Universidade do Algarve
Departamento de Ciências Biomédicas e Medicina

Extracellular matrix and angiogenic factors in hematological diseases

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RESUMO

A angiogénese consiste na formação de novos vasos sanguíneos através de vasos pré-existentes. Nos adultos, a vasculatura mantêm-se quiescente, excepto em processos altamente controlados, como nos ciclos reprodutores femininos e na cicatrização. No entanto, muitas doenças evoluem graças à angiogénese, como a artrite reumatóide, a neovascularização ocular, o crescimento tumoral e a metastização. Em tumores, a angiogénese é induzida sobretudo pelas condições de hipóxia que ocorrem nestes tecidos, levando à produção de vários factores pró-angiogénicos como o VEGF (o mais importante), PDGF, FGF, angiopoitinas, entre outros. O VEGF pode sofrer "splicing" alternativo, levando à expressão de quatro isoformas que variam no número de aminoácidos e na presença ou ausência de locais de ligação à heparina. $VEGF_{165,}$ $VEGF_{189}$ e $VEGF_{206}$ podem ligar-se às membranas celulares ou à matriz extracelular (MEC) através dos domínios de ligação à heparina; o $VEGF_{121}$ é uma proteína não ligada à heparina, apresentando-se numa forma solúvel, livremente difusível no meio. Para além destes factores pró-angiogénicos, as metaloproteases de matriz (MMPs) também são importantes na degradação da membrana basal que envolve os vasos, permitindo a migração das novas células endoteliais. A angiogénese é importante na progressão de uma patologia crónica para uma aguda, mais agressiva, estando bastante estudada em tumores sólidos, sendo, inclusivamente, um dos principais alvos terapêuticos nestas doenças. No entanto, só mais recentemente se começou a atribuir importância a este processo em tumores hematológicos, que ocorrem na medula óssea (MO) e órgãos linfáticos.

As disfunções da MO são doenças clonais resultantes da transformação neoplásica de células estaminais/progenitoras hematopoiéticas (CEPH). Entre elas, a leucemia mielóide aguda (LMA) é caracterizada por um rápido aumento de células sanguíneas imaturas, os blastos, devido a uma inibição nas vias apoptóticas destas células. Tal interfere com a homeostasia da MO e inibe a produção normal de células sanguíneas. As síndromes mielodisplásicas (SMD) são um conjunto heterogéneo de doenças hematológicas que apresentam frequentemente citopénias no sangue periférico, apesar de a MO poder apresentar-se normocelular ou hipercelular. Esta situação pode estar relacionado com um aumento da apoptose que se verifica numa

ou em várias linhagens hematopoiéticas. As SMD apresentam um risco elevado de progredir para LMA. Apesar das causas genéticas destas doenças estarem bem descritas, sabe-se que determinados componentes do microambiente medular protegem as células malignas da quimioterapia e propiciam a sua proliferação e sobrevivência. No entanto, muito falta saber acerca do microambiente na progressão, ou mesmo iniciação, das doenças hematológicas, nomeadamente na angiogénese que lhes poderá estar associada. Foi reportado um aumento da angiogénese em SMD e LMA, comparativamente a dadores saudáveis, o que sugere um papel deste processo nestas doenças. O microambiente da MO inclui o estroma (fibroblastos, células endoteliais, osteoblastos, macrófagos), factores solúveis (factores de crescimento, citocinas e quimiocinas), MMPs e a MEC. A MEC inclui várias moléculas como a fibronectina (FN), lamininas, colagéneos, glicosaminoglicanos e proteoglicanos, sendo uma estrutura bastante dinâmica com uma composição espacial e temporal bem definidas dentro da MO, o que influencia as propriedades das CEPH. A FN, as MMPs e vários factores de crescimento estão alterados quantitativamente em diversos tumores sólidos.

Assim, o objectivo deste trabalho foi compreender se o microambiente da MO é diferente entre SMD e LMA e que papel este factor poderá ter na progressão destas doenças. Através da técnica de imunoprecipitação, verificámos um aumento significativo de VEGF ligado à FN em MO de doentes com LMA. Por outro lado, a análise por ELISA do sobrenadante de MO revelou um aumento de VEGF solúvel na MO de doentes com SMD. Nesta doença, observámos um aumento da actividade das MMPs comparativamente aos doentes com LMA, que poderá ser responsável pela libertação do VEGF solúvel nas MO de SMD e, consequentemente, ao aumento da angiogénese verificado nesta patologia. As nossas experiências *in vitro* comprovaram que quando as MMPs são inibidas, há uma diminuição do VEGF solúvel. Estes resultados sugerem que a biodisponibilidade de factores angiogénicos, nomeadamento do VEGF, pode ser controlada pelo seu grau de associação à FN (dependende das MMPs) e que este fenómeno é importante na angiogénese das doenças hematológicas. Por imunohistoquímica, observámos um aumento da FN em

MO de doentes com LMA e quisemos saber o que poderia estar a regular a expressão desta proteína. Sabe-se que a via de sinalização Notch, é importante na angiogénese de tumores sólidos, mas o seu papel nas doenças hematológicas é bastante controverso. Um estudo em embriões de ratinho demonstrou que a sobrexpressão do receptor Notch leva a um aumento da FN à volta dos vasos sanguíneos em formação. Por PCR quantitativo realizado nas nossas amostras de MO, concluímos que esta via de sinalização está activa em ambas as doenças. No entanto, observámos uma forte correlação entre a expressão de elementos da via NOTCH e a expressão da FN em MO com LMA, o mesmo não se tendo verificado para as SMD. Aliando estes resultados aos anteriores relativos à regulação do VEGF, concluímos que em LMA, o VEGF encontra-se essencialmente ligado à FN, sendo a actividade das MMPs inferior à encontrada na MO com SMD. Nestas condições, a biodisponibilidade do VEGF é mais regulada, tornando a angiogénese menos exuberante. Além disso, a via de sinalização Notch poderá estar a regular a produção/deposição de FN que envolve os novos vasos sanguíneos, contribuindo para a formação de vasos mais estáveis e funcionais. Por outro lado, em SMD, a elevada actividade das MMPs leva a um aumento do VEGF solúvel na MO que, aliado a uma diminuição da FN à volta dos vasos sanguíneos em formação (dependente da via de sinalização Notch), contribui para um aumento da angiogénese e formação de vasos instáveis e menos funcionais. Assim, as diferenças encontradas no microambiente medular de SMD e LMA, nomeadamente os diferentes níveis de VEGF, MMPs e FN, deverão condicionar a angiogénese nestas doenças e, provavelmente, algumas das suas características patológicas: aumento da apoptose em SMD e sobrevivência dos blastos em LMA. Dentro do nosso conhecimento, nada foi descrito sobre a regulação da biodisponibilidade do VEGF na MO em disfunções hematológicas, assim como sobre a sinalização Notch na angiogénese destas doenças.

Palavras-chave: Medula óssea, microambiente, angiogénese, síndromes mielodisplásicas, leucemia mielóide aguda, fibronectina, VEGF, metaloproteases de matriz

ABSTRACT

Bone marrow (BM) malignancies are clonal disorders resulting from neoplastic transformation of hematopoietic stem/progenitor cells (HSPCs). Among them, acute myeloid leukemia (AML) is characterized by a rapid increase in immature blood cells numbers, due to apoptosis suppression; myelodysplastic syndromes (MDS) are characterized by peripheral cytopenia, related with increased apoptosis, and can develop to AML. Angiogenesis is an important event that mediates the progression from a chronic to a more acute and aggressive pathology, and its significance in hematological malignancies has just beginning to be explored. BM microenvironment, including soluble factors and extracellular matrix (ECM), in particular fibronectin (FN) that has been found increased in solid tumors, may be responsible for BM disease progression, but its precise role in this context has been poorly investigated. Thus, the aim of this thesis was to know if the BM microenvironment differs between MDS and AML and what role might such factor be playing in disease progression. Our results indicate that MDS BM has more soluble VEGF, a pro-angiogenic factor, and higher matrix metalloproteinases (MMPs) activity, which may be responsible for increased angiogenesis occurring in this disease. Notch pathway, known to be involved in solid tumors angiogenesis, does not regulate FN expression in MDS BM, which leads to new formed vessels instability. In AML, VEGF is kept majorly bounded to FN, and MMPs activity is lower than in MDS BM. In such conditions, VEGF bioavailability is more regulated, being angiogenesis less exuberant. In addition to this, Notch pathway regulates FN deposition around new vessels, contributing to the formation of a more functional and stable vasculature in AML. To our knowledge, nothing has been described about the regulation of VEGF bioavailability in BM diseases, as well as regarding the possible effect of Notch signaling in angiogenesis of hematological dysfunctions.

Keywords: Bone marrow, microenvironment, angiogenesis, myelodysplastic syndromes, acute myeloid leukemia, fibronectin, VEGF, matrix metalloproteinases

ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
Ang	Angiopoietin
BM	Bone marrow
BSA	Bovine serum albumin
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
DAB	Diaminobenzidine
DAPT	N-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine *t*-butyl ester
DEPC	Diethyl pyrocarbonate
Dlk	Delta-like
DLL-4	Delta-like ligand 4
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme Linked Immunosorbent Assay
ET	Thrombocythemia essential
FAB	French-American-British
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
FGFR	Fibroblast growth factors receptor

Flk-1	Fetal liver kinase-1
Flt-1	fms-like tyrosine kinase-1
FN	Fibronectin
HC	Hematopoietic cells
HGF	Hepatocyte growth factor/scatter factor
HIF1α	Hypoxia-inducible factor 1alpha
HRP	Horseradish peroxidase enzyme
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem/progenitor cell
HSPG	Heparan sulfate proteoglycans
IPSS	International Prognostic Scoring System
JAG	Jagged
KDR	Kinase insert domain receptor
LTBM	Long-term bone marrow
MAP	Mitogen-activated protein
MDS	Myelodysplastic syndrome
MDS-U	Myelodisplasic syndrome unclassified
MK	Megakaryocyte
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
MPS	Myeloproliferative syndromes
MT2-MMP	Membrane type-2 matrix metalloproteinase
NRP	Neuropilin
ON	Overnight
PBS	Phosphate buffered saline

PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PMF	Primary or idiopatic myelofibrosis
PV	Polycythemia vera
RA	Refractory anemia
RAEB	Refractory anemia with an excess of blasts
RARS	Refractory anemia with ringed sideroblasts
RCMD	Refractory anemia with multilinage dysplasia
RCMD-RS	Refractory anemia with multilinage dysplasia and ringed sideroblast
RNase out	Ribonuclease inhibitor
RT-PCR	Real-time polymerase chain reaction
RQ-PCR	Quantitative real-time polymerase chain reaction
RT	Room temperature
SDF	Stromal cell-derived factor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	Tris/Borate/EDTA
TBS	Tris Buffered Saline
TGF	Transforming growth factor
TGS	Tris/glycine/SDS
TIMP	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptors
WHO	World Health Organization

INTRODUCTION

Angiogenesis: key players and biological importance

Blood vessels are fundamentally composed of endothelial cells, which interconnect to form the tubes that direct and maintain blood flow and tissue perfusion. The development of blood vessels in embryogenesis occurs by two processes: vasculogenesis, whereby endothelial cells derive primarily from progenitor cells, and angiogenesis, in which new capillaries sprout from existing vessels. In adult mammals, new vessels are produced only through angiogenesis although a role for endothelial progenitors has been shown in several physiological and pathological situations.

In adults, the vasculature is quiescent except for highly organized processes in the female reproductive cycles (ovulation, menstruation, implantation, pregnancy) (Hanahan and Folkman, 1996). However, many diseases are driven by persistent unregulated angiogenesis, like rheumatoid arthritis (Moon *et al.*, 2010), ocular neovascularization, tumor growth and metastasis (Hanahan and Folkman, 1996; Parangi *et al.*, 1996), among others (reviewed in Carmeliet, 2005).

Major growth factors involved in the angiogenic process

The formation of new blood vessels and their permeability is primarily regulated by vascular endothelial growth factor (VEGF) or VEGF-A (after the discovery of other VEGF family members, like VEGF-B, -C, -D and -E) (Connolly *et al.*, 1989; Ferrara and Henzel, 1989). VEGF is a dimer composed by two subunits that vary 18 to 34kDa subunits (Connolly *et al.*, 1989). Four different transcripts of this protein have been identified resulting from alternate splicing of exon 6 and 7, which alters their heparin-binding affinity, and amino acid number: originating four isoforms, 206, 189, 165 and 121 (Tischer *et al.*, 1991). In addition, inclusion or exclusion of exons 6 and 7 mediate interactions with heparan sulfate proteoglycans (HSPGs) and neuropilin (NRP) co-receptors on the cell surface, enhancing their ability to bind and activate the VEGF receptors (VEGFR). This molecule had specific mitogenic effect on vascular endothelial cells, but not in adrenal cortex cells, lens epithelial cells, corneal endothelial cells, keratinocytes or fibroblasts (Ferrara and Henzel, 1989). Recently, a novel group of iso-

forms, the so-called “b-isoforms” or “VEGFxxxb” isoforms, have been described. These transcripts of the VEGF-A gene code for polypeptides with the same length as the classical ones, because exon 8 (present in all the formerly known isoforms) is substituted by an alternatively spliced exon of the same size (exon 8b) (Ladomery *et al.*, 2007; Catena *et al.*, 2010).

VEGF acts via two receptors protein tyrosine kinases, flt-1 (fms-like tyrosine kinase, VEGFR1) or flk-1 (fetal liver kinase-1; KDR, VEGFR2) (de Vries *et al.*, 1992; Quinn *et al.*, 1993). The binding of VEGF to VEGFR, in particular to flk-1/KDR, is responsible for activating several signaling cascades in endothelial cells, within different physiological functions, including survival (Gerber *et al.*, 1998), proliferation (Thakker *et al.*, 1999), and vascular permeability (Gille *et al.*, 2001). Moreover, VEGF can also bind to NRP-1, acting as a co-receptor for flk-1/KDR, and enhancing the VEGF signaling (Herzog *et al.*, 2011).

Platelet-derived growth factor (PDGF) is a family of heterodimeric or homodimeric isoforms of A- and B- polypeptide chains, synthesized as precursor molecules undergoing proteolytic maturation. These molecules are produced by different cell types and act via protein kinase receptors (Heldin and Westermark, 1999). PDGF family has a complex role in regulation of blood vessel formation (Cao *et al.*, 2002).

Fibroblast growth factors (FGFs) are a family of 20 heparin-binding growth factors. FGFs exert their pro-angiogenic activity by interacting with various endothelial cell surface receptors, including tyrosine kinase receptors, HSPGs, and integrins (Kan *et al.*, 1993). The binding of FGF isoforms to heparin sites protect growth factors from acid, heat and degradation by circulating proteases, as thrombin (FGF-1), trypsin (FGF-2) or plasmin, affecting biological signaling (Powers *et al.*, 2000). Several isoforms of this family are mitogenic for endothelial cells, like FGF-4 (Delli-Bovi *et al.*, 1988), but they have pleiotropic effects, stimulating the growth of several other cells, as smooth muscle cells, fibroblasts, and certain epithelial cells (Folkman and Shing, 1992). Furthermore, FGFRs have variable activity in promoting angiogenesis, with FGFR-1 sub-

group being associated with tumor progression and FGFR-2 subgroup with either early tumor development or decreased tumor progression (reviewed in Korc, 2009).

The angiopoietin (Ang) family of growth factors is also important for blood vessel formation (Valenzuela *et al.*, 1999). Ang-1 and Ang-4 are angiogenic factors that signal through the Tie-2 receptor tyrosine kinase, essential for normal vascular development in the mouse (reviewed in Peters *et al.*, 2004; Fukuhara *et al*., 2009, 2010). On the other hand, Ang-2 and Ang-4 bind to Tie-2 receptor and act as antagonists of Ang1, what is unusual for a family of ligands (Maisonpierre *et al.*, 1997). The biological action of angiopoietin family depends on the unique mixture of angiogenic factors and their receptors operating in tissue microenvironment (Olsen *et al.*, 2006).

Proteolytic degradation of the extracellular matrix: also part of the angiogenesis processes and the remodeling of tissues

Proteolytic degradation of extracellular matrix (ECM) is implicated in many steps of the angiogenic cascade, not only by degradation of vessel basement membranes, but also in cell migration, ECM invasion and capillary morphogenesis (Maciag *et al.*, 1982; Bellon *et al.*, 2004; reviewed in Bourboulia and Stetler-Stevenson, 2010). ECM proteolysis results from secretion and activation of matrix metalloproteinases (MMPs) in response to exogenous signals, such as cytokines, growth factors and cell-matrix interactions (Bellon *et al.*, 2004). MMP-9 is upregulated during the carcinogenic process in pancreatic islets, having an effect on the switch from vascular quiescence to angiogenesis. It mobilizes VEGF stored in ECM, leaving it available to its receptor (Bergers *et al.*, 2000). Suppression of MMP-2 reduced angiogenesis, inhibiting the transition from the pre-vascular to the vascular stage of tumor development in a chondrosarcoma model (Fang *et al.*, 2000). Moreover, several endogenous molecules are produced by partial proteolysis of ECM such as angiostatin derived from plasminogen, endostatin from collagen XVIII, anastellin from fibronectin (FN) and many others. All these molecules have pro- and anti-angiogenic activities (reviewed in Heissig *et al.*, 2003; Bellon *et al.*, 2004; Rundhaug, 2005; Milkiewicz *et al.*, 2006).

Metabolic changes underlying the angiogenic process: hypoxia as an example

Hypoxia is accepted as one of the basic mechanism that initiates and regulates angiogenesis in tumors. It mediates the up-regulation of pro-angiogenic factors, like VEGF, and the downregulation of anti-angiogenic factors (Messmer-Blust *et al.*, 2009), leading to blood vessel growth and, eventually, metastasis. The hypoxia-inducible factor 1alpha (HIF1α) is activated under hypoxia conditions and, in a glioma model, it stimulated the recruitment of CD45+ BM-derived cells to tumor areas. These cells produce MMP-9 that is essential to initiate the angiogenic switch and also regulates tumor invasiveness (Du *et al.*, 2008). Also under hypoxia, the membrane type-2 MMP (MT2-MMP) is upregulated in pancreatic cancer cells, non-small cell lung cancer cells and cervix cancer, cells and this could confer resistance to hypoxia-induced apoptosis and increase invasiveness of cancer cells (Zhu *et al.*, 2011).

Clinical relevance for angiogenesis: possible therapeutic targets

Given the biological relevance attributed to angiogenesis, in tumors but also in other diseases, soon clinical researchers began to attempt at targeting this complex process for therapeutic purposes. The inhibition of VEGF–VEGFR interactions shows a significant improved survival and disease-free survival in patients with cancer, decreasing tumor growth. However, in several cancers, this inhibition is less effective because patients acquire resistance during treatment or originally the tumor is less sensitive to VEGF signaling. Alternatively, drugs against targets related to VEGF-independent regulation of angiogenesis could be good candidates to treat these patients. This may include several molecules, such as angiostatin and endostatin that were reported to be natural anti-angiogenic proteins in the body (Matter, 2001; Shibuya, 2008). Recent studies have shown that FGFs can act synergistically with VEGF to amplify tumor angiogenesis, highlighting that targeting of both the FGF and VEGF pathways may be more efficient in suppressing tumor growth and angiogenesis than targeting either factor alone (reviewed in Korc, 2009). Even though the importance of angiogenesis was first shown in setting of solid cancer progression many years ago (Folkman, 1971), it has also been associated with hematological diseases (Perez-Atayde *et al.*, 1997; Aguayo *et al.*, 1999). VEGF and basic FGFs expression on hemato-

poietic organs (bone marrow (BM) and lymphatic organs) define some clinical characteristics in leukemias and non-Hodgkin's lymphoma, and their levels in serum/plasma patients are predictors of poor prognosis (Fiedler *et al.*, 1997; Perez-Atayde *et al.*, 1997; Aguayo *et al.*, 2000; De Bont *et al.*, 2001; Fragoso *et al.*, 2007; reviewed in Albitar, 2001; Moehler *et al.*, 2003).

In hematological tumors, MMPs expression has been shown to be upregulated in some leukemia, lymphomas and multiple myeloma (Barillé *et al.*, 1997; Kossakowska *et al.*, 1999; Kuittinen *et al.*, 2001; 2003; Pennanen *et al.*, 2008). Similar to solid tumor, in hematological tumors the BM microenvironment (stroma cells, ECM and soluble factors) must play a role in the angiogenic switch, but its actual importance on this process is not completely understood. Litwin and colleagues, comparing the low angiogenic potential of acute myeloid leukemia (AML) blasts *in vitro* with the high vessel density in BM patients *in vivo*, concluded that angiogenesis in AML likely represents a response to microenvironmental factors *in vivo*, rather than being an intrinsic property of leukemic cells (Litwin *et al.*, 2002). It is, thus, important to understand how angiogenic factor bioavailability, in particular of VEGF, can be modulated by the BM microenvironment, which can help improving anti-angiogenic therapies in hematological diseases.

BM microenvironment in hematopoiesis: creating niches for hematopoietic stem cells

The BM is the major hematopoietic organ in adult and it is found in the interior of long (e.g. humerus, femur, tibia, fibula) and flat bones (e.g. ribs, vertebrae, cranium, hip). It is a trabecular structure irrigated by large vessels and sinusoids, and filled with stromal and hematopoietic cells.

BM microenvironment comprises stromal cells (as osteoblasts, adypocytes, endothelial cells, fibroblasts, macrophages), soluble factors (growth factors, cytokines and chemokines) produced by stromal and hematopoietic cells (HCs), and the ECM. Within BM microenvironment, two well defined regions called niches were considered

to provide hematopoietic stem/progenitor cells (HSPCs) with critical instructions to self-renew, proliferate, differentiate, homing, migrate and survival (reviewed in Li and Xie, 2005; Kacena *et al.*, 2006; Moore and Lemischka, 2006; Wilson and Trumpp, 2006; Yin and Li, 2006; Arai and Suda, 2005; Scadden, 2007). HSPCs are believed to be located near bone surfaces (the osteoblastic niche) or associated with the sinusoidal endothelium (the vascular niche); the molecular signals generated by these two niches have been extensively studied (in particular for the osteoblastic niche). Osteoblasts produce important signaling molecules like osteopontin and Ang that interact with their receptors on HSPCs, keeping these cells in a quiescent state (Grassinger *et al.*, 2009; reviewed in Arai and Suda, 2007; Suda *et al.*, 2005). On the other hand, the vascular niche is considered to promote proliferation and further differentiation of HSPCs (Kopp *et al.*, 2005); it produces FGF-4 and chemokines such as stromal derived factor (SDF-1) (reviewed in Yin and Li, 2006) that recruits the HSPCs from the osteoblastic to vascular niche. Recently, with new imaging approaches, it is becoming evident that endosteal and vascular compartments may not be mutually exclusive in terms of their role on HSPC fate (Kiel and Morrison, 2008).

ECM in the BM microenvironment

The ECM component of the BM can be seen as a very dynamic structure, which spatial and temporal composition within BM creates niches that highly influence the HSPCs proprieties (Figure 1). These niches also function as a soluble factors reservoir, controlling their availability to adjacent cells. To acquire proper information from the surrounding ECM, hematopoietic cells express adhesion molecules in a regulated fashion, being integrins and proteoglycans the major receptors for ECM (reviewed in Campbell and Humphries, 2011; Kim *et al.*, 2011). Adhesion molecules participate in a range of signal transduction processes involving not only cell adhesion, but also migration, proliferation and apoptosis. In more detail, the BM ECM is an intricate network of proteins (e.g. collagens), glycoproteins (FN, laminins) glycosaminoglycans (e.g. hyaluronan) and proteoglycans (e.g. syndecans) whose turnover is tightly controlled by the adjacent cells (reviewed in Kim *et al.*, 2011). ECM molecules are pro-

duced in response to diverse environmental stimuli which are intrinsic to organ function, or may be present in stress situations involving tissue turnover such as during wound healing processes, or in malignancy (reviewed in Hynes, 2009). Nevertheless, the contribution of ECM in regulating cell-tissue-organ function, namely its role in BM homeostasis, has received little attention, and the majority of studies have focused on their receptors integrins and the signaling pathways activated through them. Also, most of studies have been made *in vitro*. The effect of ECM in hematopoiesis varies accordingly to cell lineage and maturation stage of the progenitor cell (reviewed in Lam and Adams, 2010). In long-term (LT) BM cultures, progenitors that adhere to FN proliferate significantly less than non-adherent progenitors (Hurley and Verfaillie, 1995). Collagen significantly increases the adhesion of human long term bone marrow (LTBM) cells and induces a decreased in myeloid progenitor cell production, whereas FN increases myelopoiesis (Hassan *et al.*, 1997). Laminin-10/11 is highly adhesive to lineage-committed myelomonocytic and erythroid progenitor cells and several lymphoid and myeloid cell lines, whereas laminin-8 is less adhesive. *Ex vivo* culture of murine HSCs in the presence of FN and laminin resulted in expansion of primitive stem cells and improvement in the marrow engraftability (Sagar *et al.*, 2006). Other ECM molecules seem to modulate HSPCs microenvironment. Heparan sulphate is produced by stromal cells and it presents cytokines to HSPCs, as well as promoting their ligation to the ECM molecules like thrombospondin. This leads to the formation of discrete niches, thereby orchestrating the controlled growth and differentiation of stem cells (Coombe, 1996; Gupta *et al.*, 1998). Tenascin is co-localized with other ECM molecules such as FN and collagen type III in the microenvironment surrounding the maturing hematopoietic cells and has a role in the retention of HSPCs in the stroma (Klein *et al.*, 1993). Osteopontin negatively regulates HSC numbers, as evidenced by increased of these cells in the osteopontin-null microenvironment (Calvi *et al.*, 2003; Stier *et al.*, 2005). ECM is also essential in megakaryocyte (MK) differentiation. Depending on which integrin is expressed at their surfaces, these cells exhibit different affinities for ECM ligands, which in turn influence their fates (Zweegman *et al.*, 2000; Inoue *et al.*, 2003; Balduini *et al.*, 2008; Mazharian *et al.*, 2011). Additionally, MKs are important producers of MMPs, being MMP-9 secretion essential for MK migration towards blood

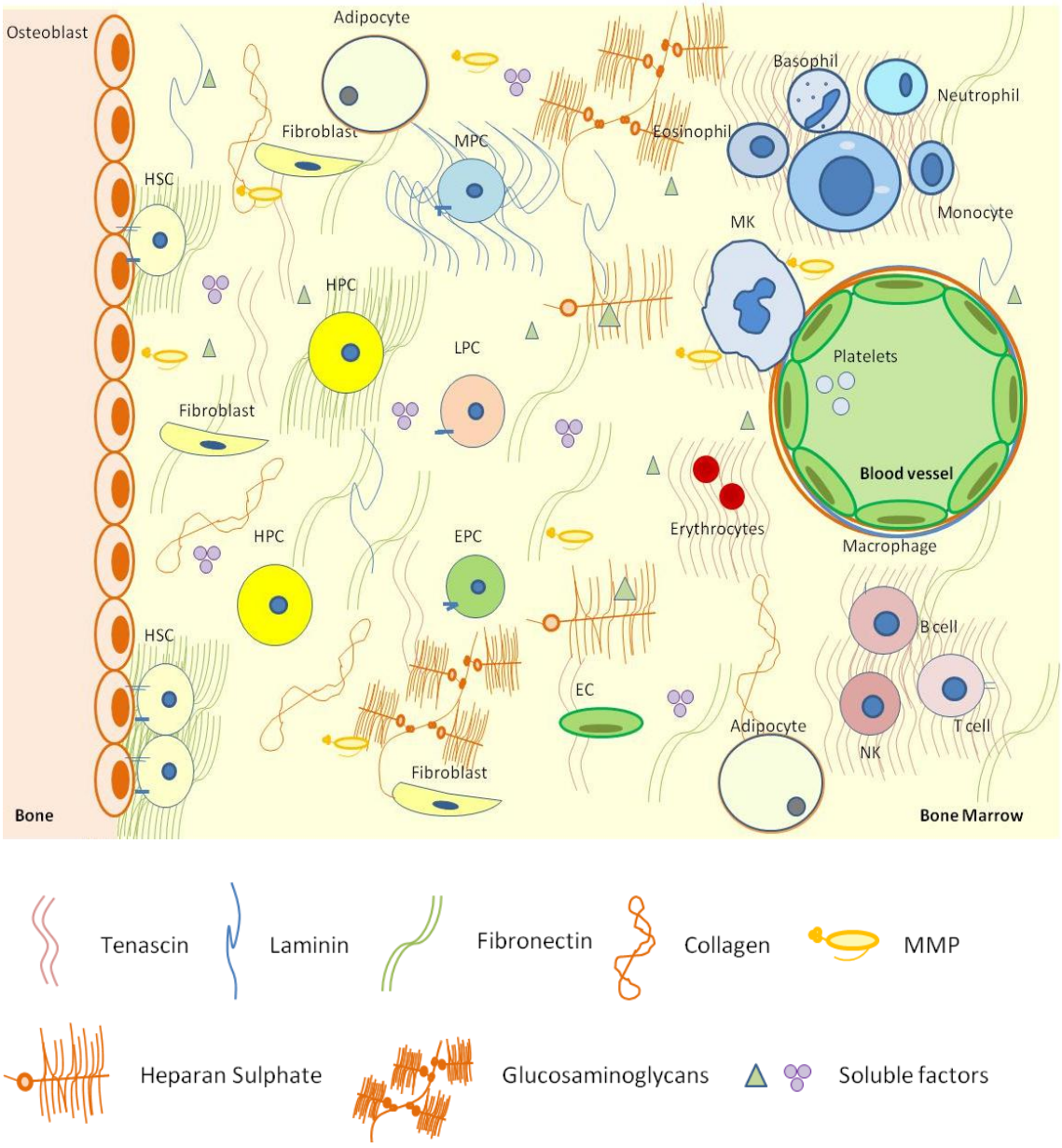

Figure 1: Schematic representation of major steps in hematopoiesis within BM microenvironment. The image represents BM niches: the osteoblastic niche, where quiescent HSPC localize, and the vascular niche, where HSPC differentiation occurs. Myeloid progenitor cells (MPC) originate basophils, neutrophils, eosinophils, monocytes, MKs (give rise to platelets), and erithrocytes, and lymphocyte progenitor cells (LPC) originate lymphocytes B and T, and natural killer cell (NK) cell. BM microenvironment is composed by ECM proteins (collagen), glycoproteins (FN and laminin) and glycosaminoglycans, soluble factors (e.g. growth factors, MMPs), and stroma (endothelial cells, osteoblasts, fibroblasts, macrophages, adipocytes).

vessels and posterior platelets release (Lane *et al.*, 2000). MMPs production by MKs may also modulate BM niches in terms of ECM remodelling, and thus, the fate of hematopoietic cells. Cell adhesion events are also very important to assure the success of transplantation (Chute, 2006; Lam and Adams, 2010). Absence of β1-containing integrins (in particular, α4β1 integrin) resulted in sequestration of HSCs in the circulation and their reduced adhesion to endothelial cells (Hirsch *et al.*, 1996; Arroyo *et al.*, 1999; Dave *et al.*, 2000; Potocnik *et al.*, 2000; reviewed in Imai *et al.*, 2010), impairing their entrance in BM.

FN, a major constituent of ECMs, plays an important role during embryogenesis, wound healing and cancer invasion by promoting cell adhesion, motility, cell cycle progression and cell survival (Hynes and Yamada, 1982; Humphries, 1989; Frisch and Ruoslahti, 1997). Reduction or loss of FN expression occur in many transformed cells in culture (Ruoslahti, 1989), but its overexpression is also associated with various human tumor cells like colorectal cancer, breast carcinoma, head and neck squamous carcinoma, and metastatic melanoma (Zhang G, 1997; Bittner *et al.*, 2000; Jiang *et al.*, 2002; Al Moustafa, 2002). FN and their integrin receptors are also key regulators of endothelial growth (Hynes *et al.*, 1999; Dvorak, 2005). While laminins and collagens appeared early in evolution, FN has been found only in vertebrates and its appearance in evolution correlates with the appearance of organisms with endothelial cell-lined vasculature (Hynes and Zhao, 2000; Whittaker *et al.*, 2006; Astrof and Hynes, 2009). It is a high-molecular weight (440KDa) molecule, consisting of two nearly identical monomers linked by a pair of disulfide bonds. The FN protein is produced from a single gene, but alternative splicing of its pre-mRNA leads to the creation of several isoforms (Figure 2). There are two types of FN: the soluble plasma FN, a major protein component of blood plasma (300µg/ml) and produced in the liver by hepatocytes, and the insoluble cellular FN, a major component of ECM (reviewed in Pankov and Yamada, 2002). Within BM, FN is localized in the osteoblastic niche, the region for which HSPCs have high affinity, but has also a wide distribution in central BM region (Nilsson *et al.*, 1998). Its broad allocation suggests that FN may be important in creating specific niches for HCs, and some roles of this molecule in hematopoiesis have been revealed, mostly on *in vitro* studies (see above).

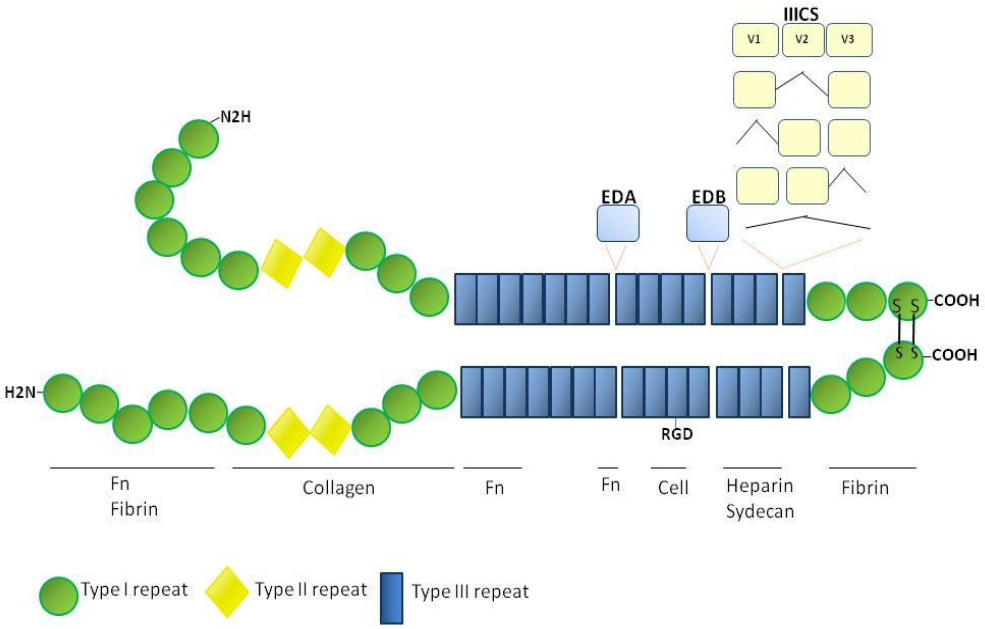

Figure 2. Schematic representation of human dimeric FN and its several binding domains. Each 220kDa FN monomere comprises multiple type I (green circles), type II (yellow lozenge) and type III (blue rectangle) repeats. Several FN isoforms can be obtained by alternative splicing: the entire EDA and EDB domains are independently included or excluded by exon skipping, whereas the IIICS domain undergoes complex splicing of mRNA transcribed from a single exon (exon subdivision is indicated). The binding sites for interaction between FN and collagen, fibrin, heparin, cells, and to other FN molecules are also represented. Cell-binding sites include the RGD domain (for α5β3 and αVβ3 integrin interaction) and the IIICS domain (for α4β1 integrin interaction).

Nevertheless, not too much is known about its importance in hematological diseases, in particular in the angiogenic processes associated with these malignancies. In this context, most of the studies only concern ECM remodeling by MMPs, but which factors regulate FN expression during this process are still to identify. Also in other biological systems, the regulation of FN has not been totally explored. Hepatocyte growth factor/scatter factor (HGF) induces FN expression and extracellular assembly on the surface of melanoma cells through activation of mitogen-activated protein (MAP) kinase pathway (Gaggioli *et al.*, 2005). The pleiotropic cytokine transforming growth factor-β (TGF-β) is the well known regulator of FN, being studied in epithelial-to-mesenchyme transition contexts, like in embryogenesis and in fibrosis (as an example, see Sureshbabu *et al.*, 2011). A recent study on mouse embryonic angiogene-

sis revealed that overexpression of the Notch ligand Dll4 increases the deposition of FN around the vessels, although the direct role of Notch signaling on FN production has not been addressed (Trindade *et al.*, 2008). In BM, nothing is known about the regulation of FN (or other ECM molecules) expression, or in homeostasis, or in disease.

BM diseases: from BM dysfunctions to malignancy

BM malignancies are clonal disorders resulting from neoplastic transformation of HSPCs. They include leukemias, lymphomas and multiple myeloma. Other related BM diseases, but not considered cancer forms, comprise myeloproliferative syndromes (MPS) and myelodisplastic syndromes (MDS).

Leukemias are classified accordingly to the hematopoietic cells which undergo the clonal transformation: lymphoblastic or lymphocytic leukemias – involving lymphocyte precursors - and myeloid or myelogenous leukemias – involving myeloid cell but also erythrocyte or platelet precursors. They are also subdivided in acute or chronic leukemias. Acute leukemias are characterized by a rapid increase in the numbers of immature blood cells, making the BM unable to produce healthy blood cells. It requires immediate treatment due to the rapid progression of the disease that leads to the exit of malignant cells into the bloodstream and spread to other organs of the body. Chronic leukemias are characterized by the excessive production of relatively mature, but still abnormal, leukocytes. Typically, they take years to progress, so they are sometimes monitored for some time before treatment to ensure maximum effectiveness of therapy. Combining these two classifications, four main types of leukemias emerge (although other forms of rare leukemias can occur): acute lymphoblastic leukemia (ALL), AML, chronic lymphocytic leukemia (CLL), and chronic myelogenous leukemia (CML) (Swerdlow *et al.*, 2008).

MPS are a group of BM clonal disorders in which excess cells are produced. They are categorized by the presence or absence of Philadelphia chromosome (t9;22) and include, among others primary or idiopatic myelofibrosis (PMF), polycythemia vera (PV),

essential thrombocythemia (ET) (Philadelphia chromosome-negative), and CML (Philadelphia chromosome-positive). CML can occur independently or progress from a PMF and is characterized by the presence of HSPC expressing the bcr-abl oncogene, with abnormal release of these clonal cells into the circulation. A complete genetic and clinicopathological classification of this group of disorders can be consulted, for instance, in Michiels *et al.*, 2007; Tefferi and Gililland, 2007, 2006; Michiels and Thiele, 2002.

MDS are a heterogenous group of clonal hematopoietic diseases characterized by peripheral cytopenia (despite a normocellular or hypercellular BM) and with a variable probability to progress to AML (Miyazato *et al.*, 2001; Braun *et al.*, 2006; Nolte and Hofmann, 2008). The incidence of these conditions has risen sharply over the past several years, making them the most common malignant BM disorders. It has become apparent that the ineffective hematopoiesis is largely caused by excessive apoptosis of myeloid precursors (Braun *et al.*, 2007; reviewed in Westwood and Mufti, 2003). Recent observations suggest that downregulation of α4β1 and α5β1 integrins on HSC, correlated with decreased *in vitro* adhesiveness to FN fragments, could be a newly identified proapoptotic mechanism in MDS (Delforge *et al.*, 2005). Inversely, the evolution of MDS from early relatively chronic phenotype to an aggressive AML is accompanied by a suppression of apoptosis in the malignant cells, mediated by changes in intracellular levels of Bcl-2-family proteins (reviewed in Westwood and Mufti, 2003). MDS-associated AML (secondary AML) is rarely cured by conventional chemotherapy (reviewed in Hamblin, 1992), which contrasts with the somewhat better outcome of *de novo* AML. The protein Delta-like (Dll), distantly related to Delta-Notch family of signaling proteins, has highly selective expression in the individuals with MDS and lower in secondary AML, being absent from *de novo* AML. This makes Dll a good candidate molecule to differentiate MDS from AML (Miyazato *et al.*, 2001).

As biopsies from several MDS patients have been used on this project, a brief classification of this disease is given due to the heterogeneity of the samples. The French-American-British (FAB) group established the first classification of MDS in 1982, defining five subtypes based on morphology, the number of blasts (in BM and periphe-

ral blood) and monocyte counts. This classification reflects the progression and the clinical course of MDS but, although providing important diagnostic information, revealed some limitations. To overcome them, in 2001, the World Health Organization (WHO) classification suggested new subtypes of MDS (table 1) (Bennett *et al.*, 1982).

Table 1. MDS classification according to WHO

WHO CLASSIFICATION	PERIPHERAL BLOOD	BONE MARROW
REFRACTORY ANEMIA (RA)	Anemia no or rare blast	Erythroid dysplasia only, < 5% blasts, < 15% ringed sideroblasts
REFRACTORY ANEMIA WITH RINGED SIDEROBLASTS (RARS)	Anemia no blast	Erythroid dysplasia only, < 5% blasts, ≥15% ringed sideroblasts
REFRACTORY ANEMIA WITH MULTILINAEGE DYSPLASIA (RCMD)	Cytopenias (bycitopenia or pancytopenia) no or rare blast no Auer rods < 1x10^9/L monocytes	Dysplasia in > 10% of cells in 2 or more myeloid cell lines < 5% blasts in marrow, No Auer rods, < 15% ringed sideroblasts
REFRACTORY ANEMIA WITH MULTILINAEGE DYSPLASIA AND RINGED SIDEROBLAST (RCMD-RS)	Cytopenias (bycitopenia or pancytopenia) no or rare blast no Auer rods < 1x10^9/L monocytes	Dysplasia in > 10% of cells in 2 or more myeloid cell lines ≥15% ringed sideroblast, No Auer rods <5% blast
REFRACTORY ANEMIA WITH EXCESS BLAST-1 (RAEB-1)	Cytopenias <5% blast No auer rods < 1x10^9/L monocytes	Unilineage or multilineage dysplasia 5% to 9% blasts No Auer rods
REFRACTORY ANEMIA WITH EXCESS BLAST-2 (RAEB-2)	Cytopenias 5-19% blast Auer rods ± < 1x10^9/L monocytes	Unilineage or multilineage dysplasia 10% to 19% blasts Auer rods ±
MYELODYSPLASTIC SYNDROME, UNCLASSIFIED (MDS-U)	Cytopenias, no or rare blast no Auer rods	Unilineage or multilineage dysplasia in granulocytes or megakaryocytes 5% to 9% blasts No Auer rods
UNCLASSIFIED (MDS ASSOCIATED WITH ISOLATE DEL (5Q))	Anemia <5% blast platelets normal or increased	Normal to increased megakaryocytes isolated del (5q) with hypolobated nuclei < 5% blasts No Auer rods Isolated del(5q)

However, this classification did not include important information on genetic abnormalities, a major prognostic factor in MDS patients. So, another classification based on significant prognostic factors, proposed in 1996 by the International Prognostic Scoring System (IPSS), defines four groups of risk (table 2).

Table 2. MDS Risk proposed by IPSS

	IPSS SCORE VALUE				
PROGNOSTIC VARIABLE	0	0,5	1	2	3
BM BLAST (%)	<5	5–10	5–10	11–20	21–30
KARYOTYPE	Good (Normal, -Y, del(5q), del(20q))	Intermediate (Complex (≥ 3 abnormalities) or chromosome 7 anomalies)		Poor (Other abnormalities)	
CYTOPENIAS	0/1	2/3			

IPSS-SUBTYPE	SCORE VALUE
Low	0
Int-1	0,5-1,0
Int-2	1,5-2,0
High	2,5

BM microenvironment in hematological diseases: the deregulation of stem cell niches

Malignant hematopoietic cells are known to express particular cell adhesion repertoires that provide them with proliferative and survival advantages within the BM microenvironment. Specific niche composition provides ideal conditions for some leukemic cells to escape from chemotherapy-induced death and acquire drug-resistance. There are several reviews on this subject (e.g. Verfaillie *et al.*, 1997; Rizo *et al.*, 2006; Konopleva *et al.*, 2009; Lane *et al.*, 2009). Most of the studies on hematological diseases refer integrins-ECM interactions as key mechanisms involved in tumor progression, whereas well identified genetic hits occurring on HSCs would be the major beginners of the oncogenic process (reviewed in Tefferi and Gilliland, 2007).

Presently, the acceptance that the BM microenvironment is important in supporting leukemia stem cells survival has conducted to the rational development of therapies that target microenvironment molecules (reviewed in Konopleva *et al.*, 2009;

Lane *et al.*, 2009). For instance, inhibition of α4β1 integrin - FN interaction in AML patients increases their sensibility to chemotherapy (Matsunaga *et al.*, 2003).

Emerging data suggests the balance between ECM production and degradation (turnover) may be crucial for normal (versus malignant) organ function, while it may also represent a way to detect tissue damage (alterations), in particular situations. In BM, mononucleated cells from healthy donors continuously produce MMP-9 and TIMP-1 (a MMP inhibitor), whereas AML and CML blast cells additionally secrete MMP-2, representing a potential marker for dissemination in myeloproliferative malignancies (Ries *et al.*, 1999). Nevertheless, the role of the BM ECM in the pathophysiology of hematological disorders has remained controversial. Although microenvironment may not initiate clonal proliferation, it should somehow favor the progression of the disease. Thus, microenvironment analysis will be important to develop effective regimens that allow for elimination of a specific clone in human patients. For that reason, we will provide some examples on the role of microenvironment, in particular ECM, in BM malignancies.

The MPS PMF is a remarkable model in which deregulation of the stem cell niche is of great importance in disease development (Lataillade *et al.*, 2008). In a typical case of PMF, hematopoietic cells in BM are replaced by collagen fibrosis (although other ECM proteins are also increased), impairing the patient's ability to generate new blood cells resulting in a progressive pancytopenia (Reilly, 1997). An ineffective megakaryocytopoiesis leads to an excessive concentration of abnormal MKs which release specific growth factors (like TGF-β) essential for fibroblast activation and, consequently, collagens, FN and other ECM molecules production (Schmitt *et al.*, 2000; Kuter *et al.*, 2007). Additionally, not only increased production of ECM occurs in PMF but there is also an imbalance between MMPs and TIMPs that may contribute to BM fibrosis (reviewed in Wang, 2005). In CML, imature CML cells have lower adhesion to stromal cells and FN as well as lower engraftment potential than normal HSC (Peled *et al.*, 2002), a feature that may account for their exit to peripheral blood.

In MDS, alterations in adhesive proprieties of myeloid progenitors (see above) are cell-autonomous and confer cell susceptibility to apoptosis (Raza et al, 1995).

However, changes in BM stroma, namely on ECM, have already been detected in some MDS patients, suggesting again a role for microenvironment in MDS progression (Tennant *et al.*, 2000; Tauro *et al.*, 2001). Remarkably, increased cell death in MDS can be followed by increased proliferation, likely of selected and well adapted cell clones, and in this case progression to AML occurs. In normal hematopoiesis, α4β1 and α5β1 integrin-mediated interactions between progenitor cells and FN are critical for progenitor cell survival. Also in AML, adhesion of blast cells to stroma via β1 (principally α4β1) and β2 integrins, seems to inhibit apoptosis in a proportion of cases (Denkers *et al.*, 1992; Liesveld *et al.*, 1993).

Angiogenesis: a link between BM microenvironment and BM diseases

As mentioned above, BM microenvironment has a role in hematological disorders progression, but may also create the appropriate conditions for disease initiation. Of particular interest is the frequent progression from an MDS to an AML, during which the specific and most relevant microenvironment factors involved are largely still unknown. This secondary AML is usually more aggressive that *de novo* AML, being of outmost importance to uncover the mechanisms behind MDS, *de novo* AML and secondary AML. These can help in finding new therapeutic approaches appropriated for each type of pathology. Angiogenesis is an important event that mediates the progression from a chronic to a more acute and aggressive pathology, and its significance in hematological malignancies has just beginning to be explored (reviewed in Shadduck *et al.*, 2007). Angiogenic factors may be produced by fibroblasts in the BM stroma and by immune cells (reviewed in Mangi and Newland, 2000), but their availability in BM may be regulated not only at gene expression level, but also by microenvironment elements. Both MDS and AML are associated with a substantial increase in BM vascularity as well as increased levels of various angiogenic factors, in cluding VEGF, basic FGFs, angiogenin, Ang-1, PDGF, HGF, epidermal growth factor (EGF), tumor necrosis factor-α (TNF-α), and TGF-α and TGF-β (Aguayo *et al.*, 2000; Master *et al.*, 2001; reviewed in Albitar, 2001). Malignant cell proliferation, angiogenesis and VEGF expression are linked in AML, as well as MMP-2 and/or MMP-9

expression (De Bont *et al.*, 2001). In addition, MMPs correlate with aggressive ALL (Kuittinen *et al.*, 2001). Increasing VEGF levels significantly correlate with shorter survival of patients with MDS and AML (Verstovsek *et al.*, 2002). Nevertheless, higher levels of cellular VEGF and lower levels of its receptor KDR are seen in MDS more than in AML (Verstovsek *et al.*, 2002; reviewed in Albitar, 2001). This fact is in agreement with a significant increase of BM microvascular density in MDS and *de novo* AML compared with healthy donors. Surprisingly, in MDS, microvascular density significantly decreases upon transformation to AML, which microvascular density was also significantly lower than in *de novo* AML (Keith *et al.*, 2007).

ECM has a key role in storing/retaining soluble factors, being proteolic enzymes important regulators of soluble factors bioavailability. For instance, VEGF levels correlate with MMP-2 and MMP-9 activity in human breast cancer (Munaut *et al.*, 2003). Also, in glioblastoma, HIF1α, the direct effector of hypoxia, induces the recruitment to tumor area of BM-derived cells that produce MMP-9, which in turn is essential and sufficient to initiate angiogenesis by increasing VEGF bioavailability (Du *et al.*, 2008).

It is not known if the type or amount of ECM molecules are important in this process, but the existence of appropriate binding sites for certain growth factor on ECM molecules strongly suggest these may be necessary. In this context, FN contains three heparin-binding and syndecan (a heparan sulfate) domains. Interestingly, the two longer forms of VEGF, $VEGF_{189}$ and $VEGF_{206}$, are not found freely in the media, because they are bound to the cell surface or ECM, via heparin-binding sites (Houck *et al.*, 1992). However, $VEGF_{121}$ is a nonheparin-binding protein, freely diffusible (Houck *et al.*, 1991). $VEGF_{165}$ has intermediated proprieties, being found in a soluble form but also bounded to ECM. Activity of the serine protease plasmin and MMP-9 can cleave the bounded forms of VEGF, in a colorectal cancer cell line, releasing a soluble factor capable of stimulating endothelial cell growth (Houck *et al.*, 1992; Hawinkels *et al.*, 2008). Also, heparin, heparan sulfate, and heparinase all induce the release of $VEGF_{165}$ and $VEGFl_{189}$, in a human embryonic kidney cell line (Hawinkels *et al.*, 2008), suggesting heparin-containing proteoglycans as candidates for VEGF-binding sites. All

these data together suggest that ECM, in particular FN, can regulate VEGF bioavailability in angiogenic processes. It remains to elucidate which signaling pathways regulate ECM and if it is an important issue in angiogenesis and tumor progression in hematological tumors.

A good candidate to regulate FN in vessel formation is the Notch signaling pathway (Trindade *et al.*, 2008). Notch signaling regulates the self-renewal of HSPCs and is also involved in various hematological malignancies (reviewed in Leong and Karsan, 2006), for example, acute T-lymphoblastic leukemia (Weng *et al.*, 2004; Tohda *et al.*, 2005; Lee *et al.*, 2007) and MDS (Länger *et al.*, 2004; Li *et al.*, 2005; Fu *et al.*, 2007; Qi *et al.*, 2008). The role of Notch signaling in oncogenesis is controversial, and because most cancers express more than one type of Notch ligand and/or receptor, the overall expression profile of these ligands/receptors may ultimately determine whether this pathway will be oncogenic or in oncosuppressive. Also cell type and the presence of specific soluble factors in tumor microenvironment influence the effect of Notch signaling in oncogenesis (reviewed Leong and Karsan, 2006). This suggests that Notch signaling may be involved in regulating BM microenvironment in hematological diseases (possibly also through FN production or release). HPCs isolated from MDS patients display a disrupted Notch signaling. Furthermore, there was a marked reduction in the plasticity of mesenchymal stem cells of MDS patients compared with those of normal BM donors, in neurogenic and adipogenic differentiation ability and hematopoiesis supporting capacity *in vitro* (Varga *et al.*, 2007).

As a final remark, BM microenvironment engages a complex mixture of cells, ECM molecules, growth factors and cytokines, which can exert reciprocal influence on each other's availability. A sum of all these factors will activate (and additionally being activated by) specific signaling pathways that will certainly contribute to hematological dysfunctions. In this Thesis, we have exploited some of the interactions between ECM molecules and angiogenic growth factor bioavailability in the setting of hematological malignancies.

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AIMS

Similar to what is known for solid tumors, angiogenesis is an important event in hematological tumors progression. In detail, the pro-angiogenic factor VEGF is overexpressed in some BM diseases (like MDS and AML), being correlated with increased vessel density. In solid tumors, the ECM and MMPs have been implicated in VEGF retention and bioavailability, thus conditioning tumor angiogenesis. Among the known ECM components, FN is frequently detected in solid tumors, where is known to regulate several cellular and structural functions. Much less is currently known concerning the role of ECM, MMPs and VEGF availability in hematological tumors.

Based on this information, the **general aim** of our study was to know if the BM microenvironment concerning ECM components, angiogenic factors availability, etc, differs between MDS and AML and what role might such factor be playing in disease progression.

To answer that, we proposed the following **specific aims**:

- Is VEGF bound to FN or is it on its soluble form in BM from MDS or AML patients?
- Are MMPs involved in VEGF bioavailability in BM from MDS or AML patients?
- Do FN amounts vary between MDS and AML and how is its expression regulated in these BM?

METHODS

Human samples

BM biopsies from 11 MDS and 9 AML (Table S1) patients were obtained after informed consent, according to IPOLFG guidelines. Biopsies were made by aspiration of BM cells from iliac crest.

Samples were centrifuged at 1400 rpm for 5 minutes, at room temperature (RT) and the supernatant was stored at -80ºC for further analysis. The pellet was ressuspended in 10ml of sterile phosphate buffered saline buffer (PBS)/ ethylenediaminetetraacetic acid (EDTA) (Table S2), added to 3ml of Lymphoprep (Axis-Shield) and centrifuged at 1800 rpm for 20 minutes, at RT. Then, a density gradient is formed and the white ring that contains the BM mononucleated cells was removed and ressuspended in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), supplemented with glutamine, antibiotic and antimicotic (complete medium), and centrifuged at 1200 rpm for 5 minutes, at RT. The pellet was ressuspended in 1ml of DMEM 10% FBS, the cells were counted in a Neubauer counting chamber and used for *in vitro* assays or ressuspend in Trizol (Invitrogen ™) (1ml/ 10^6 cells) and stored at -80ºC for further RNA isolation.

RNA isolation

To extract RNA, BM mononucleate cells stored in Trizol were brought for 5 minutes to RT to permit total dissociation of nucleoproteins complex. The next steps were performed on ice to prevent RNA degradation. Cell lysate was mixed with chloroform (1/5 volume of Trizol), vortexed for 15 seconds, incubated 15 minutes on ice and then centrifuge at 14000 rpm, 4ºC for 25 minutes. After centrifugation, three phases were formed, the one in the top is a colorless aqueous phase and contains RNA, in the middle there is an interphase with DNA and the bottom phase is red, containing proteins and lipids. The supernatant (aqueous phase) was transfer to a fresh microtube with isopropanol (1/2 Trizol volume) and this mixture incubated overnight (ON) at -20ºC. Subsequently, precipitated RNA was centrifuged at 4ºC, 14000 rpm, for 20minutes, and the supernatant discarded. The pellet was washed with

70% ethanol (diluted in diethyl pyrocarbonate (DEPC)-treated H_2O), centrifuged at 14000 rpm for 10 minutes at 4ºC, the supernatant discarded and the microtubes were left open until the remaining ethanol was completely dried. The pellet obtained was then solubilized in 10µl of RNA-free water (DEPC water), and quantified in a Nanodrop (Thermo Scientific Nanodrop 2000 Spectophetometer, program Nanodrop 2000/2000c). For that, 1,3µl from each sample was used, as well as a blank (RNA-free water).

cDNA synthesis

To synthesize cDNA from RNA, 1µg of RNA was used. A mix was prepared with the correspondent volume of RNA for 1 µg, 1µl of random hexameric primers (hexadeoxyribonucleotides; Invitrogen), (provide internal site for mRNA to synthesize a first strand of cDNA), and water for a final volume of 15µl. This mix was incubated in a Thermocycler (Biometra) 10 minutes at 70ºC, and 3 minutes at 4ºC; during this period 11µl of enzyme mix was added and the incubation remains for 1.30 hours at 42ºC, and 15 minutes at 75ºC. The enzyme mix contains 4µl First Strand 5x buffer (Invitrogen ™), 4µl DNA nucleotides dNTPs (deoxyribonucleoside triphosphates; Invitrogen™) at 5nM each, 2µl 0,1M Dithiothreitol (DTT; Invitrogen™), 1µl ribonuclease inhibitor (RNase out; Invitrogen™) and 0.5µl Reverse Transcriptase Superscript III RT (Invitrogen™). The cDNA was then store at -20ºC.

Real-time polymerase chain reaction (RT-PCR)

To validate the presence of cDNA, 18S gene was amplified by RT-PCR, in each sample.

A mix was prepared containing, for each sample, 18,15µl H_2O, 2,5µl 10x buffer (Invitrogen^{TM}), 2µl dNTPs (Invitrogen^{TM}), 0,75µl MgCl2 (Invitrogen^{TM}), 0,5µl forward and reverse primers for 18S gene (Sigma; Table S3), 0,1µl HotStarTaq DNA Polymerase (Invitrogen^{TM}), and 0,5µl cDNA. Next, mixes were incubated in a thermocycler (Biome-

tra) for 50 cycles of repeated heating and cooling of the reaction, for melting and enzymatic replication of the DNA.

Three µl of loading buffer (Table S4) were added to 10µl of each PCR product, the mix was loaded in a 2% agarose gel (Table S5) and run in an electrophoresis apparatus with 0,5x Tris/Borate/EDTA (TBE- Table S6) at 145V, for 30min. DNA bands were visualized in an imaging system under ultraviolet light (BioDocAnalyze digital, Biometra).

Quantitative real time polymerase chain reaction (RQ-PCR)

The relative expression of Notch1, Jagged1, Dll4, Hey1, Hey2 and FN was determined by RQ-PCR.

First, 10µl SYBR Green PCR Master Mix kit (contains SYBR Green I dye, ROX passive reference dye, HotStarTaq DNA Polymerase, and a dNTP mix in an optimized buffer; Applied Biosystems), 0,4µl forward primer (Sigma; Table S3), 0,4µl of reverse primer (Sigma; Table S3), 7µl of H_2O, and 0,2µl bovine serum albumin (BSA) 2% were added to each 0,2µl of cDNA sample, in a Micro-Amp optical 96-well plate. The plate was then centrifuge at 1460rpm for 2 minutes and analyzed in ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), using the SDS2.3 software. The relative expression of each gene was calculated with RQ Manager 1.2 software, using the comparative method $2^{-\Delta Ct}$.

Protein quantification

The protein content from BM supernatants was quantified with the Dye Reagent Concentrate Protein Assay (Bio-Rad). First, several dilutions of BSA were prepared in order to obtain a standard curve: 1mg/ml, 1,5mg/ml, 2,5mg/ml 5 mg/ml 10mg/ml. One µl of each BSA standard solution or 1µl of BM supernatant were diluted in 500µl of dye reagent, previously diluted 1:5 in water, vortexed, incubated 5 minutes,

at RT and their absorbance measured at 595nm (Gene Quant pro, RNA/DNA Calculator).

Enzyme Linked Immunosorbent Assay (ELISA)

To quantify VEGF protein in supernatants of BM biopsies or from BM cell cultures, an ELISA for human VEGF (Calbiochem) was used; all the solutions and protocol where provided by the manufacturer.

Initially, known concentrations of VEGF were prepared (1000, 250, 62,5, and 31,5 pg/ml), in order to construct a standard curve. Then, 100µl of Assay Diluent RD1W and 100µl of each sample (diluted 1:2 in Assay Diluent) or VEGF standards were mixed in each well of a 96-well plate coated with anti-human VEGF antibody, and incubated for 2 hours at RT with gently mixing and covered with an adhesive strip; this assay was made in duplicate. After this, wells were washed with 1x Wash Buffer, three times, and then 200µl of anti-VEGF antibody HRP-conjugated were added and incubated for 2 hours at RT, with gently mixing. Next, 200µl of Substrate Solution were added to each well and incubated for 25 minutes at RT. Finally, 50µl of Stop Solution were added to each well to stop the enzymatic reaction. The intensity of the yellow solution obtained is proportional to the amount of VEGF protein in the sample, and their respective absorbance were determined at 450nm in a Bio-Rad iMark microplate reader, program MPM6.

Western-Blotting

Analysis of protein content in BM supernatants was performed by western-blotting. First, proteins were separated by sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) that allows the sorting of proteins by their molecular weight and charge. SDS-PAGE gel composition and porosity is chosen based on the specific weight and composition of the target proteins to be analyzed. Here, an 8% gel

was used since we wanted to separate proteins of 25-200KDa size. This gel is composed of two acrylamide mixes: the resolving or separating gel (Table S7), and, on top, a stacking gel (Table S8) with large pores that compresses proteins in a thin starting zone.

As proteins have varying charges and complex shapes, they are usually denatured in the presence of a detergent (in this case SDS) that coats them with a negative charge and, therefore, they will run to the positive pole when electric current is applied to the gel.

In the next step, proteins were denatured adding Laemmli buffer (10% β-2-mercaptoethanol in loading buffer - Table S9) to equal volume of protein (equivalent to 50µg), and heating at 100ºC for 5 minutes. β-2-mercaptoethanol denatures proteins via its ability to cleave disulfide bonds, disrupting both the tertiary and the quaternary structure of the proteins. For positive control of FN staining, 2µl of 0,1% FN from bovine plasma (Sigma) was also denatured like samples. Then, samples, positive control and 5µl of protein ladder (Benchmark Pre-stained protein ladder) with molecular weights standard, were loaded in the gel, and run at 120V for 1.30 hours in an electrophoresis apparatus filled with running buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS (TGS, Bio-Rad).

The following step is the transfer of the proteins from the gel to a nitrocellulose membrane, by electric current, and the exposition of proteins on thin surface of the membrane for detection. This transfer is performed in a Criterion Blotter ™ (Biorad) filled with transfer buffer (table S10), ON, 4ºC, at 105V. To prevent the non-specific interaction of the antibody, a blocking step was performed incubating the membrane with 5% milk in TBS, Tween 0,1% (Table S11), for 1 hour, at RT, with agitation (Movil-rod, P Selecta).

Then, the membrane was incubated with primary antibody anti-FN (1:1000; F3648, Sigma) diluted in 5% milk TBS, Tween 0,1% (Table S11), at 37ºC for 2 hours, with agitation. After washing with three timers with TBS, Tween 0,1%, the membrane was incubated for 1 hour with an anti-rabbit secondary antibody conjugated with hor-

seradish peroxidase (HRP) enzyme (1:5000; 31460 Thermo Scientific), at RT, and finally wash three times with TBS, Tween 0,1%. At the end, the bands corresponded to the specific protein (FN or VEGF) were detected by incubating the membrane with the substrate for HRP enzyme (Pierce ECL - super sinal west pico chemiluminescent substrate; Thermo Scientific), and observed in a Bio-Rad molecular image system, Chemidoc XRS+, with Image Lab software. The bands obtained were quantified with ImageJ software (densitometric analysis).

Co-immunoprecipitation

Co-immunoprecipitation permits detect the presence of protein complexes, in this case, VEGF bounded to FN, in a given sample. This technique starts with the purification of FN, diluting 50µg of total protein with 500µl of PBS, BSA 0,5% and incubating it with the primary antibody, anti-FN (F3648, Sigma), ON, at 4ºC.

In the next day, protein G sepharose beads were washed with ethanol, diluted 1:1 in PBS 1x (Table S12), and centrifuged at 3000rpm, 4ºC for 2minutes. Then, supernatant was discarded; this procedure was repeated three times. At the end, the pellet (with was ressuspended in 1ml of PBS 1x. Then, 50µl of these beads were diluted with the FN-anti-FN antibody complex and incubated for 3 hours, at 4ºC, with gently mixing. Next, this mix was centrifuged at 2500rpm for 10 minutes, at 4ºC, supernatant was discarded and washed three times in PBS 1x. The pellet obtained was diluted in 20µl of Laemmli buffer (10% β 2-mercaptoethanol in loading buffer; Table S9), and heated at 95ºC for 2 minutes, to denature proteins and separate the proteins from beads. After this procedure, 50µg of purified FN from BM samples were loaded in a SDS-PAGE gel, following the Western-blotting protocol. In this case, nitrocellulose membrane was incubated with an antibody anti-VEGF (1:500; SC7269, Santa Cruz), followed by anti-mouse (1:5000; 31430 Thermo Scientific), in order to detect FN-VEGF complexes present in BM samples.

Gelatin Zymography

Gelatin zymography was performed on supernatants of BM biopsies to detect MMPs. For this technique, a 10% polyacrylamide gel with gelatin (Table S13) was used due to MMPs proteolitic activity on gelatin. A stacking gel for concentrating protein was also used as for Western-blotting.

A supernatant volume equivalent to 50 μg of total protein was diluted in equal volume of loading buffer, and run in an electrophoresis apparatus, in TGS 1x, at 140 V for 2 hours. Then, the gel was rinsed in 2.5% Triton X-100 (Table S14) and incubated in the same solution for 1 hour, at RT, with gently mix, in order to remove SDS. The gel was rinsed two times in 1x low salt collagenase buffer (Table S15) and incubated in this solution, ON at 37ºC, to optimize gelatin digestion by MMPs. At the end, the gel was washed with water and stained with Comassie brilliant blue (Sigma) working solution (a dye to stain proteins, Tables S16, S17 and S18) for 1 hour.

Imunohistochemistry

BM biopsies were included in paraffin wax, 3μm sections were obtained, rehydrated and went through a heated-induced antigen retrieval protocol (high temperature, Pt module). All these steps were performed in Anatomical Pathology Department at IPO, following their own protocols.

Sections were blocked for endogenous peroxidases with 10% H_2O_2 in methanol, for 20 minutes. Next, to reduce unspecific antibody staining, sections were incubated in PBS 0,5%, BSA 1%, FBS 2%, for 1 hour, RT Then, they were washed in water and PBS 1x and incubated ON, at 4ºC with the primary antibody, Factor VIII (1:30; Dako M0616, clone F8/86), diluted in PBS 0,5%, BSA 1%; this antibody is specific for blood vessels and MKs. After a three times wash with PBS 1x, 5 minutes, sections were incubated with the secondary antibody anti-mouse conjugated with peroxidase (DAKO), for 30 minutes, at RT and washed three times in PBS 1x. At the end, sections were incubated (at maximum, for 10 minutes) with 3,3' - diaminobenzidine (DAB), an organic substrate

for hydrogen peroxidases, which became dark brown after being oxidized. After washing in water, sections were stained for nucleus with haematoxilin for 40 seconds, and washed in tap water. At the end, section were dehydrated in 50, 70, 90, and 100% ethanol, for 1 minute each, 1 minute in xilol and the slides mounted with Entellan (Merck).

Vessel density was obtained counting the number of vessel stained with Factor VIII antibody in five different fields in each section (40x objective).

Immunofluorescence

Paraffin section were treated as for immunohistochemistry (see above) until the blocking step with PBS 0.5%, BSA 1%, FBS 2%, for 1 hour, at RT. After this, sections were incubated with primary antibody, anti-FN (1:100, F3648, Sigma) and anti-VEGF (1:500; SC7269, Santa Cruz), ON, at 4ºC. In the next day, they were washed three times with PBS 1x, 5 minutes, and incubated one hour with the appropriated secondary antibody, Alexa fluor 488 goat anti-rabbit (A-11008, Molecular Probes), or Alexa fluor 594 goat anti-mouse (A-11005, Molecular Probes). Finally, sections were washed in PBS 1x and slides mounted with Vectashield with DAPI (nuclear staining - Vector, H-1200) and examined under a fluorescent microscope (20x objective).

***In vitro* assays**

About $2x10^4$ mononucleated cells obtained from MDS and AML BM biopsies were seeded per well of a cell culture plate with 24 wells, previously coated with 0,1% FN or without coating, in DMEM 10% FBS medium. Cells grow under controlled conditions, at 37ºC, 5% CO_2. In some cases, cells grow for 3-4 weeks, and a culture of adherent cells (stromal cells) was obtained; in other situation, cells were maintained in culture for shorter periods and, in this case, BM cells from several lineages were present.

Inhibition of MMPs

Cells grow on FN for two days and then the medium was changed to DMEM 1% FBS (to avoid the effects of a higher percentage of FBS). In some wells, 2,5mM EDTA, a zinc-chelanting agent, was added in order to inhibit MMPs. In the next day, the medium was removed and stored at -80ºC until used in gelatin zymography or in ELISA assay for VEGF quantification.

Inhibition of Notch pathway

Notch pathway was inhibited in BM stromal cells with DAPT (Sigma), a γ-secretase that cleaves cell membrane proteins, including Notch receptor. One day before the assay, medium was changed to DMEM 1% FBS and in some wells were added 10µM N-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine *t*-butyl ester (DAPT) or 1µl of dimethyl sulfoxide (DMSO), in duplicated. DMSO is used as a control, since DAPT is dissolved in this organic solution. The cells were exposed to this compost by 16 hours. Next, medium was removed and stored at -80ºC, cells were isolated with trypsin (Gibco) and stored in Trizol at -80ºC for RQ-PCR.

Statistic analysis

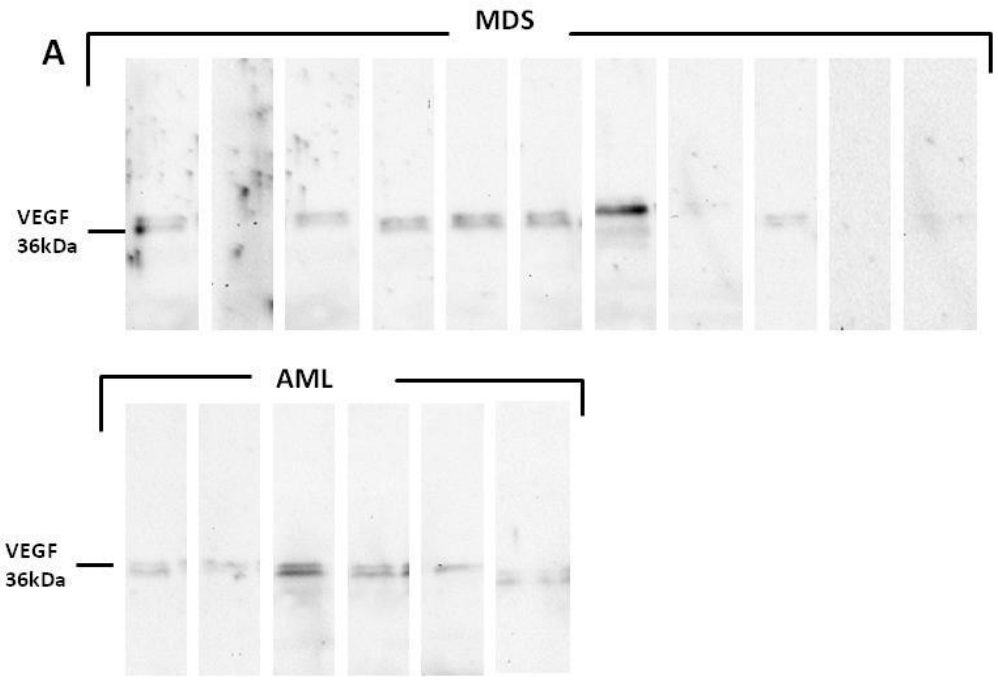
Statistic analysis was performed with GraphPad Prism v.5.0 software, using unpaired t and correlation test (both with a confidence interval of 95%).

RESULTS

VEGF is bound to FN in MDS and AML BM patient samples

ECM can function as a reservoir of soluble factors, regulating their availability in several biological events. As a first approach to study its importance in the BM microenvironment, we wanted to know if in BM malignancies VEGF, a major angiogenic factor, was bound to FN. Co-immunoprecipitation is a technique that allows the detection of protein complexes, so we used that to evaluate the association between FN and VEGF in BM MDS and AML patient samples. The antibody used to detect VEGF is specific for $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$ isoforms, being $VEGF_{189}$ and $VEGF_{165}$ able to bind ECM molecules (insoluble forms).

In fig. 1A, the bands obtained represent VEGF protein that was attached to FN in several samples of BM from MDS and AML patients. After densitometric analysis of these bands, a quantification of VEGF-FN complexes was achieved (fig. 1B).



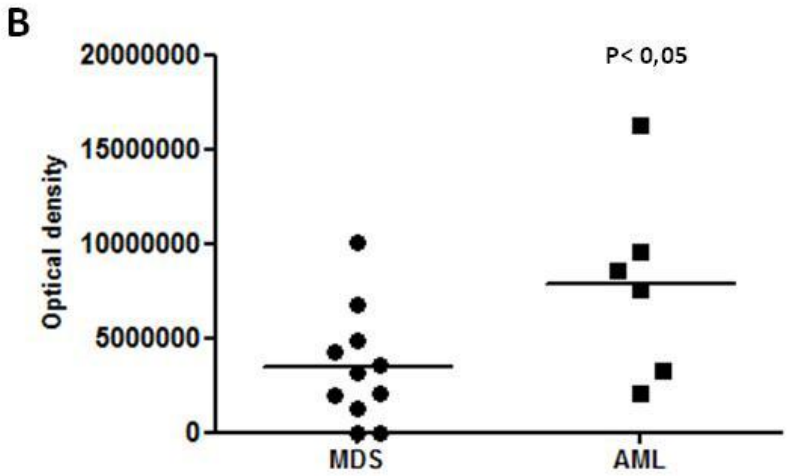


Figure 1. BM from AML patients have more VEGF bound to FN than MDS patients BM. (A) Co-immunoprecipitation of VEGF with FN obtained from AML and MDS patient samples. A band corresponding to VEGF165 dimer can be seen at approximately 36kDa. **(B)** Optical density of scanned western-blots from the same samples. p=0,0117

These results show that VEGF can be bound to FN in BM, and that this association was higher in AML patients than in MDS patients.

MMPs are important for the release of VEGF bound to FN

MMPs are zinc-dependent endopeptidases that degrade the ECM, which can lead to the release of soluble factors bound to ECM. To investigate if MMPs are important for the release of VEGF bound to FN in hematological patients BM samples, we performed an *in vitro* assay with cells isolated from BM biopsies. These cells grew in the presence or absence of EDTA (chelates ions including zinc, and therefore blocks MMP activity) and after three days in culture, the medium was analyzed in terms of MMPs activity and VEGF concentration, by gelatin zymography and ELISA assay, respectively.

By zymography we detected the inhibition of MMP-9 activity in the presence of EDTA, in both diseases, more evident for AML patients. These results also show a higher concentration of MMP-9 in the medium from AML cell cultures than from MDS

cells (Fig. 2A), which was confirmed after quantification of the bands by densitometric analysis (Fig. 2B).

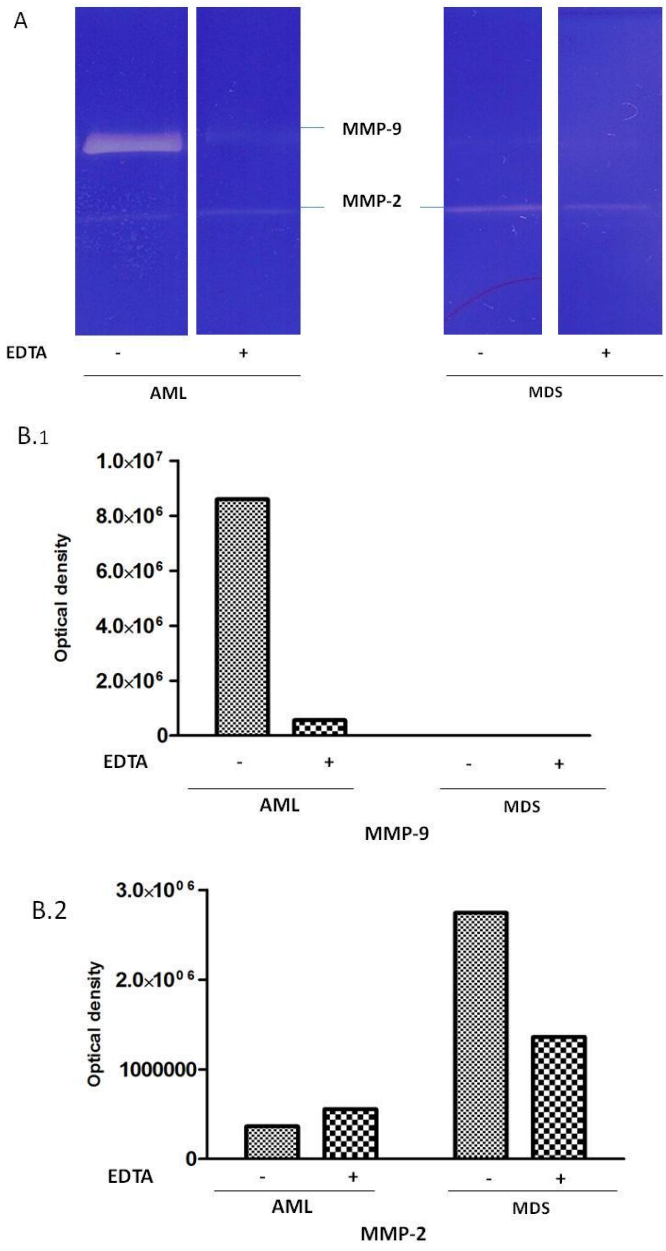


Figure 2. MMPs are inhibited by EDTA, especially in AML patient cell cultures. (A) Zymograms showing MMP-9 and MMP-2 activity in the absence (-) or presence (+) of 2,5mM EDTA in cell culture medium from AML and MDS patients. **(B)** Optical density for **(B.1)** MMP-9 and **(B.2)** MMP-2 bands shown in the zymogram.

The analysis by ELISA of VEGF levels in the medium from these cultures (fig. 3) shows that in AML patients a considerable decrease of soluble VEGF occurs when MMP activity is inhibited. The soluble VEGF in MDS cell cultures was measured, but could not be detected in these samples.

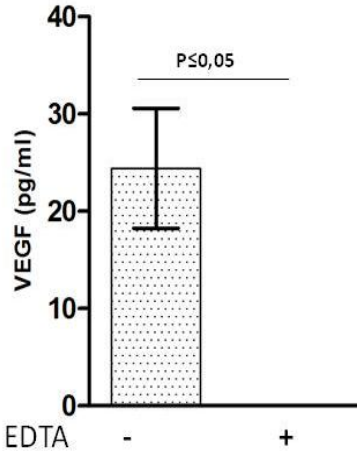


Figure 3. Levels of soluble VEGF decrease in cell culture medium from AML cells in the presence of MMPs inhibitor. VEGF was measured by ELISA assay in cell culture medium from AML patients in the absence (-) or presence (+) of EDTA. p=0,058

These results demonstrated the importance of MMPs activity in the release of VEGF produced by BM cells, and that they are active in MDS and AML BM.

Soluble VEGF is more abundant in MDS patients BM samples

Given that higher amounts of VEGF bound to FN were detected in AML cells, comparing with MDS cells, we wonder if there is also a difference in its soluble form in BM supernatants from both groups of patients. For that, soluble VEGF was measured by ELISA assay. After quantification by spectrophotometry, it is evident that MDS BM supernatants have more soluble VEGF than AML supernatants and that this difference has statistical significance (fig. 4).

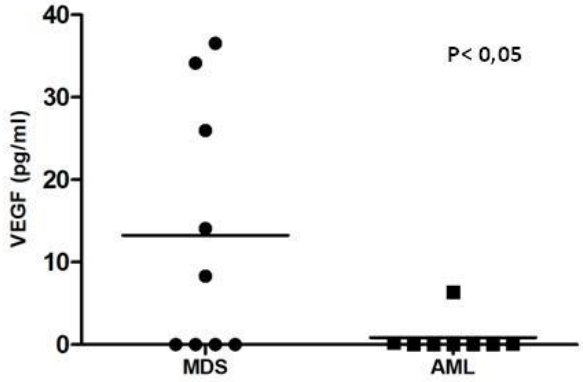
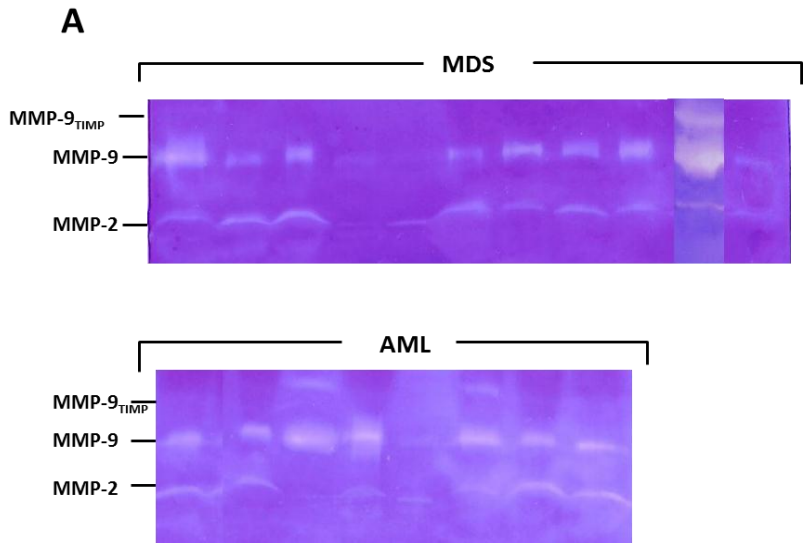


Figure 4. Soluble VEGF levels in BM supernatants are higher in MDS patients than in AML patient samples. Levels of VEGF proteins were quantified by ELISA assay, in supernatants from BM biopsies from MDS and AML BM biopsies. p= 0,0388

MMP-9 and MMP-2 are more concentrated in BM biopsies from MDS patients

Since we had observed that MMPs were important for the release of VEGF from BM cells *in vitro,* we investigated the levels of MMP-9 and MMP-2 activity in BM supernatants from AML and MDS patients.

MMPs activity from supernatants obtained from BM biopsies were measured by zymography, as above. MMP-9 and MMP-2 activity was detected in the majority of patients with both diseases (Fig. 5A).



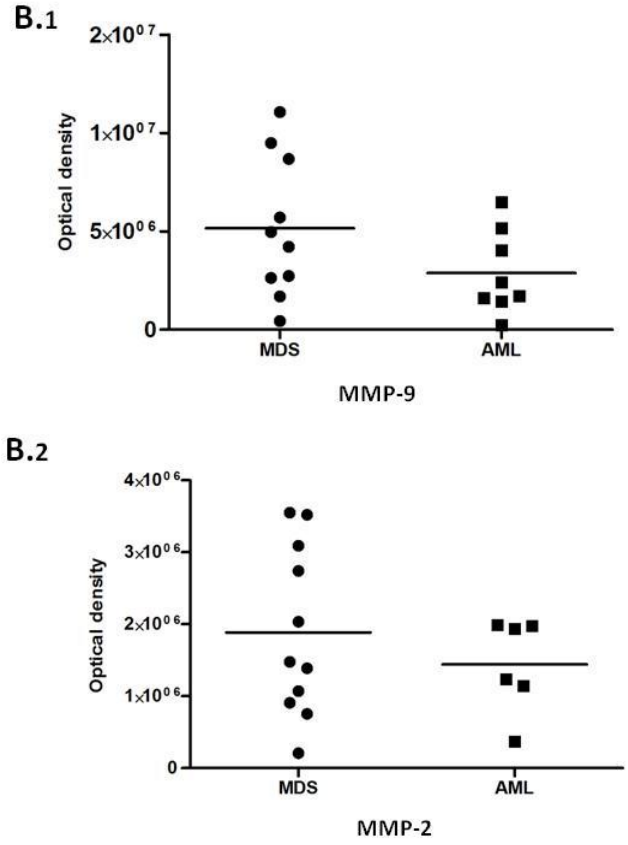


Figure 5. The amounts of MMP-9 and MMP-2 are increased in MDS patients. (A) Zymogram showing the presence of bands corresponded to MMP-9 and MMP-2 in MDS and AML BM supernatants. **(B)** Optical density for **(B.1)** MMP-9 and **(B.2)** MMP-2 bands shown in the zymogram. B.1) p=0,1312; B.2) p=0,4073

Densitometric analysis of MMP-9 and MMP2 bands obtained by zymography show a trend for an increase (not statistically significant) in the amounts of both types of MMPs in supernatants from MDS patients, comparing with AML patients (Fig. 5B) samples. This analysis also shows that MMP-9 is more concentrated in all BM patient samples than MMP2.

These results demonstrate that BM cells from MDS patients produce more MMPs than AML BM, which can be important for the release of soluble VEGF from BM.

MMP-9 levels are correlated with soluble VEGF in BM from MDS patients

A correlation test (with a confidence interval of 95%) for MMP-9 and soluble VEGF measured above demonstrated a positive relation (r) between these two factors in BM from MDS patients. This means that when the levels of MMP-9 increases in BM, soluble VEGF also increases in these patients (Fig. 6).

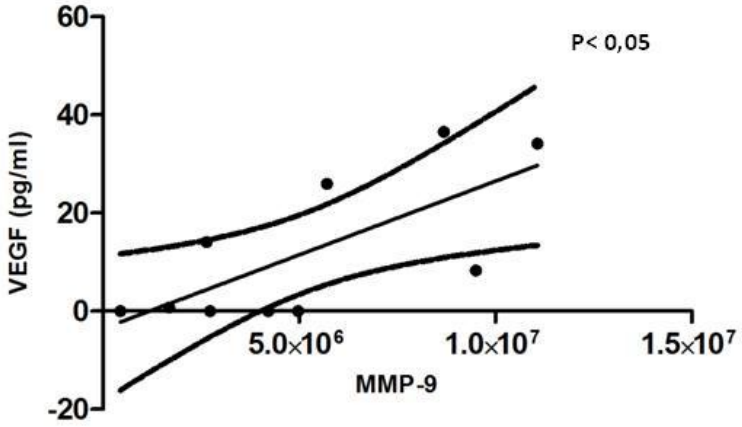


Figure 6. A positive correlation is seen between MMP-9 and soluble VEGF levels in BM supernatants from MDS patient samples. This graph was obtained by linear regression of results obtained by zymography (densitometric analysis of MMP-9 bands) and by ELISA (concentration of VEGF). p=0,0201, r=0,5114

Although done on a limited number of patient samples, together these results suggest that the higher amounts of soluble VEGF in MDS BM patients may be due to its proteolytic release from FN present in BM and that elevated concentration of MMPs contributes to such phenomena. Conversely, in AML BM, VEGF is detected mainly bound to FN, possible due to a lower concentration of MMPs produced by the BM of these patients.

MDS patients have more FN in BM than AML patients

Next, we determined by Western-blotting if the amount of FN differed between MDS and AML patient samples (Fig. 7).

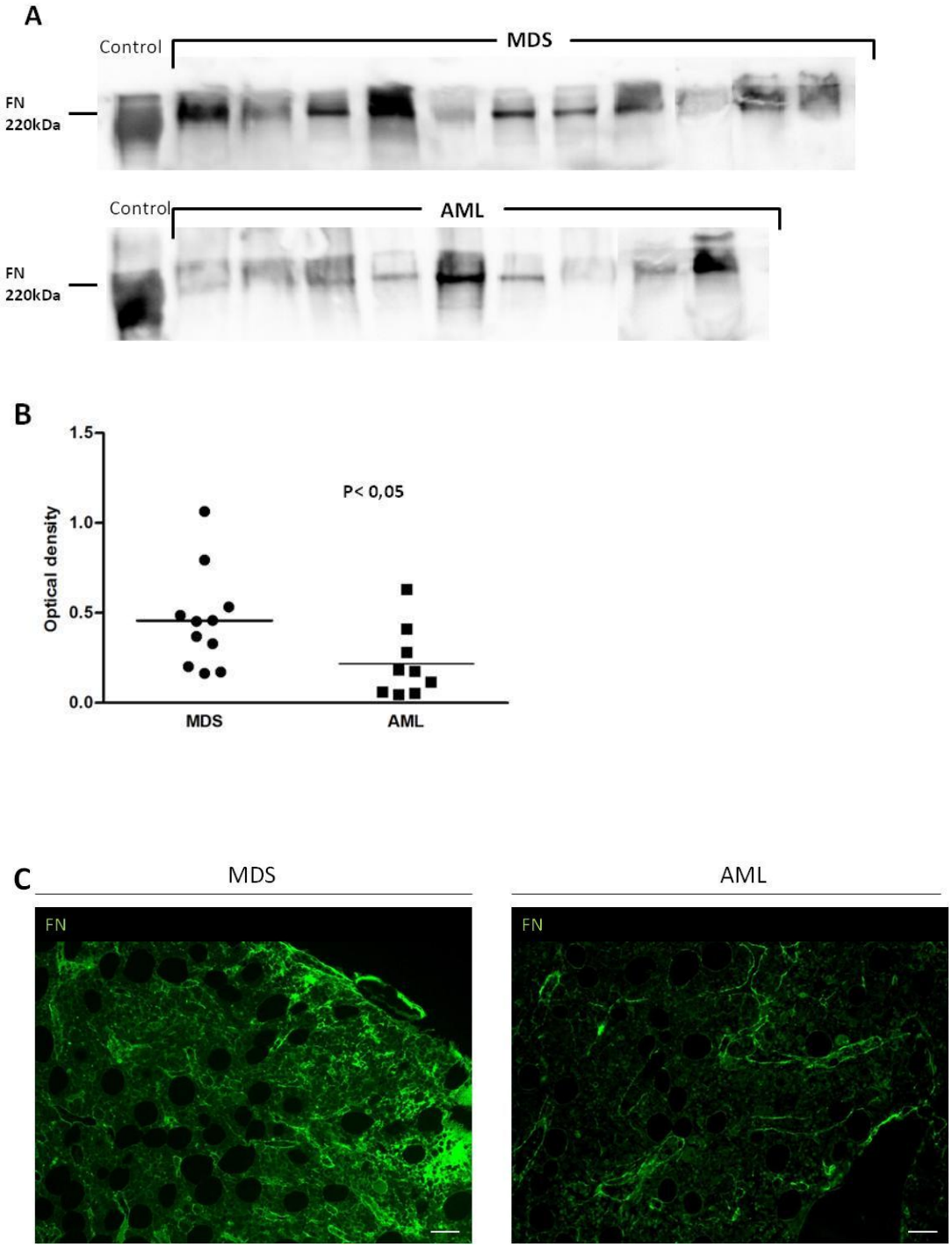


Figure 7. FN is more abundant in MDS BM samples. (A) Western-blotting for FN detection. **(B)** Optical density for the bands corresponded to FN monomer (220kDa) and **(C)** immunofluorescence staining for FN (green) in BM sections (scale bar=50µm). (B) p=0,0411

FN was present in all BM samples, but MDS patient samples have more FN protein in their BM than AML patients. This difference has statistical significance.

BM from MDS patients exhibit a more angiogenic phenotype than from AML patients

It has already been shown that both MDS and AML BM have increased vessel density and increased VEGF levels compared to normal BM, which is illustrative of the highly angiogenic and thus, aggressive phenotype of these diseases. By immunofluorescence, we saw that MDS patients BM sections have, in general, more VEGF in the cytoplasm of BM cells (Fig. 8).

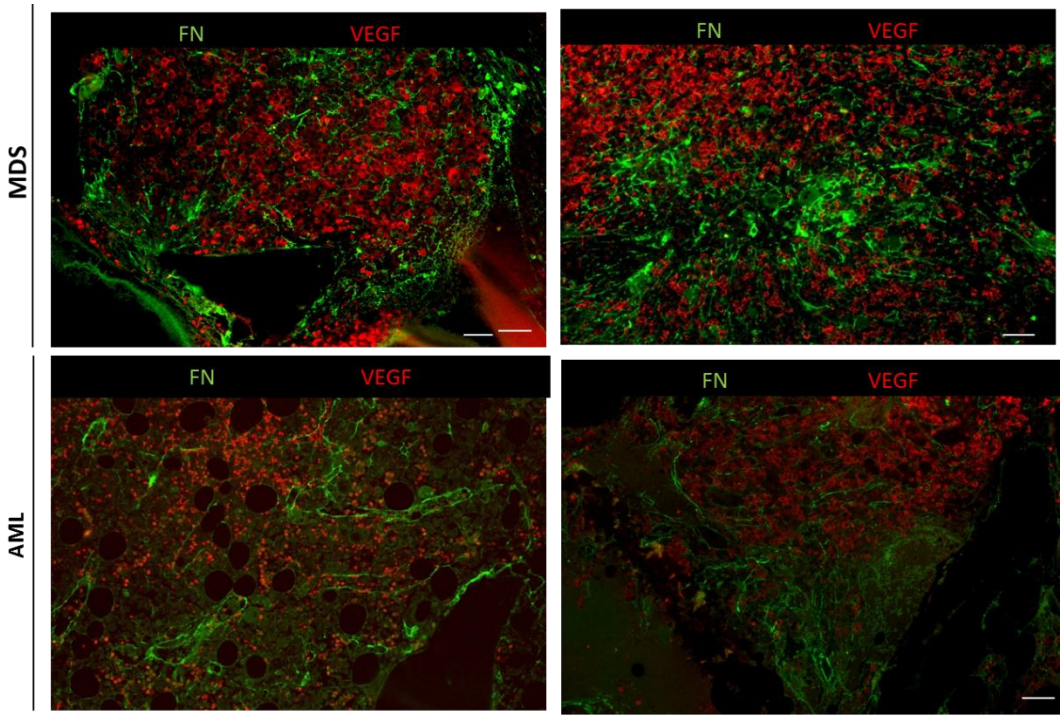


Figure 8. MDS BM have more FN and cytoplasmic VEGF than AML BM. FN (green) and VEGF (red) were detected by immunofluorescence in BM sections from MDS and AML patients (scale bar=50μm).

Although we detected more soluble VEGF in MDS BM and more VEGF bounded to FN in AML BM, the protocol used for immunofluorescence (with organic solvents) should caused the lost of soluble VEGF. These results suggest that MDS BM have more

VEGF than AML, regardless of its localization within BM.

Next we stained (fig. 9A) and counted (fig. 9B) BM blood vessels in BM sections from MDS and AML patients with an antibody against Factor VIII; this antibody also stains MKs.

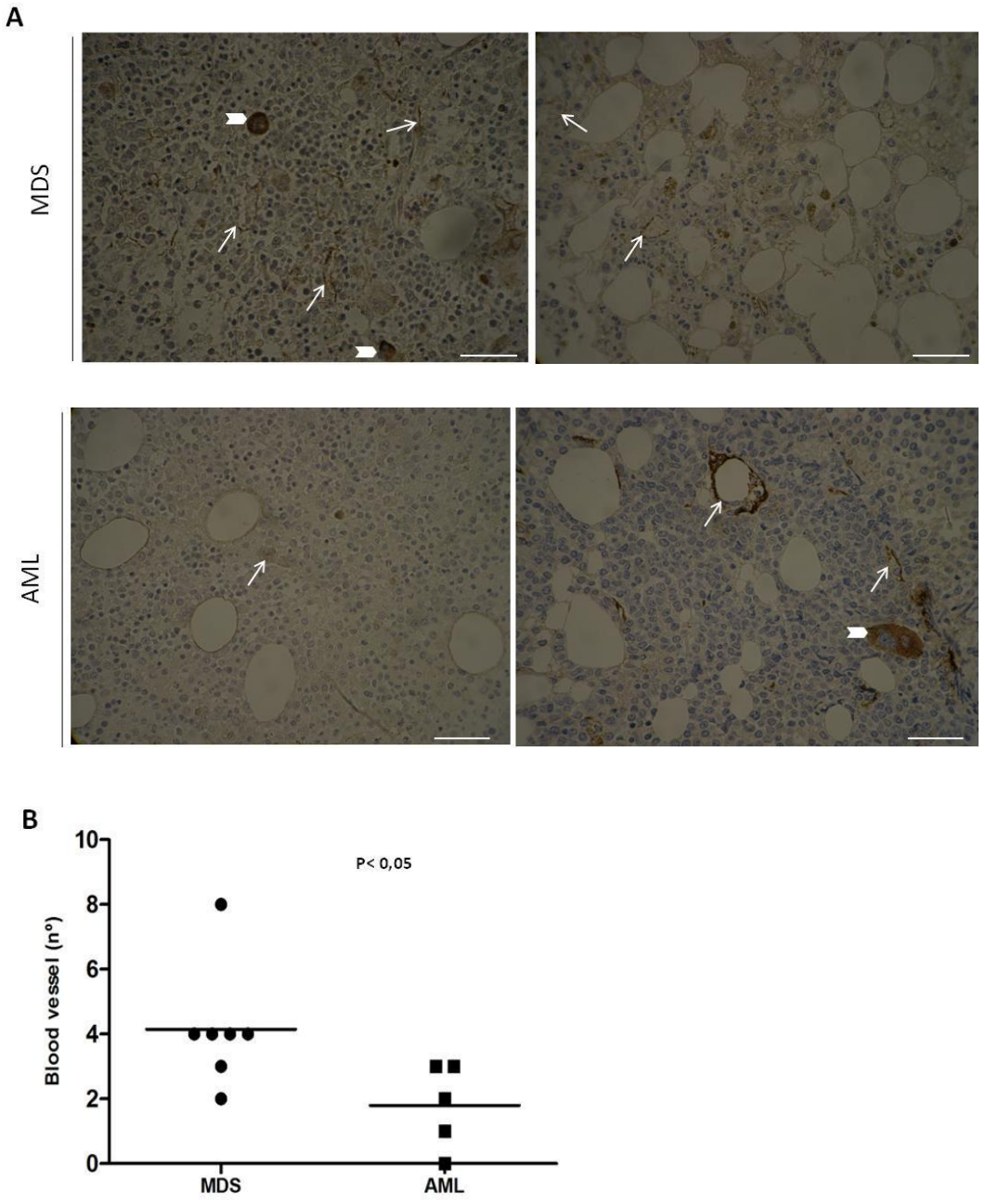


Figure 9. MDS patient samples have more BM vessels than AML patients. (A) Immunohistochemistry in BM from MDS and AML patients showing stained blood vessels (arrows) and MKs (arrowheads) (scale bar=50µm). **(B)** The quantification of these vessels was performed counting five fields with a 40x magnification lens in each BM section. p=0,0369**.**

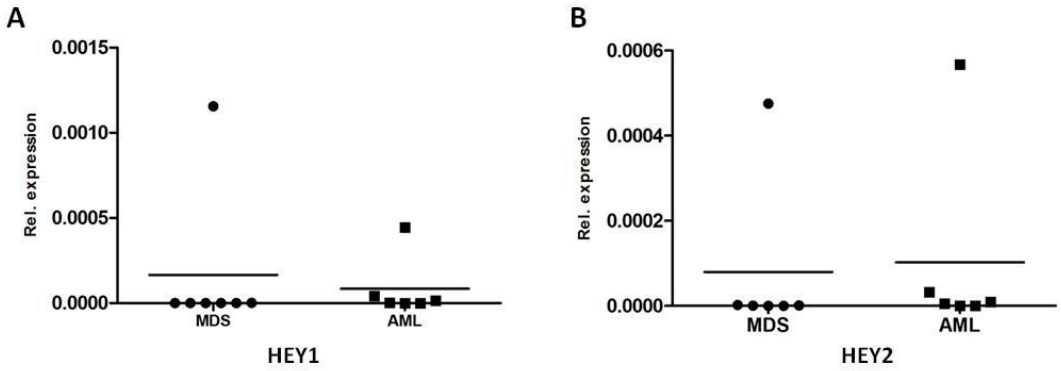
With these results we concluded that MDS patients present more BM vessels and that this angiogenic phenotype is associated with less VEGF bound to FN and consequently to more soluble VEGF present in the BM microenvironment.

Notch pathway is activated in both MDS and AML patient BM samples

Next, to exploit in more detail possible molecular mechanisms involved in the distinct angiogenesis profile and potential by MDS and AML patient BM samples, we investigated the involvement of the Notch pathway.

Notch pathway is altered in several hematological diseases. The Notch family comprises cell membrane receptors (NOTCH1, 2, 3 and 4), which are activated by Delta-like (1, 3, or 4) or Jagged (1 and 2) ligands. The primary targets genes of Notch-intracellular domain are HES and HEY (1 and 2) family, which act as transcription factors and which expression is the best indicator of the activation of this pathway.

We investigated the activation of Notch pathway in patients BM samples by RQ-PCR, detecting the expression of the following genes: HEY1, HEY2, JAG1, NOTCH1 and DLL4 (Fig.10). In addition, we analyzed the expression of FN in the same cells to see if there is any relation between this signaling pathway and FN expression (Fig. 11).



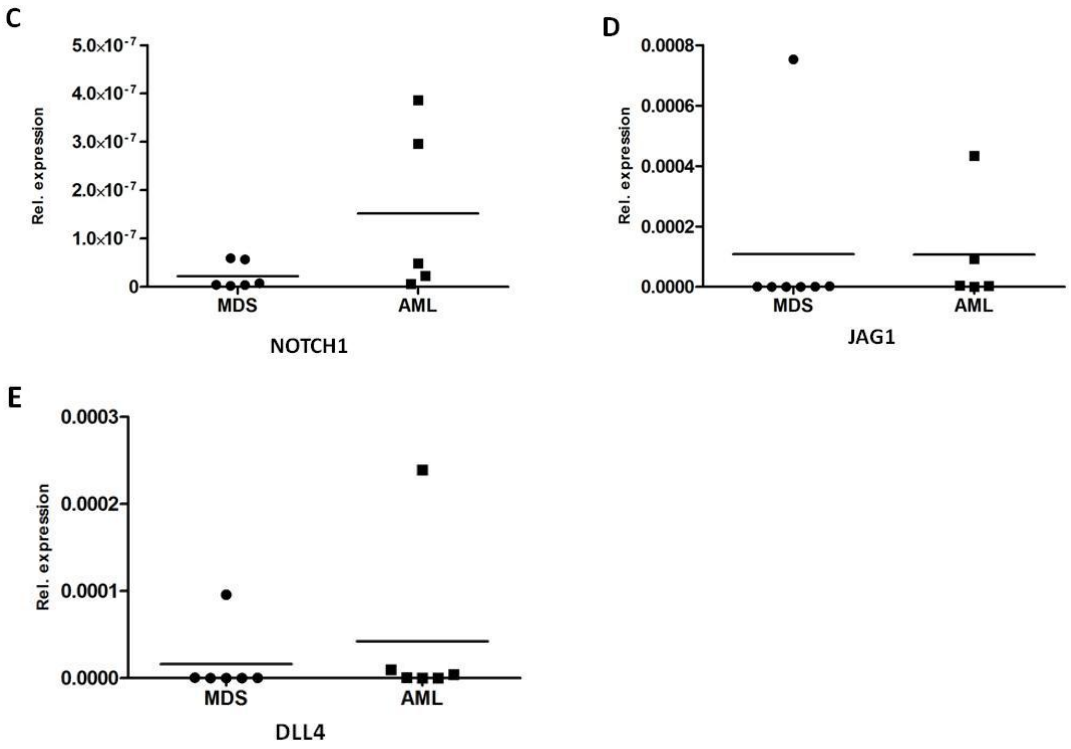


Figure 10. Members of the Notch pathway and downstream target genes show similar expression in MDS and AML patient BM samples. RQ-PCR in BM cells from MDS and AML patients for the expression of **(A)** HEY1 and **(B)** HEY2 transcriptors factors, **(C)** NOTCH receptor, and **(D)** JAG1 and **(E)** DLL4 ligands. Results are represented as mean of Δct and have no statistical significance. A) p=0,678; B) p=0,8573; C) p=0,1061; D) p=0,9934; E) p=0,5537

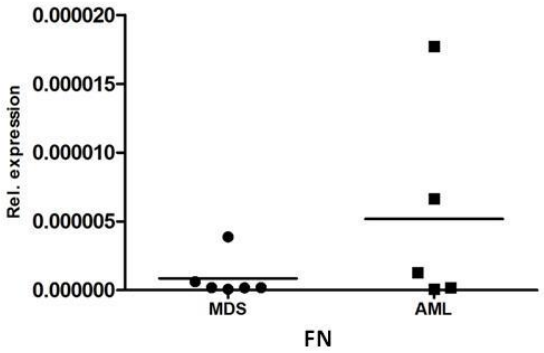


Figure 11. The expression of FN in BM cells from AML patients is higher than in MDS cells. RQ-PCR for FN expression in BM cells from AML and MDS patients. Results are represented as mean of Δct and the differences between the samples have no statistical significance. p=0,1975

Notch pathway activity correlates with FN expression in AML patient BM samples

After the analysis of the expression from several molecules of Notch pathway and FN in BM cells, we tested the correlation between the expression of these molecules, using a correlation test, with a confidence interval of 95%. The correlation coefficient (r) can be positive or negative and this shows if the expression of FN and of a given member of the Notch pathway are directly or inversely proportional, respectively (table 1). The significant correlations are represented in fig. 12 and 13.

Table 1. Correlation test. (A1, B1) indicate p value and **(A2, B2)** indicate correlation coefficient for gene expression in **(A)** MDS and **(B)** AML BM.

MDS

A. 1

P value	FN
FN	
HEY1	0,5709967
HEY2	0,05597413
JAG1	0,4037245
NOTCH1	0,6079916
DLL4	0,5232668

MDS

A. 2

Correl. Coef.	FN
FN	
HEY1	-0,2945178
HEY2	0,8687
JAG1	-0,4226907
NOTCH1	-0,2677363
DLL4	-0,38409

B. 1 **AML**

P value	Fn
Fn	
Hey1	0,000006267156
Hey2	0,001063656
JAG1	0,9486685
NOTCH1	0,02483426
Dll4	0,0001039454

B.2 **AML**

Correl. Coef.	Fn
Fn	
Hey1	0,9996991
Hey2	0,9907662
JAG1	0,05133144
NOTCH1	0,9240575
Dll4	0,9980423

In table 1 we can observe that the correlation between the expression of FN and HEY1, HEY2, NOTCH, and Dll4 is positive and has statistical significance for AML patients (table 1B, fig. 13). However, in BM cells from MDS patients the expression of FN only correlates with HEY2 expression (table 1A, fig. 12).

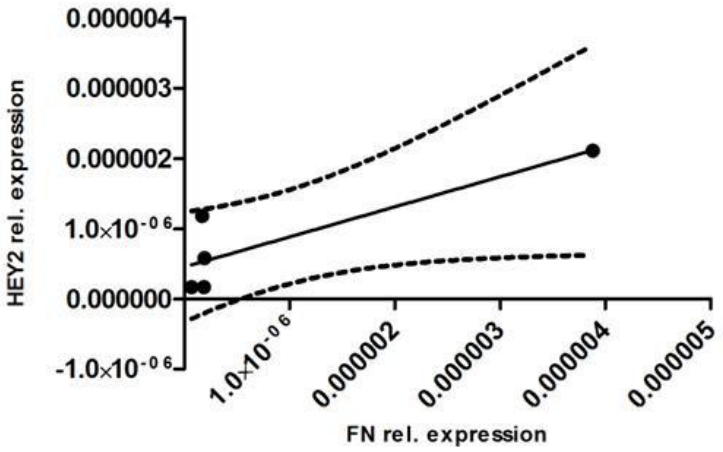


Figure 12. The expression of FN and HEY2 is positively correlated in MDS patients. Correlation curve for FN and HEY1 expression in BM from MDS patients. p=0,0560, r=0,7546

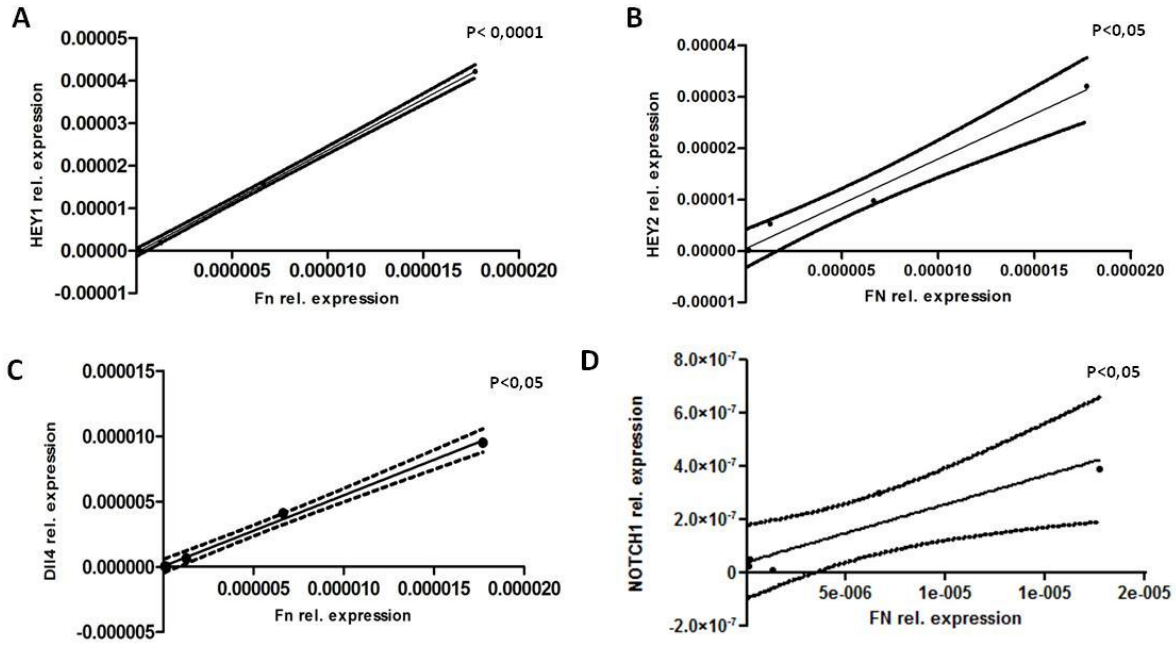


Figure 13. The expression of FN and elements of the Notch pathway are positively correlated in AML patients. Correlation curves for FN and **(A)**HEY1, **(B)**HEY2, **(C)** Dll4 and **(D)** NOTCH1 expression in BM from AML patients. A) p<0,0001, r=0,9994; B) p=0,0011, r=0,9816; C) p=0,0001, r=0,9961; D) p=0,0248, r=0,8539

These results show that in AML, when the Notch pathway is activated, there is an increase in FN expression in BM, for all patients. However, in MDS the correlation is only between FN and HEY2 expression.

Activation of NOTCH pathway increases FN expression in AML patient BM samples

To confirm the involvement of Notch pathway in FN regulation, we performed an *in vitro* assay where Notch was inhibited. To do that, we used cultures of stromal cells derived from BM biopsies of MDS and AML patients, growing in the presence or absence of DAPT for 16 hours. DAPT is a γ-secreatase that cleaves cell membrane proteins, including Notch. DMSO was used as a control. Then, we removed cells and analyzed the expression of HEY1 and FN by RQ-PCR (fig. 14).

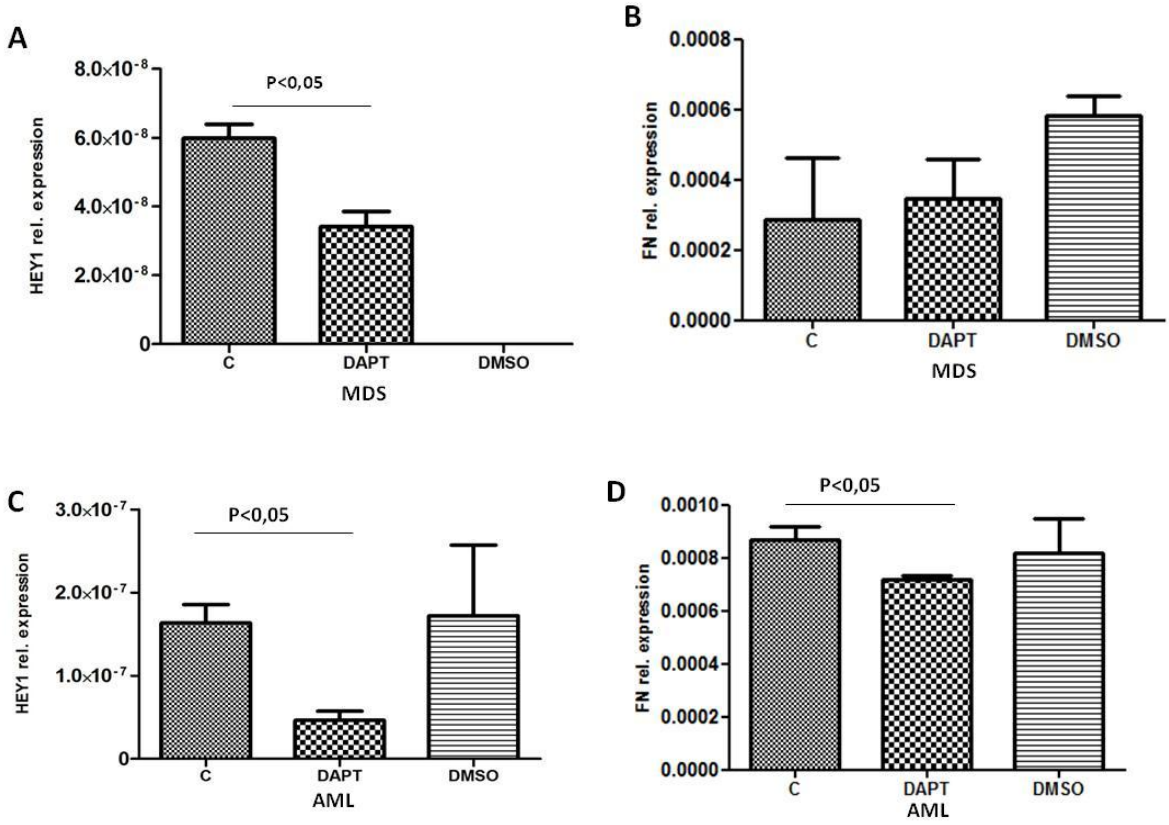


Figure 15. In MDS stromal cells the inactivation of Notch pathway does not affect FN expression , but does so in stromal cells from AML patients. RQ-PCR for **(A, C)** HEY1 and **(B, D)** FN expression 16 hours after the addition of DMEM 1% FBS (c), DMSO and DAPT inhibitor to BM stromal cells from **(A, B)** MDS and **(C, D)** AML patients. A) p=0,0225, B) p= 0,7852 C) p= 0,0036, D) p=0,0471

These results show that DAPT was able to inhibit Notch signaling, since HEY1 expression decreases in this condition. Nevertheless, this inhibition does not affect expression of FN in stroma cells from MDS patients. On the other hand, in stroma from AML patients, the inhibition of Notch signaling significantly decreases FN expression in relation to the control. This *in vitro* assay is in agreement with the positive correlation found between the expression of FN and Notch pathway molecules. All together, these results suggest that in BM from MDS patients the expression of FN may be controlled by a signaling pathway other than the Notch, which seems to be a good candidate for FN regulation in AML BM.

The propose of this study was to address if BM microenvironment differs between MDS and AML, and what role might such factor be playing in disease progression.

Angiogenesis is an important event that mediates the progression from a chronic to a more acute and aggressive pathology, and its significance in hematological malignancies has just beginning to be explored (reviewed in Shadduck *et al.*, 2007). Both MDS and AML are associated with a substantial increase in BM vascularity as well as with increased levels of various angiogenic factors including VEGF, basic FGF, angiogenin, angiopoietin-1, PDGF, HGF, EGF, TNF-α, and TGF-α and TGF-β (Aguayo *et al.*, 2000; Master *et al.*, 2001). The formation of new blood vessels and their permeability is primarily regulated by VEGF (Connolly *et al.*, 1989; Ferrara and Henzel, 1989). Three isoforms of VEGF-A, $VEGF_{165,}$ $VEGF_{189}$ and $VEGF_{206}$ can be found bounded to the cell surface or ECM, via heparin-binding sites (Houck *et al.*, 1992), whereas $VEGF_{121}$ is a nonheparin-binding protein, freely diffusible.

First, by immunoprecipitation, we detected more VEGF bounded to FN in AML than in MDS BM. Both in MDS and AML samples, VEGF was detected as a band of 36KDa that may correspond to the $VEGF_{165}$ isoform, which can be found bounded to cell surfaces or to ECM, but also as a soluble form. Nevertheless, using this technique, only the insoluble form could be detected in the BM samples. On the other hand, by ELISA, we detected a significant increase of soluble VEGF in BM from MDS samples, comparing with AML, which strongly suggest that VEGF bioavailability in these diseases may be controlled by its level of association with FN.

In hematological tumors, MMPs expression has been shown to be upregulated in some leukemia, lymphomas and multiple myeloma (Barillé *et al.*, 1997; Kossakowska *et al.*, 1999; Kuittinen *et al.*, 2001; 2003; Pennanen *et al.*, 2008). Also in a colorectal cancer cell line, the activity of serine protease plasmin and MMP-9 can cleave the bounded forms of VEGF, stimulating endothelial cell growth (Houck *et al.*, 1992; Hawinkels *et al.*, 2008). We explore if levels of MMP-9 and MMP-2 in BM was different between MDS and AML patients and if these enzymes change the amounts of soluble VEGF. After inhibiting MMPs with EDTA in BM cultures, we show a decrease in VEGF le-

vels in cell cultures medium, especially for AML. In MDS cultures, MMPs were almost absent (even without EDTA) which was not expected because we see more quantities of MMPs in BM of MDS than in AML *in vivo*. Since these *in vitro* experiments were effectuated three days after the beginning of cell culture, we suggest the cells present in MDS cultures were not the ones (or at least not present in sufficient amounts) responsible for major MMPs production *in vivo*. In fact, when we grow stromal cells from MDS and AML BM for one month, significant increased MMPs activity was detected in supernatants from MDS patients (data not shown). Three days culture of MDS BM cells have a heterogeneous cellular composition, whereas AML cultures are composed essentially by AML blasts, which secrete MMP-2 and MMP-9 two hours after culture (Matsuzaki and Janowska-Wieczorek, 1997; Ries *et al.*, 1999).

This *in vitro* approach showed that MMPs activity regulates soluble VEGF amounts in BM. Altogether, these results demonstrated that in MDS BM, VEGF is mostly present in a soluble isoform and its availability is determined by an increase in MMPs activity *in vivo*. In AML BM, VEGF is generally bounded to FN, possibly due to a reduced presence of MMPs. In accordance with this, the correlation analysis shows that increased levels of MMP-9 in BM from MDS patients correlate with increased amounts of soluble VEGF in the same samples.

Immunoflourescence staining of BM and Western-blotting analysis showed that MDS have more FN than AML. It is known that several cases of MDS have BM fibrosis (i.e. increased ECM molecules) and these can have a negative impact on patient survival (Lambertenghi-Deliliers *et al.*, 1993). Perhaps, an extensive web of FN constitutes a good reservoir for VEGF at the beginning of MDS, in a phase that do not correspond to the samples we have obtained from these patients, maybe due to absence of symptoms. At a certain point, an increase in MMPs activity releases VEGF and is this soluble form that contributes to the angiogenic switch and disease progression. In AML, blasts occupy BM in a big extent and should not be important producers of FN. In addition, our group unpublished results indicate that MKs are main producers of ECM molecules (in particular FN) and this fact may account for fibrosis associated to several hematological diseases. Interestingly, we detected a trend for in-

creased MK numbers in BM from MDS patients, comparing with AML BM (data not shown).

We also determined vessel density in our patient BM samples and confirmed a significant increased angiogenesis in MDS BM, which is in line with previous works and suggests that the different microenvironment we found in MDS BM, i.e. increased soluble VEGF and MMPs activity may be a major mechanism for angiogenesis in this disease. AML patients are also know to have an angiogenic phenotype, comparing with normal donors (reviewed in Albitar, 2001), but here this event may be controlled by other mechanism, since VEGF is mostly found bounded to FN. Alternatively, the lower amounts of soluble VEGF found in AML BM are still responsible for angiogenesis induction, but in a lesser extent, in agreement with lower vessel counts. The association of VEGF with ECM should inhibit VEGF ligation with its receptor in endothelial cells, thus, restraining angiogenesis. However, it has also been described that VEGF bounded to FN can enhance endothelial cell proliferation and migration *in vitro*, by direct association of this complex with the VEGF receptor flk-1 (VEGFR-1) and the integrin receptor α5β1, mediated by the FN heparin-II domain (Wijelath *et al.*, 2002). Nevertheless, this possible crosstalk between VEGF signaling and integrin pathway must be fewer effective in inducing angiogenesis than the pathway activated by soluble VEGF, since we (and others) show that MDS BM are more angiogenic than AML BM. In accordance to this, VEGF-KDR (VEGR-2) signaling has been demonstrated to have major impact in AML angiogenesis, in contrast with VEGF-VEGFR1 (Padró *et al*., 2002).

Notch signaling regulates the self-renewal of HSPCs, being involved in various hematological malignancies (reviewed in Leong and Karsan, 2006). This signaling pathway is also important in tumor angiogenesis. Several studies have documented the expression of Notch components in tumor vessels, most remarkably Dll4 (Mailhos *et al.*, 2001; Patel *et al.*, 2005). The overall effect is general and involves establishment and maintenance of a stabilized endothelial cell network by inhibiting the formation of new endothelial sprouts, attenuating endothelial cell proliferation or apoptosis, or decreasing VEGFR expression (reviewed in Leong and Karsan, 2006). To our knowledge,

nothing has been described about the effect of Notch signaling in angiogenesis of hematological diseases.

In the context of our study, we analyze if Notch pathway in MDS or AML alter the production of FN, influencing, in turn, VEGF availability. To do these, we examine the Notch pathway in patient BM by RQ-PCR, and show that the analyzed elements of this pathway have identical expression in cells obtained from MDS and AML BM. In addition, FN expression in both diseases did not exhibit statistical significant differences. After that, we determined correlation between FN and Dll4, Notch, HEY1 or HEY2 expression. We demonstrate that FN expression varies proportionally with Notch pathway molecules, but only in AML BM. In MDS cells, FN expression correlation is only with HEY2 transcription factor. These results suggest that Notch pathway is not coordinated with FN expression in MDS BM, but may be somehow associated with FN in AML BM. In a functional assay using stromal cells from both groups of patients, we inhibited the Notch pathway and analyzed FN expression. The results obtain were in accordance with our *in vivo* analyses, since the blockage of Notch pathway corresponded to a decrease in FN expression, but again only in AML cultures. It seems that Notch pathway, which is known to be important in embryonic and adult angiogenesis is activated in both diseases, but only in AML BM it regulates, directly or not, FN expression. During mouse embryonic angiogenesis, overexpression of the Notch ligand Dll4 increases the deposition of FN around the vessels (Trindade *et al.*, 2008), but no functional importance was given to this phenotype. In addition, activated Notch signaling can improve endothelial cell adhesion to the ECM due to an enhanced affinity state of β1 integrin receptors (Leong *et al.*, 2002). Based on our functional studies with stromal cultures (which enclose, among others, endothelial cells), we suggest that also in angiogenesis of hematological tumors, the Notch signaling may be involved, maybe inducing the deposition of FN around new forming vessel and, by this way, stabilize the vasculature. This would happen in AML, creating stable and functional vessels that contribute to tumor progression. In MDS, this pathway does not appear to regulate FN expression and this may compromise new vessels stability, allowing a more uncontrolled growth of the vasculature. This is agree-

ment with increased angiogenesis observed in our MDS samples, but also with observations made by members of our group that these vessels are unstable. On the other hand, the increased amounts of FN we observed in MDS BM should be regulated by other mechanisms (e.g. involving MKs), and by this way does not seem to be deposited around vessels. Rather, this web of FN all over BM must accumulate VEGF at the beginning of the disease, which will be released by MMPs and contribute to MDS angiogenesis.

As a general conclusion, we suggest that the differences found between MDS and AML BM microenvironment lead to different angiogenic phenotypes: in MDS, increased soluble VEGF (in association with high MMPs activity) activates uncontrolled endothelial proliferation, whereas the absence of Notch signaling regulating FN production/accumulation around new vessels contribute to dysfunctional vessels formed; in AML, VEGF is kept majorly bounded to FN, so its availability must be more regulated and the vessels formed are more functional due to FN stabilization. At the end, the different vascular niches created within BM may contribute to the dissimilar characteristics of each disease, for example, increased apoptosis of hematopoietic cells in MDS and blast survival in AML (Fig. 1).

Thus, our results have revealed to be important to better understand MDS and AML phenotypes and, hopefully, in the future, to design more accurate therapeutic strategies for these diseases.

As future work, we would like to better study the precise distribution of VEGF (soluble and insoluble forms) in relation to FN localization in MDS and AML. For that, we could performed confocal microscopy in BM sections after immunostainning and obtain three-dimensional and accurate images of BM that would allow us to better visualize VEGF-FN association *in vivo* and search for differences between MDS and AML. It would also important to know how the ligation between VEGF and FN is established, i.e., which binding domain of each molecule is involved and if other molecules are implicated. This could be achieved using inhibitory antibodies against particular binding domains in *in vitro* assays with BM isolated cells.

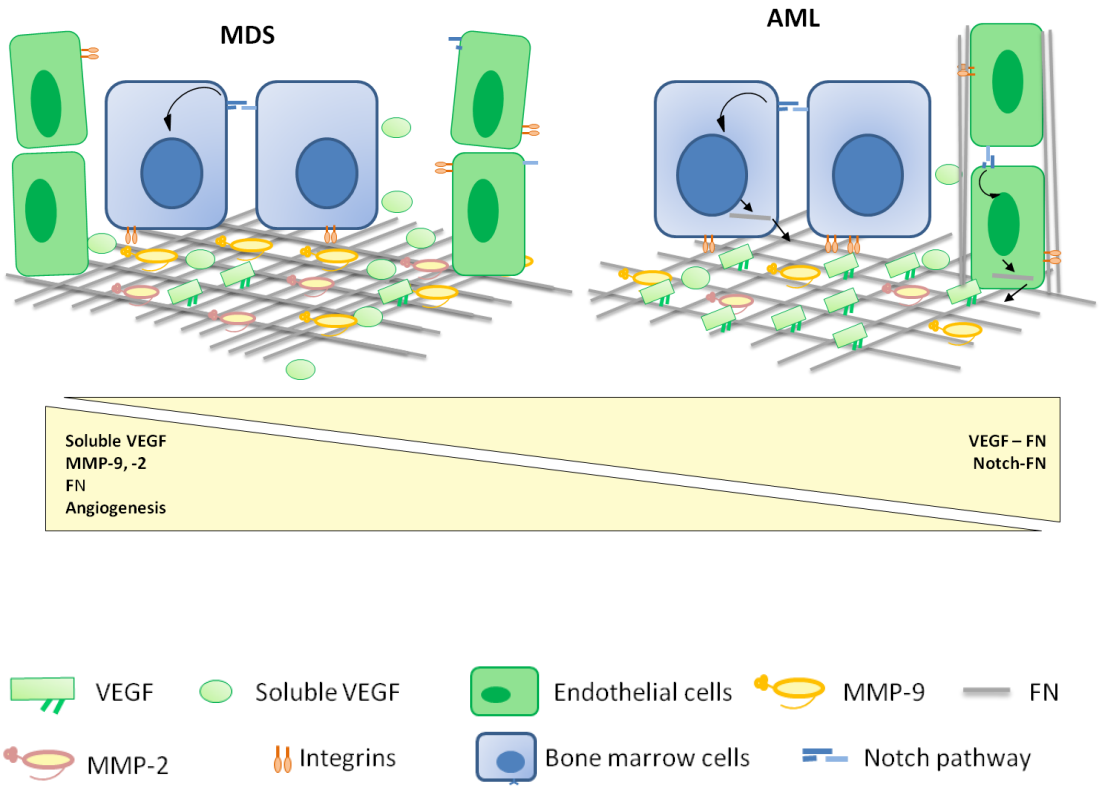


Figure 1. Schematic representation of MDS and AML BM microenvironment. The MDS BM has more soluble VEGF and higher MMPs activity, which may be responsible for increased angiogenesis occurring in this disease. Notch pathway does not regulate FN expression, which leads to new formed vessels instability. In AML, VEGF is kept majorly bounded to FN, and MMPs activity is lower than in MDS BM. In such conditions, VEGF bioavailability is more regulated, being angiogenesis less exuberant. In addition to this, Notch pathway regulates FN deposition around new vessels, contributing to the formation of a more functional and stable vasculature.

Our results concerning the Notch signaling in these diseases also rise some important questions to be answered: which cells are involved, is the regulation of FN by Notch in AML direct or indirect, what could be the role of Notch signaling in MDS, and which mechanism is regulating FN expression in MDS. Also *in vitro* assays using Notch pathways and/or FN inhibitors could provide us some clues about this. Also interesting, three-dimensional cultures of endothelial cells isolated from MDS and AML samples could be included in these studies.

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Table S1. Clinical and laboratory characteristics of patients with MDS and AML

			PERIPHERAL BLOOD			BM	
	AGE	SEX	ERITROCYTES (10^6/UL)	LEUCOCYTES (10^3/UL)	PLATELET (10^3μL)	MYELOBLASTS (%)	IPSS SCORE
MDS	76	M	2.42	4.53	223	3.2	Low
	72	F	4.01	1.77	212	2.6	Low
	30	M	3.27	3.17	71	6.4	High
	67	F	4.09	2.34	45	5.0	Int-1
	73	M	3.54	2.5	136	12	Int-2
	40	F	3.17	2.56	257	4.7	Int-1
	60	M	1.93	2.9	22	1.8	Int-1
	34	F	3.6	5.02	64	3.0	Int-1
	67	F	2.59	1.4	272	1.6	Int-1
	71	M	2.96	3.91	29	0.6	Int-1
	58	F	3.03	7.86	57	0.3	Int-1

	AGE	SEX	BM MYELOBLASTS (%)
AML	36	F	75.4
	79	F	80.7
	64	M	23.0
	79	M	20.2
	68	M	26.8
	77	M	24.8
	75	M	21
	70	M	24
	82	M	24.0

Table S2. PBS EDTA 2mM

NaCl 137mM	8g
Potassium dihydrogen phosphate (KH_2PO_4)	0,2g
Sodium phosphate (Na_2HPO_4)	1,11g
Potassium chloride (KCl)	0,2g
EDTA	0,745g

Table S3. RQ-PCR primer sequences

PRIMER	SEQUENCE 5' TO 3'
HUMAN 18S FWD	GCCCTATCAACTTTCGATGGT
HUMAN 18S RV	CCGGAATCGAACCCTGATT
HUMAN DLL4 FWD	ACTACTGCACCCACCACTCC
HUMAN DLL4 RV	CCTGTCCACTTTCTTCTTCGC
HUMAN HEY2 FWD	TCGCCTCTCCACAACTTCAG
HUMAN HEY2 RV	TGAATCCGCATGGGCAAACG
HUMAN HEY1 FWD	GAAAGTTGCGCGTTATCTGAG
HUMAN HEY1 RV	GTTGAGATGCGAAACCAGTC
HUMAN JAGGED1 FWD	CGGCTTTGCCATGTGCTT
HUMAN JAGGED1 RV	TCTTCCTCCTCCATCCCTCTGTCA
HUMAN FN FWD	CAACAGTGGGAGCGGACCTA
HUMAN FN RV	CGGCTTCCTCCATAACAAGTACA

Table S4. DNA loading buffer

Glycerol	3ml
Bromophenol blue	25mg
H_2O	10ml

Table S5. 2% agarose gel

0,5x TBE	100ml
Agarose	2g (Heat solution)
Ethidium bromide	5µl

Table S6. 5X TBE buffer

Tris base	54g
Boric acid	27,5g
0.5 M EDTA	20ml (pH 8,0)
H_2O	980ml

Table S7. 8% resolving gel

H_2O	2,3ml
30% acrylamide mix	1,3ml
1,5M Tris (pH8.8)	1,3ml
10% SDS	0,05ml
Ammonium persulfate	0,05ml
TEMED	0,006ml

Table S8. Stacking gel

H_2O	1,15ml
30% acrylamide mix	0,33ml
1,0M Tris (pH6.8)	0,500ml
10% SDS	0,02ml
Ammonium persulfate	0,02ml
TEMED	0,004ml

Table S9. Protein loading buffer

500mM Tris-base	2ml
Bromophenol blue	4ml
Glycerol	2ml
10% SDS	0,2g

Table S10. Transfer buffer

25mM Tris-base	15g
193mM Glycine	75g
100% methanol	1l
10% SDS	5ml
H_20	4l

Table S11. 10x TBS and 1x TBS-Tween

10x TBS	
Trizma HCl	24,23g
NaCl	80,06g
H_20	1l (Adjust pH to 7,6)

1x TBS-TWEEN	
TBS 10x	100ml
H_2O	900ml
Tween20	1ml

Table S12. 1x PBS

NaCl 137mM	8g
Potassium dihydrogen phosphate (KH_2PO_4)	0,2g
Sodium phosphate (Na_2HPO_4)	1,11g
Potassium chloride (KCl)	0,2g
H_2O	1l (Adjust pH to 7,2-7,4)

Table S13. 10% polyacrylamide gel with gelatin

H_2O	3ml
30% acrylamide mix	3,33ml
1.0M Tris (pH8.8)	2,5ml
1,2% Gelatin	1ml
10% SDS	0,05ml
Ammonium persulfate	0,033ml
TEMED	0,012ml

Table S14. 2,5% Triton X-100

Triton X-100	100ml
dH_20	3,9l

Table S15: 10x and 1x low salt collagenase buffer

10X LOW SALT COLLAGENASE BUFFER	
25mM Tris-base	60,6g
NaCl	117g
CaCl2	5,5g
dH_20	900ml
dH_20	Adjust to 1l (Adjust pH to 7,6)

1X LOW SALT COLLAGENASE BUFFER	
10x low salt stock collagenase buffer	100ml
dH_20	900ml
Brij-35 (30% w/v. Sigma)	670µl

Table S16. Coomassie brilliant blue stock solution

Methanol	500ml
Coomassie brilliant blue	5ml
H_2O	400ml
Acetic acid	100ml

Table S17. Destain solution

Glacial acetic acid	200ml
methanol	600ml
H_2O	1200ml

Table S18. Coomassie brilliant blue working solution

Coomassie brilliant blue stock solution	30ml
Destain solution	70ml