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PLATELET-ASSOCIATED TISSUE FACTOR EXPRESSION: INSIGHTS INTO THE MEGAKARYOCYTE-PLATELET AXIS.

BIO/14

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INTRODUCTION

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Tissue Factor biology

Tissue Factor (TF), or Thromboplastin or CD142, is the main activator of coagulation cascade. It is a 47 kDa transmembrane glycoprotein consisting of 263 amino acid organized into a 219 amino acid extracellular domain, a transmembrane region of 23 amino acid and a short 21 amino acid intracellular tail[1] (Fig. 1).

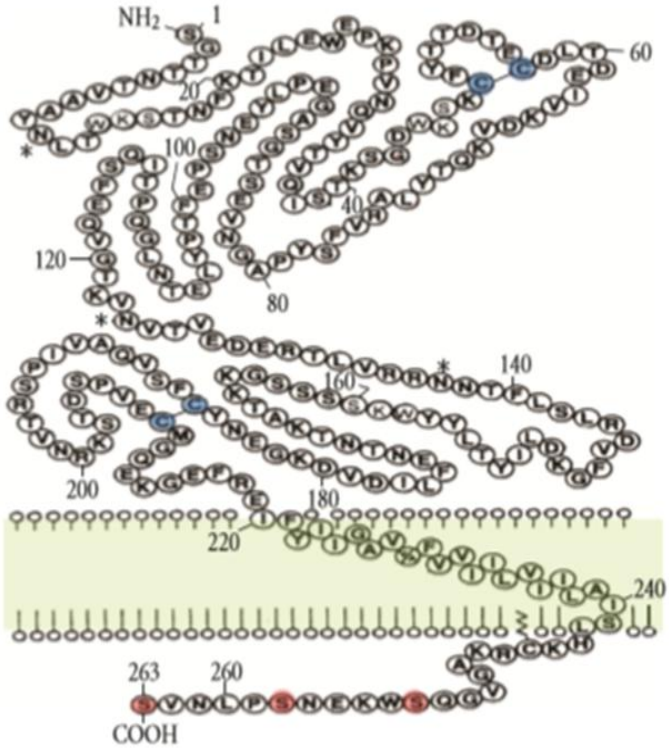

Figure 1. Tissue factor structure. The 47-kDa membrane bound single polypeptide chain consists of extracellular, transmembrane and cytoplasmic domains. There are two intrachain disulfide bridges, shown in blue, in the extracellular region where it also contains factor VII/factor VIIa binding domain. There are three serine residues, shown in red, in the cytoplasmic domain for undergoing phosphorylation.

The extracellular domain contains a catalytic site responsible for the binding with factor VII/VIIa binding domains; the intracellular region could undergo serine phosphorylations, which could modify its function[2-3]; for example, the cytoplasmic region negatively regulates TF expression, which is mediated by suppressed Erk1/2 phosphorylation.

The extracellular domain of TF is structurally homologous to interferon receptors and the X-ray crystallography confirmed the typical cytokine receptor fold consisting of two tightly aligned fibronectin type III modules (TF1 and TF2); moreover the extracellular domain contains β chains with antiparallel folding and similar to the constant domain of immunoglobulins, and presents two disulfide bonds. In human tissue factor extracellular domain there are three *N*-linked glycosylation sites and the factor VII binding site is bordered by two of these. In full-length TF the last six residues (214-219) are responsible for the connection between the extracellular region and the transmembrane domain, and this region is called "stalk region". The flexibility of the “stalk region” allows the extracellular domain to adopt various orientations with respect to the plasma membrane[4,5]. The transmembrane domain and cytoplasmic are connected by a group of four basic amino acids which represents a typical pattern of transmembrane proteins. The cytoplasmic domain presents a acylated cysteine to a palmitate or to a stearate, whose physiological significance is however still unknown, and two serine residues, which represent two putative phosphorylation sites[6].

Tissue Factor-initiated Extrinsic Pathway of Coagulation Cascade

Factor VII structure

The coagulation factor VII (FVII) is a soluble serine plasma protease synthesized by the liver and presents in the bloodstream in the form of activated FVII (1%) or in the form of the zymogen (99%), lacking of catalytic activity. The FVII consists of five domains: the first is an amino-terminal domain that contains gamma-carboxy-glutamic acid (Gla) residues, also called Gla domain which binds Ca^{++} ions and contains hydrophobic residues that confer the ability of FVII to reversibly bind anionic phospholipids of the cell membrane. Near the Gla domain there is the aromatic stack (also called the helical or hydrophobic stack) that some investigators consider to be part of the Gla domain, and close to this domain there are two epidermal growth factor-like domains and the protease domain[7,8].

TF initiates the extrinsic blood coagulation, which proceeds as Ca^{++}-dependent extracellular signaling to sequentially activate zymogens FVII, factor X (FX), and prothrombin (FII) for the generation of coagulant mediators: FVIIa, FXa and thrombin (FIIa), respectively. As a result, thrombin cleaves off fibrinogen into fibrin monomers that cross-link to produce insoluble blood clots (fig. 2). The complex TF-FVII can be activated by circulating factor VIIa complex or by the TF-VIIa, using a self-triggering mechanism, or through a positive feedback activation by other proteases (Xa, IXa, thrombin). The combination of the two factors promotes the alignment of the active site of VIIa with the cleavage site of the substrate bound to the membrane[4]. The physiological substrates of this complex are factor IX and factor X, which bind reversibly to anionic phospholipids of the

membrane[9]. Factor X can be activated by TF-VIIa complex or by the complex IXa-VIIIa-phospholipid-Ca^{++}. The factor IXa is assembled on membrane phospholipids and in the presence of its cofactor, factor VIIIa, catalyzes the activation of factor Xa, that induces platelet activation and the formation of a fibrin clot[10]. The anionic membrane phospholipids, such as phosphatidylserine, play an important role in the beginning of the TF-dependent coagulation, promoting both TF-factor VIIa complex activity and the binding of factors IX and X.

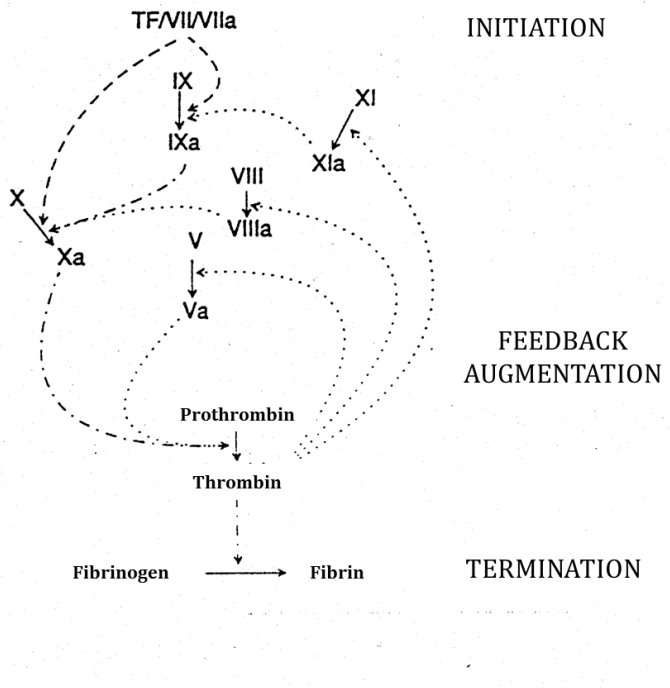

Figure 2. TF-depending pathway of coagulation cascade.

Catalytic regulation of TF-factor VIIa complex

The only physiological inhibitor of the TF-FVIIa complex is the tissue factor pathway inhibitor (TFPI). The TFPI is a serine protease consisting of a carboxyl-terminal basic domain, followed by three protease domains repeated in tandem, denominated Kunitz domains (KI-II-III), and an amino terminal acid region. The TFPI is mainly synthesized by the vascular endothelium but a small percentage is found platelets or circulating in plasma, free and/or associated with lipoproteins. The mechanism of inhibition of the extrinsic pathway of coagulation by TFPI occurs in two steps (Fig. 3):

1) TFPI binds to factor X through the KII domain and inhibits its activity. Also the carboxy-terminal end is required for the rapid and efficient inhibition of FXa;

2) TFPI-Xa complex binds to TF-FVIIa complex and neutralizes its catalytic activity: in the presence of Ca^{++} a quaternary complex TFPI-FXa-FVIIa-TF is formed[11].

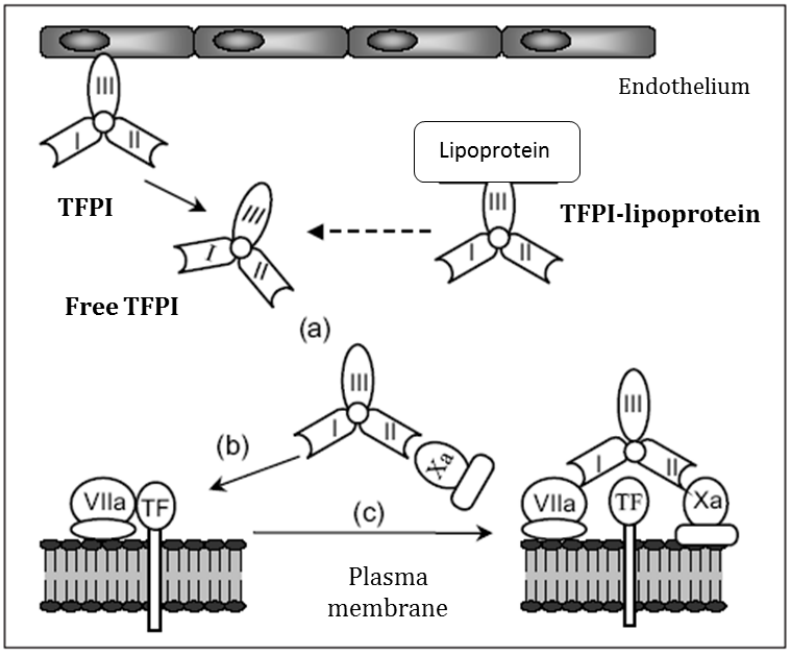

Figure 3. Regulation of TF-factor VIIa complex and mechanism of action of TFPI. The TFPI is a protease inhibitor with three domains inhibitors repeated in tandem (I, II, III) located mainly on the endothelial surface or free in plasma binding to lipoprotein. Mechanism of action: as first the free TFPI (a) binds and inhibits factor Xa (b), subsequently TFPI-factor Xa complex neutralizes the TF-factor VIIa complex forming a quaternary complex (c).

Tissue Factor: not only thrombosis and haemostasis.

Although the main physiological function of TF is the regulation of the processes of hemostasis and thrombosis, emerging evidences showed a broad spectrum of biological function of tissue factor. TF overexpression or hypercoagulability often observed in many pathological conditions expand its role in cardiovascular complications, diabetes, angiogenesis, tumor metastasis, wound repairs, embryonic development, cell adhesion/migration and in autoimmune disorders.

Cardiovascular complications are closely associated with either inflammation or thrombosis and TF plays a major role in their pathogenesis. It has been demonstrated that TF is expressed in lipid-rich human atherosclerotic plaques and it is an important determinant of the thrombogenicity of human atherosclerotic lesions[12]. Moreover TF hypercoagulability leads to cardiovascular complications and vascular disease such as arterial hypertension[13], hypertrophy[14] and acute coronary syndrome[15,16].

In conditions of hyperglycemia, such as in Type I and Type II diabetes, the excessive plasma glucose conjugates with plasma proteins, such as hemoglobin, determined the advanced glycation endproducts (AGEs) formation; the increased circulating AGEs lead to an increased expression of tissue factor making diabetes a hypercoagulable and thrombotic condition[17]. TF can also assume a pathogenic role in diabetic progression in a close relation with the inflammatory process: TF could be responsible for insulin resistance[18,19]. Many studies suggested that TF is not only essential for haemostasis, but also participates in crucial steps of blood vessel formation and remodeling[20,21]. The most interesting findings in this regard are the results of the TF gene disruption in mice, which produced an early lethality of more than 90% of TF knock out embryos in utero between E8.5 and E10.5[21]. TF has also an important role in angiogenesis and tumor metastasis[22]. In the adult animal tumor growth and especially the tendency to metastasis are influenced by the presence of TF, which plays a critical role in the processes of vascularization/angiogenesis. Increased expression of TF contributes to regulating the angiogenic properties of tumor cells, altering the production of regulatory molecules of endothelial growth, such as vascular endothelial growth factor. The

expression of TF also influences cell adhesion and motility. Its role in cell migration and adhesion explains the passage of tumor cells through the endothelial barrier. Many studies demonstrated the importance of TF in adherence and subsequent transmigration of mononuclear phagocytes through the endothelium[23].

A better understanding of the structure of TF, which shows homology with the superfamily of cytokine receptors, led to study the intracellular mechanisms triggered upon binding with FVII, while, on the extracellular surface, a series of reactions initiates the extrinsic pathway of coagulation. The initial stages of signal transduction due to the formation of the complex TF-FVIIa are still little known. However, some studies in cell lines have shown that, unlike cytokine receptors, the cytoplasmic domain of TF is not always directly involved[24]. The most accepted hypothesis is that TF does not behave like a receptor, as it causes a conformational change of FVIIa, such as to activate a membrane protein, probably identified in the receptor protease activated receptor-2 (PAR-2)[25]. PAR-2 is a G protein-coupled receptor, and its activation determines the phosphorylation of kinases, which gives rise to subsequent phosphorylation cascades that lead to an amplification of the signal and activation of specific metabolic pathways (fig. 4). The TF-FVIIa complex formation can also induce the mobilization of intracellular Ca^{++} after the activation of G protein/phospholipase C, the activation of small G proteins (such as Rac and Cdc42), the phosphorylation of tyrosine kinases of the Src family that in turn activate phosphatidylinositol 3-kinase (PI3K)[26]. The PI3K phosphorylates protein kinase B, also known as Akt, responsible for the cytoskeleton reorganization and the cell migration, and triggers by Rac and Cdc42 the phosphorylation of mitogen activated protein kinase (MAP kinase) that, once translocated into the nucleus,

promotes the expression of the so-called "immediate-early genes," that are genes whose expression occurs in the absence of a previous protein synthesis. These genes, activated by TF, encode for transcription factors, growth factors, receptors, regulators of the cellular organization and motility and for some cytokines, thus suggesting the presence of a positive feedback in increasing the expression of TF[27].

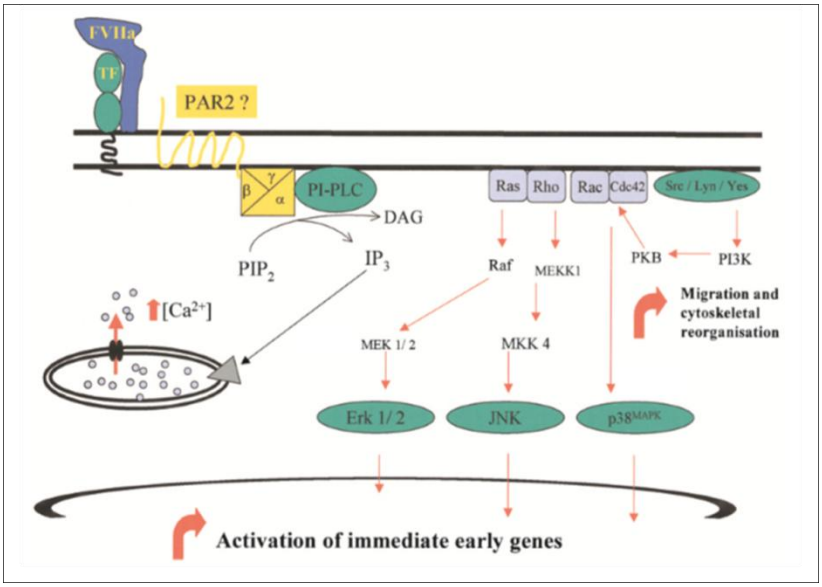

Figure 4. Schematic view of intracellular signal pathways activated by TF-FVIIa complex. The signal is transferred over the membrane by a protease-activated receptor (PAR2) that activates a trimeric G-protein and phospholipase C (PI-PLC) and, as a consequence of this, a rise in intracellular calcium concentration is observed. The mitogen activated protein kinases (MAPK) extracellular signal-regulated kinase 1/2 (Erk), c-jun N-terminal kinase (JNK), and p38MAPK are activated and the transcription of immediate early genes is increased. Members of the Src tyrosine kinase family are phosphorylated, resulting in cell migration and cytoskeletal reorganization.

However, some studies showed the evidence that the pro-metastatic role of TF is due even to the cytoplasmic domain because of the phosphorylation of at least two serine residues present within the C-terminal TF domain. The intracellular domain of TF can interact directly with the cytoskeletal components through a protein, filamin-1 (also known as actin-binding protein 280, ABP-280), forming a protein complex involved in the remodeling of the actin filaments, responsible for the morphology and cell motility[28]. The complexity of the different mechanisms by which the TF-FVIIa complex influence the morphology and cell motility and induces the expression of immediate early genes, indicates an activated status of the cell, induced by TF and relevant for the uncontrolled growth and/or the metastasis of cancer cells, that often express TF on their surface.

Vessel wall-derived Tissue Factor

In 1987 TF cDNA was cloned by four independent groups from adipose[29], fibroblast[1] and placental[30-31] cDNA libraries. However, there was no information concerning the cellular distribution of TF-producing cells within the tissue from which it has been isolated. It has been postulated that TF is not present in the vessel wall cells that come in contact with blood but must be associated with the vasculature where it could act quickly in response to vascular damage. Since the TF cDNA cloning a series of studies was conducted in order to evaluate TF expression and localization under physiological and pathological conditions. In 1989 Wilcox and colleagues reported the localization in the normal human vessel wall and in the atherosclerotic plaque of both TF messenger RNA (mRNA), by in situ

hybridization, and protein, using specific antibody in immunohistochemistry[32]. In 1996 Thiruvikraman *et al.*, using digoxigenin-labeled factors VIIa and X, showed the evidence that the lipid rich core of atherosclerotic plaques contains high levels of extracellular TF able to bind its physiological ligand. The authors concluded that this location may be responsible for the rapid initiation of thrombosis after the lipid rich atherosclerotic plaques rupture and the core contents are exposed to flowing blood[33]. In the same year the same research group further confirmed the direct contribution of TF to the procoagulant activity of human coronary atheroma showing that it was significantly reduced if the samples were pretreated with a blocking anti-TF antibody[34]. In 1997 it was demonstrated that the amount of TF present in an atherosclerotic plaque is directly correlated to the plaque thrombogenicity[35]. At the beginning of 2000 Jander *et al*. supported this concept showing that human carotid plaques with an high content of TF are most frequently found in symptomatic patients (fig. 5)[36].

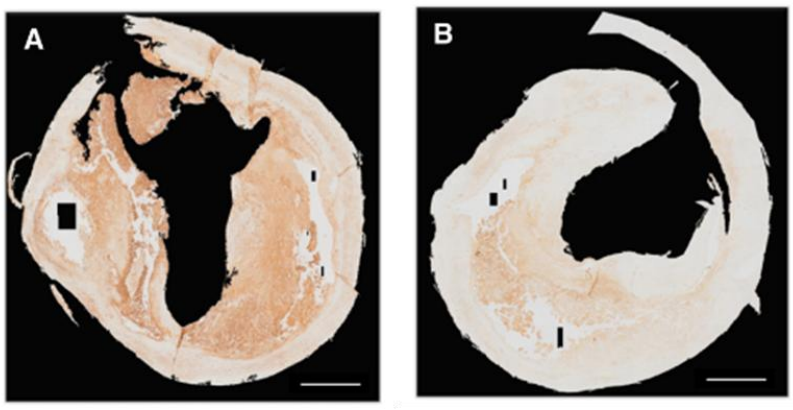

Figure 5. TF expression in human carotid endoarterectomy specimens detected by immunohistochemistry using a monoclonal mouse antibody. TF content in the plaque from symptomatic patient (A) is higher than that present in the plaque isolated from asymptomatic patients (B). Scale bar 1mm.

In pathological condition, such as in atherosclerosis, the cells of the vessel wall, endothelial cells and smooth muscle cells, and other cells, such as monocytes, can be induced to expressed TF. Indeed, in an atherosclerotic vessel TF is expressed by perturbed endothelial cells[32], by infiltrated monocytes-macrophages[15-16] and by smooth muscle cells[32,33], besides by the fibroblasts of the tunica adventitia which constitutively express the protein. The TF produced by these cells is the so-called vessel wall-derived TF[37].

In an atherosclerotic setting polymorphisms in the TF gene promoter should also be considered as factor that can induced TF expression. Four polymorphisms have been described in TF promoter: three single nucleotide polymorphisms, the -603 A7G, the -1322 C/T and the -1812 C/T, and one deletion/insertion -1208 I/D. These polymorphisms give rise to two haplotypes with almost equal frequency: the 603A and the 603G haplotype[38]. Polymorphisms in TF gene promoter can modulated TF expression and thereby its involvement in atherosclerosis and individual predisposition to atherosclerotic disease[38,39].

Based on these data, at the end of the last century, the scientific community was quite convinced that the vessel wall-derived TF plays a key role in the thrombogenicity of the atherosclerotic plaque and in the inhibition of TF pathway could open new therapeutic strategy in the prevention of acute coronary thrombosis after plaque disruption[40,41].

Platelet-associated Tissue Factor

For long time it was believed that the contribution of platelets in atherothrombotic disease was confined only to the formation of thrombus. In recent years, thanks to technological advances and new methodologies available, such as transcriptomic and proteomic, we have completely changed our knowledge of the platelet functions and activities, not only in the intravascular compartment but also in the extravascular milieu[42]. Platelets have been considered biological simple cells but recent findings indicate that platelets perform novel and complex biological functions. Platelets have been considered not able to express new genes because they don't have a nucleus; in 2003 Gnatenko and colleagues described for the first time platelet transcriptome[43]. Today we know that platelets have an average of three to seven thousand messenger RNA involved in different cellular activities (metabolisms, cell signaling) and used by platelets to do *de novo* protein synthesis. This transcriptome derived from megakaryocytes through a very well controlled mechanisms: in 2011 Cecchetti *et al.* provide the evidence that not all messenger RNA are transferred from megakaryocytes to platelets, but it depending on physiological or pathological conditions[44]. We was used to think that platelets are short-acting cells when organized into a clot, but today we know that platelets are long-lived and can mediate cell-cell interactions for many hours after initial activation.

Taking advantage of these new findings in the platelet biology, the contribution of these cells has been deeply revisited. Platelets are no more considered key player only in thrombus formation occurring upon plaque rupture, but they play an important role in the inflammatory process, playing a key role in atherothrombotic

disease in the early stages of the plaque development by releasing numerous cytokine (IL- 1β, CD40L, β-thromboglobulin, etc.), chemokines (RANTES, PF4, ENAP-78, etc.), growth factors (PDGF, TGF, EGF, bFGF, etc.) and adhesion proteins (fibrinogen, fibronectin, von Willebrand factor, etc.)[42,45].

Concomitant with this new vision of the role of platelets in atherothrombosis, at the beginning of 2000, Rauch *et al.* proposed that thrombosis occurring upon plaque rupture does not necessarily require the exposure of vessel wall-TF[46]. Platelets can be a source of active TF, the so called "blood-borne" TF or "circulating" TF, which can sustain the activation of the blood coagulation on the edge of a growing thrombus. The author's theory was based on the observation that TF antigen is circulating in the blood and its levels increased in different pathological conditions[47,48]; moreover circulating TF is associated with increased blood thrombogenicity[49] and procoagulant microparticles (MPs) was found in human blood under normal and pathological conditions[50,51].

In the 2000, Rauch and Nemerson published the finding that TF-positive microparticles released by activated monocytes and granulocytes were up taken by activated platelets through a CD15-P-selectin transfer mechanism. The authors suggest that platelet-associated TF, resulting from TF-positive MPs transfer, is an alternative source of procoagulant activity necessary for the thrombus to growth[46].

In 2003 Camera *et al*[52] and Müller *et al*[53] showed the evidence that human platelets from healthy donors express TF antigen, using different approaches (western blotting, confocal and immune electron microscopy). Moreover, Camera and colleagues reported, by using flow cytometry, that platelet activation resulted in the expression of TF besides P-selectin and all other markers of platelet activation,

on the cell surface (fig. 6). The presence of TF protein and its functional activity was further confirmed showing the binding of this protein to its physiologic ligand FVIIa, and the capacity to generate FXa[52].

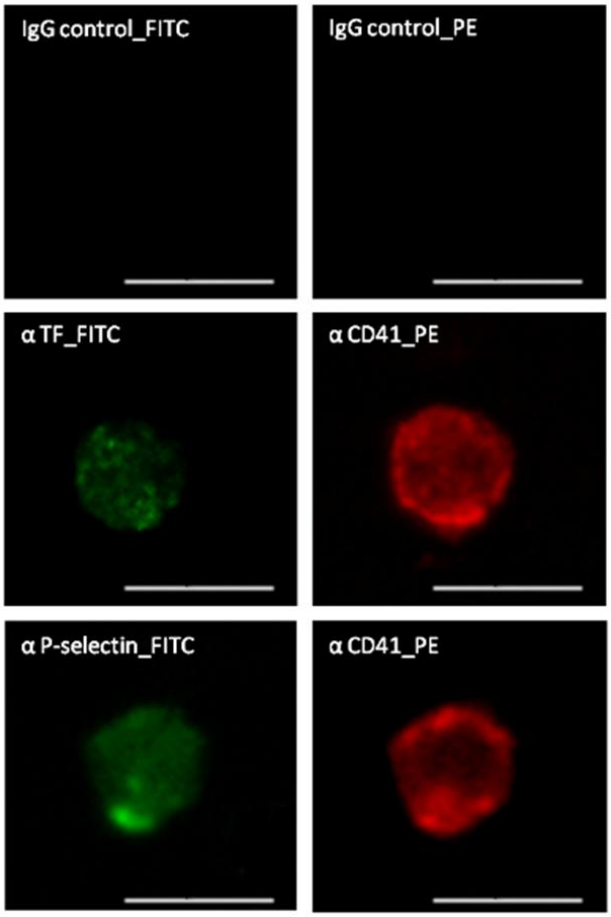

Figure 6. TF and P-selectin localization in activated platelets. ADP-stimulated platelets were stained with monoclonal mouse antibody against TF and P-selectin (green). The red staining represents the platelet population marker GpIIb (CD41). Immunolocalization was assessed by confocal microscopy. Scale bar, 5μm.

As mentioned above, the first evidence of platelet transcriptome was published in 2003[43]. In the same year, TF mRNA was found in appropriate sample resting human platelets devoided of leukocytes contamination, suggesting that platelets

could *de novo* protein synthesis[52]. Three years later, two independent groups provided the evidence that platelet activation results in the translation of TF mRNA, increasing the membrane-bound TF protein[54,55].

At present, published data suggest that three are the possible mechanisms responsible for the presence of TF in human platelets: 1) the transfer from TF-positive MPs released by activated endothelial cells or leukocytes, as proposed by Rauch and Nemerson[46]; 2) the storage of TF protein within the α-granules[35,36] and finally 3) the de novo protein synthesis from specific TF mRNA (fig. 7). All these pathways are not mutually exclusive, and one mechanism may dominate over the others depending on the pathophysiologic conditions.

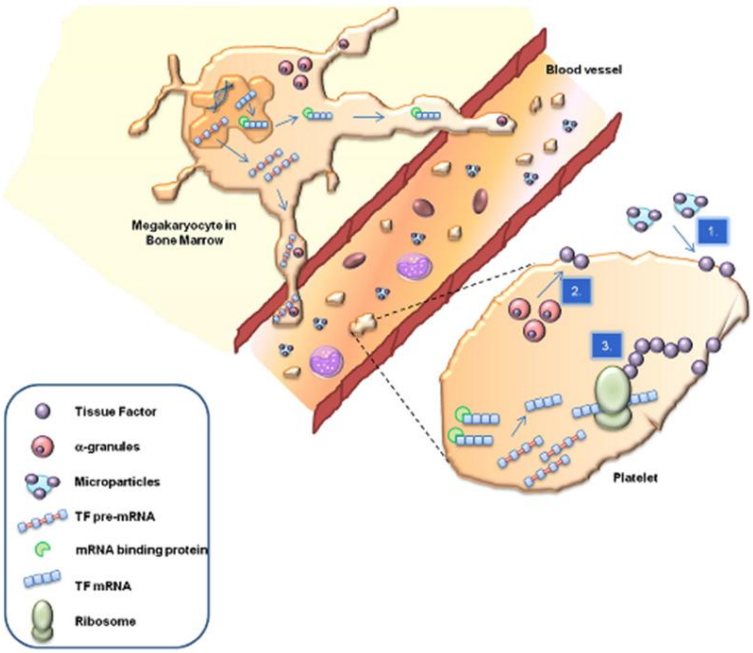

Figure 7. Schematic view of the three possible mechanisms responsible for the presence of TF in human platelets.

In the last 25 years, research in the field of atherosclerosis has clearly established the role of TF as the principal trigger for arterial thrombosis. Recent data support the concept that the thrombus propagation takes also advantage of the blood-born TF, which consists of microparticles- as well as platelet-associated TF. Moreover, in the last years data showing increased levels of platelet-associated TF in pathological conditions such as essential thrombocytopenia[56], cancer[57] and diabetes[58] have been accumulated.

Despite all these evidence it is worth of mentioning that the issue of platelet-associated TF is still a matter of intense debate among the scientific community. Many investigators do not detect TF activity in platelets and they argue that the only circulating cells capable of synthesizing TF are monocytes that may be the only source of TF-induce thrombosis when the endothelium is intact[59-61]. These investigators claim that the discrepancies of the published data depend on differences in methods, reagents and experimental conditions. The data of those able to found tissue factor in platelets, show that the expression of platelet-associated TF is the result of a dynamic process; thus its detection can considerably differ if observed at different times after *in vitro* cells stimulation.

Platelets biology

Platelets, or thrombocytes, are anucleated discoid cell fragments without nucleus and with size of 1-4 μm in diameter and 1 μm thick. Platelets are formed and released into the bloodstream by precursor cells called megakaryocytes. Megakaryocytes are rare myeloid cells that reside within the bone marrow and represents less than 0.1% of the myeloid cells[62]; they are also found in the lung and in the bloodstream. Megakaryocytes tailor their cytoplasm and membrane systems for platelet biogenesis. Before a megakaryocyte has the capacity to release platelets, it enlarges considerably to an approximate diameter of 100 μm and fills with high concentrations of ribosomes that facilitate the production of platelet-specific proteins[63]. Cellular enlargement is mediated by repeated cycles of endomitosis, a process that amplifies the DNA by as much as 64-fold[64]. The platelet release by megakaryocytes involves the development of cytoplasmic ramifications, the so called "pro-platelets" of 100-500 μm of length. This process generally starts from a single site on megakaryocyte with the emission of pseudopodal extensions that in a period of 4-10 hours stretch in pro-platelets. In the final portion of the pro-platelet, the bundles of microtubules form loops that come out and fall in pro-platelet causing the formation of bulbous tips of 3-5μm in diameter (fig 8)[65].

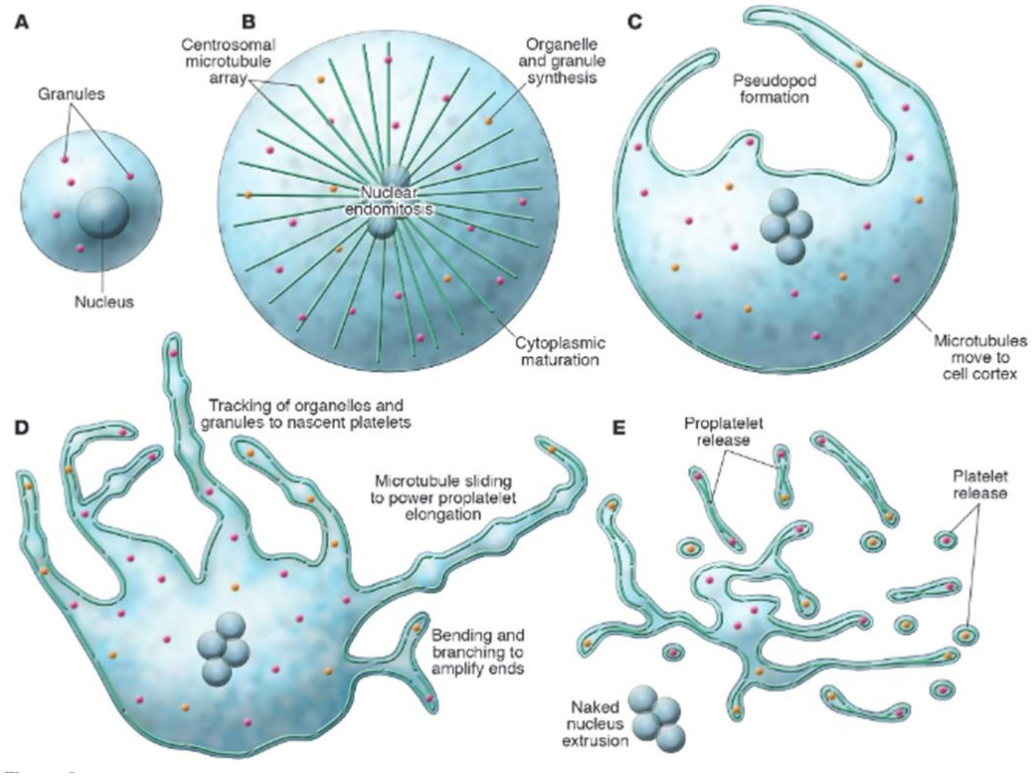

Figure 8. Schematic overview of platelets release by megakaryocytes. (B) Megakaryocytes maturation is characterized by repeated cycles of endomitosis and by a significant maturation of the cytoplasm with the formation of internal membranes, granules and organelles while the microtubules extend (C). Shortly before the formation of pro-platelets, microtubules are consolidated into a mass beneath the plasma membrane and blend in linear beams thicker branches that fill pro-platelets determining the elongation (D). In the terminal portion of the pro-platelets, the bundles of microtubules form loops that come out and fall in pro-platelets causing the formation of bulbous tips. (E) The entire cytoplasm of megakaryocytes is converted into pro-platelets that are released from the cell. The individual platelets are then released from the ends of pro-platelet.

Platelets are released by megakaryocytes, circulate in the vessels without adhering to the vascular wall. Although the prevention of bleeding is their physiological role, platelets have other important functions, for example in inflammatory processes, being a rich source of chemokines, cytokines and growth factors that are stored into the granules[66]. Platelets in the peripheral blood are approximately 250,000/μl and a decrease in their values below 100,000/μl takes the name of thrombocytopenia. Their average lifespan is about 8-10 days, after which time they reach senescence and they are removed from the bloodstream by the spleen and liver, and this mechanism also works in the case of morphological or functional abnormalities of platelets.

Structurally, platelets consist of:

- A plasma membrane rich in phospholipids including phosphatidylserine and phosphatidylinositol, negatively charged and abundant in the cytoplasmic side of the membrane where they act as a substrate for the phospholipase. The glycocalyx is rich in glycoproteins, some of which perform receptor functions in response to stimuli and agonists, giving to the platelets the ability to convey information and to give rise to a cellular response[67].
- Internal membranes formed by the open canalicular system and by the tubular dense on which are anchoring the enzymes involved in the synthesis of prostanoids.
- A cytoskeleton composed of microtubules and microfilaments, which are important in the process of platelet activation and secretion.
- Mitochondria, lysosomes and peroxisomes.

- Secretory granules. Platelets contain three types of granules: lysosomes, dense granules (also called delta granules) and α-granules. The content of the dense and alpha granules is secreted, during the platelet response, directly in the microenvironment seat of platelet aggregation. Among the molecules contained in the granules there are ADP, Ca^{++}, serotonin, content in the delta granules, fibrinogen, thrombospondin, PF- 4 and PAI-1, content in α-granules.

AIMS OF THE STUDY

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Tissue factor is the main activator of the coagulation cascade. It is a transmembrane glycoprotein with an extensive extracellular region responsible for the binding with FVII. The formation of TF-FVIIa complex results in the activation of factors X and FIX, which are responsible for the formation of fibrin from fibrinogen[4]. Unlike all the other coagulation factors that are synthesized by the liver and are released into the circulation, tissue factor is not synthesized by liver and it's not present in the bloodstream. TF is normally absent from vascular cells that come in contact with blood and it is typically expressed only by fibroblasts of the tunica adventitia[6]. However in pathological conditions the cells of the vessel wall, endothelial cells and smooth muscle cells, and other cells, such as monocytes, can be induced to express TF. Indeed in atherosclerotic vessel TF has been detected in endothelial cells, smooth muscle cells and monocytes[68]. At the beginning of 2000 Nemerson *et al.* showed the evidence that, among the circulating cells, also platelets can be a source of TF, and so they are not only involved in hemostasis but they are also cells able to trigger themselves the coagulation cascade[50]. Published data suggest that three are the mechanisms responsible for the presence of tissue factor in platelets: 1) the first and the most shared among the scientific community is the transfer from TF positive microparticles released by activated endothelial cells or leukocytes. 2) TF may also be present as a protein stored in the alpha-granules of platelets and can be exposed on the plasma membrane upon activation. 3) platelets may contain the messenger RNA coding for the TF that can be used to do *de novo* protein synthesis. Today we know that platelet have a trascriptome derived from megakaryocytes through a very well

controlled process. In 2003 our group showed the evidence that megakaryocytes derived from CD34 positive cells and human platelets have TF mRNA but the evidence that the TF mRNA and protein present in platelets are the result of a direct megakaryocyte transfer mechanism is still lacking.

Moreover, different groups have then characterized TF in platelet by using different methodologies (confocal and electron microscopy, ELISA, Western blotting, flow cytometry, functional activity assays)[52-55,70-72]. Despite this in-depth characterization, the platelet-TF is matter of controversy by some authors that, failing to identify TF in platelets, argue that the published data are artifacts, thus generating an ongoing debate[73,74].

In addition, it has to be considered that the study of human megakaryocytes and platelets is fundamentally hampered by the difficulty of finding megakaryocytes: indeed, these cells represent only the 0.01-0.1% of bone marrow cells and their location is clearly not easily accessible.

Furthermore, it is far easier to work with human platelets being certain to work with a population free of leucocyte contamination.

Based on this background the aims of this work were:

- To develop an experimental *in vitro* system of platelet production from cultured human megakaryocytes (Meg-01 cells) in order to study platelet protein expression in the absence of any cross-talk with other cellular and subcellular (microparticles) entities;
- To assess the expression of TF mRNA and protein in human Meg-megakaryoblasts, Meg-megakaryocytes and Meg-platelets;

- To assess the functional activity (i.e. capability to trigger thrombin generation) of TF expressed by human Meg-megakaryoblasts, Meg-megakaryocytes and Meg-platelets;
- To verify the capability of human Meg-megakaryocytes to transfer TF mRNA to nascent platelets;
- To confirm data obtained with Meg-01 by using human $CD34^+$-derived megakaryocytes;
- To dissect the contribution of platelet-TF to the global platelet haemostatic capacity by down regulation of TF expression in Meg-megakaryoblasts by stable silencing through a lentiviral shRNA approach.

METHODS

METHODS

Antibodies and reagents

Mouse anti human CD41-PerCP-Cy5.5, CD107 PECy7, Tissue Factor (CD142)-PE, 7-Aminoactinomycin D (7-AAD), Violet proliferation dye 450 (VPD), Annexin V-APC, BD Trucount tubes, BD Cytometer Setup and Tracking Beads and BD Comp Beads were purchased from BD Biosciences (San Jose, CA, USA). Mouse anti human CD61-PE was from Beckman Coulter (Brea, CA, USA); mouse anti human TF 4507JC FITC, mouse anti human TF 4509, goat anti human TF 4501 and Actichrome TF were from American Diagnostica Inc. (Stamford, CT, USA). Fab9012.2 anti GpVI and R27E4 anti $\alpha_2\beta_1$ were kindly provided by Prof. De Marco (Department of Laboratory Diagnostics and Cellular Therapy, IRCCS, C.R.O., Aviano, Pordenone, Italy). Lactadherin (Recombinant Mouse MFG-E8) was from R&D System (Minneapolis, MN, USA). TF 4509, GpVI, $\alpha_2\beta_1$ and Lactadherin were labeled with Alexa Fluor 488 or Alexa Fluor 633 labeling kit (Molecular Probes, Eugene, OR, USA) according to manufacturer instructions. All the antibodies have been titrated in both Meg-platelets and Meg-01 samples. Calibrated beads (1 and 4 μm) were from Molecular Probes. All the chemicals were from Sigma-Aldrich (St. Louis, MO, USA). Immunomagnetic beads were from Milteny-Biotec (Bergisch Gladbach, Germany); Stem Spam medium was from Stem Cell Technologies (Vancouver, Canada). The human megakaryoblastic cell line, Meg-01, was obtained from the American Type Culture Collection (Manassas, VA, USA). RPMI 1640 medium, L-glutamine and Penicillin-Streptomycin were from GIBCO (Life Technologies, Carlsbad, CA, USA). Fetal bovine serum (FBS) was from EuroClone (Pero, MI, Italy); valproic acid (VPA), fibrinogen, TRAP-6 and propidium iodide (PI) were from

Sigma-Aldrich; RNaseA was from Roche Applied Science (Penzberg, Upper Bavaria, Germany); thrombopoietin (TPO), interleukin-6 (IL-6) and interleukin-11 (IL-11) were from PeproTech (EC Ltd, London, UK). Human serum albumin (HSA) was from CSL Behring (King of Prussia, PA, USA). Zymutest total TF kit was from HYPHEN BioMed (Neuville-Sur-Oise, FRANCE); Bradford protein assay and iQ™ SYBR® Green supermix were from Bio-Rad Laboratories (Munich, Germany); miRNeasy Mini kit for RNA extraction was from Qiagen (Venlo, The Netherlands). All the reagents for RealTime PCR experiments were from Life Technologies (Carlsbad, California, USA); all the reagents for thrombin generation assays were from STAGO (Asnières sur Seine, France). Collagen was from Mascia Brunelli (Milan, Italy); GIPZ lentiviral shRNA particles were from DASIT Sciences (Cornaredo, MI, Italy).

Cell culture

The Meg-01 cells were cultured in RPMI 1640 with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, at 37°C in a 5% CO_2 humidified atmosphere. For megakaryocyte differentiation, Meg-01 cells were seeded in 10 cm petri dishes, 400,000 cells/ml in 15ml medium, and cultured for 8 days in the presence of 2mM VPA.

Human cord blood was collected on informed consent of the parents, with approval from the ethical committee of the Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo Foundation in accordance with the principles of the Declaration of Helsinki. Human umbilical cord blood $CD34^+$ cells were separated and cultured as previously described[76]. Briefly, $CD34^+$ cells were

separated by immunomagnetic bead technique according to the manufacturer's instruction (purity >90%) and cultured for 12 days in Stem Span medium supplemented with 10 ng/mL TPO, IL-6, and IL-11, in the presence of 1% penicillin–streptomycin at 37°C in a 5% CO_2 humidified atmosphere. The medium was changed at day 3, 7, and 10 of culture.

Meg-platelet isolation

At day 8, the medium containing the released Meg-platelets was collected, centrifuged at 130g for 5' at RT, to remove floating cells, and filtered through a PALL Purecell PL leukocyte filters for platelets (PALL Medical).

The platelet suspension, with the addition of 0.35% HSA, was then centrifuged at 1900g for 16' at RT; supernatant was discarded and platelet pellet was resuspended with HEPES-Tyrode's buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L $MgCl_2$, 12 mmol/L $NaHCO_3$, 0.4 mmol/L Na_2HPO_4, 0.35% HSA, 10 mmol/L HEPES, and 5.5 mmol/L glucose, pH 7.4) for aggregation and adhesion experiments, or human plasma for PFA-100 and Rotem experiments.

Blood Collection and preparation of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP)

Blood was collected by venipuncture of the antecubital vein of healthy volunteers who did not take antiplatelet drugs within 10 days before blood donation and who gave their informed consent to participate in the study. After the first 4 mL was discarded, whole blood (WB) was drawn with a 19-gauge needle without venous stasis into citrate-containing (1/10 volume of 0.129 mol/L sodium citrate)

evacuated tubes (Vacutainer, Becton Dickinson). Platelet Rich Plasma (PRP) was prepared by centrifugation of WB at 160g for 15', at RT with brake off. For platelet poor plasma (PPP) blood was centrifuged at 1900g, 15', at RT.

Immunocytochemistry

Meg-01 were seeded on gelatin-coated coverslip and were differentiated with VPA 2mM. At day 8 Meg-01 were fixed in 1% paraformaldehyde at room temperature for 10 min, permeabilized in 0.1% Triton X-100 in phosphate-buffered saline for 5 minutes. Immunodetection of Tissue Factor was carried out by incubating cells with mouse monoclonal anti-human Tissue Factor (4509, American Diagnostica) and by using the AlexaFluor 488 or 594-labelled goat anti-mouse IgG (Molecular Probes). Cells were also incubated with Alexa Fluor® 594 or 633 phalloidin (Molecular Probes) to selectively label actin-filaments and with Hoechst 33342 (Molecular Probes) to stain the nuclei. The staining of cells were analyzed using a confocal microscopy LSM510 (Carl Zeiss, Germany).

Flow cytometry

Analysis of megakaryocyte ploidy. In order to analyze DNA content, Meg-01 cells were harvested at day 14 of culture with VPA 2mM and fixed with cold ethanol 70% at -20°C overnight. Cells were then stained with CD41 PerCpCy5.5 (250ng) in the presence of propidium iodide 50 µg/mL and RNaseA 100 µg/mL in PBS-Triton 0.1% solution, for 2h in the dark, at 4°C. After incubation, cells were analyzed by two-color flow cytometry (FACSCalibur flow cytometer; BD Biosciences). At least

50,000 events were collected. Data were analyzed offline using BD CellQuest™ Pro software.

Meg-01 and Meg-platelet staining. All the samples were processed as follows: 50.000 Meg-01 cells or $CD34^{pos}$-MK were collected into a polypropylene tube. For Meg-platelets analysis, 100 µl of MK culture medium containing the released platelets was collected from the culture plates and transferred into a polypropylene tube. The antibodies were used at the following concentration: CD41 250ng, CD61 500ng, CD107 60ng, TF-PE 250ng, Annexin V 10ng, TF4507 250ng, TF4509 250ng, GpVI 1µg and $\alpha_2\beta_1$ 1µg. All the antibodies were incubated in presence of 10% FBS and EDTA 2mM for 15 minutes at RT, in the dark. The expression of CD61, CD41, GpVI and $\alpha_2\beta_1$ was also evaluated upon stimulation with TRAP-6 25µM added to the sample with the antibodies. For intracellular staining cells were fixed with PFA 1% for 10 minutes and permeabilized with PBS-Triton 0,1% for 15 minutes, at RT.

MP staining and analysis. MP characterization was performed on Meg01 and $CD34^+$ derived megakaryocytes supernatants. 100 µl of each supernatant sample was collected from the culture plates, transferred into a polypropylene tube and labeled 15 minutes at RT, in the dark. The labeling mix contained VPD, 7-AAD, CD61, CD107 and TF. VPD and 7-AAD staining were used to identify events with an intact plasma membrane and apoptotic bodies, respectively. Polystyrene calibrated beads (0.5, 0.9 and 3 µm) were used to set the instrument for MP detection and analysis.

By using this gating strategy, MPs were defined as 7-AAD negative, VPD and CD61 positive events and then the expression of CD107 and Tissue Factor was evaluated.

Samples were diluted in PBS and immediately analyzed by flow cytometer. Fluorescence Minus One (FMO) controls were used throughout all the stainings to assess non-specific fluorescence events.

MP and Meg-platelet quantification. MPs and Meg-platelets were counted by Trucount tubes. 50µl of cells prepared for the staining were dispensed into Trucount tubes using reverse pipetting technique and analyzed by BD FACSAria IIU™. The Flow rate was set at 1. Beads have been identified with a gate and 10,000 events have been acquired in this gate. A gate including MPs or Meg-platelets has been drawn. Calculation of MP and Meg-platelets count was obtained from the following equation: Number of beads acquired into the gate (10,000): number of events acquired into the MP or Meg-platelets gate = number of beads into the BD Trucountd tube (given by the company) : absolute number of MPs or platelets like.

Samples were processed on a BD FACSAria IIU™ cell sorter, supplied with a 100 µm nozzle, and further analyzed with a BD FACS DiVa Software™, version 6.1.3. The instrument was equipped with four lasers (laser 488, laser 633, laser 561 and laser 405) and a standard optical filter setup with a windows extension of 2. Cytometer Performances were checked by daily running BD Cytometer Setup and Tracking Beads. Compensation was performed using BD Comp Beads and manually corrected. Samples were acquired with a flow rate of 1,0 and a minimum of 10,000 events was recorded. All parameters were plotted on logarithmic scales; threshold was set on FSC. Doublets and aggregates were excluded from the analysis based on a dual parameter dot plot in which the pulse ratio (signal area/signal high; y-axis) versus signal area (x-axis) was displayed.

Enzyme-Linked Immunosorbent Assay

TF antigen levels in Meg-01, $CD34^+$-derived megakaryocyte and Meg-platelets was determined by ELISA (Total Tissue Factor, Hyphen) according to the manufacturer's instructions. Briefly, cells were lysed with β-octyl for 15 minutes at 37°C and protein concentrations determined using Bradford's method. 200µl of cell lysate were incubated in duplicate in wells coated with a mouse monoclonal antibody against human TF, overnight at 4°C. The absorbance at 450 nm was measured and compared to the values obtained from a standard curve generated using known amounts of human tissue factor.

RNA extraction and Real-time PCR

Total RNA was isolated from cells using a miRNeasy Mini kit. Total RNA (400 ng) was reverse transcribed by using 200 U SuperScript® Reverse Transcriptase and 0.5 µg oligo(dT) primer together with the manufacturer's buffer, dNTP mix (10mM each dATP, dGTP, dCTP and dTTP), and 40U RNaseOUT ™ Recombinant RNasi Inhibitor. Incubation was carried out at 50°C for 60 minutes. In order to prove that PCR products were RT-dependent and not due to contamination with genomic DNA, RNA was reverse-transcribed in the presence or absence of retro-transcriptase. Real-time quantitative PCR was carried out on iCycler Optical System (Bio-Rad Laboratories) as previously described[72]. cDNA (50 ng) was incubated in 25 µL iQ™ SYBR® Green supermix containing specific primers as listed in Table I. Amplification of GAPDH was used to correct for fluctuations in input RNA levels. A 5-step dilution series of cDNA was used to obtain a linear equation in order to calculate the amplification efficiency ($E= 10^{-1}/slope$) of each

reaction and used for Gene Expression MacroTM analysis (Biorad). The specificity of amplified products was verify by analysis of the melting curves obtained by stepwise increase of the temperature from 55°C to 95°C. Samples were run in triplicate and amplifications were confirmed three times.

Table I. Primers used in real time-PCR

Gene		Sequence (5'→3')	Exon	Amplicon length	Concentration (nM)
TF	Forw	tgatgtggataaaggagaaaactactgt	5	93 bp	500
	Rev	tctaccgggctgtctgtactctt	5		
GPIIIa	Forw	ctagtggaaagtccatcctgtatgtg	13	83 bp	700
	Rev	cactgagagcaggaccaccag	14		
COX-1	Forw	tgcccagctcctggcccgccgctt	5	302 bp	300
	Rev	gtgcatcaacacaggcgcctcttc	7		
GAPDH	Forw	ccacccatggcaaattcc	5	89 bp	300
	Rev	tcgctcctggaagatggtg	5		

Platelet adhesion

Platelet-like particles released from Meg-01 were separated by filtration as described above. After filtration Meg-platelets were centrifuged at 1500g, for 8 minutes, at RT; the supernatant was discarded and the Meg-platelet pellet was resuspended with Tyrode-EDTA-HAS buffer. Meg-platelets were seeded onto fibrinogen-coated coverslip and TRAP-6 25μM was added in order to induced platelet adhesion. Platelet adhesion was performed simultaneously on platelets from PRP as positive control.

Thrombin generation assay

Samples were lysed in β-octyl/Hepes Saline buffer at a concentration of $3x10^6$ cells/mL and tested for their capacity to induce thrombin generation using the Calibrated Automated Thrombogram (CAT) assay. Twenty μl of platelet-free normal pooled plasma (POOL NORM, STAGO) were incubated for 10 min with 20 μL of a cell sample in round-bottomed 96-well micro titer plates (Immulon 2HB, STAGO). To assess TF dependent contribution to thrombin generation, the CAT assay was performed after pre-incubation of cell samples with a neutralizing anti-TF antibody (100 μg/ml, AD4501, polyclonal goat anti-human TF). Thrombin generation was started by the addition of a $CaCl_2$/fluorogenic substrate mixture (FluCa Kit, STAGO) and fluorescence was read for 90 minutes in a Fluoroskan Ascent® reader (Thermo Labsystems, Helsinki, Finland) equipped with a 390/460 filter set. In order to correct for inner filter effects and substrate consumption, each thrombin generation measurement was calibrated against the fluorescence curve obtained in the same plasma to which a fixed amount of thrombin-α2-

macroglobulin complex was added (Thrombin Calibrator; Thrombinoscope BV). Thrombin generation curves were analyzed by dedicated software (Thrombinoscope BV, Maastricht, The Netherlands). Peak height (nM Thrombin), time-to-peak (ttPeak, minutes) and lag time (minutes) were used as main parameters determining thrombin generation.

Platelet-like particles function tests

The aggregating capacity of Meg-platelet was measured by optical aggregometry (ChronoLog model 490; Havertown, PA) using Born turbidometric method. Meg-platelets were obtained as described above and resuspended in standard Tyrode's buffer containing 0.35% BSA, to a final concentration of 250,000 platelets/µl. Cells were stimulated with collagen (10µg/ml) or TRAP-6 (50µM) in presence of fibrinogen (40 mg/ml) and $MgCl_2$ (1mM). Aggregation curves were registered and analyzed by AggroLink software (ChronoLog Corporation).

Platelet function under high shear conditions

The Platelet Function analyzer device (PFA-100®, Siemens Healthcare Diagnostics Inc., Deerfield) was used to evaluate Meg-platelet adhesion and aggregation on shear stress. Blood was collected as described above, centrifuged at 160g, for 15 min, at RT and PRP was separated in order to obtain platelet-depleted blood. 800µl of platelet-free blood were reconstituted with Meg-platelet or autologous platelets, diluted to 200,000plt/µl with PPP (prepared by centrifugation of PRP at 1900g, for 10 min, at RT) and analyzed in a collagen / epinephrine (CEPI) cartridge.

Thromboelastometry (ROTEM)

Thrombelastography was performed with the Rotem coagulation analyzer (Tem International GmbH, Munich, Germany) using the NATEM assay. Briefly, 300µl of platelet-free blood or platelet poor plasma, prepared as described above, were reconstituted with Meg-platelets (control or TF–silenced platelets) or autologous platelets. All measurements were performed at 37°C after recalcification of the samples with 20 µL of 0.2 mol/L $CaCl_2$, according to the manufacturer's instructions.

The following variables were recorded: clotting time (CT, time from addition of reagent to formation of clot with amplitude of 2 mm), clot formation time (CFT, time taken from CT until formation of clot with amplitude of 20 mm) and alpha angle (the angle of the tangent of the curve at the point that the CFT is reached) which indicate the dynamics of clot formation; CT parameter is influenced primarily by the activity of coagulation factors while CFT and alpha describe the kinetic of platelet and fibrin polymerization. The maximum clot firmness (MCF, the peak amplitude of the clot formed) gives information about clot strength and stability and is largely dependent on fibrinogen and platelets. The amplitude (A, amplitude of the clot at a specific time point) is a useful rapid indicator for the developing clot strengths, primarily influenced by platelets, fibrinogen and factor XIII.

shRNA Knockdown of Tissue Factor in Meg-01 Cells

For infection, three different target vectors were utilized: clone ID V2LHS_151505 target sequence CTGTTATTACCATTAGCAT; clone ID V3LHS_371301 target

sequence AAGTCTACACTGTTCAAAT and clone ID V3LHC_410106 target sequence: TGGAGAGCTACTG CAAATGCT. Scrambled and GAPDH-GIPZ lentiviral particles were used as control. Megakaryoblastic cell line MEG-01 were transferred into a 25 cm^2 flask and incubated for 48 hours with viral particles diluted into growth media (1:200). After 48 hours, transduced cells were selected with medium containing puromycin (10 μg/ml) using the disappearance of all GFP-MEG-01 cells, determined by FACS analysis, and elimination of all MEG-01 cells from an untransduced control culture as selection end points**.**

Statistical Analysis

Data are expressed as the means ± SD. Differences between group were analyzed by using one-way ANOVA followed by Tukey's post-hoc t test (GraphPad Prism software) or Student's paired or unpaired t-test (SPSS statistical package). A P value <0.05 was considered statistically significant.

RESULTS

RESULTS

Meg-01 cell line characterization

Meg-01 cells is a human megakaryoblastic cell line isolated from the bone marrow of a patient with Philadelphia chromosome-positive chronic myeloid leukemia and characterized for the first time in 1985 at the Faculty of Medicine of the University of Nagoya, Japan[75]. Meg-01 proliferate as a single cell suspension, only the 2% of these cells adhere (fig. 9), with an average doubling time of 50± 5,5 hours.

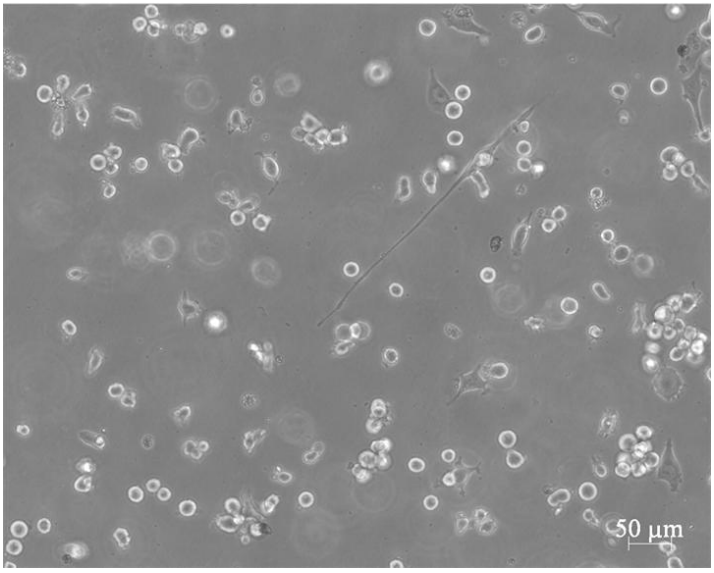


Figure 9. Phase contrast image of Meg-01 cell line photographed with a 10x lens (AxioSkop, Zeiss). In this image it is possible to appreciate how the majority of the cells grow in suspension and only a small percentage of them adhere to the culture dishes.

Meg-01 cells are mostly round shaped, with a modal diameter of 25 to 40 µm, and have large round or ovoid nuclei with fine chromatin and one or more prominent nucleoli (fig. 10a). The plasma membrane is characterized by the presence of several cytoplasmic protrusion (fig. 10b).

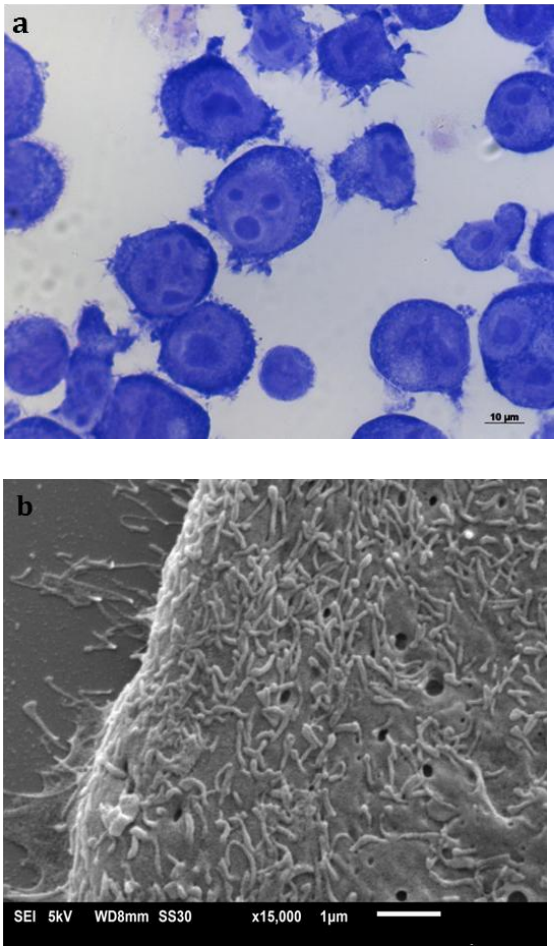


Figure 10. a) May-Grünwald Giemsa staining of Meg-01 photographed in bright field with a 40x lens showing the round shape of megakaryoblasts, the large nuclei and the numerous nucleoli. b) Scanning electron microscope image of a Meg-01 cell showing the high number of protrusion on the plasma membrane.

Treatment of meg-01 with agents, such as phorbol esters, valproic acid (VPA) or thrombopoietin, induced cell differentiation that resulted in cell adhesion (the percentage of the adherent cells increased up to 70%) (fig. 11) and in the acquisition of megakaryocyte's characteristic phenotype.

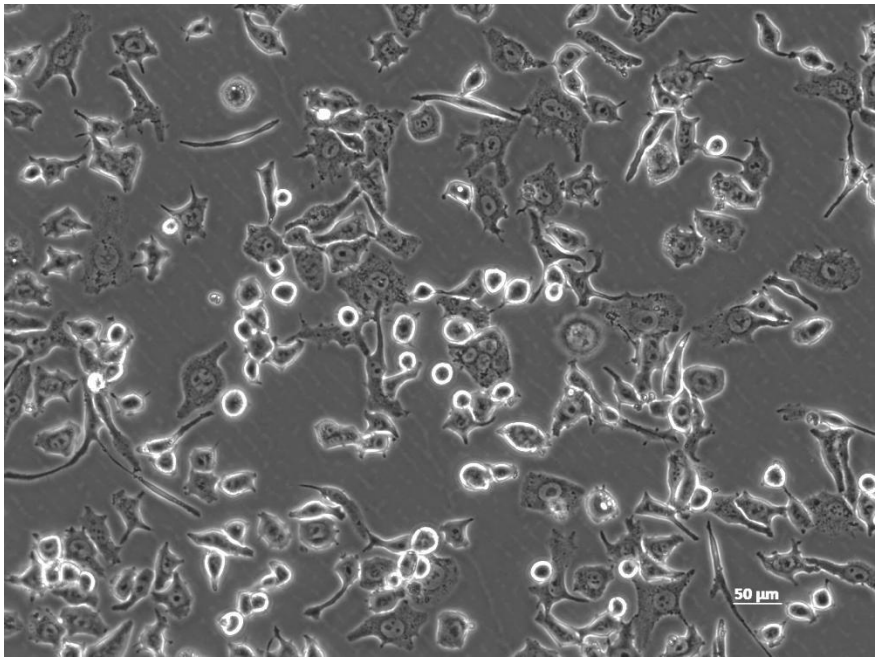


Figure 11. Phase contrast image of Meg-01 differentiated megakaryocytes by VPA 2mM differentiated to megakaryocytes (10x lens, AxioSkop microscope, Zeiss). Please note the morphological changes of the adherent megakaryocytes as compared to floating megakaryoblasts and the appearance of several nuclei.

As well as human megakaryocytes, Meg-megakaryocytes (Meg-MK) are multinucleated cells (fig. 12) indeed they don't replicated and don't do mitosis but they undergo endomitosis and become polyploid through repeated cycles of DNA replication.

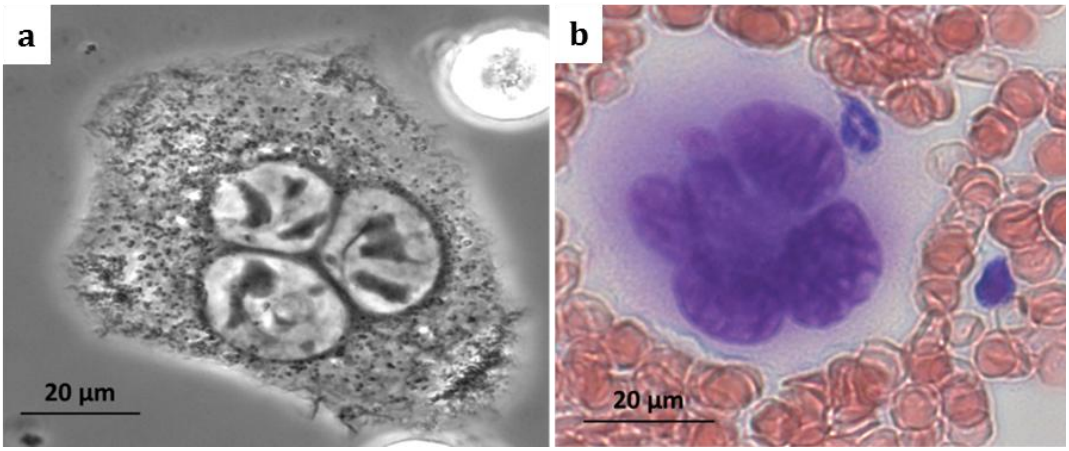


Figure 12. a) Phase contrast micrograph of a Meg-01 differentiated MK; b)bone marrow smear stained with May-Grünwald-Giemsa. Axioskop microscope (Zeiss) equipped with a 40x lens. Please note in both cells the presence of several nuclei.

Changes in ploidy during differentiation of Meg-01 was evaluated by propidium iodide staining. An example of the polyploidy analysis carried out on mature Meg-MK is reported in figure 13a: the ploidy distribution obtained by flow cytometry showed that approximately half of the Meg-MK population was 16N, 20% of cells being ≤8N and 28.2% being 32N or higher. In contrast, all megakaryoblasts were distributed among 2N and 4N (fig. 13b).

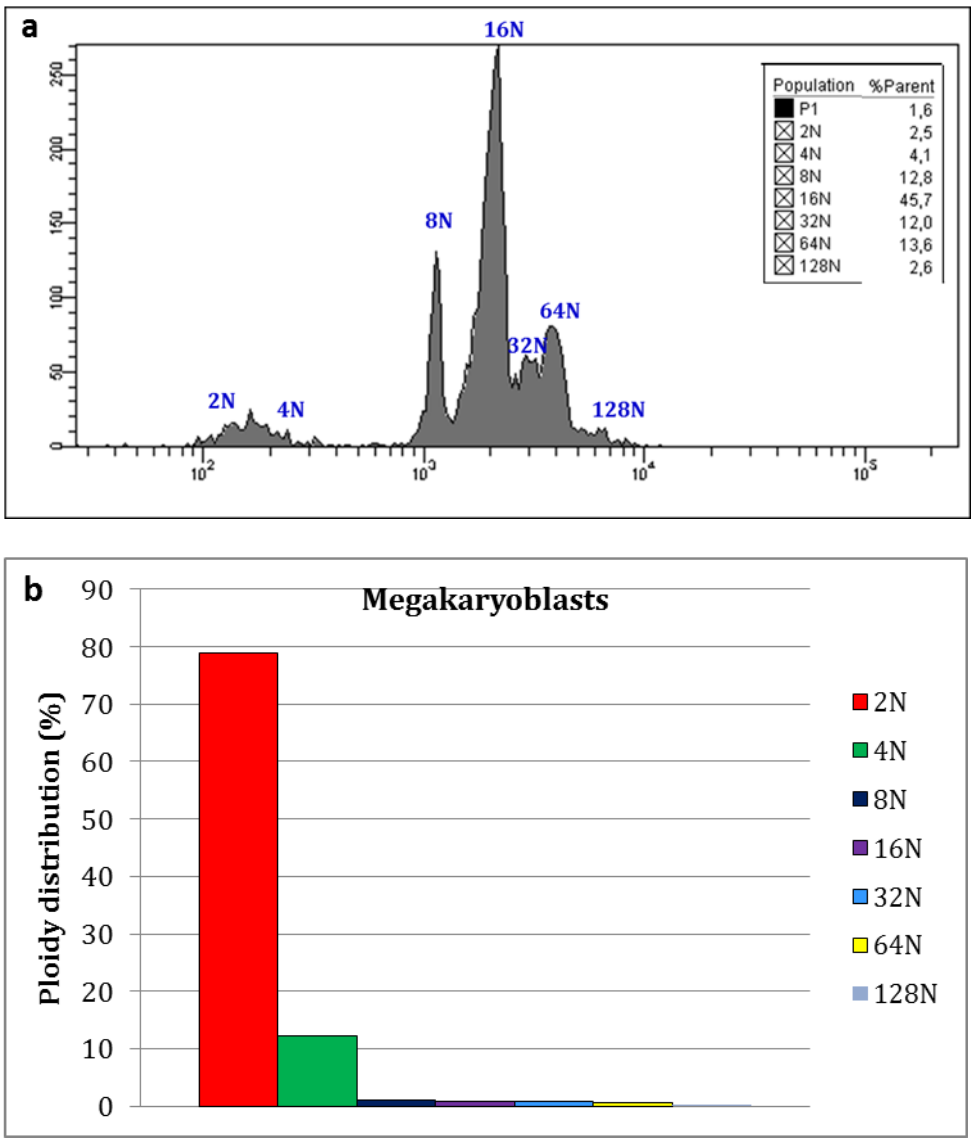


Figure 13. a) Ploidy distribution of VPA-differentiated Meg-MK. b) Ploidy distribution of Meg-01.

The expression of population markers was evaluated by flow cytometry and immunocytochemistry: all Meg-MK express GpIIb (CD41), GpIIIa (CD61) (fig. 6a and c), GpIb (CD42) (fig. 14b) and vWF (fig. 14d). Moreover, the expression of GpIIb and GpIIIa is higher in Meg-MK compared to Meg-01 (fig 15a and b).

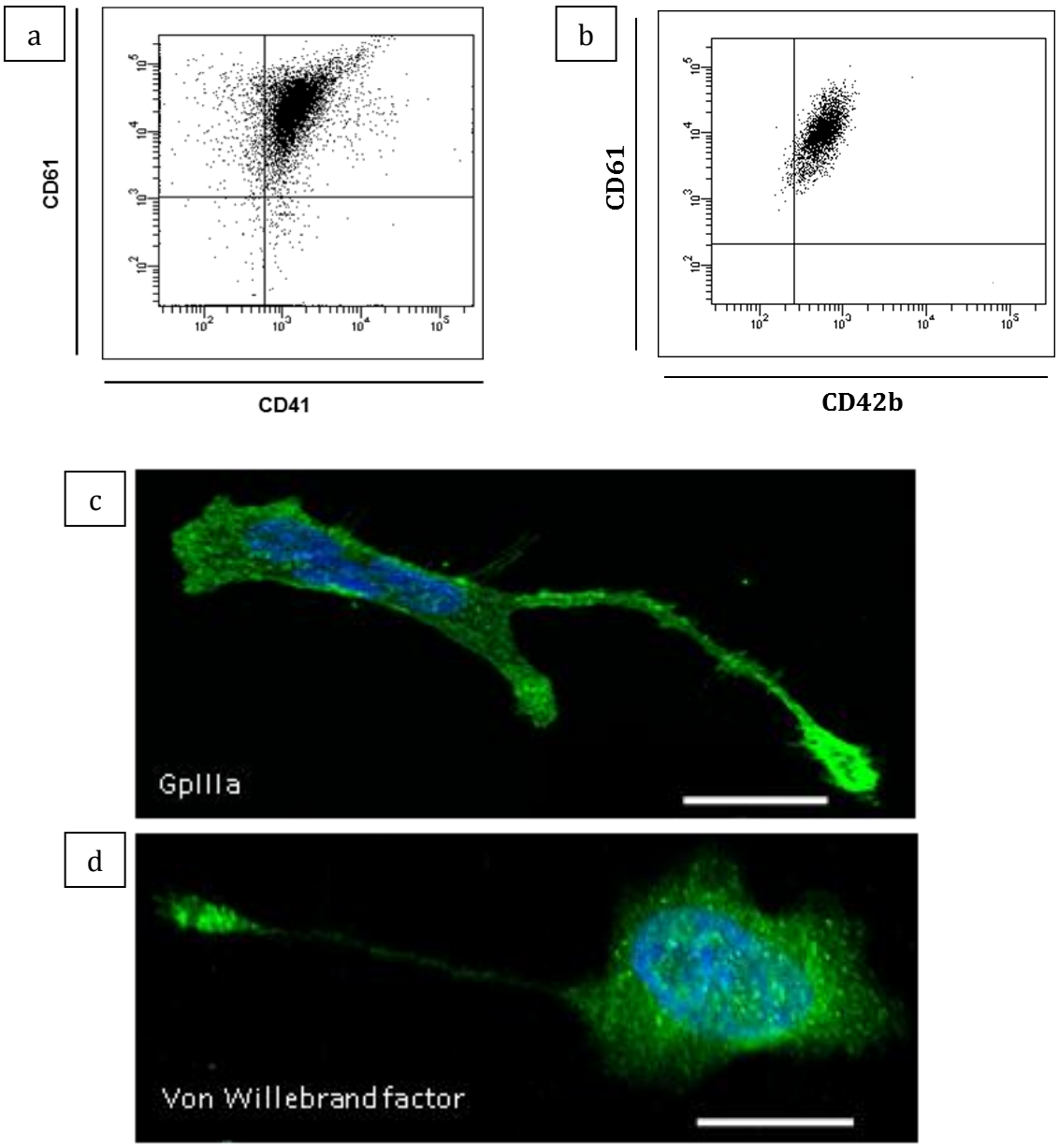


Figure 14. a) Flow cytometry evaluation of GpIIb (CD41) and GpIIIa (CD61) in Meg-megakaryocytes. b) Flow cytometry evaluation of GpIb (CD42b) in Meg-megakaryocytes. (c-d) Immunofluorescence staining and confocal microscopy analysis of GpIIIa and von Willebrand factor expression in Meg-megakaryocytes. Note the typical proplatelets extending from the cell body. Scale bar, 20μm.

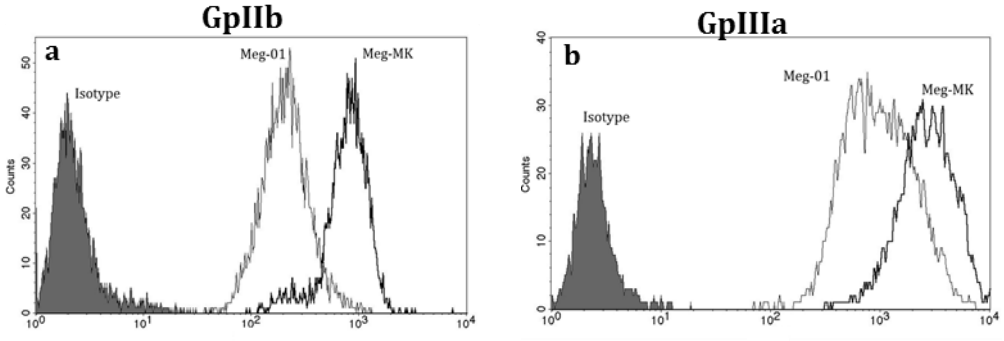


Figure 15. The expression of GpIIb (a) and GpIIIa (b) was assessed by flow cytometry both in megakaryoblasts and in megakaryocytes. The overlay shows an higher expression of both population markers in megakaryocytes compared to megakaryoblasts.

Similary to what happens in human megakaryocytes, also Meg-MK emit long (up to 500μm in length) thin cytoplasmatic processes called proplatelets (fig. 16) with the typical final tip (fig. 17) from which they release platelets. These extensions are characterized by multiple platelet-size swellings linked togheter by thin cytoplasmic bridges (fig. 16).

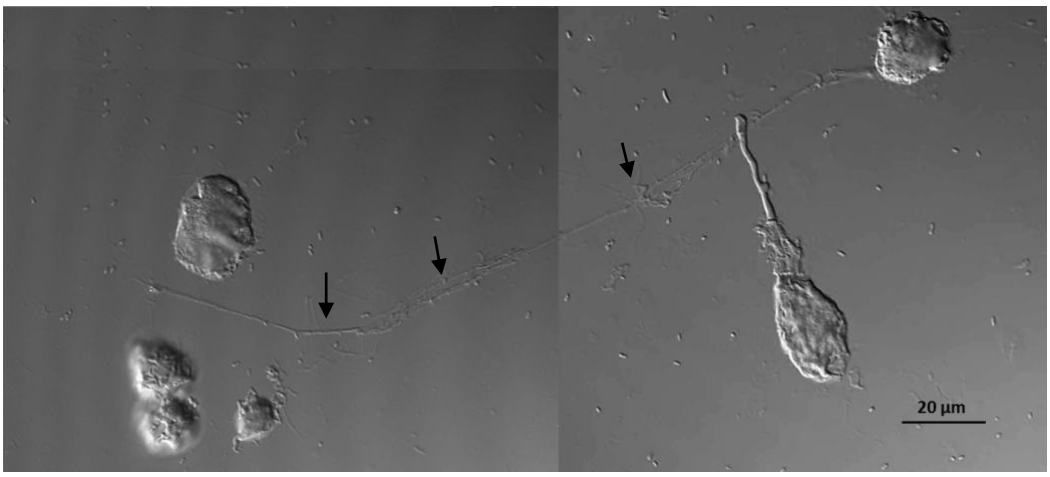


Figure 16. a) Differential Interference Contrast (DIC) image of a Meg-MK (40x lens, AxioObserver microscope, Zeiss): arrows indicate platelet-size swellings along the proplatelet.

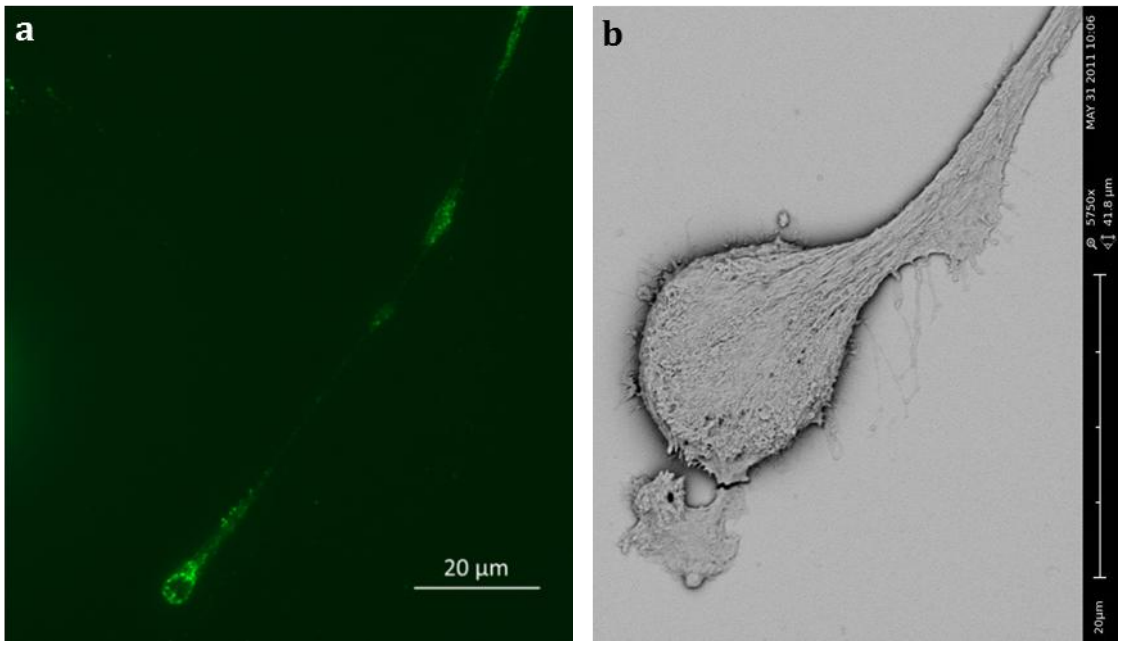


Figure 17. a) β-tubulin (green) immunostaining of a pro-platelet showing the characteristic final tip (20x lens, Apotome, Zeiss) b) Scanning electron micrograph of the final tip of a pro-platelet.

Meg-Platelets release and characterization

Meg-platelets production

VPA-differentiated Meg-MK are albe to release platelet-like particles. We counted Meg-platelets by flow cytometry using Trucount beads. The platelet release occured in a costant manner during culture: one milion of Meg-MK release from 7±3 milions platelet in one day, resulting in a total of 60-65 milions of platelets in seven days (fig. 18).

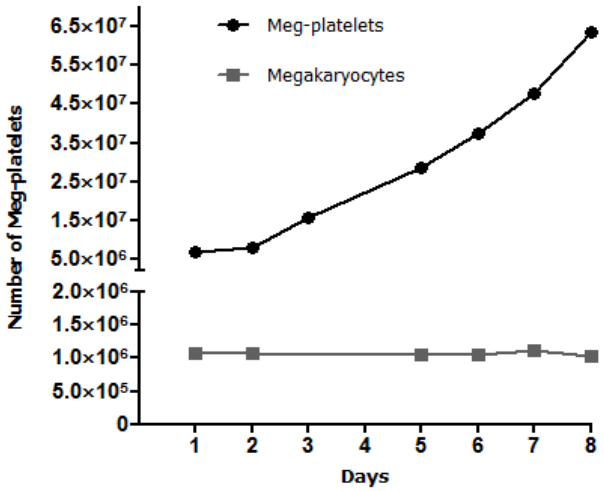


Figure 18. Daily release of platelets from VPA-differentiated Meg-MK. The number of megakaryocytes does not change during culture (grey line), while the number of platelets increased over time (black line). Counts were performed by flow cytometry with Trucount Beads.

Meg-Platelets: flow cytometric characterization

Flow cytometry analysis showed that the physical properties (side and forward scatter) of Meg-Platelets are extremely similar to those of human platelets from a platelet rich plasma (fig. 19).

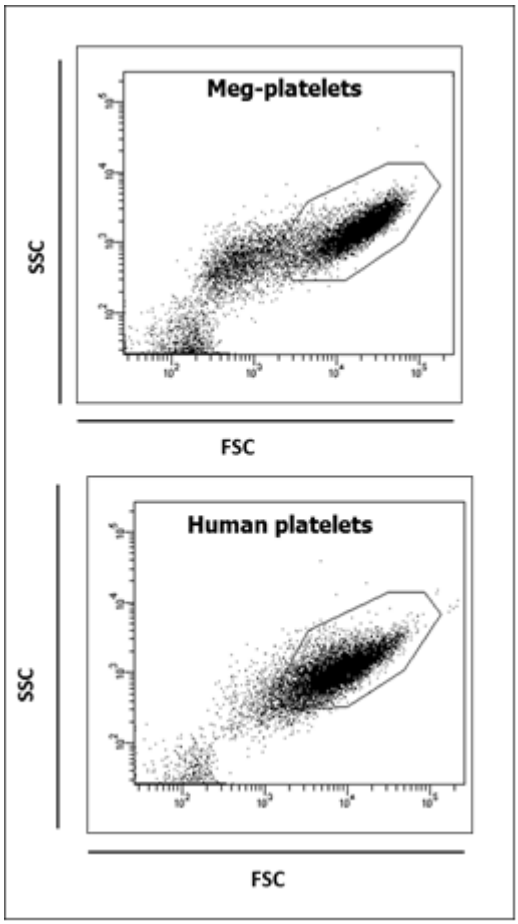


Figure 19. Physical properties (side and forward scatter) of Meg-platelets and human platelets in a platelet rich plasma.

Almost all Meg-platelets express the typical platelet markers, GpIIb and GpIIIa, but only a fraction of them expressed $\alpha_2\beta_1$ and GpVI (fig. 20a), as for human plaelets. The expression of all these platelet markers increased upon stimulation with thrombin receptor activator (TRAP-6) (fig. 20b), similarly to what occured in human platelets (fig. 20c).

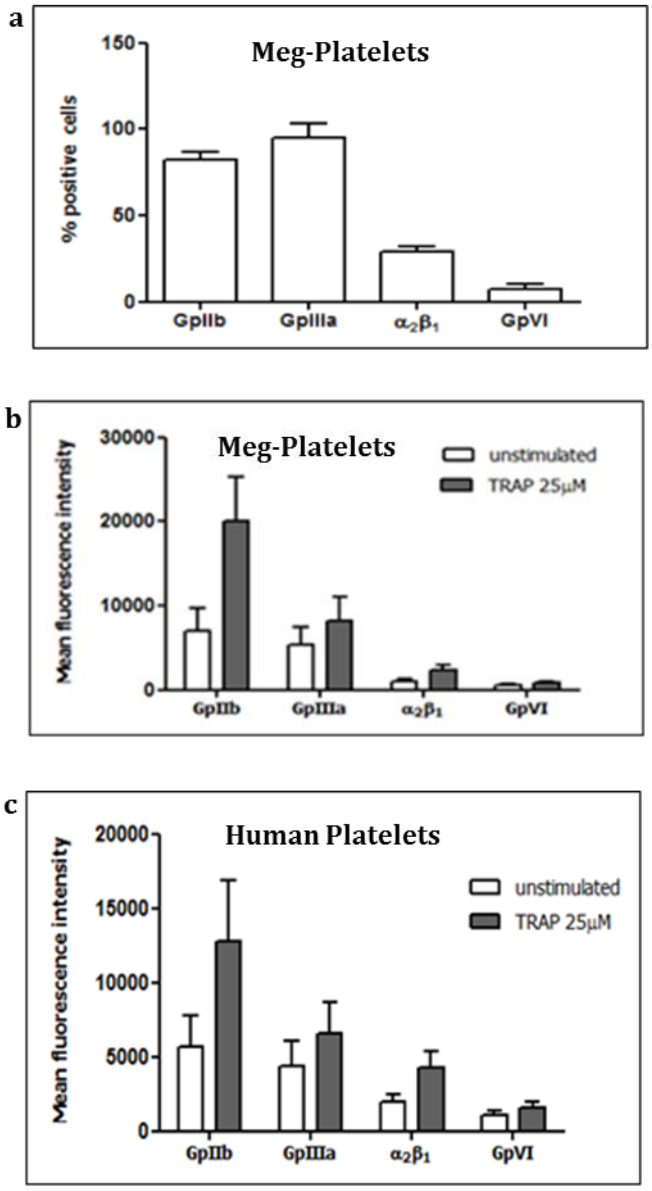


Figure 20. a) Flow cytometry evaluation of GpIIb, GpIIIa, α2β1 and GpVI expression (percentage of positive cells) in Meg-platelets. Modulation of the GpIIb, GpIIIa, α2β1 and

GpVI expression (Mean Fluorescence Intensity) in Meg-platelets (b) and in human platelets (c) upon TRAP-6 (25 μM) stimulation.

The capability of Meg-platelets to adhere to fibrinogen coated surface and to aggregate upon stimulation were also evaluated. After treatment with TRAP-6 Meg-platelets were able to adhere to fibrinogen and spread (fig. 21). Aggregation was observed in response to both TRAP-6 and collagen (fig. 22).

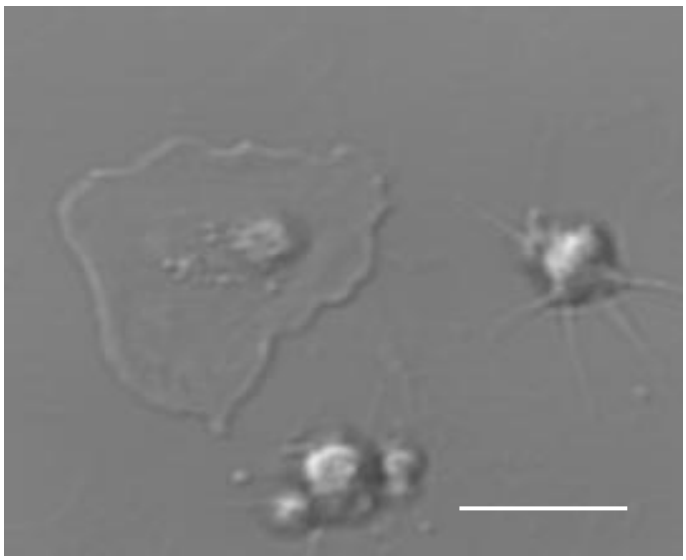


Figure 21. Differential Interference Contrast (DIC) micrograph of Meg-platelets adhering and spreading onto glass surfaces coated with fibrinogen upon stimulation with TRAP-6 (25 μM).63x lens, AxioObserver, Zeiss. Scale bar, 50 μm.

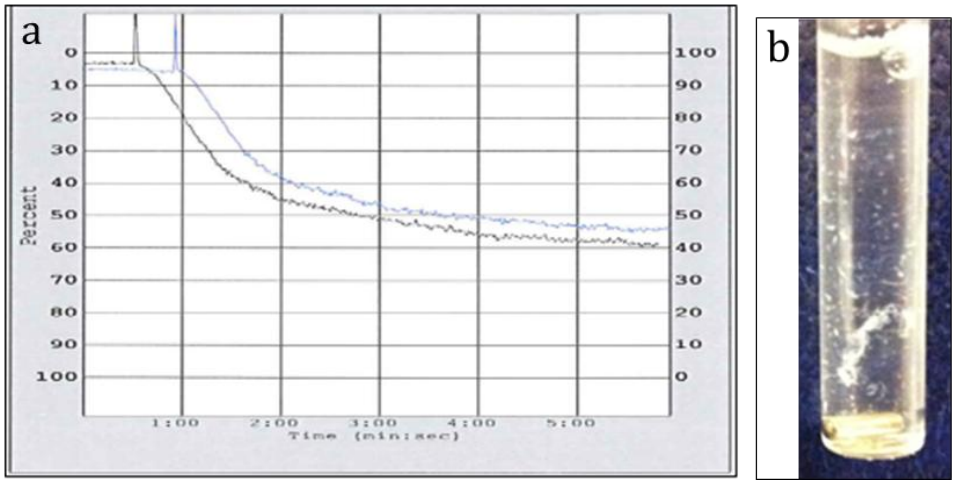


Figure 22. a) Aggregation of washed Meg-platelets performed in the presence of $MgCl_2$ (1 mM), fibrinogen (40 mg/ml) and TRAP-6 25 μM (black line) or collagen 10 μg/ml (blue line). (b) Meg-platelet aggregates at the end of the aggregation assay.

Finally, the haemostatic capacity of Meg-platelets was assessed by PFA-100 system (fig. 23) and by thromboelastometry (fig. 24). When used to reconstituted a platelet-depleted whole blood the haemostatic capacity of Meg-platelets was comparable to that obtained with whole blood reconstituted with autologous platelets. Thromboelastometry assays showed that platelet-depleted whole blood reconstituted with Meg-platelets and whole blood reconstituted with autologous platelets had similar clot formation time (CFT), α-angle and maximum clot firmness (MCF) (all parameters influenced by platelets) similar to those observed with whole blood.

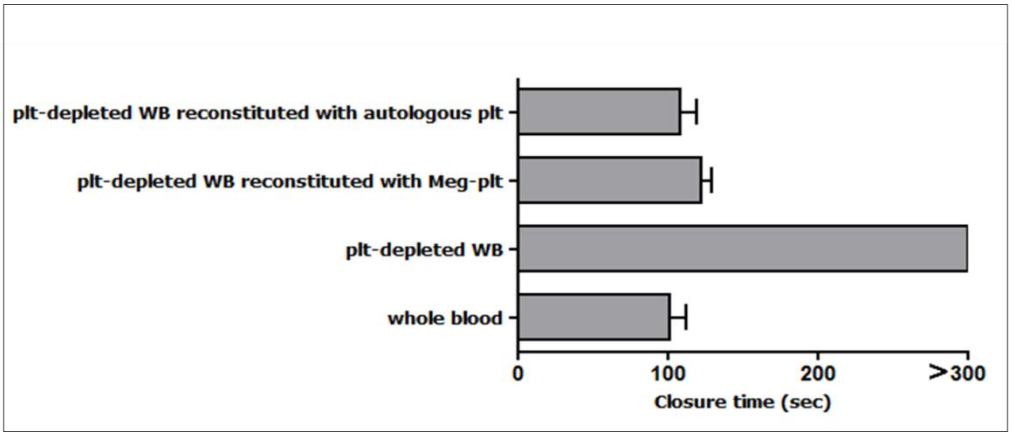


Figure 23. The haemostatic capacity of platelet-depleted whole blood (WB) reconstituted with autologous platelets or Meg-platelets assessed by PFA-100 system.

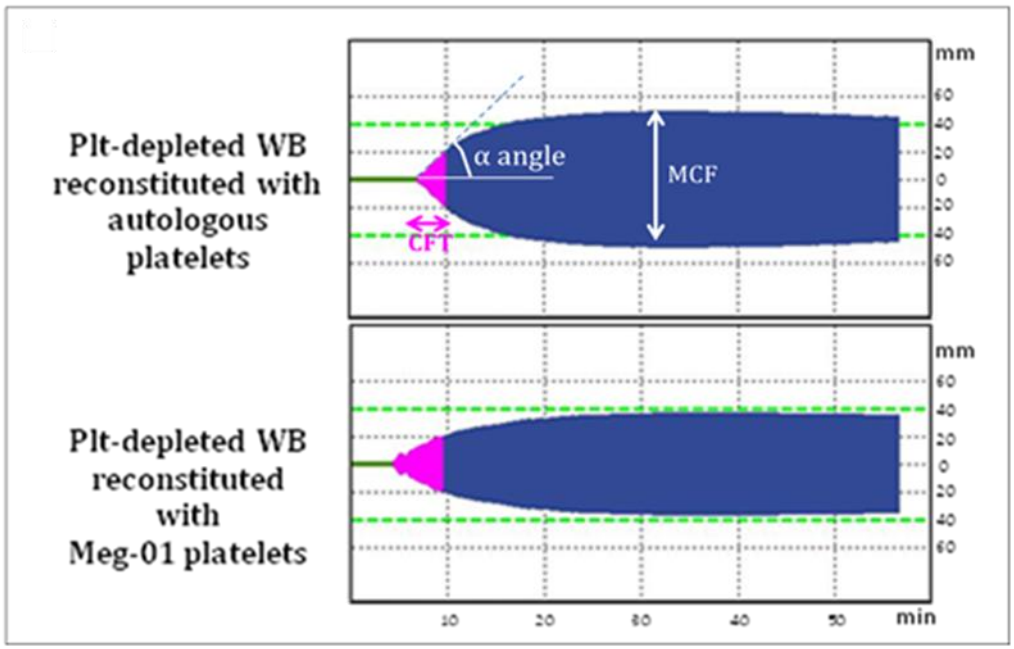
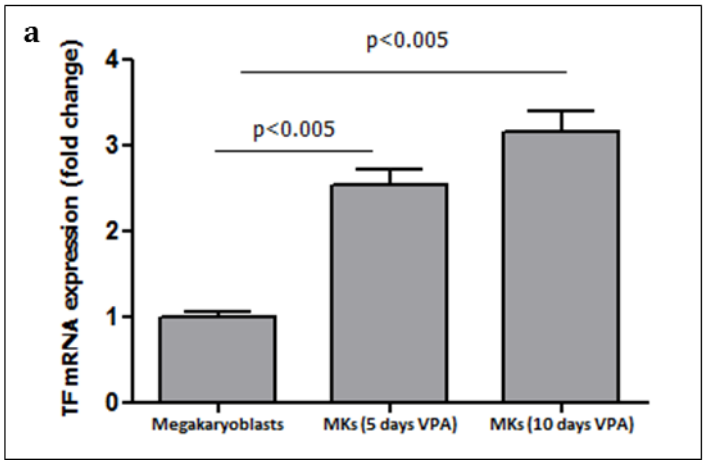


Figure 24. The haemostatic capacity of platelet-depleted whole blood (WB) reconstituted with autologous platelets or Meg-platelets assessed by thromboelastometry. MCF=maximum clot firmness; CFT=clot formation time; α-angle=angle between the tangent to the curve in the point 2 mm and the baseline.

Evaluation of Tissue Factor expression

Evaluation of Tissue factor expression and functional activity in Meg-01

In agreement with data published in 2003[52], TF mRNA expression was detected by quantitative real-time PCR in megakaryocytes. Notably, it was expressed, although at lower levels, also in megakaryoblasts and its expression gradually increased (threefold) during megakaryocyte differentiation (fig. 25a). This is a feature common to other transcripts, such as GpIIIa and cyclooxygenase-1, that showed an even much greater increase during differentiation (fig.25b).



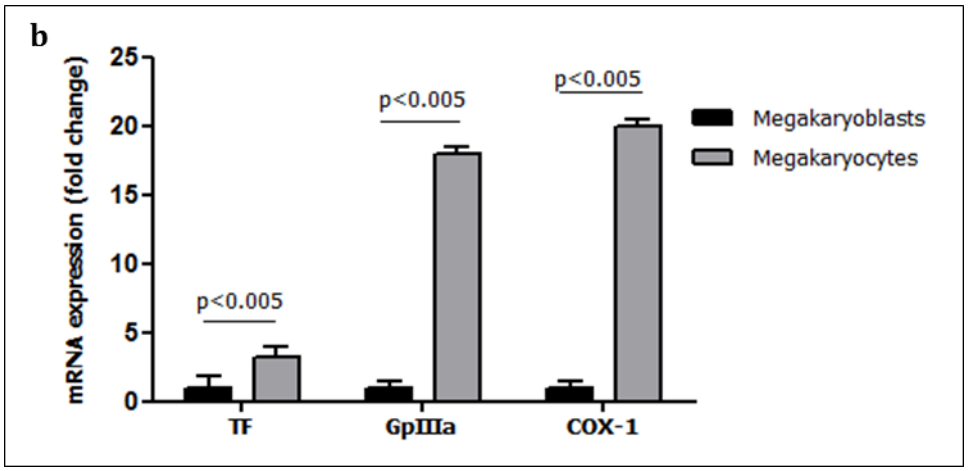


Figure 25. a)TF mRNA expression, evaluated by real time PCR, in megakaryoblasts and in Meg-MK at 5 and 10 days of differentiation with VPA. TF mRNA expression was

significantly (P<0.005) higher in megakaryocytes compared to megakaryoblasts. (b) Comparison of the relative amounts of TF, GpIIIa and COX-1 mRNAs in megakaryoblasts and megakaryocytes (setting to 1 the expression of each gene in megakaryoblasts).

In line with the PCR results TF protein, measured by Elisa, was significantly more abundant in differentiated megakaryocytes (90±30 pg/mg protein) than in megakaryoblasts (50±10 pg/mg protein) (fig. 26).

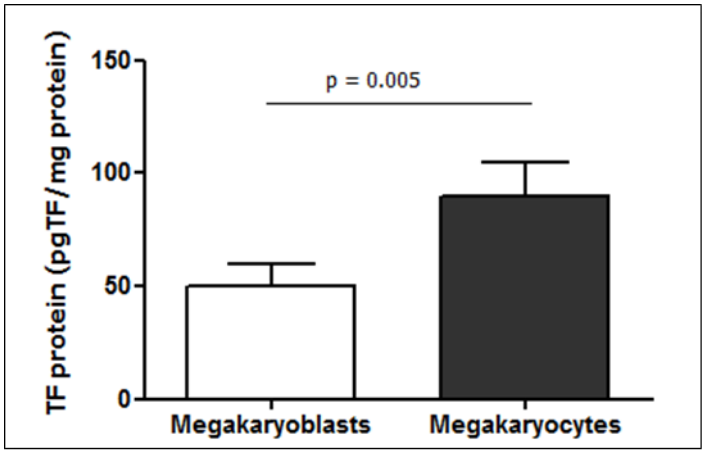


Figure 26. TF protein level assessed by Elisa. Meg-MK express roughly twice the amount of TF compared to megakaryoblasts.

To better characterize TF distribution at the cellular level, surface and intracellular staining were assessed and analyzed by flow cytometry. TF was present on the surface of ~45% of megakaryoblasts and ~70% of megakaryocytes; when analyzed intracellularly, ~75% of megakaryoblasts and ~85% of megakaryocytes were TF-positive. Saturation of the antibody with recombinant TF protein before labelling completely abolished the staining, confirming the specificity of the antibody (Fig. 27).

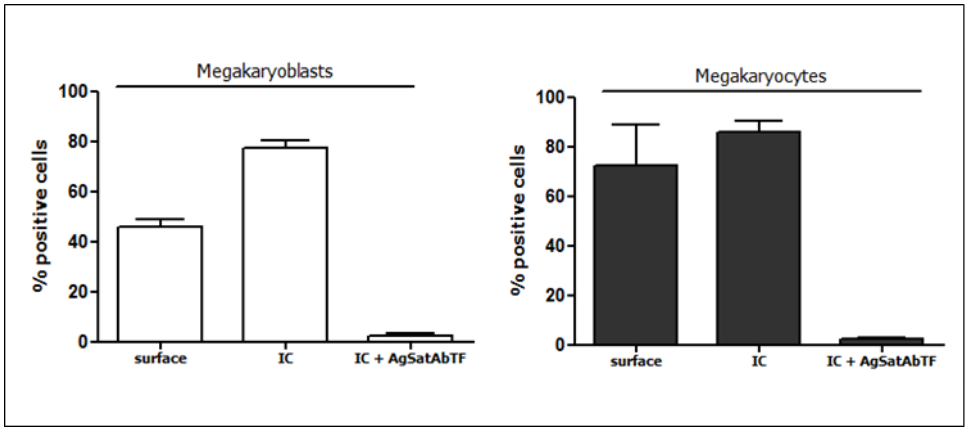


Figure 27. Surface and intracellular (IC) expression of TF analyzed by flow cytometry. TF was present on the surface of 45.8±3% of megakaryoblasts and 72.3±8% of megakaryocytes and in the cytoplasm of 77.1±3% and 86.0±4% of megakaryoblasts and megakaryocytes, respectively.

In order to assess whether TF expressed by megakaryoblasts and megakaryocytes was functionally active the thrombin generation assay was performed. Megakaryocytes showed a thrombin generation capacity higher than that of megakaryoblasts as suggested by the shorter lag time and time to peak (fig. 28). TF contribution to the thrombin generation capacity accounted for 40% and 78% for megakaryoblasts and megakaryocytes respectively, as indicated by the residual thrombin generated in the presence of a neutralizing TF antibody.

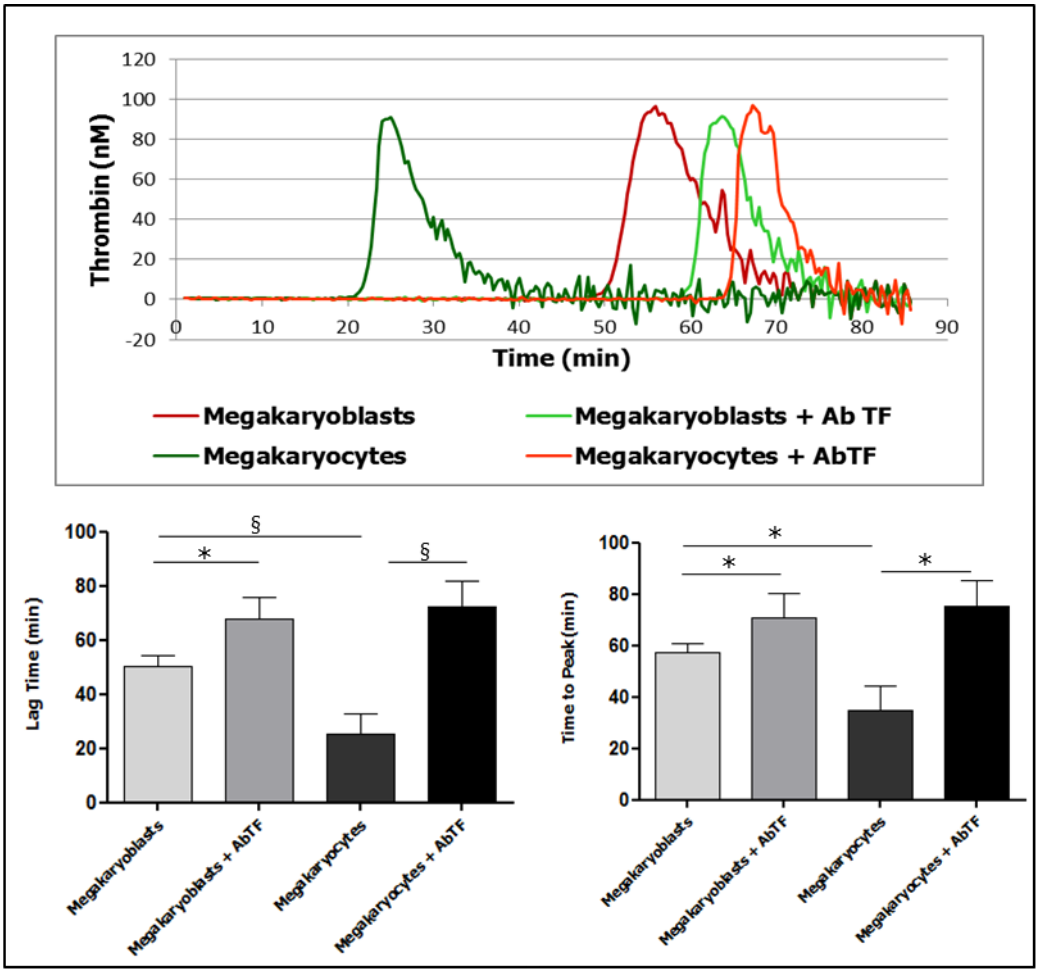


Figure 28. Thrombin generation assay performed with megakaryoblasts and megakaryocytes in the absence and presence of a neutralizing TF antibody (100 µg/ml). * P≤0.05; § P≤0.001.

Immunostaining and confocal microscopy analysis confirmed that TF was expressed on the cell surface in 70% of Meg-MK (fig. 29a), whereas almost 100% of the cells showed TF positive intracellular staining (fig. 29b). Moreover microscopy analysis showed that TF was present both in the cell body as well as in the pro-platelet tips (fig. 30).

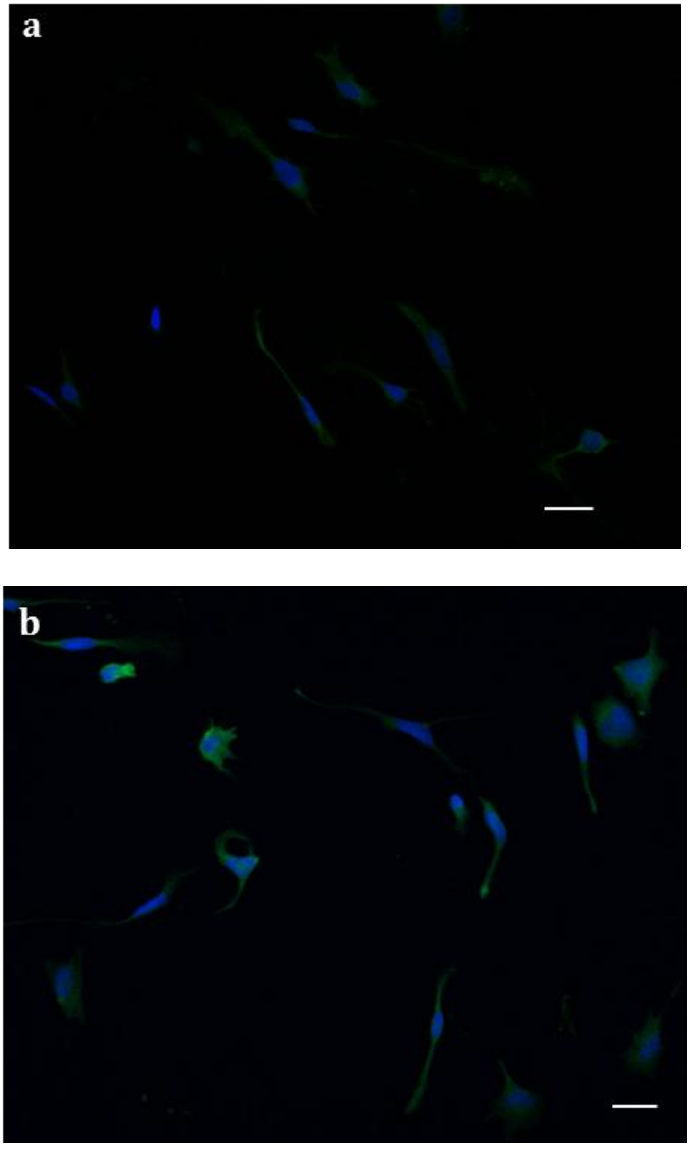


Figure 29. Immunofluorescence staining and confocal microscopy analysis of a) surface localization and b) intracellular localization of TF (green) in Meg-MK. Please note that in a) only the 70% of cells are TF positive and in b) all the cells express TF. Scale bar 50μm.

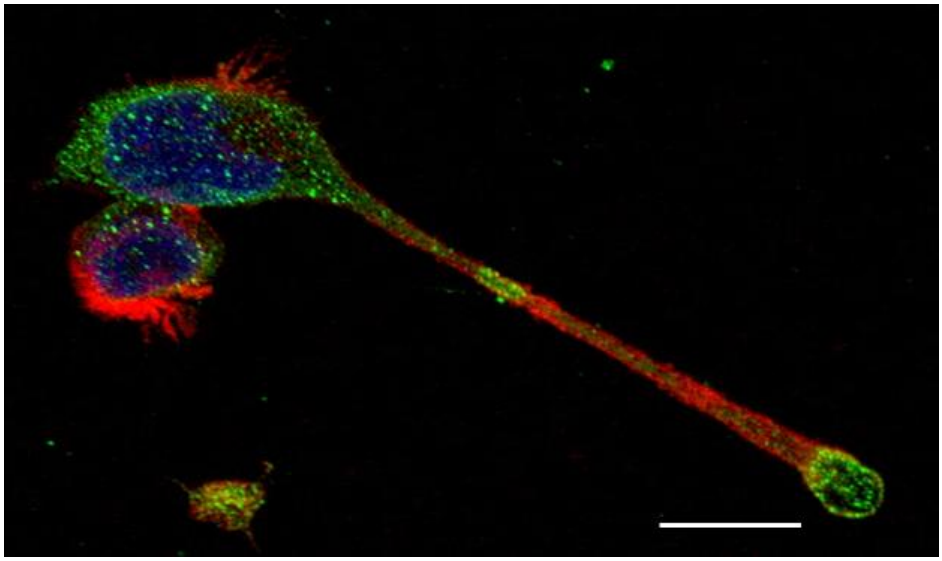


Figure 30. Intracellular immunofluorescence staining and confocal microscopy analysis of TF (green), actin filaments (red) and nuclei (blue) of a Meg-MK at day 14 of differentiation. Scale bar 20μm.

Evaluation of Tissue factor expression and functional activity in Meg-platelets

In order to verify whether the presence of TF in the final tip could support the concept of a transfer of TF from megakaryocyte to nascent platelets, TF expression in Meg-platelets was evaluated.

Flow cytometry analysis showed that TF was detectable in the cytoplasm of ~40% of Meg-platelets (fig. 31). These data reflect quite well the *in vivo* situation in humans where the percentage of TF-positive platelets in intracellular staining amounts to ~30-35% (fig. 31).

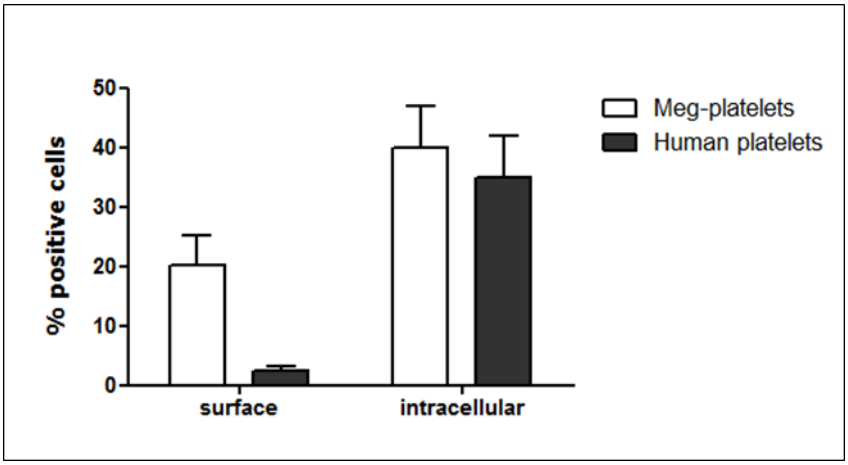
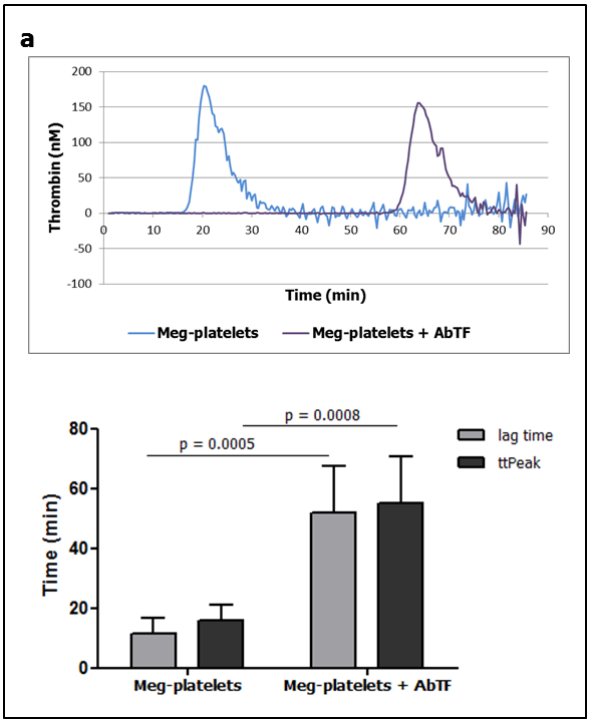


Figure 31. Surface and intracellular expression of TF in Meg-platelets and in human platelets evaluated by flow cytometry. TF was present on the surface of 20.1±5% of Meg-platelets and 2.5±0.6% of human platelets; when analyzed intracellularly, 40±7% of Meg-platelets and 35±7% of human platelets were positive for the antigen.

The thrombin generation capacity of Meg-platelet showed that TF was functionally active and its contribution was significantly blunted by a neutralizing TF antibody (fig. 32a). Experiments performed with FVII- or FX-depleted plasma showed no thrombin generation, further supporting the key role of the Meg-platelet associated TF (fig. 32b).



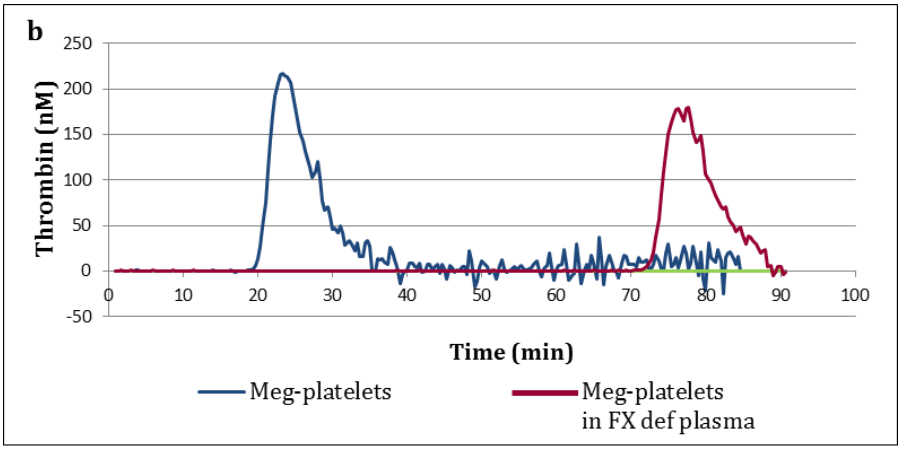


Figure 32. a) Thrombin generation assay performed with Meg-platelets in the absence and in the presence of a neutralizing TF antibody (100 µg/ml). b) Thrombin generation assay performed with Meg-platelets in factor X deficient plasma.

In agreement with previous data[52], real-time PCR experiments showed that Meg-platelets have TF mRNA (fig. 33a). This observation suggests that the possibility exists that both TF protein and mRNA are transferred from megakaryocytes to new platelets. Notably, the profile of the total RNA extracted from Meg-platelets is

virtually identical to that of human platelets (fig. 33b) and also the relative abundance of transcripts such as GpIIIa and Cox-1, besides TF, is similar in the two cell systems (fig. 33a).

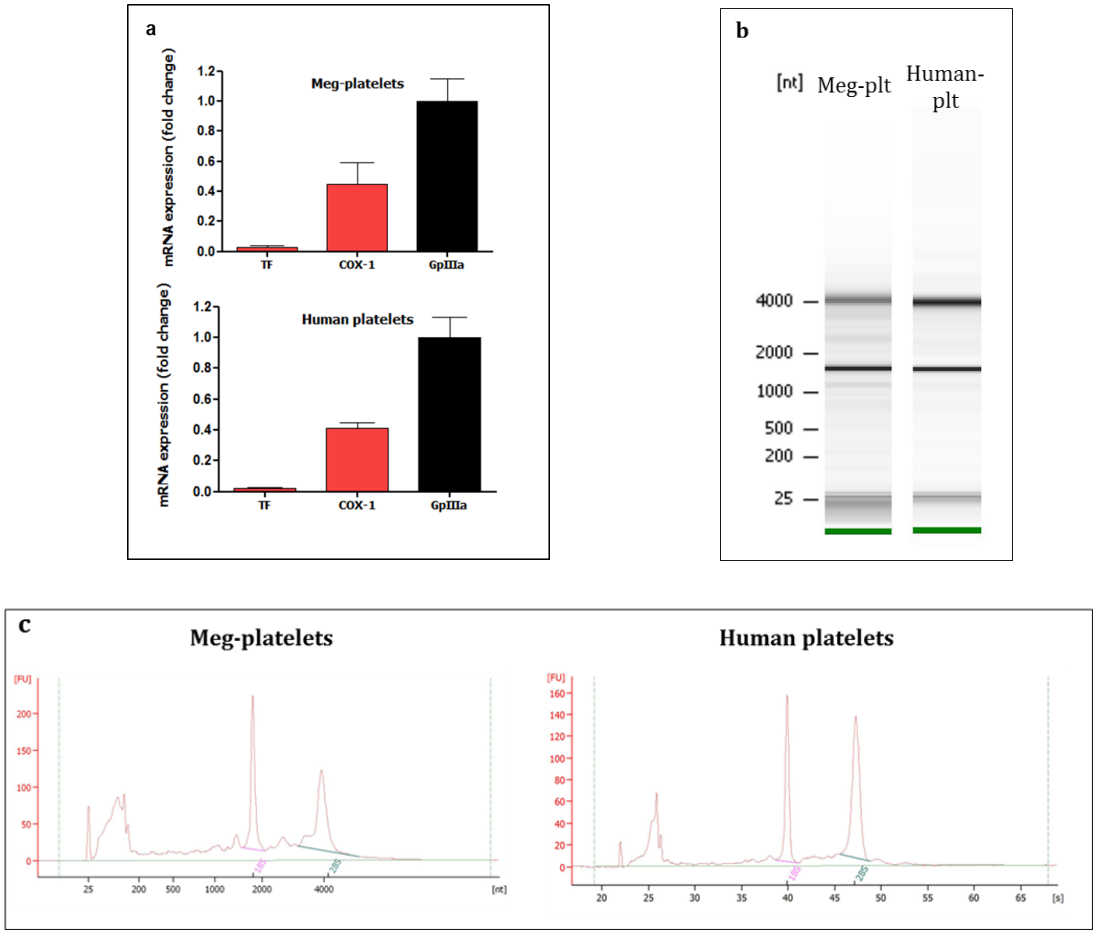


Figure 33. a) Comparison of the relative amounts of TF, GpIIIa and COX-1 mRNAs in Meg-platelets and in human platelets (setting to 1 the expression of GpIIIa in meg- and human-platelets). (b) Virtual gels of total RNA extracted from Meg- and human-platelets as provided by Bio-Analyzer. c) Meg-platelets RNA showed an electropherogram similar to that of human platelets with the 18S peak higher than that of the 28S subunit.

Evaluation of Tissue factor expression and functional activity in CD34⁺-derived megakaryocytes.

In order to confirm that the TF expression in MEG-01 was not a feature of the transformed cell line, but reflected a mechanism occurring in physiological conditions, TF expression in human CD34⁺-derived megakaryocytes (CD34⁺-MK) and in their platelets (CD34⁺-plat) was evaluated. TF protein levels in CD34⁺-MK, measured by Elisa, was similar to what observed in Meg-MK (124±27 pg/mg protein and 90±30 pg/mg protein, respectively). The thrombin generation capacity of CD34⁺-MK (fig. 34) was also comparable to that found in Meg-MK.

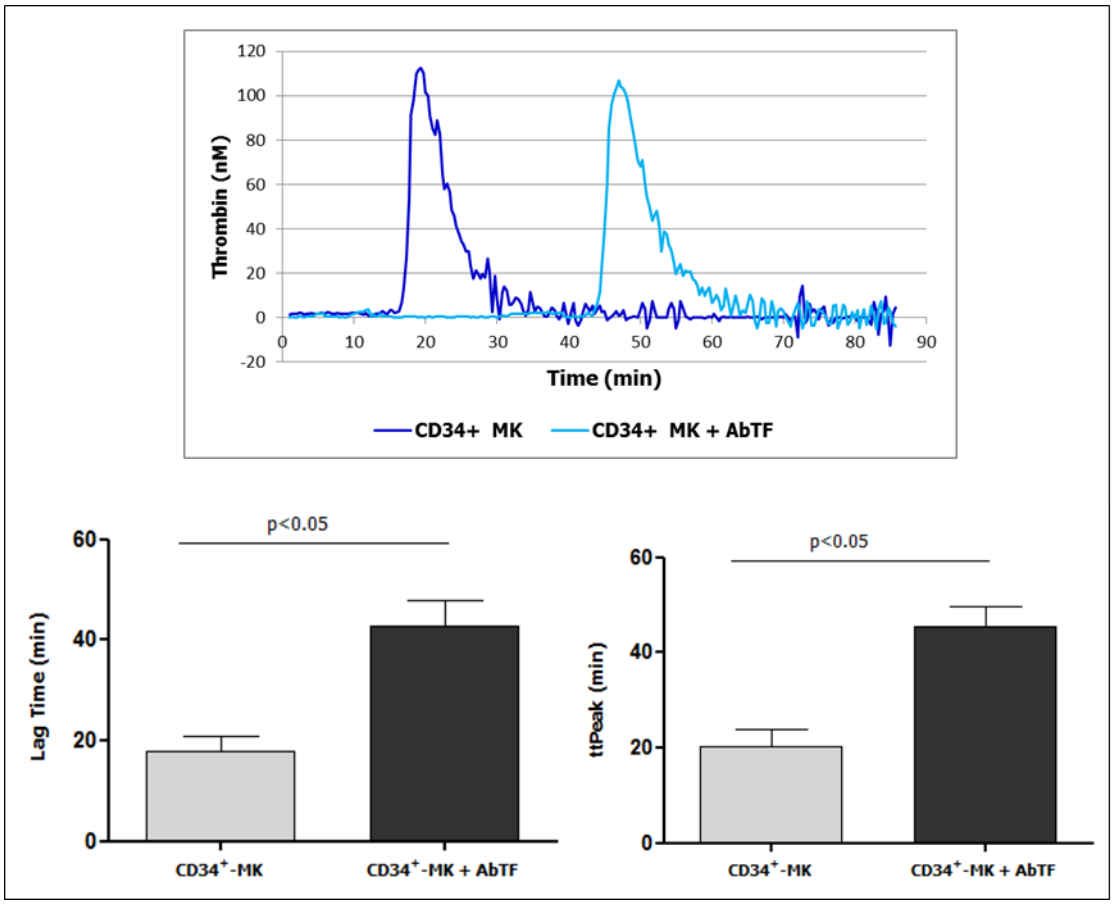


Figure 34. Thrombin generation assay performed with CD34⁺-MK in the absence and in the presence of a neutralizing TF antibody (100 µg/ml).

Flow cytometry analysis of surface and intracellular TF expression was also comparable to that observed with Meg-01 both in CD34⁺-MK and in CD34⁺-platelets. TF was present on the plasma membrane of 81±4.2% of CD34⁺-MK and 29±2.6% of CD34⁺-platelets, while the intracellular expression accounted for a 95.6±6.2% and 47±1.7% of positive cells for CD34⁺-MK and CD34⁺–derived platelets, respectively (Fig. 35).

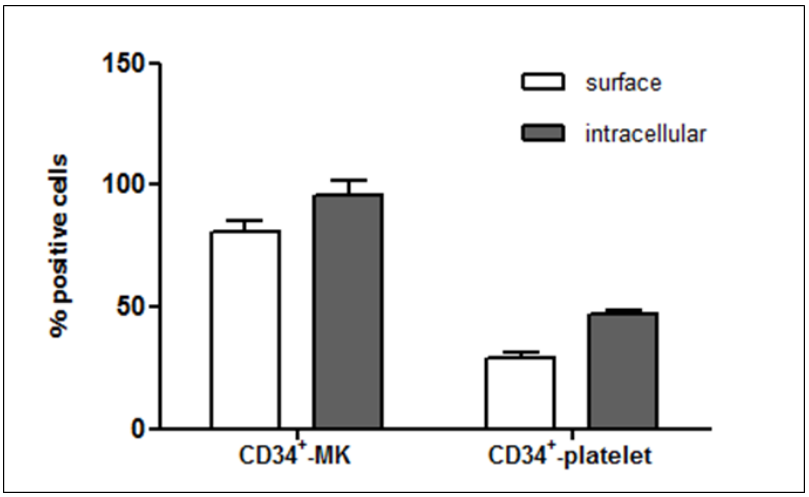


Figure 35. Surface and intracellular expression of TF in CD34⁺-MK analyzed by flow cytometry.

Immunostaining and confocal microscopy analysis performed in $CD34^+$-MK adherent to fibrinogen coated slides showed that TF was present both in the cell body as well as in proplatelet tips, as observed in Meg-MK (fig. 36).

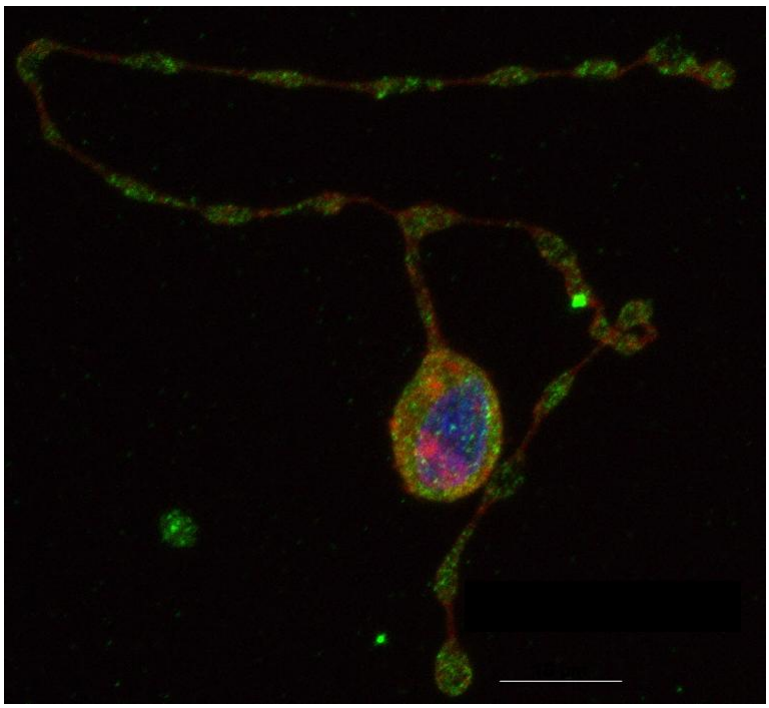


Figure 36. Intracellular immunofluorescence staining and confocal microscopy analysis of TF (green), actin filaments (red) and nuclei (blue) of a megakaryocyte at day 14 of differentiation. Scale bar 10μm.

Effect of Tissue factor silencing on Meg-01

In order to dissect the contribution of platelet-TF to the global platelet haemostatic capacity, TF was silenced in Meg-01 and its expression and function in Meg-megakaryoblast and in the megakaryocyte-shed platelets were assessed. Infection of Meg-01 with GIPZ lentiviral shRNA particles (three different target vectors) against human TF significantly prolonged the doubling time of the cells (62±6 and 145± 9 hours) underlying the pivotal role of TF not only in coagulation but also in cell proliferation. With this experimental strategy a significant reduction of TF antigen in megakaryoblasts was observed. Flow cytometryc analysis showed an almost negligible expression of TF in the silenced Meg-01(fig. 37a), and this finding is confirmed by ELISA assays (fig. 37b).

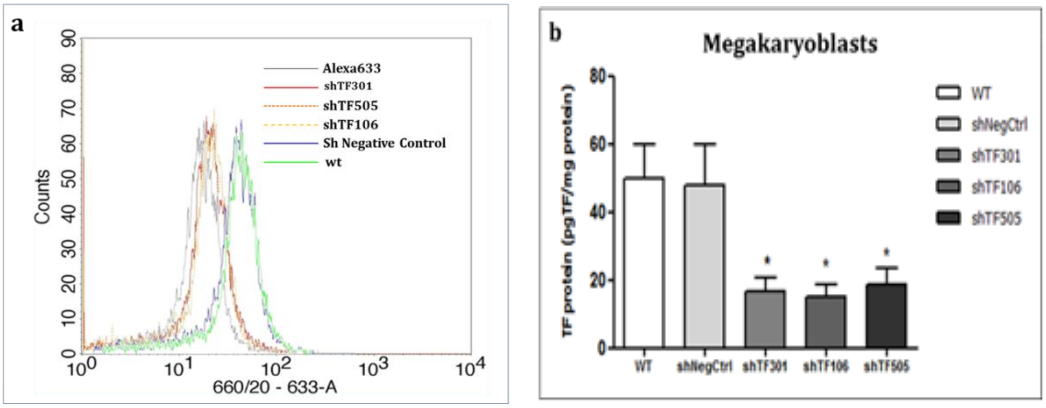


Figure 37. a) Flow cytometry evaluation of intracellular TF expression in wt Meg-01, in Meg-01 infected with GIPZ lentiviral shRNA particles to silence TF (shTF301, shTF106 and shTF505 target vectors) and in shRNA Scrambled Negative Control treated Meg-01. (b) TF protein levels, assessed by Elisa (*P=0.001), in megakaryoblasts and Meg-01 infected with GIPZ lentiviral shRNA particles to silence TF (shTF301, shTF106 and shTF505 target vectors) and shRNA Scrambled Negative Control treated cells.

Immunostaining and confocal microscopy analysis performed in Meg-MK showed no TF expression in TF-silenced Meg-MK compared to control megakaryocytes (fig. 38).

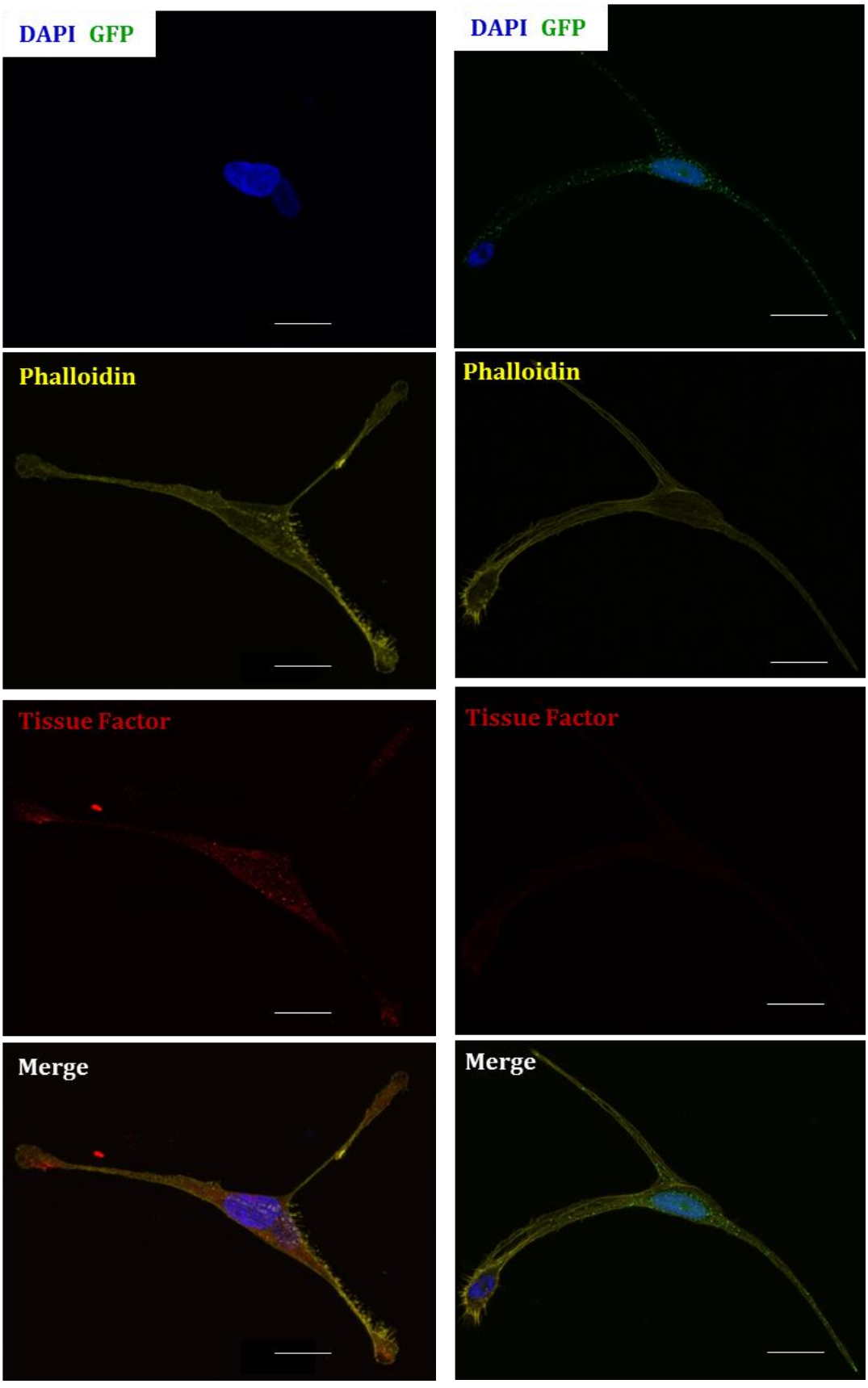

DAPI GFP
DAPI GFP
Phalloidin
Phalloidin
Tissue Factor
Tissue Factor
Merge
Merge

Figure 38. Intracellular immunofluorescence staining and confocal microscopy analysis of TF (red), actin filaments (yellow) and nuclei (blue) of a control Meg-MK (left panel) and a TF-silenced Meg-MK (right panel). Please note that TF-silenced Meg-MK are GFP positive. Scale bar 20μm.

The decreased TF expression observed in silenced megakaryoblasts was reflected in a consistent reduction of TF expression in the released platelets as assessed by flow cytometry. Only the 5% of TF silenced Meg-platelets were TF positive compared to 20% TF positive control Meg-platelets (fig. 39).

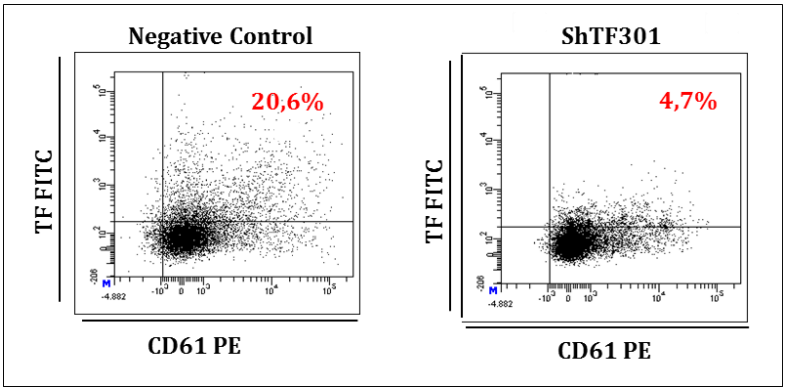


Figure 39. Flow cytometric evaluation of intracellular TF expression of platelets derived from shRNA Scrambled Negative Control treated cells and from shTF301 silenced Meg-01.

The reduction in TF expression was paralleled by a significant reduction in thrombin generation capacity of silenced megakaryoblasts, megakaryocytes and platelets. Silenced megakaryoblasts, Meg-Mk and Meg-platelets showed a delay in

the onset of the thrombin generation curve reflecting in a significant increase of lag time and time to peak (fig. 40) compared to control cells.

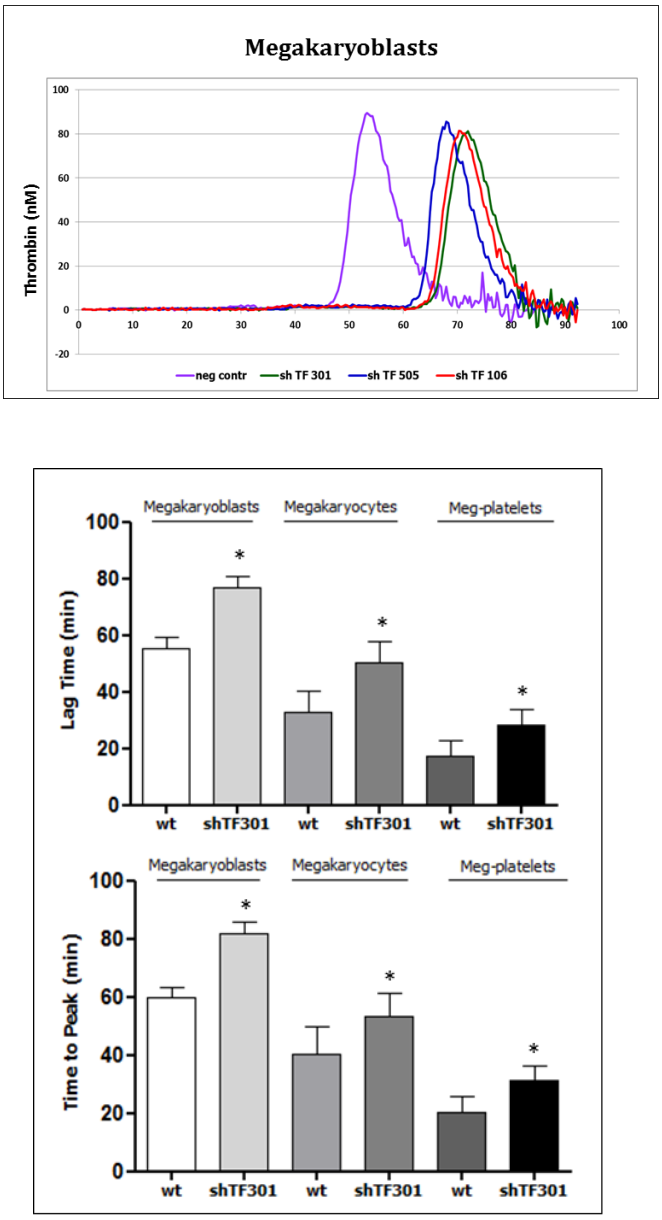


Figure 40. TF activity assessed by Thrombin Generation Assay in megakaryoblasts, megakaryocytes and Meg-platelets (*P<0.0001).

The contribution of platelet-TF to the global platelet haemostatic capacity is further emphasized when the kinetic of clot formation was assessed in a platelet-rich plasma (PRP). Platelet-depleted whole blood reconstituted with TF-silenced platelets or PRP showed a longer CFT and lower MCF and α-angle compared to platelet-depleted whole blood reconstituted with control Meg-platelets (fig. 41).

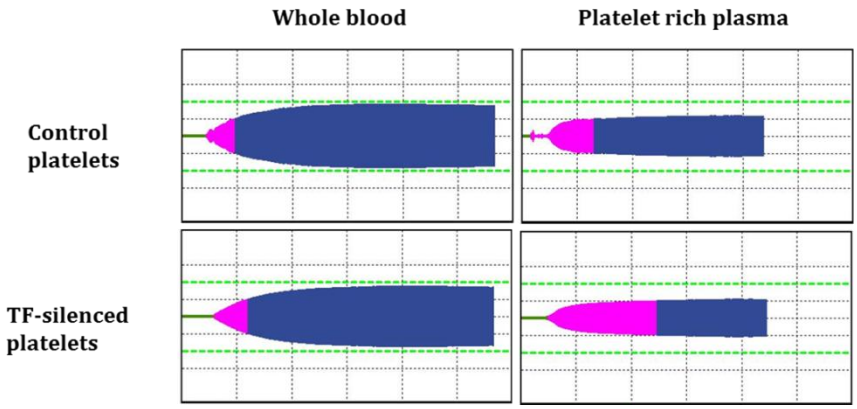


Figure 41. Global platelet haemostatic capacity analyzed by thromboelastometry in platelet-depleted whole blood (left) and plasma (right), reconstituted with control Meg-platelets or with TF-silenced Meg-platelets. Representative reaction curves (TEMograms) are shown.

Tissue factor expression in microparticles

TF-positive MPs have been found in vivo. Since both megakaryocytes and platelets release MPs, we characterized them for TF expression. First, MPs gate was assessed using polystyrene calibrated beads (0.5, 0.9 and 3 μm) to set the flow cytometer for MP detection and analysis (fig. 42a). Moreover, VPD and 7-AAD staining were used to identify events with an intact plasma membrane and apoptotic bodies, respectively. By using this gating strategy, MPs were defined as 7-AAD negative, VPD and CD61 positive events (fig. 42b) and then the expression

of CD107 and Tissue Factor was evaluated. Flow cytometry analysis showed that the majority (75 ± 7%) of MPs released in the medium were from megakaryocytes ($CD61^{pos}/CD107a^{neg}$ events). The TF^{pos} MPs accounted for 20%, 13% being from megakaryocytes and 5% from platelets (fig. 42c).

Conversely, TF expression on MPs derived from silenced Meg-01 was almost negligible (fig. 42d).

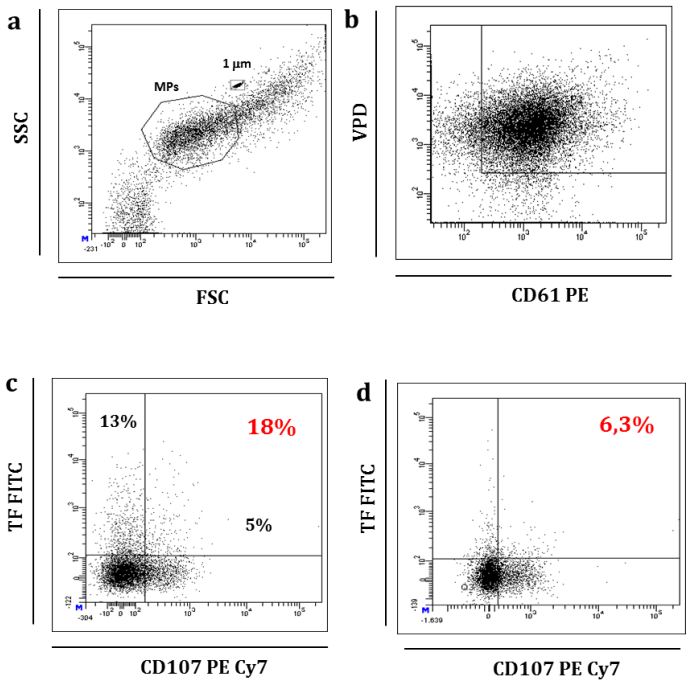


Figure 42. (a) The gating strategy for MP identification is shown: 1 µm calibrated beads were used to set the threshold size and to draw the MP gate. (b) The CD61 and VPD positive events within the MP gate were identified and analyzed for the expression of TF and CD107. CD107 positive MPs were from platelets, whereas CD107 negative MPs were from megakaryocytes. (c) Flow cytometric evaluation of surface TF expression of MPs derived from shRNA Scrambled Negative Control treated cells and (d) from shTF301 silenced Meg-01.

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DISCUSSION

DISCUSSION

In 2000 Giesen *et al.* postulated that thrombus formation and propagation, upon atherosclerotic plaque rupture, could take advantage of blood-born tissue factor: circulating platelets, carrying tissue factor (TF) derived from microparticles, may themselves trigger the activation of the coagulation cascade[50]. Since then, several papers have documented the presence of TF in human platelets, suggesting that at least three mechanisms are involved in the presence of TF in platelets: 1) the microparticle-transfer mechanism; 2) the storage within the α-granules and the open canalicular system and 3) the *de novo* protein synthesis from the TF specific messenger RNA (mRNA). A recent study showed that the transfer of mRNAs from megakaryocytes to platelets is not a random event, but occurs through a fine-tuned mechanism that select the mRNA content that will be provided to platelets. In particular, only the mRNAs that, when transcribed and transferred from the nucleus to the cytoplasm of the megakaryocyte, bind to specific RNA-binding proteins will be transferred into the nascent platelets through the complex system of microtubules. By contrast, mRNAs that did not bind to these proteins will be translated into protein that, upon storage into the granules, will be transferred to platelets[44]. In 2003 our group provided the evidence that human $CD34^+$-derived megakaryocytes express the TF mRNA[52]. Thus, it can be speculated that the TF mRNA detectable in platelets could be the result of a direct transfer from megakaryocytes, but to date the evidence for this mechanism is still lacking. Moreover, the presence of TF protein in human megakaryocyte has never been reported. Conversely, it is commonly believed that the only mechanism responsible for the presence of TF in platelets is through the uptake of TF-positive

microparticles released by activated endothelial cells or leukocytes. In order to verify whether the transfer of TF from megakaryocytes to platelets is an existing process, we took advantage from a commercially available human megakaryoblastic cell line, called Meg-01, able to differentiate into platelet-releasing megakaryocytes. We first validated this *in vitro* system, providing the evidence that Meg-platelets have the same physical (size and intracellular complexity) and antigenic characteristics of human platelets. Moreover, we reported that Meg-platelets are functionally active, in a manner comparable to human platelets, being able to aggregate in response to collagen and TRAP-6, to adhere to fibrinogen and spread onto fibrinogen-coated surface, and to contribute to the hemostatic capacity of the blood, when tested by thromboelastometry.

By using this well characterized *in vitro* model able to recapitulate megakaryocyte differentiation and platelet biogenesis, we provide consistent evidence that TF is an endogenously synthesized protein that characterizes megakaryocyte maturation. Since the cell system used allowed us to study mRNA and protein expression in the absence of any crosstalk with other cell or MP, here we provide the evidence for the direct transfer of both TF mRNA and protein from megakaryocytes to a subset of platelets where it contributes to their thrombin generation capacity. Of interest, the percentage of TF positive platelets that we observed *in vitro* (both with Meg-platelets and with $CD34^+$-derived platelets) is virtually identical to the amount found in blood from healthy individuals (30-40%). This striking data suggests that a fine-tuned mechanism, which deserves further investigation in order to dissect the molecular pathways involved in its regulation, is responsible for the controlled delivery of TF from megakaryocytes to

platelets. It is plausible that such a mechanism may account for the heterogeneity in platelet function according to the transcriptome and protein content present in different subset of platelets. Indeed, studies carried out in the last 15 years have highlighted that platelets are involved not only in hemostasis and in thrombotic events responsible for the clinical manifestation of cardiovascular diseases such as myocardial infarction, stroke and peripheral ischemia, but they are actively involved in inflammatory processes, angiogenesis and tumor metastasis[82,83], and in several pathological conditions such as Alzheimer's disease[84], systemic sclerosis[85], rheumatoid arthritis[86] and systemic lupus erythematosus[87]. Recently, Stalker and colleagues proposed a new model of thrombus formation, suggesting that the thrombus is not an homogeneous mass but it is constituted by a core of fully activated platelets overlaid by a shell of less-activated, mostly unstable platelets (fig. 43). These data further support the concept of platelet heterogenicity, showing that the haemostatic thrombus is not a homogeneous mass in which all platelets are activated to the same extent[81].

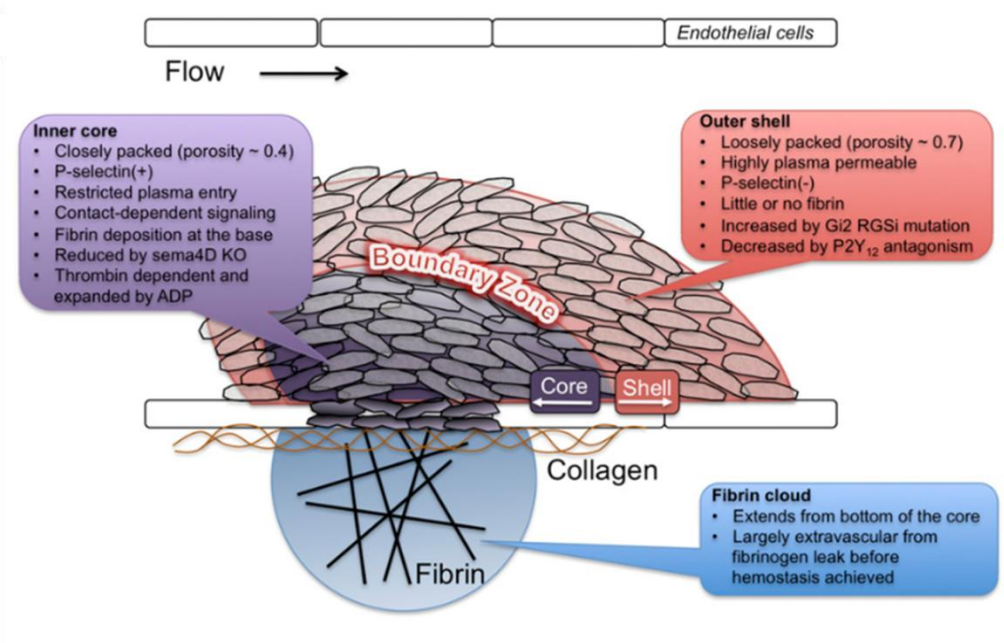

Figure 43. Structural heterogeneity in the haemostatic plug. There are distinct regions within the haemostatic thrombus where platelets are either more or less activated and where fibrin tends to accumulate. A stable core of closely packed, fully activated platelets is overlaid by an outer shell of less-activated platelets, with a boundary zone in between.

A major issue related to the finding of platelet-associated TF is the demonstration of its relative contribution to the *in vivo* thrombus formation. By taking advantage from a TF silencing approach, we developed three megakaryoblast cell lines stably under-expressing TF. This resulted in a prolonged doubling time, emphasizing the pivotal role of TF not only in the activation of the coagulation cascade but also in cell proliferation[21]. The capacity to differentiate and to release platelets were not affected. As a consequence of this manipulation the percentage of TF-positive platelets released by these cell lines significantly decreased and this was paralleled by a significant reduction in thrombin generation capacity. The contribution of platelet-TF to global platelet haemostatic capacity was further emphasized when

the kinetic of clot formation was assessed by thromboelastometry in a platelet-rich plasma.

Our data show that TF silencing in Meg-megakaryoblasts resulted in a significant reduction of TF protein expression. As a consequence we also observed a decrease in thrombin generation of whole blood reconstituted with TF-silenced platelets providing the evidence of the contribution of the platelet-associated TF expression to the thrombus formation.

TF is not the only coagulation factor present in platelets. Indeed, platelets contain several coagulation factors (FV, FIX, FXI, FXIII, fibrinogen, von Willebrand factor, TFPI, etc) which are promptly released on the cell surface upon platelet activation. Of note, for some of the above mentioned coagulation factors (FXI, FXIII, TF, TFPI, von Willebrand factor) the presence of the corresponding mRNA has also been reported and this finding is of particular relevance if the biosynthetic capacity of platelets is considered.

In conclusion all these data show that TF is expressed both by megakaryoblasts and megakaryocytes, and its expression increases during megakaryocyte differentiation. Furthermore, this *in vitro* approach, devoid of any cross-talk with other cells or microparticles, allows us to show that human megakaryocytes are able to transfer TF to a subset of platelets. Therefore platelets may express TF independently of the interaction with other cells, thus providing these cells with the capacity to control also the generation of thrombin. This is of particular relevance in view of the observation that under pathological conditions the

number of TF positive cells significantly increases and this may be due to alteration in megakaryocyte behavior[72,56-58]. This mechanism may represent a potential target for therapeutic intervention against the formation of platelet thrombi. The Meg-megakaryocytes and Meg-platelets *in vitro* model has provided novel insights into the contribution of platelets and platelet-derived TF in the activation of the blood coagulation. This model will also be useful for the assessment of molecular mechanisms responsible for the presence of an increased number of TF positive platelets in several diseases (cardiovascular diseases, cancer and autoimmunity diseases, etc). Finally, this model can be used to study megakaryocytes and platelets in diseases characterized by metabolic abnormalities associated with cardiovascular diseases, such as diabetes. Moreover, the pharmacological modulation of megakaryocyte-derived as well as platelet-derived TF can also be investigated.

ABSTRACT

ABSTRACT

Tissue factor is the main activator of the blood coagulation cascade and for this reason levels of TF are not easily detectable in cells in contact with blood under physiological conditions. By contrast, in pathological conditions endothelial cells and monocytes can be induced to express TF[68]. Induced-TF is present in atherosclerotic plaques (vessel wall-derived TF) and several studies in the past have documented its role in the plaque thrombogenicity[33,35]. At the end of the last century it was reported that, under physiological conditions, TF positive microparticles (MPs) circulate in the blood (blood-borne TF) and their number further increases in pathological conditions[47,51]. These microparticles, which arise mainly from activated endothelial cells and monocytes, could fuse with other cells conferring them the ability to activate coagulation[69].

Giesen et al. in 2000 showed the presence of TF in human platelets through this mechanism[50]. Since then several papers have documented the presence of TF in human platelets by using different approaches[52-55,70-73]. Despite this in-depth characterization, the platelet-associated TF is matter of controversy by some authors that, failing to identify TF in platelets, argue that the published data are artifacts, thus generating an ongoing debate[73,74].

To date two main "cellular entities" may be responsible for the presence of TF in platelets: TF-positive MPs derived from different activated cell types, as proposed by Nemerson's group, and megakaryocytes that transferring TF mRNA to platelets make them autonomous in the synthesis of the protein. In this regard, ten years ago our group provided the evidence that human megakaryocytes contain TF mRNA[53]. The platelet transcriptome derives from megakaryocytes through finely

tuned mechanisms[43,44]. The direct evidence that megakaryocytes transfer TF mRNA to platelets, however, is still missing; similarly, it has never been investigated whether megakaryocytes express TF protein that can be transferred to platelets; finally, the contribution of platelet TF to clot formation has never been assessed. To test these hypothesis we took advantage of a well-characterized human megakaryoblastic cell line, Meg-01, able to differentiate into megakaryocytes and to release platelets in vitro[75]. This approach allowed us to analyze TF mRNA and protein expression during the differentiation from megakaryoblasts to megakaryocytes and in the released platelets in the complete absence of any other "contaminating" cell that might be a source of microparticles. The expression of TF protein by Meg-01 and Meg-derived platelet (Meg-platelets) was confirmed in human megakaryocytes differentiated from CD34+ cells. Finally, TF mRNA was silenced in Meg-01 megakaryoblasts showing that megakaryocytes, Meg-platelets and MPs were almost devoid of TF.

Using a combination of cell biology, flow cytometry, confocal microscopy and biochemical analyses here we show evidence for the first time that functionally active TF is expressed in human megakaryocytes, and that they transfer this protein to platelets and to microparticles (MPs). TF downregulation in megakaryoblasts by lentiviral shRNA particles almost completely abolished its expression in megakaryocytes, platelets and MPs. Furthermore, TF silencing leads to a decrease of the thrombin generation by megakaryocytes which is reflected on a decrease in thrombin generation by platelets, suggesting that the platelet-associated TF may contribute to the haemostatic capacity of whole blood.

Taken together all these data shoe the evidence that human platelets and MPs carry a megakaryocyte-derived TF which is functionally active and able to trigger thrombin generation.

Finally, this *in vitro* model may be used to investigate the modification in platelet transcriptome and functionality observed in several diseases, such as cardiovascular disease, diabetes and cancer.

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