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ROLE OF novel PKCs IN PLATELET PRODUCTION AND FUNCTION

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Tutore
Prof. Silvano Capitani

Dottorando
Dott. Cecilia Carubbi

Co-tutore
Prof. Marco Vitale

Co-tutore
Prof. Giuliana Gobbi

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1. INTRODUCTION

1.1 MEGAKARYOCYTOPOIESIS

1.1.1 HAEMATOPOIESIS

Blood cells are traditionally categorized into two separate lineages: lymphoid and myeloid lineage. The lymphoid lineage consists of T, B, and NK cells, which carry out adaptive and innate immune responses. The myeloid lineage includes granulocytes (neutrophils, eosinophils, mast cells, and basophils), monocytes, erythrocytes, megakaryocytes and platelets. Because these mature blood cells are predominantly short lived, ranging from few hours for granulocytes to a couple of weeks for erythrocytes, it is demand a continued process through immature cells become differentiated blood cells which are functionally competent.

This process is named hematopoiesis, it takes place primarily in the bone marrow, and it is organized as a cellular hierarchy with a common precursor, the hematopoietic stem cell (HSC), at the apex, terminally differentiated cells on the bottom, and a series of progenitor cell intermediates that undergo a gradual fate restriction to assume the identity of a mature blood cell (Figure 1.1.1)*(Doulatov S et al. 2012)*.

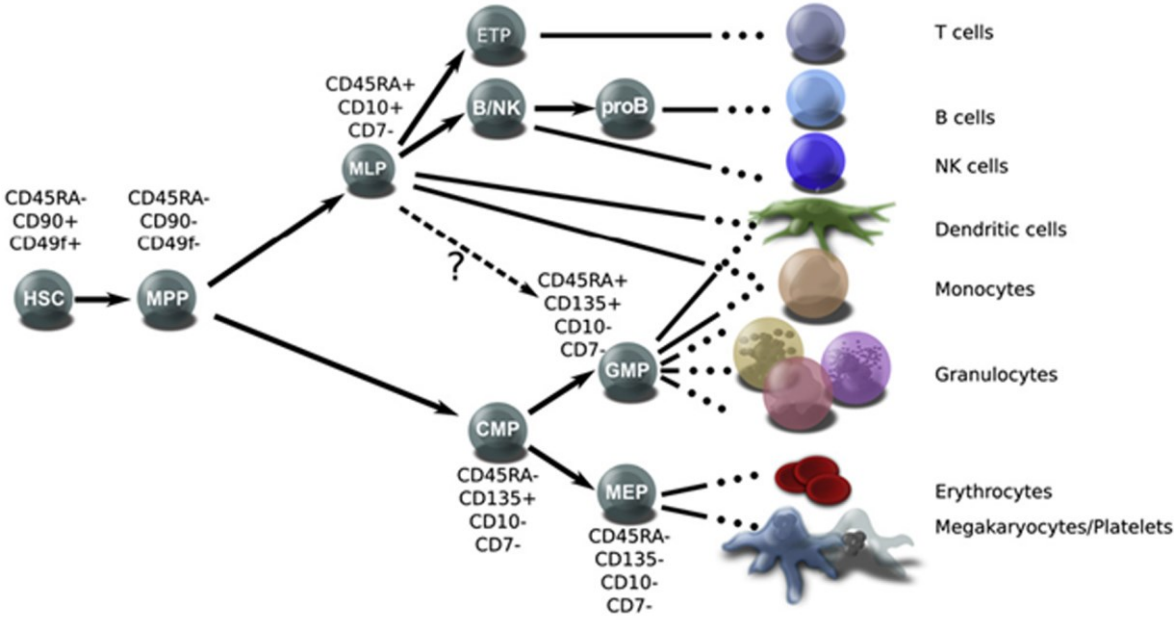

Figura 1.1.1. The diagram summarize human haematopoiesis showing lineage relationship between the different subtypes, from HSC, to mature cells; the main surface markers are displayed. (Doulatov S et al. 2012)

In human bone marrow only 1 in 10^6 cells is a transplantable HSC *(Wang et al. 1997)*, and the first marker used to identify this population is CD34 (*Civin et al. 1984),* actually, the generally accepted phenotype of human HSCs is $CD34^{+}CD38^{-}Thy1^{+}CD45^{-}$, emerged over the past decades *(Bhatia et al. 1997; Conneally et al. 1997; Lansdorp et al. 1990).* HSCs are critical for lifelong blood production and are uniquely defined by their capacity to durably self-renew, or generate daughter stem cells, while still contributing to the pool of differentiating cells. The multi potent progenitor (MPP), derived from HSC, shows high proliferative and self-renewal capacity and starts the differentiation process in hematopoiesis leading to oligopotent and later on to lineage-committed progenitors with a diminished proliferation but increased differentiation *(Notta et al. 2011)*. The contemporary model of hematopoiesis assumes that the decision for differentiation into the lymphoid/myeloid or megakaryocyte/erythrocyte lineages probably occurs very early in hematopoiesis. Several studies have demonstrated that multipotent progenitors like the

lymphoid-primed multipotent progenitor (LMPP) retain only minor megakaryocyte/erythrocyte lineage potential, whereas the vast majority of progenitors appears to be committed to the granulocyte/monocyte as well as the lymphoid lineage.

In the next step of ongoing differentiation oligopotent progenitors with differentiation capacity for several hematopoietic lineages develop from an ancestor, the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). The CLP is the earliest population in the lineage-negative fraction that upregulates the receptor for interleukin 7 (IL-7), an essential cytokine for T and B cell development. Furthermore, the CLP carries differentiation potential for all types of lymphoid cells including B cells, T cells and NK cells.

The megakaryocyte-erythrocyte progenitor (MEP) is restricted to megakaryocytes and erythrocytes.

Still a matter of dispute is the dendritic cell (DC) development, because DC mainly are the progeny of GMP, but can also be generated from lymphoid progenitors such as CLP and pro T cells under certain conditions. However, the majority of plasmacytoid DC (pDC) and conventional or myeloid DC (mDC) develop successively by several commitment steps downstream of the GMP in the bone marrow. The first step is the development of the monocyte/macrophage and DC precursor (MDP) out of the GMP that has lost differentiation potential for granulocytes. Further differentiation of MDP, which is accompanied by the loss of monocyte potential, leads to the common DC precursor (CDP) that can only give rise to pDC and mDC.

Besides the characterization of MDP and CDP by several studies, further progenitor populations for eosinophils, basophils and mast cells have been isolated downstream of the GMP and their position in the hematopoietic hierarchy is depicted.

Moreover, the monopotent megakaryocyte lineage-committed progenitor (MKP) and erythroid progenitor (EP) have been described downstream of the MEP.

Only for the monocyte/macrophage lineage and the neutrophil granulocytes, a putative committed precursor downstream of the GMP has not been identified to date.

With regard to lymphoid development one committed precursor downstream of the CLP is the bipotent T/NK cell progenitor that resides in the bone marrow and is able to generate thymic- and bone marrow-dependent NK cells as well as T cells.

Lineage commitment is to a large part mediated by small soluble extracellular protein cytokines or growth factors, but also by other extracellular factors, including direct cell-cell or cell-extracellular matrix interactions. Alternatively, it could be induced by intrinsic mechanisms, such as the stochastic up-regulation of transcription factors, or other regulatory molecules, such as microRNAs *(Laiosa CV et al. 2006)*.

Whether cytokines/growth factors can actively instruct the lineage choice of hematopoietic stem and progenitor cells or merely create a selective environment in which numbers of intrinsically committed cells are amplified by receiving survival and proliferation signals has been disputed for many years. Activation of the receptors leads to common downstream signaling events and ultimately to lineage-characteristic gene expression, depending on the intracellular context of the target cell. It is proved that most cytokine receptors activate very similar if not essentially identical downstream signaling pathways and, at the same time, cytokines can have pleiotropic effects on different cell types and even different maturation stages within the identical lineage *(Endele et al. 2012)*.

Defining the exact cocktail of cytokines required to commit HSC into lymphoid cellular subtypes is complex because several Interleukins (IL) are required: the most important and better defined cytokines involved in this process are IL-2, 4, 7, 15. IL-7 is fundamental in

the early lymphoid commitment and, with IL-2, it commits lymphoid progenitors to the T lineage; IL-4 and IL-15 are needed to commit lymphoid precursors to B and NK lineages, respectively.

Instead, stem cell factor (SCF) and Thrombopoietin (TPO) are important cytokines that enhance proliferation and of HSCs and their early commitment in CMPs; with IL-3, these cytokines also drive CMPs to differentiate into MEPs, which will be committed to the erythrocytic lineage in presence of erythropoietin (EPO) or to the megakaryocytic lineage in presence of TPO. On the other hand, granulo-monocyte colony stimulating factor (GM-CSF) pushes CMPs to differentiate in GMPs; monocyte colony stimulating factor (M-CSF) is needed to differentiate GMPs into monocytes while granulocytes colony stimulating factor (G-CSF) to differentiate GMPs into granulocytes *(Kaushansky K et al. 2006)*.

1.1.2 MEGAKARYOCYTOPOIESIS

Megakaryocytopiesis is the process by which a hematopoietic stem cell differentiates into a large progenitor cell in the bone marrow, called megakaryocyte (MK), which eventually release platelets by the extension of long, cytoplasmic branching processes, designated proplatelets, into the circulation.

Regulation of megakaryocytopoiesis

MKs develop from haematopoietic stem cells in the bone marrow and the process of differentiation is mainly regulated by thrombopoietin (TPO).

The discovery of thrombopoeitin (TPO), and its MK-specific receptor c-Mpl revolutionized the field of MK and platelet biology. In 1994, several groups discovered and cloned c-Mpl, the receptor that promotes the growth and development of MKs from their haematopoietic stem cell precursors *(Bartley TD et al. 1994; Kaushansky K et al. 1994; Kuter DJ et al. 1994; Lok S et al. 1994; de Sauvage SJ et al. 1994; Sohma Y et al. 1994; Wendling F et al. 1994)*. This discovery facilitated development of in vitro cell culture systems that reconstituted MK differentiation, proplatelet extension and platelet production, and allowed study of the mechanisms that regulate these processes *(Cramer EM et al, 1997; Lecine P et al, 1998)*. In vitro studies revealed that binding of TPO to c-Mpl results in dimerization of the receptor and activation of a number of downstream signalling pathways, including the phosphatidyli- nositide 3-kinase (PI3K), Akt, MAPK and ERK1/ERK2 path- ways *(Kaushansky K. 2005)*.

TPO was emerged as the primary regulator of thrombopoiesis. It has been demonstrated that elimination of either c-Mpl or TPO leads to severe thrombocytopenia, due to reduced MK progenitors and mature MKs as well as reduced maturation in the remaining MKs *(Solar GP et al. 1998)*. Interestingly, in most cases of thrombocytopenia, plasma TPO concentration varies inversely with platelet number *(Nichol JL et al. 1995)*. This is due to an autoregulatory loop. Platelets have c-Mpl receptors that bind to and remove TPO from plasma, and as platelet numbers rise, more TPO is removed from circulation, driving TPO levels down. Conversely, in thrombocytopenic states there are fewer platelets to adsorb TPO, allowing levels to rise and drive increased thrombopoiesis. While at first seemingly counterintuitive, this regulatory loop explains why high blood platelet levels are associated with low TPO levels, and vice versa.

More recently, it has been shown that TPO not only drives proliferation and maturation of MKs but also other bone marrow-derived progenitor cells, in particular TPO act as a

moderator of the osteoblastic haematopoietic stem cell niche expansion *(Olson TS et al. 2013)*. After clinical stem cell transplantation, haematopoietic stem cells preferentially engraft in specialized areas of bone marrow known as haematopoietic stem cell niches. Olson et al. performed radioablative conditioning in mice, and showed that host MKs are subsequently recruited to the haematopoietic stem cell niche, where they stimulate expansion and facilitate stem cell engraftment after transplantation. Interestingly, the migration of MKs to the niche is dependent on TPO signalling through the c-Mpl receptor on MKs. Likewise, inhibition of c-Mpl-dependent MK migration and function results in significantly decreased donor stem cell engraftment in murine transplantation assays *(Olson TS et al. 2013)*. This is consistent with older data showing that TPO-overexpressing mice develop a myeloproliferative phenotype similar to idiopathic myelofibrosis described in humans, with symptoms including thrombocytosis, leucocytosis and bone marrow myelofibrosis *(Yan XQ et al. 1996; Villeval JL et al. 1997)*.

Still undefined, however, is the precise cellular and molecular pathways by which TPO modulates the bone marrow environment in a way that enhances stem cell proliferation and maturation.

Other cytokines, in addition to TPO, are involved in MKs differentiation. In particular IL-3, a cytokine produced by both mast cells and T lymphocytes, can independently stimulate the early stages of megakaryocyte development up to the endomitotic phase *(Segal GM et al. 1988; Yang YC et al. 1986)*. Kit ligand, also known as stem cell factor, Cytokines such as IL-6, IL-11, and stem cell factors, also regulate stages of megakaryocyte development at multiple levels but appear to function only in concert with TPO or IL-3 *(Flanagan JG, Leder P. 1990; Briddell RA et al. 1991)*.

Megakaryocyte commitment

MKs develop from haematopoietic stem cells that reside mainly in the bone marrow, but are also found in the yolk sac, fetal liver and spleen in early development *(Long MW et al. 1982; Ogawa M.1993)*. Various classification schemes based on morphological features, histochemical staining, and biochemical markers have been used to categorize different stages of megakaryocyte development. In general, three types of morphologies can be identified in bone marrow (Figure 1.1.2)

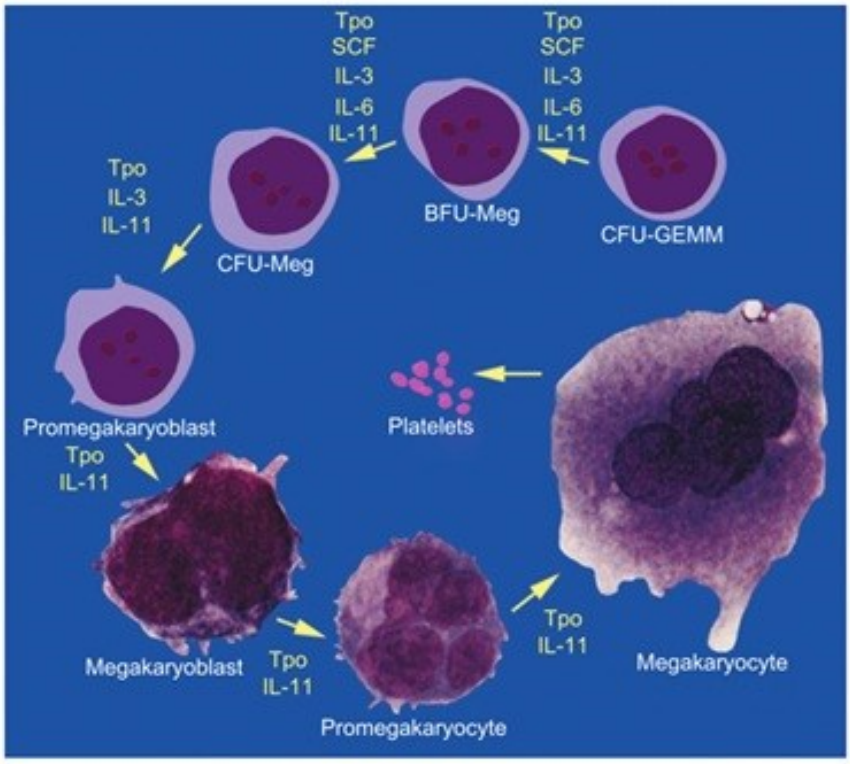

Figura 1.1.2 A schematic representation of megakaryocyte commitment. ***(Italiano JE and Hartwing JH. Platelets 3rd edition)*.**

The promegakaryoblast is the first recognizable megakaryocyte precursor. The megakaryoblast, or stage I megakaryocyte, is a more mature cell that has a distinct morphology *(Long MW et al. 1992)*. The megakaryoblast has a kidney-shaped nucleus with

two sets of chromosomes (4N). It is 10 to 50μm in diameter and appears intensely basophilic in Romanovsky-stained marrow preparations as a result of the large quantity of ribosomes, although the cytoplasm at this stage lacks granules. The megakaryoblast displays blebbing of the plasma membrane, a high nuclear-to-cytoplasmic ratio and, in rodents, is acetylcholinesterase-positive. The promegakaryocyte, or stage II megakaryocyte, is 20 to 80μm in diameter with a polychromatic cytoplasm. The cytoplasm of the promegakaryocyte is less basophilic than the megakaryoblast and contains developing granules.

In humans, MK progenitors have been characterized by clusters of differentiation markers on their surface also present in other hematopoietic progenitors, such as the CD34, CD31, and the CD133. The expression of HLA-DR enables BFU-MK (HLA-DR^{low}) to be distinguished from CFU-MK (HLA-DR^{high}) *(Briddell RA, et al. 1989)*. Most studies have focused on the main platelet glycoproteins, the GPIIb/IIIa (aIIbb3 integrin or CD41b) and the GPIb complex (GPIba, GPIbb, GPIX and GPV or CD42a, b, c and d) *(Mazur EM et al. 1981; Vinci G et al. 1984)*. CD41a (GPIIb) or CD41b (GPIIb/IIIa complex) appear relatively specific for the MK lineage, especially in adults, although mast cells can express these integrins *(Kirshenbaum AS et al. 2005)*. CD41 is present on a small fraction of marrow $CD34^+$ cells (about 3%). These $CD34^+$ $CD41^+$ cells are enriched in MK progenitors, but they do not contain all the CFU-MK. CD41 expression precedes the detection of other major platelet proteins including CD42. However, CD42 is present on a fraction of $CD34^+$ $CD41^+$ cells *(Debili N et al.1992)*. Thus, $CD34^+$ $CD41^+$ $CD42^-$ cells correspond to true CFU-MK whereas $CD34^+$ $CD41^+$ $CD42^+$ cells give rise to single MKs or clusters of less than 4 MKs. The surface expression of CD42 corresponds to a late differentiation step and is associated with a marked increase in the expression of Mpl, GPVI (collagen receptor), the a2b1 integrin (collagen receptor), CD36 and in the detection

of proteins contained in the a granules, such as PF4 or vWF. Thereafter, expression of these different platelet proteins markedly increases whereas the CD34 antigen disappears during the endomitotic process (Figure 1.1.3) *(Chang Y et al. 2007)*.

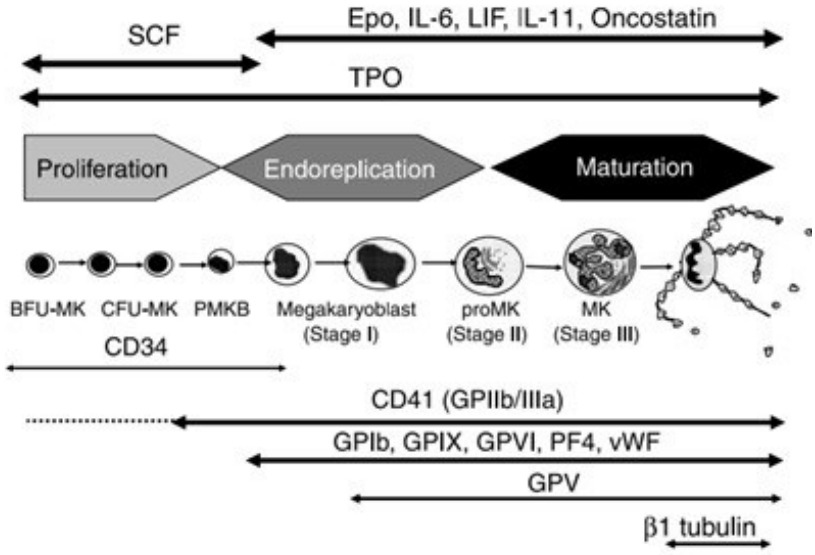

Figure 1.1.3. Expression of differentiation markers along the human megakaryocytic differentiation. (Chang Y et al. 2007)

During development, MKs increase in size, became polyploid and full of granules, expand their cytoplasmic content of cytoskeletal proteins and develop a highly invaginated membrane system.

Endomytosis and polyploidization

Endomitosis in MKs is a TPO-dependent process by which MKs become polyploid through cycles of DNA replication without cell division (Ebbe S. 1976; Gurney AL et al. 1994). During their life cycle, MKs first undergo a proliferative 2n stage in which their progression through the cell cycle is identical to other haematopoietic cells. Following which, MKs begin endomitosis and accumulate a DNA content of 4n, 8n, 16n, 32n, 64n, and even 128n in a single polylobulated nucleus before proceeding with their final

maturation and subsequent proplatelet formation (Zimmet J, Ravid K. 2000). Although the number of endomitotic cycles can range from two to six, the majority of megakaryocytes undergo three endomitotic cycles to obtain a DNA content of 16N.

Endomitosis does not result from a prematurely terminated mitosis *(Raslova H et al. 2003; Nagata Y et al. 1997; Vitrat N et al 1998) (Figure 1.1.4)*.

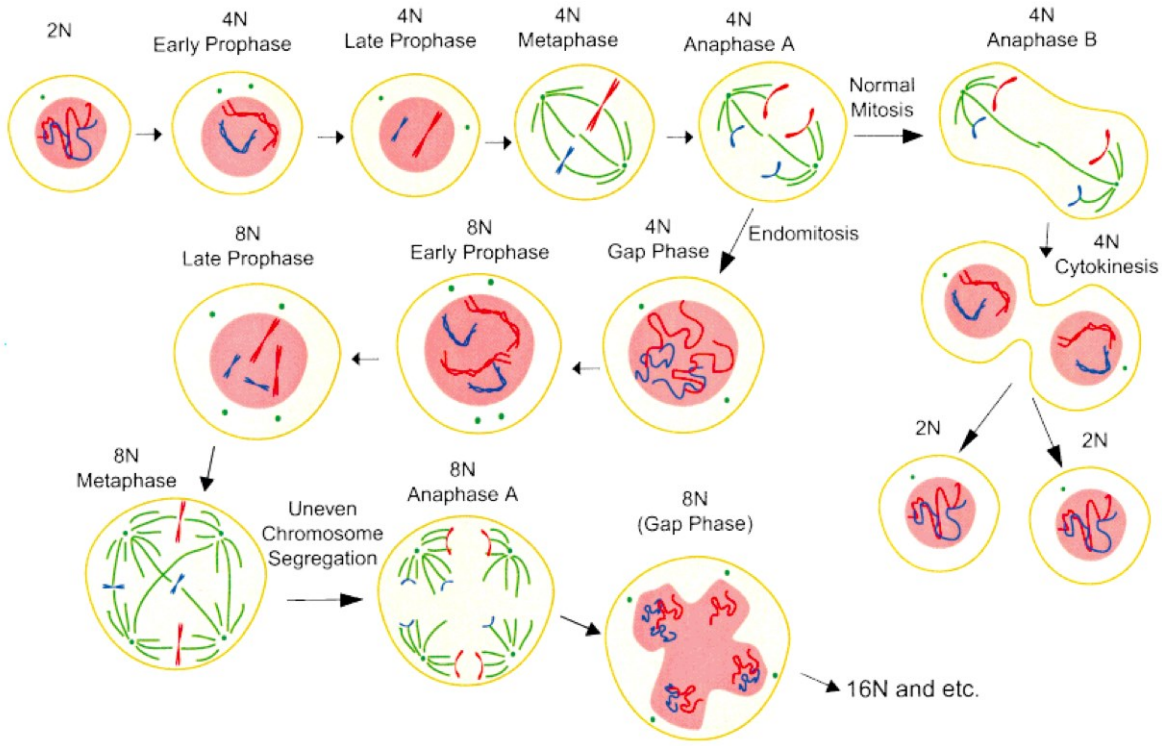

Figure 1.1.4. A schematic representation of endomitotic cell cycle in megakaryocytes. *(Ravid et al. 2002)*

Megakaryocyte progenitors initiate the cycle and undergo a short G1 phase, a typical S phase for DNA synthesis, a short G2 phase, followed by endomitosis *(Odell T et al. 1968)*. Megakaryocytes begin the mitotic cycle and proceed from prophase to anaphase A, but do not enter anaphase B or telophase or undergo cytokinesis. The nuclear envelope breaks down and an abnormal spherical mitotic spindle forms, each spindle attaches chromosomes that align to a position equidistant from the spindle poles (metaphase). Sister chromatids

segregate and begin to move toward their respective poles (anaphase A). However, the spindle poles fail to move apart and do not undergo the separation typically observed during anaphase B. Individual chromatids are not moved to the poles, and subsequently a nuclear envelope reassembles around the entire set of sister chromatids, forming a single enlarged, but lobed, nucleus with multiple chromosome copies. The cell then skips telophase and cytokinesis to enter G1. This failure to separate sets of daughter chromosomes fully may prevent the formation of a nuclear envelope around each individual set of chromosomes *(Nagata Y et al. 1997; Vitrat N et al 1998)*.

Much work has focused on identifying the signals that regulate polyploidization, highlighting a critical role for mitosis promoting factor (MPF) activity, a multiprotein complex consisting of Cdc2 and cyclin B *(Wang Z et al. 1995; Gu XF et al. 1993)*, Rho family *(Geddis AE, Kaushansky K. 2006)*, and protein-tyrosine phosphatases Shp1 and Shp2 *(Mazharian A et al. 2013)*, however a complete and definitive description of endomitosis regulator is not currently available.

Polyploidization is essential for efficient platelet production. It is theorized that MKs are polyploid in order to support cytoplasmic maturation characterized by a great production of mRNA and proteins that are packaged into granules and platelets and by a formation of invaginated membrane system (IMS) generation from which proplatelets derive *(Zimmet J, Ravid K. 2000; Machlus KR, Italiano JE. 2013)*.

Cytoplasmic maturation

After the process of endomitosis is completed, the megakaryocyte begins a maturation stage in which the cytoplasm rapidly fills with platelet-specific proteins, organelles, granules and membrane systems that will ultimately be subdivided and packaged into platelets.

In the MK, granules are derived from the budding of small vesicles containing granule cargo from the trans-Golgi network *(Blair P, Flaumenhaft R. 2009)*. Vesicles budding from the trans-Golgi network may be delivered directly to multivesicular bodies, where proteins are sorted and eventually pack- aged into granules *(Heijnen HF et al. 1998; Youssefian T, Cramer EM. 2000)*. Organelles and granules are then individually transported from the MK cell body, along the proplatelet shaft where they move bidirectionally until they are captured at proplatelet tips *(Richardson JL et al. 2005)*.

The most abundant are α granules, which contain proteins essential for platelet adhesion during vascular repair. These granules are typically 200 to 500 nm in diameter and have spherical shapes with a dark central core. They are present in early-stage megakaryocytic *(Jones OP. 1960)* and they acquire their molecular contents both from endogenous protein synthesis and by uptake and packaging of plasma proteins by receptor-mediated endocytosis and pinocytosis *(Handagama P et al. 1987)*. Endogenously synthesized proteins such as platelet factor 4, β-thromboglobulin, and von Willebrand factor are detected in megakaryocytes before endocytosed proteins such as fibrinogen. In addition, synthesized proteins predominate in the juxtanuclear Golgi area, whereas endocytosed proteins are localized in the peripheral regions of the cell *(de Larouziere V et al. 1998)*. It has been well documented that uptake and delivery of fibrinogen to α granules is mediated by integrin αIIβ3 *(Coller BS et al. 1991; Handagama P et al. 1993; Handagama P et al. 1993-2)*. Several membrane proteins critical to platelet function are also packaged into α granules, including integrin αIIbβ3, P-selectin (CD62P), and CD36. MKs also contain dense granules, approximately 250 nm in size, identified in electron micrographs by virtue of their electron-dense cores, contain a variety of hemostatically active substances that are released upon platelet activation, including serotonin, catecholamines, ADP, adenosine 5′-triphosphate (ATP), and calcium *(Youssefian T et al. 2000)*.

The maturation of MKs is characterized by the progressive formation of an elaborate membrane system that is continuous with the plasma membrane and permeates the cytoplasm. Originally, the IMS was called the demarcation membrane system (DMS) because it was thought to divide, or 'demarcate,' the MK cytoplasm into small territories from which platelets were formed *(Behnke O. 1969; Radley JM, Haller CJ. 1982)*. However, since establishing the proplatelet model of platelet release, it is more accurately referred to as the IMS to reflect its unique characteristics; the IMS is derived from the plasma membrane, retains contact with the extracellular environment and functions as a membrane reservoir for proplatelet formation *(Behnke O. 1969; Nakao K, Angrist AA. 1968)*. The IMS has also been proposed to mature into the open canalicular system (OCS) of mature platelet, which functions as a channel for the secretion of granules contents *(Nakao K, Angrist AA. 1968)*.

Platelet formation

Although it has been universally accepted that platelets derive from megakaryocytes, the mechanisms by which platelets are formed and released by these precursor cells are still largely unclear and several models of platelet formation have been proposed. (Figure 1.1.5).

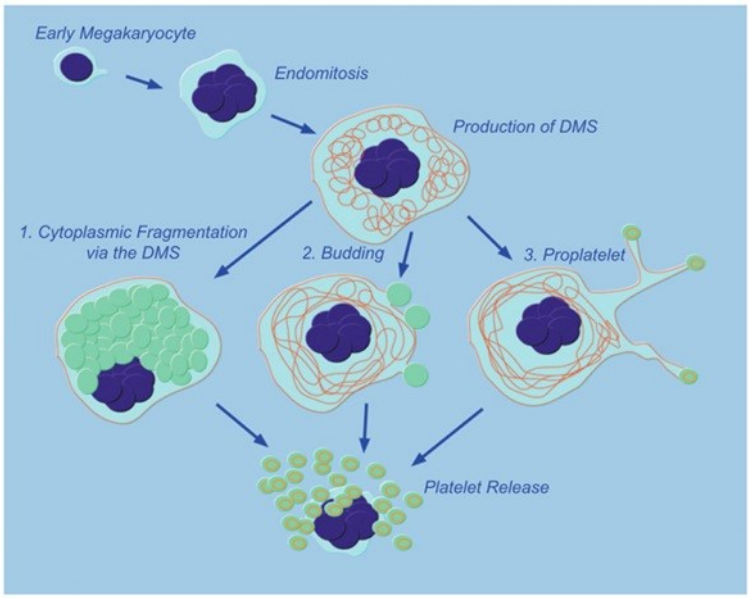

Figure1.1.5. Mechanisms proposed for platelet production *(Italiano JE and Hartwing JH. Platelets 3rd edition)*

The first model is the cytoplasmic fragmentation via the DMS. Microscopists recognized that maturing megakaryocytes became filled with membranes and platelet-specific organelles and postulated that these membranes formed a system that defined territories or fields for developing platelets *(Shaklai M, Tavassoli M. 1978)*. Release of individual platelets was proposed to occur by a massive fragmentation of the megakaryocyte cytoplasm along DMS fracture lines residing between these fields. The DMS model predicts that platelets form through an extensive internal membrane reorganization process *(Kosaki G. 2005)*. However this model has lost support because of several inconsistent observations. For example, if platelets are delineated within the megakaryocyte cytoplasm by the DMS, then platelet fields should exhibit structural characteristics of platelets, which is not the case *(Radley J, Hatshorm M. 1987)*. Platelet territories within the megakaryocyte cytoplasm lack marginal microtubule coils, the most characteristic feature of resting platelet structure. In addition, there are no studies directly demonstrating that platelet fields shatter into mature, functional platelets.

According to the second model, named platelet budding, platelets pinch off from blebs protruded at the megakaryocyte periphery. Examination of these structures by thin-section electron microscopy, however, revealed that these blebs did not contain platelet organelles, an observation inconsistent with the concept of platelet budding as a mechanism for platelet release *(Djaldetti M et al. 1979; Ihzumi T et al 1977)*.

Actually the most accreditate theory for platelet formation is the proplatelet model.

Proplatelet model

Mature MKs extend long, branching processes called proplatelets into the sinusoidal blood vessels of the bone marrow. The process commences at a single site on the MK plasma membrane. From this region, a pseudopod forms, elongates, and tapers into a tubule with an average diameter of 2– 4 um, called the proplatelet shaft. Proplatelets function as the assembly lines of platelet production, and are comprised of platelet-sized swellings in tandem arrays along the shaft *(Italiano JE et al. 1999)*. The multi-lobed nucleus remains in the MK cell body as the rest of the MK is transformed into proplatelets. After the entirety of the MK cell body is converted into proplatelets, the nucleus is extruded and degraded (Figure 1.1.6 and Figure 1.1.7).

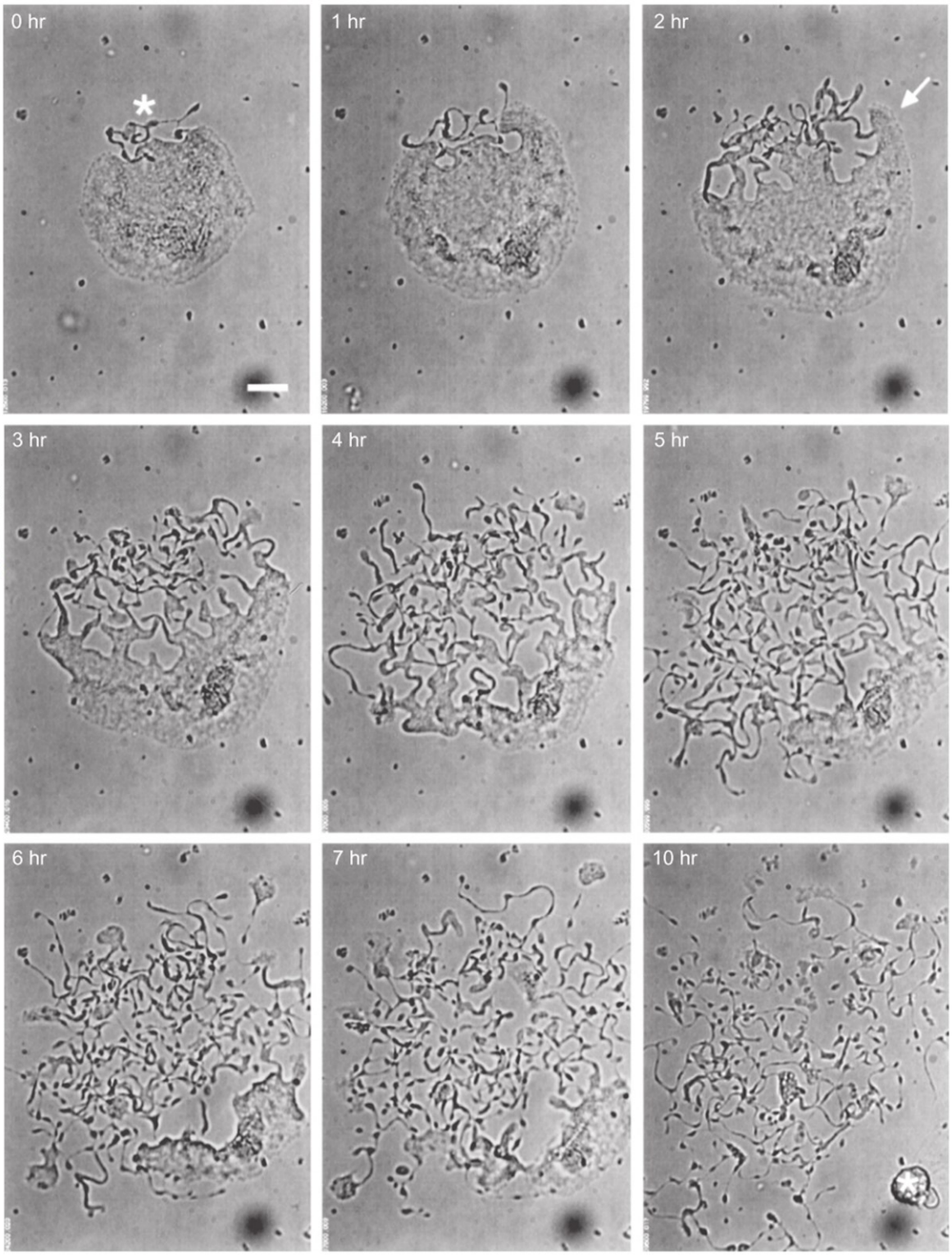

Figura 1.1.6. Video-enhanced light microscopy of mouse megakaryocyte forming proplatelet in vitro ***(Italiano JE and Hartwing JH. Platelets 3rd edition).***

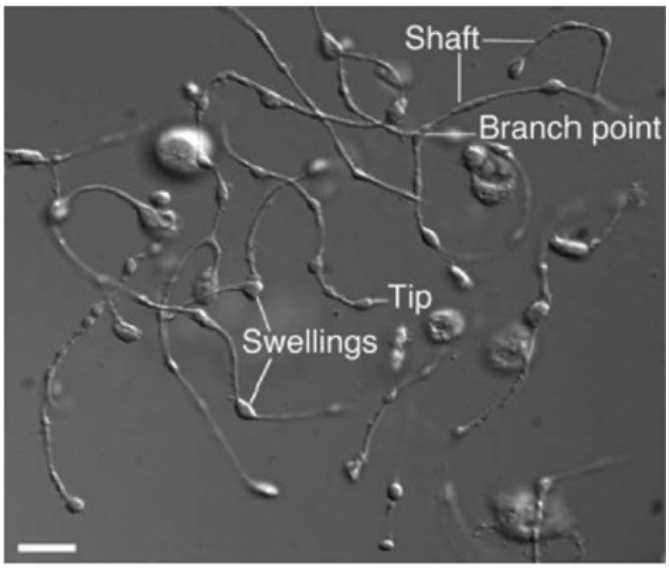

Figure 1.1.7. Proplatelet process *(Patel SR et al.2005).*

Proplatelet formation is dependent on the function of microubules. Microtubules, polymers assembled from dimers of alpha and beta tubulin, are the major components of the machine that drive proplatelet extension. Visualization of the microtubule cytoskeletons of proplatelet-producing MKs provides insights into how microtubules power platelet production. The microtubule cytoskeleton in MKs undergoes a remodelling during proplatelet production. (Figure 1.1.8).

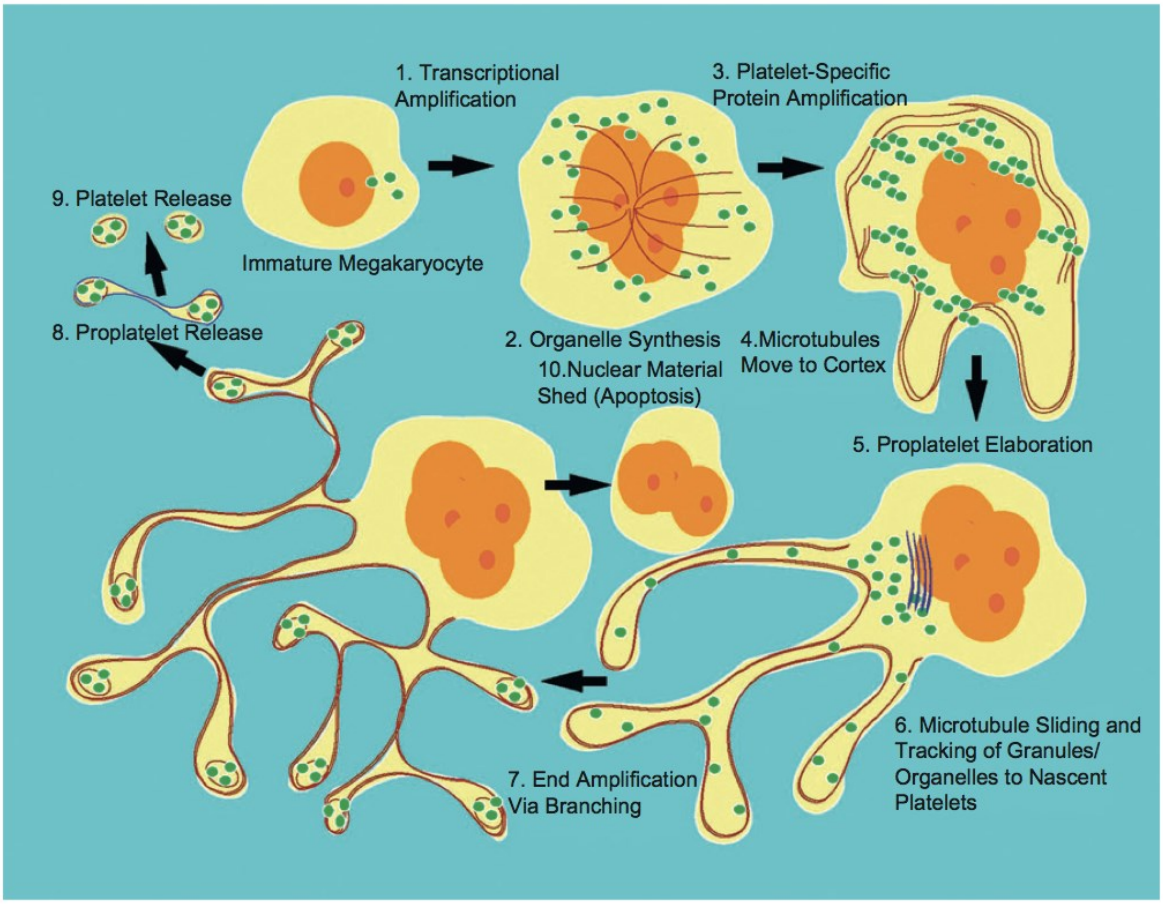

Figure 1.1.8. Propatelet model detailing some of the cytoskeletal mechanisms of platelet biogenesis ***(Italiano JE and Hartwing JH. Platelet 3rd edition)***

In immature megakaryocytes, that have not formed proplatelets, microtubules extend out from the cell centre to the cortex. As thick pseudopodia develop during the initial stages of proplatelet formation, cortical microtubules organize into thick bundles situated beneath the plasma membrane of these processes. When pseudopodia begin to elongate (at an average rate of 0.85 um/min), microtubules form thick arrays that line the entire length of the proplatelets *(Italiano JE et al. 1999)*. The microtubule bundles are thickest in the region of the proplatelet near the body of the MK, but narrow to bundles of 5–10 microtubules near proplatelet tips. The distal end of each proplatelet always has a microtubule bundle that loops just beneath the plasma membrane and re-enters the shaft to form a teardrop-shaped or tennis racket-shaped structure. Because microtubule coils

similar to those observed in blood platelets are detected only at the ends of proplatelets and not within the platelet-size beads found along the length of proplatelets, mature platelets are formed only at the tips of proplatelets *(Italiano JE et al. 1999)*. Moreover, the ends of proplatelets are amplified in a dynamic process that repeatedly bends and bifurcates the proplatelet shaft *(Italiano JE et al 1999)*. End amplification initiates when a proplatelet shaft is bent into a sharp kink that then folds back on itself, forming a loop in the microtubule bundle. The new loop eventually elongates, forming a new proplatelet shaft branching from the side of the original proplatelet. Loops lead the proplatelet tip and define the site where nascent platelets will assemble and where platelet-specific contents are trafficked. In marked difference from the microtubule-based motor that elongates proplatelets, actin-based force is used to bend the proplatelet in end amplification.

In addition to playing a crucial role in proplatelet elongation, the microtubules lining the shafts of proplatelets serve a second important function—tracks for the transport of membrane, organelles, and granules into proplatelets and assembling platelets at proplatelet ends. Individual organelles are sent from the cell body into the proplatelets, where they move bidirectionally until they are captured at proplatelet ends *(Kelley MJ et al. 2000)*.

The final step of platelet formation is represented by proplatelets extension into bone marrow vascular sinusoids, where they are released and enter the bloodstream (Figure 1.1.9)

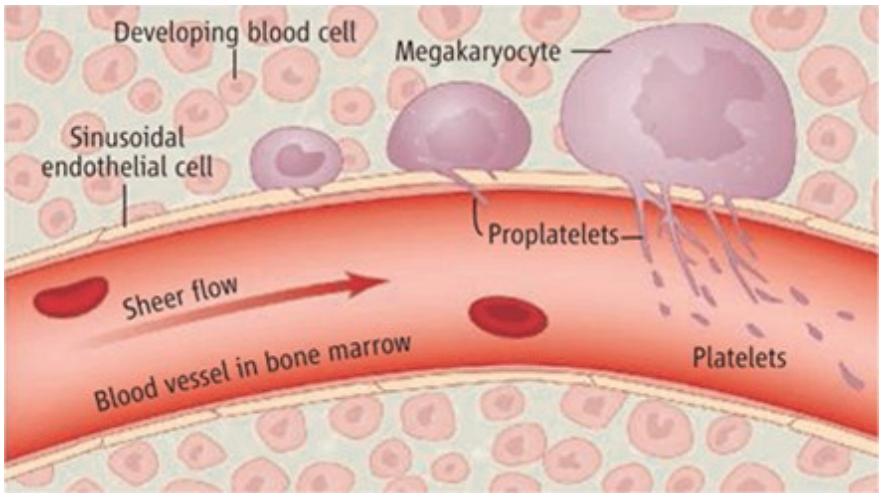

Figure 1.1.9. Megakaryocyte protruding proplatelets into bone marrow vessel. ***(Geddis AE et al. 2007)***

Notably, it has been observed that these enucleate fragments typically exceed platelet dimensions, suggesting that platelet morphogenesis continues in the circulation *(Junt T et al. 2007)*. In line with these observations, Thon and co-workers, have recently identified a previously unrecognized intermediate stage in platelet formation and release, which they termed the preplatelet *(Thon JN et al, 2010)*. Preplatelets, which appear as "giant platelets," are defined as discoid cells (3-10 microns) that retain the capacity to convert into barbell-shaped proplatelets and undergo fission into platelets. (Figure 1.1.10)

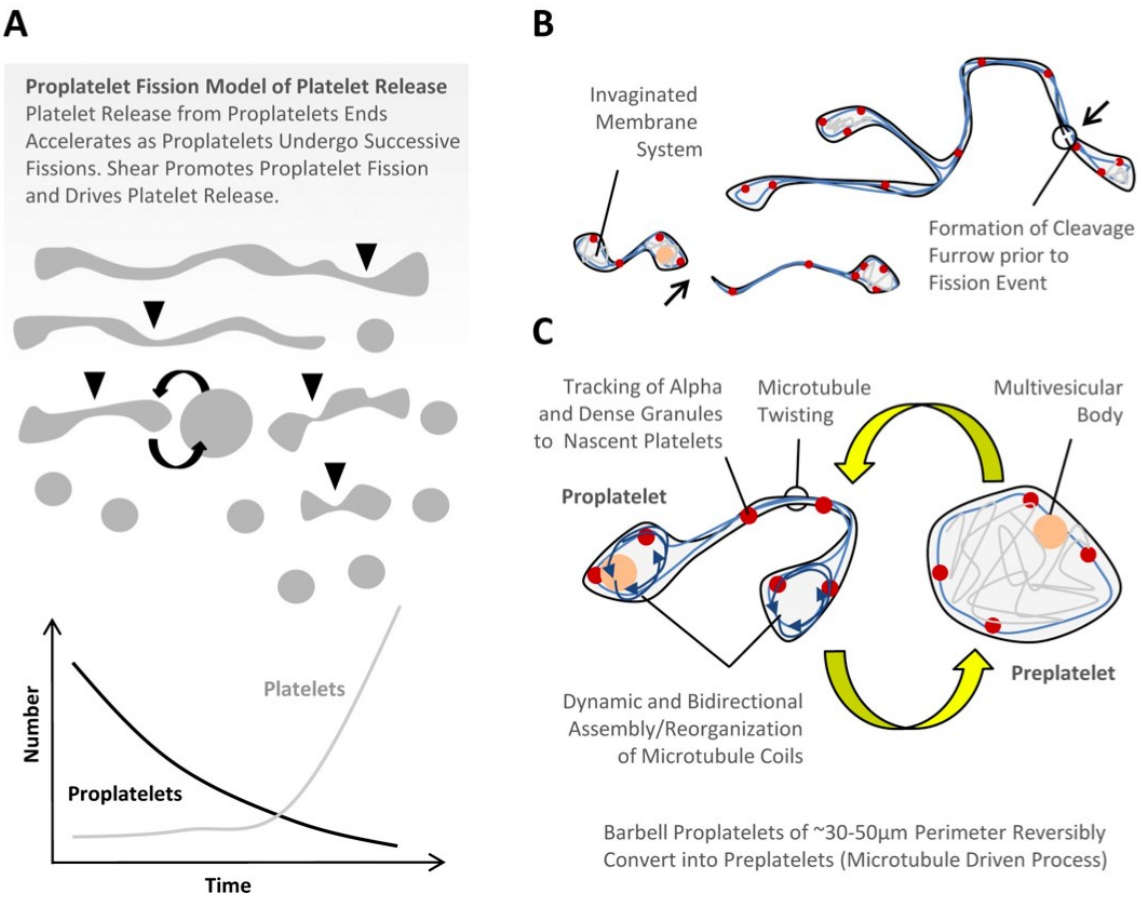

Figure 1.1.10. Proplatelet fission model of platelet release ***(Thon JL et al. 2010)***

1.1.3 BCL-2 FAMILY IN MEGAKARYOCYTOPOIESIS

There is an ongoing debate about the role of apoptosis in platelet formation, and considerable evidence has been accumulated suggesting that apoptosis is necessary for MKs to start proplatelet formation *(De Botton et al, 2002; Clarke et al, 2003)*.

Apoptosis refers to two convergent pathways of programmed cell death: intrinsic and extrinsic *(Youle & Strasser, 2008)* (Figure 1.1.11).

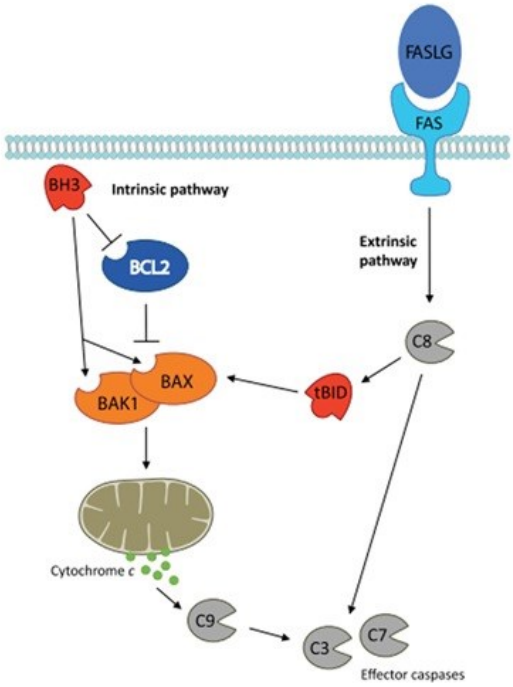

Figure 1.1.11. A schematic representation of the two pathways of apoptosis. ***(Kile BT. 2014)***

The critical regulators and effectors of the former are proteins of the BCL2 family, which are characterized by the presence of one or more BCL2 homology (BH) domains. They are divided into three groups. The first comprises the multi-domain killers BAK1 and BAX, which are the essential effectors of the intrinsic pathway. The second group are the pro-survival proteins (BCL2, BCL-XL, BCL2L2, MCL1 and BCL2A1), whose function is to prevent the activation of BAK1 and BAX. While the precise mechanisms are still being clarified, it is believed they achieve this by physically restraining BAK1 and BAX and by sequestering a third group of pro- apoptotic BCL2 family members known as 'BH3-only' proteins (BIM (BCL2L11), BID, BAD, PUMA (BBC3), NOXA (PMAIP1), BMF, BIK and HRK). In a healthy cell, BCL2 family pro-survival activity is sufficient to keep BAK1 and BAX in check. Apoptotic signals, such as DNA damage, trigger the BH3-only proteins, either by transcriptional upregulation or post-translational modification, thereby

allowing them to overwhelm the function of the pro-survival proteins and activate BAK1 and BAX. This is thought to be the result of BH3-only proteins binding to and inhibiting pro-survivals, but also by directly binding and activating BAK1 and BAX *(Willis SN et al. 2007; Ren D et al. 2010)*. Once unleashed, the latter oligomerize and induce mitochondrial outer membrane permeabilization (MOMP), facilitating the efflux of apoptogenic factors, such as cytochrome c and SMAC (DIABLO), into the cytoplasm, and eventually leading to the dissipation of mitochondrial potential. Once MOMP has occurred, the apoptotic caspase cascade is engaged, beginning with the 'initiator', or 'apical' caspase, caspase-9. Together with APAF1 and cytochrome c, the inactive caspase-9 zymogen is recruited to form the apoptosome complex, the function of which is to proteolytically activate caspase-9. Active caspase-9 then triggers the rest of the caspase cascade, culminating in the activation of the 'effector' caspases, caspase-3 and caspase-7. Caspases are the executioners of the intrinsic apoptosis pathway, mediating many of the hallmarks of apoptosis, such as DNA fragmentation, phosphatidylserine (PS) exposure and membrane blebbing. The other apoptotic pathway, the extrinsic, is activated by ligands binding to cell surface death receptors that belong to the tumour necrosis factor (TNF) receptor family, such as FAS or TNF receptor-1 (TNFR1). Once activated, the adap- tor protein FAS-associated death domain (FADD) and caspase-8 are recruited to the receptor complex. Caspase-8 is the essential mediator of the extrinsic apoptosis pathway. Following proteolytic cleavage, the activated form of caspase-8 directly triggers activation of caspase-3/7. In so-called Type I cells, such as lymphocytes, this is sufficient to induce death *(Scaffidi C et al. 1998)*. In Type 2 cells, such as hepatocytes, the additional recruitment of the intrinsic pathway is required to induce killing. This is achieved via caspase-8-mediated activation of the BH3-only protein BID, which then triggers the activation of BAK1 and BAX *(Yin MB et al. 1999; Jost PJ et al. 2009).*

In the last twenty years, the identification of thrombopoietin (TPO) and development of primary megakaryocyte culture systems led to the observation that peak platelet production by mature megakaryocytes 'corresponded to the onset of apoptosis' *(Zauli G et al. 1997)*. When mice lacking the pro-death BH3-only protein BIM *(Bouillet P et al. 1999)*, or overexpressing pro-survival BCL2 were found to be thrombocytopenic, the idea that megakaryocytes might undergo apoptosis deliberately in order to facilitate platelet shedding was born *(Ogilvy S et al. 1999)*.

Seminal work by Debili and colleagues cemented this hypothesis *(De Botton S et al. 2002)*. They reported the presence of active apoptotic caspases in the cytoplasm of cultured primary human $CD34^{+}$-derived megakaryocytes, and showedhat proplatelet formation was impaired in the presence of the fluoromethylketone caspase inhibitors Z-VAD.fmk, Z-LEDH.fmk or Z-DEVD.fmk. Moreover, transgenic megakaryocytes expressing BCL-XL under the control of the Platelet Factor 4 (PF4) promoter, produce fewer proplatelets in culture *(Kaluzhny Y et al. 2002)*.

Collectively, these data point to a productive role for the intrinsic apoptosis pathway in platelet production and, in particular, Bcl-xL expression is critical to allow the survival of the cells during the early endomitotic phase of megakaryocytic differentiation, but it must decrease to allow the platelet release process to happen, which can be considered a peculiar and productive apoptotic-like process *(Kile BT. 2014)*.

In recent years, the role of the intrinsic apoptosis pathway in megakaryocytes has been explored in detail, through studies conducted in genetically modified mice.

Spurred on by the data linking the pathway to the regulation of platelet life span, several groups have examined the requirement for BCL2 family proteins and apoptotic caspases in megakaryocyte life and death. The results to date clearly indicate that megakaryocytes, like

their anucleate progeny, possess a fully functional intrinsic apoptosis pathway. In addition to BCL-XL, megakaryocyte growth and development requires MCL1 *(Josefsson et al. 2011; Debrincat et al. 2012; Kodama et al. 2012)*. Megakaryocyte-specific deletion of BCL-XL results in severe thrombocytopenia, a reflection not only of dramatically reduced platelet life span, but aberrant platelet shedding by dying megakaryocytes *(Josefsson EC et al. 2011)*. It appears that at least in mice, megakaryocytes become dependent on BCL-XL just at the point at which they are ready to produce proplatelets. While BCL-XL-deficient megakaryocytes develop normally, they are unable to form proplatelets in vitro, and manifest striking ultrastructural defects in vivo, with large vacuolated fragments of cytoplasm being sloughed off into the sinusoids. Loss of both MCL1 and BCL-XL results in a failure of megakaryopoiesis, systemic haemorrhage and embryonic lethality, indicating that the combined functions of both pro-survivals are required for megakaryocyte growth and development *(Debrincat MA et al. 2012; Kodama T et al. 2012)*. If their role is to prevent the activation of the intrinsic apoptosis pathway, then it would be expected that deletion of BAK1 and BAX would completely rescue the defects caused by loss of MCL1 and BCL-XL, and this is indeed the case: quadruple MCL1/BCL-XL/BAK1/BAX knockout mice are healthy and exhibit normal megakaryopoiesis *(Kodama T et al. 2012)*.

A corollary of these studies is that there is little to suggest megakaryocytes require the intrinsic apoptosis pathway to produce platelets. Deletion of BAK1 and BAX does not impair thrombopoiesis, in fact platelet counts are significantly elevated due to the extended platelet life span that loss of BAK1 confers. Rather than be activated to facilitate cytoskeletal rearrangements, the intrinsic pathway must be restrained in order to survive and proceed safely through the process of platelet shedding. Treatment of mature wild-type mouse megakaryocytes in culture with ABT-737 induces mitochondrial damage and

effector caspase activation *(Josefsson EC et al. 2011)*. The result is failure of proplatelet formation and death. These effects are mediated by the intrinsic apoptosis pathway, since Bak1-/- Bax-/- megakaryocytes are resistant to ABT-737. Other apoptotic stimuli, such as the topoisomerase II inhibitor etoposide and the broad-spectrum kinase inhibitor staurosporine (STS), also trigger mitochondrial damage and caspase activation. Interestingly, however, while deletion of BAK1 and BAX can prevent etoposide- induced mitochondrial damage and caspase activation, it does not rescue proplatelet formation, suggesting that DNA damage signals suppress platelet shedding independently of the apoptotic pathway being engaged. While some differences are observed in vivo, the basic premise is the same. ABT-737 does not appear to kill wild-type megakaryocytes in situ, but in mice with a megakaryocyte-specific deletion of MCL1, it induces the fulminant apoptotic death of megakaryocytes within 3 h *(Debrincat MA et al. 2012; Kodama T et al. 2012)*. These data suggest there might be subtle differences in MCL1 expression or protein-protein interactions in cultured megakaryocytes versus their counterparts in vivo, potentially the result of the latter's exposure to a range of pro-megakaryocytic cytokines and growth factors. Regardless, activation of apoptosis is accompanied by a failure of platelet shedding and severe thrombocytopenia. As expected, deletion of BAK1 and BAX renders MCL1-deficient megakaryocytes entirely refractory to the effects of ABT-737 in vivo *(Kodama T et al. 2012)*.

Finally, data obtained with transgenic mice are in part contradictory in respect to previous observations on cultured primary human CD34+-derived megakaryocytes. This may reflect differing experimental procedures and types of MKs used in the studies, and so the role of Bcl-2 family in megakaryocytopoiesis and platelet production needs to be clarified.

1.1.4 PLATELET PRODUCTION FOR INFUSION

Platelets are essential for haemostasis, accordingly, patients with low numbers of circulating platelets or functionally hyporeactive platelets are at increased risk of spontaneous bleeding or hemorrhage following traumatic injuries or during surgical procedures. Thrombocytopenia (defined as platelet counts <150000/ul) is a major clinical problem encountered across a number of conditions, including immune thrombocytopenic purpura, myelodysplastic syndromes, chemotherapy, aplastic anaemia, human immunodeficiency, virus infection, complications during pregnancy and delivery, and surgery.

The therapeutic strategy to prevent severe bleeding is platelet transfusion. The first evidence supporting the use of platelet transfusion was reported in 1910 and since 1950 was developed the ability to prepare platelet concentrate that are currently used in patients with severe thrombocytopenia *(Duke WW. 1983; Dillard GH et al. 1951)*. The use of transfused platelets has increased more than other blood components in the last two decades *(Lu SJ et al. 2011)*, they derive entirely from human donors however, there are several limitations and challenges with platelet preparation and storage technologies. One of these limitations is the dependence upon a large donor pool. Because of the limited donor supply, there is often a shortage of available blood products. Compounding the challenges of availability is the half-life of transfused platelets (3 days), which is significantly shorter than the half-life of platelets in vivo (approximately 8-12 days) *(Dumont L et al. Rossi's principles of transfusion medicine. 4th ed. AABB/Blackwell; 2009)*. Transfused platelet units also have approximately 50% recovery *(Filip DJ, Aster RH. 1978)*. Therefore, approximately twice as many platelets need to be given to achieve the expected platelet count rise. Finally, platelet reactivity decreases with time of storage:

among the mechanisms implicated are platelet activation and apoptosis *(Ohto H, Nollet KE. 2011; Egidi MG et al. 2010).* This poor recovery and decreased platelet half-life and reactivity with time is termed the "platelet storage lesion", and it is reduced by room temperature storage *(Ohto H, Nollet KE. 2011)*. On the other hand, because of the need to store at room temperature, bacterial contamination of platelets is a significant issue10 with the result that platelets have a short shelf life of 5 days. In addition, over 10% of multiply transfused patients develop decreased responsiveness to platelet transfusion with time (platelet refractoriness). *(Bordin JO et al. 1994)*.

On these basis, a long-term goal for the field of hematology is to obtain an unlimited supply of platelets for transfusion making platelet *ex vivo* and several strategy are under study.

The ability to grow ex vivo of human megakaryocytes derived from peripheral blood stem cells was first described in 1990 when Mazur and colleagues used serum from aplastic dogs to stimulate megakaryocyte development *(Mazur EM et al. 1990)*. With the isolation and identification of thrombopoietin in 1994 *(de Sauvage FJ et al. 1994; Wendling F et al. 1994)* and the subsequent cloning and generation of recombinant TPO from both humans and mice *(Sohma Y et al 1994; Lok S et al. 1994; Kuter DJ et al. 1994)*, it became possible to culture sufficient megakaryocytes both for studies of their biology and also to contemplate harvesting ex vivo-generated platelets for clinical application. The first report of functional human platelets obtained by a two-step culture process was in 1995 *(Choi ES at al. 1995)*, showing that megakaryocytes, larger proplatelets, and smaller, platelet-sized particles could be generated in vitro from CD34+ peripheral blood cells in the presence of appropriate cytokines. These ex vivo-generated platelets have normal platelet morphology, surface expression of glycoproteins, and ultrastructural characteristics, including the presence of alpha, dense, and lysosomal granules and are responsive to agonist stimulation

(Guerriero R et al. 1995; Tao H et al. 1999). However it has been difficult to assess the in vivo half-life, functionality, ability to significantly raise platelet counts, and potential to reverse a bleeding diathesis, in large part due to the difficulty harvesting these ex vivo-generated platelets. One strategy for large-scale production of non- donor-derived platelets would be to have a continuous supply of homogenous progenitors that, upon appropriate stimulation, differentiate into megakaryocytes. The development of human embryonic stem cells (hESCs) *(Thomson JA et al.1998)* and induced pluripotent stem cells (iPSCs) *(O'Malley J et al. 2009)* provides a renewable source of cells with the potential to expand indefinitely in culture and eliminate the need for human donors. Platelet produced from hESCs, for the first time in 2006, and from iPSCs, for the first in 2010, show morphological features comparable to plasma-derived human platelet, the ability to respond to platelet agonists, and to be incorporated into a developing thrombus *in vivo (Gaur M et al. 2006; Zingariello M et al. 2010; Takayama N et al. 2008; Shattil SJ et al. 1985; Lu SJ et al. 2011)*. Despite encouraging first steps in generating clinically useful platelets beginning with hESC/iPSCs, two major roadblocks have been identified. The first is that the number of megakaryocytes generated per initial progenitor cell is orders of magnitude too low, beginning with one progenitor cell <10 megakaryocytes are obtained *(Reems JA. 2011)*. The second roadblock is the efficiency of platelets produced per megakaryocyte. In order to increase in vitro platelets production yields, recently a microfluidic platelet bioreactor has been developed: it recapitulates bone marrow stiffness, extracellular matrix composition, micro-channel size, hemodynamic vascular shear stress, and endothelial cell contacts, and supports high-resolution live-cell microscopy and quantification of platelet production. Physiological shear stresses triggered proplatelet initiation, reproduced ex vivo bone marrow proplatelet production, dramatically increasing the amount of producing platelets MKs *(Thon JN et al. 2014)*.

Nonetheless, due to the clinical and economical interests, further studies on megakaryocytopoiesis and platelets production biology need to be performed in order to discover optimal conditions of culture and achieve an increased platelets yields.

Beside bioreactors, other different approaches must be tested, such gene therapy or pharmacological approaches that are rationally designed to interact with pathways involved in the megakaryocytopoiesis and platelets production.

1.2 PLATELETS

In 1862, Giulio Bizzozero, the so-called 'father of the platelet', described a novel 'morphological element' in the blood with important roles in haemorrhage and thrombosis *(de Gaetano G. 2001)*. These elements became known as platelets, and they are the smallest, circulating, anucleate cells that are derived from megakaryocytes within the bone marrow. There are 150–400 × 10^9 platelets per litre of blood and because the lifespan of an individual platelet is only 8–10 days, 100 billion new platelets must be produced daily from bone marrow megakaryocytes in order to maintain normal platelet counts *(Kaushansky K. 2006)*.

Their primary physiological role is to sense damaged vessel endothelium and accumulate at the site of the vessel injury, where they initiate blood clotting to block the circulatory leak. Platelets circulate at high-shear rates and are activated following binding to the collagen substratum or to other extra- cellular matrix proteins that are exposed during vascular injury. Stable adhesion to collagen promotes the release of many soluble mediators from the platelet's intracellular stores, leading to further platelet recruitment and activation. These events are regulated by complex interactions involving several families of molecules, including various selectins, integrins, lipids and cytokines. Together with leukocytes and red blood cells, the activated platelets create a thrombus that arrests blood loss *(Gawaz M. 2006)*.

1.2.1 PLATELET STRUCTURE

Platelets circulate in blood with a typical discoid shape, the surface of each disc is featureless, lacking protrusions. Plug formation requires platelets to undergo rapid morphological changes as they convert from their resting discoid forms to their active

shapes. The first event that is observed as the platelet makes contact with the surface, is that the discoid shape is lost and becomes rounded or spheroid in form. Next, fingerlike projections grow from the cell periphery, then the platelet flattens over surfaces and broad lamellae are extended. (Figure 1.2.1).

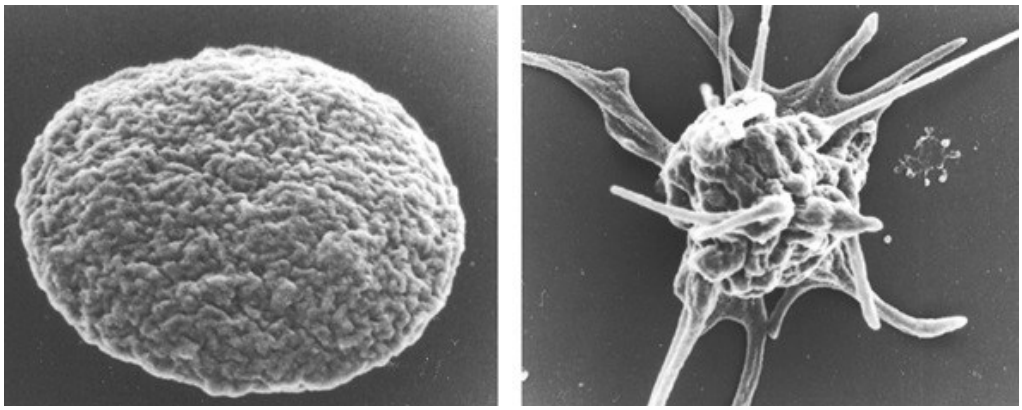

Figura 1.2.1. Platelet shape change in resting and activated states *(White JC. Platelets 3rd edition)*

Platelet structure should be divided in:

- peripheral zone
- sol-gel zone
- platelet membrane systems

Pheripheral zone

The peripheral zone is formed by an exterior glicocalix and a lipid bilayer.

The lipid bilayer is a typical unit membrane that does not differ in appearance from the membrane covering other cells *(White JG, Conrad WJ. 1973)*, it is incompressible and cannot stretch, but it is significantly different because of its role in the acceleration of blood coagulation participating to thrombin generation. Clotting is initiated at the site of vascular injury by tissue factor (TF), which serves as a cofactor for the coagulant protein,

factor VIIa, in converting factor X to Xa. Factor Xa, in association with factor Va, converts prothrombin to thrombin on the surface of the anionic phospholipid, phosphatidylserine. Platelet lipid bilayer plays a key role in this process because phosphatidylserine is provided by surface of activated platelets, and, on the other hand, platelets also contain TF encrypted with the cholesterol-rich lipid islands in the resting platelet surface membrane *(Heijnen HF et al. 1998)*.

The platelet glicocalix is thicker then in other blood cells and this exterior surface does not serve merely as a barrier to separate internal contents of platelets from the external milieu; rather, it is a very dynamic structure that serves as the site of first contact and senses changes in the vascular compartment requiring the hemostatic response of platelets at sites of vessel injury. The glycocalyx is covered with major and minor glycoprotein receptors necessary to facilitate platelet adhesion to a damaged surface, trigger full activation of the platelet, promote platelet aggregation and interaction with other cellular elements, and accelerate the process of clot retraction *(Clemetson KJ et al. 1985; Carrell NA et al. 1985; Kunicki TJ. 1988)*.

Platelet receptors include integrins, Leucine-Rich repeat family, Seven Trans-membrana Receptors and Immunoglobulin superfamily.

Among integrins, the GpIIbIIIa complex, also named αIIbβ3, is the only integrin expressed uniquely on platelets and megakaryocytes. αIIbβ3 represents 3% of the total platelet protein and 17% of the platelet membrane protein mass with 50000 to 100000 copies per platelet *(White JG, Krumwiede M. 1985; White JG et al. 1986; White JG, Sauk JJ. 1984)*. Platelet α-granule membranes also contain αIIbβ3, and this pool can become accessible and is functional upon platelet stimulation *(White JG, Krivit W. 1967; Whie JG. 1968)*. Central to the function of integrin αIIbβ3 is its capacity to undergo activation, a transition from a low-affinity state (resting state) to a high-affinity state (active state) for its extra-

cellular ligands during platelet activation and clot formation. When platelets encounter any one of a number of agonists, a series of intracellular signaling events are triggered that converge at the cytoplasmic tails (CT) of αIIbβ3. Changes in the CT are then transmitted across the platelet membrane and transform the extracellular domain of αIIbβ3 into a high-affinity state for its extracellular ligands. As a consequence, αIIbβ3 can now bind divalent fibrinogen or multivalent von Willebrand factor (VWF) *(Hynes RO. 1992)*, which can bridge platelets together to form aggregates. Recognition of other ligands, such as vitronectin, fibronectin, and thrombospondin *(Hynes RO. 1992; Plow EF et al. 2000; Plow EF, Shattil SJ. 2001; Reheman A et al. 2005)*, by αIIbβ3 may mediate platelet adhesion to components of the subendothelial matrix and regulate platelet aggregation.

The α2β1 is an integrin also known on platelets as GPIa-IIa and on lymphocytes as VLA2 *(Staatz WD et al. 1989)*. There are 2000 to 4000 copies per platelet. α2β1 is well established as a major collagen adhesion receptor on platelets and a wide range of other cells.

Other platelet integrins are α5β1 and α6β1, that represent respectively fibronectin and laminin receptor, they have a supplementary role in in platelet adhesion at injury sites *(Piotrowicz RS et al. 1988; Sonnenberg A et al. 1988)*.

The leucine-rich repeat (LRR) family is represented in platelets mainly by the GPIb-IX-V complex. The GPIb-IX-V is the second most common receptor, in platelet with approximately 50000 copies per platelet. Functions of GPIb-IX-V include regulating platelet adhesion to subendothelial matrix, endothelial cells or leukocytes, assembly of procoagulant activity on activated platelets, and signaling, mediated in part by association of GPIb-IX-V with accessory signaling receptors and localization in lipid rafts. GPIb-IX-V is the most important receptor for VWF and exposure of vascular subendothelium under

high shear results in the immediate attachment of GPIb-IX to von Willebrand factor (VWF) covering collagen fibers in the wound *(White JG. 1987).*

In platelet, Seven Trans-membrana Receptors include thrombin receptors, ADP receptors, tromboxane and prostaglandines receptors.

Thrombin receptors (PARs) are major representatives of the seven-transmembrane receptor group on platelets, because thrombin is a critical platelet agonist. Human platelets have about 2,500 copies of the PAR1 receptor, which responds to levels of thrombin of about 1 nM and a lower number of copies of PAR4 *(Andersen H et al. 1999)*, which is sensitive to concentrations of thrombin 10 times higher than PAR1.

P2Y1 and P2Y12 are platelet ADP receptors. ADP is one of the earliest identified primary platelet agonists. It has also a critical autocrine role, via secretion from dense granules. Recently, ADP receptors have been definitively identified.

One of the major member of platelet immunoglobulin superfamily, is GPVI. This protein is exclusively expressed on platelets and megakaryocytes with 4000-6000 copies per platelet. It is a low affinity collagen receptor and it actives a second collagen receptor, integrin α2β1. The binding of collagen to integrin α2β1 contributes to stable adhesion and reinforces binding to GPVI *(Furihata K et al. 2001; Best D et al. 2003; Samaha FF et al. 2005; Senis YA et al. 2009).* The immunoglobulin superfamily also includes:

- FcγRIIa (CD32), which is a low-affinity receptor for the IgG Fc domain in platelets, has a role in heparin-induced thrombocytopenia *(Carlsson LE et al. 1998).* Antibodies are developped against the heparin/platelet α-granule chemokine (platelet factor 4) complex, inducing platelet activation via FcγRIIa and, eventually, leading to thrombocytopenia.

- FcεRI, the high-affinity receptor for IgE, is a platelets activator *(Joseph M et al. 1997; Hasegawa S et al. 1999).*

Integrin-associated protein (CD47) is a receptor for the cell-binding domain of thrombospondin (TSP). TSP can mediate platelet activation and aggregation.

The endothelial cell-selective adhesion molecule (ESAM), that localizes to platelet-platelet contacts, regulates thrombus formation *in vivo (Stalker TJ et al. 2009)*.

Sol-gel zone

The sol-gel zone consist of a meshwork of fibrous material in which formed organelles are imbedded.

Fibrous material represents platelet cytoskeleton that serves as a system of molecular struts and girders that defines the discoid shape of the resting platelet and maintains cell integrity as platelets encounter high fluid shear forces generated by blood flow over the endothelium. Critical components of this system are, from the plasma membrane inward, a spectrin-based skeleton *(Boyles J et al.1985; Fox J et al. 1988)*, a microtubule coil and a rigid network of crosslinked actin filaments. (Figures 1.2.2, 1.2.3, 1.2.4).

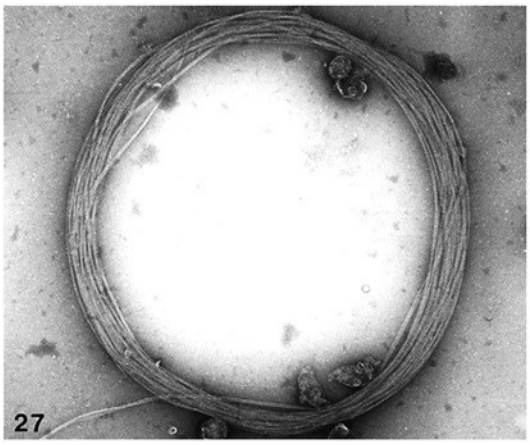

Figure 1.2.2. Microtubule coil isolated from human platelets after simultaneous fixation and detergent extraction in suspension. The coil has many loops but appears to consist of one microtubule. Mag X 13000. ***(White JC. Platelets 3rd edition)***

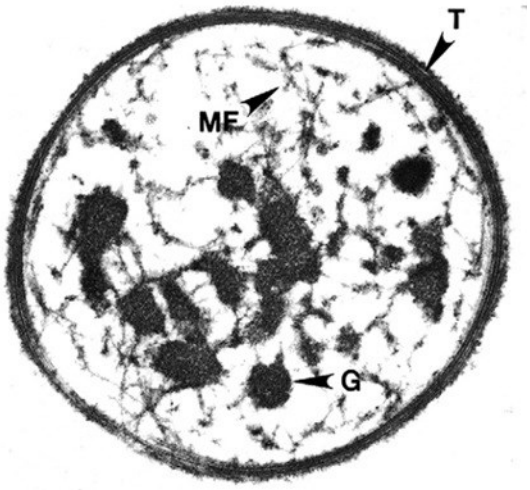

Figure 1.2.3. Thin section of a discoid platelet cytoskeleton fixed after detergent extraction in presence of lysine and phalloidin. The microtubule is well preserved (T). Remnants of α granules (G) are suspended in a matrix of microfilaments (MF) resistant to detergent extraction. Mag X 22000. ***(White JC. Platelets 3rd edition)***

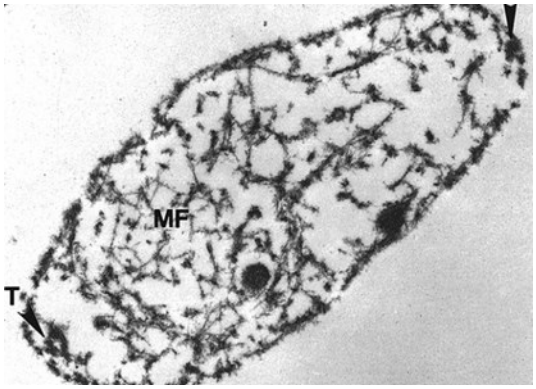

Figure 1.2.4. Cross-section of a discoid platelet cytoskeleton. The cell is from a sample of C-PRP treated with Taxol (1024 M) before extraction. Microtubule (T) profiles are evident at the polar ends of the cell. A meshwork of microfilaments (MF) replaces the cytoplasmic matrix. Although the surface membrane is gone, a fine amorphous layer remains in its place, probably submembrane filaments. Mag.X22,000. *(White JC. Platelets 3rd edition)*

A spectrin-based skeleton is a two-dimensional assembly of spectrin strands that is adherent to the cytoplasmic side of the plasma membrane. It shows both binding sites for membrane glicoprotein and for actin, connecting platelet membrane to the actin network *(Glenney J, et al. 1982; Gratzer W. 1978; Wasenius VM et al. 1989; Winkelmann J et al. 1990).*

Platelets contain a long microtubule, composed by tubulin molecules, that is approximately 100 μm in length and 25nm in diameter. To fit the long microtubule inside the resting platelet, it is wound from 8 to 12 times into a coil. The coil sits in the cytoplasm, just beneath the plasma membrane, along the thin edge of each disc. This microtubule coil is a cytoskeletal support system, responsible for distorting the cell into a discoid shape.

The third components of platelet cytoskeleton is a network of crosslinked actin filaments, also named microfilaments. Actin is the most abundant of all the platelet proteins. The concentration of actin in a platelet is 0.55 mM, which translates into approximately two million copies per platelet *(Nachmias VT. 1980)*. Of these molecules, 800000 assemble to

form the 2000 to 5000 linear actin polymers that form the cytoskeleton of the resting cell *(Hartwig J, DeSisto M. 1991)*. All evidence indicate that the filaments of the resting platelet are interconnected at various points to form a mechanically rigid cytoplasmic network. Indeed, platelets express high concentrations of actin cross-linking proteins, including filamin (FLNa and FLNb) and α-actinin *(Feng Y, Walsh CT. 2004; Stossel T et al. 2001)*. In the resting cell, the microfilament network serves as the matrix on which all organelles and other structural components are suspended and maintained separate from each other *(Escolar G et al. 1986)*. Following platelet activation, a large amount of actin molecules polymerize, and microfilaments have a unique role in contractile physiology. They constrict the circumferential microtubule coils and drives the α granules and dense bodies into close association in platelet centers. If stimulation is strong enough, granule and dense body contents are secreted to the exterior via channels of the OCS *(Escolar G et al. 1986; White JG, Krumwiede M. 1987)*, leaving behind a dense, central mass of actin.

All platelet organelles, such as mitochondria, ribosome and Golgi complex, are imbedded in this network of microtules and microfilaments; in particular platelet cytoplasm is rich of three major types of secretory organelles, α granules, dense bodies (δ granules) and lysosomes.

α-Granules are unique to platelets and are the most abundant platelet granules. There are approximately 5 α-granules per platelet, ranging in size from 200 to 500 nm *(Frojmovic MM, Milton JG. 1982)*. They constitute approximately 10% of the platelet volume and the total α-granule membrane surface area per platelet is 14 μm. Morphologic features, that have historically defined α-granules, include: (1) the peripheral membrane of the granules, (2) an electron-dense nucleoid that contains chemokines and proteoglycans, (3) a less electron-dense area adjacent to the nucleoid that contains fibrinogen, and (4) a peripheral electron-lucent zone that contains VWF *(Harrison P, Cramer EM. 1993)*. α-granules

contain both membrane bound proteins, that become expressed on the platelet surface after membrane fusion, and soluble proteins, that are released into the extracellular space. This granule content include integral membrane proteins (αIIbβ3, GPIbα-IX-V, GPVI, P-selectin), coagulant and anticoagulants proteins (Factor V, factor IX, factor XIII, plasminogen, plasminogen activator inhibitor), adhesion proteins (Fibrinogen, von Willebrand factor, thrombospondin), chemokines, growth factors, angiogenic factors. *(Berger G et al. 1996; Maynard DM et al. 2007; Nurden P et al.2004)*.

Human platelet dense bodies (DB) are smaller than the α-granules (diameter of 150nm), are fewer in number (3-8 per platelet), and have high morphological variability *(Berger G et al. 1996)*. Their most distinguishing feature is the electron-opaque spherical body within the organelle, which is however separated from the enclosing membrane by an empty space. This feature led to the term "bull's-eye" being used to describe the dense body. However, some DB are irregular in shape, have filaments extending from the dense inner core to the enclosing membrane, or contain a granule-like substance filling the usually empty space. Platelet dense granules contain Adenine nucleotides, ADP and ATP, polyphosphates, bioactive amines, such as serotonin and histamine *(Holmsen H, Weiss HJ. 1979; Ruiz FA et al. 2004)*. Dense granule membrane proteins include those that are typically sorting into lysosome-related organelles such as CD63 (granulophysin) and LAMP-2. Several platelet plasma membrane have also been identified in dense granule membranes, including GPIb and αIIbβ3 *(Youssefian T, et al. 1997)*.

Platelets contain few primary and secondary lysosomes, approximately 3 lysosmes per platelet with a diameter of 200-250nm. Platelet lysosome function is not well studied. It is possible that lysosomes may have a role in endosomal digestion, as observed in nucleated cells. Indeed, lysosomes contain protein-degrading enzymes (Cathepsins, elastase, collagenase, carboxypeptidase, proline carboxypeptidase), carbohydrate-degrading

enzymes (Glucosidase, fucosidase, galactosidase, glucuronidase, mannosidase, hexosaminidase, arabinofuranosidase), and phosphate ester-cleaving enzyme (acid phosphatase). Their membrane expresses CD63 and LAMP-2.

Membrane system

The platelet membrane system includes the Open Canalicular System (OCS) and the Dense Tubular System (DTS). The OCS is not merely connected to the platelet surface membrane; it is a continuum with the surface membrane. *(Behnke O. 1967; Behnke O. 1968; Breton-Gorius J. 1975)*. OCS channels are tortuous invaginations of surface membrane tunneling through the cytoplasm in a serpentine manner. Channels of the OCS greatly expand the total surface area of the platelet exposed to circulating plasma and provide a means for chemical and particulate substances to reach the deepest recesses of the cell *(Frojmovic MM et al. 1992; White JG. 1972)*. As a result, the OCS may be the major route to uptake and transfer products, such as fibrinogen, from plasma to platelet α granules. *(White JG, Escolar G. 1991; Escolar G, White JG. 1991)*. In addition, channels of the OCS serve as conduits for the discharge of products stored in secretory organelles during platelet activation. Channels of the OCS also have major roles in the platelets' hemostatic reaction. After adhesion to a damaged vascular surface, platelets extend filopodia to bind firmly the injured area, which is followed rapidly by the assembly of cytoplasmic actin and the spreading of the platelet to cover as much area as possible. The result is an increase of up to 420% of its exposed surface area during conversion from a discoid platelet to a fully spread cell. Evaginated channels of the OCS are the major source of membrane for the expanded surface area of the spread platelet *(Escolar G et al.1989)*.

Channels of the DTS are distinguished from clear canaliculi of the OCS by an amorphous material similar in opacity to surrounding cytoplasm concentrated within them *(Behnke O.*

1967) that stains for peroxidase *(White JG. 1972)* and binds ead, indicating calcium binding sites and enzymes involved in prostaglandin synthesis *(Gerrard JM et al. 1976)*. Like the OCS, channels of the DTS are randomly dispersed in the platelet cytoplasm. In addition, a channel or two of the DTS can be identified in close association with the circumferential band of microtubules in most thin sections of platelets. The DTS originates from rough endoplasmic reticulum in the parent megakaryocyte and is therefore residual smooth endoplasmic reticulum. There is no physical communication between channels of the OCS and DTS. The physiological role of DTS is not full understand.

1.2.2 PLATELET TRANSCRIPTOME AND PROTEOME

Proteins are the chief effectors of cell function. The total complement of proteins present in a cell at any given time is known as its proteome, which is under regulatory control and varies from cell to cell. The cell proteome is specified by information encoded by nuclear DNA, which is transcribed into messenger RNA (mRNA). The total amount of mRNA is named transcriptome and cells use template mRNAs to synthesize proteins through a process referred to as translation.

Because platelets lack nuclei, transcription of mRNA is not an option outside of the mitochondria, and so the about 3000-6000 transcripts identified in platelets, including microRNA are transcribed in megacaryocytes and then packaged into mature platelets. However, because of platelets possess a functional spliceosoma, the complex that processs pre-mRNA, their transcriptome is not static *(Gnatenko, DV, et al. 2003; Lindemann, S and Gawaz, M. 2007; Landry P et al. 2009)*.

Additionally emerging evidence indicates that megakaryocytes invest platelets with thousands of proteins, but platelets also contain ribosomes. Therefore, platelets are competent

for translation and several studies have revealed that the platelet proteome is complex, under regulatory control and subject to change in disease situations and during platelet storage *(Denis MM et al. 2005; Schwertz H et al. 2006)*.

On these basis it is evident that platelet transcriptome and proteome of newly released platelet may represent specific features of megakaryocytic precursor, but, on the other hand in mature platelet the content of mRNAs and proteins may reflect maturation process necessary for platelet functions.

Collectively analysis of platelet transcriptome and proteome allow the identification of feature linked to physiological and pathological conditions.

1.2.3 PLATELET FUNCTION

The most relevant physiological role of platelets is primary hemostasis, i.e. the timely formation of a plug at site of vascular injury in order to prevent hemorrhage.

As general overview (Figure 1.2.5), prior to vascular injury, intact endothelium provides a non-adhesive surface to platelets and platelet activation is suppressed by endothelial cell-derived inhibitory factors (prostaglandin PGI2, nitric oxide, and CD39, an ADPase on the surface of endothelial cells that can hydrolyze trace amounts of ADP that might otherwise cause inappropriate platelet activation). After endothelial damage, the development of the platelet plug is initiated by thrombin and by the collagen-von Willebrand factor complex, which capture and activates moving platelets. Platelets adhere and spread, forming a monolayer. Next, the platelet plug is extended as additional platelets are activated via the release or secretion of thromboxane A2 (TXA2), as well as ADP and other platelet agonists, most of which are ligands for G protein-coupled receptors on the platelet surface.

Activated platelets stick to each other via bridges formed by the binding of fibrinogen, fibrin, or VWF to activated αIIbβ3. Finally, close contacts between platelets in the growing hemostatic plug, along with a fibrin meshwork, help to perpetuate and stabilize the platelet plug.

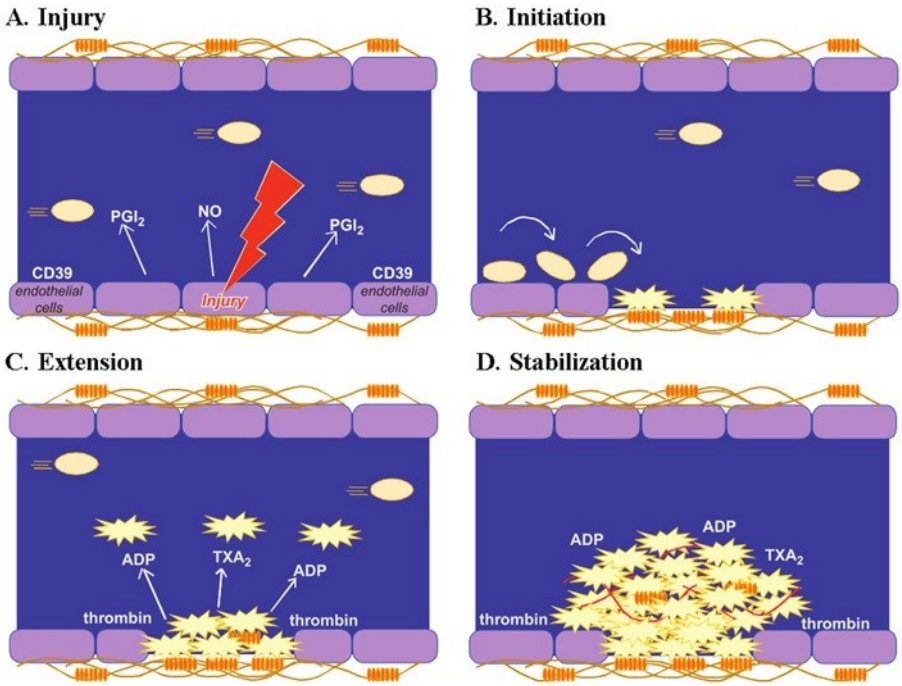

Figure 1.2.5. Stages in platelet plug formation. ***(Brass LS et al. Platelets 3rd edition)***

In more details, plug formation is usually divided into 4 phases: adhesion, activation, aggregation and thrombus stabilization.

After vascular injury the first interaction between platelet and sub endothelial matrix is mediated by VWF and platelet VWF-receptors. Subendothelial VWF binds rapidly platelet GPIb-IX-V complex. This interaction has limited lifetime and, although it cannot support stable adhesion, it allow the creation of stable adhesion between platelet and endothelium through binding of sub endothelial collagen to its receptor GPVI and α2β1.

This stable adhesion is followed by platelet activation. The interaction of platelet receptors with their ligands activate many intracellular signaling that involves primary PI3K/AKT pathway which is responsible of a broad range of biochemical and functional platelet responses. In particular, during platelet activation there is a microfilaments rearrangement followed by platelet shape change, intracellular calcium mobilization, thromboxane production, granule secretion with release of platelet agonists and conformational change of αIIbβ3 from the ligand low-affinity to the ligand high-affinity state. These mechanisms induce the following phase of aggregation, in which thromboxane and other platelet agonists interact with their receptors and amplify platelet activation, fibrinogen molecules create a bridge with its two extremities for αIIbβ3 integrins on different platelets, and other blood cells are recruited stabilizing the platelet plug. (Figure 1.2.6 and Figure 1.2.7)

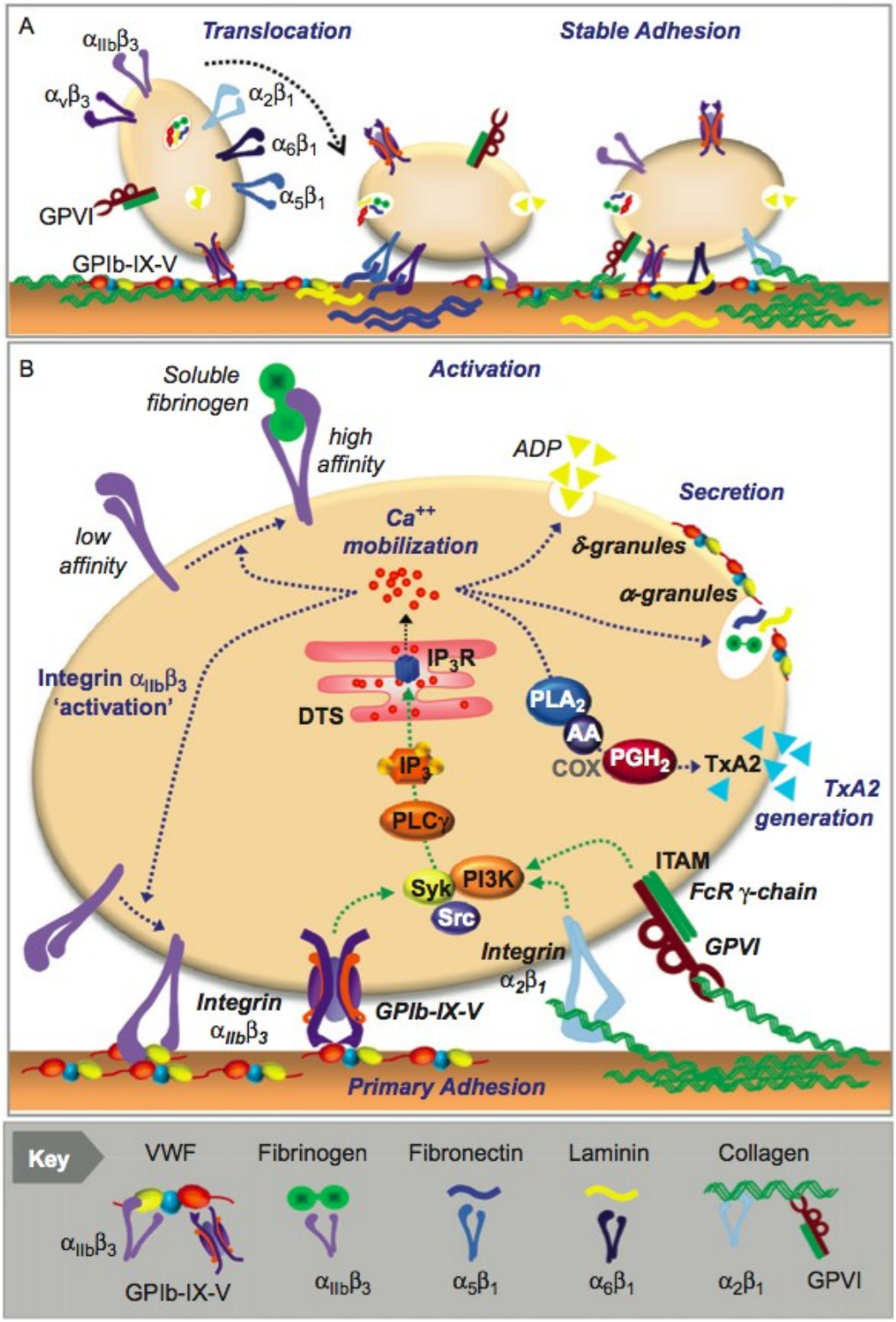

Figure 1.2.6. Platelet receptor and ligand interactions during primary adhesion and subsequent platelet activation. Schematic representation of the key ligands and receptors essential for primary platelet adhesion(A) and the subsequent signaling events following receptor ligation (B). ***(Ruggeri ZM and Jackson SP. Platelets 3rd edition)***

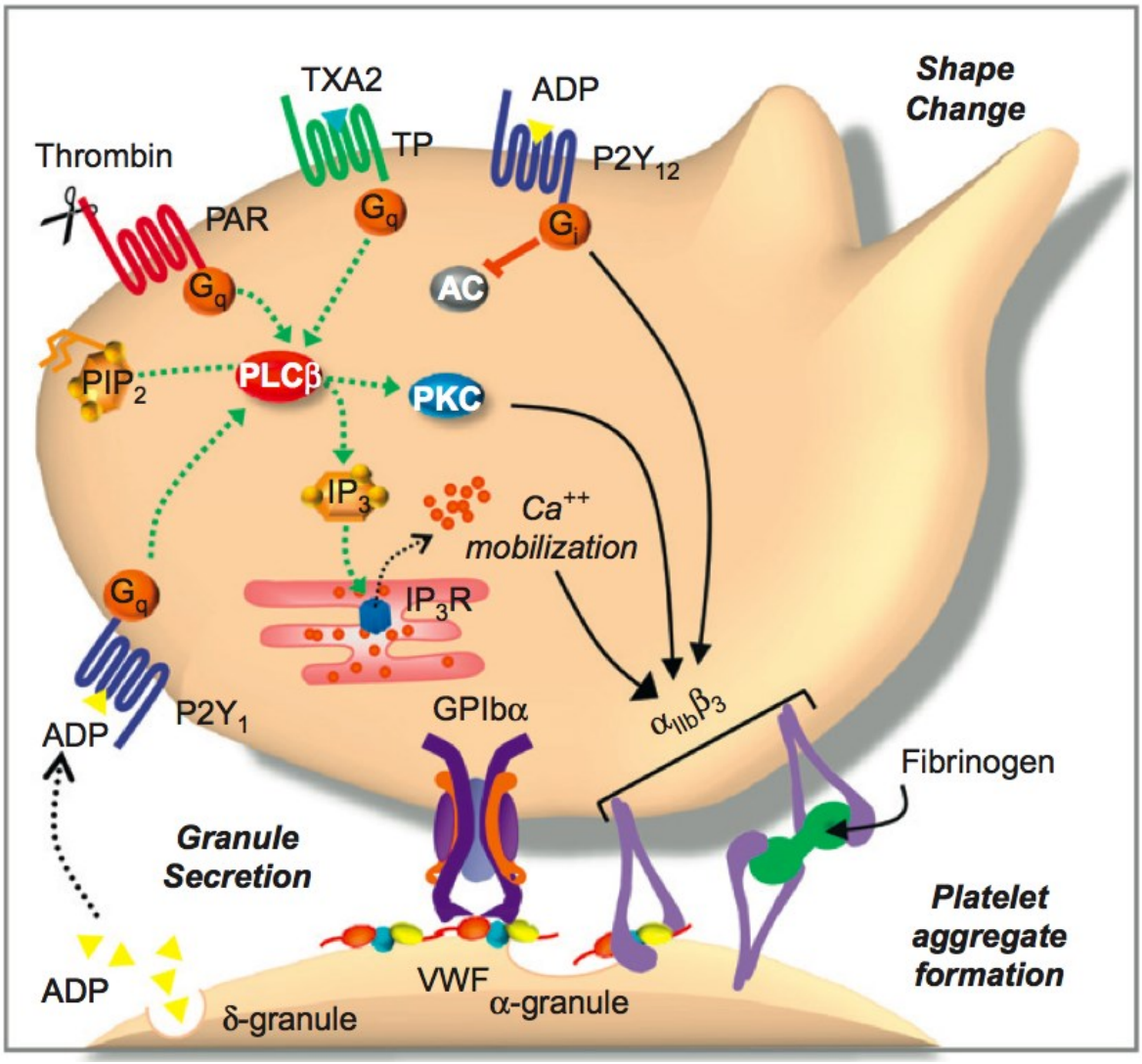

Figure 1.2.7. Platelet aggregation and co-stimulation of platelet receptors by soluble agonists. ***(Ruggeri ZM and Jackson SP. Platelets 3rd edition)***

1.2.4 PLATELETS AND ATHEROSCLEROSIS

In the last 15 years is emerged a key role of platelets in the pathogenesis of atherosclerotic disease, such as Myocardial Infarction (MI).

Atherosclerosis is the result of a complex pathological process characterized by vessel intimal lesions called atheromata (or atheromatous plaques) that protrude into the vessel lumen. Inflammation, focal intimal thickening, and lipid accumulation are key features of

this multifactorial process. Endothelial cells, monocytes and platelets are the principal cells involved in these events.

Atherosclerosis has been defined as a reaction to damage in which prolonged injury to endothelium in predisposed vessels (e.g. coronary arteries) leaded to events in which monocytes and lipids play a central role. If inflammation occurs, endothelial cells become a sticky surface to monocytes, allowing them to adhere and, in the setting of high plasma cholesterol levels, leads to lipid accumulation within the vascular wall. Lipids are oxidized and engorged by monocytes-derived macrophages, due to lipid engulfment, becomeing foam cells. Oxidized lipids have a proinflammatory effect, thus perpetuating the inflammatory state within the plaque. The vessel wall responds to injury and inflammation by inducing the migration of smooth muscle cells from the media to the intima. Moreover, smooth muscle cells proliferate and produce extracellular matrix (ECM) components, including collagen, elastic fibers, proteoglycans. Thus, the atherosclerotic lesion growths and narrows the vessel lumen. The fibrous cap formed by the ECM components prevents rupture of the plaque until destabilizing factors occur. Each plaque has a variable propensity to rupture. Recent research has shown that inflammation plays a key role in plaque rupture: many of the immune cells (macrophages, T cells, and mast cells) at sites of plaque rupture exhibit signs of activation and produce several types of molecules (inflammatory cytokines, proteases, coagulation factors, radicals, and vasoactive molecules) that can destabilize lesions *(Kovanen PT et al. 1995; Hansson GK et al. 1989; van der Wal AC, et al. 1994)*, they inhibit the formation of stable fibrous caps and attack collagen in the cap *(Hansson GK et al. 1989; Amento EP et al. 1991)*. All these reactions can conceivably induce the activation and rupture of plaque. The importance of plaque rupture in arterial thrombosis has been undoubtedly recognized *(Davies MJ and Thomas AC. 1985; Falk E. 1985)*. The acute complication that develops on the chronic lesions of

atherosclerosis after plaque rupture is atherothrombosis. In a physiological setting, self-limitation of thrombus growth occurs in order to repair damage without disturbing normal blood flow. However, when excessive thrombosis occurs, this may impair blood flow leading to occlusion of the affected vessel. What it alters the normal balance between pro- and antiaggregatory factors is not completely understood, although we know that not all lesions are equally thrombogenic. Excessive thrombus formation is plausibly due to concurrent combination of high plaque thrombogenicity and platelet hyperreactivity *(Smitherman TC et al. 1981; Langford EJ et al. 1996; Theroux P and Fuster, V. 1998; Vaitkus PT et al. 1995)*. (Figure 1.2.8)

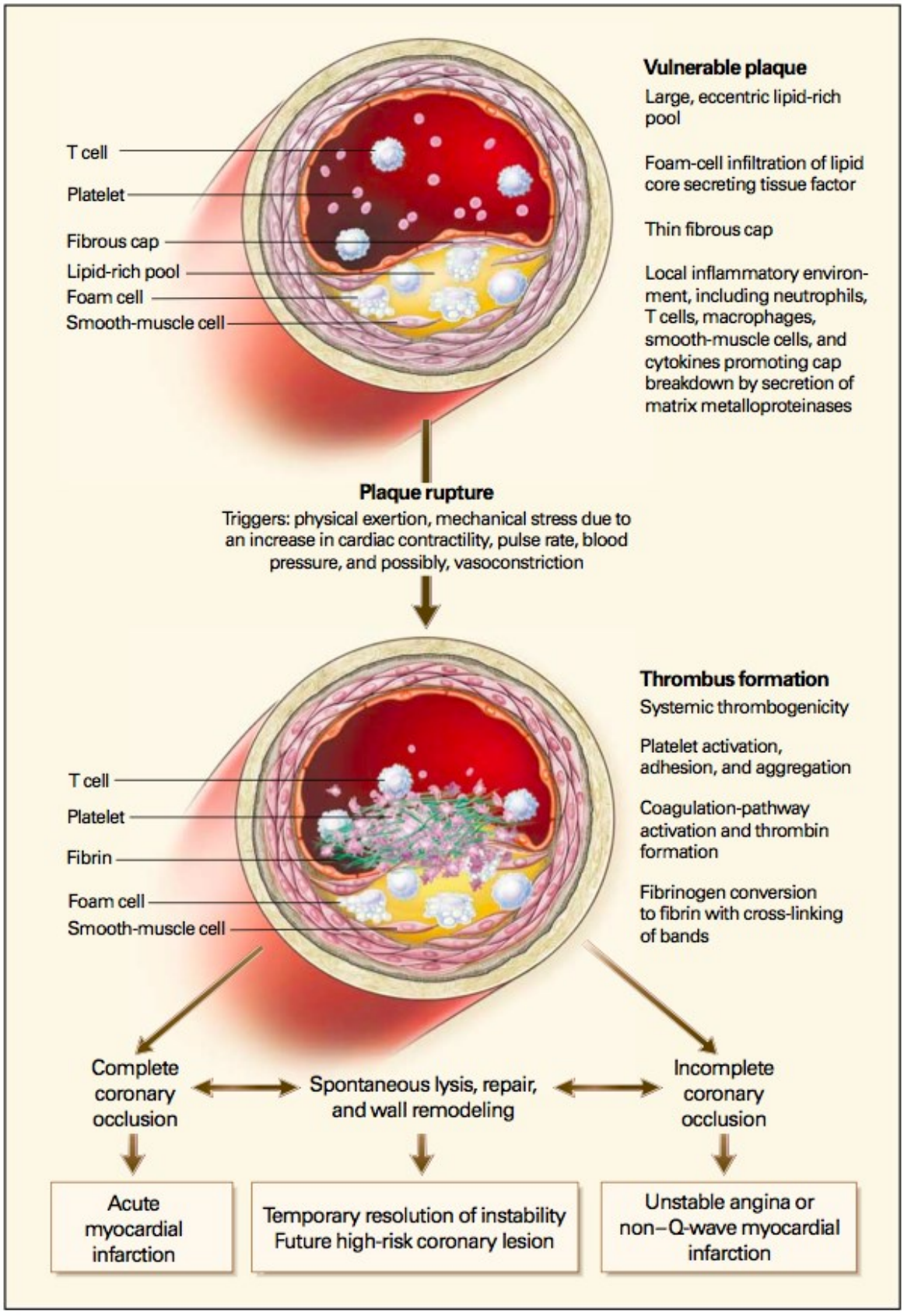

Figure 1.2.8. Pathophysiological events that culminate in thrombus formation ***(Yeghihazarians Y et al. 2011)*****.**

A platelet hyperaggregable state has been documented in many conditions that are well identified as risk-factors for cardiovascular events, such as diabetes mellitus, smoking, hypertension, hypercholesterolemia, and hyperhomocysteinemia *(Willoughby S et al. 2002)*. In addition, platelet hyperreactivity, owing to increased TXA_2 receptors in patients with myocardial infarction *(Dorn GW et al. 1990)*, lies the foundation of the clinical use of aspirin as a priority antiplatelet drug.

While it is widely accepted that platelets play a significant role in the final step of atherosclerosis, i.e. atherothrombosis, their involvement in the initiation of the atherosclerotic process has initially received scant attention. Contrary to previous thoughts, platelets take part in all stages of vascular disease by interfering with highly dynamic processes. They participate in the initiation of the disease, to its progression and acute exacerbation but also provide potential regenerative mechanisms *(Langer HF and Gawaz M. 2008)*. Abundant indirect evidences and some in vivo studies on knock-out mice support the idea that platelets play a crucial role in atherogenesis even in the earliest phases *(Davì G and Patrono C. 2007)*. For example, platelet adhere to the endothelium (Figure 1.2.9) of carotid arteries in apolipoprotein E $(apoE)^{-/-}$ deficient mice even in the complete absence of early vascular alterations, before the recruitment of monocytes *(Massberg et al. 2002)*.

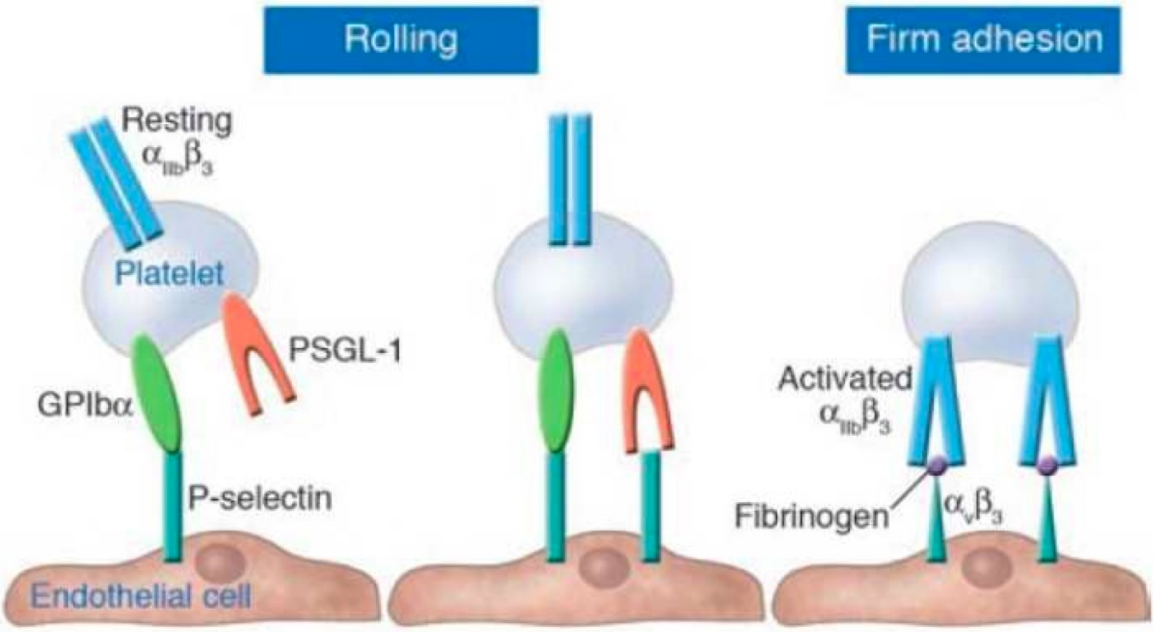

Figure 1.2.9. Platelet-endothelium adhesion. Activated endothelium surface expresses P-selectin. Platelet surface receptors GPIbα and P-selectin ligand-1 (PSGL-1) interact with endothelial P-selectin and mediate platelet rolling. Subsequent firm adhesion is mediated through β3 integrins *(Gawaz, et al. 2005)*

In addition, platelets that adhere to the vessel wall at sites of endothelial cell activation contribute to the development of chronic atherosclerotic lesion through release of chemokines (Figure 1.2.10) *(Boring L et al. 1998; Gleissner CA et al. 2008; Koenen RR et al. 2009)*.

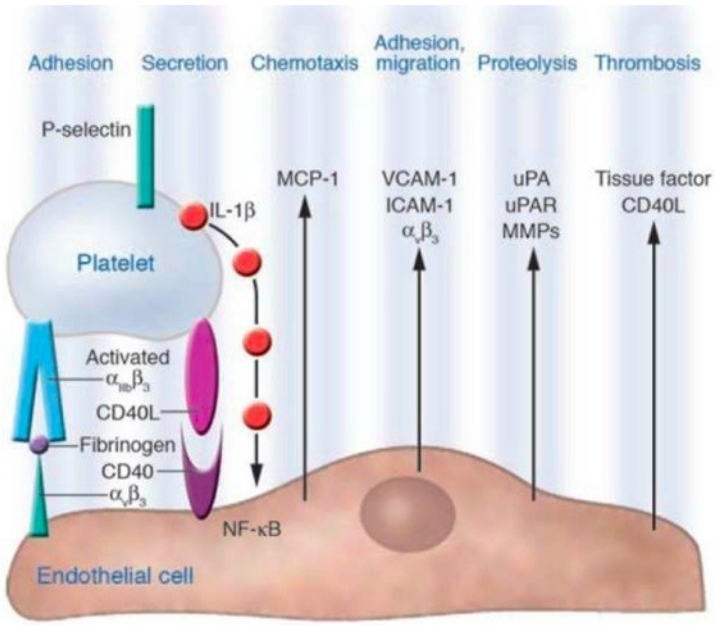

Figure 1.2.10. Adherent platelets inflame endothelial cells. Firm platelet adhesion involving αIIbβ3 induces platelet surface exposure of P-selectin (CD62P) and release of CD40L and IL-1β, which stimulate endothelial cells to provide an inflammatory milieu that supports proatherogenic alterations of the endothelium *(Gawaz, et al. 2005)*

A localized reduction of atherosclerosis in von Willebrand factor-deficient mice was observed *(Methia N et al. 2001)*. Other studies on adhesion molecules lead to the evidence that platelet P-selectin facilitates atherosclerotic lesion development *(Burger PC and Wagner DD. 2003)*. Neointimal formation after vascular injury, a typical feature of atherosclerotic lesion formation, appears to be strictly dependent on platelet, not endothelial, P-selectin *(Wang K et al. 2005; Dong ZM et al. 2000)*, and monocyte recruitment requires P-selectin to occur (Figure 1.2.11) *(Schober A et al. 2002).*

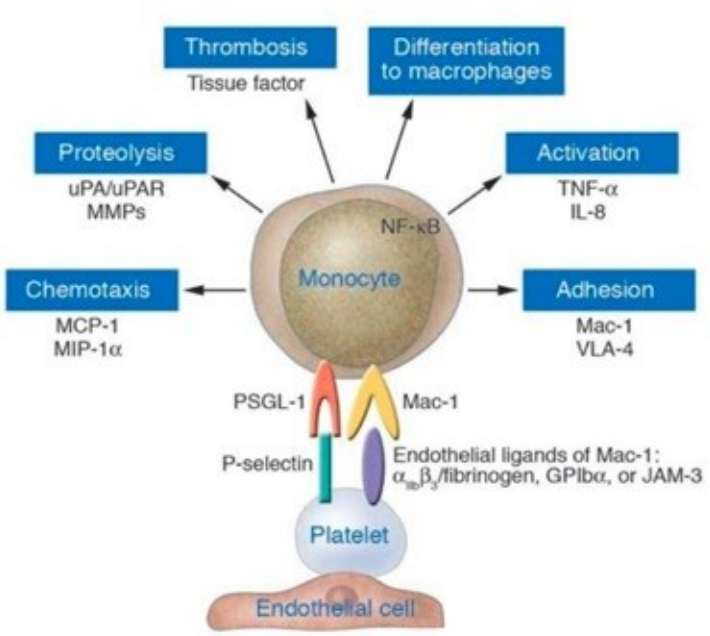

Figure 1.2.11. Adherent platelets recruit and inflame monocytes. Adherent and/or activated platelets initiate monocyte secretion of chemokines, cytokines, and procoagulatory tissue factor, upregulate and activate adhesion receptors and proteases, and induce monocyte differentiation into macrophages. Thus, platelet-monocyte interaction provides an atherogenic milieu at the vascular wall that supports plaque formation *(Gawaz, et al.2005)*

These findings were confirmed in vivo in apolipoprotein E–deficient mice, where P-selectin deficiency protects against atherosclerosis *(Collins RG et al. 2000)*. The finding that circulating activated platelets exacerbate atherosclerosis and promote foam cell formation further support this idea *(Huo Y et al. 2003; Daub K et al. 2007)*.

The amount of data available hitherto outline a complex intertwisting between thrombosis and inflammation, since many of the molecules released by platelets are indeed inflammatory cytokines. As mentioned below, platelet alpha granules contain a huge amount of different inflammatory mediators, an equipment that cannot exclusively account for platelets hemostatic function. Moreover, they express many immune receptors on their surface, including the aforementioned P-selectin, as well as integrins, CD40L, CD40, and intercellular adhesion molecule-2 (ICAM-2) *(von Hundelshausen P and Weber C. 2007)*. Platelet delivery of inflammatory mediators into the local microenvironment determines

alterations in the adhesive properties of endothelial cells. In particular, thrombin has a relevant role not only in sustaining activation of resting platelets, but also in activating endothelial cells and smooth muscle cells *(Croce K and Libby P. 2007)*.

All these alterations induce adhesion of monocytes to endothelial cells, migration and degeneration into foam cells. Thus, inflammatory, atherogenetic and thrombotic events appear to be more and more inseparable processes *(Gawaz M et al. 2005; Lindemann S et al. 2007; Langer HF et al. 2010)*.

1.3 PKC FAMILY

Protein kinase C (PKC) is a family of serine/threonine kinases identified 30 years ago. PKCs act as major mediators of signal transduction pathways and have been shown to regulate sets of biological functions as diverse as cell growth, differentiation, apoptosis, adesion, migration, transformation and tumorigenicity *(Duquesnes N et al. 2011)*.

In mammals 10 members of this family have been identified products of seven highly related genes. These members have been grouped into 3 classes according to their primary structure and activation modes: classical or conventional PKCs (α, βI, βII and γ; cPKCs), novel PKCs (δ, θ, ε and η; nPKCs), and atypical PKCs (ζ and ι ; aPKCs).

Each isozyme is expressed in a wide variety of cell types and multiple isozymes are expressed in a single cell, making it an ubiquitous family of enzymes.

PKC have been shown to be involved in numerous pathological conditions including cancer, cardiac and lung diseases, cerebral ischemic and reperfusion injury and diabetes: due to their ubiquity, PKC isozymes have therefore been selected as molecular targets for therapeutic agents. *(Mochly-Rosen et al. 2012)*

1.3.1 PKC STRUCTURE

All PKCs have a common general structure with 2 principal modules: a N-terminal regulatory domain (20-70 kDa) and a C-terminal catalytic domain (~45 kDa) separated by hinge region. Throughout the primary sequence of the enzymes, there is an alternation of conserved (C1–C4) and variable (V1–V5) regions (Figure 1.3.1).

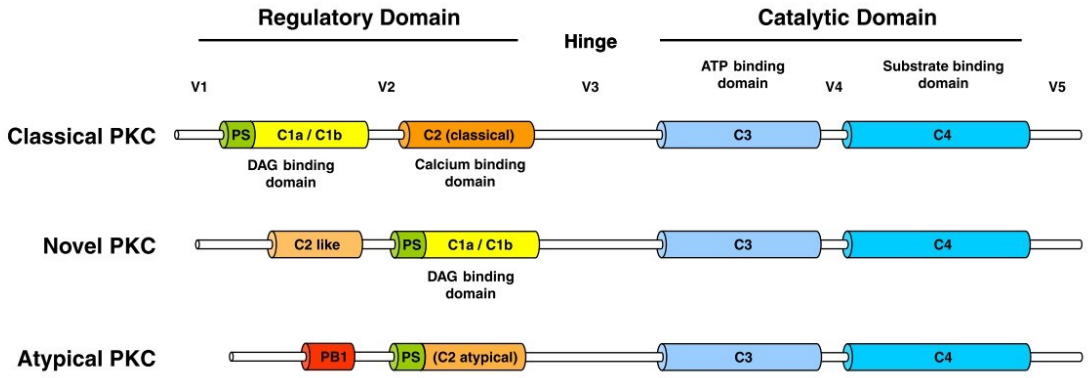

Figure 1.3.1. Schematic structure of the 3 classes of PKCs *(Duquesnes S et al. 2011)*.

The N-terminal regulatory domain primarily serves two functions. Firstly, it contains one or more modules (C1, C2, PB1 or phosphatidylserine-binding domains) that, when engaged by lipid second messengers or other interacting proteins, tether the enzyme to various locations in the cell such as the plasma membrane. In particular the C1 domain is the diacylglycerol/phorbol ester binding site, and the C2 domain contains the recognition site for acidic lipids and, in some isozymes, the Ca^{2+} -binding site. Secondly, the regulatory domain negatively regulates enzymatic activity; the C1 domain is immediately preceded by an autoinhibitory pseudosubstrate sequence that closely resembles a PKC substrate site, except that the serine/threonine residue is replaced by an alanine. This pseudosubstrate sequence binds to the substrate-binding cavity in the C-terminus and blocks catalytic activity. Removal of this pseudosubstrate sequence from the kinase domain occurs when lipid second messengers bind to the regulatory domain, resulting in substrate binding and phosphorylation. *(Freeley M et al. 2010)*

C-terminal catalytic domain interacts with substrates and is responsible for phosphotransfer activity: it contains the ATP-binding site (C3 domain), the substrate-binding site (C4 domain) and three key phosphorylation sites that were important for PKC function. These phosphorylation sites are known as the activation-loop, the turn motif and

the hydrophobic motif and while all three sites are conserved in cPKCs and nPKCs, the aPKC do not contain a hydrophobic motif. For the vast majority of PKC isoforms, phosphorylation of the activation loop is crucial for catalysis, since mutation of this residue to a non-phosphorylatable residue abolishes activity, and this phosphorylation is catalyzed by PDK-1 (Phosphoinositide-Dependent Protein Kinase-1). Phosphorylations at turn-motif and hydrophobic motif play a role in stabilization of the enzyme rather than directly controlling catalytic activity, and, in different PKC isoforms, they are autophosphorylation sites or targets for mTOR pathway (mammalian Target of Rapamycin) and chaperon proteins that include Hsp family (Heat shock protein) *(Freeley M et al. 2010).*

The hinge region is a non-conserved domain, also known as the V3 region. When PKC is active, the hinge domain may became labile to proteolytic enzymes such as caspases and Ca2+-dependent calpains. Calpains or caspases-mediated cleavage of PKC at the hinge domain produces an isolated kinases domain fragment that generally is considered to be constitutively active in the absence of any lipid second messengers, provided that these domains are phosphorylated at the activation loop *(Reyland M.E. et al. 2009; Smith L et al. 2002).*

PKC activation

The function of protein kinase C is regulated by two equally important mechanisms. First, the enzyme is rendered catalytically competent by phosphorylations that correctly align residues for catalysis and localize protein kinase C to the cytosol. Second, binding of ligands or, in some cases, substrate activates the enzyme by removing the pseudosubstrate from the substrate-binding site (Figure 1.3.2).

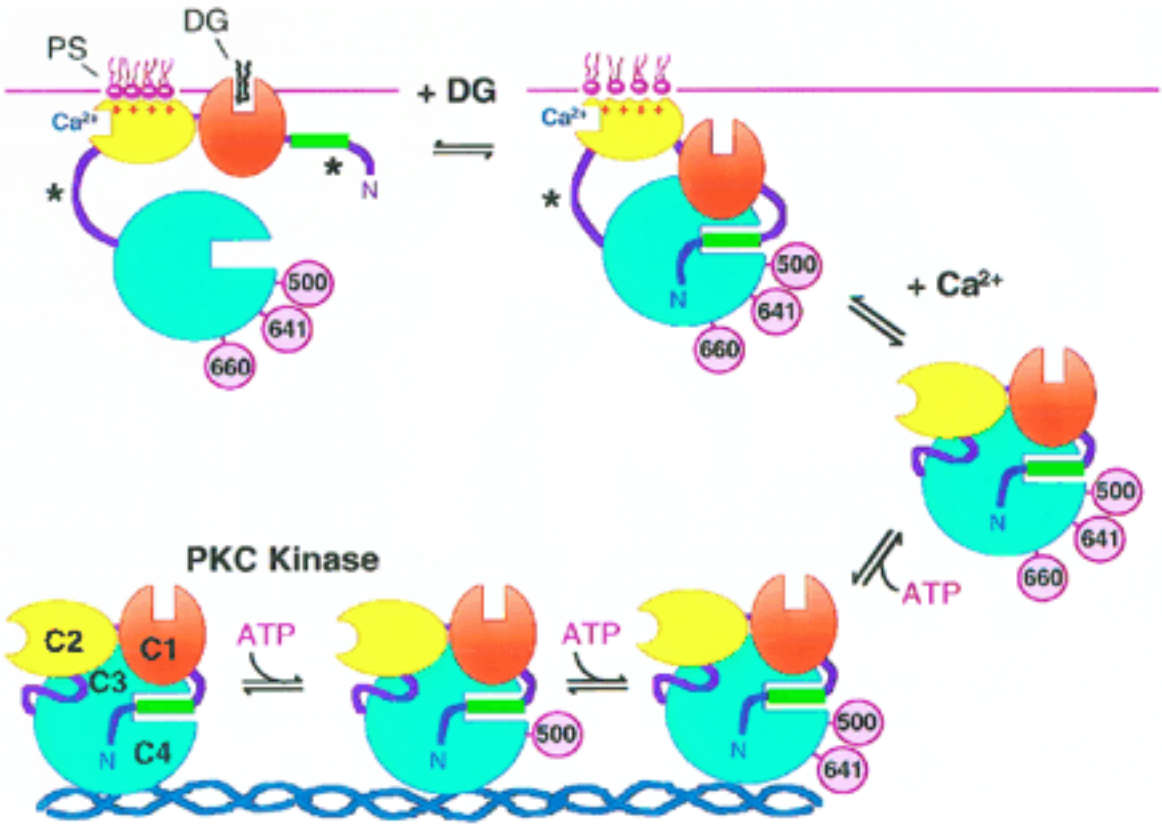

Figure 1.3.2.Model for regulation of PKC by 1) phosphorylation and 2) membrane binding and pseudosubstrate release ***(Newton AC. 1995)***

PKC is first synthesized as an inactive, dephosphorylated precursor, and phosphorylation at the activation-loop, turn-motif and hydrophobic motif sites has been described as maturational or priming events that are required for the processing of these enzymes to signalling-competent (but still inactive) forms. The first event is the transphosphorylation at the activation loop to render the kinase catalytically competent, this step is followed by autophosphorylation or mTOR pathway-dependent phosphorylation at the turn motif and hydrophobic motif that stabilize the catalytically competent conformation and that releases protein kinase C into the cytosol *(Keranen LM et al. 1995)*. The enzyme is now in an inactive state because the pseudosubstrate occupies the substrate-binding site, but this mature form is signalling-competent, it is ready to be activated by lipid second messengers such as DAG.

The complete activation of PKCs occurs when DAG or phorbol ester bind C1 domain, and Ca^{2+} binds C2 domain for cPKCs. These interactions increase PKC's membrane affinity, that is accompanied by pseudosubstrate release and PKC translocation from the cytosol to the plasma membranes as well as to other subcellular locations, where each isozyme interacts with its anchoring protein, RACK. When bound to RACK and the second messenger activators DG (and Ca^{2+} for the conventional PKC isozymes) (Figure 1.3.3), PKC is then active, phosphorylating a number of substrates that are nearby, thus leading to diverse cellular responses *(Newton AC et al. 1995).*

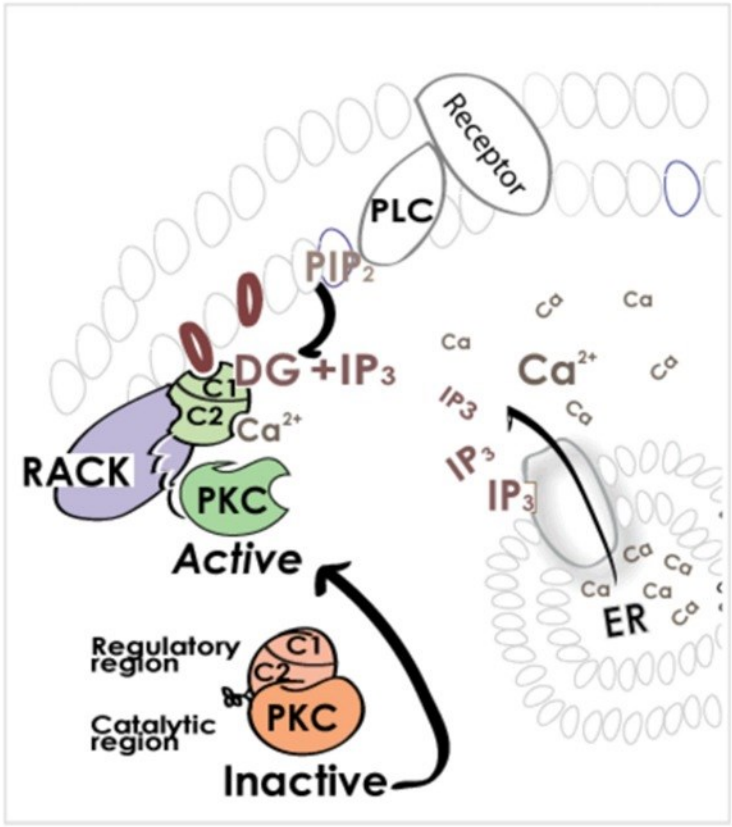

Figure 1.3.3. Interaction of PKCs with RACK *(Mochly-Rosen D et al 2011)*

A variety of hormons (adrenaline, angiotensin, etc.), groth factors (insulin, EGF, etc.) and neuro transmitters (dopamine, endorphin, etc.) when bound to their specific receptors, activate members of the Phospholipase C (PLC) family that hydrolizes the phospholipid

phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG and inositol 1,4,5-trisphosphate (IP3); in turn IP3 induces Ca^{2+} release from R lumen and increasing of Ca^{2+} concentration in the cytosol. Because of DAG production and Ca^{2+} release these molecules are considered PKC activators.

PKC signalling is terminated by the metabolism of DAG, which results in the translocation of cPKCs/nPKCs back to the cytoplasm. It has also been reported that dephosphorylation of PKCs can occur at sites such as the A-loop and HM in response to stimuli such as Tumor Necrosis Factor-α, which is likely to be a key step in turning off the signalling capacity of these kinases (*Lee JY et al. 2000)*. Prolonged PKC signaling, that is promoted by phorbol esters or sustained by DAG signaling, results in the downregulation of PKCs via the ubiquitin/proteasome system (*Lee HW et al. 1996; Lu Z et al. 1998)* and studies have shown that dephosphorylation of PKCs at the A-loop, TM and HM sites occurs prior to down regulation *(Hansra G et al. 1999).*

PKC regulation

Modulation of PKC activity presents an attractive target for clinical drug development for several reasons. 1) Isozyme-specific perturbations in PKC activity have been identified in a number of human disease states including congestive heart failure *(Bowling N et al.1999)*, bipolar disorder *(Friedman E et al. 1993; Hahn CG et al 1999)* and diabetes mellitus *(Geraldes P, King GL. 2010; Nishikawa T et al. 2000)*. PKC isozymes play an important role in critical biological processes such as cell proliferation and formation of vasculature that are important in tumor growth and metastasis *(Podar K et al. 2007; Bosco R et al. 2011)*. Animal studies demonstrate a role for individual isozymes in a particular disease and/or evidence of therapeutic effects when the isozymes are inhibited or activated *(DiazGranados N, Zarate CA Jr. 2008; Teicher BA. 2006; Budas GR et al. 2007).* A

number of pharmacologic agents that are efficacious in disease states have been shown to modulate activity of PKC, either directly or indirectly through a signaling cascade *(Pravdic D et al. 2009; Manji HK, Lenox RH. 2000)*.

Different approaches have been developed to identify selective modulator of PKC. These include development of (a) ATP-competitive small molecule inhibitors that bind to the ATP site of the kinase catalytic domain, (b) phorbol esters and derivative activators and inhibitors that bind to the C1 domain, mimicking DG-binding, and (c) inhibitor peptides that disrupt protein/protein interactions between the regulatory region and RACK blocking PKC translocation to the plasma membrane, or activator peptides that correspond to a sequence in PKC that is homologous to its RACK (Figure 1.3.4) *(Mochly-Rosen et al. 2012)*.

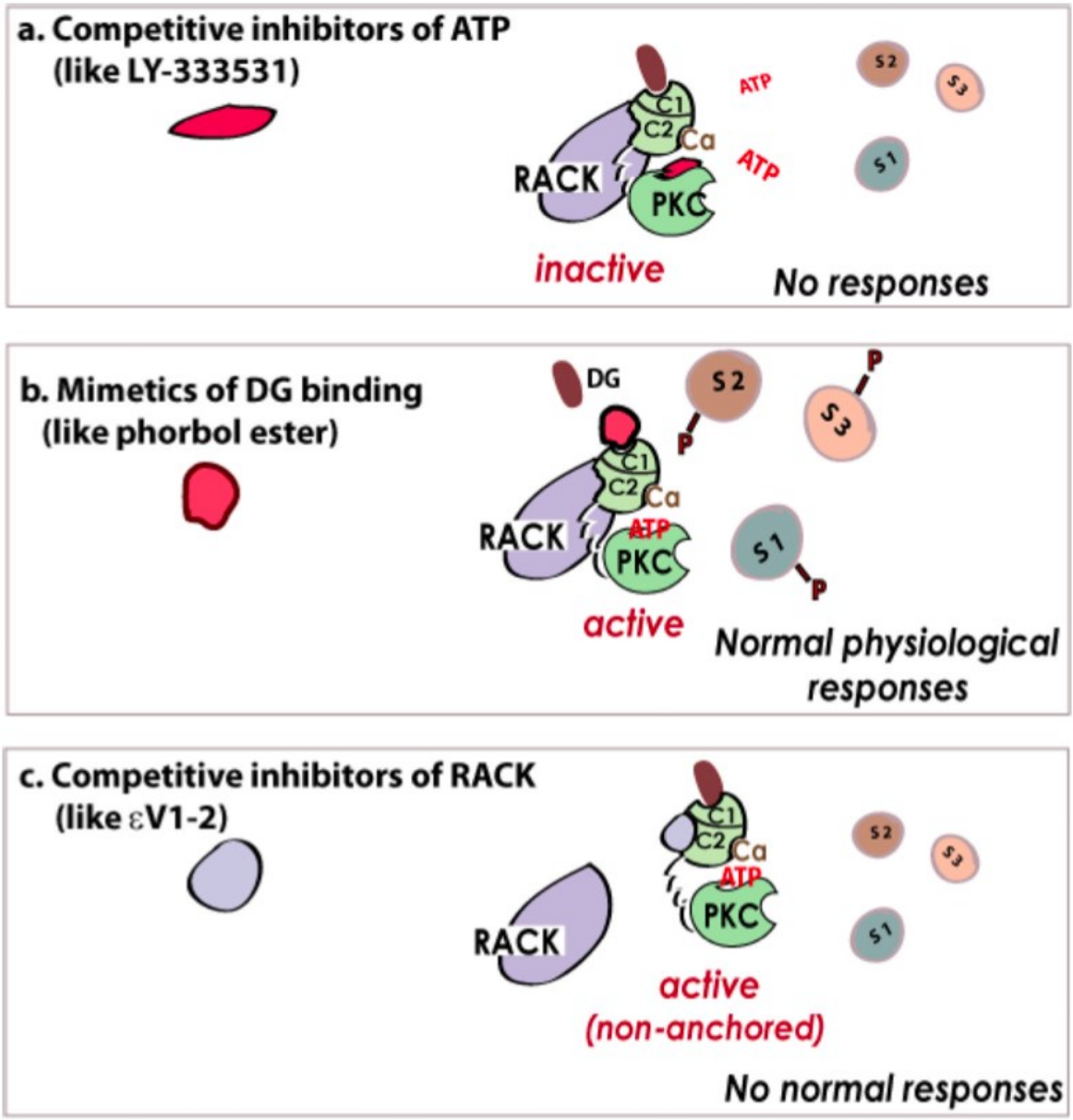

Figure 1.3.4. Inhibitors of PKCs ***(Mochly-Rosen D et al. 2011)***

Despite most PKCs are ubiquitously expressed, it is clear that specific PKC isozymes have unique and sometimes opposing roles in the same cells or in normal signaling and disease state.

In this context, the most intriguing example is represented by two novel PKC, PKCδ and PKCε that mediate contrasting and even opposing effects. In general, PKCε appears thus as the "bad" due to its oncogenic properties. It has even been proposed as a tumor marker *(Gorin MA, Pan Q. 2009)*. In contrast, PKCδ appears as the "good" with proapoptotic properties in cancers and it is an anti-oncogene. Surprisingly, for cardiologists, at least in the domain of ischemia, PKCε is the "good" since it has been shown to be a major actor in the mechanisms inducing preconditioning. In contrast, inhibition of PKCδ during reperfusion protects the heart from reperfusion-induced damage, although both were shown to have parallel effects in non-pathological cardiac hypertrophy *(Chen L et al. 2001)*. Moreover their expression is opposite in human and mouse platelet *(Pears CJ et al. 2008)*.

1.3.2 PKC EPSILON

PKCε is a novel PKC that shares many structural features with other members of the PKC family including a C1 domain containing two cysteine-rich motifs that bind DAG, a C2-like phospholipid binding domain, a pseudosubstrate domain, C3 and C4 catalytic domains that contain a purine binding site for ATP, and a substrate recognition site.

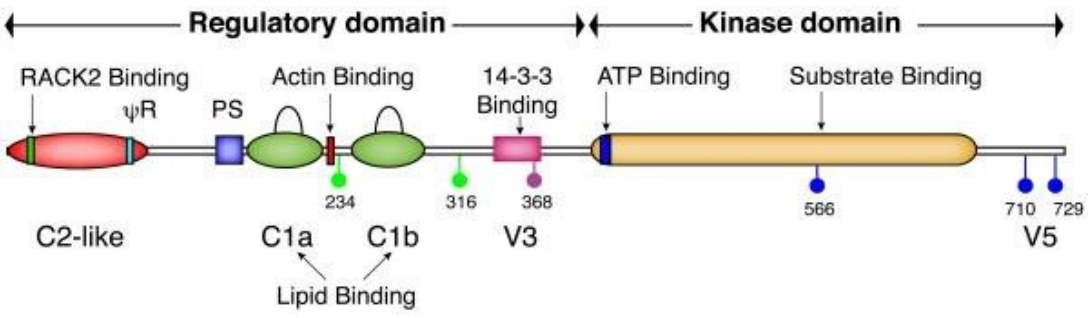

Figure 1.3.5. Schematized primary structure of PKCε ***(Newton AC et al 2011)***

A unique feature of PKCε is a 6 amino acid actin-binding motif between the C1a and C1b subdomains *(Prekeris R et al. 1996)*. Like other PKC isozymes, PKCε must be primed through phosphorylations to display full enzymatic activity and respond to allosteric regulators: the phosphorylated sites are the activation loop (Thr^{566}), the Thr-Pro turn motif (Thr^{710}), and the hydrophobic Phe-Ser-Tyr motif (Ser^{729}). PDK1 phosphorylates the activation loop, whereas the turn and hydrophobic motifs are autophosphorylated in cPKCs, and possibly in PKCε: these phosphorylations are regulated *(Cenni V et al.2002)*.

Following the maturation and the allosterical activation, due to RACK proteins, PKCε translocates to specific subcellular compartments. RACK2 is PKCε-specific, but this isoform can interact also with RACK1 to regulate integrin-mediated cell adhesion and motility *(Csukai M et al. 1997; Xu TR et al. 2007)*.

PKCε can also bind 14-3-3 proteins in V3 hinge region; during the late stages of mitosis, the formation of 14-3-3/PKCε complex is required for the separation of two daughter cells (cytokinesis) during abscission, the final stage of the cell cycle. Mutation of V3 phosphorylation site prevents this association and impairs completion of cytokinesis; likewise, knockout, depletion or inhibition of PKCε impairs abscission *(Saurin AT et al. 2008)*.

PKCε is unique among PKC isozymes in containing an actin-binding region: this six amino acid sequence (LKKQET) is located at amino acids 223-228 on PKCε. When PKCε is in the inactive state, this region is not exposed and thus actin is not bound; however, following treatment with PKC activators such as phorbol esters or DAG, PKCε undergoes a conformational change and the actin-binding region is exposed. Interestingly, binding of PKCε to actin stabilizes the active conformation of the enzyme. The PKCε-actin

interaction has profound consequences for the actin cytoskeleton, most notably promoting the formation of actin through several mechanisms, including inhibiting the depolymerization of actin and increasing the rate of actin filament elongation (*Klauck TM, et al. 1996; Prekeris R, et al. 1996; Prekeris R, et al. 1998; Nakhost A, et al. 1998)*.

Tumorigenic function of PKCε, is the best known and most studied, and PKCε is the only isoenzyme that has been considered as an oncogene. The overexpression of PKCε has been observed in a large number of cancer types like bladder, brain, breast, head lung and prostate *(Gorin MA, 2009)* and in various cellular models it confers a proliferative advantage and increases anchorage-independent growth promoting an invasive metastatic tumour-cell phenotype *(Cacace, AM. et al. 1996; Mischak, H. et al. 1993; Griner EM. 2007)*, these conditions are streakly related to chemotherapeutic resistance in various cell types overexpressing PKCε. Moreover transgenic mice overexpressing *Pkcε* develop squamous cell carcinoma with significant metastatic potential and show increased sensitivity to UV-radiation-induced carcinogenesis *(Wheeler DL et al. 2003; Reddig PJ et al. 2000).*

Its oncogenic function is related to the interaction with many signaling pathways. In particular it protects cells from apoptosis through the induction of pro-survival proteins Bcl2, Bcl-xL, XIAP (X-linked inhibitor of apoptosis), it controls PI3K-Akt activation and it induces activation of mitogen-activated protein kinase (MAPKK or MEK)–ERK pathway *(Gubina E et al. 1998; Pardo OE et al. 2006)*. PKCε overexpression prevents apoptosis induced by the tumour-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL); similarly, melanoma cells with low PKCε expression are highly sensitive to TRAIL-induced apoptosis *(Gillespies S et al. 2005)*. Moreover, in non-small-cell lung carcinoma cells, PKCε blocks mitochondrial-dependent caspase activation and inhibits

cytochrome *c* release *(Ding L et al. 2002)* while depletion of PKCε in a head and neck squamous carcinoma cell line leads to a decrease in motility and invasion through a corresponding decrease in RhoA and RhoC activity *(Pan Q et al 2006)*.

Interesting is also the role of PKCε in cardiovascular system and it has been demonstrated a cardioprotective function. In facts PKCε activation is protective when occurring prior to the ischemic event or right at reperfusion, mimicking protection induced by short ischemic period prior to the prolonged ischemic event (pre-conditioning) or right at reperfusion (post-conditioning). PKCε activation protects mitochcondrial functions, in part via activation of mitochondrial aldehyde dehydrogenase 2 (ALDH2), which removes toxic aldehydes, products of lipid per oxidation *(Inagaki K et al. 2006; Chen CH, et al. 2008; Budas GR et al. 2007; Duquesnes N et al. 2011; Yang X et al. 2010)*. ALDH2 activation via PKCε and infarct size reduction induced by PKCε activator in Myocardial Infarction model, are also associated to inhibition of fatal ventricular arrhythmia *(Ferreira JC et al. 2012).* Moreover Galli et al have demonstrated a critical role of PKCε in cardiomyocyte differentiation regulating early and late cardiac genes expression *(Galli D et al. 2013).*

PKCε is involved in the differentiation of other cell types over that of cardiomyocytes.

In particular, it is involved in the differentiation of intestinal cells, such as in colon epithelium, in which it has been described a decreasing gradient of PKCε expression from the bottom of the crypts, where intestinal stem cells are resident, to the luminal surface. This expression of PKCε at the base of the crypts, mantains the undifferentiated phenotype

of intestinal stem cell pool, while the downregulation of PKCε promotes differentiation *(Perletti GP et al. 1996; Gobbi G et al. 2012)*.

Moreover it has been demonstrated a differential levels of PKCε expression during both erythropoiesis and megacaryocytopoiesis *(Gobbi et al. 2013)*, suggesting that this isoenzyme might be a key factor in orchestrating the MEP differentiation decision along the erythroid and megacaryocitic differentiation (Figure 1.3.6).

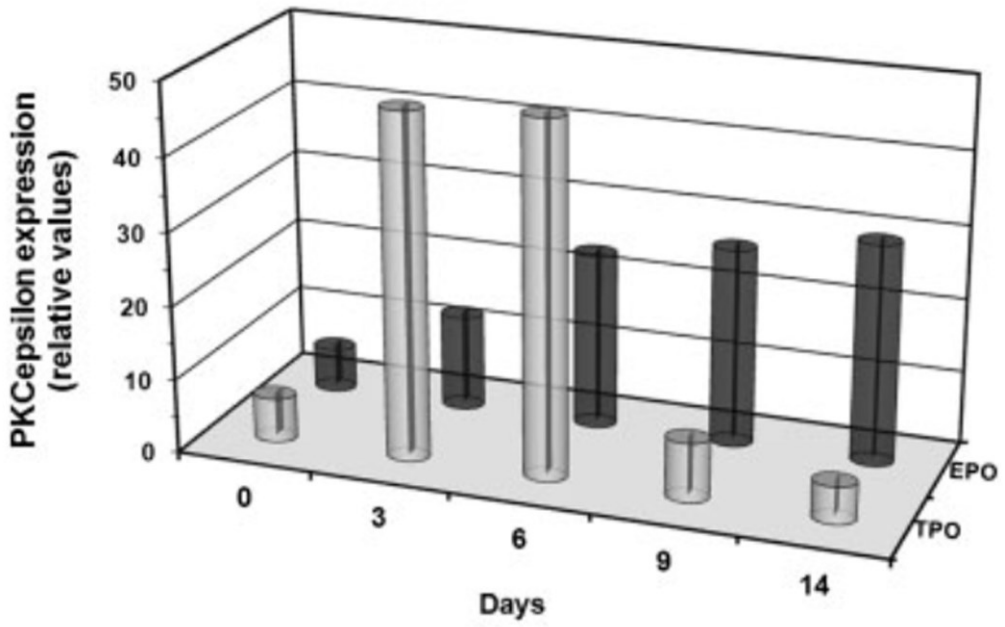

Figure 1.3.6. Histogram comparing the in vitro timing of protein kinase Cε expression levels along thrombopoietin-induced megakaryocytic differentiation (TPO) and erythropoietin-induced erythroid differentiation (EPO) ***(Gobbi G et al 2013)***

EPO induce proliferation of early erythroid progenitor poll, decreasing the level of cell cycle inhibitors and increasing transcription of cyclins, while late erythroid progenitors require EPO as a survival factor that allow them to reach terminal differentiation, promoting the expression of antiapoptotic protein of Bcl-2 family. TRAIL is a negative regulator of adult erythropoiesis, reducing the number of erythroblasts. During *in vitro* erythropoiesis of human $CD34^+$ cells, EPO induces PKCε expression, this isoenzyme

progressively reduces the sensitivity of human erythroid progenitors to TRAIL-induced apoptosis via Bcl-2 up regulation, and erythroblast can successfully complete their maturation process *(Mirandola et al. 2006)*. Also, both erythroleukemic cell line TF-1 and primary acute myeloid leukemia cells, when treated with phorbol 12,13-dibutyrate (PDBu), become sensitive to the apoptotic effects of TRAIL via PKCε down-regulation, similar to what is observed in early erythropoiesis *(Gobbi G et al. 2009)*.

1.3.3 PKC DELTA

PKCδ is the first nPKC isozyme to be identified. Its C-terminal catalytic region contains C3 and C4 domains, in common with other members of the PKC family. The C4 domain contains the activation loop (Thr^{505}), turn motif (Ser^{643}) and hydrophobic motif sites (Ser^{662}); the isozyme maturation through the phosphorylation of these three sites is regulated. Differently from other PKCs, PKCδ is a functional kinase even without Thr^{505} phosphorylation; this has been attributed to an acidic Glu^{500} in PKCδ that assumes the role of the phosphorylated activation-loop Thr in cPKCs. Nevertheless, the catalytic activity of membrane-associated allosterically activated PKCδ is increased by Thr^{505} phosphorylation *(Gschwendt M. 1999)*.

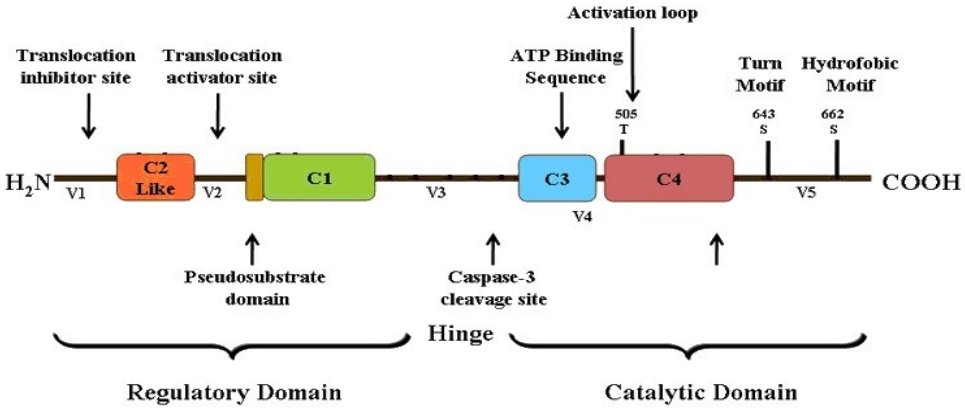

Figure 1.3.7. Schematized primary PKCδ structure ***(Atlas of Genetics and Cytogenetics in Oncology and Haematology)***

As nPKCs, PKCδ has twin C1 domains, a C2 domain and a pseudosubstrate site in his N-terminal regulatory region. The calcium-binding-like loop of the C2 domain of PKCδ contains a sequence that conforms to an optimal Scr family kinases (SFK) substrate; this sequence is unique to PKCδ and is not found in other PKC isoforms that do not become tyrosine phosphorylated in response to DAG or phorbol esters. No uniform pattern or consequence of PKCδ tyrosine phosphorylation can be extracted from the published literature, since the catalytic activity of tyrosine-phosphorylated PKCδ is variably described as decreased, increased, or even altered with regard to substrate specificity and cofactor requirements *(Denning MF et al. 1996; Acs P et al. 2000)*. After the maturation through phosphorylation and the allosteric activation by DAG/phorbol esters, PKCδ can interact with its isozyme specific RACK protein p32/gC1qBP and translocate to the membranes where it performs its catalytic activity *(Robles-Flores M et al. 2002)*.

The PKCδ hinge domain has been recognized as a caspase-dependent cleavage site, it allows the release of a catalytic domain fragment that is freed from autoinhibitory regulatory domain, leading to the activation of these PKC and thereby contributing to the proapoptotic potential of this isoenzyme *(Denning MF et al 1998)*.

PKCδ is primarily know for its tumor-soppresor rule. This isoforme has been show to be down regulated in various type of cancers and its depletion confers a malignant phenotype *(Perletti GP et al. 1999)*. Phorbol-ester-induced tumour promotion involve the downregulation of PKCδ while bryostatin 1, which protects PKCδ from downregulation, blocks phorbol-ester-induced tumour promotion, moreover, transgenic mice overexpressing PKCδ are resistant to phorbol-ester-induced tumour promotion *(Lu Z et al. 1997; Hennings, H. et al. 1987)*. This tumor-soppresor rule is closely related to pro-apoptotic and anti-proliferative PKCδ functions.

Activation of PKCδ causes proliferation defects both in G1 and G2 phases of the cell cycle through the modulation of cyclin expression or the activity of cyclin- dependent kinases, with a great degree of cell-type variability *(Black JD. 2000; Gavrielides MV et al. 2004)*. PKCδ can downregulate the expression of cyclins A, D1 and E, but can also upregulate p27 and p21 expression and PKCδ activation inversely correlates with RB phosphorylation *(Nakagawa M et al. 2005)*. However it is important to mention that, in some cell types, PKCδ can also positively regulate proliferation, for example, PKCδ is required for the proliferative effect of insulin-like growth factor and can induce post-transcriptional destabilization of p21 *(Walker JL et al. 2006)*.

During apoptosis, PKCδ can be activated by proteolysis *(Emoto Y et al. 1995)*, it is highly susceptible to caspase-3 cleavage at the hinge region, which results in the generation of a ~40-kDa C-terminal fragment that is constitutively active. Several chemotherapeutic agents, such as etoposide or cisplatin and UV radiation, induce the generation of the catalytic PKCδ fragment and, remarkably, their apoptotic efficacy is severely impaired when PKCδ function or expression is inhibited *(Denning MF et al. 1998; Reyland ME et al. 1999; Persaud SD et al. 2005)*. Most notably, cells from *Pkcδ*-null mice are defective in mitochondrial- dependent apoptosis and caspase-3 activation induced by etoposide

(Humphries MJ et al. 2006). Most evidence point to a dual involvement of PKCδ in the apoptotic cascade, both upstream and downstream of caspase-3, with PKCδ cleavage serving as a positive feedback signal to amplify caspase-3 activation. Many apoptotic stimuli can redistribute PKCδ to the mitochondria, leading to cytochrome *c* release, caspase-3 activation and initiation of the intrinsic apoptotic cycle *(Majumder PK et al. 2000; Denning MF et al. 2002)*. PKCδ pro-apoptotic function is also associated to activation of pro-apoptotic proteins Bax and Bad and inhibition on anti-apoptotic protein Bcl2. PKCδ was found to induce the expression of, and associate with, topoisomerase IIα in response to genotoxic stress *(Yoshida K et al. 2008)*. This interaction is required for the stabilization and activation of topoisomerase IIα and is confined to the S phase of the cell cycle and aberrant activation of topoisomerase IIα can trigger the intrinsic apoptotic pathway. In prostate cancer cells has determined that PKCδ can trigger an autocrine apoptotic loop through the secretion of TNFα and TRAIL *(Gonzalez-Guerrico AM & Kazanietz MG. 2005)*. In these cells, the apoptotic effect of phorbol esters is entirely dependent on PKCδ and is greatly impaired upon inhibition or RNAi depletion of TNFα and TRAIL death receptors and by caspase-8 knock-down.

Moreover, PKCδ also regulates other biological effectors that are critical for cancer biology; for example, PKCδ inhibits basal transcription of the tumor suppressor protein p53 in human myeloid leukemia cells. Since p53 is a key element in the surveillance mechanism used by cells to maintain genomic stability by eliminating cells with damaged DNA, inhibition of p53 is permissive for tumor formation *(Abbas T et al. 2004)*. PKCδ activation has also been linked to anchorage-independent tumor cell growth and enhanced tumor cell survival. These mechanisms would influence tumor invasion and metastasis, in breast cancer cells and mouse embryonic fibroblasts isolated from *Pkcd*-null mice,

downregulation of PKCδ increased cell motility via MMP9 up-regulation *(Jackson D et al. 2005)*.

Along with PKCε but with an opposite effect, PKCδ is involved in ischemic preconditioning and ventricular hypertrophy. Pharmacological PKCδ activation is reported to worsen cell damage during an ischemic insult, whereas PKCδ inhibition confers cardioprotection. Moreover, in cardiomyocytes, PKCδ has been implicated in the down-regulation of SERCA2 expression, a characteristic feature of the hypertrophic phenotype; and PKCδ over-expression is also reported to activate JNK and p38 MAPK, promote cell detachment, and induce cardiomyocyte apoptosis *(Steinberg SF, 2004)*.

In haematopoietic system it has been demonstrated an involvement of PKCδ in erytroid and granulocyte differentiation. During erythroid differentiation PKCδ is largely expressed during the early phase of proliferation, while must be down regulated to allow terminal differentiation *(Marchisio M et al. 2005; Lanuti P et al. 2006)*. Moreover U-937 cells are induced to undergo granulocytic differentiation via PKCδ phosphorylation *(Zhang HW et al. 2008)*.

Recently Sassano and co-workers have demonstrated that the pro-differentiative and antileukemic effects of statin in acute promyelocytic leukemia cells are mediated by PKCδ activation *(Sassano et al. 2012)*. Similarly antileukemic effect of INF-alpha and ingenue 3-angelate (PEP005) in chronic myelogenous leukemia cells and in primary acute myeloid leukemia (AML) cells respectively, are PKCδ dependent *(Kaur S et al. 2005; Hampson P et al. 2005)*.

1.3.4 PKCε AND PKCδ IN MEGAKARYOCYTOPOIESIS

The role of PKCε in megakaryocytopoiesis is well described in normal and pathological conditions.

Early observations demonstrated that phorbol ester induces polyploidization and platelet-specific markers expression in DAMI human MK cell line, and moreover it induces PKC ε, α, θ translocation from the cytosol to the cell membrane *(Ballen KK, Exp Hematol 1996)*. PKCε participates in MK linage commitment cooperating with GATA-1 in the activation of megakaryocytic promoters and its down-modulation inhibits megakaryocytic differentiation of K562 cells *(Goldfarb J et al. 2001)*.

Gobbi et al, have demonstrated that during *in vitro* megakaryocytic differentiation of human $CD34^+$ cells, PKCε levels are finely tuned. Progressively increase in the early phases of differentiation while decrease in the late phases. An induced persistence of elevated PKCε levels interfere with the late phase of megakaryocytic differentiation reducing polyploidization, MK-specific markers expression and platelet production. This function of PKCε is related to modulation of Bcl-xL level. It is, in fact, well-know that Bcl-xL levels must be down-modulated in the later phases of thrombopoiesis *(Sanz C et al. 2001)*, but PKCε overexpression results in an accumulation of Bcl-xL that interfere with the physiological process of thrombopoiesis *(Gobbi G et al. 2007)*.

More recently, we have demonstrated a role of PKCε regulation in Primary Myelofibrosis (PMF). In this Philadelphia chromosome-negative Myeloproliferative Neoplasm, bone marrow MKs are hyperplastic and show typical morphological abnormalities such as hypolobated nuclei, tendency to form tight clusters and impaired capacity to generate pro-PLTs in-vitro. Interestingly PMF-$CD34^+$-derived megakaryocytic express higher levels of PKCε than those from healthy subjects, these levels also correlate with disease burden.

Inhibition of PKCε function restores a normal in-vitro megakaryocytoiesis from PMF hematopoietic progenitors *(Masselli et al. In Press).*

We have also demonstrated that PKCε is differentially regulated in human and mouse megakaryocytopoiesis. Using an *in vitro* model of murine platelet production, we found that PKCε expression escalates during megakaryocytic differentiation but, contrary to the human model, it remains elevated in late phase of differentiation and in proplatelets and that down-regulation of its expression resulted in decreased proplatelet numbers and larger platelets. We propose that the mechanism by which PKCε modulates proplatelet formation is through RhoA; inhibition of RhoA reversed the proplatelet defects seen with PKCε inhibition *(Gobbi et al. 2013)*.

With regard to the role of PKCδ in megakaryocytopoiesis, it has been only observed that K562 and HEL cells show increased levels of PKCδ expression when cultured in presence of PMA to induce megakaryocytic differentiation *(Zauli T et al. 1996; Fang T et al. 2010).*

1.3.5 PKCε AND PKCδ IN PLATELET FUNCTION

PKC activity has been associated with a variety of platelet functions *(Massberg S et al. 2002)*.

PKC activity, in synergy with Ca^{2+}, regulates the secretion of dense and α-granules following platelet stimulation with phospholipase C-stimulating agonists, like collagen and thrombin *(Rinck TJ, Deutsch C. 1983; Walker TR, Watson SP. 1993; Yoshioka A et al. 2001)*. Secretion of ADP, fibrinogen, and other stored compounds, in turn, enhance the activation process *(Jin J, Kunapuli SP. 1998; Paul BZ et al. 1999)*.

PKC-mediated protein phosphorylation also induces the conformational changes of integrin αIIbβIII required for fibrinogen binding and platelet aggregation *(van Willigen G, Akkerman JW. 1991; Hers I et al. 2000)*. Activated integrins, in turn, stimulate PKC via outside-in signaling, resulting in filopodial formation and platelet spreading *(Hartwig JH et al. 1996; Buensuceso CS et al. 2005)*. Ca^{2+}-dependent PKC isoforms contribute to platelet aggregation, directly via integrin phosphorylation and indirectly via granule secretion. It has been reported that PKC is involved in Ca^{2+} flux in platelets *(Rinck TJ et al. 1983; Rosado JA, Sage SO. 2000)*, while under flow conditions PKC contributes to the stable adhesion of platelets to collagen but not to their initial attachment to the vessel wall *(Polanowska-Grabowska R, Gear AR. 1999)*. Finally, it has been demonstrated that platelet PKCs have a dual controlling role in thrombus formation, balancing the proaggregatory and procoagulant properties of thrombi *(Strehl A et al. 2007)* suggesting that the different PKC isoforms present in platelets participate to distinct activatory or suppressive pathways, the latter of which are mediated by one or more non-classical PKC isoforms *(Strehl A et al. 2007)*.

Notwithstanding these research efforts, a clear picture of the role of the different PKC isoforms in platelets is still lacking.

In particular, focusing on the two novel PKCε and PKCδ, it has been demonstrated that delta isoform is expressed in human platelet, and several studies have shown the role of PKCδ in many platelet function, such as granule secretion, thromboxane A2 synthesis and platelet aggregation.

In human platelets, collagen or thrombin induce PKCδ Tyr311 and Tyr565 phosphorylation by Src-family kinases (*Murugappan S et al. 2005; Hall KJ et al. 2007)*, which increases PKCδ activity but does not affect plasma membrane translocation (*Hall KJ et al. 2007)*. Interestingly, PKCδ tyrosine phosphorylation appears to be regulated

differently in mouse platelets, with little phosphorylation being detected after stimulation with collagen-related peptide *(Pears CJ et al. 2008)*. That PKCδ regulates granule secretion was first proposed on the basis of the reported effects of rottlerin (a PKCδ inhibitor). Rottlerin enhances GPVI-dependent granule secretion, but inhibits granule release following activation of protease-activated receptor (PAR) 1 or PAR4, suggesting that PKCδ regulates dense granule secretion in an agonist-dependent manner *(Murugappan S et al. 2004)*. Studies with PKCδ$^{-/-}$ platelets have not fully resolved the role of PKCδ. Pula and co-workers, found no difference in GPVI- dependent dense granule secretion in PKCδ$^{-/-}$ platelets as compared with the wild type. Significantly, rottlerin still enhanced granule secretion in PKCδ$^{-/-}$ platelets, under- scoring its likely off-target effects, and demonstrating that, under these conditions, it was possible to observe a potentiation of secretion *(Pula G et al. 2006)*. In contrast, Chari et al. *(Chari R et al. 2009)* found enhanced GPVI-dependent dense granule secretion in PKCδ$^{-/-}$ platelets, an effect replicated in human platelets by a specific PKCδ inhibitor peptide. The reasons for this apparent difference are not clear.

In the same paper Chari et al. *(Chari R et al. 2009)* also found that PKCδ$^{-/-}$ platelets showed partially reduced granule secretion in response to stimulation of PAR4, and dense granule secretion was also inhibited by specific PKCδ inhibitor peptide. They indicate that reduced secretion was only seen at submaximal concentrations of AYPGKF, and lost at higher concentrations, suggesting that other mechanisms may compensate for the loss of PKCδ signaling at higher agonist concentrations. The mechanisms that might underlie differential regulation of granule secretion by PKCδ by GPVI and G-protein-coupled receptor stimulation are still unclear. It has been shown recently that phosphorylation of PKCδ by the tyrosine kinase Lyn occurs downstream of GPVI but not PARs, and that Lyn and PKCδ together regulate SHIP1 phosphorylation in an agonist-dependent manner *(Chari R et al. Blood 2009)*. This is consistent with negative regulation of αIIbβIII

signaling by a Lyn–SHIP1 complex that has been previously proposed *(Maxwell MJ et al. 2004)*, and this may begin to explain the differential roles for PKCδ in signaling downstream of collagen and thrombin.

During the activation process, platelets rapidly and transiently extend filopodia, which are quickly superseded by lamellipodia to form the fully spread platelet. In PKCδ-deficient platelets, however, although both filopodia and lamellipodia do form sequentially, as in wild-type platelets, the filopodia do not subsequently disappear, unlike in the wild type, where filopodia are not apparent in the fully spread platelet. This sustained appearance of filopodia in PKCδ$^{-/-}$ platelets also results in more extensive platelet aggregation. The molecular mechanism underlying this functional change involves a physical interaction between PKCδ and the actin regulator vasodilator-stimulated phos- phoprotein (VASP). PKCδ was shown to negatively regulate phosphorylation of VASP at Ser157, reducing filopodial extension or promoting filopodial retraction, and thereby negatively controlling platelet–platelet interaction to limit aggregation.

In contrast, although PKCδ$^{-/-}$ platelets show enhanced collagen/collagen-related peptide-induced aggregation *(Pula G et al. 2006)* and, under some conditions, may show slightly enhanced GPVI- dependent dense granule secretion, the two are possibly unrelated. Inhibition of $P2Y_1$ and $P2Y_{12}$did not prevent enhanced aggregation in PKCδ$^{-/-}$ platelets *(Pula G et al. 2006)*. Furthermore, aggregation of PKCδ$^{-/-}$ platelets could not be further enhanced by rottlerin, whereas dense granule secretion was further potentiated. Instead, we have proposed that the enhanced aggregation results from increased filopodia formation in PKCδ$^{-/-}$ platelets *(Pula G et al. 2006)*.

Regarding to the PKCε, its expression is still a matter of debate. Most Authors do not find it *(Buensuceso CS et al. 2005; Pears CJ et al. 2008)* while other report its presence

(Crosby D, Poole AW. 2003; Murugappan S et al. 2004); although is now well established that mouse platelet express PKCε *(Pears CJ et al. 2008),* functional data are still contradictory.

In platelets from PKCε null mouse, Pears et al showed a marked inhibition of aggregation and dense granule secretion in response to GPVI agonists but no significant functional change in response to ADP *(Pears CJ et al. 2008)*. At the opposite, recently Bynagari-Settipalli et al *(Bynagari-Settipalli YS et al. 2012)* showed an increase in ADP-induced aggregation and secretion in platelets from PKCε null mice. Indeed, signaling through GPVI suggests a role for PKCε in the initial steps of thrombus formation in mouse platelets.

On these basis, functional role of novel PKC, and in particular PKCε, in human platelet still needs large studies to be clearly defined.

2. AIM OF THE THESIS

2. AIM OF THE THESIS

Among PKC family, PKCε and PKCδ could be considered the yin and yang of novel PKCs, because of their antithetical role in many cellular mechanisms such as proliferation, apoptosis, tumor growth and cardioprotection.

In this contest, my research aimed to investigate a possible opposite role of these two PKC isoforms in unexplored fields such as (i) circulating platelets function and properties and (ii) regulation of in-vitro human megakaryocytopoiesis and platelet production. Data available from the literature suggest that

In mature platelets circulating in the pheripheral blood, the levels of expression of PKCε are opposed to those of PKCδ. In fact, it is well documented that human platelets express PKCδ and that this isoform is involved in platelet activation, degranulation and aggregation.

By contrast, PKCε expression is still a subject of debate. Anyway, the majority of data shows that, on the contrary to PKCδ, it is absent in human platelets, while it is expressed in murine platelets where it plays a proaggregant function.

On these basis, during the first period of my doctoral fellowship, I focused my research on PKCε expression in human platelets, exploring whether its levels could be associated to platelet hyper-reactivity and related diseases (i.e. acute myocardial infarction), in order to clarify PKCε role in platelet function.

Since platelets are enucleated cell fragments, their trascriptome and proteome reflect that of megakaryocytes they derive from. Indeed, PKCε expression in mature human platelets (i.e. virtually absent) is consistent with its down-modulation during the later phases of megakaryocytopoiesis. Although it is well established that PKCδ is present in human platelets regulating their function, little is known about its expression during megakaryocytopoiesis

Given this background, during the second period of my doctoral fellowship I sought to demonstrate whether PKCδ expression in human platelets could be specific, and timely modulated during megakaryocyte differentiation, similarly to what described for PKCε. Therefore I studied PKCδ expression and function using *in vitro* model of human megakaryocytopoiesis.

Finally, given the growing interest of the scientific community on ameliorating *in vitro* platelet production for transfusion purposes, I studied whether the modulation of PKCε/PKCδ balance could affect platelet production in vitro

3. MATERIALS & METHODS

PART I:

PKCε IN PLATELETS FUNCTION

To investigate PKCε expression in human platelets of peripheral blood and its function on platelet reactivity, I performed *ex vivo* tests on platelets obtained from healthy donors and patients affected by acute myocardial infarction and stable coronary artery disease.

3.1.1 PATIENTS

Three groups of subjects were studied: 1) twenty-four acute myocardial infarction patients (MI) with an ST-segment elevation; 2) twenty-four patients with newly-diagnosed stable CAD (sCAD) and 3) twenty-four healthy subjects (HD). Patients were enrolled at the Cardiology Division of the Azienda Ospedaliero-Universitaria of Parma after written informed consent was obtained and the study was performed according to the Declaration of Helsinski. The protocol was approved by the unique Local Ethical Committee of the Ospedale Maggiore of Parma and University of Parma. Blood collection from MI patients was accomplished within 12 hours from the acute event (in most cases within 3– 4 hours) and before any invasive procedure or pharmacological treatment was performed. Previous antiplatelet therapy was an exclusion criteria in all groups.

Fifty ml of citrate anti-coagulated blood samples were taken from patients and controls (collected in Vacutainers, 3.8% sodium citrate final concentration; BD Vacutainer, Becton Dickinson, San Diego, CA) for subsequent analyses.

3.1.2 PLATELET ACTIVATION

Aliquots of whole blood samples were stained with anti-CD62p monoclonal antibody (mAb) as a marker of platelet activation and a-granule release and analyzed by flow cytometry *(Solinas E et al. 2009; Carubbi C et al. 2014)*. Briefly, 1:100 PBS-diluted whole blood was incubated with the mAb CD62p-FITC (anti P-selectin, Pharmingen Becton Dickinson, San Diego, CA) in the presence of incremental doses of ADP (0; 1,25; 2,5; 5 mM). After 20 min at room temperature, 400 ml of 2% buffered paraformaldehyde was added for fixation.

Analysis of the samples was performed by an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA) and the Expo ADC software (Beckman Coulter). In some experiments, the absolute number of surface antigens expressed/cell was calculated. To this purpose, the flow cytometer was calibrated with a set of standardized beads (DAKO, Glostrup, Denmark) each with a known amount of fluorochrome (either FITC or PE) expressed in units of MESF (Molecules of Equivalent Soluble Fluorescein). Thus, a standard curve was constructed by plotting MESF values for the beads against the median channel in which the peak was displayed.

3.1.3 PLATELET PURIFICATION

All the remaining blood samples were centrifuged at 160 g for 20 minutes at room temperature (RT), to obtain platelet rich plasma (PRP). Platelets were then purified by negative separation using magnetic beads coated with anti-CD45 antibodies (Dyna-beadsH, Invitrogen, Carlsbad, CA), to deplete nucleated cells. Briefly, PRP were stained with the magnetic beads-coated mAb anti-CD45 for 20 min at RT on a rotator. Purified

platelets were washed 3 times in PBS/BSA solution, counted, tested for purity by anti-CD41 staining and flow cytometry analysis (only samples where CD41+ cells .98% were used), and finally processed for RNA extraction.

3.1.4 RNA ISOLATION

Higly purified platelets were treated with an appropriate amount of TRIzolTM (Invitrogen) for cell lysis and RNA extraction, following the manufacturer's protocol. Briefly, chloroform was added to TRIzolTM-treated samples and centrifuged at 12.000 g for 15 min at 4°C. The acqueous phase, containing RNA, was transferred in a new tube and added with an equal volume of isopropanol. After incubation, the samples were centrifuged at 12.000g for 15 min at 4°C, to obtain RNA pellets that were washed and resumed in DEPC-treated water for quantification by spectrophotometer.

3.1.5 AMPLIFICATION OF RNA FOR PKCε GENE EXPRESSION ANALYSIS

The isolated RNA was both positively and negatively tested for cell population purity by Reverse-Transcription PCR (RT-PCR). A standard set of primers was used to test platelet RNA (amplification of CD41) or contaminant cells RNA (amplification of CD45 for nucleated cells).

Platelet RNA purification was followed by reverse transcription and RT-PCR to yield complementary DNA (cDNA). From the cDNA sample, cRNA was synthesized by in vitro transcription (IVT), and then analyzed for PKCε gene expression. Briefly, 1 μg total RNA was reverse transcribed with MMV reverse transcriptase and subjected to PCR amplification to detect CD41, CD45 and PKCε cDNA.

PCR were performed under the following reaction conditions: 95 ºC for 30 sec, 56 ºC for 30 sec, 72 ºC for 30 sec and a final extension at 72 ºC for 5 min. We used 35 cycles of amplification. The sequences of primers used for PCR were: CD41: 5'-GCAAT GTCGA GGGCT TTGAG-3' (sense) and 5'-GGCTG TTCTT GCTCC GTATC-3' (antisense); CD45: 5'-GGAAG TGCTG CAATG TGTCA TT-3' (sense) and 5'-CTTGA CATGC ATACT ATTAT CTGAT GTCA-3' (antisense); PKCε: 5'-CAATG GCCTT CTTAA GATCA AAA-3' (sense) and 5'-CCTGA GAGATC GATGATC ACATAC-3' (antisense). Primers used for PKCε RT-PCR amplify exon 1 and exon 2 sequences flanking the first intronic sequence of the gene, which is 190,000 bp.

3.1.6 QUANTITATIVE ANALYSIS FOR PKCε GENE EXPRESSION BY REAL-TIME PCR

Equal quantities of RNA for each sample were retro-transcribed with Malone Murine Leukemia Virus Reverse Transcriptase (Promega, Madison WI, USA) according to manufacturer'instructions. Two µl of 1:1 cDNA dilution were used to perform real-time PCR with GoTaq® qPCR master mix (Promega) and 200 nM of each primer in Applied Biosystems StepOne real-time machine (Applied Biosystems, Carlsbad, CA). Each reaction was performed in triplicate and mean Ct values were considered for quantitation. Relative gene expression was analysed using comparative Ct experiment software subset following manufacturer's instructions.

The sequences of primers used for PCR were: CD41: 5'-GCAAT GTCGA GGGCT TTGAG-3' (sense) and 5'-GGCTG TTCTT GCTCC GTATC-3' (antisense); CD45: 5'-GGAAG TGCTG CAATG TGTCA TT-3' (sense) and 5'-CTTGA CATGC ATACT

ATTAT CTGAT GTCA-3' (antisense); PKCε: 5'-CACCA TCCAG TTTGA GGAGC-3' (sense) and 5'-CGACC CTGAG AGATC GATGA -3' (antisense).

3.1.7 PROTEIN EXTRACTION AND WESTERN BLOT

Total proteins were extracted from 1 ml (450x103/μl) of purified platelets. Briefly, 1 ml of isolated platelets from each sample was collected and centrifuged at 1700 rpm for 10 min. The pellets were then suspended in a cell lysis buffer (50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mM Na3VO4; 1 mM NaF) supplemented with fresh protease inhibitors and protein concentration was determined using BCATM protein assay kit (Pierce, Rockford, IL). Fifty μg of proteins from each sample were then migrated in 5% SDS-acrylamide gels and blotted onto nitrocellulose filters.

Blotted filters were blocked and incubated with specific primary antibodies diluted as described in maunfacturers' protocols. Specifically, rabbit polyclonal anti-PKCε (Upstate, Lake Placid, NY) and mAb anti-β-actin (Sigma, Saint Luis, MO) were diluted 1:5000. Filters were washed and further incubated for 1.5 hours at room temperature with 1:5000 peroxidase-conjugated anti-rabbit or with 1:2000 peroxidase-conjugated anti-mouse IgG (Pierce) in the primary antibody working solution at RT. Specific reactions were revealed with the ECL Supersignal West Pico Chemiluminescent Substrate detection system (Pierce).

3.1.8 PKCε PROTEIN EXPRESSION IN PLATELETS BY FLOW CYTOMETRY

Aliquots of PRP samples were stained with: i) anti-CD41a monoclonal antibody (mAb), as a marker of platelet population; ii) Thyazole Orange (TO), to identify reticulated platelets; iii) rabbit anti-PKCε monoclonal antibody, to test the presence of PKCε protein; and analyzed by flow cytometry. Briefly, 200 µl of PRP were washed and treated with IntraPrepTM Permeabilization Reagent (Immunotech, Marseille, France), following manufacturer's protocol. One-hundred µl of Reagent 1 (fixation reagent) were added to the samples. After 15 min of incubation at room temperature, samples were washed and incubated with 100 µl of Reagent 2 (permeabilization reagent), 10 µl CD41a-Cy5 (Becton Dickinson, San Diego, CA) and 1 µl of affinity purified rabbit anti-PKCε Ab (Novus Biologicals, Littelton, CO); negative control was incubated with 100 µl of Reagent 2, 10 µl isotype-matched IgG-Cy5 (Immunotech) and 1 µl of an isotype-matched Ab (rabbit IgG, Sigma) for 45 min at room temperature in the dark. After a washing step, 1 µl of affinity purified goat anti-rabbit-PE Ab (Beckman Coulter) was added and the samples were incubated for 45 min at room temperature in the dark. Finally, the samples were washed and a solution10 ng/ml of TO (Sigma) was added, in the presence or absence of 10 mg/ml RNAsi (negative control), and incubated for 30 min at room temperature in the dark and analysed by Flow Cytometry. Working dilutions of all reagents were previously determined with serial dilution tests. All samples were analysed on an FC500 flow cytometer (Beckman Coulter).

3.1.9 PLATELET TRASFECTION WITH PKCε PROTEIN

Human recombinant PKCε (rhPKCε; GenWay Biotech, San Diego, CA) was transfected into purified platelets using Proteojuice protein transfection reagent (Novagen, Podenzano,

Italy), according to manufacturer's protocols. For each transfection, 1ml of PRP was centrifuged at 1800 rpm for 7 min, the supernatant was removed and pellets were washed with serum-free medium. Subsequently, samples were centrifuged at 1800 rpm for 7 min and medium was completely removed. The transfection mixture was prepared as follows: 25µl of serum-free medium were added to 1µg of PKCε protein (or nothing, as negative control) and 1,25 µl of ProteoJuice protein transfection reagent. After 20 min of incubation at room temperature, 225 µl of serum-free medium were added to the transfection mixture. Finally, the platelet samples were incubated with the mix at 37°C, 5% CO2 for 3,5 hours. After transfection, the samples were washed twice with serum-free medium to remove excess protein.

In some experiments, transfected and control platelets were re-added to platelet-deprived whole blood and aliquots were then used for platelet activation analysis by flow cytometry, as described above.

3.1.10 PLATELET ADHESION ANALYSIS IN SHEAR RATE SYSTEM

Isolated PRP was treated for rhPKCε transfection, as described above. The procedures to prepare a washed erythrocyte suspensions have been described previously in detail *(Mazzucato M et al. 2004)*. PRP containing 2-8 x108 loaded platelets/ml was mixed with washed erythrocytes, to obtain a suspension with hematocrit of 42-45%, and apyrase (grade III; 142 ATPase U/mg of protein; Sigma) was added at the final concentration of 5 ATPase U/ml. The mixture was centrifuged at 1000 g for 15 min, the supernatant was discarded and the cell pellet was suspended in plasma. Suspensions of acid-insoluble fibrillar type I collagen from bovine achille tendon (Sigma) in 0.5M acetic acid (pH 2.8) were prepared as previously described *(Folie BJ et al 1988)* and two hundred microliters was used to coat glass coverslips for 60 minutes at 22-25°C in a humidified box. After coating, coverslips were washed with PBS and kept in a moist environment until assembled in a modified Hele-Shaw flow chamber *(Savage B et al 1998)*. The flow chamber was positioned on the stage of an inverted microscope equipped with epifluorescent illumination (Diaphot-TMD; Nikon Instech, Shinagawaku, Japan), an intensified CCD videocamera (C-2400-87; Hamamatsu Photonics, Shizuoka, Japan), and appropriate filters. The total area of an optical field corresponded to approximately 0.007 mm2. Blood cells were aspirated through the chamber with a syringe pump (Harvard Apparatus, Hollistone, MA) at a flow rate calculated to obtain the desired wall shear rate at the inlet. Platelet adhesion was measured using blood containing 10 μM mepacrine to render platelets fluorescent.

Three negative controls were run for each sample: i) untreated platelets; ii) platelets treated with a PKCε traslocation inhibitor peptide (Merck KgaA, Darmstadt, Germany) added to the trasfection mixture (1ug of inhibitor peptide) with or, iii) without rhPKCε. Experiments were recorded in real time on videotape at the rate of 25 frames/s, which

resulted in a time resolution of 0.04 s. Selected video sequences were also digitized in real time using a Matrox-Digisuite board (Matrox Graphics Inc., Dorval, Quebec, Canada).

Single frame images were captured from videotapes after an initial blood perfusion for 3 minutes on the different substrates. A threshold was applied to distinguish platelets from the background and the area occupied by all platelets in an image was measured using MicroImage (image-processing software; Tesi-Imaging srl, Venice, Italy).

3.1.11 STATISTICAL ANALYSIS

The variables were compared between the three groups of patients using One Way Anova and Bonferroni t-test for multiple comparisons. Where indicated the variables were compared using Mann-Whitney test. T-test for independent or correlated samples was used to compare some indicated data. All the results are expressed as means plus or minus SD. Chi-square analysis of contingency tables were used for PKCε mRNA expression analysis of frequency comparison in the three groups. All the statistical tests were performed at the 0.05 p-value.

PART II:

PKCδ/ε BALANCE IN HUMAN THROMBOPOIESIS

To investigate PKCδ expression during megakaryocytopoiesis and platelet production and the role of PKCδ/ε balance in human thrombopoiesis, I used an *in vitro* model of MKs differentiation from human CD34+ haematopoietic stem cells.

3.2.1 $CD34^+$ CELL ISOLATION AND CELL CULTURE

Primary $CD34^+$ cells were isolated from peripheral blood of healthy donors and primary myelofibrosis (PMF) patients. Samples were collected in sodium citrate tubes after written informed consent and approval by the Ethical Committee of Parma University Hospital (according to the Helsinki declaration of 2004 and informed consent was obtained from the donors). Primary $CD34^+$ cells were purified by immunomagnetic positive selection using the $CD34^+$-cell isolation Kit (Milteny Biotech, Gladbach, Germany) in the magnetic field of autoMACS Pro Separator (Milteny Biotech), according to the manufacturer's protocol. Purity of $CD34^+$ cells was immediately checked by a R-phycoerythrin (RPE)-conjugated anti-CD34 mAb (Immunotech, Beckman Coulter, Miami, FL) and flow cytometry; only samples exceeding 95% purity were used for subsequent experiments.

Purified human $CD34^+$ cells were cultured up to 14 days, at an optimal cell density of 1x106 cells/ml, in serum free X-vivo medium (Lonza Group, Switzerland) supplemented with 200 ng/ml of recombinant human thrombopoietin (TPO), 50 ng/ml of recombinant

human stem cell factor (SCF) and 3 ng/ml of recombinant human interleukin-3 (IL-3) (Peprotech, London, UK) renewed every 3 days.

3.2.2 shRNA CELL INFECTION

For shRNA-based gene silencing, pLKO.1 lentiviral vector encoding short hairpin RNAs (shRNA) against human PKCdelta (transcription variant 1, NM_212539 and transcription variant 2, NM_006254; shPKCdelta) were obtained from Open-Biosystem (Thermo Fisher Scientific Inc, Waltham, MA USA). As control (shCT), we used the MISSION pLKO.1-puro Non-Target shRNA Control Plasmid, containing an shRNA insert that does not target any known genes from any species (Sigma-Aldrich, St. Louis, MO, USA). shRNA expressing viruses were produced in 293TL cells according to standard protocols. Cells were infected at Day 8 of TPO-culture and then cultured in the presence of puromycin, to select infected, puromycin-resistant cells.

At Day 14 of culture, infected MKs were analysed for morphological evaluation by light microscopy and an aliquots from each cultures were collected for flow cytometry and western blot analysis.

3.2.3 PHARMACOLOGICAL INHIBITION AND ACTIVATION OF PKCδ AND PKCε

PKCδ activity was modulated by the δV1-1 (SFNSYELGSL) and by ψδRACK (MRAAEDPM) peptides coniugated to TAT47-57 (CYGRKKRRQRRR) by a cysteine disulfide bound (synthesized by Selleckchem, Huston, TX, U.S.A). PKCε activity was modulated by the εV1-2 (CEAVSLKPT) and by ψεRACK (CHDAPIGYD) peptides coniugated to TAT47-57 (CYGRKKRRQRRR) by a cysteine disulfide bound (synthesized

by AnaSpec Inc, Fremont, CA, U.S.A). TAT47-57 (CYGRKKRRQRRR) peptide was used as control (synthesized by AnaSpec Inc, Fremont, CA, U.S.A). Briefly, δV1-1 and εV1-2 are peptides corresponding to the least conserved sequences between isozymes within the C2 domain that selectively inhibit the corresponding isozyme preventing PKC translocation and substrate phosphorylation. Instead ψδRACK and ψεRACK are PKC allosteric activator derived from the C2 region sequence, implicated in auto inhibitory intramolecular interactions *(Mochly-Rosen D, Nature reviews 2012; 11: 937-957).*

MK cultures of primary CD34+ cells were splitted into four treatments from day 8: i) cells treated with 1μM ψδRACK and 1μM εV1-2 (ψδRACK / εV1-2); ii) cells treated with 1μM δV1-1 and 1μM ψεRACK (δV1-1 / ψεRACK); iii) cells treated with 2μM TAT47-57 as control (TAT47-57); iiii) untreated cells (Untreated). Peptides were renewed every 48 hours, cells were cultured up to 13 days and then collected for further analysis.

3.2.4 MORPHOLOGICAL EVALUATION OF MKs DIFFERENTIATION

Cell plates at 14 days of culture were analysed using Leica DM IL phase contrast microscope (40X/0.5NA) and images were obtained by Leica ICC50 HD camera (Leica Microsystems, Wetzlar, Germany).

Percentage of megakaryocytes extending proplatelets and cell diameter were determined using ImageJ software analizing 100 cells for each treatment from at least four independent experiments.

3.2.5 FLOW CYTOMETRIC ANALYSIS

Flow cytometry was used to analyse the viability and the ploidy of the differentiating cell populations at day 14 of culture.

Cell culture viability was assessed by propidium iodide (PI) (Sigma-Aldrich) staining, briefly aliquot of cells were incubated with 2,5 ug/ml of PI in PBS for 15 minutes in dark at room temperature and then analysed by flow cytometry.

For ploidy analysis, aliquots of cells were permealized with 70% ethanol overnight at 4°C, washed with PBS and incubated in PBS containing 50 μg/ml PI and 100 μg/ml RNAse-A (Sigma-Aldrich) for 15 minutes in dark at room temperature before flow cytometry analysis. $CD34^+$ cells at day 0 were used as marker for the identification of the diploid (2N) peak.

Finally, to quantify the platelet production in culture, fixed volumes of the culture supernatants were collected, incubated with anti-CD41-RPE and calcein AM (to exclude fragments; Sigma-Aldrich) and added to a fixed volume of calibration beads (DAKO, Agilent Techonolgies, Santa Clara CA, U.S.A) at known concentration. Both the platelets and beads populations were simultaneously identified in flow cytometry on the Forward Scatter (FS) vs logarithmic Side Scatter (SS) dot plot. The absolute platelets count was performed on the gated CD41+/Calcein AM+ platelet population *(Gobbi G. 2009; Nurden P. Blood 2010)*.

Analysis of the samples was performed by an FC500 flow cytometer (Beckman Coulter) and the Expo ADC software (Beckman Coulter).

3.2.6 PROTEIN EXPRESSION ANALYSIS

Cultured cells were counted and 1.5 x 106 cells were collected on Days 0, 3, 6, 9, 14 for healthy donors and on day 14 for PMF and ITP patients. Cells were washed in PBS and centrifuged at 200g for 10 min. Pellets were resuspended in a cell lysis buffer (50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mM Na3VO4; 1 mM NaF) supplemented with fresh protease inhibitors and protein concentration was determined using BCATM protein assay kit (Pierce, Thermo Scientific). Twenty five μg of proteins from each sample were ran on 4-20% SDS-acrylamide gels (Biorad), blotted onto nitrocellulose membranes, blocked and incubated with specific primary antibodies diluted as described in maunfacturers' protocols. Specifically, mouse monoclonal anti-PKCdelta antibody (BD Pharmingen, Cat. #610397) was diluted 1:500, rabbit polyclonal anti-PKCepsilon antibody (Merk Millipore, Cat.# 06-991) was diluted 1:1000, rabbit polyclonal anti-Bax antibody and rabbit polyclonal anti-Bcl-xL antibody (Cell Signaling Technology, Inc. Lake Placid, NY, Cat. # 2772 and Cat. # 2762) were diluted 1:500 and 1:1000 respectively and monoclonal anti-GAPDH antibody (Merk Millipore, Cat. # MAB374), 1:5000 dilution was used as protein loading control.

Membranes were washed and incubated for 1.5 hours at room temperature with 1:5000 peroxidase-conjugated anti-rabbit (Pierce, Thermo Fisher Scientific Inc) or with 1:2000 peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich) at room temperature and resolved by ECL Supersignal West Pico Chemiluminescent Substrate detection system (Pierce, Thermo Fisher Scientific Inc). Protein densitometric analyses from western blot assays were performed by using the Image J software system, normalized for GAPDH expression levels.

3.2.7 STATISTICAL ANALYSIS

Statistical analysis were performed by using t-Test to compare two samples, Anova and Tukey Tests for multiple comparisons.

4. RESULTS

PART I:

PKCε IN PLATELETS FUNCTION

PKCε is virtually absent in human platelets and its expression is precisely regulated during human megakaryocytic differentiation. In mice platelets, PKCε shows a proaggregant function. Platelet hyperactivation play crucial roles in the pathophysiology of thrombosis and myocardial infarction (MI). On these basis, I hypothesized that platelets from myocardial infarction patients might ectopically express PKCε with a pathophysiological role in the disease.

4.1.1 MI PATIENTS CARRY HYPER-RESPONSIVE PLATELETS

It is well known that patients with acute coronary syndromes carry hyper-responsive platelets *(Smitherman TC et al.1981; Langford EJ et al. 1996)* and show systemic platelet activation *(Langford EJ et al.1996; Theroux P, Fuster V. 1998; Vaitkus PT et al.1995; Hamm CW et al. 1987; Dorn GW, 2nd, 1990).* Therefore, I first studied the activation state of platelets and their sensitivity to agonistic stimuli in a randomly selected sub-group of patients. In agreement with the current literature, I found that platelets from MI patients express significantly higher surface levels of p-selectin than those from sCAD patients and healthy donors (Fig. 4.1.1A). Moreover, in MI patients CD62p expression was induced at significantly higher levels by sub-optimal doses of ADP (Fig. 4.1.1B).

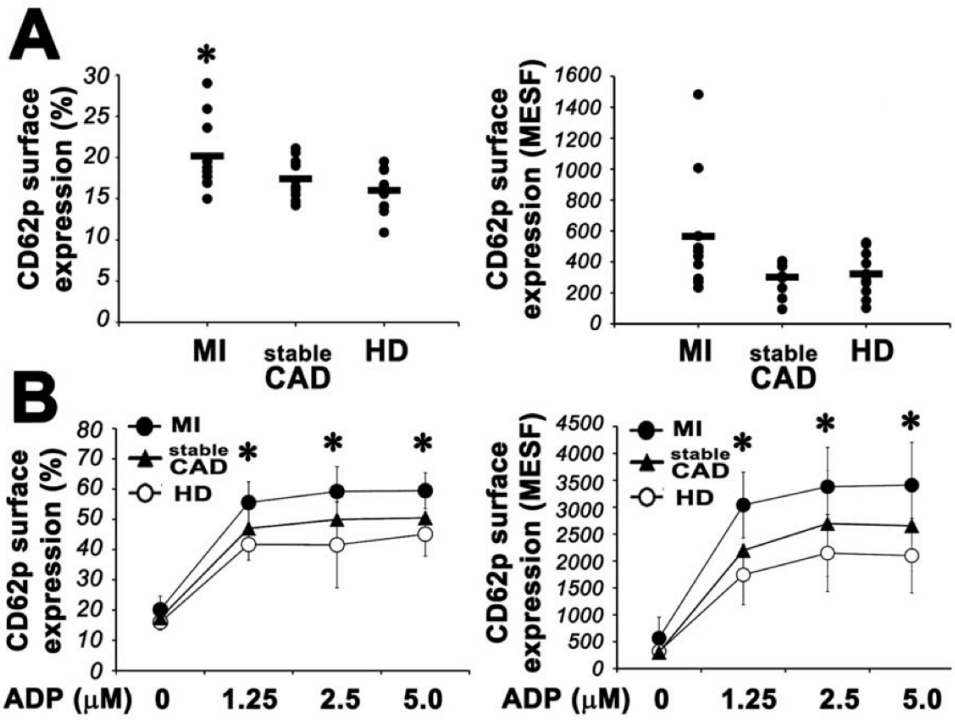

Figure 4.1.1.Platelet activation in MI patients. Panel A: Left. Flow cytometric analysis of platelet CD62p surface expression in patients with MI, sCAD and healthy donors. Cells were stained with specific mAb anti P-selectin, as described in Materials and Methods. Ten patients were analyzed for each group. Right. Quantification of CD62p expression on the surface of platelets from MI, sCAD and HD. Absolute numbers of surface antigens expressed/cell (MESF). (Anova and Bonferroni t-test; * P<0.05 MI *vs* HD; no significant differences were found between stableCAD and HD). Panel B: Platelet surface expression of CD62p in MI (●), sCAD (▲) and HD (○) patients in the presence of increasing doses of ADP. Left panel shows the percentage of positive cells. Right panel shows the absolute numbers of surface antigens expressed/cell (MESF). Data from 7 patients of each group are expressed as means ± S.D. (Anova and Bonferroni t-test; * P<0.05 MI *vs* HD; no significant differences were found between stable CAD and HD).

4.1.2 PLATELETS FROM MI PATIENTS CONTAIN mRNA FOR PKCε AND EXPRESS PKCε PROTEIN

Since PKCε is finely modulated during megakaryocytopoiesis *(Gobbi G et al. 2007)* and it is known to increase the response of mouse platelets to GPVI-mediated activation *(Pears CJ et al. 2008)*, I reasoned that it could be a good candidate to account for the hyper-responsiveness of platelets from MI patients. Therefore, I extracted total mRNA from highly purified platelets and studied the expression of mRNA for PKCε by RT-PCR. The absence of nucleated cells contamination in the isolated platelet population was assessed by RT-PCR for CD45 expression (Fig. 4.1.2A). Moreover, given the dimensions (190 Kb)

of the first intronic sequence of the PKCε gene and the positioning of the primers I used for PKCε RT-PCR, I could reasonably exclude the presence of PKCε pre-mRNA in platelets. In agreement with previous data *(Pears CJ et al. 2008)*, expression of PKCε mRNA was found at low frequency both in platelets from normal subjects and sCAD patients. On the contrary, platelets from the majority of MI patients (21 out of 24; 87.5%) had a clear-cut expression of PKCε mRNA (Fig. 4.1.2B, C), as confirmed also by qPCR (Fig. 4.1.2D).

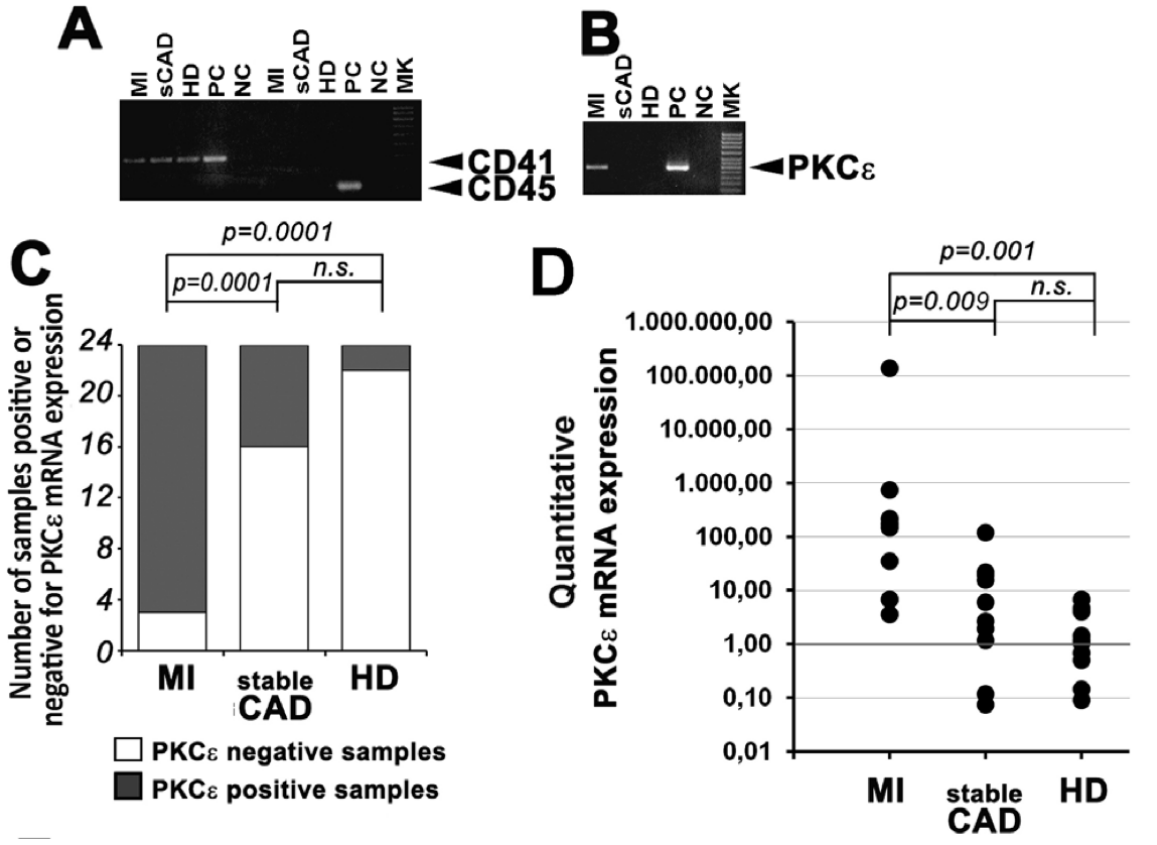

Figure 4.1.2 Platelets from MI patients contain mRNA for PKCε. Panel A: Representative RT-PCR analysis of CD41 and CD45 expression in MI, sCAD and healthy donor platelets. Equal amounts of total cDNA were amplified by PCR to detect the indicated mRNA expression. CD45 is not expressed in isolated platelets, indicating the absence of nucleated cell contaminants. Panel B: Representative RT-PCR analysis of PKCε expression in MI, sCAD and healthy donor platelets. Equal amounts of total cDNA were amplified by PCR to detect PKCε mRNA. Platelets from MI patients express PKCε. PC: positive control; NC: negative control. Panel C: Analysis of PKCε RNA expression in MI, sCAD and HD platelets. The samples positive to PKCε mRNA expression in MI, sCAD and HD were respectively 21, 8, 3 out of 72 (24 each group) (Chi-square test: p = 0.0001 MI *vs* HD; p = 0.0001 MI *vs* sCAD; no significant differences were found between sCAD and HD). Panel D: Quantitative analysis of PKCε mRNA expression by real-time PCR. Equal amounts of total cDNA were amplified by PCR. The expression level of PKCε mRNA in each patient was compared with the mean expression in the HD group. Data from 10 patients of each group are shown (Mann Whitney test: p = 0.001 MI *vs* HD; p = 0.009 MI *vs* sCAD; no significant differences were found between sCAD and HD).

I consequently studied the expression levels of PKCε protein by Western Blot selecting PKCε mRNA-positive patients from each group (10 patients from the MI group; 5 patients from the sCAD group; 3 subjects from HD group). As expected, accordingly with qPCR results, PKCε expression in MI patients was about three fold greater then sCAD and healthy subjects (Fig. 4.1.3A, B).

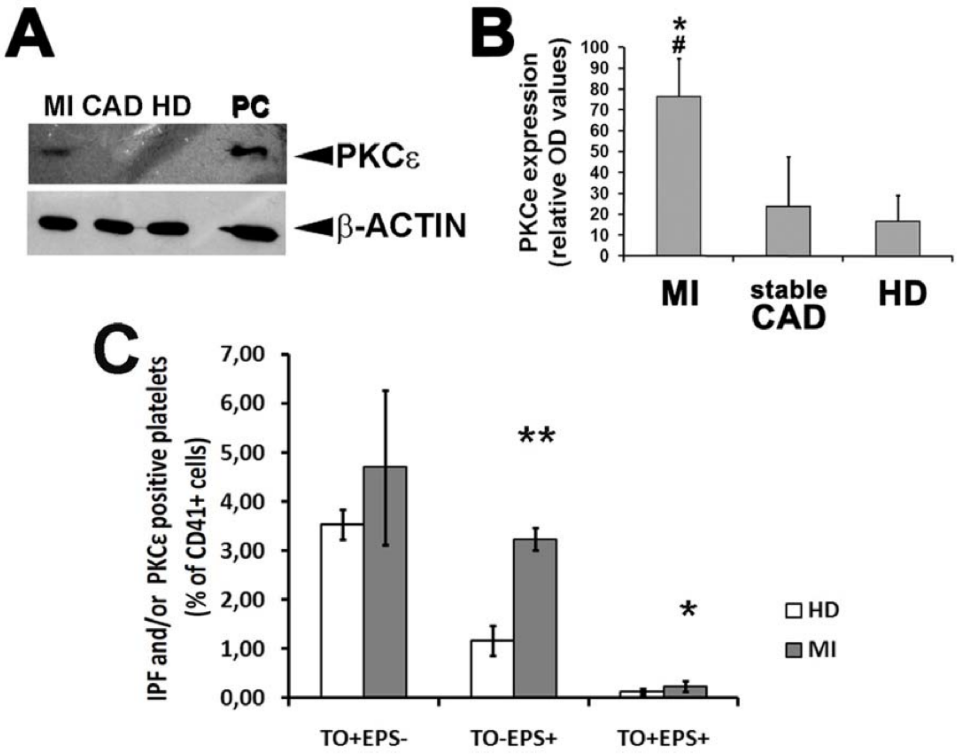

Figure 4.1.3 . Platelets from MI patients express PKCε protein. Panel A: A representative Western blot assay for the detection of total PKCε protein expression in platelets from PKCε mRNA-positive MI , sCAD and HD. β-Actin was assayed for protein loading. Panel B: Densitometric analysis of PKCε protein expression normalized against β-actin in PKCε mRNA-positive MI , sCAD and HD platelets. Data are expressed as means ± S.D. (Anova and Bonferroni t-test; * P<0.05 MI *vs* HD; # P<0.05 MI *vs* sCAD; no significant differences were found between sCAD and HD). Panel C: PKCε protein expression in mature and immature platelets from healty donors (HD) and MI patients. Cells were simultaneously labelled with Thyazole Orange (TO) -to visualize the immature platelet fraction (IPF) - and anti-PKCε mAb, and analyzed by flow cytometry. Three populations were identified within the CD41+ cell subset: PKCε negative reticulated platelets (TO+EPS-); PKCε positive reticulated platelets (TO+EPS+); PKCε positive mature platelets (TO-EPS+); (Data from 3 patients/group, expressed as means ± S.D. t-Test ** P<0.001; * P<0.05).

To test if this ‘‘ectopic’’ expression of PKCε in the platelets from MI patients could be attributed to the immature platelet fraction, I analysed by flow cytometry the population of reticulated platelets from MI patients and HD. As reported in Figure 4.1.3C, the percentage of platelets from MI patients expressing PKCε protein was significantly increased both in mature (TO-EPS+) and immature (reticulated) (TO+EPS+) platelet fractions, as compared to HD. More specifically, the ratio between PKCε positive platelet subsets from MI vs HD subjects was 2.9±0.73 in mature platelets (TO-EPS+) and 2.17±1.44 in immature platelets (TO+EPS+) ($p = 0.48$, ns).

4.1.3 PLATELETS FROM MI PATIENTS DURING FOLLOW UP BECAME NEGATIVE FOR PKCε mRNA

To test whether the expression of PKCε in MI was transient or stable, 11 randomly selected MI patients positive for platelet PKCε mRNA expression were re-called between day 15 and day 30 from the acute episode and the platelets were tested again for PKCε expression. All platelet samples were found negative for PKCε mRNA expression as soon as 15 days from the acute MI episode (not shown).

4.1.4 PKCε-EXPRESSING PLATELETS ARE HYPER-RESPONSIVE

To study the correlation between PKCε expression and platelet activation, I compared the p-selectin (CD62p) cell surface expression in PKCε positive vs PKCε negative platelets in all the analysed groups (Fig. 4.1.4A). The general trend is a significant higher p-selectin expression in PKCε positive cells. Subsequently, I tested the platelet reactivity to the stimulation with sub-optimal doses of ADP. As expected, both PKCε negative and positive

platelets were activated, as compared to controls (resting), but the positive cells were significantly more reactive than the negative (Fig. 4.1.4B).

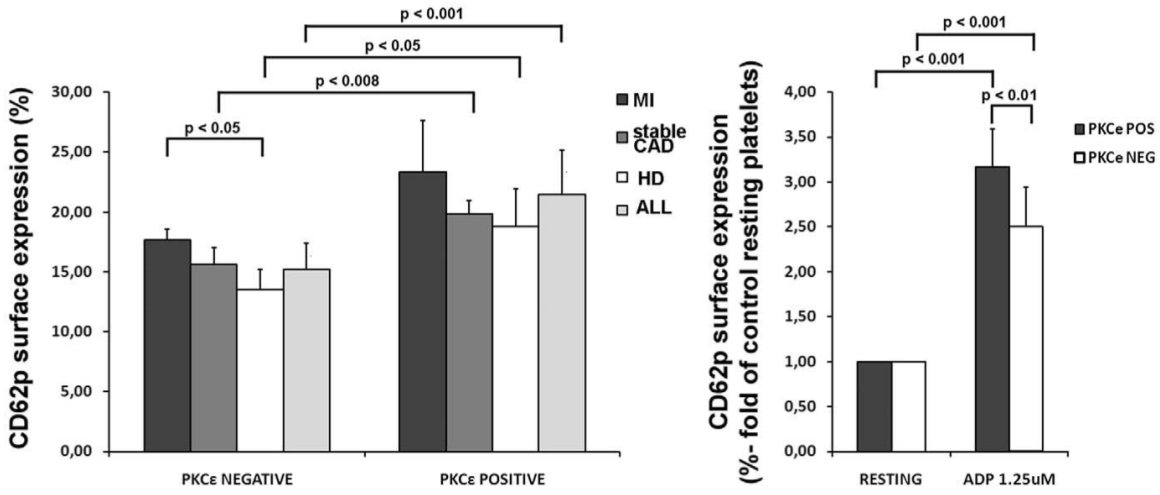

Figure 4.1.4 PKCε protein expression in platelets correlates with their activation levels. Panel A: Flow cytometric analysis of platelet CD62p surface expression in patients with MI, sCAD, healthy donor and in all the sample (ALL), on the basis of PKCε expression. Cells were stained with specific mAb anti P-selectin (CD62p). Seven patients were analyzed for each group (MI: 2 PKCε negative and 5 PKCε positive samples; sCAD: 4 PKCε negative and 3 PKCε positive samples; HD: 4 PKCε negative and 3 PKCε positive samples). Data is expressed as mean ± S.D (Anova and Bonferroni t-test). Panel B: Flow cytometric analysis of CD62p surface expression in PKCε negative and positive platelets. Cells were treated with ADP and compared with untreated platelets (resting). Ten patients were analyzed for each group. Data is expressed as mean ± S.D (Anova and Bonferroni t-test).

4.1.5 PKCε-OVEREXPRESSING PLATELETS ARE HYPER-RESPONSIVE AND SHOW ENHANCED ADHESION TO COLLAGEN

To study the functional role of PKCε in platelets, I subsequently forced its expression in platelets from normal healthy donors in vitro mimicking the in vivo situation. rhPKCε protein was therefore successfully transfected in healthy donor platelets (thus originally negative for PKCε expression) (Fig. 4.1.5A). Subsequently, $PKC\varepsilon^{+}$ platelets were functionally assayed for ADP-induced activation and shear stress adhesion, using mock-transfected normal platelets as negative controls.

First, PKCε$^+$ normal platelets and control platelets were treated with minimal doses of ADP (0.3 mM) and analyzed for CD62p surface expression by flow cytometry. There were no significant differences in the surface expression of CD62p between PKCε$^+$ normal platelets and control platelets in the absence of ADP stimulation. On the contrary, PKCε$^+$ platelets treated with 0.3 mM ADP express significantly higher levels of CD62p than control platelets (Fig. 4.1.5B).

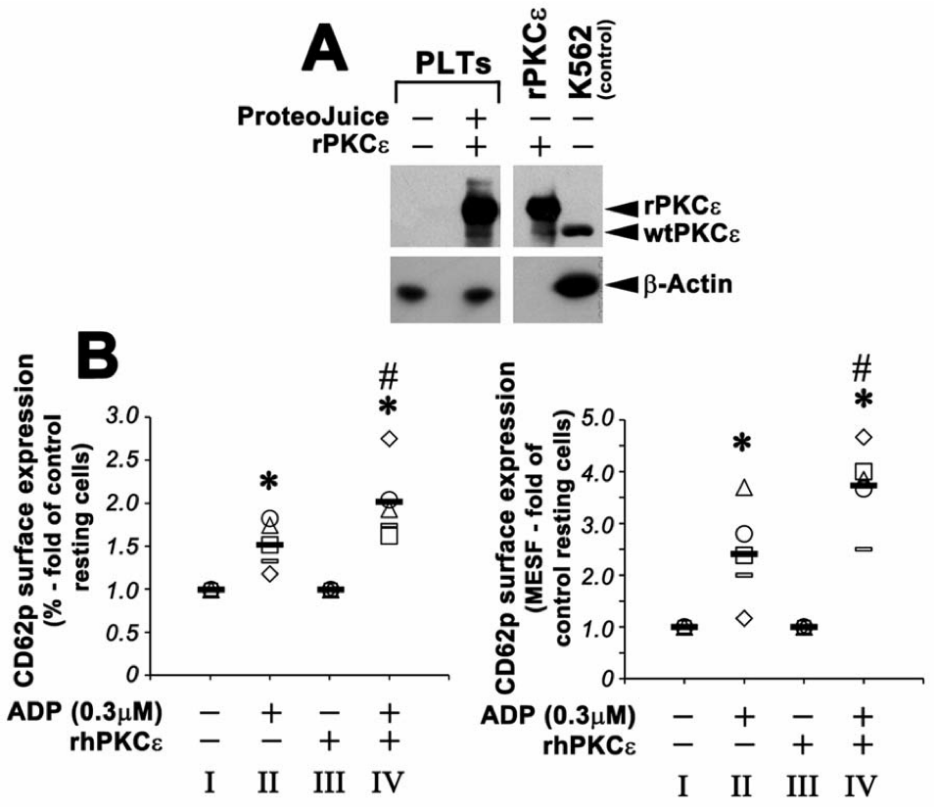

Figure 4.1.5 PKCε protein transfection in normal platelets induces hyper-responsiveness to ADP-mediated activation. Panel A. Western blot detection of total PKCε protein expression in transfected platelets. Healthy donor platelets were incubated with ProteoJuice medium in the presence or absence (negative control) of recombinant PKCε (rhPKCε). K562 cells were used as positive control. β-Actin was assayed for protein loading. Panel B: The expression of CD62p on the surface of activated platelets was compared to the expression of CD62p on resting platelets. rhPKCε-transfected platelets were significantly more reactive than activated control platelets. Left panel shows the percentage of positive cells. Right panel shows the absolute numbers of surface antigens expressed/cell (MESF). Data from 5 independent experiments (each symbol is related to one experiment) are expressed as means ± S.D. (Anova and Bonferroni t-test; * $P<0.05$ activated platelets *vs* resting platelets – II *vs* I and IV *vs* III; # $P<0.05$ rhPKCε-transfected platelets *vs* activated control platelets – IV *vs* II).

Second, I studied the adhesion to collagen-coated surfaces of PKCε-overexpressing normal platelets under controlled physiological low (600 sec21) or high (1500 sec21) shear rates. Adhesion to collagen at 1500 sec21 was significantly increased in normal platelets over-expressing PKCε, particularly after 3 minutes testing, (Fig. 4.1.6). Although hampered by a high variability, adhesion at low shear rate also showed a trend to increase. Adhesion of PKCε-transfected platelets treated with the PKCε inhibitor was similar to that of untreated platelets, demonstrating the specificity of the observed enhancement.

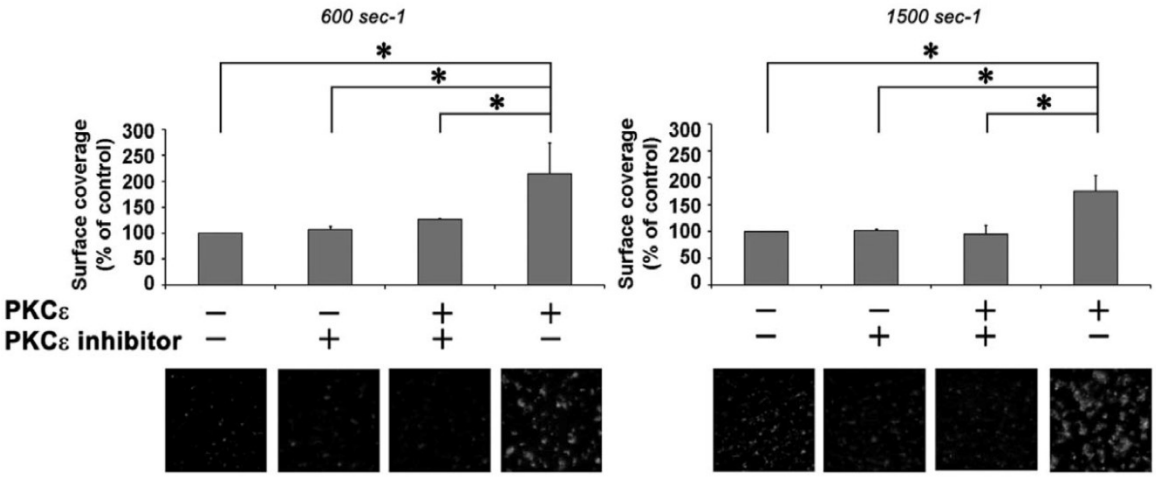

Figure 4.1.6. PKCε enhances platelet adhesion to fibrillar type I collagen under flow conditions**.** rhPKCε-transfected and control platelets were reconstituted in whole blood, previously deprived from PRP, and tested for their adhesion capacity under flow. Mepacrine-loaded platelets (5-7x10^8/ml) and washed erythrocytes (hematocrit 42-45%) suspended in plasma, were perfused for 3 minutes over immobilized fibrillar type I collagen. Surface coverage was measured on an area of 0.07 mm^2 after 3 minutes of perfusion at 600 s^{-1} or 1500 s^{-1} and is shown as mean ± 95% confidence intervals of at least 3 separate experiments. Results are shown relative to the values observed in untreated blood cell suspensions (control) (Anova and Bonferroni t-test; * $p< 0.01$). Representative single-frame images of each surface are also shown.

PART II:

PKCδ/ε BALANCE IN HUMAN THROMBOPOIESIS

It has been demonstrated that in human megakaryocytic differentiation, PKCε modulates MK maturation and platelets release and that it is down-regulated in the final step of megakaryocytpoiesis, in agreement with its absence in human platelets. It's also well known that PKCδ mediate contrasting and even opposing effects as compared to PKCε in several non-hematopoietic models. On these basis I hypothesized that, during the process of platelets production, PKCδ might has an opposite role than PKCε and that the PKCδ/ε balance could be a key factor for human thrombopoiesis.

4.2.1 PKCδ/PKCε and Bax/Bcl-xL EXPRESSION LEVELS ARE DIFFERENTLY MODULATED DURING MK DIFFERENTIATION

In agreement with previous studies in human megakaryocyte cultures *(Gobbi G et al. 2007)*, PKCε protein expression increases during the early phases of MK differentiation, declining on the final steps of this process. On the contrary, human PKCδ rises at the beginning of megakaryocytopoiesis, remaining highly expressed throughout the entire maturation process (Fig. 4.2.1A, B).

Besides, both Bcl-xL and Bax expressions are significantly modulated in differentiating MKs, with a kinetic similar to PKCε and PKCδ respectively (Fig. 4.2.1A, C).

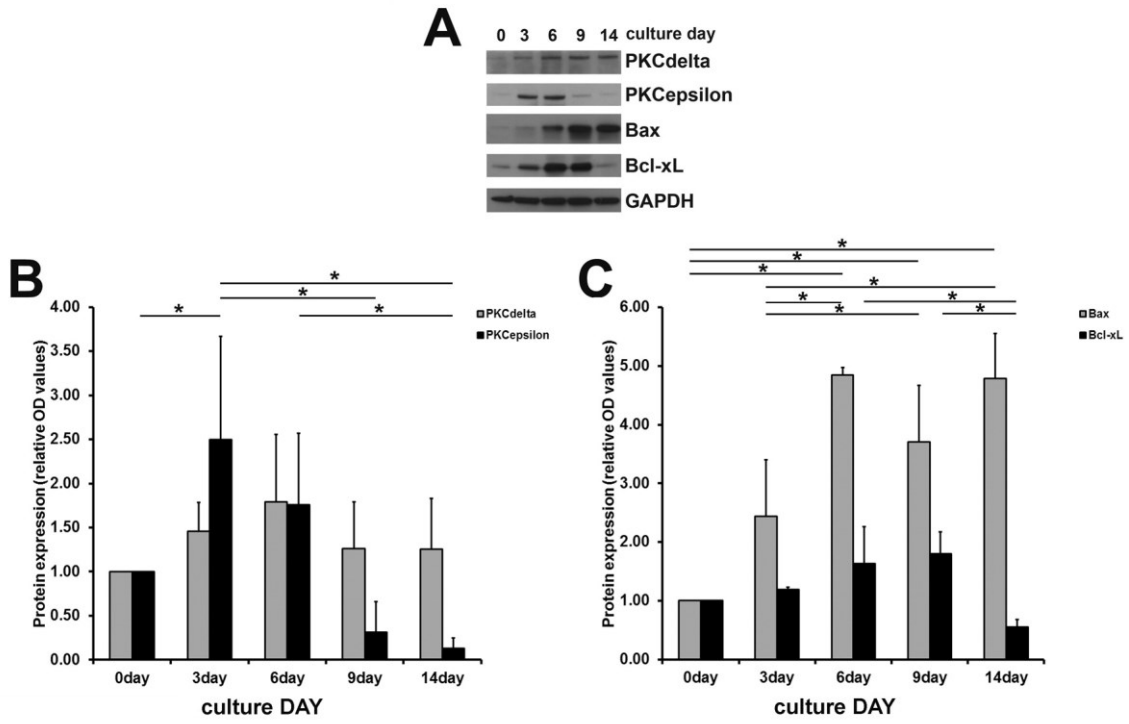

Figure 4.2.1 PKCdelta/PKCepsilon and Bax/Bcl-xL expression levels are differently modulated during MK differentiation. Panel A: Western blot detection of PKC delta, PKCepsilon, Bax, Bcl-xL protein expression in MK cultures. GAPDH was monitored for protein loading. Panel B: Relative PKC delta and PKCepsilon protein expression during megakaryocytic differentiation human $CD34^+$ cells normalized for GAPDH expression levels. Panel C: Relative Bax and Bcl-xL protein expression during megakaryocytic differentiation human $CD34^+$ cells normalized for GAPDH expression levels. Densitometric measurements of Western blots from 3 replicates were performed by ImageJ software (means ± SD; * $p < 0.05$ Anova and Tukey tests).

4.2.2 PKCδ DOWN-REGULATION REVERSES THE NORMAL EXPRESSION OF Bcl-xL AND Bax

It has been previously demonstrated that during late phases of MK differentiation the PKCε-forced expression induces Bcl-xL up-regulation *(Gobbi G et al. 2007)*. Based on PKCδ-specific shRNA data, I sought to determine whether PKCδ expression is necessary for regulating normal levels of Bcl-xL and Bax expression at the end of megakaryocytopoiesis. Therefore, I used recombinant lentiviral vectors to introduce and

stably express shRNA molecules that specifically target PKCδ into MK differentiating cells at Day 8 of culture. Analysis of megakaryocyte cultures at Day 14 (Day 5 post-infection) revealed that the abrogation of PKCδ was specific, as it did not modify the expression of PKCε (Fig. 4.2.2A, B). However, the selective down-regulation of PKCδ dramatically reduces Bax and boosts Bcl-xL expression (Fig. 4.2.2A, B). The densitometric analysis (panel B) of western blot assays clearly indicate a significant modulation of the tested proteins only in the presence of PKCδ-specific shRNA (shPKCdelta), as compared to the samples infected with control shRNA (shCT), which are fully similar to those uninfected.

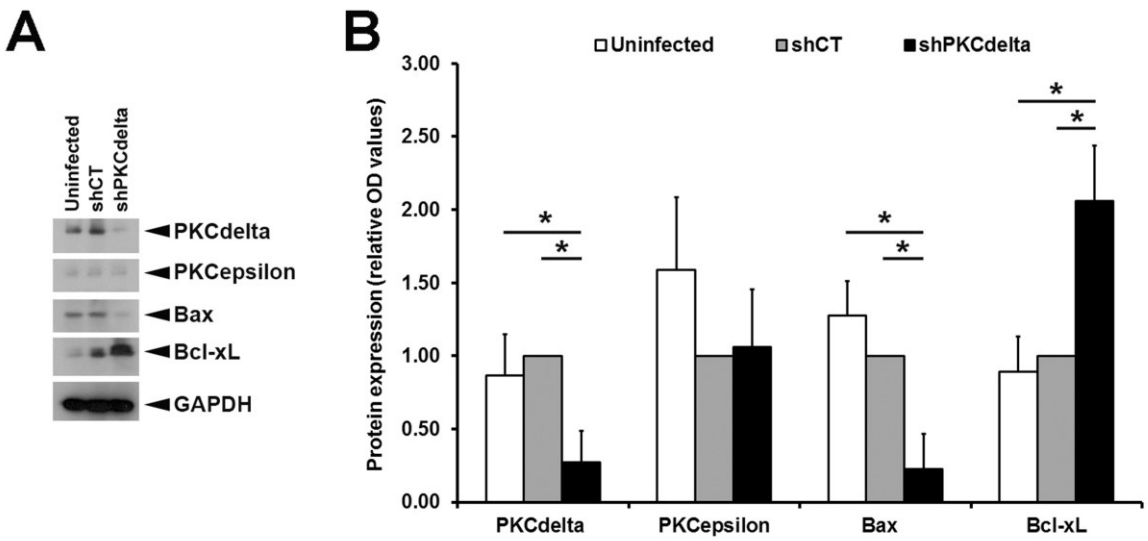

Figure 4.2.2 . PKCdelta down-regulation reverses the normal expression of Bcl-xL and Bax. Panel A: Western blot detection of PKCdelta, PKCepsilon, Bax, Bcl-xL in human MK uninfected (UN) or infected with PKCdelta-specific shRNA (shPKCdelta), and Non-Target shRNA control (shCT). GAPDH was monitored for protein loading. Panel B: Densitometric analysis of proteins expression, normalized for GAPDH expression levels, were performed using ImageJ software. Densitometric measurements of Western blots from 4 replicates (means ± one SD; *$p<0.05$ ANOVA and Tukey Tests).

4.2.3 PKCδ DOWN-REGULATION IMPAIRS MK DIFFERENTIATION AND PLATELET FORMATION

Analysis of puromycin-selected MK cultures at Day 5 post-infection revealed that abrogation of PKCδ affected MK differentiation (Fig. 4.2.3). Indeed, PKCδ-specific shRNA (shPKCdelta) infected cells resulted more viable (Fig. 4.2.3A), smaller (Fig. 4.2.3B) and less polyploid (Fig. 4.2.3 C, D), as compared to controls (Uninfected and shCT).

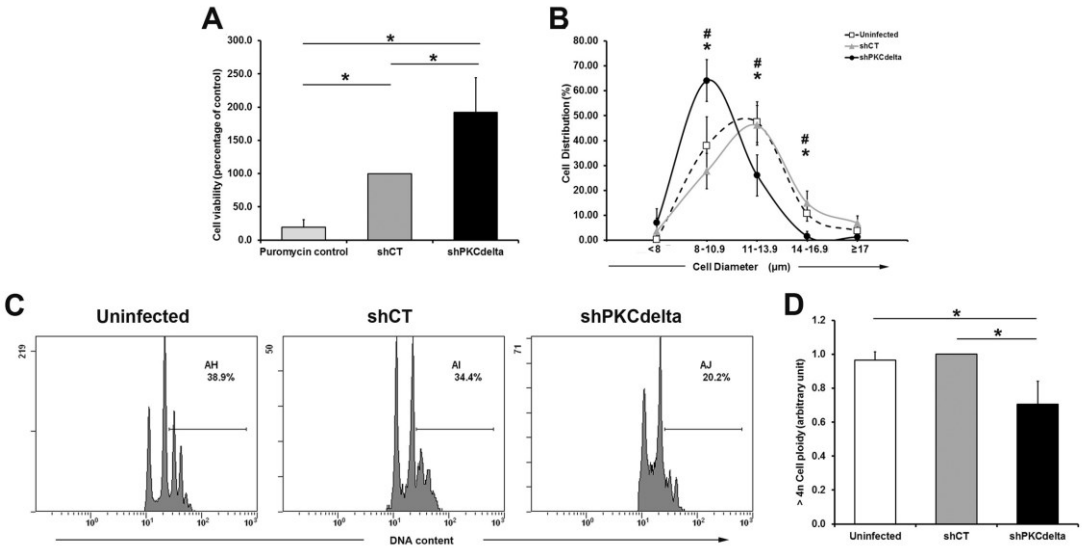

Figure 4.2.3 PKCdelta down-regualtion impairs MK differentiation. Panel A: Cell viability analysis of puromycin- selected MK cultures at day 5 post-infection. Percentage of PI negative cells from 3 replicates (cells data express as percentage of shCT; means ± one SD; *p<0.05 ANOVA and Tukey tests). Panel B: Analysis of size distribution within uninfected MK and puromycin- selected MK cultures at day 5 post-infection. Percentage of cells with a given diameter range of 3 replicates. (mean ± one SD; *p<0.05 shPKCdelta *vs* Uninfected, #p<0.05 shPKCdelta *vs* shCT, ANOVA and Tukey tests). Panel C: Representative histogram of cell ploidy analysis of puromycin- selected MK cultures at day 5 post-infection. Panel D: Percentage of puromycin- selected MK cultures at day 5 post-infection with a DNA content >4N from 3 replicates (cells data express as arbitrary unit of shCT; means ± one SD; *p<0.05 ANOVA and Tukey tests).

Although few residual branched protrusion could still be observed, proPLTs generated by shPKCdelta-infected cells were characterized by few abortive branches and virtual absence of the discoid structures (Fig. 4.2.4A). On the contrary, shCT-infected MKs, as well as uninfected samples, were characterized by the presence of platelet formation (Fig. 4.2.4A).

Moreover, I observed a greater than 50% reduction in platelet numbers in cultures where I introduced PKCdelta shRNA (Fig. 4.2.4B).

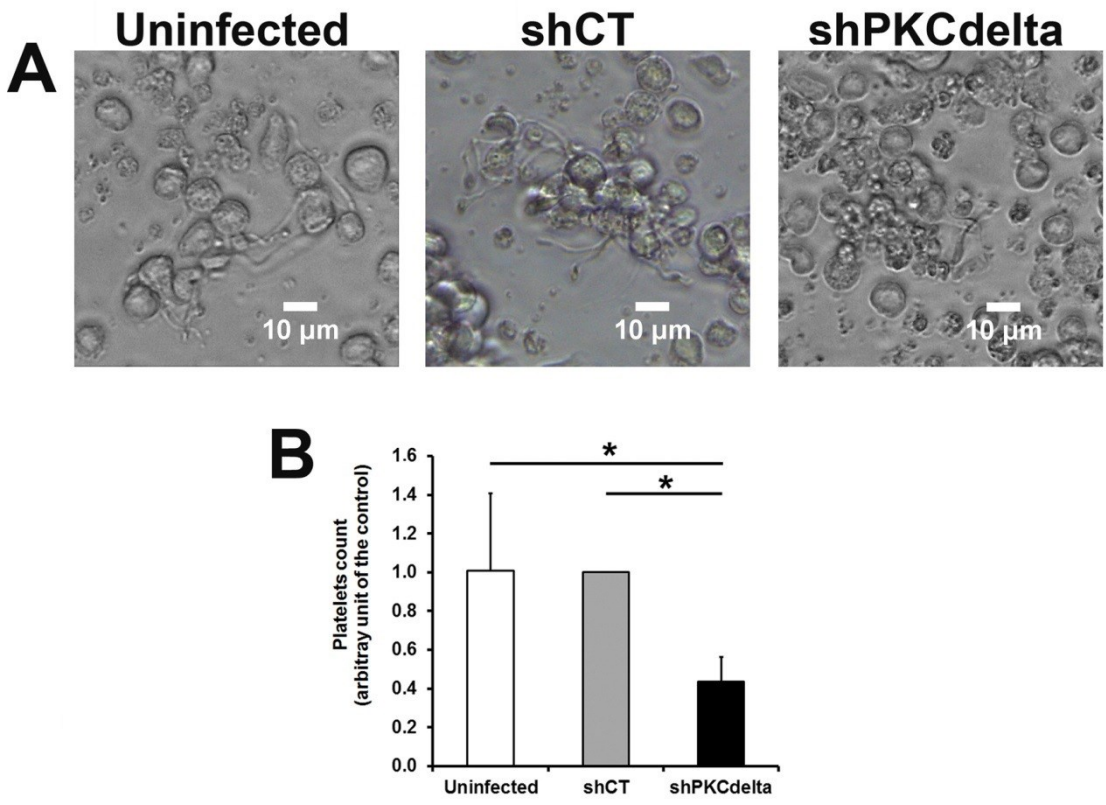

Figure 4.2.4. PKCdelta down-regualtion impairs pro-platelets formation. Panel A: representative images of pro-platelet forming MK of uninfected, infected with specific shRNA for PKCdelta (shPKCdelta) or control (shCT) at day 5 post-infection. Panel B: Analysis of platelet production of uninfected, infected with specific shRNA for PKCdelta (shPKCdelta) or control (shCT) at day 5 post-infection from 5 replicates. (data were normalized for shCT, means ± one SD; *$p<0.05$ ANOVA and Tukey tests).

4.2.4 PKCδ/PKCε BALANCE REGULATES HUMAN THROMBOPOIESIS

In this *in vitro* MK-differentiating model, PKCδ and PKCε showed different kinetics, reaching opposite expression levels at Day 14 of colture (Fig. 4.2.5).

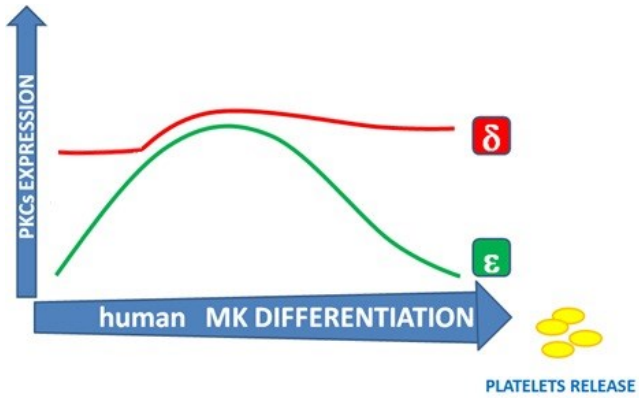

Figure 4.2.5. A schematic rapresentation of PKCδ and PKCε protein expression levels during MK differentiation

I hypothesized that the normal expression or even the rate of these two PKC isoforms could be pivotal for usual megakaryocytopoiesis and platelet production. In order to test this speculation, $CD34^+$ cells were isolated from peripheral blood of patients affected by primary myelofibrosis (PMF) a disease characterized by abnormal platelet production. Isolated cells were cultured up to Day 14 in the presence of TPO and then collected for further western blot analysis (Fig. 4.2.6A). As shown in Figure 4.2.6B, TPO-treated cells from PMF were characterized by an abnormal expression of PKCδ and PKCε, as well as Bcl-xL and Bax. Indeed, accordingly with previous data *(Masselli E et al. In Press)*, in PMF samples the expression levels of PKCε and Bcl-xL were considerable higher than in healty donors (HD) (Fig. 4.2.6B). As expected, the expression levels of PKCδ and Bax were almost halved in PMF, as compared to HD (Fig 4.2.6B).

Afterwards, HD-derived MK differentiating cells were treated with specific PKCε and PKCδ activatory or inhibitory peptides at Day 8 and then cultured for further 5 days. As assumed, the simultaneous inhibition of PKCδ and activation of PKCε significantly reduced the amount of platelets released in colture (Fig. 4.2.6.C).

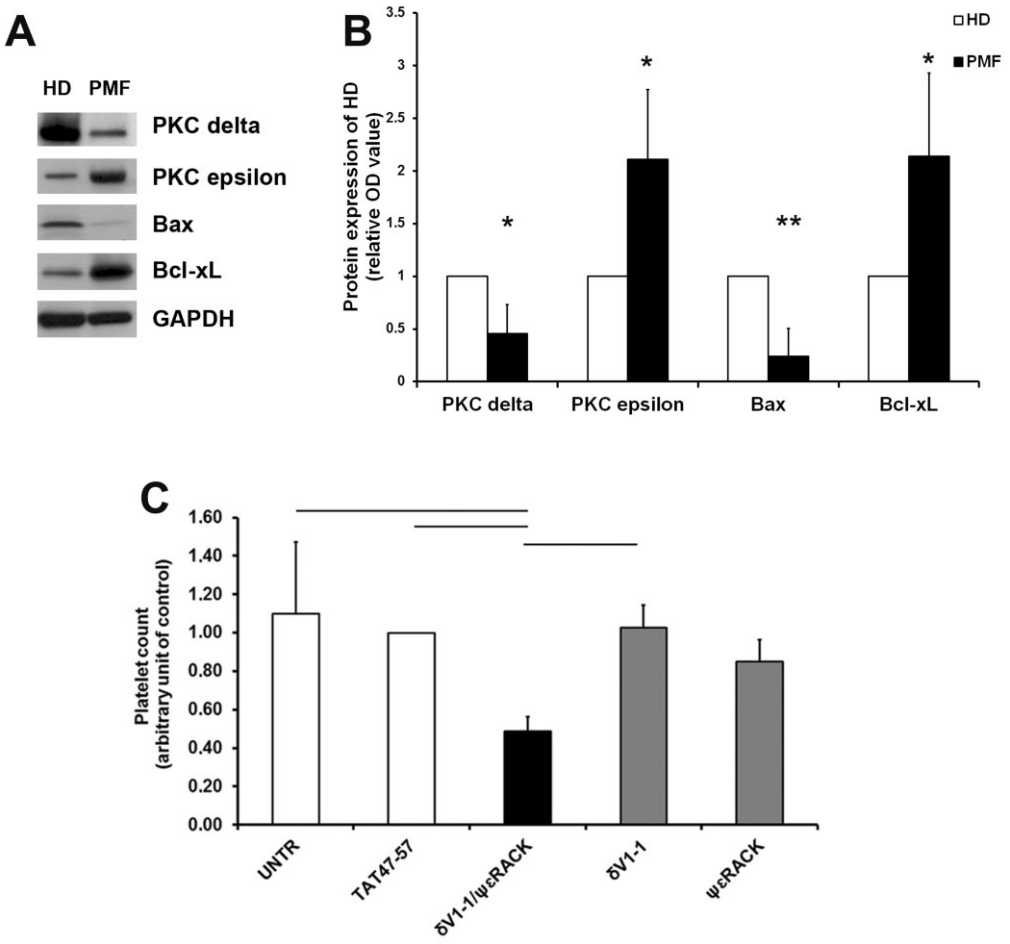

Figure 4.2.6. An imbalance between PKCδ and PKCε reduces platelet release. Panel A: Western blot detection of PKCdelta, PKCepsilon, Bax, Bcl-xL in human MK at day 13 of differentiation of Healthy donor (HD) and PMF patient. GAPDH was monitored for protein loading. Panel B: Densitometric analysis of proteins expression, normalized for GAPDH expression levels, were performed using ImageJ software. Densitometric measurements of Western blots from 4 replicates (means ± one SD; *p<0.05 t-Tests) Panel C: Analysis of platelet production of untreated (UNTR), treated with TAT_{47-57} peptide (TAT_{47-57}), PKCdelta inhibitor peptide, PKCepsilon activator peptide alone (δV1-1 and ψεRACK respectively) or in combination (δV1-1/ ψεRACK) cultures, at day 5 post treatment. Data were expressed as arbitrary unit of control (TAT_{47-57}) from at least 3 independent experiments. (means ± one SD; ANOVA and Tukey Tests).

Conversely, the concurrent pharmacological PKCδ activation and PKCε inhibition clearly demonstrated the possibility to potentiate platelet production, increasing proplatelet formation and platelet delivery, as shows in figure 4.2.7. As a matter of fact, the combination of PKCδ-activator peptide and PKCε-inhibitor peptide, was able to boost both the percentage of MK producing proplatelet formation (Fig. 4.2.7 A) and the number of released platelet in culture media (Fig. 4.2.7. B), as compared to controls.

Collectively, thee data show that platelet production can be in vitro forced by modulating PKCδ/PKCε expression and function.

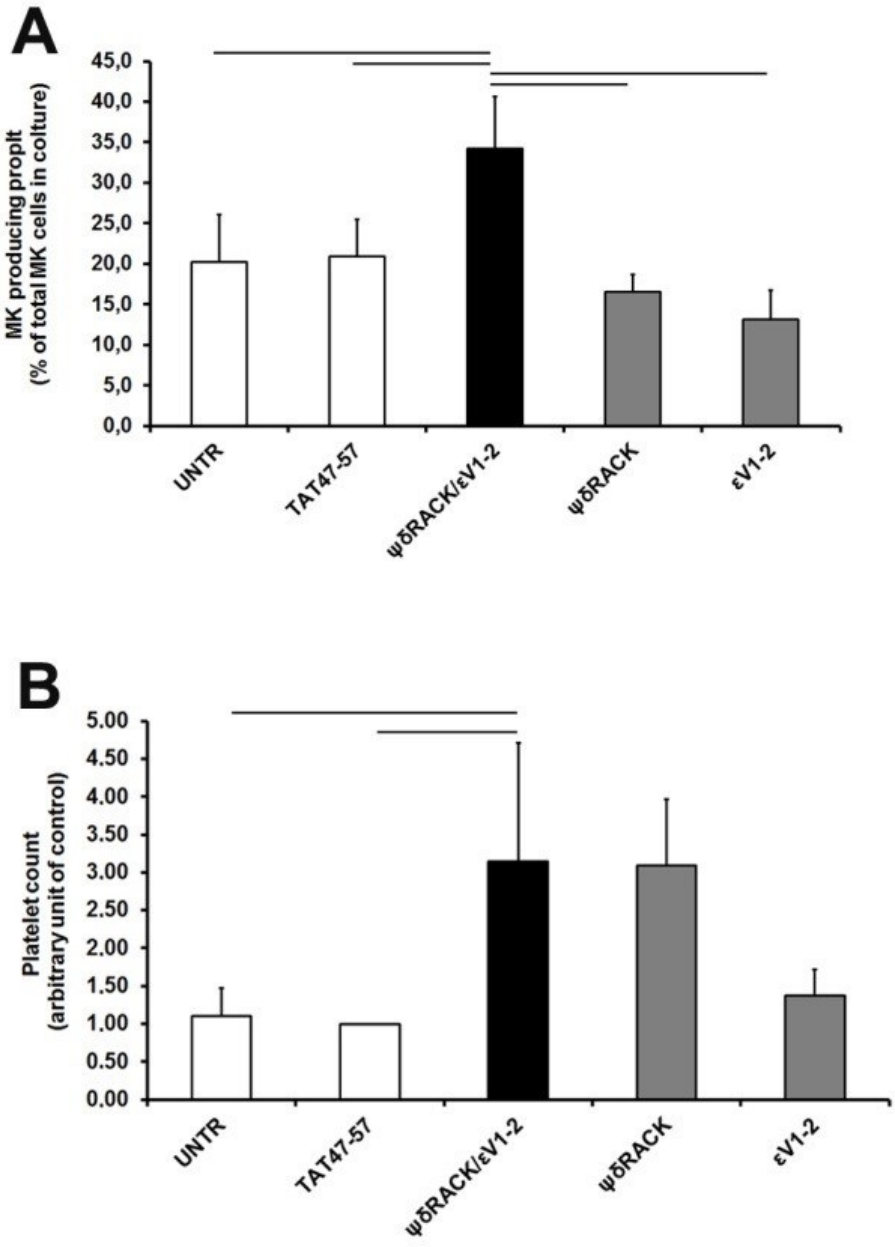

Figure 4.2.7. The amount of platelet production can be revised by modulating PKCepsilon/PKCdelta expression. Panel A: Analysis of pro-platelet producing megakaryocytes of untreated (UNTR), treated with TAT_{47-57} peptide (TAT_{47-57}), PKCdelta activator peptide, PKCepsilon inhibitor peptide alone (ψδRACK and εV1-2 respectively) or in combination (ψδRACK / εV1-2) cultures, at day 5 post treatment. Data were expressed as % of megakaryocyte extending pro-platelet analizing 100cells for each treatment from at least 3 independent experiments. (means ± one SD; ANOVA and Tukey Tests).Panel B: Analysis of platelet production of untreated (UNTR), treated with TAT_{47-57} peptide (TAT_{47-57}), PKCdelta activator peptide, PKCepsilon inhibitor peptide alone (ψδRACK and εV1-2 respectively) or in combination (ψδRACK / εV1-2) cultures, at day 5 post treatment. Data were expressed as arbitrary unit of control (TAT_{47-57}) from at least 3 independent experiments. (means ± one SD; ANOVA and Tukey Tests).

5. DISCUSSION

Protein kinase C (PKC) is a family of serine-threonine kinase involved in many cellular functions, including cell death, proliferation and differentiation *(Corbalán-García S et al. 2006).*

In particular PKCδ and PKCε are two isoforms with opposing roles in many cell models. PKCε is largely considered as an oncogene because of its anti-apoptotic and pro-proliferation functions *(Newton PM and Messing RO 2011)*, conversely PKCδ generally slows proliferation, induces cell cycle arrest and apoptosis *(Steinberg SF 2004).* In the heart, they are among the most widley expressed PKC isozymes and they play an antithetical role in the ischemic-reperfusion preconditioning process (i.e.: enhancing role forPKCε and inhibiting role for PKCδ (*Chen L et al. 2001)*. Moreover, the importance of these two PKC isoforms in pathological conditions has been proven by clinical trials based on specific PKCε and PKCδ inhibitor peptides *(Mochly-Rosen D et al. 2011)*.

Platelets are the smallest blood cells with a primary physiological role in hemostasis. They are produced by megakaryocytes as anucleated cells that contain proteins and mRNA derived from megakaryocyte. Platelets retain also the protein synthesis machinery, therefore, platelet mRNAs can be efficiently translated during platelet life, during around 10 days.

PKCs have been established as important regulators of several platelet functions. The physiological expression of the different PKC isoforms varies significantly in mature platelets, with relevant differences in humans and mice.

Concerning novel PKC isoforms, it has been demonstrated that PKCδ is expressed in human platelets and regulates the activation response to GPVI agonists and adhesion to collagen *(Hamm CW et al. 1987)*. This behavior of PKCδ in humans is similar to the one described for PKCε in mice, where it plays a relevant role in the activatory signaling

cascade emanating from the GPVI receptor *(Pears CJ et al. 2008)*. On the other hand, PKCε expression and function in human platelets is still very controversial *(Crosby D and Poole AW. 2003; Muruggapan S et al. 2004; Beunsuceso S et al. 2005; Pears CJ et al. 2008)*.

The results of my research showed that the majority of HD-derived platelets did not express PKCε. This is in agreement with previous data *(Gobbi G et al. 2007)* describing the down-modulation of PKCε expression in *in vitro* human megakaryocytopoiesis from day 6 onward of TPO-driven MK differentiation of CD34 precursors.

Platelets play a central role in the genesis and propagation of atherothrombosisis and the development of platelet thrombus is a critical, final phase in myocardial infarction.

I demonstrated that in human platelets, PKCε is selectively *de novo* expressed in myocardial infarction, but not in stable coronary artery disease patients, and its expression returns negative after 15 days of follow-up after the acute event. Additionally, by functional experiments, I demonstrated that PKCε-transfected normal human platelets enhance their adhesion properties to collagen-coated surfaces under physiologically high shear forces. Myocardial infarction patients express PKCε mRNA at significantly higher frequency than healthy donors and stable coronary artery disease. Considering the dimensions of the first intronic sequence of the PKCε gene, that would virtually preclude the persistence of a potential PKCε pre-mRNA in the platelet, my findings suggest that platelet generations produced before the acute event of myocardial infarction might retain PKCε-mRNA that is not down-regulated during terminal MK differentiation. An alternative explanation would be an anticipated release of platelets, before physiological PKCε down-modulation. This possibility is however unlikely, as PKCε down-modulation takes place around day 6 of *in vitro* MK differentiation, that would be too early. Besides, the analysis conducted on the reticulated platelets of some myocardial infarction patients,

randomly selected from the recruited cohort, did not show any difference in terms of PKCε expression as compared to mature platelets, excluding the possibility that the appearance of PKCε positive platelets in myocardial infarction patients could be selectively ascribed to newly formed platelets.

These results suggest that the ectopic expression of PKCε in platelets could be used as a marker of probability to anticipate the acute event in patients at risk.

Since many pathological conditions depend on an abnormal platelet function, the study of molecular mechanisms involved in platelet activation and thrombus formation, has always been a matter of great interest. On the other hand, many diseases are related to abnormal platelet production.

Thrombocytopenia is a major clinical problem encountered across a number of conditions, including immune thrombocytopenic purpura, myelodysplastic syndromes, chemotherapy, aplastic anaemia, human immunodeficiency, virus infection, complications during pregnancy and delivery, and surgery. Patients with a low platelets number are at increased risk of spontaneous bleeding or hemorrhage and, to prevent them, they are treated with platelet transfusion. However the use of apheresis-equivalent units derived from human donors, shows several limitations and challenges with platelet preparation and storage technologies , such as clinically significant immunogenicity, associated risk of sepsis and inventory shortages due to high and 5-days shelf life *(Thon JN et al. 2014)*.

On these basis, two strategies are possible: i) to potentiate an *in vitro* platelet producing system to obtain platelets for infusion and ii) to develop pharmacological treatments able to modulate *in vivo* megakaryocytopoiesis and platelet production. Therefore, a deeper understanding of molecular mechanisms that control

megakaryocytopoiesis is a key element to regulate *in vitro* and *in vivo* platelet production for clinical applications.

PKCε is involved in human and mouse megakaryocytopoiesis.

In human $CD34^+$-derived MKs, PKCε is down-modulated in the later phases of differentiation and its forced overexpression reduces cell polyploidization and platelet production via Bcl-xL up-regulation *(Gobbi G et al 2007)*.

Using the same model, I found that, conversely to PKCε levels, PKCδ is constantly expressed and its forced down-modulation results in reduced MKs differentiation and platelet release via Bcl-xL up-regulation and Bax down-modulation.

The importance of PKCε and PKCδ balance in human thrombopoiesis is additionally proven by my findings in pathological conditions, ie. myeloproliferative disorders. Primary myelofibrosis is a chronic myeloproliferative neoplasm characterized by bone marrow hyperplastic MKs with an impaired capacity to generate proplatelets in vitro. Interestingly, I found that PMF-$CD34^+$-derived megakaryocytes show an imbalance between PKCδ/ PKCε expression, with an increase in PKCε levels- in agreement with our more recent data *(Masselli et al. In press)* – and a decrease in PKCδ levels, than those from healthy subjects. Moreover, these expression levels of PKCs are associated with similar imbalance between their down-stream effectors Bcl-xL and Bax, respectively.

Finally, using a pharmacological inhibitor and activator of PKCδ and PKCε function, I obtained a modulation of *in vitro* platelet production. The concomitant PKCδ inhibition and PKCε activation reduces platelet release; conversely, PKCδ activation associated with PKCε inhibition clearly demonstrates the possibility to potentiate platelet production.

Collectively, these data suggest that novel PKCs i) should be adequately expressed in human circulating platelets and PKCε ectopic expression could be associated to cardiovascular deseases, suggesting its possible use as a risk marker; ii) have a crucial role during normal human megakaryocytopoiesis and platelet production, through a mechanism involving apoptotic pathway (specifically Bcl-xL and Bax); iii) might be modulated, as protein expression levels, during in vitro megakaryocytopoiesis and by modifying their rate is possible to revise platelet production, suggesting future strategy for platelet diseases therapy and infusion-aimed platelet expansion.

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