e-005	Ariadne Pathways
positive regulation of transcription from RNA polymerase II promoter	Group	1.24272e-005	biological_process
cell-matrix adhesion	Group	1.24424e-005	biological_process
ProstaglandinFR -> ATF1/ELK-SRF/CREB signaling	Pathway	1.24896e-005	Ariadne Pathways
vesicle-mediated transport	Group	2.00596e-005	biological_process
RNA processing	Group	2.17154e-005	biological_process
protein complex assembly	Group	2.39186e-005	biological_process
AdrenergicRa -> ELK-SRF signaling	Pathway	2.51617e-005	Ariadne Pathways
IL8R -> CREB/EGR signaling	Pathway	2.69459e-005	Ariadne Pathways
NTRK -> AP-1/CREB/ELK-SRF/MYC/SMAD3/TP53 signaling	Pathway	3.17944e-005	Ariadne Pathways
intracellular protein transport	Group	3.42739e-005	biological_process
ThromboxaneR -> CREB signaling	Pathway	3.57738e-005	Ariadne Pathways
AdenosineR -> AP-1 signaling	Pathway	4.5588e-005	Ariadne Pathways
Notch Pathway	Pathway	5.07402e-005	Ariadne Pathways
DNA recombination	Group	5.18688e-005	biological_process
PTAFR -> AP-1/ATF1/CREB/ERK-SRF signaling	Pathway	5.46443e-005	Ariadne Pathways
AdrenergicRb -> CREB signaling	Pathway	7.31138e-005	Ariadne Pathways
DNA repair	Group	7.89122e-005	biological_process
TNFRSF1A -> AP-1/ATF/TP53 signaling	Pathway	8.19938e-005	Ariadne Pathways
CholecystokininR -> ELK-SRF signaling	Pathway	9.06278e-005	Ariadne Pathways
endocytosis	Group	9.27979e-005	biological_process
CCR1 -> STAT signaling	Pathway	9.88798e-005	Ariadne Pathways
chromatin modification	Group	0.000103728	biological_process

Like in any cancers malignant cells in MM escape from apoptosis and cell cycle regulation so we can understand these results. The expansion of a malignant populations results when cell cycle is deregulated or apoptosis is inhibited (Nadav *et al.* 2006; Runge *et al.* 2006).

Several pathways involving Stat genes are present. Jak-Stat pathway (Fig.2) is an important mechanism involved in gene activation, proliferation and differentiation of cells (Heinrich *et al.* 1998) and is well documented and targeted for gene therapy in MM (Li *et al.* 2010; Monaghan *et al.* 2011; Zhang *et al.* 1992).

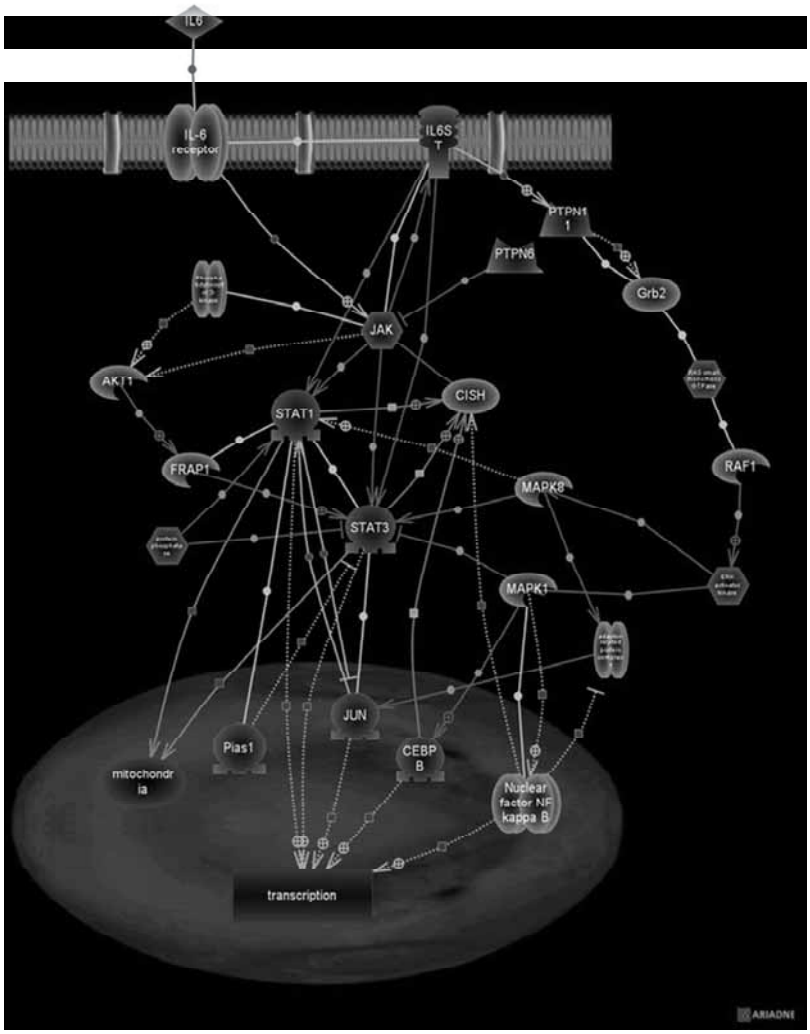

Figure 2. *Jak-Stat pathway starting from Il-6 (Ariadne Genomics Pathway Studio)*

Genes from cell to cell adhesion biological group and focal adhesion regulation pathway in particular (Fig.3) are also present in GSEA analysis of the adherent fraction. These genes could explain why we have an adherent group of L363 cells.

Signaling through adhesion molecules is another important factor for survival and proliferations of MM malignant cells (Runge *et al.* 2006) and we could see here that adherent group of L363 benefit from this.

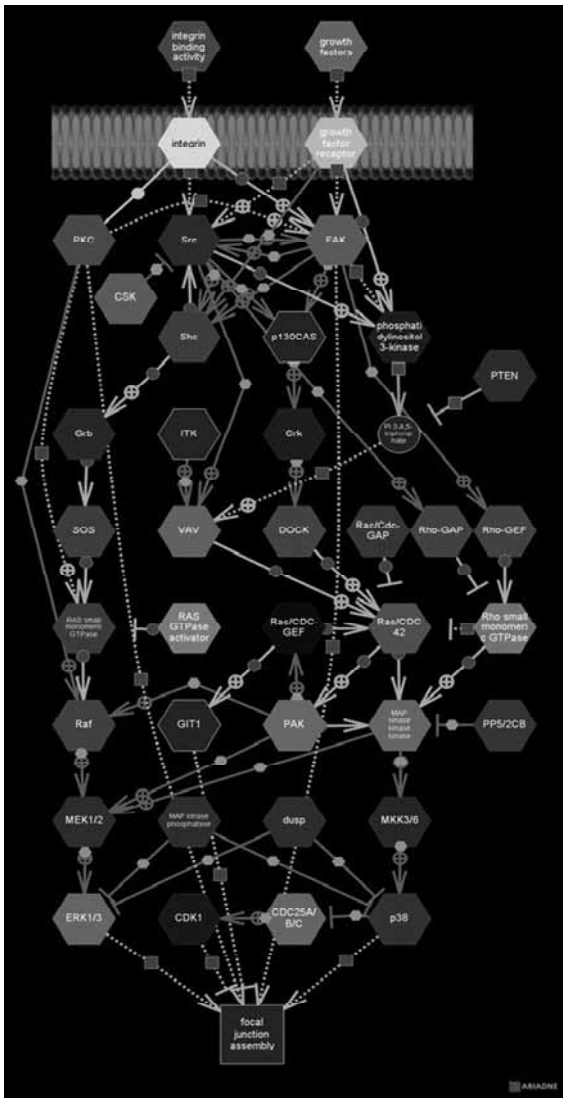

Figure 3. *Focal Adhesion Regulation pathway (Ariadne Genomics Pathway Studio)*

What is interesting is the listing of chromatin remodeling group of genes. We could speculate that during co-culture L363 adhere to BMSCs and this contact is followed by chromatin modifications which should lead to activation and inactivation of other genes.

The GSEA analysis of non-adherent fraction shows similar results but is a little less enriched in groups of genes and pathways (Table 2).

Table 2. *GSEA analysis for non-adherent fraction of L363.Groups of genes and pathways are sorted according to p-value.*

Name	Type	p-value
Actin Cytoskeleton Regulation	Pathway	9.91471e-013
actin cytoskeleton organization and biogenesis	Group	7.7315e-011
T Cell Activation	Pathway	1.02541e-010
protein amino acid phosphorylation	Group	2.06896e-009
intracellular signaling cascade	Group	2.08824e-009
Focal Adhesion Regulation	Pathway	7.22823e-008
collagen fibril organization	Group	1.02218e-007
antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	Group	3.5503e-007
Notch Pathway	Pathway	1.03183e-006
EphrinR -> actin signaling	Pathway	1.39943e-006
heart development	Group	1.41878e-006
Mast Cell Activation	Pathway	2.80055e-006
negative regulation of transcription from RNA polymerase II promoter	Group	4.4219e-006
NK Cell Activation	Pathway	6.28418e-006
apoptosis	Group	6.72629e-006
GFR -> AP-1/CREB/CREBBP/ELK-SRF/MYC signaling	Pathway	1.26576e-005
GFR -> NCOR2 signaling	Pathway	2.18771e-005
response to cytokine stimulus	Group	2.23038e-005
cell migration	Group	5.00859e-005
FcIgER -> ELK-SRF signaling	Pathway	5.51718e-005
T-cell receptor -> ATF/CREB signaling pathway	Pathway	5.87207e-005
AngiotensinR -> CREB/ELK-SRF/TP53 signaling	Pathway	6.01854e-005
NTRK -> AP-1/CREB/ELK-SRF/MYC/SMAD3/TP53 signaling	Pathway	6.51755e-005
Gonadotrope Cell Activation	Pathway	6.75204e-005
regulation of cell growth	Group	7.11294e-005
regulation of apoptosis	Group	7.49006e-005
IL4R -> ELK-SRF/HMGY signaling	Pathway	7.97987e-005
ICAM1 -> AP-1/CREB/ELK-SRF signaling	Pathway	0.000121752
negative regulation of cell cycle	Group	0.000138059
cell adhesion	Group	0.000141082
KIT -> MITF signaling	Pathway	0.000157054
AdrenergicRa -> STAT3 signaling	Pathway	0.000167192

CONCLUSIONS

This experiment gives an insight of genes and pathways in L363 MM cell line affected by microenvironment represented here by BMSCs. Our results are in concordance with other people findings in this field and offer a good image of effects at molecular level.

We could see that there are many genes involved in this pathology ranging from apoptosis to cell to cell adhesion and interaction, genes that regulates cell cycle, cell migration, cell survival and replication.

There are more genes and pathways affected in the adherent fraction which means that intimate contact of malignant cells with stroma together with signals from soluble factors of microenvironment is more important than paracrine signalling alone.

Knowing what genes and pathways are involved in MM pathology is important for the elaboration of therapeutic strategies.

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