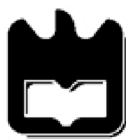

Dissertação de Mestrado em
Biotecnologia Molecular

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O papel do DDR1 na migração de megacariócitos

The role of DDR1 in megakaryocyte migration

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação científica da Doutora Alessandra Balduini, Professora da Universitá degli studi di Pavia, e Doutor António Correia, Professor catedrático do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Matriz extracelular; Colagénio tipo I ; Megacariócito; Receptores de colagénio; Discoidin Domain Receptor; SHP1 fosfatase; Syk tirosina cinase; Migração celular

resumo

A regulação do desenvolvimento dos megacariócitos (MKs) é fundamentalmente mediada pelas matrizes extracelulares (ECMs). No entanto, apenas 2 dos diferentes receptores ECM têm sido alvo de foco dos estudos em plaquetas e MKs, a integrina alpha2beta1 e a glicoproteína GPVI. Neste trabalho, demonstramos a expressão do receptor de colagénio Discoidin Domain Receptor 1 (DDR1) em MKs humanos, quer a níveis proteicos quer a níveis de mRNA. Apresentamos também evidências do envolvimento do DDR1 na regulação da mobilidade dos MKs na presença do colagénio tipo I através dum mecanismo baseado na actividade da fosfatase SHP1 e tirosina cinase Syk. Mais detalhadamente demonstramos que a inibição da ligação do DDR1 ao colagénio tipo I, preservando a interacção dos outros receptores de colagénio (GPVI, alpha2beta1 e LAIR-1), leva à diminuição da migração devido á redução na actividade da fosfatase SHP1 e consequentemente o aumento da fosforilação dos níveis da Syk. Por sua vez, a inibição da actividade da Syk restaurou a migração dos MKs em colagénio tipo I após inibição do DDR1. Concluindo, relatamos a expressão e função de um receptor de colagénio diferente em MKs humano. Enfatizo que o aumento do nível de complexidade é necessário para a melhor compreensão das interacções entre MKs e colagénio no ambiente da medula óssea.

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keywords

Extracellular matrix; Type I collagen; Megakaryocyte; Collagen receptors; Discoidin Domain Receptor; SHP1 phosphatase; Syk tyrosine kinase; Cell migration

abstract

The regulation of megakaryocytes (MKs) is fundamentally mediated by extracellular matrices (ECMs). Although between different collagen receptors only integrin alpha2beta1 and GPVI have been the focus of studies in platelets and MKs. In these work, we show the expression of the different collagen receptor Discoidin Domain Receptor 1 (DDR1) in human MKs at both protein and mRNA levels. We present also evidences of the DDR1 participation in the mediation of MK mobility on type I collagen through a mechanism based on the SHP1 phosphatase activity and Syk tyrosine kinase. In detail, we demonstrate that inhibition of DDR1 binding to type I collagen, conserving the engagement of the other collagen receptors (integrin alpha2beta1, GPVI and LAIR-1), leads to a decrease of MK migration due to the reduction in SHP1 phosphatase activity and subsequent increase of phosphorylated Syk levels. Consistently, the inhibition of Syk activity restored the MK migration on type I collagen upon DDR1 inhibition. In conclusion, we report the expression and function of a novel collagen receptor on human MKs. Besides we emphasize that increasing level of complexity is necessary to better understand MK-collagen interactions in the bone marrow environment.

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List of abbreviations

ADP – Adenosine diphosphate

AFM – Atomic Force Microscopy

AGM – Aorta Gonad Mesonephros

AML/RUNX1 – Acute myeloid leukemia/runt-related transcription factor 1

Ang-1/Tie2 – Angiopoietin-1/Tie2

ATP – Adenosine triphosphate

BCR – B cell antigen receptor

BFU-MK – MK burst-forming unit

BM – Bone marrow

BSA – Bovine serum albumin

CaR – Ca^{2+}-sensing receptor

CD – Cluster of differentiation

CFU-GEMM – Colony-forming unit- granulocyte, erythrocyte, megakaryocyte and monocyte

CLP – Common lymphoid progenitor

CMP – Common myeloid progenitor

c-Mpl – Thrombopoietin receptor

Csk – C-terminal Src kinase

DC – Dendritic cell

DDR – Discoidin domain receptor

DMEM – Dulbecco's modified eagle medium

DMS – Demarcation membrane system

ECM – Extracellular matrix

EPO – Erythropoietin

EPOR – Erythropoietin receptor

ERK – Extracellular signal regulated kinase

ET – Essential thrombocythaemia

ETS – E-twenty six

FAK – Focal adhesion kinase

FBS – Fetal bovine serum

FcRγ – Fc receptor subunit γ

FGF – Fibroblast growth factor

FN – Fibronectin

FOG – Friend of GATA

FXIII – Factor XIII

GCV – Ganciclovir

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GMP – Granulocyte/Macrophage progenitor

GP – Glycoprotein

HRP – Horseradish peroxidase

HSC – Hematopoietic stem cells

Ig – Immunoglobulin

IG – Immunoglobulin-like extracellular domain

IL – Interleukin

ITAM – Immunoreceptor tyrosine-based activation motif

JAK2 – Janus kinase 2

JM – Justamembrane

LT-HSC – Long term hematopoietic stem cells

MAPK – Mitogen-activated protein kinase

MEK – Extracellular signal-related kinase

MEP – Megakaryocyte/Erythrocyte progenitor

MIDAS – Metal ion-dependent adhesion site

MK – Megakaryocyte

MPP – Multi-potent progenitor

MSC – Mesenchymal stem cell

NF-E2 – Nuclear factor erythroid 2

NK – Natural killer

NLS – Nuclear localization sequence

NMHC – Non-muscle myosin heavy chain

OD – Optical density

PBS – Phosphate-buffered saline

PF4 – Platelet factor 4

PI3K – Phosphoinositide-3 kinase

PLC – phospholipase C

PP – Protein phosphatase

P-NPP – *para*-Nitrophenylphosphate

PTB – Phosphotirosine binding

PTH – Parathyroid hormone

PTHrP – PTH-related protein

PTK – Protein tyrosine kinases

PTP – Protein tyrosine phosphatase

pTyr – Phosphotyrosine

RANKL – Receptor activator of NF-κB ligand

RKT – Receptor tyrosine kinase

SCF – Stem cell factor

SDF-1 – Stromal-derived factor-1

SDS – Sodium dodecyl sulphate

SH2 – Src homology-2

SHP – Src homology region 2 domain-containing phosphatase

SNO – Spindle-shaped N-cadherin^{+}CD45^{-} osteoblastic

STAT – Signal transducer and activator of transcription

ST-HSC – Short-term hematopoietic stem cells

Syk – Spleen tyrosine kinase

TCR – T cell antigen receptor

TF – Transcription factor

TGF-β_1 – Transforming growth factor β_1

TMAFM – Tapping-mode atomic force microscopy

TPO – Thrombopoietin

VCAM – Vascular cell adhesion molecule

VLA – Very late antigen

vWF – Von Willebrand factor

β-TG – β-thromboglobulin

1. Introduction

1.1 From hematopoietic stem cells to megakaryocytes

MKs are large polyploid cells that reside primarily in the bone marrow but are also found in the lung and peripheral blood, representing only 0.05% to 0.1% of bone marrow cells [1]. The process of their formation is called megakaryocytopoiesis. In the presence of TPO, megakaryocytes mature by polyploidization and cytoplasmic maturation, undergo drastic morphological changes, and form proplatelets, which give rise to circulating platelets, also known as thrombocytes. MKs are derived from HSCs, which evolve from the multi-potential hemangioblast. These cells give rise to all blood and blood vessel precursor cells [2].

The first site where is found HSCs is the intra-embryonic yolk sac at around day 21 and 28 of gestation. The *de novo* hematopoeisis in placenta and AGM occurs at nearly similar wave of gestation (around day 28 and 40) before it circulates into fetal liver where there is the large HSC pool during gestation. At around day 40, the HSCs migrate and reside within the bone marrow which finally becomes the source of HSC in adult life [3, 4]. HSC can be discriminate into two different classes: ST-HSCs that have reconstitution ability, which is restricted to more than a few weeks, and LT-HSCs that are capable of contributing to hematopoiesis for months or even life time [5].

The origin of all blood cells in hematopoietic system is believed to be derived from HSCs that contain self-renewal capacity and give rise to MPPs which lose self-renewal potential but remain fully differentiate. The HSC maintenance depends at the ability of HSCs self-renew and differentiation. For example if self-renewal was augmented without measurements the population would expand excessively, leading to tumor genesis. On the other hand, if cell differentiation overwhelmed self-renewal, the HSCs population would vanish. So it is crucial that HSCs regulate the balance between the cellular self-renewal and differentiation for tissue homeostasis [5, 6].

MPPs further give rise to oligopotent progenitors which are CLPs and CMPs. All these oligopotent progenitors differentiate into their restricted lineage commitment: CMPs advance to MEPs, GMPs, and DC progenitors; CLPs give rise to T cell progenitors, B cell progenitors, NK cell progenitors and DC progenitors. Notably, DC progenitors (CD8α+

DC, CD8α− DC, and plasmacytoid DC) could be derived from both CMPs and CLPs [6, 7].

The early CMP arises from the HSC that can be cloned as the multi-lineage CFU-GEMM. The differentiation of CMP is modulated by molecular signals controlled by regulatory TFs. Two major TFs involved in CMP differentiation are GATA-1, which leads to differentiation into MEP, and PU.1, which regulates GMP [8]. The downregulation of PU.1 expression in the CMP is the first event associated with the restriction of differentiation to erythroid and MK lineages [9, 10]. The bi-potential MEP can develop into the highly proliferative, early BFU-MK, or the more mature smaller CFU-MK, depending on environmental factors, cytokines and chemokines [11].

Like other primitive hematopoietic cells, bipotent MEPs resemble small lymphocytes but can be distinguished by a specific pattern of cell surface protein display, IL-7Rα^-/Lin$^-$/c- Kit$^+$/Sca-1$^-$/CD34$^-$/FcRγ^{lo} [12].

The CFU-MK is a cell that develops into a simple colony containing from 3 to 50 mature megakaryocytes. In the other hand, BFU-MK derive to larger, more complex colonies that include satellite collections of megakaryocytes and contain up to several hundred cells [12].

Through maturation these cells discontinue CD34 antigen expression and begin to express specific MK’s membrane protein such as CD41 and CD61 (integrin αIIbβ3), CD42 (glycoprotein Ib) and glycoprotein V.

Alternatively, MEPs can progress to early and late erythroid progenitors, the BFU-E and CFU-E [13]. These that are committed to the erythroid lineage begin to express the transferrin receptor (CD71), and as they mature they lose CD41 expression but express the thrombospondin receptor (CD36), glycophorin, and ultimately globin [12]. The proliferating diploid MK progenitors (megakaryoblasts) lose their capacity to divide, but retain their ability for DNA replication (endoreduplication/endomitosis) and cytoplasmic maturation.

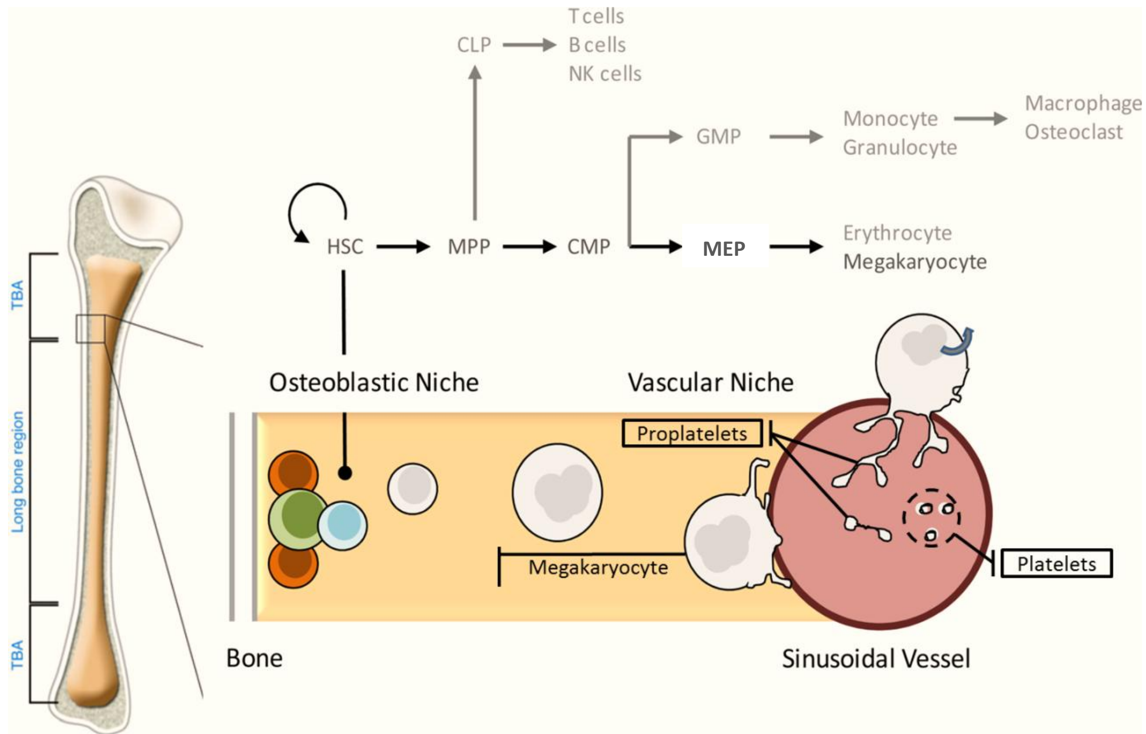


Figure 1 – Summary of megakaryocyte maturation and platelet production.

HSCs that contain self-renewal capacity give rise to MPPs, which migrates from the endosteal bone surface to blood vessels at the center of the BM cavity. Therefore these cells proliferate and differentiate into two distinguish lineages – CLP and CMP. Furthermore the MKs, which differentiate from CMP, maturate – nuclear amplification and protein production – to develop proplatelet elongations into the sinusoidal blood vessels of the BM. Finally the MKs release the platelets and proplatelets that will continue to mature and give rise to platelets. TBA – trabecular bone area. Adapted from *Platelet formation* [14].

1.2 Regulation of megakaryocytopoiesis

The bone marrow is a complex environment where chemokines, cytokines as well adhesive interactions play an important role in the processes of megakaryocytopoiesis and platelet production since is the niche for MKs maturation. These factors and the involving mechanisms regulate megakaryocytopoiesis in many ways such proliferation, differentiation and platelet release. As the MKs supply at least 10^{11} platelets every day, they have to respond to changes in requirements for circulating platelets, increasing more than 10 fold under conditions that demand platelet production [15].

The discovery of TPO has contributed greatly to platelet biology, because it permits relatively large and pure cultures of megakaryocytes to be generated in vitro. TPO remains

under development as a potential clinical thrombopoietic and/or hematopoietic agent and as a drug to stimulate ex vivo expansion of HSCs [16].

C-Mpl ligand, or as known TPO, is the primary physiological growth factor for the MK lineage, which also plays a vital role in the survival and proliferation of HSCs [17, 18]. TPO is the most potent cytokine for stimulating the proliferation and maturation of MK progenitor cells. It stimulates MKs to increase in cell size and ploidy, and to help the platelet formation [15]. In adults, TPO is produce constitutively, and its circulating levels are regulated by the extent of binding to c-Mpl receptors on circulating platelets and bone marrow MKs resulting in the elimination of TPO-c-Mpl complexes, therefore blood and marrow levels of TPO are usually inversely related to bone marrow MK mass and platelet counts [19]. As a consequence, megakaryocytopoiesis is regulated by plasma levels of free TPO, where overall platelet production plays an incisive role. In the steady state, TPO is synthesized predominantly and constitutively in the liver [19]. TPO is encoded by a single human gene, located on chromosome 3q26·3–3q27, which produces a 353 amino acid precursor protein. The mature molecule, composed of 332 amino acids, is acidic and heavily glycosylated [20]. This powerful cytokine affects mature platelets, reducing the level of ADP, collagen, or thrombin necessary for aggregation [21, 22], and stimulates platelet adhesion [23]. In these cells the α-granule secretion and aggregation are also enhanced by TPO that is induced by thrombin in a PI3K [24]. TPO can as well act in synergy with other hematopoietic cytokines and has been utilized effectively to expand human HSC and MK progenitor cells in vitro [17, 25-27].

TPO initiates the signal transduction by binding to the c-Mpl at the distal part, which leads to the activation of homodimer of c-Mpl [16]. Consequently, JAK2 can phosphorylate tyrosine residues within the receptor itself which at least two tyrosine residues, Tyr625 and Tyr630, are phosphorylated on c-Mpl [16, 28], thereby stimulating the downstream cascade STATs, PI3K and the MAPKs, ERKs-1 and -2. Therefore, TPO/Mpl/JAK2/Lnk pathway can be concluded as a gatekeeper for HSC quiescence [29, 30].

TPO shares high homology with EPO in its N-terminal half, reflecting a close evolutionary relationship between their receptor signaling pathways. Exceptions to steady state platelet production occur during ET [31, 32] and in acute inflammation when

circulating TPO levels are increased [28, 33, 34] due to enhanced hepatic expression driven by the acute phase protein, IL-6 [35].

As told before, though TPO plays a main role in regulation of megakaryocytopoiesis it usually conjugates with other factors which can affect this activity. Some of these growth factors, that can stimulate MK growth by them self or in conjunction with TPO, are stem cell factor, FLT ligand, FGF, EPO, IL-3, IL-6, IL-11 and GM-CSF [36, 37].

EPO is the major growth factor for erythroid cells, which interacts with the EPOR, a cell surface receptor expressed in erythroid, megakaryocytic, and mast cells, triggering signaling cascades leading to the proliferation, differentiation, and survival of erythroid progenitors [38].

However there are negative regulators of megakaryocytopoiesis, such factors as transforming growth factor-β1, IL-4 and PF4 are known to inhibit MK development [39, 40].

On the other hand, chemokines, cytokines and cellular interactions have a vital role in megakaryocytopoiesis and thrombocytopoiesis influencing the survival and proliferation of MKs. For instance SDF-1 enhances both megakaryocytopoiesis and homing of HSCs to the bone marrow during fetal development [41-43]. This activity of SDF-1 enhances the movement of megakaryocyte progenitors from the proliferative “osteoblastic niche” to the “vascular niche” thus driving to thrombopoiesis. Thrombopoiesis initiates during endothelial migration of $CXCR4^{+}$ MK in response to SDF-1 [44]. CXCR4 signaling in MK includes the serine/threonine MAPKs, MAPK and AKT MAPK pathways, which comprise MEK and ERK1/2, to control cell differentiation and proliferation [45].

For last but not less important, the regulation of megakaryocytes and thrombocytopoiesis by TFs, which can specifically activate the genes of MK lineage precursors and/or repress gene expression for other cell types by arranging complexes that coordinately control chromatin association. Two of the most influent TFs in Mk-specific genes regulation are the GATA and FOG, which also interacts with AML/RUNX1 and ETS proteins [46]. The GATA-1 is a zinc-finger protein that is upregulated during MK development due to is important role in gene regulation, at the same time the PU.1 is downregulated. In this way, GATA-1 acts early in megakaryocyte development, where it is involved in lineage commitment of megakaryocyte from its progenitor cells [10]. GATA-1

also functions later in megakaryocyte development, controlling proliferation. Yet the 9-zinc finger protein FOG-1 is also a crucial cofactor, which interacts with GATA-1, for embryonic hematopoeisis and MK development [46].

Besides the refereed TFs, the NF-E2 has been recognized as major controller of platelet biosynthesis. It is a heterodimer of p45 subunit, MK-erythroid specific, and p18 subunit, non-lineage specific, that assumes the basic leucine zipper motif [47]. As a FT, NF-E2 plays a crucial role on controlling an array of MK genes which are vital for platelet production processes [48]. NF-E2 has also been shown to interact with the promoter for Rab27b, a small GTPase identified in platelets. Rab27b expression is high in terminally differentiated megakaryocytes, and its inhibition in megakaryocytes results in attenuated proplatelet production, which suggests a role for Rab27b in proplatelet formation [48].

1.3 Maturation of MKs

The MK maturation is mainly characterized by two phenomenon: endoduplication (polyploidization) and expansion of cytoplasmic mass. To give rise to circulating platelets, MKs enlarge significantly reaching, approximately, 100μm diameter and produce a high concentration of ribosomes to help the production of platelets-specific proteins. This enlargement is matched by multiple rounds of endomitosis, in other words, the cells enter mitosis and begin to condense chromosomes, but do not segregate chromosomes to daughters. Instead, they enter a G1-like state and re-enter S phase. This process begins with the binding of TPO to the c-Mpl receptor. TPO, the primary regulator of thrombocytopoiesis, is currently the only known cytokine required for megakaryocytes to maintain a constant platelet mass [49]. TPO is thought to act in conjunction with other factors, including IL-3, IL-6, and IL-11, although these cytokines are not essential for megakaryocyte maturation [50].

During endomitosis, chromosomes replicate and the nuclear envelope breaks down. Although interconnected mitotic spindles assemble, the normal mitotic cycle is arrested during anaphase B. The spindles fail to separate, and both telophase and cytokinesis are bypassed. Nuclear envelope reformation results in a polyploid, multi-lobed nucleus with DNA contents ranging from 4N up to 128N within each MK.

1.3.1 Endomitosis

Mature megakaryocytes are invariably polyploid and contain from two (4N) to 54 (128N) times the normal diploid amount of DNA [51, 52], being the ploidy mean in human MK of 16N [52]. Unlike the small percentage of hepatocytes and macrophages that have two to four folds the normal diploid content of DNA and whose DNA is contained in multiple separate nuclei, all of the DNA in megakaryocytes is contained within one highly lobulated nuclear envelope where each nuclear lobule represents one diploid amount (2N) of DNA. In general there is a relationship between increased ploidy and increased megakaryocyte size, but given the time needed for the cytoplasm of megakaryocytes to mature, not all small megakaryocytes are of low ploidy. In fetal life megakaryocytes are less polyploid; cultured mature megakaryocytes from fetal liver at 8 to 10 weeks of gestation are only 2N and 4N, while 8N megakaryocytes are detected at 20 weeks of gestation [53].

At some unclear point in the megakaryocytic differentiation pathway, mitosis ceases and the unusual process of endomitosis (also called polyploidization or endoreduplication) commences [54]. Endomitosis is a process in which DNA replication occurs but neither the nucleus nor the cell undergoes division (cytokinesis). Morphologically, endomitosis is associated with the dissolution of the nuclear membrane and the formation of a multipolar mitotic spindle [54]. While initially it was assumed that endomitosis was simply the absence of mitosis after each round of DNA replication, studies in mice [55] showed that megakaryocytes indeed enter mitosis and progress through normal prophase, prometaphase, metaphase, and up to anaphase A, but not to anaphase B, telophase, or cytokinesis. After anaphase, the nuclear membrane is reassembled about the sister chromatids as a single nucleus skipping telophase and cytokinesis, and the cells enter the next round of DNA replication.

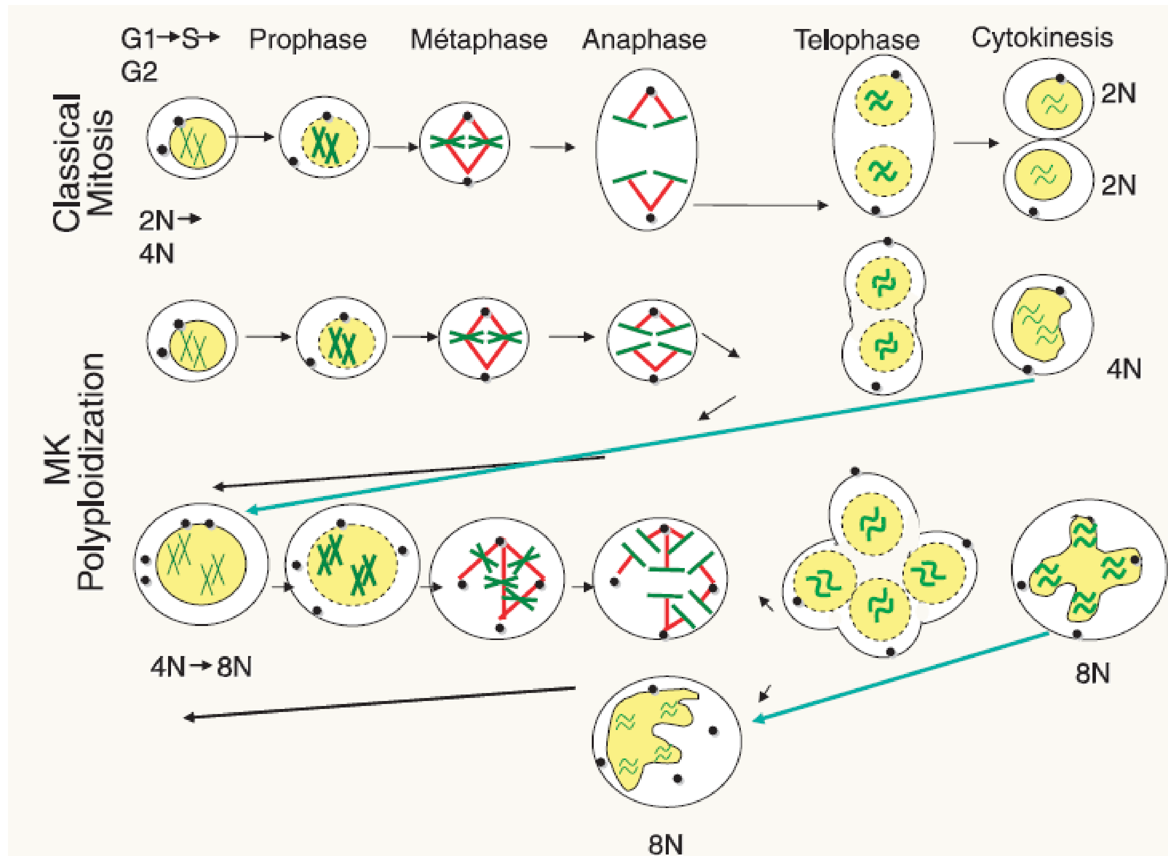


Figure 2 – Representation of endomitosis.

Adapted from *From hematopoietic stem cells to platelets* [56]

Cessation of mitosis in the diploid megakaryocyte progenitors is apparently directly coupled to the start of endoreduplication, and it is generally assumed that most cells greater than 4N in ploidy are committed to endomitosis and rarely divide mitotically. The probability of entering the endomitotic pathway is hierarchically dependent upon the state of differentiation of the progenitor [57]. Thus, the more primitive the progenitor is, the more likely it is to remain a mitotic cell.

1.3.2 Demarcation membrane system

Furthermore to expansion of DNA, there are other significant maturations processes as internal membrane systems, granules, and organelles are assembled in bulk during the MKs development. MK ultrastructure reveals an abundant pool of cytoplasmic membranes that constitute the DMS. Due to its origin in tubular invaginations of the plasma membrane, the DMS maintains continuity with the extracellular space, and whole-cell patch-clamp recordings reveal the DMS to be a single electrophysiologic entity. DMS functions, however, remain controversial, and its name recalls early theories on platelet

biogenesis. Prior to proplatelet-based models of thrombopoiesis, nascent platelets were believed to assemble within the MK cytoplasm, partitioned or demarcated by these membranes. Subsequently, the DMS was proposed to function instead as the membrane reservoir required to extend proplatelets. Although DMS membranes have continuity with the plasma membrane [58], so are now thought to function primarily as a membrane reservoir for the formation of proplatelets, the precursors of platelets. Ultrastructural studies of the DMS reveal that it invaginates from the plasma membrane and presents profiles of fenestrated tubules and cisternae.

In any case, the side of the invaginated DMS that faces the extracellular space must emerge as the proplatelet surface, much like the turning of a glove inside out. Contents on the cytoplasmic side include the contents of future platelets and factors responsible for proplatelet morphogenesis such as oriented microtubule bundles. In one of the last visible events in the MK-cell body, dispersed microtubules reorganize in thick bundles at the cell cortex and enable pseudopod formation. At or about the same time, portions of the DMS dilate considerably and migrate into the cell periphery, presumably to prepare for microtubule-powered eversion as proplatelets [58].

1.3.3 Cytoplasmic rearrangements

Before the assembly of proplatelets begins there is also the formation of a dense tubular network [59] and the open canalicular system, a channeled system for granule release. Simultaneously specific proteins associated with platelets, such as GPs including the integrin $\alpha_{IIb}\beta_3$ (CD41a or GPIIb/IIIa complex), CD41b (GPIIb), CD61 (GPIIIa), CD42a (GPIX), CD42b (GPIb) and CD51 (aV), vWF and fibrinogen receptors, are synthesized and sent to the megakaryocyte surface, while others are packaged into secretory granules with such factors as vWF, coagulation factor VIII, factor V and PF4, which are loaded into α-granules [60]. Still other proteins, such as fibrinogen, β-TG, are collected from plasma through endocytosis and/or pinocytosis by megakaryocytes and are selectively placed in platelet-specific granules [60, 61]. As well, mitochondria and dense granules, which, like α-granules, derive from Golgi complexes, are accumulated during megakaryocyte maturation. Thus, as terminally differentiated megakaryocytes complete maturation, they are fully equipped with the elements and machinery required for the major task of platelet biogenesis.

Final step of MKs maturation is the release of platelets that occurs when the MKs cytoplasm is transformed into proplatelets.

1.3.4 Megakaryocyte granules

Also, during the maturation of the MKs, are produce four distinct granules with different internal elements: alpha (α), dense, lysosomal and microperoxisomal. These granules will migrate to platelet offering proteins and other constituents required for maintenance.

α granules are the most numerous granules. The α granule body itself is made early in megakaryocyte development before the demarcation membrane system and first appears in the Golgi apparatus of megakaryoblasts [61]. These granules have several platelet proteins, including vWF, PF4, and thrombospondin, which are synthetize in the MKs [62].

Other proteins such as fibrinogen undergo GPIIb/IIIa receptor-mediated endocytosis from the plasma into the α granules of both megakaryocytes and platelets [63]. As well others, such as albumin and IgG are pinocytosed from the plasma into the α granules of megakaryocytes and platelets [64]. The location of megakaryocytes in close proximity to vascular sinuses may facilitate uptake of these circulating proteins [65].

Dense granules accumulate and store serotonin, calcium and adenine nucleotides (synthetize by MKs) but do not acquire their content of serotonin and calcium until platelets are released into the circulation and then uptake calcium and most of the body's circulating serotonin [66, 67].

Lysosomal granules are a unique class of granules that has arylsulfatase, acid phosphatase and cathepsin D [68, 69].

Microperoxisome granules are small granules (90 nm) that arise prior to α granules formation and contain catalase. These granules look like the microperoxisomes of other cells.

1.4 Proplatelet and platelet formation

Proplatelets are created by the outflow and evagination of extensive internal membrane system of the mature MKs to pull together platelets. The assembly of platelets from megakaryocytes comprises an elaborate process that transforms the cytoplasm into 100 to 500 μm long branched proplatelets where the individual platelets rise. The proplatelet and platelet development process generally begins from a unique site on the megakaryocyte where 1 or more wide pseudopodia form. These processes continue to

elongate, taking 4 to 10 hours, and become conical into proplatelets with an average diameter of 2 to 4 μm. The microtubules arrays are crucial for proplatelet formation [70]. Microtubules, hollow polymers composed from αβ-tubulin dimers, are the main structural component of the engine that leads the elongation of proplatelets, as the shape maintenance [70-72]. Just before proplatelet formation, microtubules consolidate in a mass just under the cortical plasma membrane. These microtubules align into bundles and fill the cortex of the first blunt process extended by megakaryocytes, signaling the beginning of proplatelet development. The microtubules join into thick linear bundles that fill the proplatelet shafts when the proplatelets lengthen and taper. At the proplatelet end, the microtubule bundles form loops, which reenter the proplatelet shaft. This process gives rise to the bulbous tips of the proplatelets, each measuring 3 to 5 μm in diameter [70].

The extension of proplatelets is due to a bipolar array of microtubule bundles distributed along the proplatelet shaft that lengthen via continuous polymerization of tubulin at their free plus ends, and dynein powered sliding of overlapping microtubules at a rate of ~4-5 μm/min [73].

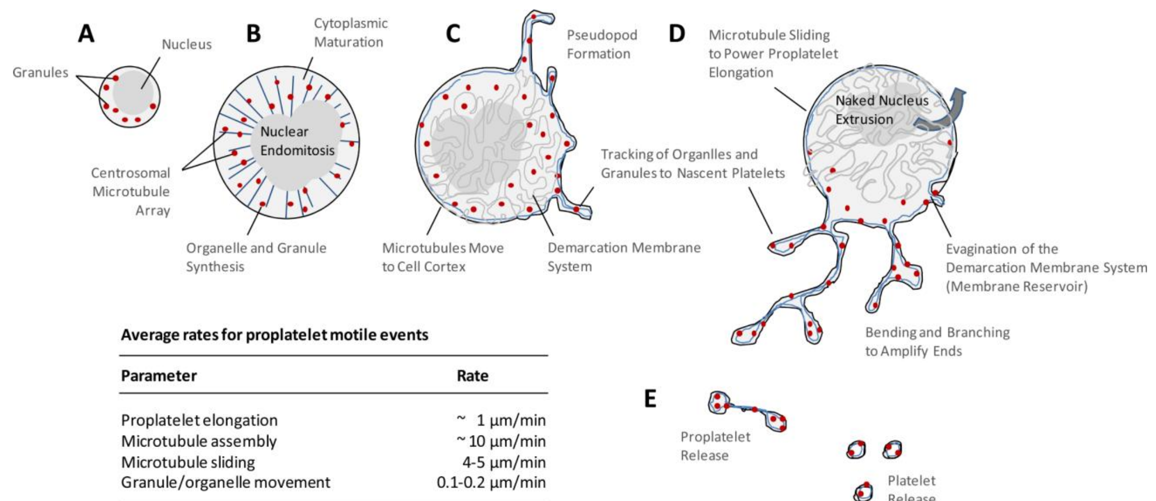
Proplatelets are arbitrarily decorated with several bulges or swellings, all similar in size to a platelet, which gives them the appearance of beads connected by thin cytoplasmic strings. The development of further proplatelets continues at or near the original site of proplatelet formation and spreads in a wavelike fashion throughout the rest of the cell until the megakaryocyte cytoplasm is completely transformed into an extensive and complex network of interconnected proplatelets [70].

Repeated actin-dependent bending and branching that bifurcates the proplatelet shaft is common, and serves to increase the number of free proplatelet ends from which platelets are thought to be released [74]. Branching occurs when a portion of the proplatelet shaft becomes bent, from which some of the microtubules within the loop separate from the bundle to form a new bulge in the shaft [70].

The multi lobed nucleus of the megakaryocyte cell body is compacted into a central mass with little cytoplasm and is ultimately extruded and degraded. Platelet-sized swellings also develop at the proplatelet ends and are the primary sites of platelet assembly and release, as opposed to the swellings along the length of the proplatelet shaft.

Microtubule bundles in the proplatelet shaft serve as tracks along which organelles and granules move at a rate of 0.1 μm/min [75]. This process is sequential and

bidirectional, culminating at the proplatelet tips where the cargo becomes trapped. The general reduced speed of traffic compared to other cell types, and bidirectional nature of granule/organelle movement suggests that the underlying goal of this transport process is to mix the various granules/ organelles within the proplatelet before it is driven to the proplatelet ends, from which platelets are presumably released.



Average rates for proplatelet motile events

Parameter	Rate
Proplatelet elongation	~ 1 µm/min
Microtubule assembly	~ 10 µm/min
Microtubule sliding	4-5 µm/min
Granule/organelle movement	0.1-0.2 µm/min

Figure 3 – Representation of MKs maturation from immature cells (A) to released platelets (E) (B) MKs expansion: nuclear endomitosis and organelles and granules synthesis. (C) Microtubules translocation to cortex cell, formation of DMS and pseudopod formation. (D) Proplatelet elongation and amplification of the ends. Adapted from *Platelet formation* [14].

It is known to be two major proteins responsible for the organelles and granules transportation to proplatelets ends. The cytoplasmic dynein plays a vital role on the mechanism of sliding proplatelet microtubules relative to one another in proplatelets. This mechanism permits that the organelles and granules, which are attached to the microtubules, travel alongside with the microtubules like a "piggyback" way. On the other hand kinesin is responsible for transporting these elements along the microtubules.

It is thought that the platelets release is mediated by a specialized apoptotic process for the reason that has been identified a number of apoptotic factors on MKs. This process consists on the retraction of MKs cytoplasm that had been complete converted in proplatelets releasing free proplatelets or platelets [70]. Proplatelets are released as chains of platelet-sized particles, resembling a narrow shaft with two teardrop-shape tips, which most commonly will release platelets.

1.5 Bone and bone marrow

The bone confines structure, protection and movement being fundamental for the body. Mammalian bone is made of bone cells at diverse stages of development (osteoblasts, preosteoblasts and osteocytes) and mineral deposits for example calcium and phosphate and collagen fibrils. BM retains, in bone cavity, the HSCs, which are surrounded by stromal cells, until they have matured and are released into vascular system. Additionally MSCs also exist in the bone cavity and it is though that these cells give rise to the mainstream of marrow stromal cell lineages, including osteoblasts, fibroblasts, chondrocytes, adipocytes, myocytes and endothelial cells [76].

Therefore there is a closed relationship between osteogenesis and hematopoiesis, revealing the crucial role of osteoblasts in the regulation of hematopoiesis.

A stem cell niche is a specific site in adult tissues where stem cells reside and undergo self-renewal and produce large numbers of progenitors. The niche is composed by supporting cells that provide a microenvironment for stem cells and also the signals emanating from the supporting cells [77, 78].

In mammals, a percentage of HSCs can be found in the BM close to the endosteal bone surface, where the mainly cells that reside are osteoblasts. This anatomic organization suggests reciprocal communication between the two cell types and a potential role for osteoblasts (responsible for bone growth) in regulating HSCs (responsible for blood formation) [79]. This notion is supported by the observation that osteoblasts are known to produce a variety of hematopoietic growth factors [80, 81], including the RANKL, which plays an essential role in induction of osteoclast differentiation.

However the HSCs and their progenitors, can be found also near to the sinusoidal endothelium suggesting that these cells also interacts with the endothelial cells, which provide a different microenvironment designated as vascular niche.

In both niches, osteoblastic and vascular niche, there are molecules that mediate signaling and adhesive interactions between stem cells and these niches. The diverse molecules involved in these interactions cell-niche add distinguish characteristics to each niche's function. At the present time the well-studied signaling molecules involved in niche regulation comprise Ang-1/Tie2, CaR, SCF/c-Kit and Jagged/Notch [82-84]. The Ang-1, which is expressed in osteoblasts, enhances the ability of HSCs to become quiescent and can induce adhesion to osteoblastic cells through Tie2, a tyrosine kinase

receptor that is expressed in HSCs and endothelial cells [83]. Likewise CaR enables retention of HSCs on the endosteal bone surface. On the other hand, SCF signaling through is receptor, c-Kit, promotes proliferation and survival of HSCs. This signaling plays also an important role in regulation of HSCs activation and release from the osteoblastic niche. Has also been demonstrated the expansion of the HSCs population mediated by the Jag1/Notch activity [85]. There are another interactions, already cited, that plays a crucial role in cell proliferation and differentiation, such as the TPO signaling conjugate with IL-3 and IL-6.

In the past years several studies have being made to understand in a better way the relationship between the osteoblastic niche and the HSC maintenance. A study, realized in transgenic mouse, demonstrates that the overexpression of PTH and PTHrP receptor converges to increase of osteoblasts number increasing also the number of HSCs [82]. Additionally was shown that an increase in the number of SNO cells results in the augment of the LT-HSCs number [86]. Furthermore, studies of transgenic mice that specifically express the herpesvirus thymidime kinase gene in developing osteoblasts under the control of the collagen α_1 promoter. The usage of these mice permits to ablate osteoblasts with GCV. The mice treated with GCV presented loss of osteoblast and consequently led to substantial decreases in the number of HSCs and reduced the BM cellularity [87].

It is supported that the osteoblastic niche has a more prominent role in maintaining HSC quiescence and that the vascular niche promotes proliferation and differentiation of stem and progenitor cells by providing a more nutrient-rich microenvironment, with higher concentrations of oxygen and growth factors and in which mature blood cells are ultimately released into the peripheral circulation [88, 89]. For example HSCs leave the osteoblastic niche and translocated to the vascular niche, where they differentiated into megakaryocyte progenitors for further megakaryocyte maturation as well as platelet release [88].

Has already described, a portion of HSCs can leave the osteoblastic niche, move to the vascular niche and enter in the vascular system, and possibly returning to BM [90]. It is proposed that this mobilization can be due to stress conditions on osteoblastic niche or to proliferate, as showed by Weissman et al [91]. However, the underlying physiological function of these events is not well understood.

The vascular niche depends on FGF-4 and chemokines for instance SDF-1 to recruit HPCs [88, 91]. The transendothelial migration of $CD41^+$ MKs is highly potentiated by the chemotactic factor SDF-1, while the FGF-4 increases the adhesion of the progenitors of MKs to the vascular niche.

These two molecules are involved in a multiple downstream signaling cascades, where P13K, PKC and p38 MAPK play a vital role [88]. FGF-4 and SDF-1 enhance the expression of the adhesion molecule VLA-4 on megakaryocytes as well as VCAM-1 on endothelial cells. It is also known that vascular endothelial-cadherin is essential for both proper assembly and mediation of adhesion of megakaryocyte progenitors to the vascular niche for survival and further maturation.

As the MKs migrates through the BM the microenvironment will change confining the best condition for each stage of MKs maturation. Recent studies point to the importance of the microenvironment of BM, especially the ECM proteins that play a critical role on HSCs proliferation and differentiation, as also in regulation of MKs. ECM proteins such as fibrinogen, fibronectin, VWF, laminin and type IV collagen, which are present at vascular niche promotes the proplatelet formation [92, 93]. While, type I collagen completely suppresses proplatelet formation and is the most abundant extracellular protein of the osteoblastic niche [94]. These peculiar differences between the 2 niches in the BM insure the properly maturation of MKs and proplatelet formation. The type I collagen binds to integrin $\alpha_2\beta_1$, suppressing the proplatelet formation in osteoblastic niche, is essential for the MKs well maturation and prevents the premature proplatelet formation and platelet release. More specifically the integrin $\alpha_2\beta_1$ involves the Rho/ROCK pathway that is involved in the phosphorylation of the myosin light chain, which suggests that the actin-myosin cytoskeleton, specially the ATPase activity of myosin-IIA, plays a role in the regulation of proplatelet formation [95]. Although the adhesion of MKs to the other collagens expressed in the bone marrow (type III, IV and VI) don’t show the same effect as type I, contrarily support proplatelet formation. These differential effects may be explained by the peculiar structural properties of the diverse collagens, along with distinct receptor engagement [96]. In a recent study the type I collagen was modified by N-acetylation of lysine side chains to change the structural properties. It was shown that decreasing the type I collagen stiffness in company with a nonorganyzed fibrillar structure completely reverted type I collagen-dependent functions of MKs, including proplatelet

formation inhibition. With these findings we can say that the MKs maturation and PPF isn't strictly regulated by biochemical factors but also physical factors are crucial for these processes.

The FN is a main component of BM ECM that can moderate the homing of hematopoietic progenitor cells, the organization and composition of BM ECMs and cell-matrix adhesion sites [97].

Recently has been observed that FXIII activity affect MKs spreading and FN matrix assembly on type I collagen, demonstrating the crucial role of FXIII in stabilizing MKs-type I collagen interaction within BM [97].

vWF is a large glycoprotein that is found in its inactive form in circulation, the subendothelial matrix lining the vessel wall, platelet α granules and the endothelial Weibel-Palade bodies [98]. Under conditions of high shear stress, conformational changes within vWF occur, allowing binding of the platelet GP Ib–V–IX receptor complex to the arrested vWF.

Underlying these complex regulatory mechanisms is the development of mechanical forces and the activation of biochemical signaling pathways, which may be modulated by biophysical signaling. Oxygen tension, for example, is a modulator of cell activity within the bone marrow, and an oxygen gradient exists between the osteoblastic and vascular niches, with more mature cells migrating towards the higher oxygen vascularized compartment of the bone marrow [99].

In sum the microenvironment properties as ECMs, physical parameters (i.e. oxygen tension) and soluble factors (cytokines and chemokines) are vital for the right MKs maturation and proplatelet formation.

1.6 Collagen

In mammals, the collagens are the most abundant proteins found. Not only, collagen-rich extracellular are fundamental for the biochemical properties of tissues, as well plays a crucial role in cell adhesion and migration during growth, differentiation, morphogenesis and wound healing.

A typical mammal has more than 30 structural variants of collagen, particular to certain tissues and each somewhat different in sequence and function [100]. Some human genetic defects in collagen structure illustrate the close relationship between amino acid sequence and three-dimensional structure in this protein. *Osteogenesis imperfecta* is

characterized by abnormal bone formation in babies; Ehlers-Danlos syndrome is characterized by loose joints [100]. Both conditions can be lethal, and both result from the substitution of an amino acid residue with a larger R group (such as Cys or Ser) for a single Gly residue in each chain (a different Gly residue in each disorder). These single-residue substitutions have a catastrophic effect on collagen function because they disrupt the Gly–X–Y repeat that gives collagen its unique helical structure. Given its role in the collagen triple helix (Fig. 4), Gly cannot be replaced by another amino acid residue without substantial deleterious effects on collagen structure. The collagen helix is a unique secondary structure quite distinct from the helix. It is left-handed and has three amino acid residues per turn (Fig. 4). Collagen is also a coiled coil, but one with distinct tertiary and quaternary structures: three separate polypeptides, called chains (not to be confused with helices), are supertwisted about each other (Fig. 4 – c). The superhelical twisting is right-handed in collagen, opposite in sense to the left-handed helix of the chains. There are many types of vertebrate collagen. Typically they contain about 35% Gly, 11% Ala, and 21% Pro and 4-Hyp (4-hydroxyproline, an uncommon amino acid).

The unusual amino acid content of collagen is related to structural constraints unique to the collagen helix. The amino acid sequence in collagen is generally a repeating tripeptide unit, Gly–X–Y, where X is often Pro, and Y is often 4-Hyp. The glycine is required at every third position to allow the close packing of α chains within the triple helix (Fig. 4 – d) [101, 102]. The Pro and 4-Hyp residues permit the sharp twisting of the collagen helix. The amino acid sequence and the supertwisted quaternary structure of collagen allow a very close packing of its three polypeptides. The tight wrapping of the chains in the collagen triple helix provides tensile strength greater than that of a steel wire of equal cross section. Collagen fibrils are supramolecular assemblies consisting of triple-helical collagen molecules (sometimes referred to as tropocollagen molecules) associated in a variety of ways to provide different degrees of tensile strength [101, 102]. The chains of collagen molecules and the collagen molecules of fibrils are cross-linked by unusual types of covalent bonds involving Lys, HyLys (5-hydroxylysine), or His residues that are present at a few of the X and Y positions in collagens. These links create uncommon amino acid residues such as dehydrohydroxylysinonorleucine. The increasingly rigid and brittle character of aging connective tissue results from accumulated covalent cross-links in collagen fibrils [103].

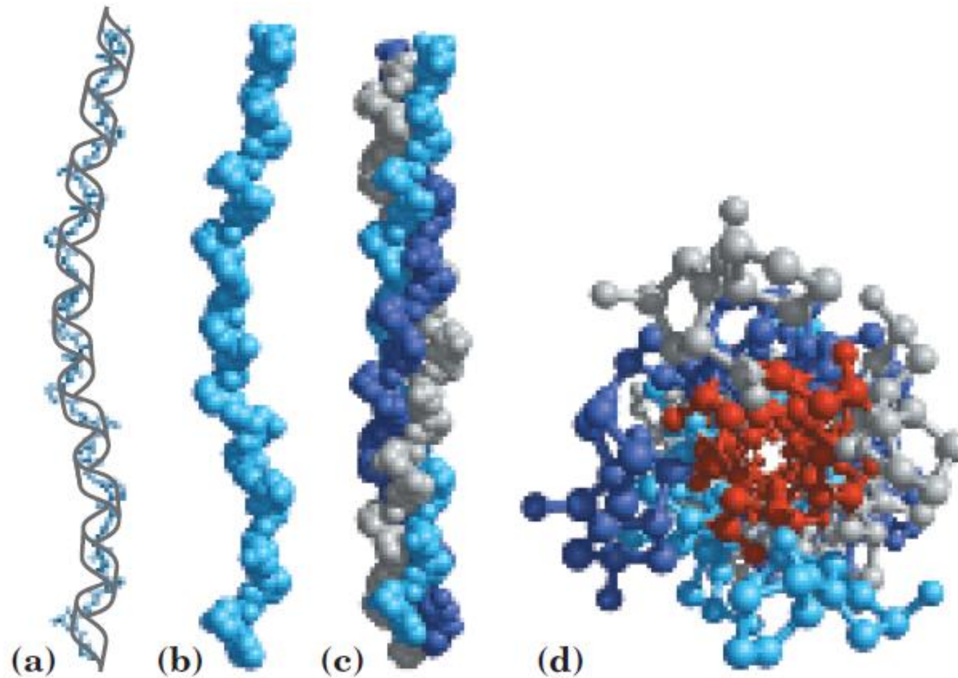


Figure 4 – Structure of collagen.

a – The chain of collagen has a repeating secondary structure unique to this protein. The repeating tripeptide sequence Gly–X–Pro or Gly–X–4-Hyp adopts a left-handed helical structure with three residues per turn. The repeating sequence used to generate this model is Gly–Pro–4-Hyp. b – Space-filling model of the same chain. c – Three of these helices (shown here in gray, blue, and purple) wrap around one another with a right-handed twist. d – The three-stranded collagen superhelix shown from one end, in a ball-and-stick representation. Gly residues are shown in red. Glycine, because of its small size, is required at the tight junction where the three chains are in contact. The balls in this illustration do not represent the van der Waals radii of the individual atoms. The center of the three-stranded superhelix is not hollow, as it appears here, but is very tightly packed [104].

1.6.1 Mammalian collagen receptors

The mammalian collagen receptors are transmembrane proteins that interact directly with the collagen triple helix. Following ligand binding, the cytoplasmic domains of collagen receptors connect to the cytoskeleton and trigger the assembly of signaling complexes. Thus, a large variety of complex signaling events can be transduced by collagen receptors in a bidirectional manner across the cell membrane. These events serve to modulate and coordinate many aspects of cell behavior, such as proliferation, survival, shape, polarity, motility, gene expression, and differentiation that are required for such

fundamental processes as development, tissue morphogenesis, and wound healing within multicellular organisms.

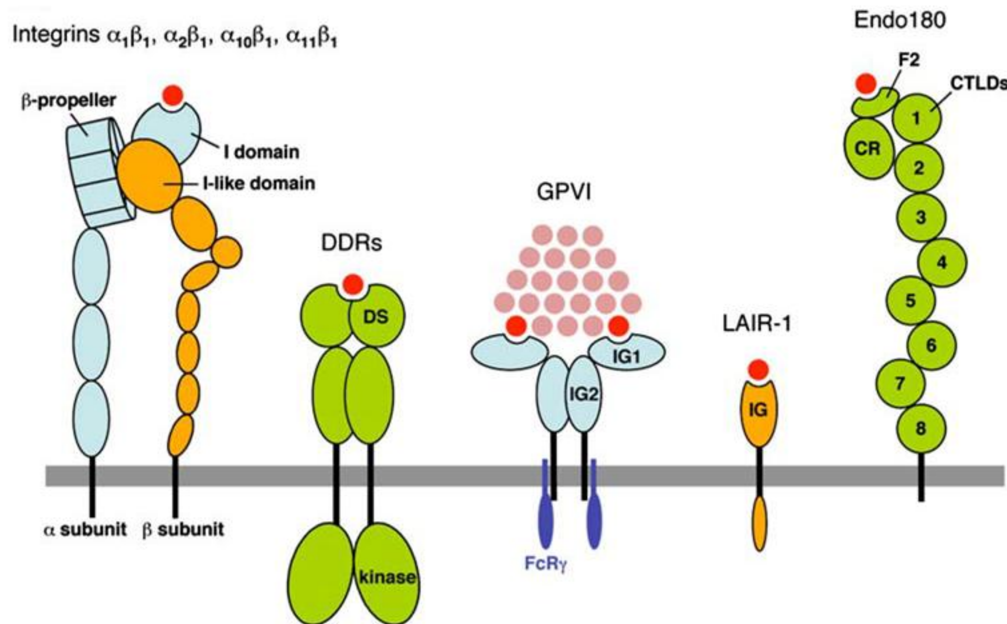


Figure 5– Mammalian collagen receptors.

Schematic domain structures, in which the collagen-binding sites are indicated by grooves containing red circles [105].

1.6.2 Integrins

Integrins are the major mammalian receptors for cell adhesion to extracellular matrix [106]. These receptors are noncovalently linked dimers consisting of an α- and a β-subunit (Fig. 5), at the moment there are 18 α- and 8 β-subunits that have been identified in humans combine to form 24 different receptors. These subunits are composed by a large modular extracellular domain, tracked by a single transmenbrane helix and a short cytoplasmic domain that mediates interactions with the cytoskeleton. Both subunits fold into a N-terminal extracellular globular head that creates the ligand binding surface sitting on two membrane-spanning legs. All α subunits contain at the N-terminal a seven-bladed β propeller structure. The α2 extracellular domain furthermore contains a unique inserted (I) domain, with a conserved cation binding site known as the MIDAS with a clear preference for $Mg2^+/Mn2^+$. Structural studies of the isolated I-domain with and without collagen peptide demonstrate the importance of the MIDAS motif and adjacent side chains for ligand binding and show a conformational change of the I-domain, from a closed to an open state, upon ligand binding [107].

Many integrins are not constitutively active, and adhesion of cells to matrix proteins needs to be strictly controlled and regulated in response to environmental changes. Integrins exist in at least three states, inactive, active, and ligand-bound; the switching of integrins from an inactive to an active state involves conformational changes not only in the ligand-binding pocket, but also across the whole of the extracellular domain and in the cytoplasmic face. Thus, the activation of the ligand-binding domain in the head and the binding of ligand are coupled via long-range conformational changes to signaling events in the cytoplasm. Conversely, intracellular events effect changes in the cytoplasmic domains that are translated in the opposite direction to the integrin head, allowing activation and ligand binding. This bidirectional communication is termed outside-in and inside-out signaling [107].

Among the extracellular matrix and basal membrane ligands for integrins are FN, fibrinogen, laminin, various collagens, vitronectin and vWF [108]. Two groups of integrin binding extra cellular ligands are identified: receptors for collagen and laminin (α_1, α_2, α_3, α_6, β_4) and receptors for FN and vitronectin (α_4, α_5, α_V, β_3).

The adhesion system of the integrins is redundant. Individual integrins can often bind to more than one ligand. Equally, individual ligands are, more often than not, recognized by more than one integrin. The first described recognition site is the tripeptide RGD (Arg- Gly-Asp) present on fibronectin and vitronectin and a variety of other adhesion molecules. This RGD tripeptide is a ligand for α_5, $\alpha_{IIb}\beta_3$ integrins and most of the α_V integrins ($\alpha_5\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_V\beta_6$). The conformation of the RGD site appears to determine which integrin an RGD protein or RDG peptide will bind [108]. Others peptide sequences have been recognized as integrin ligand. The KQAGDV sequence (Lys-Gln-Ala-Gly-Asp-Val) associated with fibrinogen is bound by the $\alpha_{IIb}\beta_3$ integrin. The $\alpha_2\beta_1$ integrin binds the DGEA (Asp-Gly-Glu-Ala) sequence present on the type I collagen molecule [108].

The cell surface integrin repertoire is subjected to significant changes during differentiation [109], inflammation [110], tissue healing process [111], and malignant transformation [112]. Agents that affect growth and differentiation can modulate integrin expression [113]. The TGF-β_1 is the first recognized regulator of integrin expression [114-116]. In various cell lines TGF-β_1, for example, upregulates the synthesis or cell surface

expression of at least α_1–α_6, α_V, β_1, β_3, β_5, and β_6 integrin subunits. In some cell lines, TGF-β_1 completely changes the cellular integrin pattern [114].

Besides expressing specific integrins the cells are able to modulate the binding properties of integrins. The modification of the integrin intracellular domains regulates the ligand-binding function of the extracellular part. For example, macrophages express $\alpha_6\beta_1$ laminin receptor on their surface but the cells are incapable of binding to laminin before $\alpha_6\beta_1$ is phosphorylated as the result of macrophage activation [117]. In addition the integrins play a crucial role in cell migration and differentiation by the induction of certain genes through signaling.

1.6.3 Discoidin domain receptors

The DDR1 belongs to a subfamily of RTKs that function as collagen receptors independent of β1 integrins [118-120]. The DDRs (DDR1 and DDR2) are unique among RTKs in being activated by an extracellular matrix component; usually RTKs are triggered by growth factors. The homologous DDRs are single-span transmembrane proteins, with an extracellular domain comprising an N-terminal discoidin homology domain [121] trailed by a region of approximately 200 amino acids exclusive to DDRs. The cytoplasmic domain contains an unusually large JM domain tracked by the C-terminal catalytic tyrosine kinase domain [122]. Interestingly, monomeric DDR proteins do not bind collagen, whereas dimeric DDRs bind collagen I with high affinity [122]. Nevertheless, not all dimeric DDR constructs bind collagen [123, 124], suggesting that collagen recognition requires a specific arrangement of the discoidin domains within the dimer. A later study confirmed that the homologous loops are also involved in collagen binding by DDR1 [125].

There are five DDR1 isoforms, three of them, DDR1a, DDR1b, and DDR1c, encode full-length, functional receptors while DDR1d and DDR1e encode truncated or kinase inactive receptors.

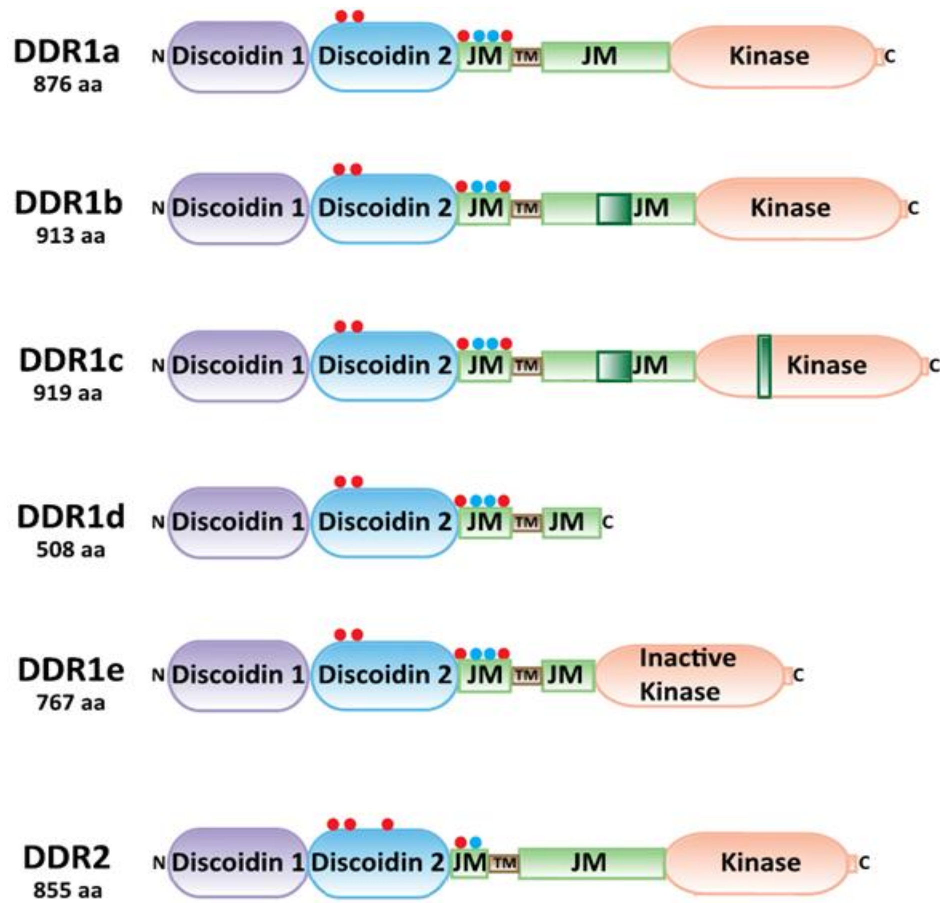


Figure 6 – Domain structure of DDRs.

Residues that are added as a result of alternative splicing are indicated by dark green boxes within the corresponding domain. Red and blue circles indicate putative N-glycosylation and O-glycolation sites, respectively [126].

DDR1a is generated as a result of deletion of exon 11 in the cytosolic JM domain [127]. DDR1c is generated as a result of the use of an alternate 5′ splice acceptor site at the 5′ intron/exon boundary of exon 14 within the kinase domain, giving rise to an additional 18 bp without open reading frame disruption [127].

The DDR1d isoform is generated through deletion of exons 11 and 12 in the cytosolic JM domain, resulting in a frame-shift mutation and a pre-maturely truncated protein [128]. In DDR1e, exons 11 and 12 are deleted, but the open reading frame is not disrupted due to an additional deletion at the beginning of exon 10 (via an alternate 5′ splice acceptor site in exon 10). However, in DDR1e, the ATP binding site within the kinase domain (at the end of exon 12) is missing, rendering this isoform inactive [128].

Like all RTKs, the DDRs undergo receptor autophosphorylation upon ligand binding, however in the DDRs this process is abnormally slow and sustained [118, 119].

Noordeen et al have recently showed that collagen binds to preformed DDR dimers on the cell membrane [129], indicating that the paradigm of ligand-induced receptor dimerisation [130] does not apply to the DDRs. It is though that the activation of the cytosolic kinase domains involves substantial conformational changes along the entire molecule, as proposed for another constitutively dimeric RTK, the insulin receptor [131]. Furthermore a leucine zipper motif in the transmenbrane domain is required for DDR1 activation, suggesting that the conformational alteration upon collagen binding could involve a rotation of the transmembrane domains within the dimer [129].

These receptors have important role in control developmental processes [132, 133] and regulate cell adhesion, migration, proliferation, and remodeling of the extracellular matrix by modulating the expression and activity of matrix metalloproteinases [134, 135].

Biochemical studies have shown that DDR2 activation requires the phosphorylation by Src of specific tyrosines in the activation loop of the DDR2 tyrosine kinase domain [136, 137]. Following phosphorylation by Src, the activated kinase domain of DDRs is believed to autophosphorylate several tyrosines in the JM region, which then recruit adaptor proteins such as Shc1 [119, 136] and Nck2 [138], the protein tyrosine phosphatase SHP2, and members of the STAT family of transcription factors [139]. Studies made with recombinant DDR extracellular domains by Leitinger et al showed that the DDR is necessary and sufficient for collagen binding [122].

Upon collagen binding, DDRs undergo autophosphorylation at multiple tyrosine residues within the cytosolic JM and kinase domains. Phosphorylation of tyrosine residues in a precise order recruits cytoplasmic signaling molecules containing SH2 and PTB domains, which in turn assemble protein complexes that serve to transduce receptor signals. In DDR1b and DDR1c, the cytosolic JM region and the kinase domain possess 15 tyrosine residues that can potentially undergo phosphorylation and serve as docking sites for SH2/3 or PTB containing adaptor proteins (Fig. 7). Several adaptor proteins have been shown to bind DDR1 in response to collagen stimulation, and in some instances, the phosphotyrosine residues involved in binding were also identified (Fig. 7). For example, ShcA, the p85a subunit of PI3K, Nck1/2, SHP2, Csk [140], NMHC-IIA, and the FAK homolog, Pyk2, were all found to associate with activated DDR1. Most of these interactions were confirmed in a recent proteomic study, which investigated the phosphotyrosine interactome of DDR1 in pervanadate treated SF268 human glioblastoma

cells overexpressing DDR1b and in human placenta using immobilized DDR1 phosphotyrosine peptides [141]. This study also showed that Stat1a/b, Stat3, and Stat5 directly bind to various phosphotyrosine residues in DDR1, suggesting that activated DDR1 possibly brings SHP2 and its substrates in close proximity. Other DDR1 binding proteins identified by this approach include RasGAP, the guanine nucleotide exchange factors Vav2 and Vav3, the adaptor protein CRKII, and the phosphatase SHIP2. Several DDR1-interacting partners that bind DDR1 regardless of its phosphorylation status, have also been identified. These include DARPP32, KIBRA, Syk, Notch1, E-cadherin, and the Par3/Par6 cell polarity proteins. In the case of DDR2, ShcA was reported to bind at pY471 following collagen I stimulation [137], and indirect evidence suggests that DDR2 may constitutively interact with Src [136], which may also be phosphorylated by DDR2.

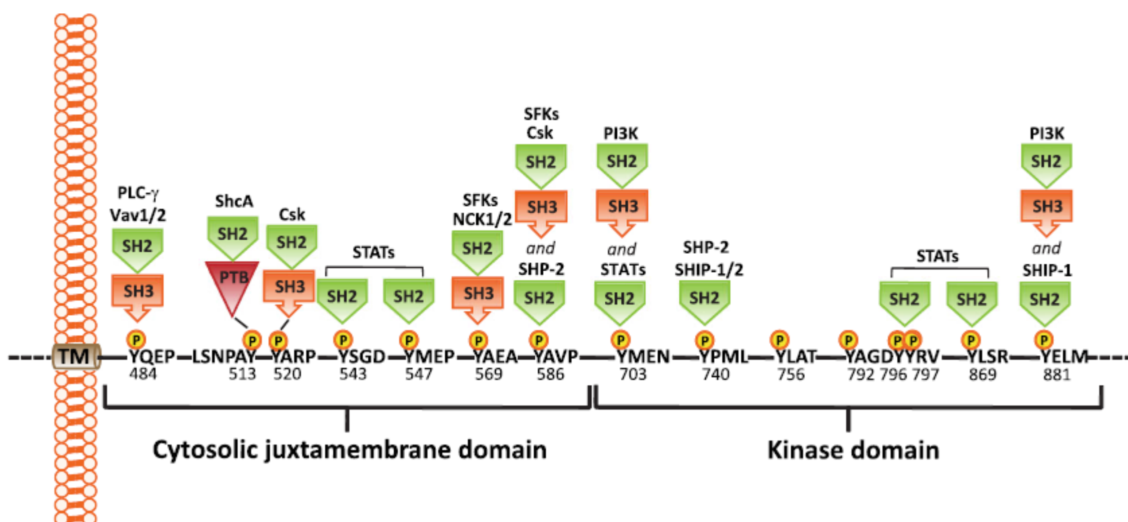


Figure 7 – Identified phospho-DDR1 interactions.

The indicated amino acid annotations refer to the DDR1b isoform [126].

1.6.4 Glycoprotein VI

GPVI is a member of the immunoglobulin superfamily, expressed uniquely on platelets and megakaryocytes. The GPVI polypeptide consists of two immunoglobulin-like extracellular domains (IG1 and IG2), oriented 90º apart, which are connected by a heavily *O*-glicosylated mucin-like stalk to a hydrophobic transmenbrane helix that is tracked by a short cytoplasmic domain containing with a proline-rich sequence that binds Scr kinases Fyn and Lyn, and a calmodulin binding region. The transmembrane domain contains a single arginine allowing association with a disulfide-linked FcRγ dimer, which is important for GPVI surface expression and signaling [142].

Collagen binding further relies on the highly specific recognition of the collagen Gly–Pro–Hyp sequence [143], which induces tyrosine phosphorylation of the ITAM of FcRγ and binding of tyrosine kinase Syk, which generates formation of a signalosome, including LAT, SLP-76, and PLCγ2, leading to a series of downstream pathways that culminate in platelet activation[144].

This membrane glycoprotein forms a dimeric structure by fusing IG1IG2 to the IgG Fc domain exhibited high affinity to collagen, although monomeric IG1IG2 itself showed little collagen affinity. Thus, the GPVI dimer was suggested to have a unique conformation that gives rise to high affinity binding to collagen. The crystal structure of the GPVI IG1IG2 domain indicated that two IG1IG2 domains can form a dimer with a dimerization interface located on the IG2 domain, providing direct evidence for dimer formation, albeit in a nonphysiological setting [142, 145].

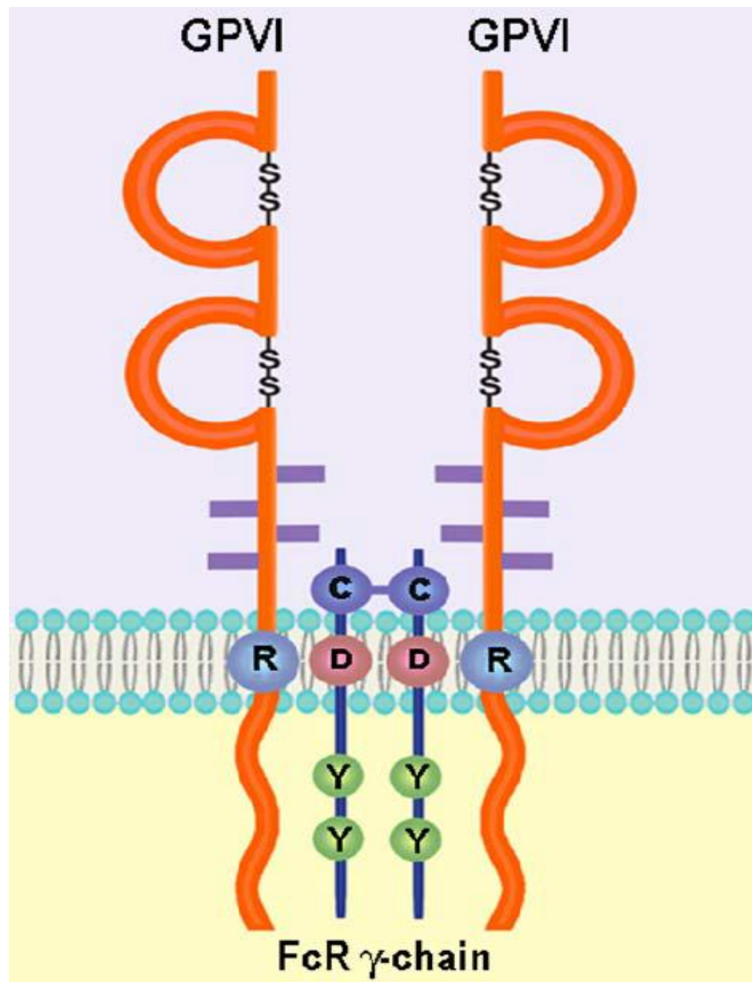


Figure 8 – Model of GPVI structure.

GPVI is present as a complex with the FcRγ chain. Megakaryocyte maturation occurs in the marrow, whereas proplatelet formation and platelet release takes place in the blood stream either in marrow sinusoids or in the general circulation. It is thus likely that specific mechanisms exist for the adhesion of progenitors to bone marrow stroma cells or to extracellular matrix proteins during maturation. On the other hand, to release platelets, megakaryocytes have to migrate through the endothelial barrier and cross the extracellular matrix, where they may interact with adhesive proteins such as FN and collagen. In platelets, the synergy of the two collagen receptors α2β1 and GPVI is important in mediating adhesion and in subsequent platelet secretion and aggregation [142].

1.7 Tyrosine kinases and tyrosine phosphatases

The process of reversible phosphorylation is perhaps the cell's most prevalent means of regulation at the molecular level. It has been estimated that up to 30% of all cellular proteins are phosphorylated, and phosphorylation has been shown to play a crucial regulatory role in such diverse cellular events as metabolism, growth and differentiation, vesicular transport, and gene transcription. Phosphorylation and dephosphorylation are carried out by kinases and phosphatases, respectively. There are currently predicted to be 518 kinases and 125 phosphatases encoded in the human genome, further underscoring the overall importance of phosphorylation in molecular regulation [146]. Phosphatases are generally divided into two main families based on their catalytic mechanism and substrate specificity: the PPs, which exclusively desphosphorylate serine and threonine residues, and the PTPs, which can dephosphorylate tyrosine residues. PTPs can be further classified into subfamilies based on (1) subcellular location (receptor versus intracellular), (2) substrate preference, and (3) three-dimensional topology [147].

The tyrosine kinase family is of profound importance due to its involvement in the signalling processes associated with mitogenic stimulation, cell growth, and oncogenesis. The tyrosine kinases fall into two major classes, those that are cell membrane receptors and integral membrane proteins, and those that are intracellular and function downstream of the receptors.

The megakaryocytopoiesis is highly dependent of the growth factor dependent tyrosine kinase receptors including c-kit, FGF receptor, DDR1, the RON receptor, and the GM-CSF receptor [148]. Binding of growth factors to their respective receptors results in receptor dimerization and subsequent autophosphorylation on tyrosine residues. Tyrosine autophosphorylations become sites of association for cytoplasmic signaling molecules via

their SH2 domains. These molecules can be cytoplasmic tyrosine kinases such as the Src kinases, TEC, CHK and Syk. Others are molecules such as PLC-γ, PI3K, Shc, GTPase-activating protein [149], and the SH2-containing tyrosine phosphatases SHP1 and SHP2[147]. These molecules generate second messengers, regulate the phosphorylation of other downstream molecules, and also regulate the phosphorylation of the receptor itself. The different cytoplasmic components activate pathways involved in either changes in cell growth or changes in the cytoskeleton that affect maturation of the cell.

1.7.1 Src

The Src family of tyrosine kinases is the largest of the nonreceptor tyrosine kinase families and is formed from a group of proteins that all share a high homology with the oncoprotein, v-Src, and the cellular counterpart, c-Src. Members of the Src family include Src, Yes, Fyn, Lck, Lyn, Blk, Hck, Fgr, and Yrk, which range in size from 53 to 64 kDa [150, 151]. These kinases, which associate with the plasma membrane and often physically link to receptors, become activated via a poorly understood mechanism that involves clustering of receptors. Src family kinases were the first nonreceptor tyrosine kinases to be well characterized with regard to their structure and biochemical mechanism of activation [152-154]. Src kinases are defined by their conserved domain structure consisting of an N-terminal membrane localization motif, a unique region, a SH3 domain, a SH2 domain, a kinase domain, and a C-terminal, regulatory tail.

Activation of Src family members occurs by two potential mechanisms: the co-presence of another protein containing a specific phosphotyrosine residue that has a higher affinity for the SH2 domain than the negative regulatory phosphotyrosine within the Src member; or the dephosphorylation of the negative regulatory phosphotyrosine by a specific protein tyrosine phosphatase. In vertebrates, the kinase activity of Src family members is normally inhibited by the phosphorylation of a tyrosine residue within the kinase domain [155, 156].

The crystal structure of the Src and Hck kinases determined the relationship between these domains for inactive Src family kinases [157]. In these structures, a phosphorylated tyrosine (Tyr 527 in Src) within the tail is bound to the SH2 domain through an intra-molecular interaction. Furthermore, a segment of the SH2 domain-kinase linker binds the SH3 domain and packs against the back of the kinase domain. Together, these interactions appear to distort the kinase domain preventing full activity. Biochemical

and enzymatic characterizations of Src family kinases supported the notion that both the SH2-tail and SH3-linker interactions are important for down-regulation of kinase activity [153, 158].

1.7.2 Syk

The Syk, family of PTKs, encode essential signaling components required for normal immunity. Their functions have been most intensely studied within mammalian immune cells. This family of PTKs is represented earlier within the phylogenetic tree, in the *hydra vulgaris*, as a single gene product expressed in epithelial cells and plays an important function in the recognition of foreign cells. In mammalian systems, this family of PTKs appears to have evolved from a gene duplication event to give rise to its two family members – Zeta-Chain-Associated Protein of 70K Mr (ZAP-70) and Syk. Genetic studies in humans and mice have demonstrated their essential roles in the function and development of T cells, B cells, mast cells, monocytes/macrophages, and the lymphatic system. Studies further underscore their importance in TCR, BCR, IgG and IgE receptors (FcRs) and integrin receptor signaling [159, 160].

In the past years has been shown the crucial role of Syk kinase in integrin-mediated activation of PLC2 and phosphorylation of MLC, which is likely to contribute to the changes in MK morphology and actin structure, both of which are required for MK migration [161].

Syk plays an important role in regulating migration of neutrophils and macrophages, both in chemokine signaling and integrin mediated adhesion. Syk is activated after integrin ligation on neutrophils, monocytic cell lines and platelets. The activation of Syk in migrating neutrophils is required for the establishment of stable lamellipodia and subsequent directed migration of the cells. Stabilization of the leading edge in migrating cells is associated with the accumulation of PIP3 at the lamellipodium in a positive feedback loop requiring phosphatidyl-inositol-3-kinase, Rac, and actin polymerization [162].

1.7.3 SHP

SHPs have two N-terminal SH2 domains (N-SH2 and C-SH2), a classic PTP domain and a C-terminal tail (‘C-tail’). SHP1 and SHP2 have two tyrosyl phosphorylation sites in their C-tails, which are phosphorylated differentially by receptor and non-receptor

PTKs [163]. SHP2 also has proline-rich domains that might bind SH3 domain-containing proteins, but specific interacting proteins have not been reported. SHP1 lacks a proline-rich domain, but has a basic sequence that can function as a NLS [164, 165].

SHP1 has two tandem SH2 domains at the N-terminus, followed by a catalytic domain, and an inhibitory C-terminus. SHP1 has a negative impact on lymphocyte signaling ([166]). By contrast, its cousin SHP2, which is widely expressed, in general promotes signaling pathways that lead to differentiation, cell growth, and migration

SHP2 is a cytosolic protein tyrosine phosphatase that shares many structural and regulatory features with SHP1. These include the presence of two adjacent amino-terminal SH2 domains followed by the phosphatase domain and a carboxyterminal domain that contains two conserved tyrosine phosphorylation sites. In contrast to SHP1, which is expressed mainly in hematopoietic cells, the distribution of SHP2 is quite broad. The role of SHP2 phosphatase activity in signaling is unclear. However, accumulating evidence suggests that it plays a positive role downstream to various receptors [167, 168].

SHP2 is required for integrin-evoked cell spreading, migration and Erk activation. How SHP2 mediates these effects remains unclear. SHPs1 becomes tyrosyl phosphorylated following integrin activation, probably by a SFK, and recruits SHP2 [169, 170], raising the possibility that the effects of SHP2 on adhesion are mediated by SHP1–SHP2 complexes. However, although fibroblasts lacking the SHPs1 cytoplasmic domain have increased stress fibers and/or defective migration, they also have decreased Rho activation [171]. Some workers report no effect of dominant negative SHP2 on Fak tyrosine phosphorylation, but others find enhanced phosphorylation. Studies of SHP2 mutant fibroblasts also disagree. One report shows that SHP2 is required for normal integrin-evoked Src and Fak phosphorylation [169], whereas another finds decreased Fak dephosphorylation upon de-adhesion [172]. These discrepancies might reflect differences in experimental details or design, reagents or experimental systems. SHP2 also might help to integrate RTK and integrin signals because stimulation of the RTK EphA2 was reported to cause SHP2-mediated FAK dephosphorylation and to inhibit integrin function [173].

The SH2 domain PTP SHP1 regulates multiple signal transduction events by dephosphorylation. These comprise signaling of cytokine receptors such as the erythropoietin receptor, and the interleukin-3 receptor, and of receptor tyrosine kinases such as c-Kit, the colony-stimulating factor-1 receptor, and the epithelial kinase Ros. SHP1

modulates also the function of immunoreceptors, and cytoplasmic tyrosine kinases such as Lck or Syk [174]. In these and many other cases, SHP1 regulates signaling in a negative manner. In other pathways, SHP1 may also exert a positive function. Thus, a role of SHP1 for differentiation of glia cells, and for Ras-dependent activation of mitogen-activated protein kinase have been reported. Also, SHP1 has the capacity to activate Src kinase by dephosphorylation of the inhibitory phosphotyrosine in the Src C terminus and may thus stimulate Src-dependent phosphorylations in certain cell types [175].

1.8 Objective

In this work our main goal is to investigate the role of DDR1 in regulation of MKs migration upon adhesion to type I collagen. To reach it we aim to understand the mechanisms underlying this regulation, essentially the role of SHP1, SHP2 and Syk that have been already shown to be involve on migration signaling on other cells [161, 171].

In a personal matter I am aiming to improve and learn new procedures and methodologies, and above all acquire new knowledge. Besides the knowledge I am targeting to get laboratory routines and laboratory co-working skills.

2. Results

2.1 Human MKs expresses and synthesizes DDR1

In first place we analyzed the expression of DDR1 by RT-PCR and western blot, on human MKs derived from umbilical cord blood progenitor cells. As we can see in Fig. 9A-B, at day 13 of culture DDR1 expression was spotted in mature MKs, and in human fibroblasts as positive control, at both mRNA and protein levels. On the other hand, DDR1 was also expressed in peripheral blood platelet lysates, as shown by western blot in Fig. 9C. As we can see through the Fig. 9D, the red stain, corresponding to the anti-DDR1 antibody, is found mostly at the cell surface, indicating that DDR1 was expressed largely on MK cellular membrane where is displayed the transmembrane protein CD61 (stained with green).

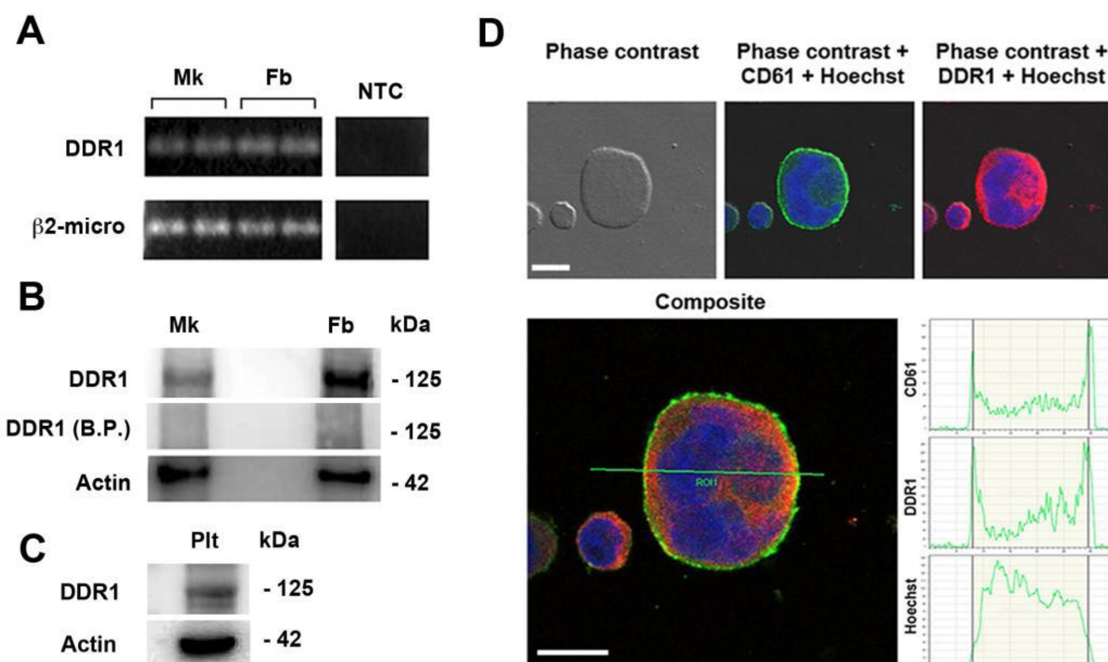


Figure 9 – Human MKs expresses and synthesizes DDR1 tyrosine kinase.

A – total cellular RNA was extracted from MKs and Fibroblasts (Fb) as positive control – microglobulin was used as housekeeping gene. NTC, are No template controls in the reverse trascriptase and PCR steps. RT-PCR products were loaded in duplicates for each cell type. B – MK and fibroblast lysates were subjected to western blot analysis using an anti-DDR1 antibody. The anti-DDR1 blocking peptide (B.P.) was used to confirm the specificity of the antibody. Actin was probed to show the equal loading. C – DDR1 expression was demonstrated in peripheral blood

platelet lysate (Plt) by western blot. Shown here are representative western blots out of three independent experiments. D – MKs were cytospun on polylisine coated glass coverslips, fixed and stained with an anti-DDR1 antibody (red) and an anti-CD61 antibody (green). The graphics report the intensity of the fluorescence signal along the x axis for each fluorochrome on the optical section. (Immunofluorescence staining, DM IRBE inverted microscope, magnification 40x). Scale bars are 25 μm. Nuclei were counterstained with Hoechst 33288 (blue).

2.2 Type I collagen-dependent DDR1 activation

To better understand the catalytic activity of DDR1 upon type I collagen binding was measure by the phosphotyrosine probing of DDR1 immunoprecipitates (Fig. 10A, panels i and ii). In order to evaluate the role of DDR1 activation in modulating MK function on type I collagen, we took advantage of a recombinant chimera protein (DDR1:Fc), which had been shown to block DDR1-collagen interactions [176]. This protein binds through its extracellular domain of DDR1 to type I collagen, on the specific binding site of DDR1, preventing the binding of DDR1 present on MKs membrane, impeding the DDR1 activation. We demonstrate the DDR1:Fc ability to bind to our type I collagen (derivation of the collagen) by solid phase binding assay (Fig. 10B). Additionally, we proved, by AFM, that the pretreatment of type I collagen with DDR1:Fc did not modify the structural properties and the normal banding of the extracellular protein (Fig. 10C). To finish, we verified if the DDR1 blocking molecule could influence the function of the other collagen receptor expressed by human MKs, α2β1 integrin and GPVI. As shown in Fig. 10D, panels i by western blot analysis, the DDR1:Fc molecule works specifically blocking the activation of DDR1 while the activation of α2β1 integrin and of GPVI is unprejudiced. To determine the levels of activated integrin we used a beta1 integrin antibody (clone HUTS-4) recognizing epitopes in the 355-425 region (hybrid domain), whose expression parallels the activity of beta1 integrin [177]. As mentioned the GPVI may form a dimer that has functional impact. The activation of GPVI was determined using an antibody that binds to the dimeric form of the receptor with an affinity 200-fold higher than to the monomeric form [178]. Further, the activation of the collagen receptor LAIR-1, which expression in mature MKs was recently demonstrated (38), appear unaffected by the treatment of megakaryocyte with DDR1:Fc (Fig. 10D, panels ii). With all these data we demonstrate that the use of this chimera highly prevents the interaction of DDR1 with type

I collagen without interfering with another collagen receptors expressed by human MKs, and therefore none other signaling pathways.

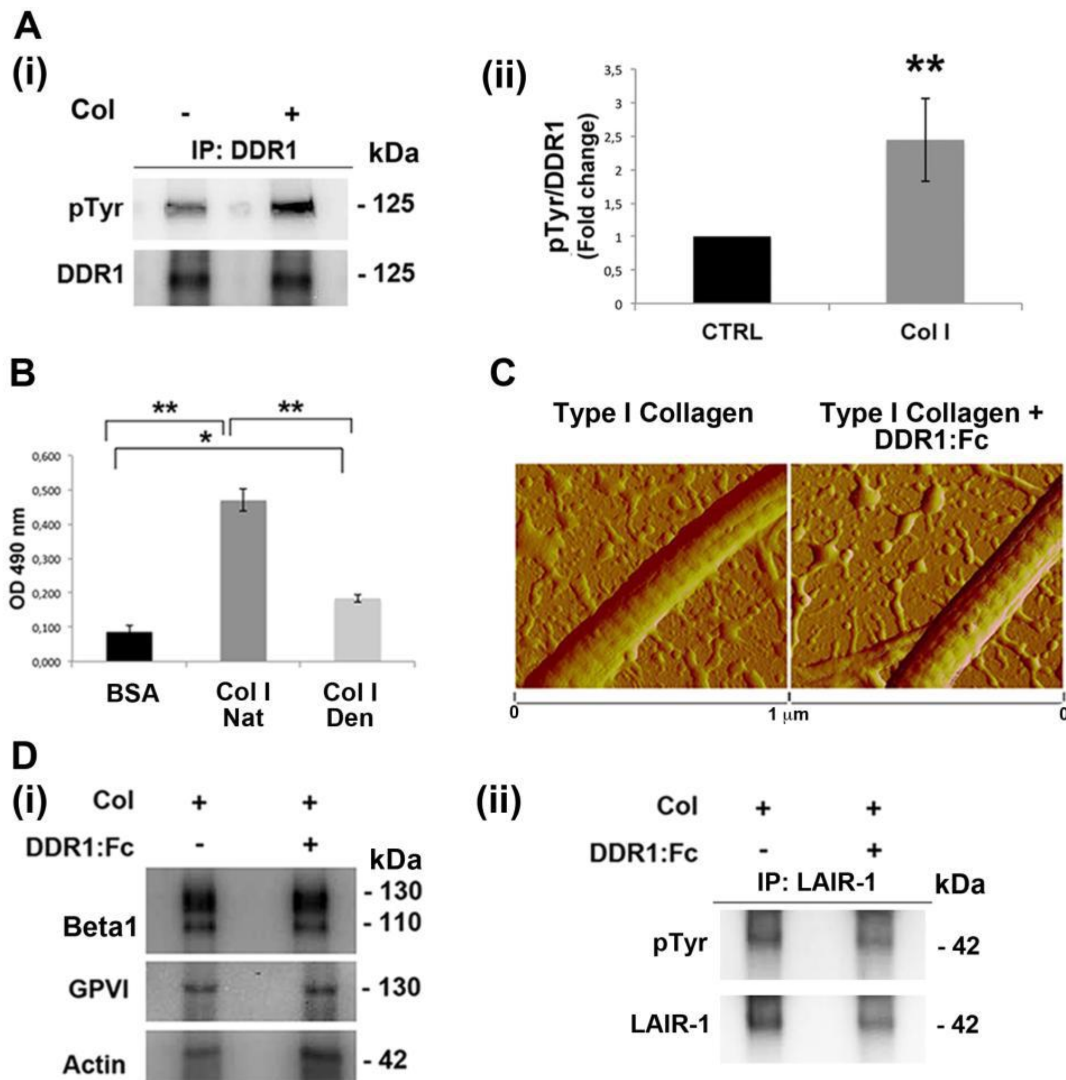


Figure 10 – Type I collagen dependent DDR1 activation.

A – panel i, lysates of MKs, plated on tissue culture plastic or on type I collagen for 16 hours, were immunoprecipitated with an anti-DDR1 antibody and subjected to western blotting. Membranes were stained with a monoclonal antibody against phosphotyrosine and with anti-DDR1 antibody. Panel ii, densitometry analysis of the western blots of 125 Kda pTyr band on DDR1 immunoprecipitates. B – binding assay to evaluate the DDR1:Fc binding affinity on native type I collagen (Nat Col I) with respect to denatured type I collagen (Den Col I). BSA was used as negative control. OD was measured at 490 nm. C – Atomic Force Microscopy images of dehydrated collagen coated cover-slips in the presence or not of DDR1:Fc showing similar banded fibrils. D – panel i MK were plated, for 2 hours, on type I collagen, in presence or not of DDR1:Fc.

Lysates were subjected to western blot analysis of active beta1 integrin (HUTS-4) and dimeric GPVI. Actin was probed to show the equal loading. Panel ii, lysates of megakaryocyte treated as above described in D were immunoprecipitated with anti-LAIR-1 antibody and subjected to western blot. Membranes were stained with antibodies anti-pTyr and anti-LAIR-1. Shown here are representative western blots out of three independent experiments. Data are presented as mean ± SD. (n = 5, 4 independent experiments). *p < .05. **p < .01.

2.3 DDR1-dependent regulation of MK migration

To study DDR1 involvement in MKs adhesion, we plated mature MKs on type I collagen coated cover-slips, having or not the DDR1 blocking molecule (DDR1:Fc) or an IgG as control. Besides we pretreated a number of cells with a FcR blocking solution to evade a possible binding of the Fc receptor (FcR) with the chimera (DDR1:Fc). We stained the cells, after 16 hours, with anti-CD61+ antibody and then we counted the adherent cells with fluorescence microscopy with a 20X/0.5 UPlanF1 objective. The Mk adhesion on type I collagen showed to be unaffected by DDR1 activation after counting analogous number of CD61+ cells per field in all conditions (Fig. 11A). Furthermore, to study the MKs migration mediated by DDR1 activation, we seeded in the upper well of a trans-well plate, after coating the trans-well filter with type I collagen mixed with DDR1:Fc or with IgG as control. Besides, in order to control the binding of the Fc cells with the chimera (DDR1:Fc) we pretreated the cells with the FcR blocking solution. After 16 hours of migration, we counted the number of cells that had passed, through the filter, to the lower well. We found a denotable reduction, of about 40%, on the number of migrated cells in the presence of DDR1:Fc as compared to control samples (Fig. 11B). Moreover, the samples which have been blocked with the FcR didn’t alter the number of migrated cells on type I collagen mixed to DDR1:Fc. Furthermore, in order to corroborate these effects we stained the lower side of the coated trans-well filters with an anti CD61 antibody to identify MKs that had invaded the type I collagen coated filters (Fig. 11C-D).

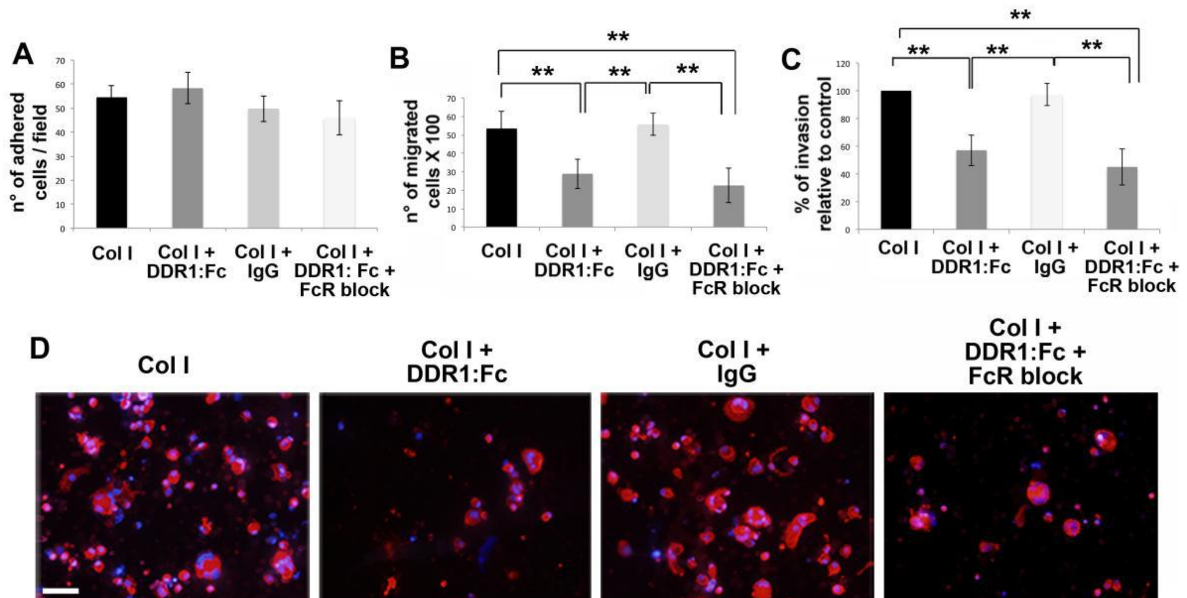


Figure 11 – DDR1-dependent regulation of MK migration.

A – MKs were plated on type I collagen coated cover-slips, in the presence of the DDR1:Fc blocking molecule or an IgG as control. Where indicated MKs were pretreated with FcR blocking solution. After 16 hours adherent MKs were fixed, stained with anti-CD61 antibody and then counted by fluorescence microscopy. B – MKs were left to migrate in a trans-well plate, upon coating the trans-well filter with type I collagen mixed with the DDR1:Fc blocking molecule or an IgG as control. Where indicated MKs were pretreated with FcR blocking solution. After 16 hours MKs that had passed in the lower chamber were collected and counted by phase contrast microscopy. C – MKs adherent on the lower side of the trans-well filter were fixed and stained with anti-CD61 antibody and then counted by fluorescence microscopy. D – representative images of MK invasion of type I collagen. Cells, adherent to the lower side of the trans-well coated filter, were fixed and stained with anti-CD61 antibody (red) antibody. (Immunofluorescence staining, Olympus BX51 microscope, magnifications 20X). Scale bars 100 m. Nuclei were stained with Hoechst 33288 (blue). Reported results are the means ± SD. (n = 6 independent experiment). **p < .01.

2.4 Syk is involved in DDR1-dependent MK migration

In order to understand the happenings underlying the reduction of MK migration on type I collagen subsequent of DDR1 inhibition, we explored the association and phosphorylation-dependent activation of tyrosine kinases Syk, the MAPK ERK and of the myosin light chain (MLC2), acknowledged to be involved in DDR1 signaling as well as in DDR1-dependent regulation of cell migration processes [179-181]. We decided to show, in

first place, the interaction between DDR1 with Syk and with myosin IIA dependently on MK-type I collagen interaction (Fig. 12A). In the next step, by inhibiting the DDR1 activation with DDR1:Fc, we found an increase in the phosphorylation levels of Syk kinase and, to a lesser magnitude, of MLC2 in type I collagen adhering-MKs after 16 hours, on the other hand ERK activation was alongside reduced (Fig. 12B). We also analyzed the phosphorylation upon 3 hours adhesion, although we did not detected noteworthy differences (data not shown), most possible due to the low kinetics of DDR1 activation, as told in introduction. In sum these results displayed a noticeable increase of phosphorylated Syk after DDR1 inhibition, which suggest a DDR1 dependent negative regulation of Syk activation. To clarify the role of Syk in reducing MK migration upon DDR1 inhibition, we used the specific Syk inhibitor R406 in new migration assays performed. As we can see in Fig. 12C that it is evident the MK reestablished migration to a level comparable to untreated control upon Syk inhibition, overcoming DDR1 inhibition. Furthermore, in order to corroborate these effects we stained the lower side of the coated trans-well filters with an anti CD61 antibody to identify MKs that had invaded the type I collagen coated filters (Fig. 12D and 12E).

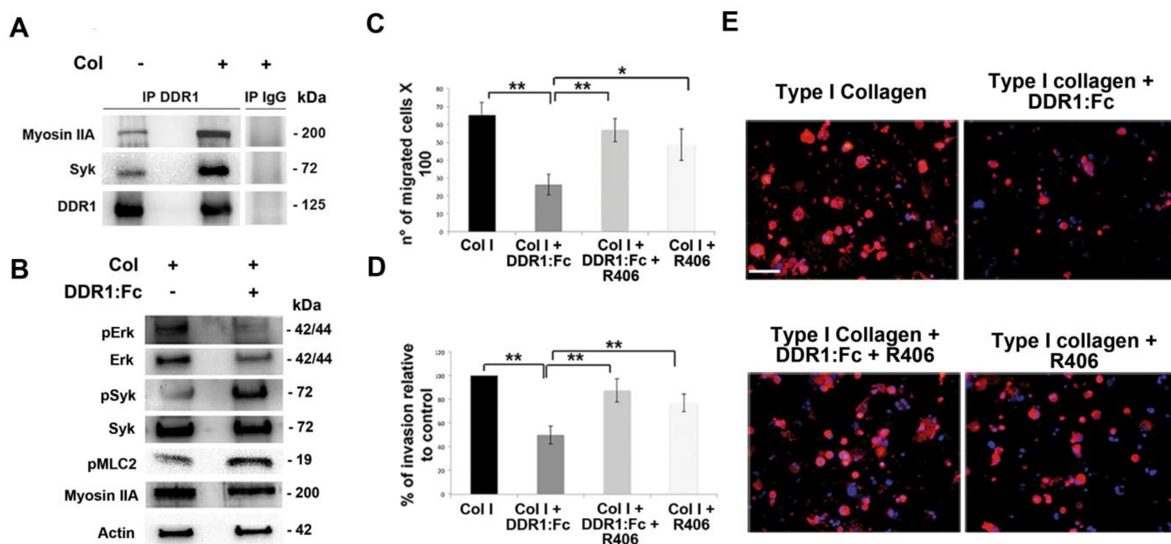


Figure 12 – Syk kinase is involved in DDR1 mediated MKs migration on type I collagen.

A –DDR1 was immunoprecipitated in cell lysates of MKs plated for 16 hours on type I collagen, or on tissue culture plastic. A control sample was immunoprecipitated with an unrelated antibody (IgG). Membranes were probed with anti Syk and anti-myosin IIA antibodies to show DDR1 interacting protein, and re-probed with anti DDR1 antibody to show the equal loading. B – total

cellular lysates of MKs plated for 16 hours on type I collagen, inhibiting or not DDR1 activation, were subjected to western blot analysis. Membranes were probed with the indicated antibody, with p indicating the phosphorylated form. Actin was probed to show the equal loading. C – transwell migration assay of mature MKs through type I collagen, in the presence of the DDR1:Fc blocking molecule and of Syk specific inhibitor compound R406 (5 μm) either mixed or used singularly. After 16 hours, MKs that had passed in the lower chamber were counted by phase contrast microscopy. D – MKs adherent to the lower side of the trans-well filter were fixed and stained with anti-CD61 antibody and then counted by fluorescence microscopy. E – representative images of MK invasion of type I collagen. Cells, adherent to the lower side of the trans-well coated filter, were fixed and stained with anti-CD61 antibody (red). (Immunofluorescence staining, Olympus BX51 microscope, magnifications 20X). Scale bars are 100 m. Nuclei were stained with Hoechst 33288 (blue). Data are presented as mean ± SD.(n = 5, 5, 5 independent experiment). *p < .05. **p < .01

2.5 SHP1 modulates Syk activation upon type I collagen-DDR1 binding in MKs

As DDR1 did appear to negatively mediate the Syk phosphorylation, we decide to explore the mechanisms underlying by focusing on a probable participation of phosphatase enzymes. For that propose we made phosphatase activity assays on cell lysates of MKs plated on type I collagen for 16 hours, treated or not with DDR1:Fc. The results demonstrate a considerably decrease of total tyrosine phosphatase activity upon inhibition of DDR1 activation (73.3 ± 6.6% relative to controls). Since the two Src homology-2 (SH2) domain-containing tyrosine phosphatases, SHP1 and SHP2, have shown to dephosphorylate Syk [175], we decided to investigate the interaction between DDR1 and this two tyrosine phosphates. Therefore, we analyzed, by western blot, the co-immunoprecipitation of DDR1 with both SHP1 and SHP2 in MKs plated on type I collagen or on tissue culture plastic, as negative control of DDR1 activation. Despite SHP2 hasn't been detect in any of tested conditions, contrarily to already shown in previous works [139], the DDR1-SHP1 interaction looked to be highly augmented upon adhesion of cells on type I collagen (Fig. 13A), most likely due the DDR1 activation and, consequently, autophosphorylation of its tyrosine residues recruiting SHP1, as described before on 1.6.3 Discoidin domain receptors. Furthermore to better understand this interaction we performed the SHP1 phospho-specific probing of western blot analysis of MK lysates, cultured in the presence or not of DDR1:Fc blocking molecule. As we can see

on Fig. 13B the SHP1 phosphorylation was significantly decreased after inhibition of DDR1. We also verified, by phosphatase activity assays of SHP1 immunoprecipitates a reduction of around 20% in SHP1 enzymatic activity when DDR1 activity was blocked (Fig. 13D). Besides we submitted to western blot, for the same loadings, the corresponding immune complexes, bound to protein A-sepharose (data not shown). In order to confirm our results we explored the relation between SHP1 phosphatase reduction and Syk activation. A western blot analysis was made from Mks treated with the SHP1 specific inhibitor sodium stibogluconate, revealing an augment in Syk phosphorylation in treated MKs relative to control, reproducing the DDR1 blocking condition (Fig. 13C). For last we tested the migration of Mks treated with sodium stibogluconate, resulting in a reduction of cell migration (Fig. 13E). In sum these results demonstrate that DDR1 activation increases SHP1 activity that, sequentially, dephosphorylates Syk preventing the Syk-mediated inhibition of MK migration on type I collagen.

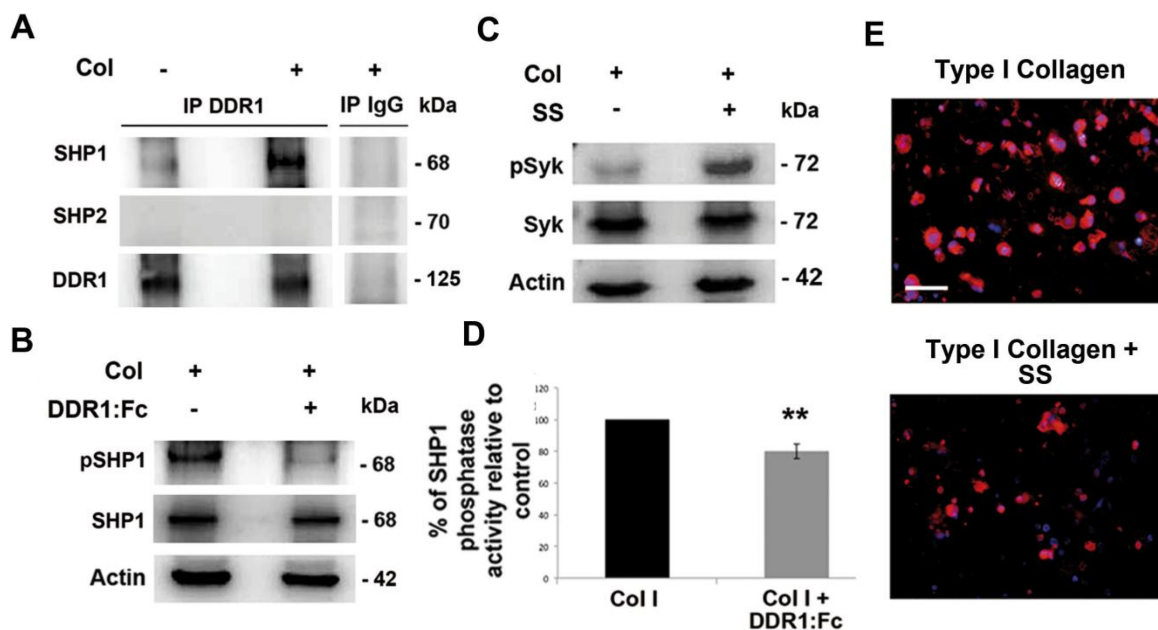


Figure 13 – SHP1 modulates Syk activation upon type I collagen-DDR1 binding in MKs.

A – DDR1 immunoprecipitates from MKs plated for 16 hours on type I collagen, or on plastic, were analyzed by western blot. A control sample was immunoprecipitated with an unrelated antibody (IgG). Membranes were probed with anti-SHP1 and anti-SHP2 antibodies to show the co-immunoprecipitation, and with anti-DDR1 antibody to show the equal loading. B –western blot analysis of cell lysates of MKs plated for 16 hours on type I collagen mixed or not with DDR1:Fc blocking molecule. Membranes were probed with the indicated antibodies with p indicating the

phosphorylated form. Actin was probed to show the equal loading. C – MKs were treated with the SHP1 specific inhibitor sodium stibogluconate (SS) (13.4 M) and plated for 3 hours on type I collagen. Cell lysates were subjected to western blot analysis. Membranes were probed with anti-phospho Syk (Tyr 525/526) antibody, anti-Syk and anti-actin antibodies to show equal loading. D – SHP1 phosphatase activity measured in a phosphatase assay on SHP1 immunoprecipitates from MK lysates, using P-NPP as substrate. MKs were plated for 16 hours on type I collagen mixed or not with DDR1:Fc blocking molecule. E – representative images of MK invasion of type I collagen. Cells, adherent to the lower side of the trans-well coated filter, were fixed and stained with anti-CD61 antibody (red) antibody. (Immunofluorescence staining, Olympus BX51 microscope, magnifications 20X). Scale bars are 100 m. Nuclei were stained with Hoechst 33288 (blue). Data are presented as mean ± SD. (n = 5, 4, 4, 4, 3, 3 independent experiments). $*p < .05$ $**p < .01$.

3. Discussion

In the past years, a growth of evidence demonstrates the crucial role that the ECM proteins have in MKs regulation. As already described on introduction the bone marrow presents 2 distinct environments, the osteoblastic niche and the vascular niche, although both niches have completely diverse roles on MK maturation. The migration of MKs from the osteoblastic niche to the vascular niche is fundamental for the well maturation of those and, subsequently, the well platelet production. These different microenvironments are vital to lead the migration of MKs to the vascular niche where they can properly release platelets in the vascular sinusoids.

The type I collagen is most abundant protein in the osteblastic niche, this structural protein inhibits the maturation of MKs, preventing the early proplatelet formation and platelet release. Thus the MK interaction with type I collagen is essential for the MK development. Consequently various studies have been made to understand this interaction MK-type I collagen, comprehending the role of the principal mammalian collagen receptors, such as GPVI and integrin α2β1 on the MKs regulation. Though these works much, is still unknown, particularly the interaction, expression and function of other matrix receptors.

In the last decade number of studies focused on the tyrosine kinase receptors: discoidin domain receptors (DDR1 and DDR2). It has been demonstrate that these receptors are stimulated in presence of fibrillar and basement membrane collagens and mediate migration and cell adhesion in different tissues. In this work we studied the mechanism underlying the migration of MKs on type I collagen mediated by DDR1.

In first place we demonstrate that the DDR1 was in fact expressed on mature MKs using RT-PCR and western blot. We also showed that the DDR1 is mainly expressed on MK cellular membrane as expected by immunofluorescense and confocal microscopy. In the other hand, we detected the expression of the receptor in peripheral blood platelet lysates revealing also a possible role in mediating platelet adhesion on collagen. With this we could affirm that the DDR1 is a tyrosine kinase receptor well expressed in mature MKs.

Knowing that the DDR1 was present on human MKs we proceed to evaluate the DDR1 activation on type I collagen. We saw that the interaction between type I collagen and this receptor leaded to an increase of its activation (fig. 10A, panel i), confirming the results of previous works [140]. Then we used the chimera protein DDR1:Fc to block the interactions DDR1-collagen to find if the activation of DDR1 would modulate MK

behavior on type I collagen. The results show that upon blockage of type I collagen with the chimera there wasn't any increase of DDR1 activation. Furthermore we evaluate that the DDR1:Fc didn't modified the type I collagen structure and also didn't alter the activation of the other collagen receptors, making sure that all the results obtained are influenced by only the DDR1-type I collagen interactions.

Furthermore we checked that the MK adhesion remains without significantly changes upon blocking DDR1 on type I collagen. Further we tried to understand the implications of the blockage of DDR1-collagen on the migration of MKs. As we can see by the results that on DDR1:Fc treated type I collagen MKs migration had a decrease of 40% relative to control, which shows that the DDR1 has a crucial role in the modulation of MKs migration. To better understand this regulation we look forward to the signaling pathways underlying.

We then evaluated the phosphorylation-dependent activation of some tyrosine kinases that could be related with DDR1 activation, as previous studies had described [179-181]. We focused on phosphorylation of Syk, due to be already demonstrated its involvement on cell migration [161, 162]. We conferred the interaction between DDR1 and Syk with the results of co-immunoprecipitation, where we could see an increased interaction between the two proteins upon type I collagen adhesion. As the data shows (Fig.12 B), the most evident change between the different tyrosine kinases was the phosphorylation increase of Syk upon blocking DDR1 on type I collagen.

Additionally, by performing new migration assays, we observed that using the specific Syk inhibitor R406 restored the migration. With these migration assays we demonstrated the predominant role of Syk on MK migration, suggesting that DDR1 activation negatively regulates the Syk activity.

To better understand the DDR1-Syk interaction and regulation, we explored the activation of a specific group of phosphatases, SHP1 and SHP2, since these two phosphatases have been demonstrated to interact with DDR1 [140] and to dephosphorylate Syk [174, 175]. So we focused on the direct interaction between this two phosphatases and DDR1. The co-immunoprecipitation assay demonstrated an increased interaction between only DDR1 and SHP1, while SHP2 was not detected at all. The results (Fig. 13 A) indicate that the activation of DDR1 leads to an increase of SHP1 phosphorylation. Instead the blockage of DDR1 leads to a decrease of phosphorylated SHP1, indicating that the DDR1 activation correlates with SHP1 phosphorylation. Finally we showed that the use of the specific SHP1

inhibitor SS lead to strong increase of Syk phosphorylation and to the consequent decrease of MK migration, equal to the results presented after blockage of DDR1 activation with DDR1:Fc. We then conclude that the SHP1 phosphatase downregulate the Syk activation via dephosphorylation.

With all put together, we acknowledge that the interaction MK-type I collagen leads to an increase of DDR1 activation that increases the activation of SHP1 which leads to Syk dephosphorylation preventing the Syk-mediated inhibition of MK migration on type I collagen.

4. Conclusion

The microenvironment has a crucial role on cells behavior, being the interaction with ECMs, via cell receptors, a vital factor for cell development.

With this, diverse studies have been made about the relation between cell receptors, as DDR1 [140], cell regulation in different kind of cells as well in different ECMs. With our work we proved that DDR1 had a fundamental role in mediating the migration of MKs on type I collagen.

Also recently have been proved that SHP1 interacts with Syk which is involved on cell migration [162, 174]. Furthermore we correlated the SHP1, Syk and DDR1 in a signaling pathway, which regulates the MK migration. We demonstrated as well that this regulation involve the SHP1 capacity to dephosphorylate Syk preventing the negatively intervention on migration of MKs.

In the end, for the first time we demonstrate the expression of the tyrosine kinase receptor DDR1 by mature human MKs and that the DDR1 activation upon type I collagen stimulates MKs migration, involving the interaction of SHP1 with Syk.

Although with this work we extended the list of receptors involved in MKs-microenvironment interactions, there is still many to learn in order to fully understand the different mechanisms underlying the MKs maturation.

5. Materials and methods

5.1 Reagents and antibodies

5.1.1 Culture reagents

- Type I collagen was purified as described previously [182].
- The anti-DDR1 blocking peptide was from Santa Cruz Biotechnology.
- FcR blocking solution was from Miltenyi Biotec (Bergisch Gladbach, Germany).
- DMEM and FBS were from Gibco (Invitrogen).

5.1.2 Inhibitors

- DDR1:Fc recombinant protein from R&D systems (Minneapolis, MN, USA).
- Sodium Stibogluconate from Calbiochem (San Diego, CA).
- R406 from Axon Medchem (Groningen, NL).

5.1.3 Other reagents

- Precision Plus protein standard from Biorad.
- Protein A-Sepharose from Staphylococcus aureus was from Sigma Aldrich (St. Louis, MO, USA).

5.1.4 Antibodies

- Rabbit polyclonal anti-DDR1 (C-20) (Santa Cruz Biotechnology; Santa Cruz, CA, USA).
- Mouse monoclonal anti-CD61 (clone SZ21) (Immunotech; Marseille, France).
- Alexa Fluor–conjugated antibodies (Invitrogen; Burlington, ON, USA).
- Rabbit polyclonal anti-Non Muscle Myosin IIA (clone BT-567) (BTI; Oklahoma City, OK, USA).
- Rabbit polyclonal anti-pospho-MLC2 (Ser19) (Cell Signaling; Danvers, MA, USA).
- Mouse monoclonal anti-phosphotyrosine (clone 4G10) (Millipore; Billerica, MA, USA).
- Rabbit monoclonal anti-phospho-Syk (Tyr525/526) (clone C87C1) (Cell Signaling).
- Rabbit polyclonal anti-Syk (N-19) (sc-1077) (Santa Cruz Biotechnology).

- Rabbit monoclonal anti-phospho-MAPK 1/2 (ERK 1/2) (Thr185/Tyr187) (clone AW39) (Millipore).
- Mouse monoclonal anti-MAPK 1/2 (ERK 1/2) (clone 3A7) (Cell Signaling).
- Rabbit polyclonal anti-SHP1 (sc-287) (Santa Cruz Biotechnology).
- Mouse monoclonal anti-SHP2 (sc-7384) (Santa Cruz Biotechnology).
- Rabbit polyclonal anti-phospho-SHP1 (Tyr536) (Abcam; Cambridge, UK).
- Mouse monoclonal anti β actin (Sigma-Aldrich).
- Mouse monoclonal anti-activated β1 integrin (HUTS-4) (Millipore).
- Mouse monoclonal anti-LAIR-1 (sc-59281) (Santa Cruz Biotechnology).
- The anti-GPVI antibody (clone 9E18) was kindly provided by M. Jandrot-Perrus (Inserm, U698, Hôpital Bichat, Paris).
- HRP conjugated anti-rabbit and anti-mouse secondary antibodies were from Biorad (Milan, Italy).

5.2 CD34+ Extraction

Upon informed consent of the parents, in accordance with the ethical committee of the IRCCS Policlinico San Matteo Foundation and the principles of the Declaration of Helsinki, human umbilical cord blood was collected after normal pregnancies and deliveries, and processed within 48 h. Mononuclear cells were separated by layering CB onto Lympholyte (<1077 g/ml, Cedarlane, Hornby, Canada) and centrifuging for 30 min at 1500 rpm (425 × g) at 20°C. The resulting nucleated cells were washed twice in phosphate-buffered saline (PBS) and suspended in Stem Span medium (Stem-Cell Technologies, Vancouver, Canada). CD34+ cells obtained from nonadherent, low-density (<1077 g/ml) fraction were separated by immunomagnetic bead technique (Miltenyi-Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. CD34+ cells were 98% pure.

5.3 Cell culture

Liquid cultures were initiated by plating 1×10^6/ml low-density cells or 1×10^4/ml CD34+ cells in Stem Span medium containing 10 ng/ml TPO, interleukin(IL)-6, IL-11, Flt3-L (all from PeproTech EC Ltd., London, UK).

Skin human Fibroblasts were cultured in DMEM supplemented with 10% FBS. Cultures were maintained for up to 12 days at 37°C in a humidified atmosphere of 5% CO_2.

5.4 Immunofluorescence and confocal microscopy analysis

To evaluate DDR1 distribution on MKs surface, MKs were fixed in 3% paraformaldehyde for 20 min at RT, permeabilized by 0.5% Triton X-100 for 5 min, and subsequently blocked with 3% BSA and 10% FBS in PBS for 1 h at RT. Cells were then incubated with the following primary antibodies diluted in PBS for 1 h at RT with anti-DDR1 and anti-CD61 antibodies both diluted 1:100. After washing with PBS, cells were incubated with 10 µg/mL of the appropriate secondary antibody conjugated with either Alexa Fluor 594 or Alexa Fluor 488 (Invitrogen, Milan, Italy) in PBS for 1 h at RT. Nuclear counterstaining was performed with Hoechst 33258 (100 ng/mL in PBS). Specimens were mounted in Mowiol 4–88. Negative controls were routinely performed by omitting the primary antibody. Analysis by conventional fluorescence and confocal microscopy were performed with an Olympus BX51 microscope using a 20X/0.5 UPlanF1 objective (Olympus; Watford, UK) and a TCS SPII confocal laser scanning microscopy system equipped with a Leica DM IRBE inverted microscope using a 40x oil NA 1.25 objective (Leica; Bensheim, Germany). For each specimen at least 20 megakaryocytes were examined.

To analyze matrix structures in different conditions, glass covers-lips were coated with type I collagen, as described in Materials and Methods, in the presence or not of DDR1:Fc, for 16 hours at 37°C in PBS and then observed by TMAFM on a Digital Instruments Multi-Mode Nanoscope III/a SPM (Digital Instruments, Santa Barbara, California, USA) with Olympus OTR 8 oxide-sharpened silicon nitride probes.

5.5 Binding assays

96-well microtiter plates were coated overnight at 4 °C with the solutions of collagenous samples in NaCl/Pi (200 L per well). Control wells were coated with 200 L containing 5 g of BSA in NaCl/Pi. All analyses were done at least in triplicate. After rinsing with 0.15 M NaCl, 0.05% (v/v) Tween-20, the wells were incubated with 200 L of 1% (w/v) BSA in NaCl/Pi, for 1 h at room temperature. After rinse as above described, the coated wells were incubated for 2 h at room temperature with 20 g/mL of DDR1:Fc blocking molecule in 200 L of NaCl/P_i, 0.05% (v/v) Tween-20. For every solid- phase experiment, control for dose-dependent, nonspecific binding to coated BSA wells was performed, under identical conditions. Bound DDR1:Fc were incubated for 2 h with HRP-conjugated anti-human IgG1 antibodies for detection. Horseradish peroxidase was diluted

1:1000 in 2 mg/mL of BSA solution, followed by a rinse and by the substrate solution (0.04% o-phenylenediamine dihydrochloride and 0.04% (v/v) hydrogen peroxide in a buffer containing 514 mM disodium hydrogen phosphate, 24.3 mM citric acid, pH 5). Color development was stopped by adding 100 L of 3 M HCl and the absorbance measured at 490-655 nm using a microplate reader.

5.6 RT-PCR and qRT-PCR

CD61+ MKs at day 13 of culture were separated, using the immunomagnetic beads technique (Miltenyi Biotec), and total cellular RNA was extracted using the Mammalian GeneElute Total RNA Kit (Sigma-Aldrich) and reverse transcribed to cDNA using MuLV reverse transcriptase (Applera; Monza, Italy) following the manufacturer's instructions. For DDR1 and beta2-microglobulin expression 1/10 of the resulting cDNA was amplified using the following primers: DDR1 5' TCTATGCTGGGGACTATTACCG 3' and 5' GTCACTCGCAGTCGTGAACTT 3', beta 2 microglobulin 5' CCCCCACTGAAAAAGATGAGT 3' and 5' TGATGCTGCTTACATGTCTCG 3'. Twenty µL of the PCR products were loaded on ethidium bromide–stained 1% agarose gel. For quantitative expression analysis of DDR1, real-time PCR was performed using the Rotorgene 6000 (Eurogentec, San Diego, CA, USA), and 1/20 of the resulting cDNA was amplified in triplicate using the MESA GREEN qPCR MasterMix Plus for SYBR assay no ROX sample (Eurogentec). Rotorgene 6000 series software Version 1.7 was used for the comparative concentration analysis [97]. For quantitative expression analysis of TGs, real-time PCR was performed using the Rotorgene 6000 (Eurogentec), and 1/20 of the resulting cDNA was amplified in triplicate using the MESA GREEN qPCR MasterMix Plus for SYBR assay no ROX sample (Eurogentec). Rotorgene 6000 series software Version 1.7 was used for the comparative concentration analysis.

5.7 Preparation of platelet lysates from washed human platelets for western immunoblotting analysis

Peripheral blood from healthy subjects was collected in citric acid/citrate/dextrose (ACD) as anticoagulant (blood:ACD, 6:1, v/v) in the presence of apyrase 0.2 U/mL and PGE1 1 µM. Platelet-rich plasma was prepared by centrifuging blood samples at 200xg for 10 min. The platelet-rich plasma was then aspirated, centrifuged at 1200xg for 10 min and washed with PIPES buffer (20mM PIPES and 136 mM NaCl, pH 6.5). Washed platelets

were then lysed in SDS buffer (Tris 0.5M, pH 6.8, 2% SDS, 10% glycerol), in the presence of a 2% protease inhibitor cocktail (Sigma), on ice for 30 min. Lysates were clarified by centrifugation at 15700 x g at 4°C for 15 min.

5.8 Immunoprecipitation and Western blotting

MKs, 1 x 10^6 cells/condition, were seeded on 25 μg/mL type I collagen coated 6 well plates or on plastic. Where indicated type I collagen was incubated with 20 μg/mL of DDR1:Fc for 4 hours at 4 °C. Cells were left to adhere for 16 hours at 37 °C and 5% CO2 and then lysed in Hepes-glycerol lisys buffer (Hepes 50 mM, NaCl 150 mM, 10% glycerol, 1% triton X-100, MgCl2 1,5 mM, EGTA 1 mM, NaF 100 mM, PMSF 1 mM, Na3VO4 1 mM, 1 g/mL Leupeptin, 1 g/mL Aprotinin). Lysates were centrifuged at 14.000 x g at 4 °C for 15 min. For immunoprecipitation cellular lysates were precleared by incubation with protein A sepharose. Precleared lysates were incubated with 2 μg of anti-DDR1 or anti-LAIR-1 or anti-SHP1 (Santa Cruz Biotechnology) for 4 hours at 4 °C on a rotatory shaker followed by adding 100 μL of 50 mg/mL protein A sepharose and incubation overnight at 4 °C on a rotatory shaker. Beads were washed three times in lysis buffer. Protein were eluted with Laemmli buffer at 90 °C for 5 minutes and separated by non-reducing SDS-PAGE for activated β1 integrin and for dimeric GPVI, or reducing SDS-PAGE for other proteins, and transferred to a polyvinylidene fluoride (PVDF) membrane. Western blots were developed with western immobilon (Millipore). Densitometric analyses were performed after ECL chemiluminescence detection using quantity one software (Bio-Rad).

5.9 Evaluation of cell adhesion

To analyze MK adhesion onto type I collagen, 12-mm glass cover slips were coated with 25 μg/mL type I collagen (provided by Prof. Tira and Dr Gruppi, University of Pavia or by Sigma) overnight at 4 ºC, and subsequently blocked with 1% BSA for 1 h at RT. Where indicated, type I collagen was incubated with 20 μg/mL of DDR1:Fc for 4 hours at 4°C, or with an unrelated IgG as control. Cells at day 13 of culture were harvested and allowed to adhere for 16 hours (1 x 10^5 cells/well) at 37 °C and 5% CO_2. Where indicated cells were pre-incubated with FcR blocking solution for 15 minutes, according to manufacturer's instructions, before being seeded. Adhering cells were washed with PBS, and after being fixed and stained with anti CD61 antibody and Hoechst 33258, were counted by fluorescence microscopy Conventional fluorescence microscopy was performed through an Olympus BX51 (Hamburg, Germany) microscope, using a 63x/1.25

or a 100x/1.30 UplanF1 oil-immersion objective. For each specimen at least 100 megakaryocytes were observed.

5.10 Migration assays

MK migration and invasion assay were performed in trans-well migration chambers (8 µm, Millipore) coated with 25 µg/mL of type I collagen containing or not 20 µg/mL of DDR1:Fc, or of an unrelated IgG as control. Briefly 50 L of collagen solution was overlaid on polycarbonate insert overnight at 4°C. MKs (25 x 10^3 in 100 µL) were seeded on the upper well in 100 µL of StemSpan and incubated at 37°C and 5% CO_2, in some experiments 5 µm R406 or 13.4 µm sodium stibogluconate were added to the medium. Where indicated cells were pre-incubated with FcR blocking solution for 15 minutes before being seeded. After 16 hours, MKs that had passed through the trans-well to the other side of the filters and in the outer wells, which contained StemSpan medium with 100 ng/ml of SDF1-α (Peprotech, London, UK), were recovered and counted by inverted microscope. Thereafter, the upper side of the filters was carefully washed with cold PBS, and cells remaining on the upper face of the filters were removed with a cotton wool swab. Trans-well filters were fixed in 3% paraformaldehyde for 20 min at RT, stained using a monoclonal antibody against CD61 and with Hoechst 33258, cut out with a scalpel, and mounted onto glass slides, putting the lower face on the top. The total number of cells that had migrated was counted and images acquired using a Olympus BX51 using 20X/0.5 UPlanF1 objective. Each experiment was performed in triplicate. Data are expressed as numbers of total migrated cells per insert or as percentages of cells related to that of the control.

5.11 Phosphatase assay

Tyrosine phosphatase assay was performed using P-NPP as substrate. Cells were lysed with Hepes-glycerol lysis buffer without sodium orthovanadate and sodium fluoride. Cell lysates and SHP1 immunoprecipitation was performed as described in "Immunoprecipitation and western blotting". Sample volumes were adjusted to analyze equal protein concentrations. For total phosphatase activity, phosphatase assay buffer was added to have a final volume of 600 L and a final concentration of 25 M of P-NPP. Samples were incubated for 150 minutes at 37 °C under shaking. The optical density (OD) was measured at 405 nm.

For SHP1 phosphatase activity, the immune complexes were washed twice with Hepes-glycerol buffer without phosphatase inhibitors and twice with phosphatase assay buffer (10 mM HEPES; 0,2 mM EDTA; 0,5% BSA; 1 mM dithiothreitol; pH 7,5). Immune complexes were incubated in 600 µL of phosphatase assay buffer with a final concentration of 25 M of P-NPP for 150 minutes at 37 °C under shaking. After centrifugation (14000 x g, 4 °C) for 1 minute, the OD of supernatants was measured at 405 nm. The corresponding immune complexes, bound to protein A–sepharose, were submitted to Western blotting to confirm the equal loading.

6. Bibliography

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