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Study of the factors that regulate the expression of Jagged-2

Ana Raquel Machado Duarte

DISSERTAÇÃO

MESTRADO EM BIOLOGIA HUMANA E AMBIENTE

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Dissertação orientada pelo Doutor Sérgio Jerónimo Dias, Unidade de Neovascularização, Instituto de Medicina Molecular da Faculdade de Medicina da Universidade de Lisboa e pela Professora Doutora Ana Maria Crespo; Departamento de Biologia Animal; Faculdade de Ciências da Universidade de Lisboa.

2013

Pai.
As adversidades da vida nunca te fizeram desistir.
Hoje dedico especialmente a ti a minha tese, pois a mais ninguém pode ela significar tanto.

Mãe.
Pelo simples e grandioso facto de seres minha mãe, única, insubstituível, inigualável e incansável.

Vocês são, sempre foram e para sempre serão o meu exemplo e o meu orgulho para a vida.

Hardships often prepare ordinary people for an extraordinary destiny

S. C. Lewis

NOTA PRÉVIA

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NATURE REVIEWS | CANCER

O trabalho prático da dissertação foi elaborado na Unidade SDias, no Instituto de Medicina Molecular da Faculdade de Medicina da Universidade de Lisboa.

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PALAVRAS-CHAVE

Células derivadas da medula óssea; EMT; Jagged-2; metástases

RESUMO

Todos os dias, o organismo humano produz milhares de células, através de um processo denominado hematopoiese. As células hematopoiéticas estão envolvidas em processos fisiológicos e patológicos. A hematopoiese é regulada por factores genéticos e pelo ambiente circundante. É esta interacção que determina o destino de cada tipo celular, bem como regula a sua proliferação, diferenciação, a autorrenovação, a apoptose, ou se permanecem em repouso (quiescência). Assim, o controle inadequado de manutenção da hematopoiese está intimamente ligado ao desenvolvimento de neoplasias hematológicas. Compreender a biologia da hematopoiese e a regeneração da imunidade celular, pode resultar na diminuição da morbidade, mortalidade associada a patologias hematológicas e na descoberta de novas terapias.

A angiogénese, outro dos processos envolvidos na manutenção e crescimento celular, é indispensável para o crescimento tumoral e formação de metástases envolvendo a formação de novos vasos sanguíneos capilares a partir de vasos pré-existente. Outros mecanismos de formação de vasos sanguíneos têm sido sugeridos, nomeadamente a vasculogénese, envolvendo o recrutamento de células derivadas da medula óssea.

Na última década foi ainda sugerido que a formação de metástases tumorais possa envolver a contribuição de células não-malignas nomeadamente por populações de células derivadas da medula óssea. Numa tentativa de compreender a formação de metástases, como um evento "sistémico" do cancro, vários foram os autores que estudaram o papel das células derivadas da medula óssea, capazes de induzir angiogénese no tumor primário e também na formação do nicho pré-metastático. No entanto, o papel directo das células derivadas da medula óssea na formação de metástases do tumor primário não tinha sido descrito até recentemente, por estudos do nosso laboratório.

Jagged-2 é uma proteína transmembranar que se caracteriza por um terminal-N de domínio DSL (Delta, Serrate, LAG- 2) que é essencial para a interacção com o receptor Notch. Este ligando pertence à via de sinalização Notch, uma via conservada evolutivamente que é necessária para o desenvolvimento embrionário, a regulação da homeostase dos tecidos e para a manutenção de células estaminais em adultos. Esta

via está entre os canais de comunicação mais utilizados em células animais. A via de sinalização Notch tem vários papéis na especificação do destino final da célula, na padronização dos tecidos e na morfogénese através dos efeitos sobre a diferenciação, proliferação, sobrevivência e apoptose. Um aspecto interessante da via Notch é a sua função aparentemente contraditória no desenvolvimento de tumores, pois pode actuar como um oncogene ou como um gene supressor do tumor. Devido à importância da via de sinalização Notch no desenvolvimento embrionário e adulto, há várias doenças humanas associadas a defeitos nos genes que estão envolvidos na sinalização Notch. Mutações na via de sinalização Notch podem causar o desenvolvimento de fenótipos que afetam o fígado, o esqueleto, o coração, os olhos, o rosto, os rins e o sistema vascular. Outra condição muito grave causada pela desregulamentação da atividade de sinalização da via Notch resulta da transformação de células-T que causam leucemia linfoblástica aguda. Com a possível exceção de algumas neoplasias epidérmicas humanas, a inibição da via de sinalização Notch é uma estratégia viável para o tratamento de certos tumores sólidos e hematopoiéticos.

Novas evidências indicam que para se controlar efetivamente o cancro é preciso considerar o mesmo como uma doença que envolve complexas interações multicelulares heterotípicas dentro de um tecido recém-formado, o tecido cancerígeno. A progressão do tumor envolve várias etapas, até as células cancerígenas adquirirem a capacidade de invasão para outros locais mais distantes do corpo.

A formação de metástases é o resultado de um processo complexo, que possui múltiplos passos e é muitas vezes denominado de cascata de invasão de metástases. Um dos principais processos que regulam a invasão local de tumores epiteliais é denominado epitelial-mesenquimal (EMT). A progressão tumoral está associada à aquisição do fenótipo mesenquimal, que é acompanhada pela perda de expressão de marcadores epiteliais e aumento de marcadores moleculares mesenquimatosos. Assim, EMT é um processo caracterizado por uma transdiferenciação ao nível das células do tumor, e por uma diminuição nos marcadores epiteliais, tais como sub-regulação e relocalização de E-caderina; perda de adesão célula-a-célula; polaridade apical-basal; reorganização do citoesqueleto de actina bem como a regulação negativa e a translocação de β-catenina da membrana da célula para o núcleo e da aquisição de marcadores mesenquimatosos, tais como vimentina; fibronectina e N-caderina associados com o aumento da motilidade celular e a capacidade de invasão.

Através de Jagged-2, ligando da via de sinalização Notch, envolvido nas junções célula-a-célula e, consequentemente, na formação de metástases, foram estudados os

factores envolvidos na expressão de Jagged-2. O objetivo principal deste trabalho foi investigar os fatores que regulam a expressão de Jagged-2 e que possam estar presentes no microambiente tumoral. Para atingir este objectivo, fomos estudar as células mononucleares do sangue periférico e do sangue do cordão umbilical de indivíduos saudáveis, na tentativa de determinar quais as células circulantes com maior expressão de Jagged-2 e descobrir os factores envolvidos na regulação da expressão de Jagged-2.

Começamos por determinar qual a expressão de Jagged-2 em circulação, no sangue periférico de pessoas saudáveis e no sangue do cordão umbilical. No sangue periférico e no sangue do cordão umbilical de pessoas saudáveis foram encontradas diferenças significativas a nível da expressão de Jagged-2, tanto por RT- qPCR como por citometria de fluxo.

Para percebermos quais os factores que regulam a expressão de Jagged-2, utilizamos alguns ensaios *in vitro.* Submetemos células leucémicas a várias condições de hipoxia. Os nossos resultados sugerem que o $CoCl_2$ que induz hipoxia em células leucémicas e regula a expressão de Jagged-2. Outro dos nossos ensaios *in vitro* foi a presença ou ausência de FBS nas mesmas células, estes resultados também sugerem que a ausência de FBS regula a expressão de Jagged-2.

Este é apenas um ponto de partida para uma futura investigação, tanto a nível dos *"ligandos*" da via de sinalização Notch, nomeadamente o Jagged-2, como na formação de metástases presentes num grande número de cancros. Se todos os factores que regulam a expressão de Jagged-2 forem estudados e combinados com alguns tipos de cancro metastático pode ser possível criar terapias específicas que atrasem o aparecimento de metástases.

KEYWORDS

Bone marrow derived cells; EMT; Jagged2, metastasis

ABSTRACT

Over the last decade there has been an increase in the understanding of the regulate factors in the tumor progression and metastasis formation. In an attempt to understand metastases formation as a “systemic” event of cancer, several authors have reported the role of Bone Marrow (BM) derived cells in inducing primary tumor angiogenesis and also in the formation of the pre-metastatic niche.

The main objective of this thesis was to investigate the factors that regulate the expression of Jagged-2. Jagged-2 is a transmembrane protein and it is a ligand of the Notch signaling pathway, required for embryonic development, the regulation of tissue homeostasis, and the maintenance of stem cells in adults. The Notch pathway has multiple roles in cell fate specification, tissue patterning, and morphogenesis through effects on differentiation, proliferation, survival, and apoptosis. An interesting aspect of the Notch pathway are it apparently opposite functions in tumor development, because it can act as an oncogene or as a tumor suppressor. In many individuals, the endpoint of multistep tumor progression involves, unfortunately, the acquisition by cancer cells of the ability to invade and to metastasize from the primary tumor to distant sites in the body - the manifestations of high-grade malignancy. One of the major processes regulating local invasion in epithelial tumors is termed Epithelial to Mesenchymal Transition (EMT) which is associated with the acquisition of mesenchymal phenotype, which is accompanied by the loss of epithelial marker expression and up-regulation of mesenchymal molecular markers.

Through the expression of Jagged 2 ligand of Notch pathway involved in junction cell-to-cell and consequently the formation of metastases, we studied the factors involved in the expression of Jagged 2. Our findings suggested that hypoxia-induced $CoCl_2$ in leukemic cells regulates the expression of Jagged2 and that the absence of the FBS also regulates the expression of Jagged2. Using both by qRT-PCR and flow cytometry methods, differences in the expression levels of Jagged 2 were found in peripheral and umbilical cord bloods of healthy people. This could be a starting point for a viable strategy for treatment and new targeted therapies.

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LIST OF ABBREVIATIONS

ADAM – a Disintegrin and Metalloproteinase

APC – Allophycocyanin

ATCC – American Type Culture Collection

BM – Bone Marrow

BSA – Bovine Serum Albumine

CADASIL – Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy

CB – Cord Blood

CBMC's – Cord Blood Mononuclear Cells

CO_2 – Carbon Dioxide

$CoCl_2$ – Cobalt (II) Chloride

DEPC – Deithylpyrocarbonate

Dll – Delta-like

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

dNTPs – deoxynucleotides

DSL – Delta; Serrate; LAG-2

EDTA – Ethylenediamine Tetraacetic Acid

EGF – Epidermal Growth Factor

EMT – Epithelial to Mesenchymal Transition

EPC's – Endothelial Progenitor Cells

FACS – Fluorescense-activated cell sorting

FBS – Fetal Bovine Serum

FITC – Fluorescein

Flk1 – Fetal liver kinase 1

FWD – Forward

H - Hours

Hes – Hairy/Enhancer of Split

HSC's – Hematopoietic Stem Cells

IPS – Instituto Português do Sangue

Jag – Jagged

KDR – Kinase insert Domain Receptor

Mam1 – Mastermind-like 1

MET – Mesenchymal-Epithelial Trasition

mL – mililitre

mRNA – Messenger Ribonucleic Acid

NICD – Notch Intracellular Domain

Nrarp – Notch regulated ankyrin repeat protein

PB – Peripheral Blood

PBS – Phosphate Buffered Saline

PBMC's – Peripheral Blood Mononuclear Cells

PE – R-Phycoerythrin

PerCP – Peridinin Chlorophyll Protein Complex

PFA – Paraformaldehyde

qRT-PCR – quantitative Real-Time Polymerase Chain Reaction

RCLB – Red Cell Lysis Buffer

REV – Reverse

RNA – Ribonucleic Acid

RPMI – Roswell Park Memorial Institute

RT- Room Temperature

SD – Spondylocostal Dysostosis

T-ALL – T-cell malignancies

TAMs – Tumor associated macrophages

VEGF – Vascular Endothelial Growth Factor

VEGFR-2 – Vascular Endothelial Growth Factor Receptor 2

µl – Microlitre (10^{-3} ml)

ºC – Degree Centigrade

1 INTRODUCTION

1.1 HEMATOPOIESIS

Every day, the human organism produces thousands of cells, this process is called hematopoiesis. These cells (white blood cells, red blood cells, and platelets) are involved in physiological and pathological events[2].

Hematopoiesis is in detail the process by which all lineages of blood cells are generated in a hierarchical and stepwise manner from immature cells present in the Bone Marrow (BM) and subsequently released into circulating blood and peripheral organs for further maturation steps and/or effector function [3, 67]. The hematopoietic system consists in many cell types with specialized functions (Figure 1), such as the red blood cells (erythrocytes), whose carry oxygen to the tissues, the platelets, that help prevent bleeding, the granulocytes (neutrophils, basophils and eosinophils) and macrophages (known as myeloid cells), that fight infections from bacteria, fungi, and other parasites. Also, B-lymphocytes produce antibodies, while T-lymphocytes can directly kill or isolate inflammated cells recognized as foreign to the body, including many virus-infected cells and cancer cells. Some of these cells are also involved in tissue and bone remodeling as well as in the removal of dead cells [3, 5]. Also important is the contribution of the bone marrow stroma in the control and regulation of hematopoiesis. The bone marrow microenvironment, thus consists in many different cell types which interact and modulate the outcome of the hematopoietic process (Figure 1).

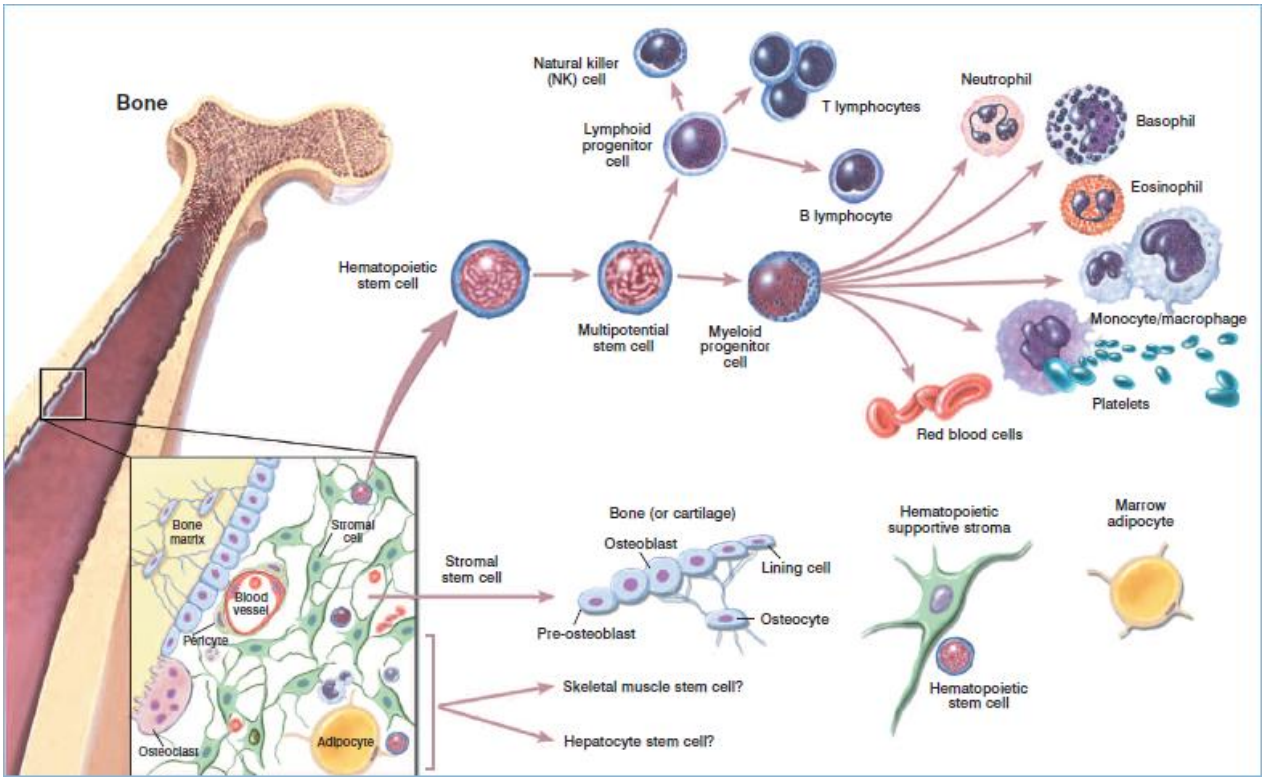


Figure 1| Hematopoietic and stromal cell differentiation.[5]

The hematopoietic process requires a careful regulation of the cells genetic factors and those exerted by the surrounding environment. It is this interaction that determines cell fate (hematopoietic stem cells (HSCs), progenitors, and mature blood cells) as cells proliferate, differentiate, self-renew, undergo apoptosis, or remain quiescent. So, improper control of HSCs maintenance is intimately tied to the development of hematological malignancies. [2, 3, 67].

Understanding the biology of hematopoiesis and the generation of immunity may result in decreased morbidity, mortality as a consequence of hematological diseases and may contribute towards the discovery of new therapies.

1.2 THE VASCULAR SYSTEM

Blood vessels supply oxygen and nutrients to all tissues and organs in our body and provide a gateway for immune surveillance. The network of blood vessels includes arteries, veins, arterioles, venules and capillaries. Large vessels are responsible for blood transport and smaller vessels, specifically capillaries, for exchange of gases and metabolites over the vessel wall [23].

The urge to explore the secrets about blood and lymph vessel development and behavior has exploded parallel to the increasing knowledge about their role in a number of pathologic and physiologic conditions [24, 25]

1.2.1 VASCULOGENESIS

Vascularization is critical for embryonic development and for normal physiological functions in large multicellular organisms [25].

In the embryo, the first vessels arise by de novo formation of blood vessels from precursor cells, angioblasts or vascular precursor cells, that shape blood islands which later fuse to create a primitive plexus of vessels. This process is referred to as vasculogenesis [23].

The primitive vascular network is modified by the process of angiogenesis, leading to maturation, branching and formation of a complex vascular network. Vasculogenesis has long been thought of being restricted to the pre-natal stage

occurring exclusively in the developing embryo. However, recent data has shown that neovascularization in adult life, both in pathological and physiological conditions, can also occur by vasculogenesis, and is particularly important in certain solid and hematological cancers [24, 25].

1.2.2 ANGIOGENESIS

In contrast to vasculogenesis, angiogenesis refers to the formation of new blood vessels from pre-existing ones. Angiogenesis depends on cell adhesion and proteolytic mechanisms that involve the activity of growth factors, extracellular matrix proteins, proteases and adhesion molecules [28, 29]. This process occurs throughout our lifespan. Normal (physiological) angiogenesis is vital for wound healing and for the development of the endometrium during the uterine cycle [26, 27]. This process is involved in various physiological processes such as organ growth, wound healing, revascularization of ischemic tissue, ovulation, menstruation, implantation and pregnancy [25].

When angiogenic growth factors are created in greater amounts than angiogenesis inhibitors, the balance is tilted in favour of the growth of new blood vessels. When inhibitors are present in greater amounts than stimulators, angiogenesis is stopped. In a healthy status, the body maintains a balance of angiogenesis regulators. [25, 27]. Also, angiogenesis is indispensable for metastatic growth. [26].

1.3 THE CONTRIBUTION OF BONE-MARROW DERIVED CELLS IN ANGIOGENESIS AND METASTASES FORMATION

Several studies have demonstrated that Bone Marrow - derived cells are recruited to angiogenic sites to support the establishment of new vessels, namely through the involvement of endothelial progenitors cells [34, 69, 70].

Endothelial progenitor cells, which are mainly located in the bone marrow niche postnatally, play a crucial role in angiogenesis. These cells are phenotypically characterized as CD34+, CD133+ and VEGF receptor 2+ (VEGFR-2+, also known as KDR or Flk1), among other less consensual markers. The process of neovascularization involves the release of endothelial progenitor cells from the bone marrow to the blood circulation in response to various signals [26].

In the last decade there has been increasing evidence suggesting that tumor metastasis is also regulated by non-malignant cells of the tumor microenvironment,

namely by bone marrow - derived cell populations [35]. In fact distinct BM-derived populations such as Tumor Associated Macrophages (TAMs) [36, 37, 38], pre-metastatic niche cells [39, 40] and Endothelial Progenitor Cells (EPCs) [36] have been shown to enhance metastization via multiple processes. Nevertheless a direct role of BM-derived cells in promoting early metastases-promoting changes at the primary tumor, namely the induction of Epithelial to Mesenchymal Transition (EMT, described in the next section) at the primary tumor had not been described.

1.4 METASTASIS FORMATION

A possible endpoint of the multistep tumor progression model involves the acquisition by cancer cells of the ability to invade and to metastasize from the primary tumor to distant sites in the body—the manifestations of high-grade malignancy. Indeed, the formation of are responsible for 90% of cancer-associated mortality [9, 10]. Despite significant advances in the treatment of primary tumors, metastases remain a significant clinical problem, likely reflecting our limited knowledge of the mechanisms governing this complex process (Figure 2) [10].

The formation of metastases is the result of a complex, multistep process that is often termed the invasion-metastasis cascade. These steps include: local invasion by tumor cells; entry into systemic circulation ('intravasation'); invasion of the target organ ('extravasation'); and finally proliferation and growth of the secondary tumor [11]. One of the major processes regulating local invasion in epithelial tumors is termed Epithelial to Mesenchymal Transition (EMT) [12, 13].

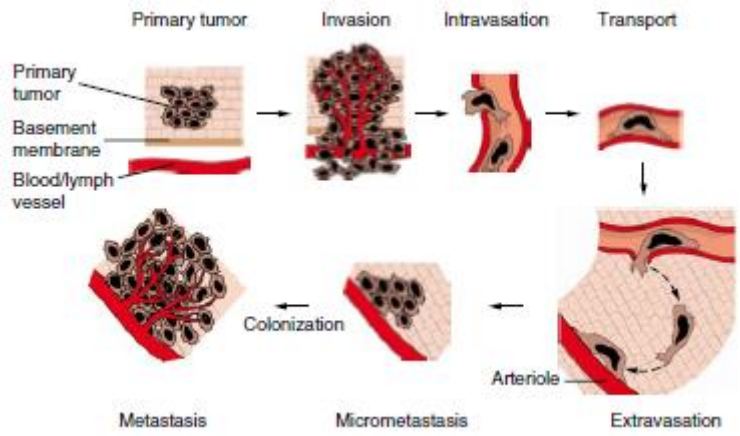


Figure 2| The invasion-metastasis cascade. Cancer cells in the primary tumor acquire the ability to invade adjacent tissue, enter into the vessels of the blood and lymphatic systems (intravasation), travel in

these channels to distant sites in the body, escape from these vessels (extravasation) into nearby tissues, and establish small tumor colonies (micrometastases) in these tissues. On occasion, the cells forming a micrometastasis will acquire the ability to proliferate vigorously, resulting in the formation of a macroscopic metastasis—the process termed "colonization [9].

To explain how cancer cells can migrate from a primary site to colonize specific distant sites to grow and from metastases, Stephen Paget [70] proposed the "seed and soil" hypothesis more than a century ago. Paget proposed that the formation of metastasis depends both on the properties of the tumor cells (seed) as well as the permissive role of environment (soil) at the distant site [8]. The Metastasis formation is a multifactorial process that dictate how cells migrate, survive, and proliferate in "foreign" environments, as well as on the cellular and cytokine profile of the tissue from which the cells initially egress and of the cytokine and cellular composition of the tissue(s) to which the cells home to form a metastasis [14].

1.4.1 EPITHELIAL TO MESENCHYMAL TRANSITION (EMT)

Epithelial-to-Mesenchymal Transition (EMT) was first recognized as a feature of embryogenesis, which is vital for morphogenesis during embryonic development. Recently it has also been implicated in the conversion of early stage tumors into invasive malignancies [15, 72, 73, 74].

Increasing evidence suggests that tumor progression involves the acquisition of an EMT phenotype, which allows tumor cells to acquire the capacity to infiltrate surrounding tissues, and to metastasize to distant sites. Progression of most carcinomas is associated with the acquisition of mesenchymal phenotype, which is accompanied by the loss of epithelial marker expression and up-regulation of mesenchymal molecular markers [15, 16]. Thus, EMT is a transcriptionally regulated process characterized, at the tumor cell level, by a decrease in epithelial markers such as down-regulation and relocation of E-cadherin, loss of cell-cell adhesion, apical-basal polarity, actin cytoskeleton reorganization as well as down-regulation and translocation of β-catenin from the cell membrane to nucleus [15, 17], and acquisition of mesenchymal markers such as vimentin, fibronectin and N-cadherin associated with as increase in cell motility and invasion capacity [18, 19, 20].

Epithelial cells form a sheet or layers of cells that are tightly connected laterally by specialized junction structures, including adherent junctions, desmosomes, tight junctions, and gap junctions. Among these, adherent junctions play a particularly

important role in assembling and constructing lateral cell-cell adhesions in epithelial cell sheets. Epithelial cells establish an aligned apical-basal polarity through their association with a lamina layer at their basal surface, often called the basement membrane[16]. In contrast to epithelial cells, mesenchymal cells exhibit a front-back end polarity and rarely establish direct contacts with neighboring mesenchymal cells [21]. Unlike epithelial cells, mesenchymal cells can invade as individual cells through ECM constructed by epithelial sheets and by mesenchymal cells themselves (Figure 3) [16, 17, 21].

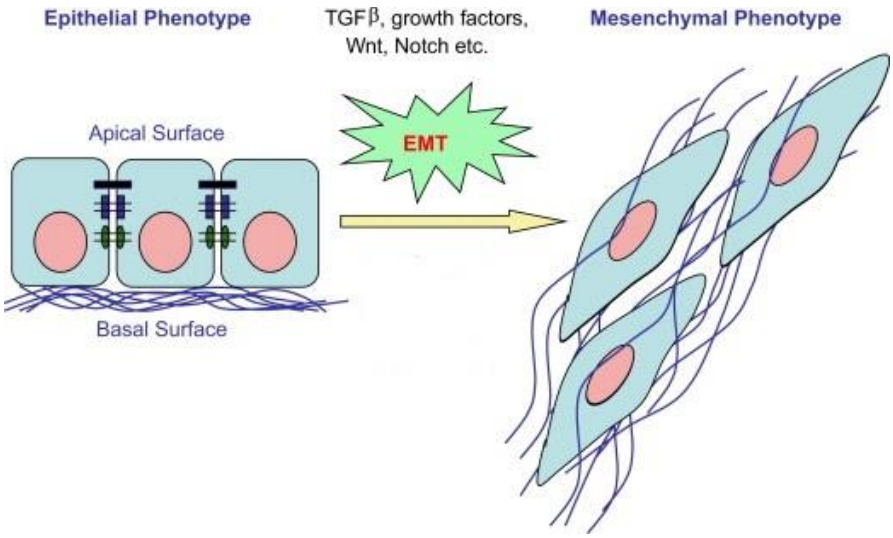


Figure 3| Epithelial-to-mesenchymal transition (EMT). EMT occurs when epithelial cells lose their epithelial cell characteristics, including dissolution of cell–cell junctions, and acquire a mesenchymal phenotype, characterized by cell skeleton reorganization [22].

The EMT program is activated at multiple steps of embryonic development to enable the conversion of various types of epithelial cells into mesenchymal cells. The activation of EMT, however, does not necessarily represent an irreversible commitment of cells to different lineages. Thus, the reverse program, termed Mesenchymal-Epithelial Transition (MET), also occurs both during embryonic development and during several pathological processes and is believed to be involved in the establishment of metastases. The reversibility of EMT underscores the enormous plasticity of certain embryonic and adult cells that participate in disease pathogenesis [16, 21].

1.5 NOTCH-DELTA SIGNALING PATHWAY

The Notch-Delta signaling pathway is an evolutionarily conserved signaling pathway that is required for embryonic development, the regulation of tissue homeostasis, and the maintenance of stem cells in adults. It is among the most commonly used communication channels in animal cells [1]. Notch pathway has multiple roles in cell fate specification, tissue patterning, and morphogenesis through effects on differentiation, proliferation, survival, and apoptosis [43, 41, 28]. In mammals, there are five canonical DSL (Delta, Serrate, LAG-2) ligands: Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged-1 (Jag1), and Jagged-2 (Jag2). These ligands are type I cell-surface proteins with multiple tandem Epidermal Growth Factor (EGF) repeats in their extracellular domains [4]. DSL ligand bind to Notch receptors, which are single-pass, type I transmembrane receptors. In mammals, there are four Notch receptors, Notch 1 to Notch 4. Binding of a DSL ligand to the extra cellular domain of Notch receptor triggers a series of proteolytic cleavages of Notch, first by a member of the disintegrin and metalloproteases (ADAM) family within the juxtamembrane region, followed by gamma-secretase within the transmembrane domain. The final cleavage releases the Notch intracellular domain (NICD) from the cell membrane, which translocates to the nucleus and directly interacts with the transcription factor CSL (CBF1/RBPjk/Su(H)/Lag-1) [28, 44, 42, 7] (Figure 4).This binding converts CBF1 into a transcriptional activator, which leads to transcription of target genes [7]. In the absence of NICD, CSL represses transcription through interactions with a corepressor complex that contains a histone deacetylase [7, 45]. Binding of the NICD to CSL displaces the corepressor complex and replaces it with a transcriptional activation complex that includes the NICD, Mastermind-like (Mam1), and histone acetyltransferase to turn on the expression of Notch target genes such as the basic helix-loop-helix proteins Hairy/Enhancer of Split (Hes), Hes-related proteins, and Notch regulated ankyrin repeat protein (Nrarp). Proteins encoded by the Hes and Hey genes are, in turn, transcriptional repressors of both their own expression and further downstream genes [46].

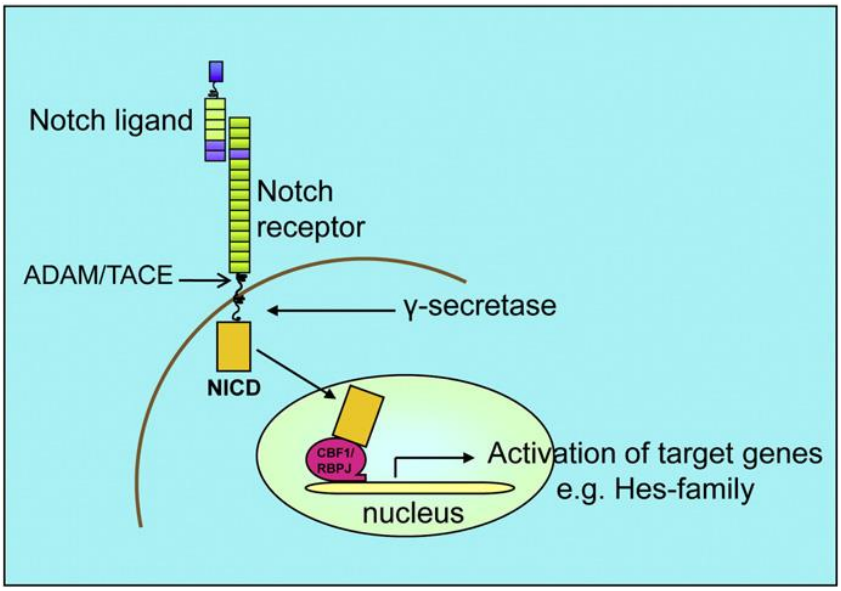


Figure 4| Schematic representation of the Notch-Delta signalling pathway. Binding of the Notch ligand to the membrane-bound Notch receptors leads to a sequence of proteolytic events resulting in cleavage of the Notch extracellular domain by the ADAM protease, followed by cleavage of the intracellular domain by γ-secretase [7].

1.5.1 Receptors and Ligands of the Notch pathway

Both the Notch receptor and its ligands, Delta and Serrate (known as Jagged in mammals), are transmembrane proteins with large extracellular domains that consist primarily of epidermal growth factor (EGF)-like repeats [28, 66].

Notch is a single-pass transmembrane receptor that can be activated by different transmembrane ligands. This receptors consist of a signal peptide and an extracellular domain that is responsible for ligand interaction [4, 29, 7]. The mature Notch receptors is produced through a furin cleavage during biosynthesis. Notch extracellular domains contain 29-36 EGF repeats, 3 cysteine rich LIN repeats and a region that links to the transmembrane and intracellular fragment [28, 7].

Notch receptors have broad expression patterns in many tissues, but analyses of where cleavage occurs or where target genes are expressed reveal a limited profile of activation. The activity of the receptor must also be regulated through post-transcriptional mechanisms [28, 32].

The Notch ligands as well as Notch receptors, are transmembrane proteins that are characterized by an N-terminal DSL (Delta, Serrate and LAG-2) domain that is

essential for interactions with the Notch receptor. The extracellular domains of the ligands contain varying numbers of Epidermal Growth Factor (EGF)-repeats [4, 7, 28].

The ligands are subdivided into classes. Delta or Delta-like (Dll) and Serrate (Jagged in mammals), depending on the presence or absence of a Cysteine Rich (CR) domain [28, 32]. This ligand expressed on one cell binds to a Notch receptor expressed on neighboring cells that are in direct contact [7, 29, 30]. Expression of Notch ligands during development is quite dynamic and contributes significantly to differential activity of the pathway. In some development contexts, the ligand is produced by a distinct population of cells [28, 31, 32].

There are at least five functional Notch ligands in vertebrates: 3 orthologs of the Drosophila Delta (Delta or Delta-like [Dll] 1, 3 and 4) and 2 of the Drosophila Serrate (Jagged1 and Jagged2). All ligands are able to interact with the Notch receptor and induced the second cleavage at the extracellular level. However, all ligands have different expression patterns and specific deletion/inhibition of specific ligands results in a very diverse outcome [4, 7, 32].

1.5.2 THE NOTCH PATHWAY IN DISEASE

Due to the importance of Notch signalling in embryonic and adult development, there are several human diseases linked to defects in the genes that are involved in Notch signalling (Table 1) [31, 66].

Mutations in the Notch signalling pathway cause developmental phenotypes that affect the liver, skeleton, heart, eye, face, kidney, and vascular system. Notch signalling associated disorders include the autosomal dominant, multi-system, Alagille Syndrome caused by mutations in both a ligand (Jagged 1) and receptor (Notch 2) and Autosomal Recessive Spondylocostal Dysostosis, caused by mutations in a ligand (Delta-like 3), as well as several other members of the Notch signalling pathway. Mutations in receptor Notch 3 cause the dominant adult onset disorder Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), a vascular disorder with onset in the 4th or 5th decades [32, 33].

Another very serious condition caused by deregulation of Notch signaling activity is T-cell linked acute lymphoblastic leukaemia, when mutations are of the receptor Notch 1 or Notch 3 [31, 32, 33].

Table 1| Diseases caused by altered Notch signalling.

Disease	Symptoms	Cause
Alagille syndrome	Kindney, eye, heart and skeleton developmental problems and also defects in bile duct formation leading to liver problems.	Mutations on the Jagged 1 gene
CADASIL syndrome	Autosomal vascular disorder linked with a variety of symptoms ranging from migraines to premature death.	Mutations on Notch 1 and 3
T-cell acute lymphoblastic leukaemia	Aggressive tumor derived from T-cell progenitor due to increased Notch signalling activation.	Mutations involving either the Notch heterodimerization domain or the PEST domain. Translocation of a truncated from of Notch resulting in signalling hyperactivation
Spondylocostal dysostosis (SD)	Rib defects causing abnormalities in vertebral segmentation and trunk size.	Mutations in Delta-like 3

1.4.3 NOTCH PATHWAY IN CANCER

Mutations in the Notch pathway often lead to tumorigenesis. An interesting aspect of the Notch pathway are its apparently opposite functions in tumor development, because it can act as an oncogene or as a tumor suppressor [30, 33].

Notch signalling activity depends on signal strength, timing, cell type, and context. The results of altered Notch signalling depends on its normal function in a given tissue. Notch thus acts as an oncogene if its normal function acts as a regulator of precursor cell fate; however its tumor suppressor activity is detected in tissues in which Notch signalling initiates terminal differentiation events [30, 33]. Table 2 summarizes the involvement of abnormal Notch signalling in cancer.

Table 2| Abnormal Notch signalling in turmorigenesis.

Tumor type/process	Function
Hematological tumors	
T-cell malignancies (T-ALL)	Oncogenic Notch signalling
B-cell malignancies	Oncogenic Notch signalling
Solid tumors	
Breast cancer	Oncogenic Notch signalling and Tumor suppressive Notch signalling
Gut cancer	Oncogenic Notch signalling
Skin cancer Keratinocyte-derived carcinoma	Tumor suppressive Notch signalling
Skin cancer Melanocyte-derived carcinoma	Oncogenic Notch signalling
Cervical cancer	Tumor suppressive Notch signalling

With the possible exception of some human epidermal malignancies, Notch signalling inhibition is a viable strategy for treatment of certain solid and hematopoietic tumors [30, 33].

1.6 OBJECTIVES OF THIS THESIS

The goal of this thesis was to study the factors that regulate the expression of Jagged-2 on hematopoietic, bone marrow derived cells.

In order to achieve this main objective we specifically proposed to:

1. Study peripheral and cord blood mononuclear cells from healthy individuals in order to define a profile of Jagged-2 expression on normal hematopoietic circulating cells.
2. Test whether putative tumor factors are involved in the regulation of the expression of Jagged-2 by *in vitro* assay.

In summary, with this study we expected to gain a better insight into the mechanisms involved in the regulation of Jagged-2 expression.

2 MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

Peripheral blood (PB) samples from healthy patients were kindly donated by Instituto Português do Sangue (IPS), Lisbon, Portugal. Cord blood (CB) samples were collected according to informed consent and following the guidelines from Hospital Santa Maria, Lisbon, Portugal from Doctor Diana Martins. PB and CB samples were collected in buffy-coat with EDTA to a total volume of 70 *ml* in sterile conditions.

Samples were diluted in Phosphate Buffer Saline (PBS) (1:1). PB mononuclear cells and CB mononuclear cells were isolated according to the manufacturer's protocol.

2.2 ISOLATION OF MONONUCLEAR CELLS

PB and CB samples were centrifuged at 2000 rpm for 20 minutes at Room Temperature (RT) to separate the PBMC's from erythrocytes and granulocytes through Ficoll (Lymphoprep^{TM}). This method allows the separation of cells according to density of the cells.

The remaining fraction was lysed using 50 *ml* of Red Cell Lysis Buffer (RCLB), for 15 minutes at RT. The resulting mononuclear cell fraction was washed in PBS + 1% BSA and used for further analysis.

Assessment of cell viability was performed by Trypan blue exclusion method, using a BÜrker hemocytometer.

2.3 CELL CULTURE

In this work, two mammalian cell lines derived from peripheral blood were used.

Jurkat cell line (ATCC – TIB-159) has a lymphoblastic morphology and was grown in RPMI 1640 Medium (Gibco) supplemented with 10% FBS, L-glutamine and antibiotic. On the other hand, THP-1 cell line (ATCC – TIB-202) has a monocytic morphology and was grown in RPMI 1640 Medium (Gibco) supplemented with 10% Fetal Bovine Serum (FBS), L-glutamine and 1% Streptomycin/penicillin.

Both cells lines were maintained in a 5% CO_2 humidified incubator at 37ºC. The concentration of cells was maintained between 5 x 10^5 and 1 x 10^6 viable cells/ml. Cells in exponential growth phase were plated and ressuspended in fresh growth medium (RPMI) at 37ºC. After centrifugation, the supernatant was discarded and the cellular pellet ressuspended in 1 *ml* of freezing medium (FBS + 10% DMSO) and transferred to

criotubes. Tubes were maintained at -80ºC. All media and supplements were purchased from Invitrogen.

2.3.1 VIABILITY

Viability was determined using trypan blue method. Trypan Blue is a vital dye. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable.

2 *µl* of trypan blue were used for 10 *µl* of sample. Cells were routinely counted manually with a BÜrker hemocytometer. This procedure was repeated for all time points and during cell growth.

2.3.2 IN VITRO ASSAYS

Jurkat cells ($1x10^6$ cells/ml) were incubated in 9-well plates in the presence or absence of FBS in a total volume of 5 *ml* of medium (RPMI) at 37ºC ,5% CO_2. Cells were harvested at 24h, 48h and 72h and analyzed by FACS and qRT-PCR.

These cells ($1x10^6$ cells/ml) were incubated with 100 µM and 200 µM $CoCl_2$ in a total volume of 5 *ml* of RPMI in 9-well plates at 37ºC, 5% CO_2. Cells were harvested at 24h, 48h and 72h and analyzed by FACS and qRT-PCR. This in vitro assay is used to test the effect of hypoxia.

2.4 RNA EXTRACTION

RNA was extracted using TRIzol reagent® (Sigma) method. TRIzol reagent® maintains the integrity of the RNA, while disrupting cells and dissolving cell components during sample homogenization or lysis.

All the samples were centrifuged and the supernatants were discarded. Pellets were frozen in TRIzol reagent and stored at – 80ºC. Tubes containing cells and TRIzol were thawed on ice and 100 *µl* of chloroform (0,2 *µl* of chloroform per 1 *µl* of TRIzol) were added. The mixture was centrifuged at 13200 rpm for 20 minutes at 4ºC. The top aqueous phase of each tube, containing the RNA, was transferred to new tubes, mixed with 250 *µl* isopropanol (0,5 *µl* per 1 *µl* of TRIzol), and kept overnight at -20ºC.

After 24h the tubes were centrifuged at 13200 rpm for 20 minutes at 4ºC to precipitate the RNA. Supernatants were discarded and the pellets were washed with ethanol 80%. Pellets were ressuspended in DEPC-treated water and quantification was performed on NanoDrop 1000 (Thermo Scientific) spectrophotometer.

2.5 cDNA SYNTHESIS

The cDNA for quantification of transcripts was synthesized from 500 *ng* of total RNA using Random Hexamers, dNTPs 10 mM and DEPC-treated water up to 12 *µl*. Tubes were incubated at 65^{O}C for 5 minutes and a mixture containing First Strand Buffer, DTT 0,1 M and RNAse Out was added. Tubes were incubated at 25^{O}C for 2 minutes, and then 1 *µl* of SScript enzyme was added, and incubated at 25ºC for 15 min, followed by 50 min at 42ºC and 15 min at 70ºC. Reagents and respective quantities are listed in Annex 1.

2.6 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

Levels of mRNAs were measured by qRT-PCR using Viia 7 System (Applied Biosystems) and SYBR-Green fluorescent dye in 384-well plates. This method allows both the amplification and quantification of a housekeeping gene, obtained through light emission of a fluorescent dye that intercalates into the DNA. Relative expression was calculated using relative quantification (2^(-ΔCt)). The housekeeping gene used to normalize human samples was 18s. Amplification reaction mixture contains SYBR-Green, water, 0,15 *µl* of both forward (FWD) and reverse (REV) primers, followed by addition of 2 *µl* of cDNA template per well. qRT-PCR data were analyzed by Viia 7 ™ software (Applied Biosystems).Sequences of the primers used in this study are listed in Annex 2 while reagents and their respective quantities are listed in Annex 3.

2.7 FLOW CYTOMETRY

5x10^{5} cells from each time point were blocked for 10 minutes at 4ºC with FcR fragment (1:100 dilution) for reduction of non-specific reactions. Cells were then incubated with Jagged-2 antibody (PE anti-human Jagged-2, 346903 BioLegend) in a 2,5:100 dilution in PBS+BSA 0,5% for 45 minutes in the dark, with rotation, at 4ºC.

The PB and CB mononuclear cells were stained with different antibodies (CD11b, CD45, CD34, CD19 and Jagged-2). The same procedure was used. Briefly, $5x10^5$ cells were blocked for 10 minutes at 4ºC with FcR fragment in a 1:100 dilution and then incubated with anti-CD11b (APC), anti-CD45 (PerCP), anti-CD19 (FITC), anti-CD34 (FITC) and Jagged-2 (PE). The antibodies were diluted in PBS+BSA 0,5% for 45 minutes in the dark with rotation at 4ºC.The list of antibodies description, respective dilution and fluorochrome are in Annex 4.

Flow cytometry was performed on FACSCalibur and analyzed with FlowJo 8.7 Software.

2.8 STATISTICAL ANALYSIS

All data analysis was performed using Microsoft™ Office Excel and Graphpad® Prism 5.0 software.

Results are expressed as mean ± standard error and the statistical significance was determined using Student's T test. In all comparisons, the significance level to reject the null hypothesis was 5%, that is, p values < 0.05 were considered statistically significant.

3 RESULTS

3.1 MAINTENANCE OF MONONUCLEAR CELLS IN DIFFERENT CONDITIONS

In order to understand how circulating mononuclear cells may be maintained without compromising cell staining, various in vitro assays were performed and then the cells were analyzed by flow cytometry. The aim of these experiments was therefore to assess the time frame during which cell staining against Jag2, for instance, is not compromised. Assays were performed in duplicate and data is presented as mean and standard deviation.

3.1.1 STANDARD MEDIUM CULTURE

After PBMCS cells were isolated they were incubated in RPMI medium at 37ºC for 24 hours.

In Figure 5 it is possible to observe that the CD11B cells gown in RPMI medium have approximately 45% of staining, and therefore are logically alive after 24 hours. However, Jagged-2 expression is not significant like it was expected.

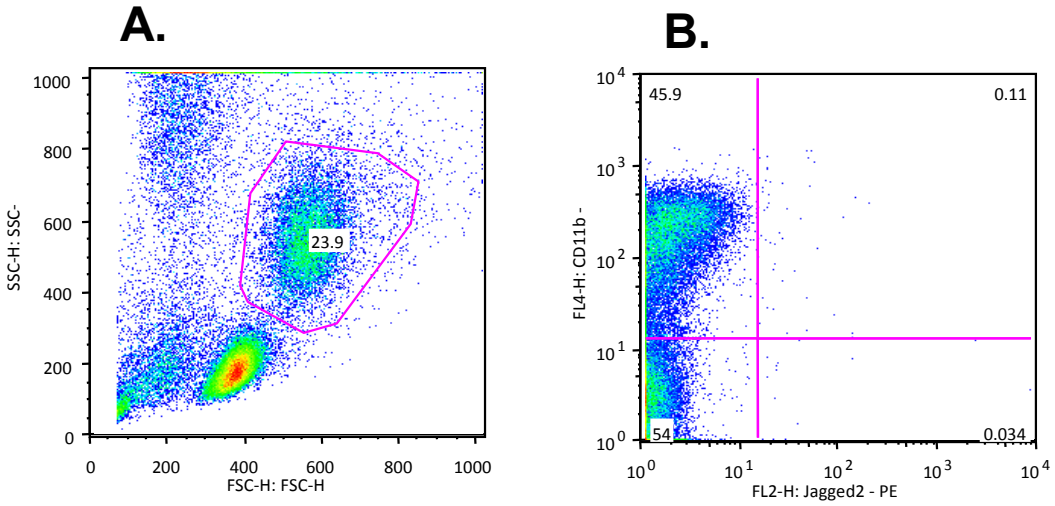


Figure 5| PBMCs in culture expressed CD11b/Jagged2 by flow cytometry. Percentage of CD11b+Jagged2+ cells in the mononuclear cell fraction of PB samples in culture of RPMI medium, by flow cytometry. A) Total population of PBMCs staining with anti-CD11b and anti-Jagged2, presented to the linear scale FSC and SSC. B) Population of PBMCs staining with anti-CD11b and anti-Jagged2, presented to the logarithmic scale Jagged2 and CD11b, where approximately 45% of PBMCs expressing CD11b.

3.1.2 PFA FIXATION

PBMCs were fixed with paraformaldehyde (PFA) after isolation for 24h at 4°C in RPMI medium with 4% PFA and then stained for CD11b and Jag2, as described above.

The results show that cells fixed with 4% PFA fail to express the antigen CD11b (expressed on monocytes) (Figure 6) and also Jag2 is no longer detectable.

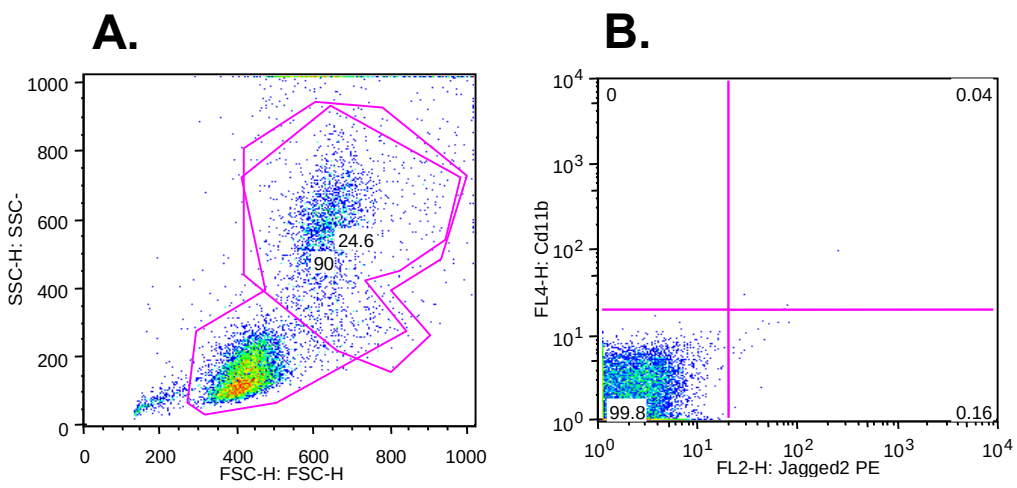


Figure 6| PBMCs fixed with 4% PFA expressed CD11b/Jagged2 by flow cytometry. Percentage of CD11b+Jagged2+ cells in the mononuclear cell fraction of PB samples when incubated with 4% PFA, by flow cytometry. A) Total population of PBMCs staining with anti-CD11b and anti-Jagged2, presented to the linear scale FSC and SSC. B) Population of PBMCs staining with anti-CD11b and anti-Jagged2, presented to the logarithmic scale Jagged2 and CD11b.

3.1.3 PBS

An alternative way of maintaining the PBMCs integrity was to incubate cells with PBS 1x following isolation at 4ºC during 24h. The results show that cells maintained in PBS express CD11b consistently, although Jag2 expression is almost undetectable (Figure 7).

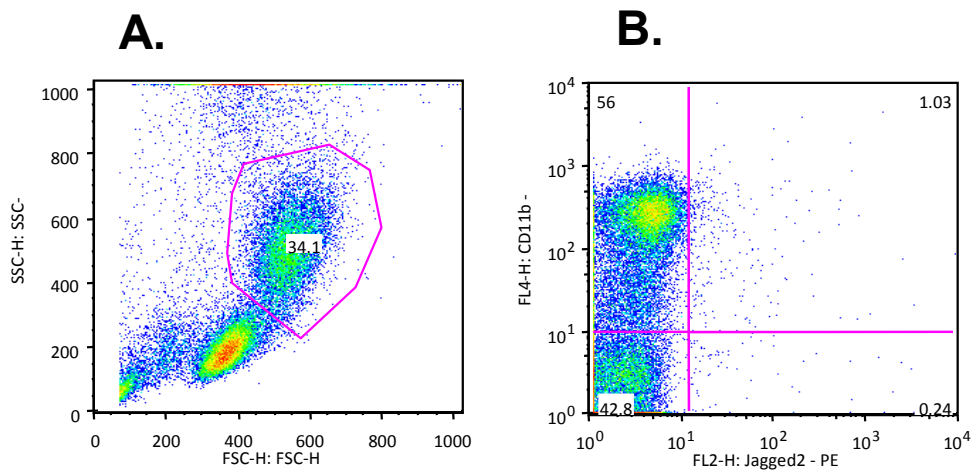


Figure 7| PBMCs in PBS expressed CD11b/Jagged2 by flow cytometry. Percentage of CD11b+Jagged2+ cells in the mononuclear cell fraction of PB samples incubated with PBS 1x, by flow cytometry. A) Total population of PBMCs staining with anti-CD11b and anti-Jagged2, linear scale FSC and SSC. B) Population of PBMCs staining with anti-CD11b and anti-Jagged2, presented to the logarithmic scale Jagged2 and CD11b, where approximately 50% of PBMCs expressing CD11b.

3.2 CD11B AND JAGGED-2 EXPRESSED IN PERIPHERAL BLOOD

3.2.1 FLOW CYTOMETRY RESULTS

To study the expression of Jagged-2 in peripheral blood cells by flow cytometry the cells were co-stained with anti-CD11b since we had previously shown this was the main source of Jagged-2 in circulating cells.

A total of 5 peripheral blood samples were used for this analysis (n = 5 buffy coat samples, kindly donated by the “Instituto Português do Sangue”). The PBMCs were isolated by Ficoll - Lymphoprep method (described in Materials and Methods) and then the cells were stained with two antibodies (CD11b and Jag2). It was possible to verify the presence of monocytes (± 30%) in peripheral blood of every sample, although the number of Jagged2 or CD11b/Jag2 positive cells was almost non-existent (Figure 8).

Assays were performed in triplicate and data is presented as mean and standard deviation.

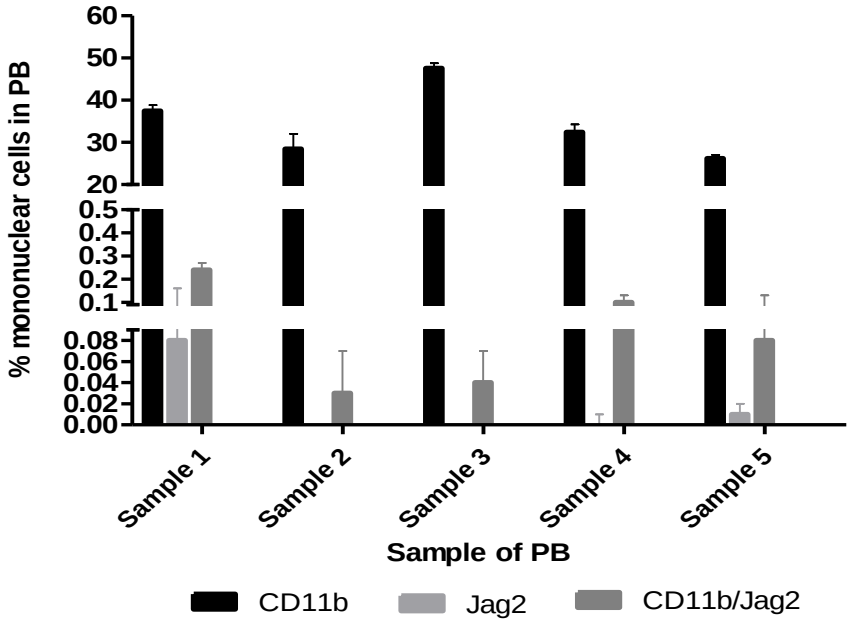


Figure 8| Mononuclear cells stained (CD11b and Jag2) present in PB. Flow cytometry based quantification of CD11b+Jag2+; CD11b+ and Jag2+ percentage in PB. Data are means ± s.d.

The results show that between 30 to 50% of monocytes is found in normal peripheral blood samples. The percentage of cells expressed Jagged-2 is extremely low or almost non-existent following this methodology.

3.2.2 qRT-PCR RESULTS

To further study the expression of Jagged-2 in peripheral blood at the mRNA level, qRT-PCR was used. For this purpose, mRNA was extracted from the same samples used in Flow cytometry (n=3).

The data shows that Jagged-2 is detectable at the mRNA level with varying results (Figure 9).

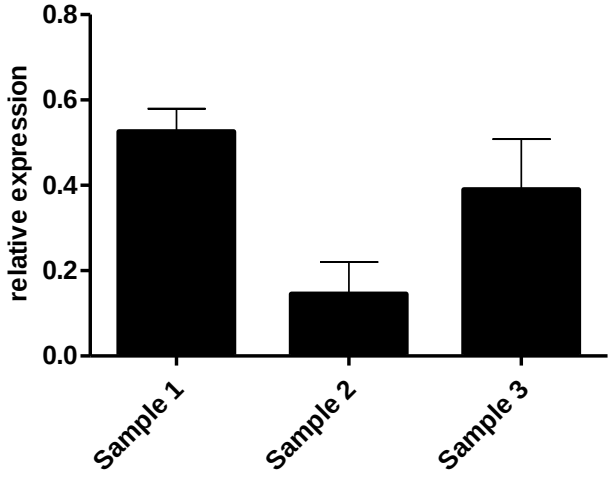


Figure 9| Jagged2 expressed in peripheral blood mononuclear cells. Quantification of the samples that expressed Jagged2. Data are means ± s.d.

3.3 CD11B AND JAGGED-2 EXPRESSED IN UMBILICAL CORD BLOOD SAMPLES

In the latest years, the function of Notch in embryonic hematopoiesis has been extensively studied. However the activity of all Notch ligands it is still unclear. [47, 48, 49].

Given the accessibility and therapeutic potential of cord blood samples, next we studied the expression of the CD11b and Jagged 2 in human umbilical cord blood. These experiments aimed at demonstrating the levels of Jagged2 expressing cells in normal blood samples.

3.3.1 FLOW CYTOMETRY RESULTS

A total of 4 samples of Human umbilical cord blood samples (n=4) were collected for isolation of mononuclear cells. The mononuclear cells of the umbilical cord bloods were isolated by Ficoll - Lymphoprep method (described in chapter 2) and the mononuclear cells were stained with two antibodies (CD11b and Jag2).

Assays were performed in triplicate and data are presented as mean and standard deviation (Figure 10).

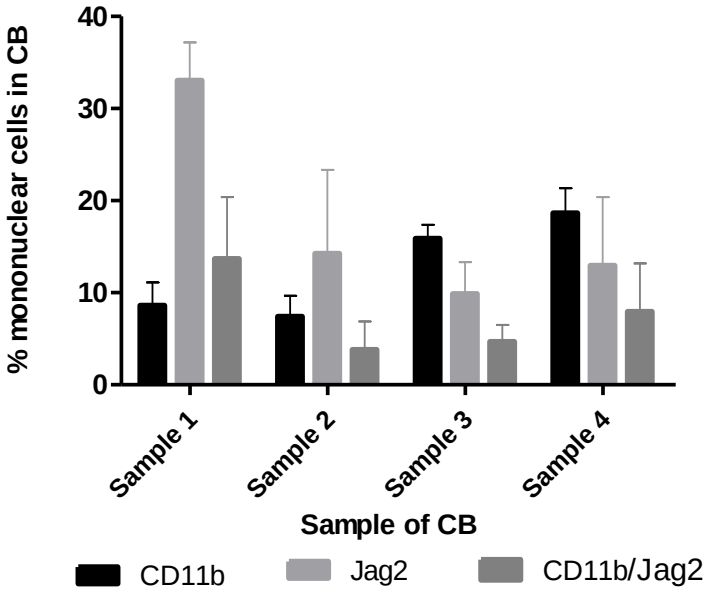


Figure 10| Mononuclear cells stained (CD11b and Jag2) present in CB. Flow cytometry based quantification of CD11b+Jag2+; CD11b+ and Jag2+ percentage in CB. Data are means ± s.d.

In contrast to the peripheral blood samples, in the umbilical cord blood there is an important proportion of cells stained both for CD11b and for Jagged-2 (CD11b/Jagged2). Despite some inter-sample heterogeneity, our results clearly demonstrate cord blood samples have an important proportion of CD11b monocytes/myeloid cells that expresses Jagged-2.

3.3.2 QRT-PCR RESULTS

The mononuclear cells obtained from the cord blood samples were used to extract mRNA (described in chapter 2) to determine the expression of Jagged-2 at the mRNA level.

Similarly to what was obtained from the peripheral blood samples, our data shows the cord blood mononuclear cells express Jagged-2 at varying levels (Figure 11).

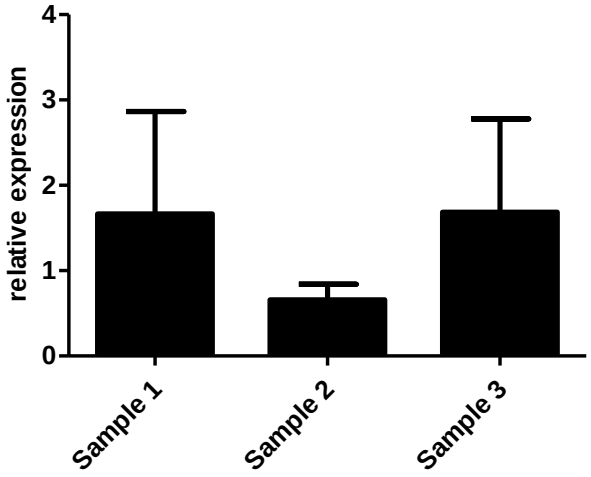


Figure 11| Jagged2 expressed in peripheral blood mononuclear cells. Quantification of the samples that expressed Jagged2. Data are means ± s.d.

3.4 JAGGED-2 EXPRESSION ON LEUKEMIA CELL LINES

The expression of Jagged-2 were analysed in a malignant cellular context. For this purpose, were cultured and studied leukemia cell lines. The purpose of these experiments was to determine the expression of Jagged-2 on malignant circulating mononuclear cells and also to establish an in vitro model to test putative signals that might regulate Jagged-2 expression. We started by isolating mRNA from 3 different leukemia cell lines and to assess the expression of Jagged-2 by qRT-PCR (Figure 12).

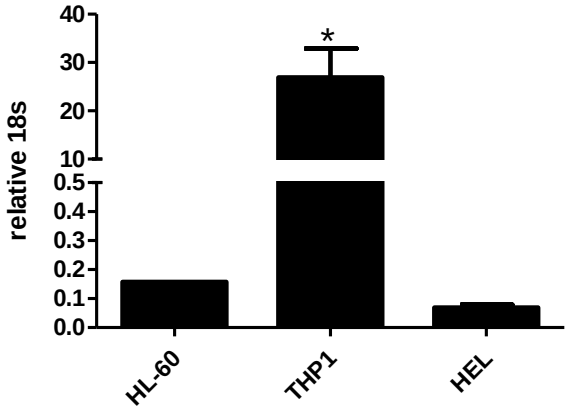
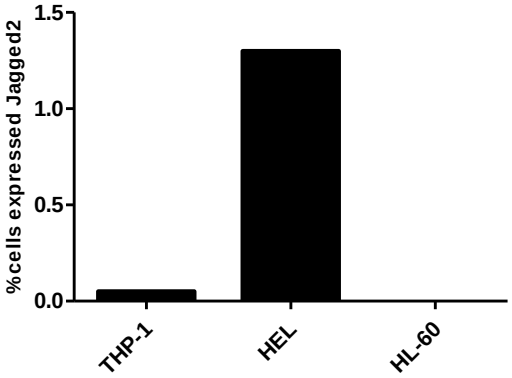


Figure 12| Jagged2 expressed in different cells. Quantification of the cells that expressed Jagged2 by RT-qPCR.

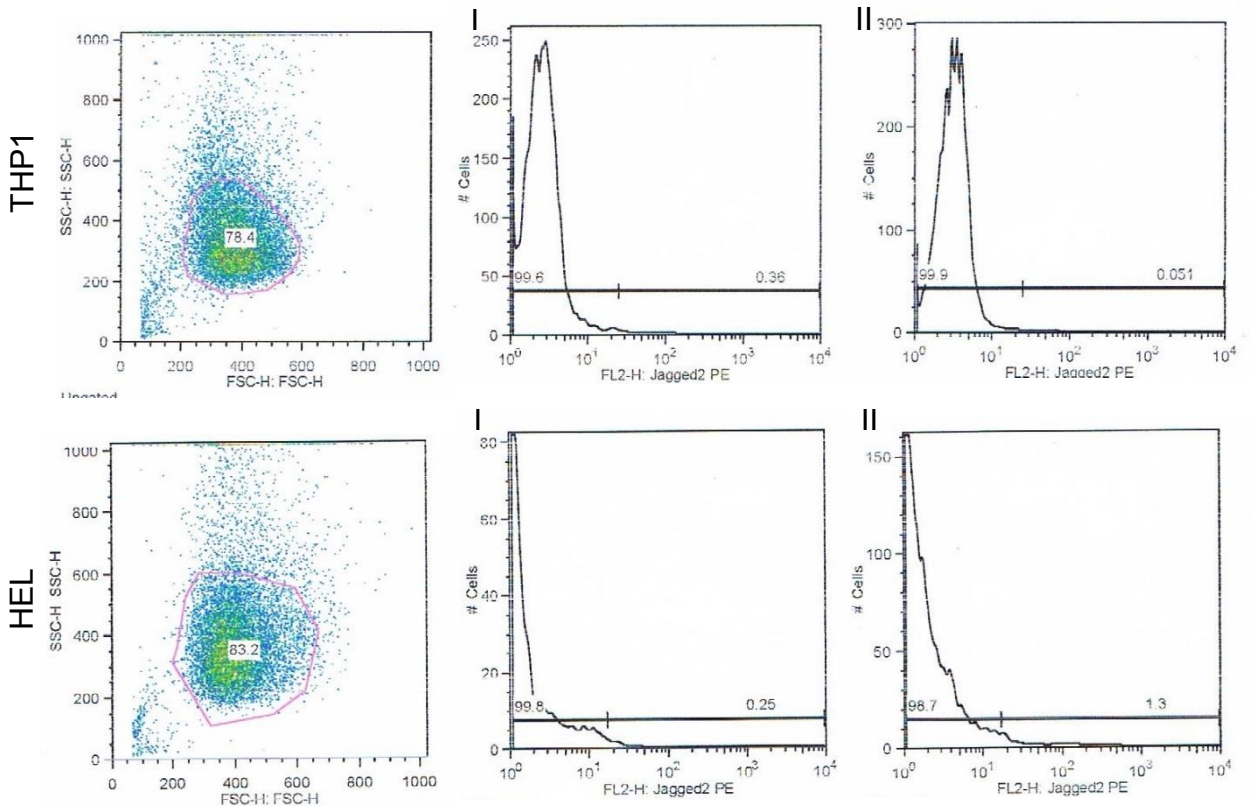
The data suggests that there are significant differences in the expression of Jagged-2 between the 3 cell lines studied. THP-1 cells have a higher expression of

Jagged-2 (p=0,001). The same experiment was realized by flow cytometry, but the results obtained were either all negative or incoherent with the qRT-PCR (Figure 13 – A and - B).

A)



B)



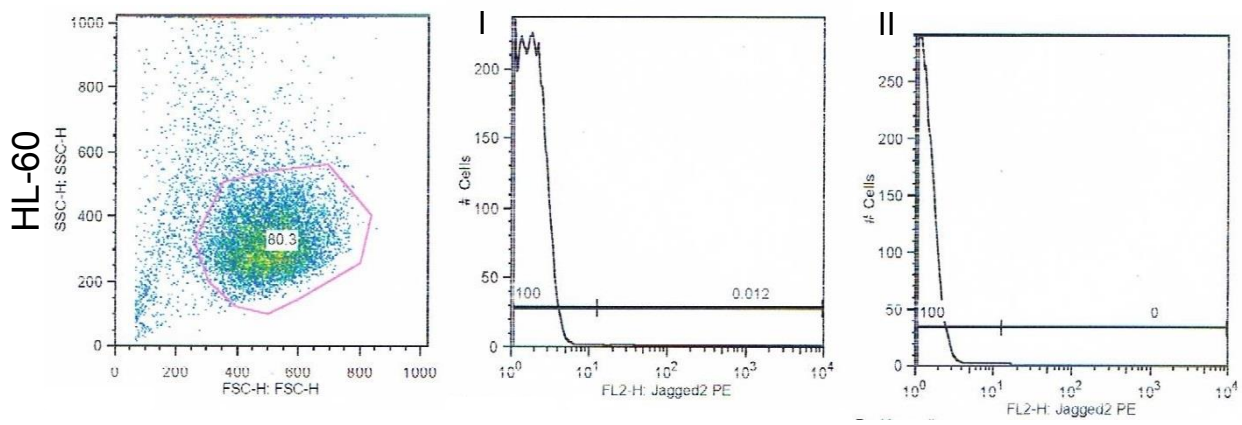


Figure 13| Jagged2 expressed in different cells. A) Quantification of the cells that expressed Jagged2 by flow cytometry. B) Graph that quantifies the expression of Jagged2 obtained from FlowJo. I – unstained cells of the different cell lines. II – Stained cells of the different cell lines.

3.4.1 JURKAT CELLS LINE

Given the heterogeneity and lack of reproducibility in Jagged-2 expression by the two leukaemia cell lines studied, we decided to use Jurkat cells, previously shown in the laboratory to clearly and consistently express Jagged-2. These cells were here cultured under different culture conditions and the effects of such conditions in cell survival and in Jagged-2 expression was assessed.

The cells were maintained in standard culture conditions until a volume of $1x10^6$ cells/ml was achieved. When this cell number was exceeded, cells were frozen at -80ºC or used for other tests (Figure 14).

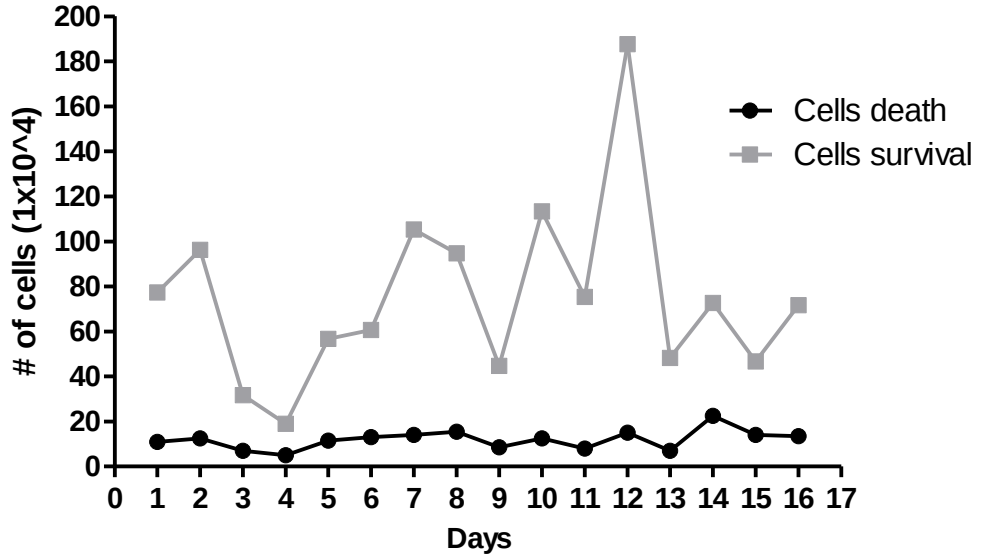


Figure 14| Jurkat cells survival under standard culture conditions.

The data shows standard culture conditions are adequate to maintain Jurkat cells in the same culture flask for up to 16 consecutive days. This allows the control of cell expansion from a single vial, thus reducing possible variations in gene expression in follow up experiments.

3.4.2 Influence of the FBS in cell viability

Next, Jurkat cells were subjected to different culture conditions and studied at different time points. In a volume of 3 *ml* with concentration 1 x 10^6 viable cells/ml, the cells were incubated in the absence or presence of 10% FBS, during 24 and 48 hours (Figure 15 and 16A and B). Assays were performed in triplicate and data is presented as mean and standard deviation.

Our data shows the number of viable cells is reduced in the absence of FBS at the different time points (Figure 15).

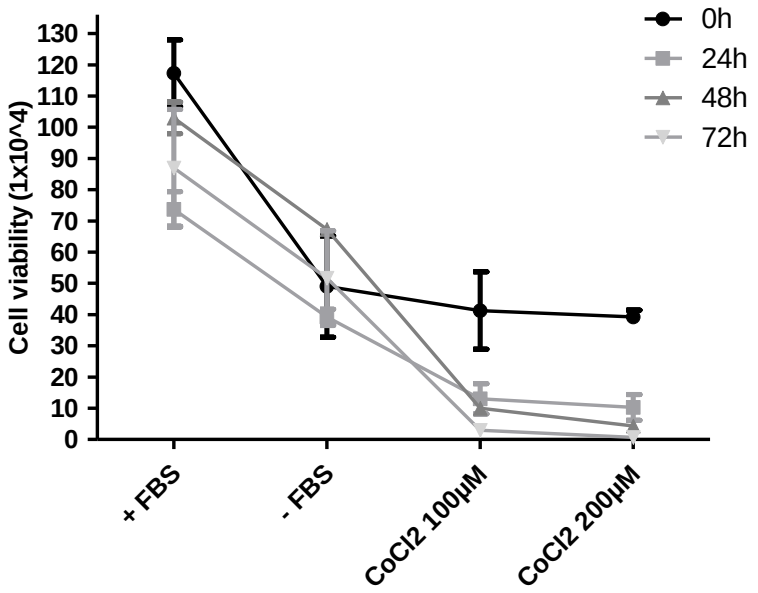


Figure 15| Effect of FBS and CoCl2 in Jurkat cells viability. Jurkat cells were incubated with different concentrations of CoCl2 and with presence or absence of FBS for 24h, 48h and 72h. Data is reported compared with control (0h) and represent the mean ± SD of experiment performed in triplicate.

3.4.3 Influence of the Hypoxia on cell viability

All the hypoxia-dependent events in cells appear to share a common denominator: hypoxia-induced factor (HIF), which is a heterodimer transcription factor [47, 48]. When cells in vitro are treated with a well-characterized hypoxia mimicker cobalt chloride (CoCl2), intracellular changes are similar to those upon hypoxia [49, 50]. Oxygen plays a key role in stabilizing HIF-1a and its function. When the oxygen tension is normal, HIF-1a is rapidly oxidized by hydroxylase enzymes, but when cells become hypoxic, HIF-1a escapes the degradation and starts to accumulate, triggering the activation of a large number of genes [50, 51].

The Jurkat cells were submitted a different conditions, in different time points. In a volume of 3 *ml* with concentration 1 x 10^6 viable cells/ml, incubated the cells with 100 µM and 200 µM of $CoCl_2$, during 24, 48 and 72 hours (Figure 15 and 16A and B). Assays were performed in triplicate and data are presented as mean and standard deviation. We found that $CoCl_2$ exposure decreased Jurkat cells viability. Exposure to both concentration (100 µM or 200 µM $CoCl_2$ from 24h), markedly decreased cell viability (Figure 15).

3.4.4 Effect of FBS and Hypoxia on Jagged-2 expression by Jurkat cells

In the presence of FBS the expression of Jagged-2 does not change over time, but as shown in Figure 16A, the absence of FBS showed a significant increase in the expression of Jagged-2 (p=0,37), at 48h. Moreover, Figure 16A also shows a significant increase in the expression of Jagged-2 (p=0,007) when the cells were cultured in the presence of 100 µM of $CoCl_2$ for 24h and 48h. However, if cells were cultured in the presence of 200 µM $CoCl_2$ for 48hrs, expression of Jagged2 was reduced.

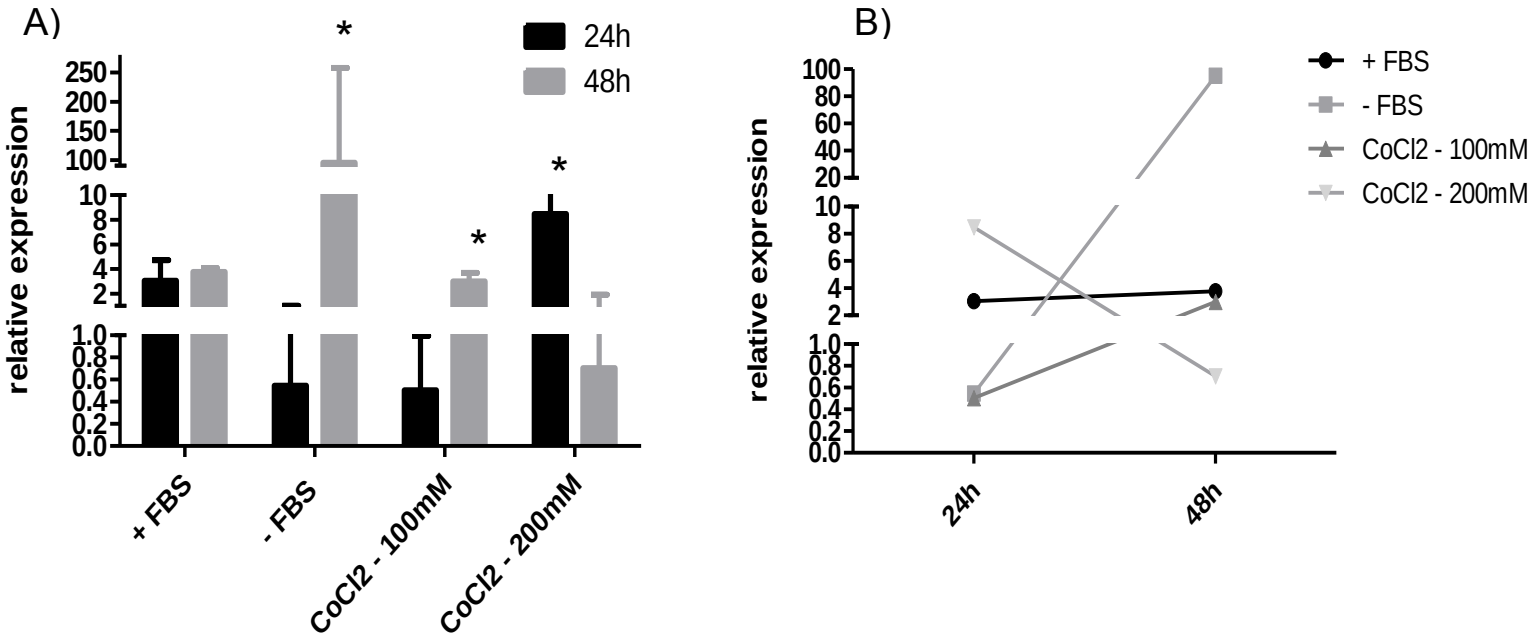


Figure 16| Jagged 2 expression by Jurkat cells under different culture conditions. A) Quantification of Jurkat cells cultured in the presence or absence of FBS, 100 µM or 200 µM of $CoCl_2$ for 24h and 48h. B) Changes in the expression of Jagged2 after 24h and 48h under the different culture conditions by quantitative real-time PCR.

4 DISCUSSION

Studying the factors that influence and regulate the expression of Jagged-2 on circulating cells is of significant importance, since this molecule is believed to act during normal development and also during tumor growth. Therefore, this study may contribute in the future for the development of more specific and targeted therapies, as well as to the use of a non-invasive cellular biomarker to monitor cancer progression, regression or response to therapies.

Our first approach was to define the best methods to fix isolated mononuclear cells for subsequent analysis by flow cytometry. Unexpectedly, PFA fixation (routinely used for fixing cells prior to flow cytometry) proved to be less efficient than PBS alone; the expression of CD11b and of Jagged-2 was almost completely lost in the former. This result, although apparently of less importance is critical in a clinical setting: it is important to define the best conditions to recover cells and to fix them so that the methods are reproducible and consistent.

Recent studies in our laboratory [53, 68, 69, 70] showed that quantification of circulating (peripheral blood) and tumor-derived CD11b+Jag2+ cells in colo-rectal cancer patients was significantly correlated with the presence of metastatic disease [53].

The formation of metastasis is a process that involves various steps which ultimately culminate in cancer cells escaping from the primary tumor. One of the first steps of this process is EMT, which involves rate-limiting stages/events that are influenced by non-malignant cells of the tumour microenvironment. Many of these cells are derived from the bone marrow, particularly the myeloid lineage, and are recruited by cancer cells. These cells, such as macrophages, monocytes, neutrophils and mast cells contribute to tumour angiogenesis through the production of growth factors, cytokines and proteases. Some studies have demonstrated that some of these cells are implicated also in the later stages of tumour progression, namely invasion and metastasis [52, 53, 54].

The Notch pathway has also been associated with EMT and metastasis formation. In detail, the notch pathway has been shown to be up regulated in breast cancer brain metastasis [55, 56]. Interestingly Jagged-2 expression in tumor cells has been recently described as a major regulator of EMT and metastases in lung adenocarcinoma via a miR-200 dependent downstream mechanism [60, 61, 62].

In the present studies we analysed the expression of Jagged-2 on normal circulating mononuclear cells. This was done by studying peripheral blood and cord blood samples. The results varied significantly between normal peripheral blood samples and cord bloods. Interestingly, while the overall CD11b (monocyte) content was similar between the 2 blood types, Jagged-2 expression was very low in peripheral blood

samples but elevated in cord bloods. These results were observed despite some inconsistency between Flow cytometry and qRT-PCR data, which may be partially due to technical issues and also due to a small sample size. However, though the number of samples was small, the results suggests the presence of CD11b+Jagged2+ cells in cord bloods may be a reflection of the overall process of EMT taking place during embryonic development. Moreover, this result also assures the quantification of circulating CD11b+Jagged2+ circulating mononuclear cells in cancer patients is not random since these are almost undetected in normal peripheral blood samples. Taken together, these data provided novel and important control data that support the use of CD11b+Jagged2+ cells as a potential biomarker for EMT. Were analyzed a sample of peripheral blood from a patient with colorectal cancer without metastases (results not shown) where it was observed the presence of CD11b+Jagged2+, giving a greater relevance to the potential Jagged-2 as a biomarker for EMT.

To study the factors which may affect the expression of Jagged-2 and are present in the tumor microenvironment, several leukemic cell lines were used. After RNA extraction we did an qRT-qPCR analysis where we used THP1, HL-60 and HEL cells to test the expression of Jagged-2 (Figure 12). Our results indicate that the THP1 cells have a significantly higher expression than the other cell lines tested, as seen in Figure 12. Although this experiment was repeated using the same cells in flow cytometry, our results have been inconclusive (Figure 13A and B) since the level of protein expression is low and not coherent with the qRT-PCR data. As above, this may be due to technical issues and to a small sample size.

We tested whether hypoxia influenced the expression of Jagged-2. To test this hypothesis, we used lymphoid leukemic cells (Jurkat cells, previously shown to be positive for Jagged-2), since several studies have suggested that Notch signalling is augmented under hypoxic conditions in human cervical, colon, ovarian and breast cancer cell lines [57, 58, 60, 61]. These reports suggested that activation of the hypoxia-mediated Notch pathway in tumors promotes cell survival and invasiveness [57, 60, 61].

$CoCl_2$ used to create hypoxic conditions in the cells, at two different concentrations (100 μl and 200 μl) and at different time points (24h and 48h). In the first 24 hours and with a concentration of 100 μl $CoCl_2$, the Jagged-2 expression in Jurkat cells is very small, but increases at 48h. However at a concentration of 200 μl $CoCl_2$ in the cells in the first 24 hours the Jagged2 expression was significantly higher both in relation to the 48h following with the same concentration, as in compared to the concentration of 100 μl $CoCl_2$ (Figure 16A).

As it was observed in other studies, these findings suggest that in hypoxic conditions the expression of Jagged-2 is increased. Other studies have shown that hypoxia induced Notch activation is essential to promote EMT and cell survival at the hypoxic invasive front and that the hypoxia-induced Jagged-2 expression in bone marrow stromal cells promotes self-renewal of cancer stem-like cells by activating Notch signalling. There are a lot of signals that can regulate EMT through cell-cell contacts mediated by families of transmembrane receptors and ligands expressed on adjacent cells [64], such as the Notch pathway. It has also been demonstrated that Jagged1-induced Notch activation promotes EMT of breast epithelial cells [63]. Nevertheless, the expression of Jagged-2 on circulating mononuclear cells has been less studied, and our data suggests the exposure of such cells to an hypoxic environment such as the tumor microenvironment modulates Jagged2 expression.

In the last decade it was discovered that the Notch pathway is constitutively activated in cancer cells originating from various tissues, due to gain-of-function mutations in Notch genes and overexpression of Notch ligands [65].

We also tested the effect of serum starvation on Jagged-2 expression by Jurkat cells. As expected, cell viability in the absence of FBS decreases over time, although an apparent maintenance of viability is observed at 48h (Figure 15). These results may be questionable, because in order to determine cell viability trypan blue dye was used, which indicates the number of viable cells and nonviable cells. The nonviable cells do not have an intact and functional membrane, but this method does not differentiate between apoptotic and necrotic cells [59]. Jagged-2 expression decreases after 24h, if the cells are in absence of FBS but after 48h, expression Jagged-2 is increased (Figure 16A and B). Since expression of Jagged-2 increases significantly with the absence of FBS for 48 hours, we can say that one factor that influences the expression of Jagged2 and probably induces activation of Notch is the absence of nutrients (FBS) in the microenvironment.

5 CONCLUSION

Taken together, the data presented in this Thesis provided clues to the expression of Jagged-2 on normal circulating mononuclear cells and also suggested signals from the tumor microenvironment may regulate the expression of Jagged-2. These data thus provide novel information and validation of Jagged-2 expression by circulating normal mononuclear cells, supporting the use of this cellular biomarker in a cancer setting.

Moreover, the suggestion that Jagged-2 is upregulated by hypoxia and by nutrient starvation suggests the infiltration of circulating mononuclear cells into tumors and their accumulation in necrotic (hypoxic) sites may be a trigger for EMT. This has implications in our understanding of the onset of EMT (and of the metastatic process).

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7 ANNEXES

Annex 1. **cDNA synthesis for mRNA quantification**

Reagents (1st reaction)	**Volume (µl) per reaction**
Random Hexamers 10 mM	1,0
dNTPs 10 mM	1,0
DEPC H_2O	8,0
Final volume	10,0

Reagents (2nd reaction)	**Volume (µl) per reaction**
5x First Strand Buffer	4,0
DTT 10 mM	2,0
RNAseOUT® Ribonuclease Inhibitor (40 u/µl)	1,0
Final volume	7,0

Reagents (3rd reaction)	**Volume (µl) per reaction**
SuperScript® II Reverse Transcriptase (200 u/µl)	1,0
Final volume	1,0

Annex 2. **Primers for validation of targets genes**

Primer	Sequence
18s FWD	GCCCTATCAACTTTCGATGGTAGT
18s REV	CCGGAATCGAACCCTGATT
Jagged-2 FWD	GTCGTCATCCCCTTCCAGTTC
Jagged-2 REV	CTCATTCGGGGTGGTATCGTT

Annex 3. **qRT-PCR for housekeeping genes**

Reagents	Volume (µl) per reaction
Bidistilled water	2,7
SYBR Green	3,5
FWD Primer	0,15
REV Primer	0,15
Final Volume	6,5

Annex 4. **Description of Antibodies**

Antibody	Clone	Concentration	Fluorochrome	Emission Max (nm)
Anti-CD11b BioLegend	ICRF44	2,5:100	APC	660
Anti-CD19 R&D Systems	4G7-2E3	1:100	FITC	525
Anti-CD34 Miltenyi Biotec	AC136	1:100	FITC	525
Anti-CD45 eBioscience	HI100	5:100	PerCP	675
Anti-Jagged2 BioLegend	MHJ2-523	2,5:100	PE	575