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Modulazione della regolazione ipossica come target terapeutico nel mieloma multiplo

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Multiple myeloma

Pathophysiology

Multiple myeloma (MM) is a neoplasm of post–germinal center, terminally differentiated B cells. It is characterized by a multifocal proliferation of clonal, long-lived plasma cells within the bone marrow (BM) and associated skeletal destruction, serum monoclonal gammopathy, immune suppression, and end-organ sequelae.. Unlike the genomes of most hematological malignancies, and similar to those of solid-tissue neoplasms, MM genomes are typified by numerous structural and numerical chromosomal aberrations as well as mutations in a number of oncogenes and tumor-suppressor genes, some of which have been linked to disease pathogenesis and clinical behavior. Recent studies have also defined the importance of interactions between the MM cells and their BM microenvironment, dysregulation in signaling pathways and in a specialized subpopulation of cells within the tumor for tumor cell growth and survival, and the development of resistance to therapy

The MM genome is characterized by a distinctive combination of gains and losses of whole chromosomes, by nonrandom chromosomal translocations that cause dysregulation of the genes at the breakpoints, and by point mutations. In addition, a constellation of small focal areas of chromosomal amplifications and deletions have recently been identified via high-resolution technologies such as array–comparative genomic hybridization (aCGH) and single-nucleotide polymorphism arrays.

MM can be subdivided into two groups according to the pattern of chromosomal gains and losses. Approximately 55–60% of MM primary tumors are characterized by a hyperdiploid karyotype with a number of chromosomes ranging from 48 to 74 and trisomies of odd-numbered chromosomes including 3, 5, 7, 9, 11, 15, 19, and 21. The remaining cases make up a nonhyperdiploid group, which includes tumors with a hypodiploid, near-diploid, pseudodiploid, or near-tetraploid chromosome number (i.e., fewer than 48 or more than 74 chromosomes). The mechanisms underlying this pattern have not been elucidated; importantly, ploidy status rarely changes during disease progression and patients with hyperdiploid MM tend to have a better prognosis than do those with nonhyperdiploid disease. On the basis of recent genomic and GEP studies, the hyperdiploid and nonhyperdiploid groups have been further subclassified

. Two translocations directly increase the expression of cyclins: (*a*) t(11;14)(q13;q32), which occurs in 15% to 20% of MM patients, induces cyclin D1 overexpression; and (*b*) t(6;14)(p21;q32), which is present in 5% of MM cases, increases expression of cyclin D3. Another translocation, t(4;14)(p16.3;q32), is present in approximately 15% of patients (61) and dysregulates the expression

of the Wolf-Hirschhorn syndrome candidate 1 gene [*WHSC1*, also known as multiple myeloma set domain (*MMSET*)], which encodes a protein with homology to histone methyltransferases; and the receptor tyrosine kinase fibroblast growth factor receptor 3 (*FGFR3*) gene. Finally, t(14;16)(q32;q23) dysregulates the oncogene *MAF*, a basic leucine-zipper transcription factor, in 5% to 10% of patients and t(14;20)(q32;q11) affects another member of this family, *MAFB*, in 5% of cases. These rearrangements generally seem to be mutually exclusive, although in 5% of MGUS and 25% of advanced MM cases two independent translocations may be found in the same patient. The oncogenic consequences of these translocations on MM pathogenesis—with the exceptions of those caused by *MAF* and *FGFR3* deregulation—have not yet been extensively explored. MAF promotes MM cell proliferation and increases MM cell adhesion to bone marrow stromal cells (BMSCs). Inhibition of FGFR3 in t(4;14) MM induces PC differentiation and apoptosis. However, FGFR3 is not expressed in 25% of patients with t(4;14) MM, which suggests that it may not be universally relevant as a therapeutic target in this group of patients. Importantly, these chromosomal translocations more often affect nonhyperdiploid patients and are linked to prognosis. Specifically, t(14;16) and t(4;14) translocations are associated with a poor prognosis, whereas patients presenting with t(11;14) translocations have a longer survival time relative to all other genetic subtypes .

It is well established that the physical interaction between MM cells and the BM microenvironment, which can be modeled in vitro and in vivo, plays a crucial role in MM pathogenesis. The BM microenvironment consists of a variety of ECM proteins (e.g., fibronectin, collagen, laminin, and osteopontin), and cells (e.g., hematopoietic stem cells, progenitor and precursor cells, immune cells, erythroid cells, SCs, endothelial cells, adipocytes, osteoclasts, and osteoblasts). Normally, BM cells secrete paracrine factors including IL-6, insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), B cell–activating factor, FGF, SC-derived factor 1α (SDF-1α), and TNF-α, which are further upregulated following the adhesion of MM cells to the BM milieu. Direct interaction between MM cells and BM cells along with secreted chemokines activates pleiotropic signaling pathways that mediate growth, survival, drug resistance, and migration of MM cells, as well as BM osteoclastogenesis and angiogenesis.

Constitutive genetic alterations in MM cells are not the sole determinants of the biological behavior of MM cells in their local microenvironment. Instead, the molecular profiles of MM cells show a wide range of inducible alterations at the transcriptional and proteomic levels that mediate the protective effects of the BM microenvironment on MM cells, enhance adhesion of MM cells to BMSCs and BM accessory cells, recruit new blood vessels to provide nutrients and growth factor

support for MM cells, and/or trigger increased osteoclastic bone resorption For example, the interaction of MM cells with BMSCs and BM accessory cells upregulates transcripts for cytokines such as IL-6, HGF, and IGF; the antiapoptotic protein Mcl-1 and heat shock proteins (Hsps), which regulate the conformation and function of proliferative/antiapoptotic proteins. This increased autocrine production of cytokines, along with paracrine cytokine production from the BMSCs, stimulates proliferative/antiapoptotic signaling cascades in MM cells. Upregulation of Mcl-1 or Hsps contributes to increased MM cell survival and drug resistance in the BM milieu. Cytokine-driven signaling events also lead to transcriptional upregulation of adhesion molecules, such as integrins and ECM proteins, and enhances the binding of MM cells to BMSCs, which in turn promotes the viability of MM cells in the BM milieu by activating antiapoptotic signaling cascades (e.g., the FAK/PI3K axis) both directly (via adhesion molecules and cell-cell contact) and indirectly (by increasing the secretion of antiapoptotic cytokines). These inducible molecular alterations in MM cells that interact with their microenvironment also influence MM-associated neoangiogenesis and bone resorption. Finally, constitutive chromosomal translocations, amplifications, deletions, or gene mutations in MM cells modulate the transcriptional, proteomic, and functional sequelae triggered in MM cells that interact with their local BM milieu. For example, IL-6 secretion is upregulated by the MM-BMSC interaction and may be particularly important in MM cells that overexpress IL-6 receptor due to amplifications in chromosome 1q21. These studies suggest that oncogenomics can be used to show MM subtype–specific interactions with the BM milieu.

Interestingly, magnetic beads coated with antibodies against CD138 are broadly used to isolate normal and MM PCs for ex vivo functional and molecular studies, including GEP analysis. Considering the biological role of CD138, it will be important to learn whether its engagement during the processes of purification with anti-CD138 antibodies has any biological consequences on the cells, including in gene expression, given that GEP analysis comparing normal PCs with MM cells is performed following CD138 purification.

Treatment and novel agents

During the last 10 years, several randomized controlled trials in different clinical settings of multiple myeloma (MM) have shown that bortezomib thalidomide or lenalidomide containing regimens increase the progression-free and, in many cases, overall survival compared to regimens of the pre-thalidomide era. Consequently, the improved rates and depth of response achieved with these novel agents or their combinations are not merely "cosmetic" effects on monoclonal immunoglobulin levels. Instead, they reflect a tangible impact on the biology of the disease.

Therefore, the improved overall survival of MM patients diagnosed between 2001-2006 vs. prior to 2000, should be attributed, at least in part to the introduction of novel agents
However, so far, the overall survival curves in the post-thalidomide lack of an identifiable plateau and their early steep drop off. These features respectively suggest that currently available treatment options for myeloma are unlikely to be curative. Therefore, substantial therapeutic challenges still remain in MM. Functional studies identifying novel "drivers of tumour development have to be investigated to identify and select novel class of therapeutic.

HIF 1α and tumour cells

Hypoxia-inducible factors (HIFs) play a key role in the cellular response experienced in hypoxic tumors, mediating adaptive responses that allow hypoxic cells to survive in the hostile environment. Tumor hypoxia is one of the most important factors determining the response of solid tumors to surgery, chemotherapy and radiation therapy. Studies on the tumor microenvironment have identified tumor hypoxia as an important factor negatively affecting clinical outcome by promoting genetic instability, tumour cell metastasis and invasiveness. Cell clones within the tumour may react to the experienced hypoxic stress by undergoing genomic and proteomic alterations that allow cells to survive and proliferate despite the environment, forming a vicious circle, with a high selection pressure towards malignant cells capable of making sufficient adaptations. Through gene expression modifications, the hypoxia-surviving cells can change to an aggressive phenotype-promoting tumour expansion and metastatic potential. The aggressive behavior and treatment resistance observed in hypoxic tumours are primarily mediated by hypoxia-inducible factors (HIFs). Within the hypoxic cells, the diminished level of oxygen stabilizes and activates the transcription of HIFs. Identification and understanding of HIF expression and function is therefore of key interest for the development of hypoxia-targeting agents.

HIF 1α: synthesis and regulation

HIFs are heterodimeric transcription factors, consisting of an oxygen-sensitive HIF1-α subunit and an oxygen-insensitive HIF-*β* subunit. The HIF1-α subunit is a novel protein, whereas the HIF-*β* subunit is identical to the mammalian protein aryl hydrocarbon nuclear translocator (ARNT). The HIF-*β* subunits are constitutively expressed in all cells, whereas the HIF- α subunits are subject to oxygen-dependent degradation conducted by oxygen-dependent prolyl-4-hydroxylase domain proteins (PHDs), giving HIF- α a half-life of less than 5 min under normoxic conditions Under hypoxic conditions the HIF- α protein is stabilized and rapidly transferred to the cell nucleus,

directed by a nuclear localization sequence situated in its C-terminus. Following the dimerization of the HIF- α and HIF-*β* subunits, the complex binds to an HIF-binding DNA sequence, termed the hypoxia response elements (HRE), in a myriad of genes and induces or suppresses their transcription.

In normoxic cells, cytoplasmic HIF- α becomes earmarked for degradation by hydroxylation in two oxygen-dependent degradation domains (ODD), mediated by three cytoplasmic PHDs (PHD1, 2 and 3). The PHDs hydroxylate two proline residues using molecular oxygen and 2-oxoglutarate (2-OG) (α -ketoglutarate) as cosubstrates, and ferrous iron [Fe(II)] and ascorbate as cofactors.. Following prolyl hydroxylation, HIF-α becomes polyubiquitinated by the von Hippel–Lindau protein (VHL)/Elongin-C E3 ubiquitin complex. This interaction between HIF- α and the VHL and Elongin C is stabilized by the spermidine/spermine N1-acetyltransferase-1 (SSAT1) protein, which thereby facilitates ubiquitination. Finally the 26S proteasome, a barrel-shaped multiprotein proteolytic complex, degrades the hydroxylated and ubiquitinated HIF- α subunit. Under hypoxic conditions (<5%O_2) the PHDs become non-functional and the cytoplasmic HIF- α is stabililized. Hypoxic conditions will allow the HIF- α subunit to escape proteasomal degradation, and allow HIF- α to dimerize with a *β*-subunit and form active HIF. Dimerized, potentially active, HIF is subject to further oxygen-dependent regulation of its transcriptional activity.

HIF-1 α protein synthesis is, independently of oxygen levels, regulated by activation of the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. The increased expression of HIF-1 α mediated by growth factor signalling is, unlike hypoxia, which induces HIF-1 α stability and transcription in all cells types, cell type specific. HIF accumulation can therefore be regulated by hypoxia-independent mechanisms, following oncogene activation of RAS, PI3K or inactivation of tumour suppressors such as the PI3K inhibitor PTEN. The PI3K pathway mediates the translation of HIF-1 α via the Akt (protein kinase B) and the mammalian target of rapamycin (mTOR), whereas the MAPK pathway (RAS–MEK–ERK) initiates the translation via activation of extracellular signal-regulated kinase (ERK). Both HIF-1 α and HIF-2 α activation induce the expression of transcriptional growth factors, which initiates the activation of RTK and their downstream pathways, potentially also forming a positive autocrine feedback loop. The increased expression of different transcription factors mediated by HIF-1 α and HIF-2 α will influence the regulation of multiple cellular functions including metabolism, cell cycle progression, anti-apoptosis and angiogenesis.

HIF and multiple myeloma

While numerous studies have examined the role of hypoxia in solid tumour progression, few is known regarding the role of hypoxia and the HIF transcription factors in hematological malignancies such as MM. Initial interest in this area stemmed from the well-established association between angiogenesis and MM disease progression, and the knowledge that hypoxia is a critical regulator of angiogenesis in other pathologies Two studies have examined HIF-1a and HIF-2a protein expression in BM biopsy specimens taken from MM and monoclonal gammopathy of undetermined significance patients at clinical presentation of MM cells. In the second, much larger study, strong HIF-1a and HIF-2a expression was observed in 33 and 13.2% of MM cases, respectively, and a significant association was observed between the expression of HIF-1a and HIF-2a in MM cells. As already mentioned, aberrant expression of either HIF-1a or HIF-2a has been documented in many solid tumours and when either or both are found to be overexpressed, it indicates a highly aggressive tumour with a much poorer prognosis. As yet, the association between aberrant HIF expression, and patient survival and prognosis has not been examined in MM. It has been proposed that in MM cells, HIF-1 predominantly coordinates immediate, 'up-front' responses to hypoxia, whereas HIF-2 coordinates responses to more prolonged, chronic hypoxia, as has been reported previously in other cell types. As yet, the biological implications of the different expression patterns and induction kinetics of HIF-1a and HIF-2a in MM remain unknown. In addition to being induced by environmental hypoxia, aberrant HIF expression in MM cells can arise through genetic abnormalities such as loss of tumour suppressor activity. The helix–loop–helix transcription factor, c-Myc, is a protooncogene, which is de-regulated in 30–50% of patients with advanced MM. Of note, recent studies have documented an important relationship between HIF-1 and c-Myc in MM PCs, which results in the upregulation of HIF-1a independent of hypoxia. Under normoxic conditions, deregulated c-Myc promotes constitutive HIF-1 protein activation, promotes aberrant VEGF production by MM PCs and angiogenesis, and is associated with a poor prognosis in MM patients. Hypoxia-activated pro-drugs combine a potent DNA-alkylating agent with a hypoxia-activated molecular trigger. These agents are designed to deliver cytotoxic agents to hypoxic tumour zones and minimize drug-related toxicities in normoxic tissues.

HIF-1α inhibitor as a therapeutic target in multiple myeloma

Background

Despite clinical development of new generations of immunomodulatory agents (IMIDs), and proteasome inhibitors and the introduction of innovative drugs combinations, multiple myeloma (MM) remains an incurable disease[1-2]. Intrinsic mechanism of resistance[3] and microenvironment influence[4] have been suggested to significantly contribute to disease progression and chemotherapy failure, therefore identification of additional therapeutic agents interfering with MM survival pathways is warranted.

The hypoxic microenvironment in which myeloma PCs reside, it is emerging as a pro survival zone[5-6]; therefore target pathway induced by hypoxic[7] and inhibit hypoxia-driven disease processes[8] will be ultimately providing the much needed therapeutic advances for MM patients.

In solid tumours, preclinical data have showed that HIF-1α over expression resulted in increase tumour growth, whereas loss of HIF activity results in decreasing: moreover, the expression of the HIF family members has been associated with an higher risk of progression and mortality. HIFs activate transcription of genes that play key roles in critical aspects of cancer biology, including stem cell maintenance[9], cell immortalization, genetic instability[10], vascularisation[11], glucose metabolism[12] pH regulation[13] immune evasion[14] invasion and metastasis[15] and radiation resistance[16] .Given the extensive validation of HIF-1 as a potential therapeutic target, drugs that inhibit HIF-1 have been identified and shown to have anticancer effects in xenograft models. In solid tumour, the response to hypoxia microenvironment or cytokine stimuli, mediated by up regulation of hypoxia inducible transcription factors, including HIF1α and 2α, represents a mechanism of pro-survival conferring a positive selection to tumour cells[17]. Furthermore, abnormal activation of HIF1α, independent by physical or biological stimuli, has been observed due to impaired activity of tumour suppressor genes, and has been associated with an aggressive phenotype[18-19].

In this contest, the identification of HIF family members as crucial drivers of a more aggressive phenotype in tumour cells make investigational studies intriguing also in the contest of multiple myeloma.

While numerous studies have examined the role of HIF family members in solid tumour, in MM limited data are available[20]. HIF1a has been described to be constitutive expressed in MM cell lines, in hypoxia and normoxic condition[21], and pro-survival cytokines, as IGF1α and IL6, have been shown to further enhance HIF1-level[22]. Strong HIF1α expression has been described in

$CD138^+$cells within the bone marrow of MM patients and gene expression profiling has shown a significant upregulation of pro angiogenic genes related to HIF1α. Furthermore, protein expression in MM cell lines sensitive to bortezomib and lenalidomide has shown an early down regulation of HIF1α

These data let us to hypothesize that a selective HIF1α inhibitor could trigger survival pathway in MM[23] and therefore provide insight into the biology of MM suggesting a novel therapeutic approach.

In this study we provide evidence that HIF-1α, even in the absence of hypoxia signal, is constitutive expressed in MM plasma cells and is further inducible by bone marrow milieu stimuli. Using EZN-2968[24], a selective antisense oligonucleotide antagonist of HIF1 α, we showed that HIF inhibition is sufficient to induce a permanent cell cycle arrest through p21 upregulation at clinical achievable doses, while sparing non malignant cells and overcoming the protective effect conferred to MM cells by stromal cells or bone marrow cytokines.

Our data support the hypothesis that HIF-1α inhibition can suppress tumor growth by preventing proliferation of plasmacell and blocking pro-survival signaling pathways, and therefore provide the framework to test HIF-1α as a novel target for multiple myeloma treatment.

Material and Methods

Cell culture

Human MM cell lines MM1S, U266, RPMI8226 and OPM-2 were obtained from DMSZ Germany and cultured in RPMI-1640 media (BioWhittaker, Walkersville, MD, USA) with 10% of FBS (BIOCHROM^{AG}, Leonornstr., Berlin, Germany), 2 mM of L-Glutamine (BIOCHROM^{AG}, Leonornstr., Berlin, Germany), streptomycin 100 U/ml, penicillin 100 U/ml and maintained in 5% CO_2 at 37°C. For culture under hypoxic conditions, we used an hypoxic chamber INVIVO2 400 apparatus (Biotrace International).

Mononuclear cells from bone marrow aspirates or peripheral blood samples were isolated by density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ, USA). $CD138^+$ and $CD34^+$ cells were then isolated by immunomagnetic bead positive selection in a Mini MACS LS column following the manufacturer's protocol (Milteny Biotech, Auburn, CA). The purity of MM cells was confirmed by flow cytometric analysis using phycoerytrin-conjugated anti CD138/CD34 antibody (Milteny Biotech, Auburn, CA). Patients samples were collected after informed consent.

Compounds

EZN2968 and EZN3088 were gently obtained from EZON Pharmaceuticals (Inc., Piscataway, New Jersey). The sequence of EZN2968 is 5-TGGcaagcatccTGTa-3, where uppercase and lowercase indicate LNA or DNA residues, respectively. All internucleotide linkages are phosphorothioate linkages. EZN2968 has complementarity to residues 1,197 to 1,212 of the sequence encoding human HIF-1α. The sequence of the scrambled oligonucleotide (Mock), EZN3088, is 5-CGTcagtatgcgAATc-3.

Recombinant human Interleukin 6 (IL6) and Insulin like Growth Factor I (IGF-I) were purchased from R&D System (Minneapolis, MN, USA), where phytohemagglutinin (PHA-P) and Cobalt Chloride ($CoCl_2$) from Sigma-Aldrich (St Louis, MO, USA).

Apoptosis Assay

Cellular apoptosis was evaluated using FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™), according to the manufacturer's instructions. Briefly, 10^6 cells were washed in PBS and incubated in Annexin binding buffer with FITC-labeled annexin V and PI for 15 minutes at room temperature before analysis. Cell fluorescence and PI uptake were measured by mean of a

FacScan flow cytometer (set on 488 nm excitation and 530 nm bandpass filter wave length for fluorescing detection or 580 nm for PI detection) and a dedicated software (BD Pharmingen™).

Cell cycle analysis

Cell cycle analysis was evaluated using the PI/RNase Staining Buffer (BD Pharmingen™), according to the manufacturer's instructions. Briefly, 10^6 cells were incubated with PI/RNase buffer for 15 minutes at room temperature before analysis. PI uptake was measured by mean of a FACScan flow cytometer set at 580 nm and analyzed with ModFitLT version 3.1 (Verity software).

Cell viability assessment

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) colorimetric survival assay. In brief, MM cell lines were plated in 96 well plates at density of 50.000 cells/well. EZN2968/EZN3088 were added at the concentration indicated and compared with the controls. Cultures were then incubated for 24h, 48h and 72h in a 37°C incubator with 5% of CO_2. MTT was added with a final concentration of 5 mg/ml to each well for 4h followed by addition of isopropanol containing 0.1 N HCl to dissolve the crystals. Optical absorbance of the culture medium was then measured at 570/620 nm using a spectrophotometer (Multiskan Ex Microplate Photometer, Thermo Scientific, Meridian Rd, Rockford, USA). Each experiment was done in triplicate measurements.

Peripheral blood samples, $CD34^+$ cells from healthy donors, and $CD138^+$ cells from myeloma patients were collected and plated at density of 40.000 cells/well and treated with EZN2968/EZN3088 for up to 48h.

Cell death commitment assay

The minimum exposure of MM cells to EZN2968 required to commit them to death was evaluated by incubating cells in 6 well plates with EZN2968 (20 μmol/L) for up to 96h. Following incubation, the cells were washed with drug-free medium to remove any residual drug, and incubated in drug-free medium for an additional 3 days, resulting in equal length of incubation for all experimental conditions. MM cell survival was quantified by MTT survival assay and expressed as percentage of the value obtained from respective controls.

RNA isolation and Quantitative real-time PCR

Total RNA was isolated using RNeasy mini kit (Qiagen, Valencia, CA) with an automated RNA extraction method according to the manufacturer's instructions (QIAcube, Qiagen, Valencia,

CA). 100 ng of total RNA was reverse transcribed using SuperScript™ III First-Strand Synthesis System and random hexamers (Invitrogen Life Technologies). Real-time PCR (qRT-PCR) was performed by adding 2 µl of 20 µl cDNA to an universal master Mix (Lightcycler probe Master mix, Roche, Applied science), primers (0.5 µmol/L of each primer) and universal probes, UPL (0.2 µmol/L of each probe). UPL probe #66, #60 and #17 were used to quantified HIF-1α, GAPDH and HIF-2α respectively. The following primers pairs have been used: HIF-1α forward 5'-TTTTTCAAGCAGTAGGAATTGGA-3' and reverse 5'-GTGATGTAGTAGCTGCATGATCG-3'; HIF-2α forward 5'-GACATGAAGTTCACCTACTGTGATG-3' and reverse 5'-GCGCATGGTAGAATTCATAGG-3'; GAPDH forward 5'-AGCCACATCGCTCAGACAC-3' and reverse 5'-GCCCAATACGACCAAATCC-3'. All reactions were performed in triplicate using the LightCycler® 480 instrument (Roche, Applied science) in a total volume of 20 µl.

Western Blotting

For Immunoblotting analyses, cells pellets were lysed by using radio-immunoprecipitation assay (RIPA)-lysis buffer modified (5 mM EDTA, 2 mM Na_3VO_4, 5 mM NaF and 1 mM PMSF) in combination with complete protease inhibitor cocktail (all from Sigma-Aldrich, St Louis, MO, USA). SDS-polyacrylamide gel electrophoresis (10%-12%) was performed (50 µg of protein per lane), and proteins were electroblotted onto PVDF membranes. The primary antibodies used for Immunoblotting were purchased from: Novus Biologicals, (HIF-1α), Cell Signaling Technology (Autophagy Antibody Sampler Kit and ER stress Antibody Sampler Kit).

ELISA Assay

VEGF levels secreted by MM cells treated with EZN2968 or control oligonucleotide (20 µmol/L), were quantified, using Human VEGF Quantikine® kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Supernatants were harvested from 24h, 48h, 72h and 96h cultures. All standards and samples were tested in triplicate.

Electron microscopy

Culture cells were fixed with 2% glutaraldehyde in culture medium (15 min) and in 2% glutaraldehyde-0.1 M NaCacodylate/HCl, pH 7.4 (30 min), washed three times in 0.2 M NaCacodylate/HCl, pH 7.4, and then post fixed with 1% OsO_4-0.15 M NaCacodylate/HCl, pH 7.4 (30 min). After dehydration in a growing gradient of ethanol (30, 50, 70 and 95%, 5 min for each step), impregnation steps and inclusion were performed in Epon and finally polymerized at 60°C for

48h. The section were cut, contrasted with uranyl acetate and lead citrate before analysis with a JEOL 100CS electron microscope.

Statistical analysis

Data were analyzed by means of the two-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. Statistical analysis was performed by running the GraphPad Prism® 4.0 software (GraphPad Software, Inc.) statistical package on a personal computer. Data are reported as mean values ± SD. Two-tailed P values of less than 0.05 were regarded as statistically significant.

Results

Constitutional and inducible expression of HIF-1α in human multiple myeloma cells

Baseline expression of HIF-1α was initially investigated on MM cell lines and primary samples from MM patients. Under normoxia culture condition, HIF-1α mRNA and protein expression was detectable in all MM cell lines tested (MM1S, RPMI8226, U266 and OPM2) (Fig. 1a) and in $CD138^+$ cells from newly diagnosed MM patients samples.

Significant up-regulation of HIF-1α protein expression was observed after a short incubation with IL6 (50 ng/ml), IGF-I (100 ng/ml) or clore chloride ($CoCl_2$ 100 μM) confirming that HIF-1α can be further induced by biological stimuli (Fig. 1b).

EZN2968 efficiently inhibit HIF-1α expression in normoxic and hypoxic conditions of culture

To assess the specificity of EZN2968 toward HIF-1α mRNA, MM cell lines were culture in normoxia (pO_2 21%) or hypoxia (pO_2 1%) condition, with or without EZN2968 (20 μM) or the control oligonucleotide (20 μM). EZN2968 efficiently down modulated HIF-1α mRNA and protein expression after 6 hours of incubation either in normoxia or hypoxia conditions (Fig. 2a). Subsequently, HIF-1α and HIF-2α mRNA expression was evaluated at 24, 48 and 72 hours. As shown in Fig. 2b, the inhibition of HIF-1α was lastingly observed in a long time culture experiments, while HIF-2α expression was not affected, suggesting a highly specificity of EZN2968 for the HIF-1α isoform.

Finally we tested whether vascular endothelial growth factor (VEGF), a key transcription factor downstream target of HIF-1α, was modulated by EZN2968. The secretion of VEGF, released by MM cell lines cultured in the presence or absence of EZN2968, decreased in a time dependent manner in the treated compared to untreated samples (Fig. 2c).

Effect of EZN2968 on cells survival of MM cell lines and primary cells

We next explored the effects of EZN2968 on the growth and survival of MM cells. Under normoxia (pO_2 21%) condition of culture, the viability of MM1S cell line, tested by MTT colorimetric survival assay, after 24, 48, and 72 hours of treatment with EZN2968 was 80%, 77% and 62% compared to untreated cells, respectively; while under hypoxia (pO_2 1%) the viability was 62%, 48% and 50% compared to untreated cells, respectively (Fig. 3a). At the same culture condition, comparable percentage of viability was observed in U266, RPMI8226 and OPM2 cell lines treated with EZN2968 compare to untreated cells (Data not shown). Subsequently, MM cells were exposed to EZN2968 or control oligonucleotide for up to 96 hours, followed by drug washout and

incubation in drug-free medium for an additional 24, 48 and 72 hours. MM cells, pre-treated for 48 hours, showed a viability of 79% , 73%, 67%, after 24h, 48h, and 72h of culture in free media compare to untreated cells, respectively, suggesting irreversible modulation of viability induced by HIF deletion as early as 48 hrs (Fig. 3b).

We next evaluated the effect EZN2968 on primary samples from MM patients. In $CD138^+$ cells, treated with EZN2968 (20 μmol/L) after 24 and 48 hours of treatment, we observed a reduction of cells viability of about 28% and 14% compared to control, respectively while $CD34^+$ cells and peripheral blood mononuclear cells (PBMCs) derived from healthy donors treated with EZN2968 for 24, 48 and 72 hours resulted in no effect on cell viability and proliferation compared to control, suggesting that non malignant cells were barely sensitive to EZN2968 treatment (Fig. 4a-d).

In order to address whether HIF-1α inhibition was able to affect cell proliferation, we examined cell cycle profile. Treatment with EZN2968 (up to 72 hrs) gave rise to a progressive accumulation of cells in S-phase (from 37,04% at 24 hrs to 57,56% at 72 hrs in treated cells compared to control cells), a concomitant reduction of G2/M phase (from 5,69% at 24 hrs to 1,35% at 72 hrs in treated cells compared to control cells), and a moderately increased of the subG0 phase (from 4,38% at 24 hrs to 11,48% at 72 hrs in treated cells compared to control cells). These data let us to suggest that EZN2968 triggers arrest in S-phase (Fig.3c).

EZN2968-induced arrest in S-phase was further confirmed by cell cycle analysis on previous synchronize S-phase cells treated with EZN2968 for 48 hrs (Data not shown).

To evaluate if HIF-1α depletion affected survival of MM cells, we analysed AnnexinV/PI staining at 24h, 48h, and 72h After 72hours of incubation we observed about 20% of Annexin V positive cells compared to untreated cells at 72 hours (Fig. 3d). Taken together these data providing suggestions that HIF-1α inhibition blocks cell cycle progression while slighting enhanced a delayed apoptosis.

Cellular response induced by inhibition of HIF-1α

In order to determine the mechanism behind the cell cycle arrest, whole cell lysed was analysed for the level of key markers of cell cycle progression. Western blott analysis revealed that HIF-1α inhibition caused slightly increased in Cyclin D2, while no change were observed in Cyclin E. and Cyclin B1Significantly, after 24 hrs of treatment, the cyclin-dependent kinase inhibitors p21 showed dramatic increased in protein level lastingly unregulated for at least for 72 hrs (Fig. 5a).

Subsequently, to investigate the mechanism of cell death induced by HIF-1α deletion, we tested both apoptosis and autophagy markers. Western blott analysis showed PARP cleavage after 48 hrs of treatment while no change in Beclin1 or LC3A /B level was observed.

Interestingly, gene expression profiling analysis of the significantly modulated transcription factors induced by EZN2968 treatment showed that SP1-induced genes were the most significant modulated. SP1, a transcription factor regulated by hypoxia inducible factors, has been shown to induced p21 expression in HIF-1α knowdown cells (Fig. 5b).

HIF-1 α and bone marrow microenvironment

To assess if EZN2968 was able to block HIF-1α upregulation induced by hypoxia, we evaluated on several MM cell lines HIF-1α mRNA level in the presence or absence of stroma cell with or without EZN2968 treatment. As shown in Fig 6a, HIF-1α was significantly down regulated by EZN2968 even in the presence of stromal cells. We next examined whether EZN2968 has an impact on MM cell viability and proliferation in the presence of BM stroma cells and cytokines IL6 and IGF1. Cell viability was tested on MM.1S, U266, OPM-2 and RPMI8226 cells, cultured in the presence of IL-6 (10 ng/L), IGF-1 (50 ng/L) and stromal cells for 24-72 h with or without EZN-2968 (20 μmol/L) for up to 72 hrs. As shown in Fig 6b, EZN2968 strongly inhibited viability and proliferation of MM cell lines in the presence of stroma cells as well as IL-6 and IGF-1 as early as 24 hours. Importantly, these data indicate that EZN2968 is more potent against MM cell growth and proliferation in the presence of BM cells and cytokines than against MM cells alone.

HIF-1 α blocks MAPK kinase signalling pathway and cytokines secretions induced by bone marrow microenvironment

Several signaling pathways mediate the pleiotropic proliferative and antiapoptotic response of MM cells upon interaction with the BM microenvironment. We therefore tested whether EZN2968 affected the following major MM pathways: (*a*) the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, (*b*) the Ras/Raf/MAPK kinase (MEK)/extracellular signal–regulated kinase (ERK) pathway, and (*c*) the Janus kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) pathway. Western blot analysis were performed on proteins lyses from MM1S, cultured in the presence of stroma cells-culture supernatant with or without EZN2968. Baseline and phosphorylated form of AKT, STAT3 and ERK1/2 was evaluated. Of note, ERK phosphorylation was strongly inhibited by EZN2968, while no change in the phospho-forms of STAT3 and AKT was observed (Fig. 7a).

Many of the growth factors secreted by MM cells and BMSCs stimulate osteoclastogenesis and angiogenesis. Thus, targeting MM-BMSC interactions and related growth factors may provide the basis for the development of novel treatment strategies for MM and associated bone disease. We

tested whether EZN2968 affected cytokine secretion. Supernatant of MM1S cells cultured in the presence or absence of stroma cells with or without EZN2968 treatment was tested by ELISA assay. As shown in Fig.7b, IL6, IL8, IL10 and IFNγ, were significantly reduced in the presence of the HIF-1α inhibitor.

Discussion

Despite major therapeutic advances, MM still remains incurable[25-26]. Compelling evidence suggests that BM microenvironment factors promotes MM cell growth and drug resistance. In solid tumors, hypoxia is one of the microenvironmental factors that drives tumor progression and treatment resistance and preclinical and clinical studies provides evidence that HIF-1α inhibition affects tumour survival[27-28].

MM develops in a specialized hypoxic BM microenvironment and abnormal activation of HIF-1, has been reported, however few is known about the role of HIF in promoting and sustain tumour growth[20].

In the present study, we examined the role of HIF-1α in MM using an antisense oligonucleotide specific for HIF-1α mRNA (EZN2968).

We identified constitutive expression of HIF-1α in established myeloma cell lines and in primary myeloma cells, in addition IGF-1, IL6 and stroma cells significantly enhanced HIF-1α expression, confirming that HIF-1α lies downstream signalling pathway involved in MM survival.

In agreement with previous studies on solid tumours[3], inhibition of HIF-1α gave rise to a progressive accumulation of cells in S-phase, while only a small percentage of cells entered in apoptotic phase. Of note, we observed that HIF-1α inhibition induced upregulation of p21 and Cyclin D2. Our preliminary observation of most significant modulated transcription factors induced by HIF inhibition suggests the involvement of SP1 transcriptional genes, mainly related to cell cycle regulation. More data are needed to confirm the modulation of SP1-P21 network induced by HIF inhibition.

Importantly we observed that HIF inhibition showed stronger efficacy in reducing cell viability in the presence of bone marrow stroma cells compared to plasmacells alone , suggesting that hypoxic pathway is fundamental in sustain survival and progression of MM cells.

Moreover HIF inhibition blocks MAPK signaling pathway s while did not affect PI3-K/AKT and STAT3 signaling. Interestingly, proangiogenic (VEGF) and pro-inflammatory citokines (IL6, IL8) were suppressed after treatment even in the presence of stroma cells.

Theses data suggest that targeting HIF in multiple myeloma allow not only to induced a cell cycle arrest , but strongly affect the signaling mediated by bone marrow microenvironment

We conclude that HIF-1 inhibition may be an attractive therapeutic strategy for multiple myeloma..

Figures

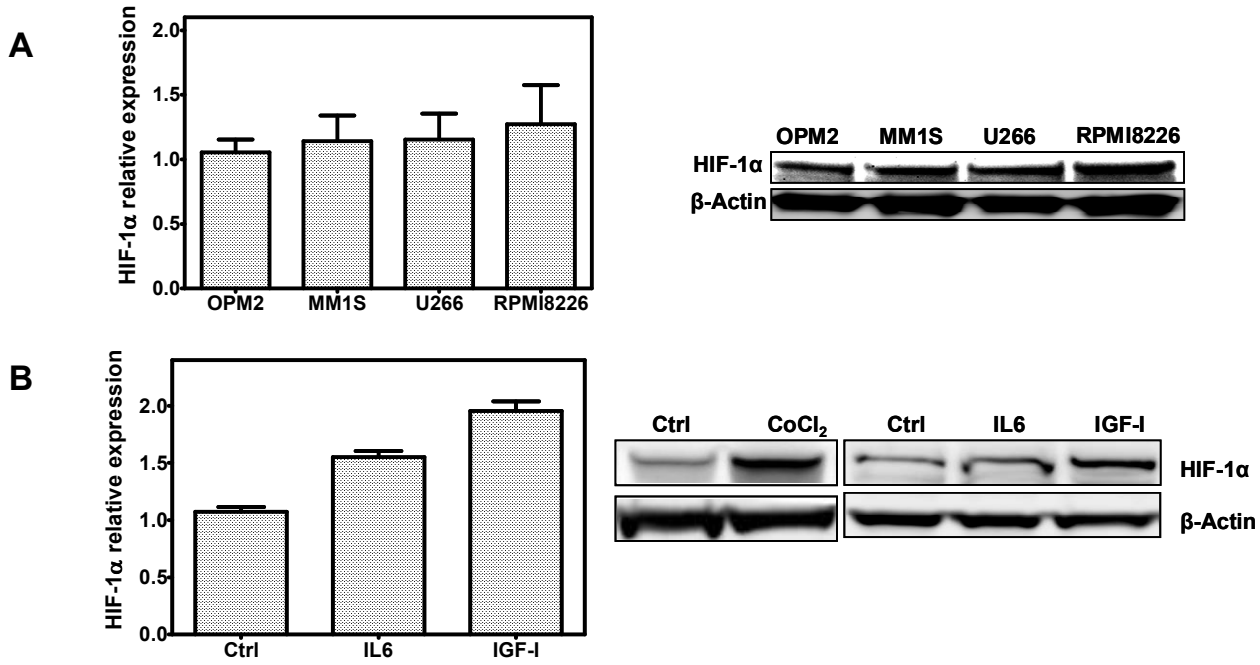


Figure 1. HIF-1α expression in multiple myeloma cells. (**A**) Baseline level of HIF-1α in normoxia culture condition was assessed by Western Blotting analysis in four MM cell lines. To evaluate if our system was able to induce HIF expression, MM1S was treated with Interleukin 6 (50 ng/ml) or Insulin like Growth Factor I (100 ng/ml) for 4h, or $CoCl_2$ (100 μM) for 24h. (**B**) mRNA expression of HIF-1α was measured by qRT-PCR and normalized to GAPDH level.

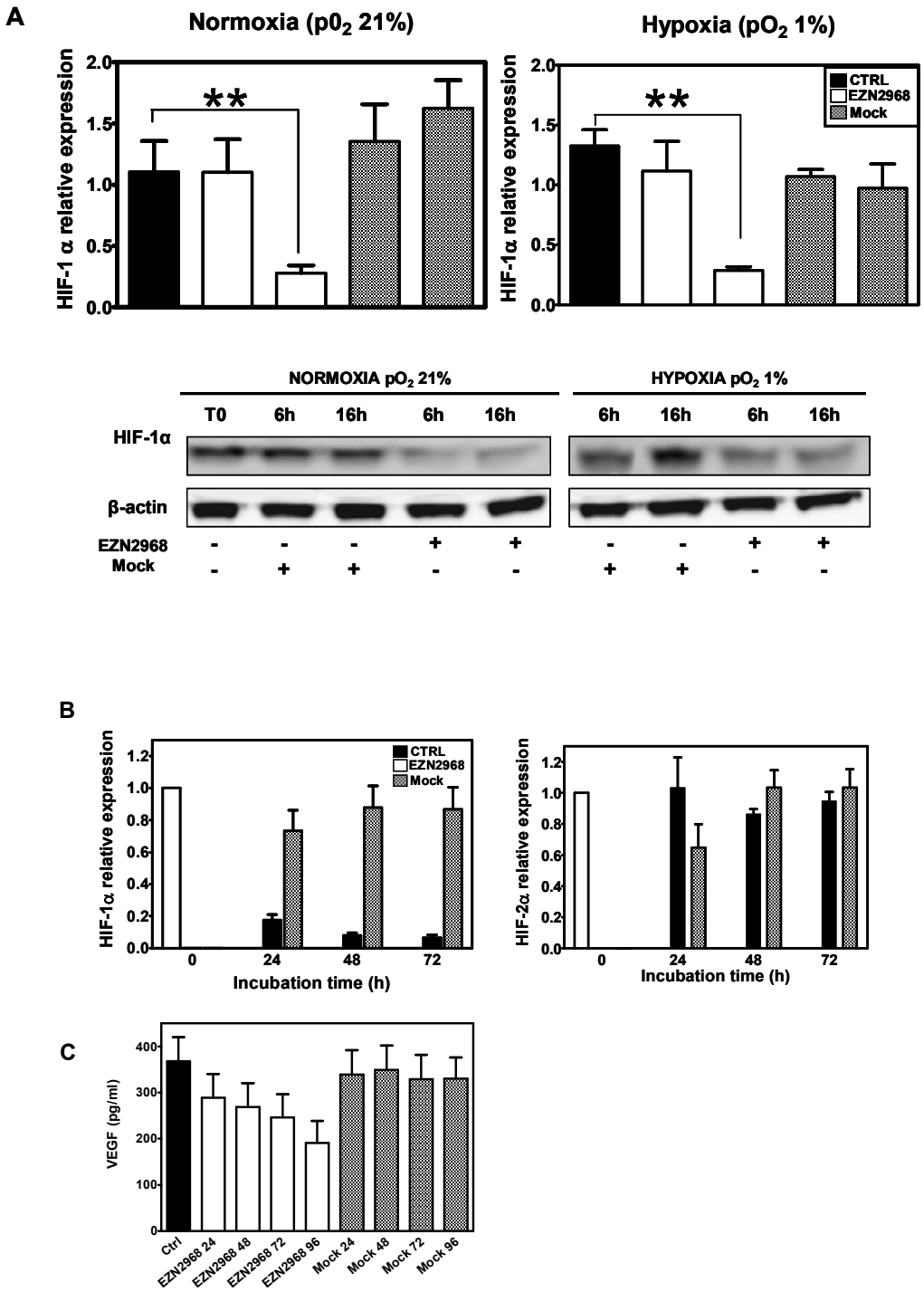


Figure 2. Effect of HIF-1α antagonist on mRNA and protein level in MM cells. (**A**) MM1S cell line was incubated in the presence of EZN2968 or control oligonucleotide (20 μmol/L) for 6h and 16h in normoxic or hypoxic conditions respectively. The HIF-1α mRNA expression and protein level was then analyzed by qRT-PCR (normalized to GAPDH level) and Western Blotting (β-actin served as a control fro equal protein loading). (**B**) MM1S cells were incubated in the presence of EZN2968 or control oligonucleotide (20 μmol/L) for 24h, 48h, and 72h respectively, and mRNA expression of HIF-1α and HIF-2α was evaluated by qRT-PCR. (**C**) VEGF level secreted by MM cells alone or treated in the presence or absence of EZN2968, were quantified by ELISA test. Values shown in **A** and **B** are mean ± SD calculated based on triplicate measurements (** $p<0.01$)

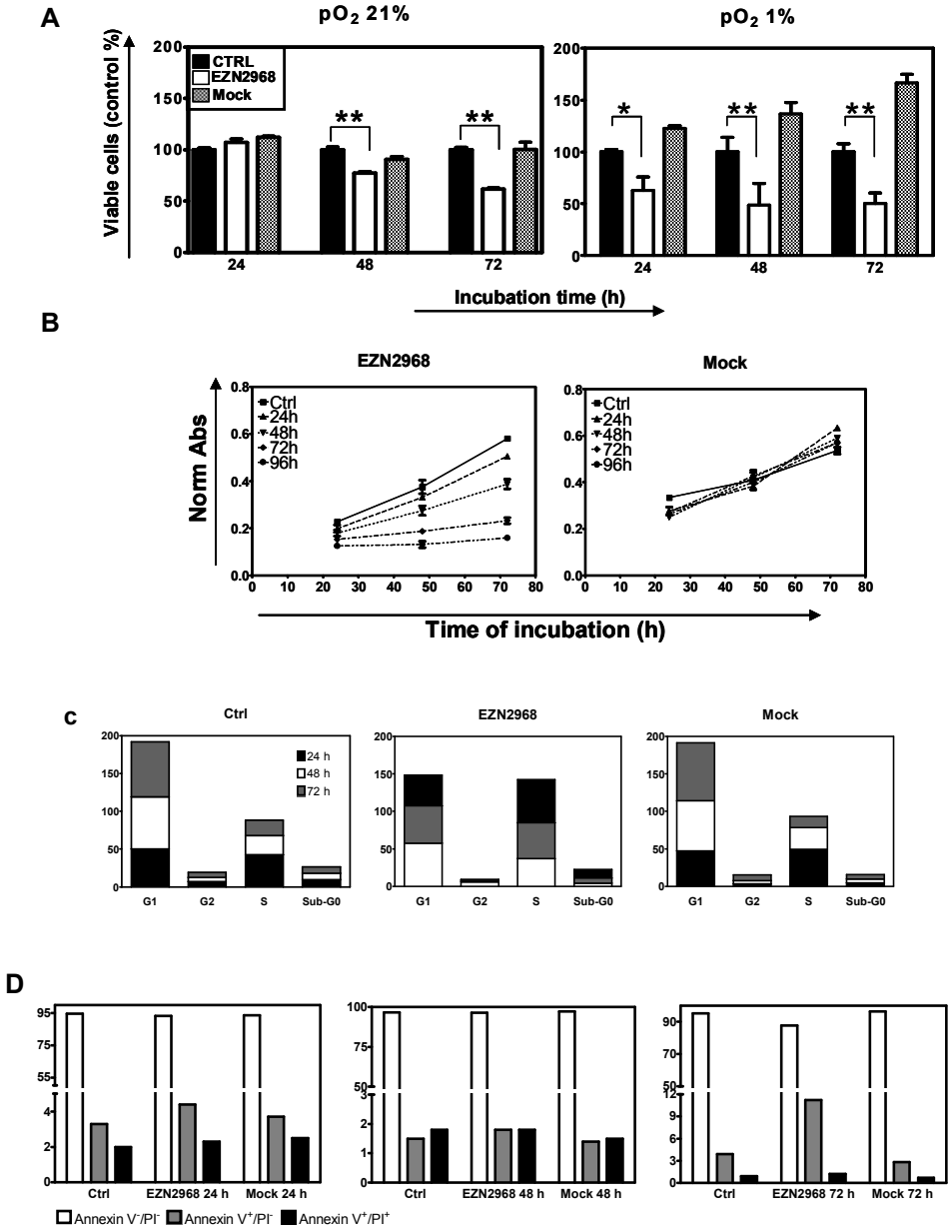


Figure 3. EZN2968 causes cell death. (**A**) The viability of MM1S cells in normoxia and hypoxia conditions was evaluated at 24h, 48h and 72h by MTT colorimetric survival assay. (**B**) A cell death commitment assay was performed to evaluate the irreversible impact on cell viability of EZN2968. MM1S cells were exposed to EZN2968 for up to 96h followed by drug washout and additional incubation in drug free medium for up to 72h.(**C**) MM1S cells were exposed to 20 μmol/L of EZN2968 or control oligonucleotide for 24h and 72h and their cell cycle profiles were compared to the control cells cultured for 72h in absence of the drug, using propidium iodide (PI) staining. (**D**) MM1S cells were exposed to EZN2968 and Mock (20 μmol/L) for 24h, 48h and 72h collected, washed, stained for Annexin V and PI, and analyzed using flow cytometry. Exposure to EZN2968 resulted in an increased fraction of Annexin V^-PI^+ events indicating a cell death event. Values shown in **A** and **D** are mean ± SD calculated based on three independent experiments (** $p<0.01$).

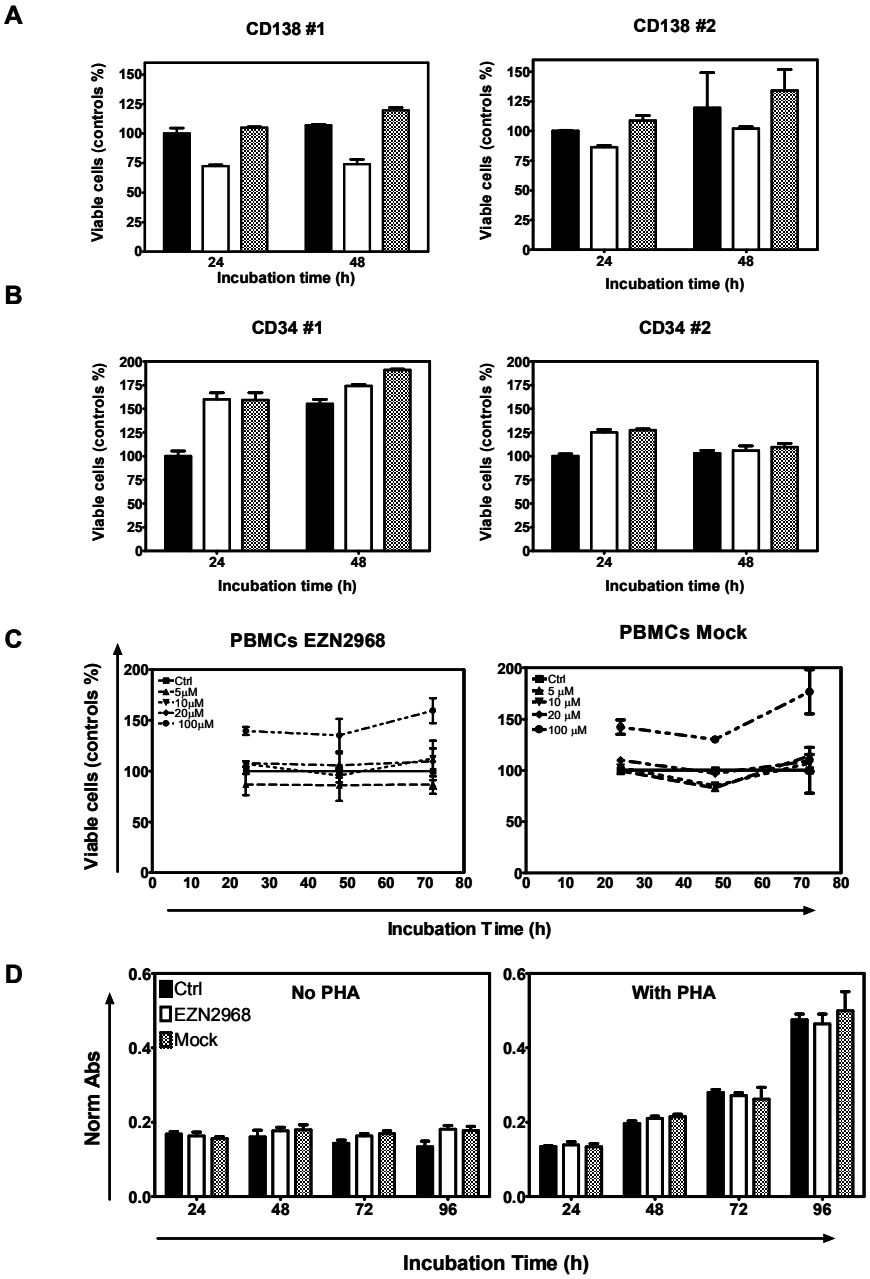
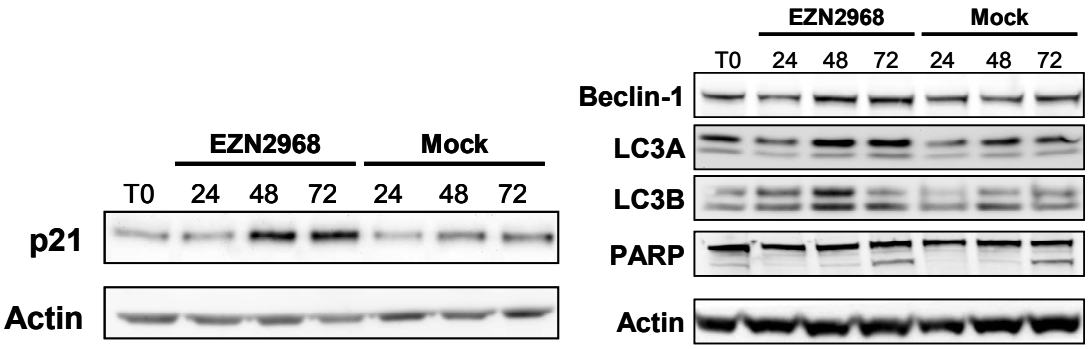


Figure 4. Effect of EZN2968 on primary and non-malignant cells from MM patients. $CD138^+$ cells from MM patients and $CD34^+$ from healthy donors were purified and treated with or without EZN2968 for 24h and 48h. MTT colorimetric survival assay was performed. PBMC cells were incubated in the presence or absence of EZN2968 (20 μmol/L) for up to 96h with or without *phytohemagglutinin* stimulation (1 μg/μl *PHA*). Values shown in **A**, **B** and **C** are mean ± SD calculated based on triplicate measurements.



	Network	Go processes	P-value	zScore	gScore
1	SP1	regulation of localization (36.8%; 3.318e-07), organ development (44.7%; 2.425e-06), multicellular organismal process (68.4%; 3.637e-06), multicellular organismal development (55.3%; 6.032e-06), regulation of transport (28.9%; 8.268e-06)	7,170E-94	108,27	108,27
2	HNF4-alpha	carbohydrate homeostasis (13.8%; 3.669e-05), glucose homeostasis (13.8%; 3.669e-05), anatomical structure homeostasis (13.8%; 1.688e-04), mRNA transcription (6.9%; 1.960e-04), lipid homeostasis (10.3%; 3.644e-04)	3,560E-73	95,5	95,5
3	AP-1	locomotion (37.5%; 2.363e-06), defense response (37.5%; 4.080e-06), regulation of smooth muscle cell proliferation (16.7%; 1.234e-05), inflammatory response (25.0%; 1.316e-05), response to external stimulus (37.5%; 2.325e-05)	8,560E-58	84,67	84,67
4	C/EBPbeta	positive regulation of peptidyl-tyrosine phosphorylation (18.2%; 8.573e-06), response to external stimulus (40.9%; 1.008e-05), regulation of peptidyl-tyrosine phosphorylation (18.2%; 2.566e-05), chemotaxis (27.3%; 4.190e-05), taxis (27.3%; 4.258e-05)	3,050E-55	82,73	82,73
5	NF-kB	cellular response to lipopolysaccharide (20.0%; 4.078e-06), inflammatory response (30.0%; 4.095e-06), cellular response to molecule of bacterial origin (20.0%; 5.082e-06), cellular response to biotic stimulus (20.0%; 8.052e-06), response to wounding (40.0%; 9.175e-06)	1,310E-47	76,62	76,62
6	GATA-1	positive regulation of exit from mitosis (10.5%; 1.942e-05), regulation of exit from mitosis (10.5%; 1.310e-04), positive regulation of neutrophil chemotaxis (10.5%; 1.447e-04), regulation of neutrophil chemotaxis (10.5%; 2.063e-04), response to external stimulus (36.8%; 2.204e-04)	4,510E-45	74,47	74,47
7	GCR-alpha	response to stimulus (89.5%; 3.360e-06), response to external stimulus (42.1%; 2.478e-05), regulation of response to external stimulus (26.3%; 2.690e-05), regulation of localization (42.1%; 3.781e-05), regulation of low-density lipoprotein particle receptor biosynthetic process (10.5%; 3.809e-05)	4,510E-45	74,47	74,47
8	CREB1	cellular response to peptide hormone stimulus (29.4%; 8.744e-06), response to external stimulus (47.1%; 9.074e-06), regulation of localization (47.1%; 1.395e-05), regulation of cell migration (29.4%; 1.703e-05), regulation of cell motility (29.4%; 2.143e-05)	5,200E-40	69,98	69,98

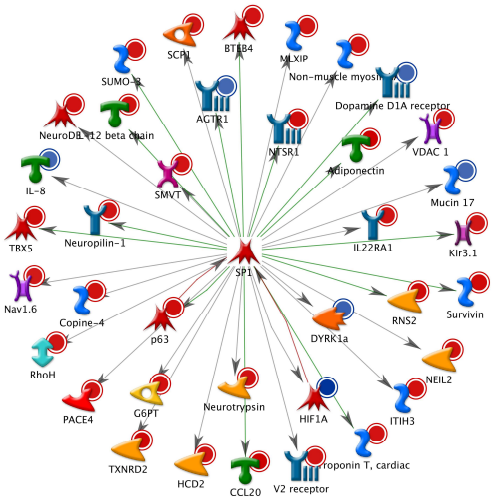
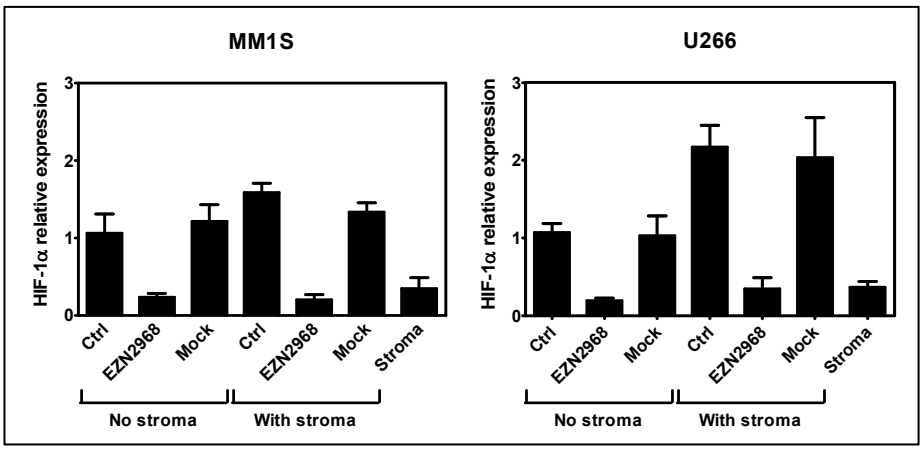


Figure 5. MM1S were incubated with EZN2968 or control oligonucleotide for up to 72h. Whole cell lysates were prepared and analyzed by Western blot for the expression of proteins involved in autophagy and apoptosis. (**A**). Gene expression profiling analysis of the significantly modulated transcription factors induced by EZN2968 was analysed by *GeneGo process*(**B**);most significant modulated. SP1-induced genes were represented by *GeneGo Process* analysis(**C**).

A



B

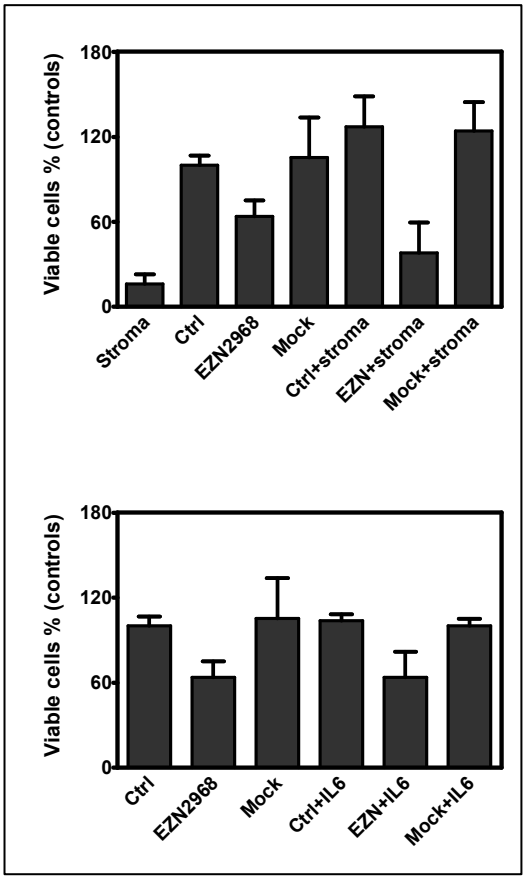
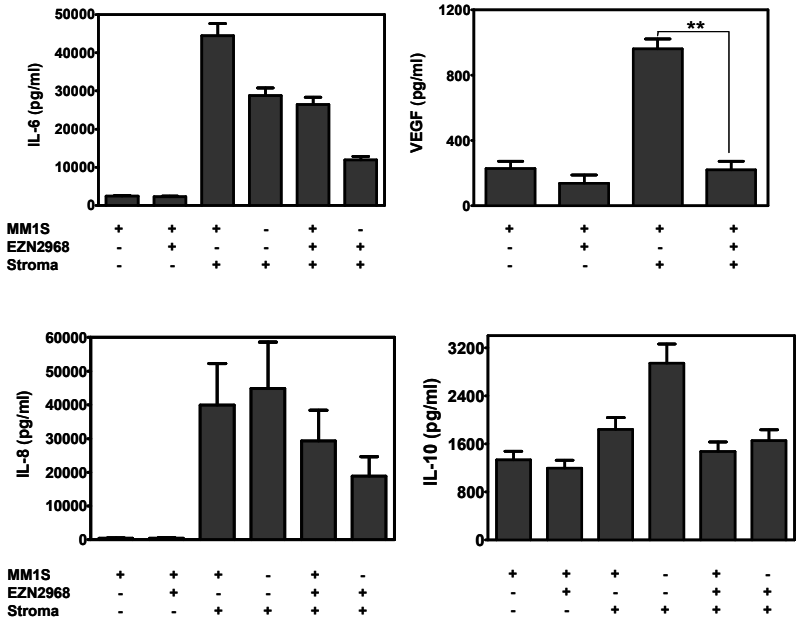


Figure 6 HIF-1α and stroma cells. MM1S were incubated for 24hrs with or without bone marrow stroma cells, in the presence of EZN2968 or control oligonucleotide (20 μmol/L) in normoxic conditions.HIF-1α mRNA expression was analyzed by qRT-PCR (normalized to GAPDH level) and Western Blotting (β-actin served as a control fro equal protein loading)(**A**). Viability of MM1S cells in normoxia and hypoxia conditions was evaluated at 24h by MTT colorimetric survival assay(**B**)



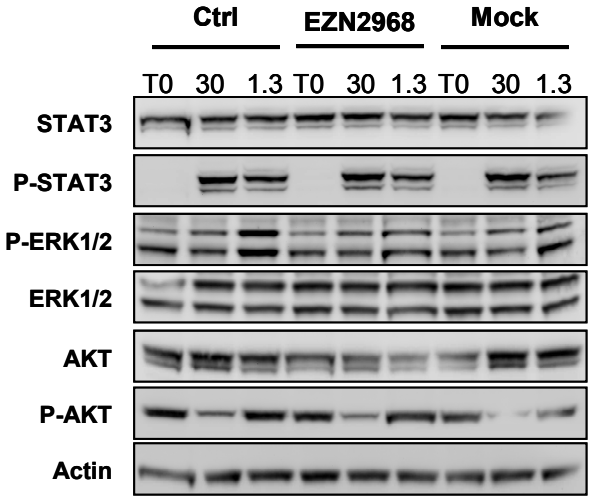


Figure 7 HIF-1α inhibition block survival pathways and inflammatory cytokines induced by stroma cells. MM1S were incubated for 24hrs with or without bone marrow stroma cells, in the presence of EZN2968 or control oligonucleotide (20 μmol/L) in normoxic conditions. Supernatant ,media was analysided by Elisa assay for testing proinflammatory cytokines(**A**); western blot analysis of Baseline and phosphorylated form of AKT, STAT3 and ERK1/2 was evaluated.(**B**);

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