Role of membrane microparticles in angiogenesis

Raffaella Soleti

UNIVERSITÉ D'ANGERS

UNIVERSITA' DEGLI STUDI DI BARI

Année 2008 **N° d'ordre 900**

ROLE OF MEMBRANE MICROPARTICLES IN ANGIOGENESIS

RÔLE DES MICROPARTICULES MEMBRANAIRES DANS L'ANGIOGENÈSE

THÈSE DE DOCTORAT EN COTUTELLE

Spécialité : Pharmacologie expérimentale et clinique

ÉCOLE DOCTORALE BIOLOGIE SANTÉ

Présentée et soutenue publiquement

le 18 décembre 2008 à Bari

par **Raffaella SOLETI**

Devant le jury ci-dessous :

Professeur Bernard MULLER	Rapporteur externe
Professeur Dario Domenico LOFRUMENTO	Rapporteur externe
Professeur Georges LEFTHERIOTIS	Examinateur
Professeur Antonella MUSCELLA	Examinateur
Professeur Maria Antonietta PANARO	Co-directeur de thèse
Docteur Carmen MARTÍNEZ	Directeur de thèse

Laboratoire de Biologie Neuro-Vasculaire Intégrée
UMR CNRS 6214-INSERM U771
Faculté de Médecine

Ai miei genitori

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

Questa tesi è un lavoro collettivo. E' perciò naturale esprimere la mia riconoscenza a tutti i coloro che hanno guidato i miei passi, mi hanno offerto la loro esperienza e il loro sapere, insegnato il rigore, la perseveranza e la pazienza, donato consigli giudiziosi, evitato le insidie, motivato nei momenti di debolezza, protetto dai miei errori, sostenuto nelle avversità, offerto la loro amicizia, le loro orecchie e le loro spalle per i miei sfoghi ed anche per avermi aperto il loro cuore!!

Ringrazio il Prof. Vincenzo Mitolo che mi ha offerto possibilità di scoprire nuovi orizzonti, permettendo la mia partenza ad Angers.

Ringrazio il Dott. Carmen Martinez che ha diretto questo lavoro, per avermi seguito con consigli e confronti che mi hanno aiutato ad intraprendere, ogni volta, le scelte più appropriate, per la continua disponibilità e prontezza nei chiarimenti e suggerimenti, per la lettura critica della tesi e per avermi guidato con i suoi suggerimenti fino alla conclusione di questo percorso formativo.

Ringrazio il Dott. Ramaroson Andriantsitohaina che, con il suo ottimismo, i suoi incoraggiamenti e consigli, l'entusiasmo contagioso e la passione per la ricerca, ha saputo orientarmi verso direzioni piene di risultati e sostenuto la realizzazione di questo lavoro e la sua revisione.

E' grazie a Carmen e Naina che ho scoperto le microparticelle!! Ed è loro che ringrazio per l'affetto dimostratomi, il tempo trascorso fuori dal laboratorio ed il loro appoggio certo.

Ringrazio la Prof. sa Maria Antonietta Panaro che ha sostenuto la mia permanenza ad Angers, per la continua disponibilità e gentilezza che mi ha mostrato in ogni momento.

Ringrazio il Dott. Daniel Henrion per avermi accolto nel sua unità di ricerca.

Ringrazio il Prof. Bernard Muller, il Prof. Dario Lofrumento, il Prof. Georges

Leftheriotis e la Prof. sa Antonella Muscella per aver accettato di giudicare questo lavoro.

Ringrazio Chiara, collega, ma soprattutto amica, che ha costruito le solide basi di questo lavoro e che con estrema pazienza ha sopportato i miei sbalzi di umore e le mie paranoie. Se ho raggiunto questo traguardo lo devo anche alla sua continua presenza.

Ringrazio Tarek, il mio compagno di viaggio, con cui condivido questo studio e traguardo!!

Ringrazio "le italiane" Mariele, Angela,Mirella e Daniela con cui ho trascorso la maggior parte del mio tempo, allegre serate, ed a cui ho esternato i miei nervosismi più estremi, ma soprattutto per avermi mostrato affetto e amicizia.

Ringrazio Simon, Matthieu, Ahmed, Abdel, Vannina e Silvia per gli scambi, gli aiuti ed i bei momenti trascorsi insieme.

Ringrazio Emmannuelle per la sua gentilezza e cortesia in ogni momento.

Desidero ringraziare Tita, Rosetta, Tonia, Pasqua, Angela, Annamaria, Margherita, Sabrina, con le quali ho iniziato questo cammino, scambiato pensieri e risate, trovato sostegno e amicizia.

Per ultimi, ma di certo non per importanza, ringrazio la mia famiglia e gli amici che mi sono stati molto vicini in tutto questi periodo.

Il mio primo pensiero, ovviamente, va ai miei genitori, a cui dedico questo lavoro di tesi: senza il loro aiuto non avrei mai raggiunto questa meta. Sono davvero grata per tutto il loro sostengo! Spero tutti i sacrifici spesi siano in questo modo, almeno parzialmente, ripagati.

Ringrazio i miei fratelli che,seppure lontani, mi sono stati vicini ed hanno sempre tifato per me!!

Ringrazio i miei amici, vecchi e nuovi, che hanno reso piacevole il soggiorno angevino

ed ancor più i miei rientri.

A tutti voi spero di riconsegnarvi almeno in parte quello che mi avete donato!!

ABBREVIATIONS

ActD: actinomycin D;

Ang: angiopoietin;

bFGF: basic-fibroblast growth factor;

cGMP: Guanosine 3',5'-cyclic monophosphate;

Cav-1: caveolin-1;

COX-2: cyclooxygenase-2;

ECM: extracellular matrix;

EC(s): endothelial cell(s);

EGFR: epidermal growth factor receptor;

EMP(s): endothelial microparticle(s);

eNOS: endothelial nitric oxide-syntase;

EPC(s): endothelial progenitor cell(s);

ERK: extracellular-regulated kinase;

FAK: focal adhesion kinase;

Flt: VEGFR;

FGF: fibroblast growth factor;

FGFR: fibroblast growth factor receptor;

GP: glycoprotein;

HGF: hepatocyte growth factor;

HIF: hypoxia induced factor;

HIV: human immunodeficienty virus;

HSP: heat shock protein;

ICAM-1: intracellular adhesion molecule-1;

IGF-1: insulin-like growth factor 1;

IL: interleukin;

iNOS: inducible nitric oxide-syntase;

LMP(s): lymphocytic microparticle(s);

JNK: jun N-terminal kinase;

MAPK: mitogen-activated protein kinase;

MCP-1: monocyte chemotactic protein 1;

MEK: MAPK-ERK-kinase;

MMP(s): matrix metalloproteinase(s);

MP(s): microparticle(s);

MPs^{Shh+}: microparticles bearing sonic hedgehog;

NADPH: nicotinamide adenine dinucleotide phosphate;

NF-κB: nuclear factor κB;

NO: nitric oxide;

NOX: NADPH oxidase;

PC: phosphatidylcholine;

PDGF: platelet-derived growth factor;

PE: phosphatidylethanolamine;

PECAM 1: platelet-endothelium adhesion molecule 1;

PGE2: prostaglandin E2;

PHA: phytohemagglutinin;

PI3K: phosphatidyl-inositol 3 kinase;

PKC: protein kinase C;

PMA: phorbol-myristate-acetate;

PMP(s): platelet microparticle(s);

PS: phosphatidylserine;

ROCK: Rho-associated kinase;

ROS: reactive oxygen species;

S1P: sphingosine 1-phosphates;

Shh: sonic hedgehog;

SM: sphingomyelin;

SMC(s): smooth muscle cell(s);

SOD: superoxide dismutase;

TF: tissue factor;

TGFβ: transforming growth factor β;

TIMP(s): tissue-localized inhibitors of metalloproteinase(s);

TNF-α: tumour necrosis factor-α;

TSP1: thrombospondin 1;

T_XA_2: thromboxane A_2;

tPA: tissue plasminogen activator;

uPA: urokinase plasminogen activator;

uPAR: urokinase plasminogen activator receptor;

VEGF: vascular endothelial growth factor;

VEGFR: vascular endothelial growth factor receptor.

PUBLICATIONS

Publications

S. Lisi, M. Sisto, **R. Soleti**, C. Saponaro, P. Scagliusi, M. D'Amore, M. Saccia, AB. Maffione, V. Mitolo. Fcγ receptors mediate internalization of anti-Ro and anti-La autoantibodies from Sjögren's syndrome and apoptosis in human salivary gland cell line A-253. J Oral Pathol Med 2007 36 (9), 511–523.

M. Pricci, JM. Bourget, H. Robitaille, C. Porro, **R. Soleti**, A. Mostefai, FA. Auger, MC. Martinez, R. Andriantsitohaina, L. Germain. Applications of human tissue engineered blood vessel models to study the effects of shed membrane microparticles from T-lymphocytes on vascular function. Tissue Eng.doi:10.1089/ten.tea.2007.0360.

Review:

C. Porro, **R. Soleti**, T. Benameur, AB. Maffione , R. Andriantsitohaina, MC. Martinez MC. 2009. Sonic hedgehog pathway as a target for therapy in angiogenesis-related diseases. *Current Signal Transduction Therapy.* In press.

Publication in revision:

R. Soleti*, T. Benameur*, C. Porro, MA. Panaro, R. Andriantsitohaina, MC. Martínez. Microparticles harbouring Sonic Hedgehog promote angiogenesis through the up-regulation of adhesion proteins and pro-angiogenic factors. *These authors participated equally in this work.

Publication submitted:

ML. Mastronardi, HA. Mostefai, **R. Soleti**, A. Agouni, MC. Martinez, R. Andriantsitohaina. Microparticles from apoptotic monocytes enhance NO and reactive oxygen species in human endothelial cells concomitantly with angiogenesis.

Publication in preparation:

T. Benameur*, **R. Soleti***, C. Porro, MA. Panaro, R. Andriantsitohaina, MC. Martínez. *In vivo* pro-angiogenic effects exhibit by Sonic Hedgehog carried by microparticles. *These authors participated equally in this work.

PART I

MICROPARTICLES

I. General aspects

Transfer of information between cells is pivotal for proper functioning of multi-cellular organisms, which have evolved elaborate communication strategies to coordinate cell activities. Much of this crosstalk occurring at cell-cell contacts, is regulated by complex structural interfaces and also involves secreted extracellular bioactive molecules. In addition to this classical and well characterized network, in the last years, microparticles (MPs) have been considered as efficient vectors of biological information from one cell type to another and hence, it has been proposed that MPs may account for long-range intercellular communication (Mostefai *et al.* 2008a).

MPs are plasma membrane vesicles released from various cell types during activation by agonists or physical or chemical stress, including apoptosis (Martinez *et al*. 2005). They expose at their surface phosphatidylserine (PS) and express antigenic profile characteristic of the phenotype of the cell they stem from. The most abundant particle species in peripheral blood are platelet-derived MPs (PMPs), but circulating MPs can also arise from lymphocytes, monocytes, endothelial cells (ECs), and other cell types. Besides, they are normal constituents of blood plasma, but their concentration increases during pathological states.

The variable composition and amount may explain, at least in part, the beneficial or deleterious effects elicited by MPs in physiological or pathological conditions (Freyssinet 2004). Moreover, the greater ability to circulate throughout the vasculature confers them another characteristic that aids in transmission of biological messages. Additionally, the ways by which they may mediate intercellular communication are

different. They can bear combinations of ligands that would engage different cell-surface receptors simultaneously. Thus, MPs may provide interaction between cells without the need for direct cell contact. They can bind to target cell membrane, which would then bear new surface antigen and acquire new biologic properties and activities. Alternatively, MPs can also fuse with target cells and transfer bioactive molecules.

Altogether, these evidences support the hypothesis that MPs constitute vectors able to transport transcellular messages and thus, they can be considered as real actors in the regulation of physiological and pathological process.

I.1. Generation of Microparticles

Microparticles are a heterogeneous population of small membrane-coated vesicles released virtually from any cell types during activation or apoptosis. Their generation seems to be a well-regulated process, although these vesicles are highly variable in size (0.05-1 μm), composition and function. In eukaryotic cells, the transbilayer distribution of lipids across biological membranes is asymmetric (Bretscher 1972).

The choline-containing lipids, phosphatidylcholine (PC) and sphingomyelin (SM), are enriched primarily on the external leaflet of the plasma membrane. In contrast, the amine-containing glycerophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are located preferentially on the cytoplasmic leaflet. Loss of transmembrane phospholipid asymmetry, with consequent exposure of PS in the external monolayer, occurs in both normal and pathologic conditions. PS externalization is induced early in the process of apoptosis (Fadok 1992) and during cell activation

(Bevers *et al.* 1982). This perturbation results in a change in cell surface properties, including conversion to a procoagulant state (Lubin *et al.* 1981), increased adhesion (Schlegel *et al.* 1985), increased aggregation (Wali *et al.* 1987), and recognition by phagocytic cells (Fadok *et al*. 1998; 2001). While these processes are essential for normal cell development and homeostasis, unregulated loss of PS asymmetry may contribute significantly to heart disease and stroke and has been associated with diseases that have high cardiovascular risk (Wali *et al*. 1988; Wilson *et al.* 1993). Because passive lipid transbilayer diffusion is slow, a number of proteins have evolved to either dissipate or maintain this lipid gradient. These proteins fall into three classes: cytofacially-directed, ATP-dependent transporters, translocases; exofacially-directed, ATP-dependent transporters, floppases; and bidirectional, ATP-independent and Ca^{2+}-dependent transporters, scramblases. The translocase is highly selective for PS and functions to keep this lipid sequestered from the cell surface. Floppase activity has been associated with the ATP-binding cassette class of transmembrane transporters. Scramblases are inherently non-specific and function to randomize the distribution of newly synthesized lipids in the endoplasmic reticulum or plasma membrane lipids in activated cells (Bevers *et al.* 1999). The combined action of these proteins and the physical properties of the membrane bilayer that generate and maintain transbilayer lipid asymmetry (Fig. 1).

During cell activation by different agents (thrombin, collagen, ADP, calcium ionophores), the asymmetrical distribution of phospholipids is lost. The intracellular mechanisms underlying the release of MPs seem to be associated to sustained increase of Ca^{2+}, which favours scramblases and floppases activities and concomitantly inhibits the translocase. As consequence, became the exposure of PS at the external leaflet of the

plasma membrane and, probably, this is the most prominent feature of the collapse of transbilayer asymmetry in mammalian cells. These modifications are followed by kinase activation, phosphatase inhibition, cytoskeleton degradation by Ca^{2+}-dependent proteolysis and, an increase in bleb formation takes place (Fig. 2).

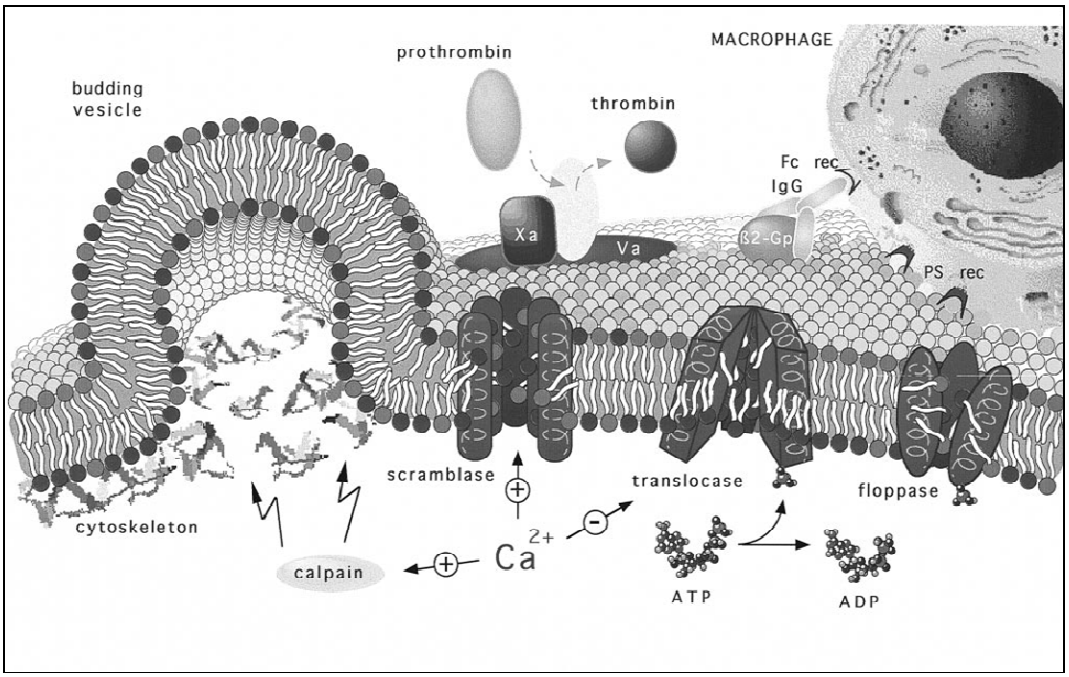


Fig. 1: Regulation and physiology of membrane phospholipids asymmetry. This model describes how membrane phospholipid asymmetry is generated and maintained. Membrane lipid asymmetry is regulated by the cooperative activities of three transporters. Translocase, which rapidly transports PS and PE from the cell outer-to-inner leaflet; floppase, which slowly transports lipids from the cell inner-to-outer leaflet; and scramblase, which allows lipids to move randomly between both leaflets. The model predicts that the translocases are targets for Ca^{2+} that directly regulates the transporter activities. Elevated intracellular Ca^{2+} induces PS randomization across the cell membrane by providing a stimulus that positively and negatively regulates scramblase and translocase activities, respectively. At physiologic Ca^{2+} concentrations, PS asymmetry is promoted because of an active translocase and floppase but inactive scramblase. Increased cytosolic Ca^{2+} result in cell and/or calpain activation, which facilitate membrane blebbing and the release of PS-expressing MPs. The appearance of PS on outer leaflet promotes coagulation and thrombosis and marks the cell as a pathologic target for elimination by phagocytes. Recognition of the PS-expressing targets can occur by both antibody-dependent and direct receptor-mediated pathways (Fom Zwaal and Schroit 1997).

MP formation during apoptosis results from Rho-associated kinase (ROCK I) activity, due to caspase 3 activation. ROCK I promotes increased actin-myosin force

generation, couples actin-myosin filaments to the plasma membrane and, as consequence, leads to disruption of membrane skeleton structure and formation of membrane blebs (Coleman *et al.* 2001) (Fig.2).

Conversely, other authors have shown that MP shedding induced by thrombin from ECs involves ROCK II activation via caspase 2 pathway, despite an absence of cell death (Sapet *et al.*2006), illustrating the complexity of pathways that lead to the formation of MPs.

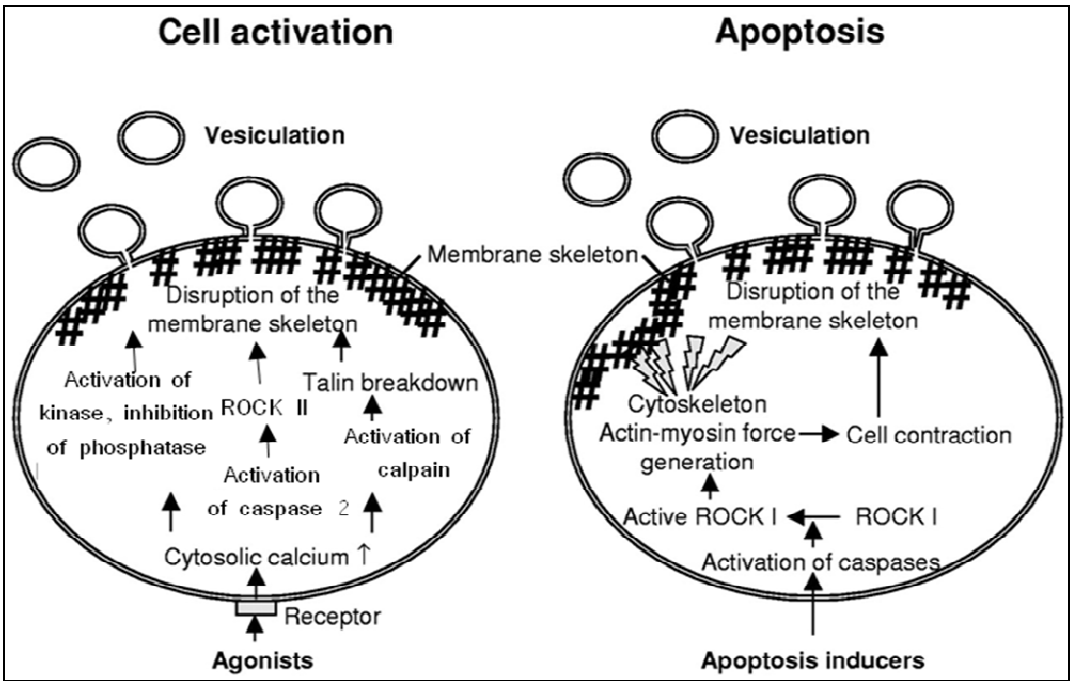


Fig. 2: Schematic representation of general mechanisms involved in MP formation during cell activation and apoptosis (Modified from VanWijk *et al*. 2003).

I.2. Composition of Microparticles

Microparticle bilayer consists mainly of phospholipids and proteins and results negatively charged because of presence of PS and PE. Their composition differs

between cell types and process triggering their formation.

As examples, the phospholipids composition of MPs from healthy humans consists mainly of PC (60%) (Weerheim *et al.* 2002); whereas MPs from synovial fluid of inflamed joints of arthritis patients contain PC, PE, SM, lysophospholipids (all 20-25%) and small amounts of PS (Fourcade *et al*. 1995). Also, MPs from ECs exposed to an oxidative stress present oxidized phospholipids, whereas there are absent in the same cells stimulated with calcium ionophore (Huber *et al.* 2002).

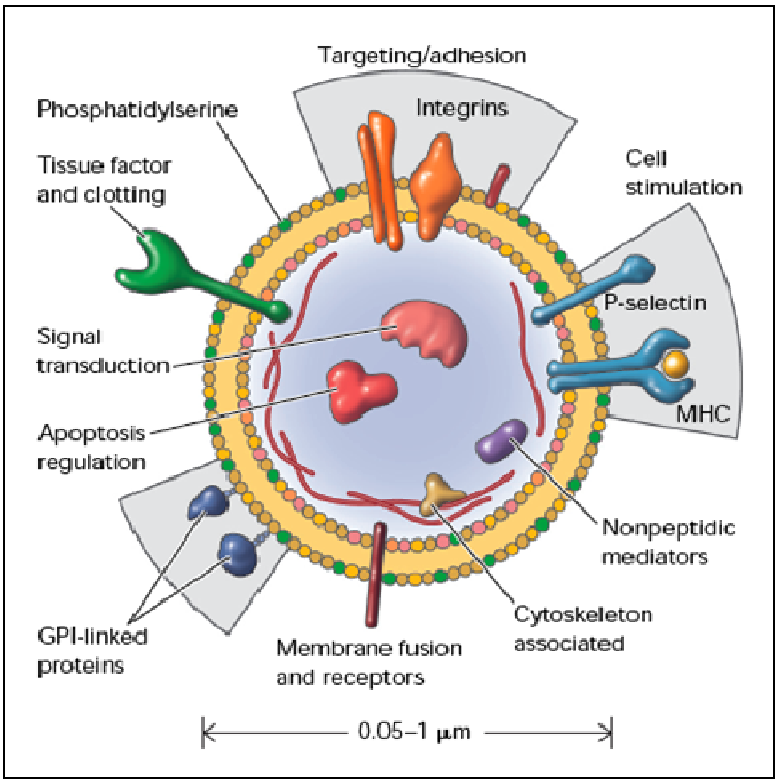


Fig. 3: Cellular MPs: a disseminated storage pool of bioactive effectors. MPs are shed from the plasma membrane of stimulated cells. They harbor membrane and carry cytoplasmic proteins as well as bioactive lipids implicated in a variety of fundamental processes. This representation does not intend to be exhaustive with respect to the different hijacked components. MHC, major histocompatibility complex; GPI, glycosylphosphatidylinositol (From Hugel *et al*. 2005).

In addition, on the surface, MPs bear antigens characteristic of the cell from which they are released and carry other membrane and cytoplasmic constituents (Fig. 3). Besides, these antigens can be used for determination of cell origin using antibodies directed against specific epitopes. PMPs expose glycoproteins (GP)Ib (CD42b), platelet-endothelium adhesion molecule-1 (PECAM 1; CD31) and the fibrinogen receptor, the integrin αIIbβ3 (GPIIb-IIIa). In addition, they can expose markers of activated cells such as P-selectin (CD62P) (Diamant *et al*. 2004).

Global composition of MP proteins can be related to stimulus at their origin. MPs generated from activated (by phytohemagglutinin, PHA and phorbol-myristate-acetate, PMA) and apoptotic (actinomycin D, ActD) CEM T lymphocytes (cell line) or lymphocytes from diabetic patients expose on their surface the morphogen Sonic Hedgehog (Shh) (protein implicate in embryonic and adult development). Whereas treatment of same cells with PHA alone, PMA alone and ActD alone generates MPs lacking in Shh, as well as diabetic patient MPs elicited under apoptotic conditions (Martinez *et al.* 2006). Moreover, the comparison of protein composition obtained from the CEM T-cell line MPs either in mitogenic (PHA) and apoptotic (ActD) conditions shows several differences. In total, 390 proteins were identified in MPs, among which 34% were described to be associated or localized in the plasma membrane. Only very few nuclear, mitochondrial, Golgi, or endoplasmic reticulum proteins were detected (less than 10% in total). Histone proteins (H2A, H2B, H3) were only identified in apoptotic conditions, as expected following cell death induction. Half of the detected proteins are intracellular proteins.

They can be grouped into cytoskeleton or cytoskeleton-associated proteins (actins, actinins, tubulins, myosins, ezrin, filamins, ARP2/3 proteins, destrin), heat shock proteins (HSP90 and 71), translation-associated proteins (ribosome proteins and elongation factors), and metabolism enzymes (*e.g.*, lactate dehydrogenases, peroxiredoxins, glyceraldehyde 3-phosphate dehydrogenase). The differentially expressed proteins were essentially cytoplasmic proteins: ribosomal proteins, which were dramatically increased under apoptotic stimulation, elongation factors, and nuclear histones. Only three plasma membrane CD antigens were differentially detected, (CD81, CD99, CD107) among which CD99 can be attributed to apoptosis triggering (Miguet *et al.* 2006).

II. Microparticles in health and disease

Despite being previously considered inert dust without specific function, MPs actively orchestrate important physiological and pathophysiological processes in vascular diseases (table 1). MPs have been implicated in hemostasis and thrombosis, diabetes, inflammation, atherosclerosis, angiogenesis, tumour progression, apoptosis, vascular cell proliferation and outgrowth of transplanted haematopoietic stem cells (Baj-Krzyworzeka *et al.* 2002; Janowska-Wieczorek 2001; Azevedo *et al.* 2007).

In vitro, the release of MPs has been shown from ECs, smooth muscle cells (SMC), platelet, leukocytes, lymphocytes and erythrocytes. Some of these MP populations occur in the blood of healthy individuals and patients. There are obvious alteration in number, cellular origin and composition of MP population in various disease states. However, the real impact of these changes on their *in vivo* effect is still

not fully understood.

MP ORIGIN	EFFECTS	PATHOLOGY
PLATELET	Endothelium activation Vascular hyperreactivity Hematopoietic cell proliferation Angiogenesis enhancement	Hypertension Myocardial infarction Diabetes Cancer
ENDOTHELIAL	Angiogenesis enhancement (low concentration) Angiogenesis inhibition (high concentration) Endothelial dysfunction	Acute coronary syndrome Type I diabetes mellitus Lupus anticoagulant
LEUKOCYTE	Vascular hyporeactivity Endothelial stimulation Endothelial dysfunction	AIDS Preeclampsia Type II diabetes mellitus Severe trauma, sepsis

Table 1: Depending on their origin, examples of MP-evoked effects and associated pathologies.

II.1. Microparticles in coagulation

Negatively charged phospholipids, mainly PS, exposed by MPs promote the aggregation of coagulation cascade system. PMPs exhibit these anionic phospholipids, which yield high-affinity binding sites for coagulation factors factor VIII, factor IXa and factor Va (Gilbert *et al.* 1991; Hoffman *et al.* 1992) and giving PMP procoagulant properties. PMPs harbour major membrane glycoproteins, including functional adhesive receptors and consequently disseminate procoagulant potential (Fox 1994). The procoagulant ability is also extended to monocytes, lymphocytes and endothelial MPs (EMPs), which present PS at their surface.

Moreover, monocytic, fibroblast and EMPs expose tissue factor (TF), which is the trigger of coagulation cascade that culminate in generation of fibrin clot (Morrissey 2001). Besides, on EMP surface other effectors are detected, for instance von Willebrand factor and E-selectin. In addition to their support of the fluid phase of coagulation, MPs also have a role in the recruitment of cells to developing thrombi. Furthermore, under certain conditions, MPs can also exhibit anticoagulant properties dependent on their origin and the stimulus to release. Accordingly, MPs may contribute to the complex regulation of balance between an anti- or prothrombotic vasculature (Lynch and Ludlam 2007).

II.2. Microparticles in inflammation

MPs play a role as actors in inflammatory process because they promote cells to produce potent proinflammatory mediators and they carry them. MPs can harbour interleukin-1β (IL-1β) (MacKenzie *et al.* 2001), a proinflammatory cytokine, or substrates of phospholipase A_2 for the generation of lysophosphatic acid, a potent proinflammatory mediator and platelet agonist (Fourcade *et al.* 1995). PMPs which deliver arachidonic acid to ECs may initiate inflammation, encouraging the up-regulation of intracellular adhesion molecule-1 (ICAM-1) and cyclooxygenase-2 (COX-2) which modulates the vascular and platelet functional interaction and activate a membrane-liked signalling (Barry *et al.* 1997; 1999).

Blood-derived MPs can stimulate release of cytokines from ECs and up-regulation of TF expression at their surface. PMPs also enhance expression of cell adhesion molecules in monocytic and ECs and induce production of IL-8, IL-1β and IL-

6 by ECs as well as IL-8, IL-1β and tumour necrosis factor-α (TNF-α) by monocytes (Nomura 2001).

II.3. Effects of Microparticles on cardiovascular system

Effects elicited by MPs are not restricted to inflammation and coagulation, but also involve changes on alteration in endothelial function and vascular contraction. In fact, they can affect EC (Martin *et al.* 2004) and SMC (Tesse *et al.* 2005) responses and vasoreactivity, as well as, angiogenesis (Tarabolletti *et al*. 2002; Kim *et al.* 2004). In addition, MPs may attenuate or exacerbate cardiovascular disorders. Moreover, endothelial responses trigger by MPs can be acute, by releasing several factors, or delayed, implying changes in expression of genes involved in structural and functional regulation of vascular wall (Martinez *et al.* 2005).

Under several pathological conditions the level of circulating EMPs increases and manifests EC damage and dysfunction, which may be also aggravated by MPs themselves. EMPs impair endothelium-dependent relaxation in rat aorta by altering nitric oxide (NO) production. This effect results in enhancing production of superoxide anion that reduces the bioavailability of NO (Brodsky *et al.* 2004).

Moreover EMPs, which bear protease activities, can elicit angiogenesis inducing the degradation of extracellular matrix (ECM), an essential step of neovascular structure formation (Tarabolletti *et al.* 2002).

PMPs stimulate platelets and ECs through modification of arachidonic acid metabolism and generation of thromboxane A_2 (TxA_2). They induce expression of the proinflammatory inducible isoform of COX-2 and the generation and release of

prostacyclin (Barry *et al.* 2007). Furthermore they enhance arachidonic-induced contraction in the aorta and methacholine-induced contraction in rabbit pulmonary arteries (Pfister 2004). PMPs from healthy individuals promote proliferation, migration and tube formation in cultured ECs and these effects are mediated by their lipid component, notably sphingosine 1-phosphates (S1P) (Kim et al. 2004). Another study shows that proangiogenic effect exerted by PMPs is mediated by vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) and also via activation of phosphatidylinositol 3-kinase (PI3K), Src kinase and extracellular-regulated kinase (ERK) pathways (Brill *et al*. 2005).

Lymphocytic MPs isolated from diabetic patients lead to endothelial dysfunction, especially by decreasing the endothelial NO-syntase (eNOS) expression and increasing caveolin-1 (Cav-1) expression (Martin *et al.* 2004). Little information is available regarding the effect of MPs on the regulation of vascular tone via direct action on SMCs.

Nevertheless, *in vitro* generated MPs from apoptotic lymphocytes are also able to act directly on SMCs through the activation of the transcription nuclear factor κB (NF-κB), leading to enhanced expression of inducible NOS (iNOS) and COX-2 with subsequent increased NO and prostacyclin production respectively, ending in vascular hyporeactivity (Tesse *et al.* 2005). These MPs are able to decrease NO production and increase oxidative stress in ECs. They active multiple pathway related to NO and reactive oxygen species (ROS) production, mainly though PI3K. Beside, PI3K controls the activation of ERK cascade, which counteracts the increase of xanthine oxidase-derived ROS production by the former. Furthermore, the NF-κB pathway regulates both NO and ROS production associated with the effects of MPs. All these effects lead to

endothelial dysfunction (Mostefai *et al.* 2008a). Additionally, MPs from apoptotic lymphocytes potently suppress neovascularization *in vivo* and *in vitro* by augmenting ROS generation via NADPH oxidase (NOX) and interfering with the VEGF signalling pathway (Yang *et al.* 2007). MPs generated *in vitro* from human activated/apoptotic T-lymphocytes, express Shh morphogen. This morphogen is involved in many biological processes during embryonic development, but it may be recruited postnatal mainly in response to tissue injury (Porro *et al.* 2009). MPs^{Shh+} are able to induce NO production from cultured ECs and organs taken from MPs^{Shh+}-treated mice; and this effect was mediated directly by Shh pathway. Shh cascade is complex, as provide by its cross-talk with other pathways. In fact, it can activate non canonical signals, like PI3K and Akt and, at the same time, it is subjected to regulation by other signal cascades, like protein kinase C (PKC)δ/MEK-1 pathway (Kessaris *et al*. 2004).

In ECs, MPs^{Shh+} exert the concomitant effect of enhancement of NO and decrease of ROS production, which might result in an increasing of the bioavailability of generated NO by reducing oxidative stress and the subsequent scavenging of NO. The increase in NO release is associated with an enhancement of eNOS expression and activity, as reflected by the increase in eNOS phosphorylation, and with changes in the expression and phosphorylation of Cav-1. All of these effects of MPs^{Shh+} on pathways involved in NO production, except on Cav-1 expression, are dependent on the PI3K pathway. By contrast, the reduction in ROS induced by MPs is dependent on PI3K and ERK pathways (Agouni *et al.* 2007). Moreover, in a model of mice coronary arteries subjected to ischemia/reperfusion MPs bearing Shh restore endothelial dysfunction, probably through their dual ability to increase NO and reduce ROS (Agouni *et al.* 2007). Accordingly, modulation exerted by MPs^{Shh+} on different pathways leads to

beneficial potential effect on the cardiovascular system. In addition, MPs^{Shh+} are involved in differentiation process which concern either developmental and regenerative phenomena of vascular cells, in fact they addressed human K562 pluripotent erythroleukemic cells toward megakaryocytic lineage and facilitate the progression from S to G_2/M phase of cell cycle (Martinez *et al.* 2006).

Few studies have shown the role of MPs shed from SMCs. Probably these MPs fuse with injured arterial wall and atherosclerotic plaques and release TF harboured at their surface (Schecter *et al.* 2000). Moreover the ability of apoptotic smooth muscle MPs to enhance thrombus formation correlates to the functional TF carried (Brisset *et al.* 2003).

The majority of *in vivo* circulating MPs derive from platelet compared with MPs from other circulating or vascular cells (Martinez *et al.* 2005). Under several pathological conditions, the number of total MPs, as well as the proportion of their different origins, can change (VanWijk *et al.* 2003). Thus, in diseases such as atherosclerosis, congestive heart failure, diabetes, preeclampsia and cancer, the level of circulating MPs is considerably enhanced compared with healthy patients.

Moreover, the phenotype of circulating MPs is altered in different pathological states. Indeed, PMPs are enhanced in myocardial infarction (Mallat *et al.* 2000), hypertension (Preston *et al.* 2003), diabetes (Nomura *et al.* 1995), metabolic syndrome (Agouni *et al.* 2008), sepsis (Mostefai *et al.* 2008c) and cancer (Kim *et al.* 2003), whereas EMPs are most abundant in acute coronary syndromes (Mallat *et al.* 2000), metabolic syndrome (Agouni *et al.* 2008), sepsis (Mostefai *et al.* 2008c) and type I diabetes mellitus (Sabatier *et al.* 2002). In diabetic patients, the number of MPs of leukocyte origin is threefold higher than in healthy donors (Sabatier *et al.* 2002). In

addition, human immunodeficient virus (HIV)-infected patients show elevated levels of MPs bearing CD4 antigen (Aupeix *et al*. 1997). Elevated levels of MPs from granulocytes and lymphocytes have been reported in preeclampsia (VanWijk *et al*. 2002). Also, in severe trauma, circulating levels of MPs generated from activated leukocytes and harbouring adhesion marker are enhanced (Fujimi *et al*. 2003).

Because of the variety of MPs, it is plausible that they may exert pleiotropic effects on the vascular wall. Moreover, depending on the MP composition, one can speculate that different subpopulations of MPs may serve as vectors of exchange of specific message in regulating vascular function and dysfunction (Martinez *et al*. 2005).

Below are briefly described such pathologies in which MPs may exert deleterious or beneficial effects in regarding endothelial and/or vasomotor function.

II.4. Microparticles and preeclampsia

Preeclampsia is a pregnancy disorder due to association of hypertension and proteinuria. Total number of circulating MPs from healthy and preeclamptic pregnant women is not significantly altered despite an increase in number of T cells and granulocytes in blood. Moreover, a close relationship between endothelial dysfunction and circulating level of EMPs is reported in preeclamptic patients (Redman *et al.* 2007). Women affected by preeclampsia display elevated levels of MPs from lympho-monocytes and platelets in their blood stream compared with normal pregnancy. Furthermore, MPs from preeclamptic women are able to induce reduced vascular responsiveness to a vasoconstrictor agent, because of up-regulation of proinflammatory protein expression, iNOS and COX-2. Among MPs from preeclamptic women, the

platelet subset is able to stimulate only the release of NO, whereas the non-platelet ones induced both the release of NO and COX-2 vasoconstrictor products.

Thus, MPs in preeclampsia could act as vectors to stimulate intracellular cascades in vascular cells, leading to an enhanced NO production to counteract increased COX-2 vasoconstrictor metabolites by taking into account pregnancy (Meziani *et al*. 2006; Tesse *et al*. 2007).

II.5. Microparticles and sepsis

Sepsis is an acute and systemic immune response mainly to bacterial infection. During sepsis, it has been reported the generation of procoagulant MPs from endothelial, platelet, erythroid and leukocyte origin (Itakura Sumi *et al.* 2003; Nieuwland *et al.* 2000). MPs active inflammatory process, cellular apoptosis and promote multiorgan failure (Kobayashi *et al.* 2001). Also, MPs contribute to increase of TF expression and activity, which support the spread of coagulopathy, microcirculatory thrombosis, and tissue hypoxia and then generation of lactates. Furthermore MPs generated during sepsis participate in modulation of oxidative status in several tissues, because of greater production of ROS originated by NOX subunits present on PMPs (Ogura *et al.* 2001; 2004). However, recent data show that MPs from septic patients exert protective effects. Firstly, it has been described that, in patients with severe sepsis, high levels of platelet, leukocyte and endothelial MPs are associated with higher survival rate. Moreover, a negative correlation between MPs and organ dysfunction exists, suggesting decreased cell activation in patients with higher morbidity and mortality (Soriano *et al.* 2005).

Secondly, Mostefai *et al.* (2008c) have shown that MPs from septic patients are able to counteract sepsis-associated hyporeactivity through a mechanism sensitive to the TxA_2 receptor antagonist, SQ-29548. Then, although septic MPs may be link between inflammation and thrombosis observed in sepsis, they may rather protective against vascular hyporeactivity in order to maintain a tonic pressor response in septic shock patients. These data bring mechanistic basis of the correlation between increased circulating MPs and better survival rate in early phase of septic shock patients.

II.6. Microparticles and diabetes

The development of vasculopathies in diabetes involves multifactorial process including pathological activation of vascular cells, which may explain the elevated levels of circulating MPs found in blood of patients affected by both type I and type II diabetes.

The cellular origin and the procoagulant activity of circulating MPs differ according to the type of diabetes (Sabatier *et al.* 2002). PMPs, as well as monocyte MPs, are elevated in patients with type I diabetes mellitus. Especially, monocyte MPs were highly elevated in patients with diabetic nephropathy and could be an indicator of vascular complications in diabetes (Nomura *et al.* 1995; Omoto *et al.* 2002). By contrast, type II diabetic mellitus patients present significantly higher amount of PMPs and EMPs. Indeed, in type II diabetic patients only the total PS-positive blood cell MPs is increased, whereas in type I diabetic patients the total MPs, EMPs and PMPs and the procoagulant activity are also elevated.

Moreover, the large increase of circulating EMPs in type I diabetes suggests that

EMPs could be markers of the endothelial damage associated with microvascular complications and nephropathy in these patients (Sabatier *et al*. 2002).

II.7. Microparticles and hypertension

High blood pressure is often associated with high concentrations of circulating MPs, in particular monocytic MPs and PMPs. In such patients, treatment with an inhibitor of calcium voltage-dependent channels, significantly reduces the levels of circulating MPs. This reduction is important in the prevention of cardiovascular complications caused by adhesion molecules, activated platelets and monocytes, and high levels of circulating PMPs and monocytic MPs (Nomura *et al.* 2002). By contrast, Preston *et al*., (2003) have shown that in severe uncontrolled hypertension an increased pressure-dependent release of EMPs and PMPs takes place.

II.8. Microparticles and cancer

Tumour cells are able to generated MPs both *in vitro* and *in vivo*. Through proteins, such as urokinase, CD147 or SM, harbored by MPs from tumour cells, MPs can modify the adhesive and invasive properties of tumour target cells (Angelucci *et al.* 2000), or the angiogenic activity of ECs (Millimaggi *et al.* 2007). Moreover, it has been shown that PMPs enhance the *in vitro* invasive potential of breast cancer cell lines, and induce metastasis and angiogenesis in lung cancer.

Indeed, injection of PMPs resulted in metastatic foci in lung mice (Janowska-Wieczorek *et al*. 2005). These data suggest that MPs transfer a transcellular signal that

could allow tumour progression. Accordingly, it has been show that epidermal growth factor receptor (EGFR) carried by MPs from glioma cells can merge with the plasma membrane of cancer cells lacking this receptor. As consequence, transfer of oncogenic activity occurs, including activation of EGFR downstream signalling pathways, such as mitogen-activated kinase (MAPK) and Akt cascades, increased production of VEGF, and expression of the anti-apoptotic protein Bcl-x_L, decreased level of p27/kip1 cyclin-dependent kinase inhibitor, morphological transformation and increase in anchorage-independent growth capacity. These events lead to propagation of an enhanced proliferative, survival, motogenic and angiogenic capacity (Al-Nedawi *et al*. 2008).

Circulating MPs have also been studied in patients with cancer so their diagnostic and prognostic utility has been suggested. In patients with stage IV gastric cancer, PMPs were significantly elevated when compared with patients with stage I or II/III. PMPs count had also more than 90% sensitivity and specificity in the prediction of distant metastasis in these patients (Kim *et al*. 2003). Another study showed that PMPs and monocyte MPs are elevated in patients with lung cancer as compared with control subjects. Also, these MPs are significantly higher in patients with non-small cell lung cancer as compared with patients with small cell lung cancer (Kanazawa *et al*. 2003).

In addition, levels of P-selectin associated to PMPs and TF generated from cancer cells are increased indicating that proteins involved in hemostasis are elevated in patients with cancer (Yu and Rak 2004) and may represent a tool for exacerbated thrombosis.

II.9. Microparticles and AIDS

MPs seem to play several roles in AIDS, including implication in HIV infection, as well as in the propagation of the virus and its escape from classical vaccine strategies.

Few years ago, a mechanism allowing HIV to infect cells lacking the chemokine receptor CCR5 was suggested. CCR5 is released through MPs from the surface of CCR5-positive cells and is transferred to deficient peripheral blood mononuclear cells, rendering them CCR5 positive and susceptible to HIV-1 infection (Mack *et al*. 2000). A recent study confirms the existence of such a process by showing that platelet- and megakaryocyte-derived MPs can also transfer CXCR4 receptor to CXCR4-null cells (Rozmyslowicz *et al.* 2003).

In addition, one cannot exclude a role for CD4+-derived MPs observed in augmented proportions in some HIV-positive patients, especially in a situation in which individuals with high levels of circulating MPs and low circulating CD4+ cell counts seem to be protected from the classical complications of AIDS (Aupeix *et al*. 1997).

PART II

ANGIOGENESIS

I. General aspects

In multicellular organisms, all cells require a dependable, finely controlled supply of oxygen and nutrients. During the early stages of embryonic development, in absence of vascularization, this need takes place by diffusion, but rapidly has evolved an elaborate network of capillary plexuses and blood vessels. The initial events in vascular growth in which endothelial progenitor cells (EPCs), also called angioblasts, migrate to discrete locations, differentiate *in situ* and assemble in solid endothelial cords, forming later a plexus with endocardial tube, is referred to as vasculogenesis (Noden 1989). The subsequent growth, expansion and remodelling of primitive vessels into mature vessels, in embryonic life, as well as the formation of new blood vessels by sprouting of capillaries from existing vasculature, in pre- and postnatal life is referred to as angiogenesis. This process is based on endothelial sprouting or intussusceptive (non-sprouting) microvascular growth (Ausprunk and Folkman 1977; Risau 1997). The latter represents an additional and/or alternative mechanism and is not dependent on local EC proliferation or sprouting: a large sinusoidal capillary divides into smaller capillaries, which then grow separately (Djonov *et al*. 2000). The functional modifications of largest arteries, such addiction of a thick muscular coat concomitant with acquisition of viscoelastic and vasomotor properties, are referred to as arteriogenesis.

New vessels in the adult arise mainly through angiogenesis, although vasculogenesis also may occur. Even if, as a general rule, establishment of the vasculature of most organs occurs by angiogenesis, development of the vascular network of certain endodermal organs, including the liver, lungs, pancreas, stomach/

intestine and spleen, occurs by vasculogenesis (Pardanaud and Dieterlen-Lievre 1999). The existence of a postnatal vasculogenesis is supported by the evidence that both ECs and EPCs co-exist in the circulation. Moreover, EPCs are also recruited to sites of neovascularization in mature mammals from a circulating, marrow-derived population of progenitor cells (Asahara *et al*. 1997). The distinction between vasculogenesis and angiogenesis is not absolute and they overlap. Both require EC proliferation, migration, three-dimensional reorganization of newly formed aggregates and use similar extracellular matrix adhesive mechanisms (Drake *et al*. 1995). Moreover, vasculogenesis and angiogenesis are not mutually exclusive, inasmuch as angioblasts can be incorporated into expanding pre-existing blood vessels (Auerbach and Auerbach 1997).

Sprouting angiogenesis develops through a highly orchestrated multi-step process. The initial vasodilatation of existing vessels is accompanied with increasing of endothelial permeability and basement membrane degradation by the action of proteolytic enzymes, such as matrix metalloproteinases (MMPs) and plasminogen activators secreted by ECs, resulting in the formation of tiny sprouts penetrating the perivascular stroma. This allows migration of the ECs at the sprout tip toward the angiogenic stimulus and proliferation of the ECs below the sprout. The subsequent canalization, branching, and formation of vascular loops, lead to the development of a functioning circulatory network. Finally, perivascular apposition of pericytes and SMCs to support the abluminal side and *de novo* synthesis by ECs and pericytes of the basement membrane constituents lead to vessels stabilization.

Angiogenesis is subject to a complex control system with proangiogenic and antiangiogenic factors. In adults, angiogenesis is tightly controlled by this "angiogenic

balance", a physiological balance between the stimulatory and inhibitory signals for blood vessel growth; this switch depends on a local change in the balance.

In normal circumstances, the formation of new blood vessels occurs during wound healing, organ regeneration and in the female reproductive system during ovulation, menstruation, and the formation of the placenta (Hoeben *et al*. 2004). Also, a large number of different and non-related diseases are associated with impairment or excess of new vasculature formation. Among the pathologies in which angiogenesis is impaired, tissue damage after reperfusion of ischemic tissue or cardiac failure or diabetes needs formation of new collateral vessels to improve disease conditions (Carmeliet *et al*. 1999; Ferrara and Alitalo 1999). In several diseases, excessive angiogenesis is part of the pathology. These diseases include cancer (both solid and hematologic tumours), cardiovascular diseases (atherosclerosis), chronic inflammation (rheumatoid arthritis, Crohn's disease), diabetic retinopathy, psoriasis and endometriosis (Griffioen and Molema 2000).

Angiogenic growth factors, under both physiological and pathological conditions, induce, promote and/or interfere with all steps of angiogenesis (Losordo and Dimmeler 2004; Ng and D'amore 2001; Post *et al*. 2001). A variety of growth factors plays significant role in cell proliferation, maturation and differentiation leading to the formation of mature blood vessels. These factors act as signalling molecules between cells, and bind to specific receptors on the surface of their target cells. Also, hypoxia is the most important environmental factor that leads to neovascularization.

I.1. Angiogenic mediators

Angiogenesis is driven by numerous mediators produced by different cells under a large variety of conditions. These mediators are either soluble, ECM or membrane bound growth factors, or components of ECM themselves. The best-known factors with proven angiogenic potency are the family of VEGF and FGF (Tunyogi-Csapo *et al.* 2007; Jacobs 2007), hepatocyte growth factor (HGF) (Tong *et al*. 2006), NO (Lau and Ma 1996), and angiopoietins (Ang1 and 2) (Asahara *et al*. 1998).

I.1.1. Vascular endothelial growth factor

The VEGF family comprises seven members. The most abundant and potently mitogenic is the VEGF-A. The two VEGF-specific tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR-Flk-1), are expressed on vascular endothelium. Activation of VEGFR-2 by VEGF interaction is a critical requirement to induce the full spectrum of VEGF responses. VEGF transcription is stimulated greatly by hypoxia, as a result of hypoxia inducible factor (HIF-α) binding to a hypoxia response element within the VEGF promoter (Semenza 2001). VEGF production is also increased by inflammatory mediators, such as IL-1α and IL-1β, transforming growth factor β (TGFβ), prostaglandin E2 (PGE2), or COX-2 activation (McColl *et al.* 2004), as well as mechanical forces of shear stress and cell stretch (Milkiewicz *et al*. 2001; Li *et al* 1997). VEGF promotes EC survival through activation of PI3K/Akt pathway and through association with $\alpha_v\beta_3$ integrin and activation of focal adhesion kinase (FAK) (Zachary 2003). VEGF induces EC proliferation and migration through numerous pathways, including activation of the MAPK, ERK, p38 and c-jun N-terminal kinase (JNK), and RhoGTPase family members (Zachary 2003).

I.1.2. Fibroblast growth factor

Members of the FGF family are also potent inducers of angiogenesis. Cellular responses mediated by FGFs include cell migration, proliferation and differentiation (Kanda *et al*. 1997). The FGF family consists of nine structurally related polypeptides, of which FGF-1 (acid-FGF) and FGF-2 (basic-FGF) are most extensively studied. The cellular effects of FGFs are mediated via specific binding to high-affinity tyrosine kinase receptors (FGFRs) (Klein 1996). Binding of FGF-2 to these receptors initiates PKC dependent signalling and/or GRB2/SOS mediated activation of MAPK pathway. FGFR1 signalling stimulates migration and differentiation whereas FGFR2 only migration (Kanda *et al*. 2004). A major contribution of FGF signalling may be through the recruitment of other growth factor pathways.

I.1.3. Hepatocyte growth factor

HGF is secreted by mesenchyme-derived cells as an inactive precursor that is activated by proteolytic cleavage by urokinase or tissue plasminogen activator (uPA or tPA) (Zhang *et al.* 2003). HGF binds to a tyrosine kinase receptor, c-met, found predominantly on epithelial and ECs. Through activation of c-met, HGF is a potent mediator of angiogenesis, in part because it induces production of VEGF by the endothelium (Reisinger *et al*. 2003; Zhang *et al*. 2003). In addition, HGF can induce angiogenesis independently of the VEGF pathway (Sengupta *et al*. 2003). HGF-α dependent activation of the transcription factor Ets-1 causes further production and release of HGF, creating a positive feedback loop that perpetuates the state of EC activation (Hashiya *et al.* 2004). HGF also activates sphingosine kinase, increasing production of S1P, a molecule known to increase cell motility (Duan *et al.* 2004).

Another effect of HGF signalling is the negative regulation of thrombospondin 1 (TSP1) in tumour cells (Zhang *et al.* 2003). TSP1 is an extracellular matrix protein associated with anti-angiogenesis signalling, including activation of EC apoptosis.

I.1.4. Nitric oxide

NO is an important mediator of angiogenesis, in addition to its well-recognized vasodilatory properties. NO triggers capillary EC growth and differentiation via cyclic GMP-dependent gene transcription. The regulation of capillary growth by NO is complex because both angiogenic and angiostatic effects of NO have been demonstrated (Lau and Ma 1996; Murohara *et al.* 1998; RayChaudhury *et al.* 1996; Ziche *et al*. 1994).

Low concentrations of NO stimulate capillary-like tube formation and cell migration through activation of PKC and ERK and c-Jun phosphorylation, whereas high NO concentrations inhibit these angiogenic responses (Jones *et al*. 2004). NO is a component of the pathways underlying VEGF-activated EC proliferation. However, whether NO acts as an upstream or a downstream mediator of VEGF is controversial. VEGF induces the expression of eNOS and promotes the release of NO via activation of the MAPK cascade (Papapetropoulos *et al.* 1997; Parenti *et al*. 1998). NO has been shown to both increase and decrease VEGF production via modulation of HIF-1α expression. Some authors report that NO mediates suppression of hypoxia-induced production of VEGF by decreasing HIF-1α DNA binding activity (Huang *et al*. 1999; Sogawa *et al*. 1998), whereas others describe enhanced HIF-1α binding activity in response to NO (Dulak *et al*. 2000; Kimura *et al*. 2000). Interestingly, while VEGF-induced angiogenesis is mediated by NO, the capillary growth stimulated by FGF-2 can be both NO-independent and inhibited by NO (RayChaudhury *et al*. 1996; Ziche *et al*. 1997).

I.1.5. Angiopoietins

The two isoforms of angiopoietins (Ang), Ang1 and 2, play a role in vascular stabilization. The former is associated with developing vessels and its absence leads to defects in vascular remodeling (Thurston 2003); the latter antagonizes Ang1 action, causing destabilization of preexisting vessels. Ang2 is found in tissues such as ovary, uterus, and placenta that undergo transient or periodic growth and vascularization, followed by regression (Maisonpierre *et al*. 1997).

Ang and receptor tyrosine kinase Tie1 and Tie2 play critical role in the later stages of angiogenesis as well. Ang1 and Ang2 are Tie2-specific ligands that activate or antagonize Tie2 signalling in endothelium, respectively (Asahara *et al*. 1998). They are required for communication of ECs with the surrounding mesenchyme to establish stable cellular and biochemical interaction (Maisonpiere *et al*. 1997). Tie1 function is related to EC differentiation and the establishment of blood vessel integrity. Tie2, on the other hand, is particularly important for vascular network formation (Dumont *et al*. 1994; Puri *et al*. 1995; Sato *et al*. 1995).

I.1.6. Other angiogenic mediators

Other angiogenic factors involve in the switch are TGF-β1 and PDGF. When mesenchymal cells are treated with TGF-β1, they express SMC markers, indicating differentiation toward a SMC lineage, and the differentiation can be blocked by antibodies against TGF-β1 (Hirschi *et al*. 1998). TGF-β1 has been also reported to direct neural crest cells toward a vascular SMC lineage (Shah *et al*. 1996). PDGF-B is secreted by ECs, presumably in response to VEGF and facilitates recruitment of mural cells. PDGF-B gene mutation may cause failure of pericyte recruitment (Lindhal *et al*.

1997).

It is important to note that the activity of an angiogenesis-regulating cytokine depends on the presence and concentration of other factors or cytokines in the environment of the responding endothelium (Pepper *et al.* 1998). As example, exogenous factors such as hormones can affect condition leading to angiogenesis (Schiffenbauer *et al.* 1997). Moreover it is well appreciated that immune system cells such as monocytes/macrophage, lymphocytes and mast cells can affect pro- and antiangiogenic balance (Sunderkotter *et al.* 1996; Blair *et al.* 1997).

In recent years, evidence has accumulated that, in addition to the classic factors, many other endogenous peptides (erythropoietin, angiotensin II, endothelins, proadrenomedullin-derived peptides, urotensin II, adipokines, neuropeptide-Y, vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide and substance P) play an important regulatory role in angiogenesis, especially under pathological conditions (Ribatti *et al.* 2007).

Moreover, recent data show that MPs are implicated in modulation of neovascularization. PMPs promote proliferation and survival of ECs as well as tube formation (Kim *et al.* 2004); they induce angiogenesis and improve revascularization after chronic ischemia *in vivo* (Brill *et al.* 2005). Furthermore, MPs derived from tumour cells also promote tumour angiogenesis (Kim *et al.* 2002), and MPs from endothelial origin may contribute to angiogenesis because of the MMPs activities they carry (Taraboletti *et al.* 2002). Major details on MPs involvement in angiogenic process are discussed below.

I.2. Angiogenesis process

I.2.1. Vasodilatation, increased endothelial permeability and extracellular matrix (ECM) degradation

Vasodilatation of existing vessels is one of the earliest steps in angiogenesis. VEGF is a major player in neovessels initiation; based on its ability to induce vasodilatation via endothelial NO production and its EC permeability increasing effect (Ziche *et al*. 1997). The observation that VEGF production is under control of HIF-α strengthens the suggestion of an early involvement of VEGF in the angiogenic response. Moreover, VEGFR expression is up-regulated under hypoxic or ischemic conditions as well (Forsythe *et al.* 1996). This allows redistribution of intracellular adhesion molecules, including PECAM-1 and vascular endothelial (VE)-cadherin, and alteration in cell membrane structure via induction of a series of kinases (Eliceiri *et al.* 1999a; Gale and Yancopoulos 1999). Then, the extravasion of plasma proteins follows, which creates a provisional network support (Dvorak 1986) that leads subsequently to the migration of activated ECs. Consequently permeability changes must be tightly regulated. For instance, Ang1 is a natural anti-permeability factor, which provides protection and balance against excessive plasma leakage (Thurston *et al*. 2000).

Endothelial sprouting is further enhanced by Ang2, which, appearing at angiogenic and vascular remodelling sites, is involved in detaching SMC and loosening underlying matrix, thereby allowing ECs to migrate as inter-EC contacts are relieved (Gale and Yancopoulos 1999; Maissonpierre *et al*. 1997).

Degradation of ECM involves an array of proteinases which not only provides way for the migrating cells, but also results in the liberation of growth factors, including bFGF, VEGF and insulin-like growth factor-1 (IGF-1), which otherwise remain sequestered within the matrix. Over twenty MMPs have been described and implicated in angiogenesis, tumourogenesis and cell proliferation (Nelson *et al.* 2000). Inhibitors of MMPs include circulating protease inhibitors, such as tissue-localized inhibitors of metalloproteinases (TIMPs) (Brew *et al.* 2000). It is, at least partly, through the secretion of MMP-2, MMP-3 and MMP-9, and suppression of TIMP-2 that Ang1 induces sprouting (Kim *et al.* 2000). Similarly, MMP-3, MMP-7 and MMP-9 have been shown to induce angiogenesis in neonatal bones and tumours (Vu *et al.* 1999). However, MMPs do not uniformly enhance angiogenesis; temporal and spatial factors likely dictate their function. An interaction with other proteins also alters their roles in angiogenesis. TSP1 is believed to be antiangiogenic by preventing activation of MMP-2 and MMP-9 (Bein and Simons 2000). Other proteinases, such as plasmin, have also implicated in matrix degradation enabling endothelial migration (Pepper 2001) (Fig. 4).

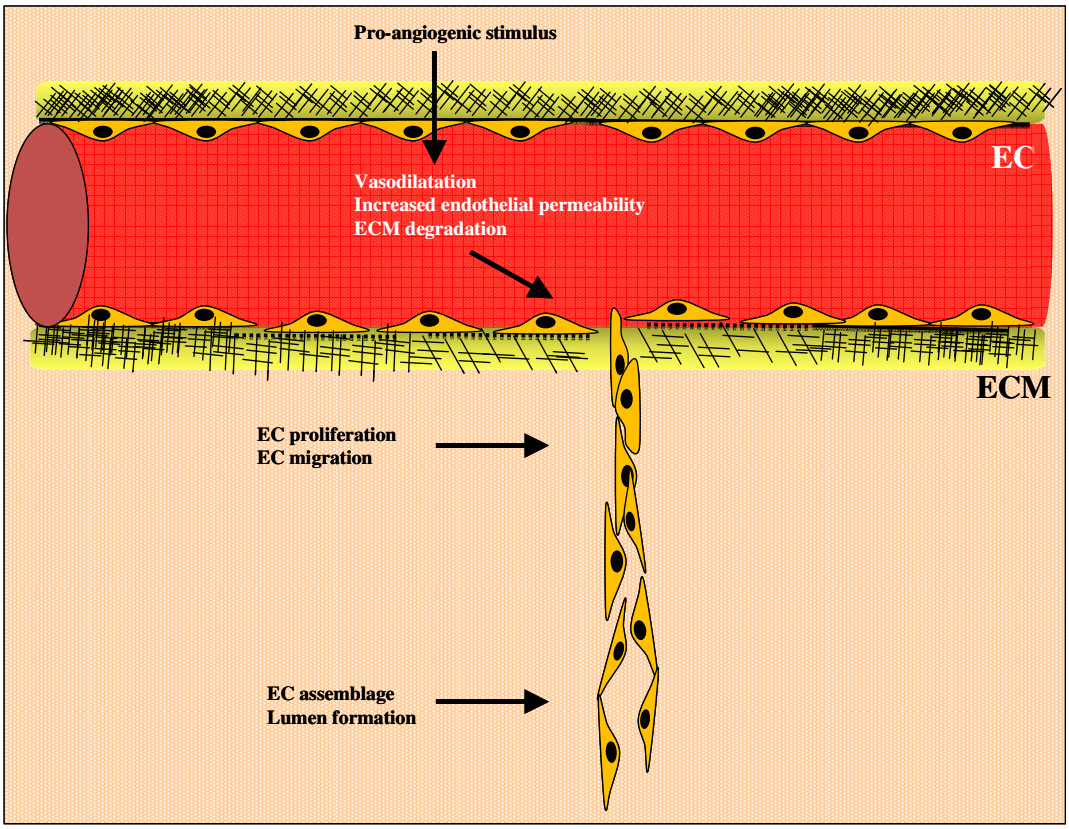


Fig. 4: Early events implicated in new vessel formation. Angiogenic stimuli (hypoxia, growth factor, inflammation or mechanical factors) cause vasodilatation, and increased endothelial cell (EC) permeability. Degradation of extracellular matrix (ECM), controlled mainly by MMPs, promote EC invasion into surrounding interstitial matrix concomitantly with remodelling of cytoskeleton, which provides directional migration of EC sprouting. EC proliferation occurs early in angiogenesis and continues as new capillary sprout elongates. Once migrate, ECs assemble to form a multi-cellular structure, which forms a lumen. New vessel will be formed when the sprout anastamoses with a pre-existing capillary.

I.2.2. Endothelial cells proliferation and migration

When the physical barriers are dissolved, proliferating ECs are free to migrate to distant sites. This stage involves interplay between the various forms of VEGF, Ang, bFGF and their receptors, all of which are responsible in mediating angiogenesis, although additional factors have also been implicated. Besides its effect on angiogenesis initiation, VEGF also affects EC proliferation. This effect can be partly attributed to the ability of VEGF to release NO and activate MAPK family through cGMP (Yu and Sato 1999). Ang1, via phosphorylation of Tie2, is chemotactic for ECs, induces sprouting and stimulates the interaction between endothelial and peri-endothelial cells (Gale *et al.* 2002; Suri *et al.* 1996). Ang2, in concert with VEGF is also angiogenic, although in absence of VEGF may induce vessel regression (Maisonpiere *et al.* 1997). FGF stimulate EC growth and recruit mesenchymal and/or inflammatory cells, producing many angiogenic factors (Carmeliet 2000). PDGF is angiogenic for microvascular sprouting ECs and recruits pericytes and SMCs around nascent vessel sprout (Lindhal *et al.* 1998; Hellström *et al.* 1999). Several chemokines, including monocyte chemotactic protein 1 (MCP-1), have been demonstrated to induce endothelial growth (Belperio *et al.* 2000). When the ECs proliferate and migrate, in part by signalling through integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (Eliceiri and Cheresh 1999b), PECAM-1 (Ilan *et al.* 1999) and Eph/ephrin receptor-ligand pairs (Huynh-Do *et al.* 1999; Shima and Mailhos 2000; Wilkinson 2000), they contact with other ECs (Fig. 4).

EC junctions are established with gap proteins such as VE-cadherin and members of the connexin family (Corada *et al*. 1999; Knudsen *et al*. 1998).

I.2.3. Endothelial cells assemblage, lumen formation and stabilization

Once ECs migrate into the ECM, they assemble in solid cords. Sprouting of one or two cells may form a lumen by intracellular canalization, which occurs through fusion of cytoplasmic vesicles, or by the alternative process in which a lumen is created by the membrane apposition of two different cells (Eggiton Gerritsen 2003). Lumen diameter is tightly regulated by interactions between various VEGF isoforms, Ang, and their receptors as well as different integrins ($\alpha_v\beta_3$ and $\alpha_5\beta_1$) (Suri *et al*. 1998; Bayless 2000). Finally, there are also several endogenous inhibitors of lumen formation, including TSP1 (Gendron *et al*. 2000).

EC interaction with ECM and mesenchymal cells is a prerequisite to form a stable vasculature. Therefore, after EC proliferation and maturation, and the formation of endothelial tube structures, a surrounding vessel layer composed of mural cells (pericytes in small vessel and SMCs in large vessels) is required. ECs may accomplish this via the synthesis and secretion of PDGF, a mitogen chemoattractant for a variety of mesenchymal cells. Subsequent differentiation of mural precursor cells into pericytes and SMC is believed to be a cell-cell contact dependent process (Griffioen Molena 2000). On endothelial cell-mural cell contact, a latent form of TGFβ, producing by both endothelial and mural cells, is activated in a plasmin-mediated process.

Activated TGFβ can induce changes in myofibroblasts and pericytes, which may contribute to the formation of a quiescent vessel, ECM production and maintenance of growth control.

II. Angiogenesis and Microparticles

It has been shown that different stages of angiogenesis might be mediated by MPs. The effects elicited by MPs depend on the mechanics of stimulation and the activation status of the cell from which they originate (Fig. 5) and also on their concentration.

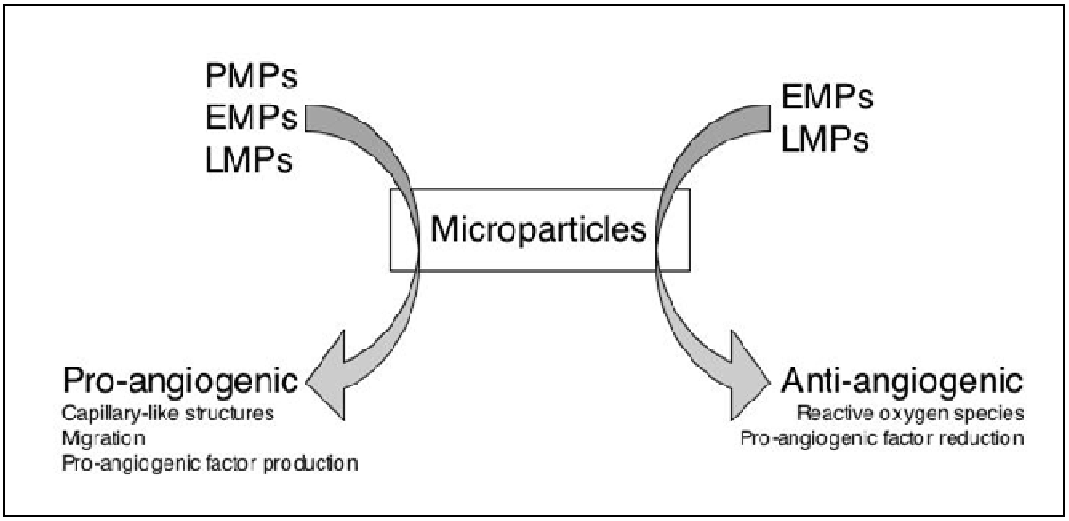


Fig. 5: Differential effects of MPs on angiogenesis depending on their origin. MPs from platelets (PMPs) display pro-angiogenic properties by promoting capillary-like structures and pro-angiogenic factor production. By contrast, both endothelial- and lymphocyte-derived MPs (EMPs, LMPs) possess pro- or antiangiogenic properties depending on the stimuli used for their production (Mostefai *et al.* 2008a)

ECs shed protease-containing vesicles that might play a role in endothelial proteolitic activity during angiogenesis. EMPs contain MMP-2 and MMP-9 in both active and proenzyme forms. TIMP-1 and TIMP-2, endogenous inhibitors of MMPs, are present in the same MPs and could exert a regulatory effect on the activity of the protease. Low concentrations stimulated cord formation, higher ones being inhibitory. Small amounts of MMPs are essential for the onset of morphogenetic program, whereas an excess of proteolytic activity prevents tubulogenesis and cause the re-absorptions of the formed vasculature. In this respect, the need for a delicate balance between proteases and their inhibitors, in each phase of the angiogenic process, could provide an additional functional explanation for the presence of TIMPs in vesicles shed by ECs (Taraboletti *et al*. 2002). By contrast, another study shown that EMPs are able to impairs angiogenesis *in vitro* by affecting all parameters of the capillary network formation by uniformly decreasing total capillary length and numbers of meshes and branching points and increasing mesh area. One of the possible mechanisms of this phenomenon may be the increase in oxidative stress in cells treated with EMPs. This hypothesis is supported by the finding that treatment with the cell-permeable superoxide dismutase (SOD) mimetic restores all parameters of angiogenesis affected by EMPs (Mezentsev *et al.* 2005). The different concentration of MPs used in these two studies may explain the differences observed. Physiological concentration of EMPs, such as those found in the circulation of healthy subjects, do not affect any of the parameters of angiogenesis *in vivo*. At low concentration, MPs do not affect the endothelium (Brodsky *et al*. 2004; Taraboletti *et al.* 2002).

However, when the number of circulating EMPs exceeds a certain threshold, the EMPs became an important factor in pathophysiology of disease, directly affecting the

endothelium and other circulating cells (Horstman *et al*. 2004; Jy *et al*. 2004). Moreover, EMPs provide a catalytic surface for the conversion of plasminogen into plasmin by expressing uPA and its receptor (uPAR). They are able to modulate angiogenic responses of EPCs *in vitro*. EMPs affect EPCs angiogenesis in Matrigel® in a concentration-dependent manner. While low amounts of EMPs increase tube formation, higher concentrations inhibit it (Lacroix *et al*. 2007).

Furthermore, MPs derived from EPCs are able to trigger an angiogenic program. In fact, they are incorporated in ECs by interaction with integrins expressed on MP surface. *In vitro*, these MPs promoted EC survival, proliferation and organization in capillary-like structures. *In vivo*, MP-stimulated human ECs organize in patent vessels. When incubated with RNase MPs failed to induce *in vitro* and *in vivo* effects, suggesting that they are able to transfer angiogenic program also through mRNA. In fact, analysis of mRNA extract indicate that MPs are shuttling a specific subset of cellular mRNA, such as mRNA associated with PI3K/Akt signalling pathway and with eNOS, known to be involved in angiogenesis (Deregibus *et al*. 2007).

Another study has shown that PMPs induce an angiogenic response, both *in vitro* and *in vivo*. In rat aortic ring model, this effect is mediated by cytokines, such as VEGF, bFGF and PDGF. PMPs exert their effect via PI3K, Src kinase and ERK. Moreover PMPs induce invasion of ECs through Matrigel®, and this effect is also mediated by VEGF, heparanase and PDGF.

Furthermore, these authors have shown that in a rat model of *in vivo* chronic myocardial ischemia, injection of PMPs into the myocardium induces angiogenesis indicating their strong implication on development of blood vessel *de novo* (Brill *et al*.

2005).

On the other hand, it has been shown that MPs shed by apoptotic lymphocytes, inhibit *in vitro* and *in vivo* angiogenesis, by suppressing vascular cell survival, proliferation and migration. They induce ROS production by enhancing both NOX activity and expression. Increased ROS levels occur upstream of induction of CD36 (TSP1 receptor) with subsequent suppression of VEGF/VEGFR2 signalling pathway. Moreover, in a model of *in vivo* neovascularization, these MPs are able to antagonize the pro-angiogenic effects induced by VEGF, and then, inhibit cell migration (Yang *et al.* 2008). Furthermore, in our laboratory it has been shown that MPs from apoptotic lymphocytes decrease NO production via PI3K pathway. Decreased NO generation is associated with enhanced phosphorylation of eNOS on its inhibitory site and overexpression of Cav-1 (Mostefai. *et al*. 2008b). Indeed, antiangiogenic effects of LMPs are linked to oxidative stress and reduced NO release from ECs (Mostefai *et al*. 2008a).

THE AIM OF STUDY

MPs exhibit abrupt properties, because of their complex composition, only partially elucidate, and because of mechanisms triggered by the interaction with target cells, not fully understood. However, their undisputed capacity to transfer biological information among cells confers them an active role in development of physio-pathological processes.

Their implication in modulation of angiogenesis is described in the literature, even if the data regarding angiogenic effects trigger by MPs are antithetical. The several responses elicited by MPs are linked to stimulus at their origin, status of cell from which they shed, and their concentration.

In this study we discuss about the vascular effects of engineering human T lymphocytes undergoing activation and apoptosis in order to generate MPs bearing Shh (Martinez *et al*. 2006).

Shh is a crucial morphogen implicates, not only, in the course of embryonic development, but also in adult life. In fact, during post-natal life, Shh pathway orchestrates diverse processes such as cell proliferation, differentiation and angiogenesis (Porro *et al*. 2009). Regarding angiogenesis, it has been described that activation of Shh cascade induces formation of capillary-like structures *in vitro* (Kanda *et al*. 2003) and new vessel generation *in vivo* (Pola *et al*. 2001). Moreover, the effect promoted by Shh affects modulation of VEGF and eNOS activities (Podlasek *et al*. 2005) and involves PI3K/Akt pathway, which also belongs to intracellular mechanism for endothelial NO release. Furthermore, in ECs MPs harbouring Shh trigger changes in the expression and phosphorylation of enzymes related to the NO pathway and these effects are directly

mediated by Shh. In addition, in the same cells, they are able to reduce oxidative stress by PI3K and ERK-dependent mechanisms (Agouni *et al*. 2007).

At light of these evidences, we tested the hypothesis that engineered human MPs bearing Shh have a crucial role in modulation of angiogenesis. Accordingly, the first part of our work concerns investigations of angiogenesis process *in vitro* with respect to the steps linking to ECs ability to form capillary-like structure, adhesion, proliferation, and expression of pro-angiogenic factors. The second part probes the capacity of MPs carrying Shh to modulate neo-vascularization using *in vivo* model of mouse hind limb ischemia. In order to verify the effects elicited by these MPs, we performed experiences concerning the recovery of blood flow, the implication of pathways which regulate NO production and the expression of key factors that belong to the complex control system under which angiogenesis is submitted.

MANUSCRIPT I

Microparticles harbouring Sonic Hedgehog promote angiogenesis through the up-regulation of adhesion proteins and pro-angiogenic factors

Raffaella Soleti[1]*, Tarek Benameur[1]*, Chiara Porro[1], Maria Antonietta Panaro[2], Ramaroson Andriantsitohaina[1] and Maria Carmen Martínez[1]

[1]CNRS, UMR 6214, INSERM, U771, Université d'Angers, Faculté de Médecine, Angers, F-49045 France; [2]Department of Human Anatomy and Histology, University of Bari, Bari, Italy.

*These authors participated equally in this work.

This work was supported in part by grants from Fonds Européen pour le Développement Régional (R.A. n° 8891), Agence Nationale pour la Recherche (M.C.M. n°ANR-07-PHYSIO-010-01), CNRS, INSERM et Université d'Angers. R.S. and T.B. are recipients of a doctoral fellowship from Italian and French Education Ministry (MENRT), respectively.

Reprint requests should be sent to M.C. Martínez, Biologie Neuro-Vasculaire Intégrée, CNRS UMR 6214-INSERM U771, Faculté de Médecine, Rue Haute de Reculée, 49045 Angers (France). Phone: + 33 2 41 73 58 57; Fax: +33 2 41 73 58 95.

E-mail: carmen.martinez@univ-angers.fr

Running title: Shh carried by plasma microparticles promotes angiogenesis

Keywords: Microvesicles, angiogenesis, morphogens, vascular endothelial growth factor.

Microparticles are small fragments generated from the plasma membrane after cell stimulation or apoptosis. We have recently shown that microparticles harboring the morphogen Sonic Hedgehog (MPs^{Shh+}) correct endothelial injury by release of nitric oxide from endothelial cells (Agouni et al., FASEB J. 11: 2735-2741, 2007). Here, we show that MPs^{Shh+} induce the formation of capillary-like structures in an *in vitro* model using human endothelial cells, although they inhibited cell migration. Besides, MPs^{Shh+} regulate cell proliferation as evidenced by the increase of cyclin D1 expression. Both cell adhesion and expression of proteins involved in this process such as Rho A and phosphorylation of focal activated kinase were increased by MPs^{Shh+}, via a Rho kinase inhibitor-sensitive pathway. We demonstrate that MPs^{Shh+} increase mRNA levels of pro-angiogenic factors (VEGF, HGF, ICAM-1, MMP1, IL-1beta, and FLT-1) as measured by qRT-PCR. Interestingly, the effects induced by MPs^{Shh+} on the formation of capillary-like structures, expression of adhesion molecules and pro-angiogenic factors were reversed after silencing of the Shh receptor, using siRNA or when Sonic Hedgehog signaling was pharmacologically inhibited with cyclopamine. Taken together, we show that Sonic Hedgehog carried by MPs^{Shh+} regulate angiogenesis, and we propose that MPs harboring Sonic Hedgehog may contribute to the generation of a vascular network in pathologies requiring wound healing.

INTRODUCTION

The morphogen Sonic Hedgehog (Shh) pathway is critical for normal growth. In addition to its role in embryonic development, it has been shown that, in adults, Shh network can participate in cell differentiation, proliferation and angiogenesis (1). Regarding angiogenesis, it has been reported that Shh pathway activation promotes *in vitro* capillary-like structures on Matrigel® (2) but also *in vivo* neo-vascularization (3). Moreover, recent data showed that the effects of Shh recombinant protein in both angiogenesis and specification of neuronal fates are mediated by the phosphatidylinositol 3-kinase (PI3-kinase)/Akt but not protein kinase C pathways (2,4,5).

Taking in consideration that angiogenesis is an essential process of cells from cardiovascular system during diseases associated with ischemia or for tumor growth, the role of Shh-induced angiogenesis in these pathologies is critical. Thus, it has been shown that Shh gene therapy may have considerable therapeutic potential by improving cardiac function in either ischemia or infarct models and wound healing in diabetes (6,7). Independently of effects of Shh cascade in angiogenesis, uncontrolled activation of the Hedgehog signaling pathway is a causal factor in many cancers (8) and upregulation of Shh-related proteins has been shown in human tumors such as pancreatic adenocarcinoma, basal cell carcinoma, glioma and prostate cancer (9-12).

Tumor cells but also blood and vessel wall cells are able to release large amounts of small plasma membrane fragments called microparticles (MPs). MPs are responsible, at least in part, to cancer-associated thrombosis because they harbor at their surface tissue factor and other components necessary for thrombus formation (13). Thus, MPs may represent a sign of vascular complications in patients with lung and gastric cancer (14,15). These authors have reported enhanced circulating monocyte- and platelet-

derived MPs in patients with lung cancer. In addition, levels of P-selectin associated to platelet MPs and tissue factor generated from cancer cells are increased indicating that proteins involved in hemostasis are elevated in patients with cancer (16) and may represent a tool for exacerbated thrombosis. Also, through proteins carried by MPs from tumor cells, such as urokinase, CD147 or sphingomyelin, MPs can modify the adhesive and invasive properties of target cells (17), or the angiogenic activity of endothelial cells (18). Moreover, it has been shown that platelet MPs enhance the *in vitro* invasive potential of breast cancer cell lines, and induce metastasis and angiogenesis in lung cancer (19,20). These data suggest that MPs transfer a transcellular signal that may allow tumor progression. In this context, MPs from acute myelogenous leukemia patients functionally transferred CXCR4 to HL-60 cells and increased their chemotaxis and homing to the bone marrow of immunodeficient mice (21).

We have recently shown that MPs generated *in vitro* from human apoptotic/stimulated lymphocytes express Shh (MPs^{Shh+}) at their surface and induce cell differentiation (22). In addition, this type of MPs is able to stimulate nitric oxide (NO) production from endothelial cells by direct activation of the Shh and PI3-kinase pathways (23). Thus, MPs^{Shh+} could represent a potential tool to modulate angiogenesis through their direct action on endothelial cells. Here, we studied the effects of MPs^{Shh+} on *in vitro* angiogenesis using a human model of endothelial cells. For this, we have investigated MPs^{Shh+} effects on migration, proliferation, adhesion and formation of capillary-like structures as well as the angiogenic factors produced by endothelial cells under MPs^{Shh+} treatment.

MATERIALS AND METHODS

MPs production

The human lymphoid CEM T cell line (ATCC, Manassas, VA) was used for MP production. Cells were seeded at 10^6 cells/ml and cultured in serum-free X-VIVO 15 medium (Lonza, Walkersville, MD). MPs were produced as previously described (22,23). Briefly, CEM cells were treated with phytohemagglutinin (5 µg/mL; Sigma Aldrich, St Louis, MO) for 72 hours, and then with phorbol-12-myristate-13 acetate (20 ng/mL; Sigma Aldrich) and actinomycin D (0.5 µg/mL; Sigma Aldrich) for 24 hours. A supernatant was obtained by centrifugation at 750 *g* for 15 minutes, then at 1500 *g* for 5 minutes to remove cells and large debris, respectively. MPs from the supernatant were washed after three centrifugation steps (45 minutes at 14000 *g*) and recovered in 400 µL NaCl (0.9% w/v). Washing medium for the last supernatant was used as control. Determination of the amount of MPs was carried out by measuring MP-associated proteins, using the method of Lowry, with BSA (Sigma Aldrich) as the standard.

Cell culture

The Eahy 926 endothelial cell line was maintained at 37°C in a humidified incubator gassed with 5% CO_2 in air and was cultured in growth medium (DMEM, Ham's F-12, 1:1; Lonza) supplemented with 1% L-glutamine, 1% NEAA, 1% Na-pyruvate, 1% streptomycin/ penicillin (Lonza), 1% HAT (Sigma Aldrich), and 10% of heat-inactivated FBS (Invitrogen, Cergy Pontoise, France). Also, freshly delivered umbilical cords were obtained from a nearby hospital. Human umbilical vein endothelial cells (HUVECs) were obtained as previously described (24) and grown on plastic flasks in MCBD 131 medium containing 1% L-glutamine, 1% streptomycin/ penicillin, 500 ng/L EGF, 1 µg/L b-FGF, supplemented with 10% of heat-inactivated FBS. HUVECs were

used at the 2nd to 4th passage. Cells were grown for 24 hours in the absence or presence of 10 µg/mL MPs pre-incubated or not with Shh inhibitor cyclopamine (30 µM; Biomol International, Plymouth Meeting, PA). All agents were used at concentrations at which no cytotoxicity was observed, as deduced from Trypan blue exclusion. In our previous study, treatment of endothelial cells *in vitro* with 10 µg/mL MPs for 24 hours corresponded to the concentration and time required to obtain NO release from endothelial cells (23). Thus, all the experiments of the present work were performed under these conditions.

In vitro capillary network formation on Matrigel®

After 24 hours of incubation with MPs in the absence or in the presence of either the inhibitor cyclopamine or siRNA, Eahy 926 cells with siRNA transfection (see below) were detached with trypsin EDTA (Lonza). Cells were seeded with a density of $150x10^3$ cells/well pre-coated with Matrigel® (Sigma Aldrich). Briefly, 100 µL of Matrigel® substrate diluted with serum-free medium (1:1 dilution) was added into a 4-well plate and allowed to solidify for 1 hour at 37 °C. Then, cells incubated with medium containing 10% of FBS, and allowed to adhere for 1 hour after which the different stimuli were added. Tube formation was examined by phase-contrast microscopy (MOTIC AE21) after 4 and 24 hours.

RNA interference and transient transfection

In order to silencing Patched-1, the Shh receptor, siRNA duplexes specific for human Patched-1 and control, non-silencing siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Transient transfection of Eahy 926 endothelial cells was done according to the manufacturer's protocol. Briefly, cells were seeded in 6-well

plates, grown for 24 hours (60% confluence), and then transiently transfected with 100 nM of Patched-1-specific or control siRNA using the transfection reagent provided, which also served as control without siRNA. Medium was replaced 24 hours later by fresh medium and cells were grown for an additional 24 hours, prior to either western blot analysis of Patched-1 expression or functional studies. After siRNA transfection, Patched-1 downexpression was >80% (not shown) as previously illustrated by Agouni et al. (23).

Western blot

After treatment, cells were homogenized and lysed. Proteins (20 µg) were separated on 10% SDS-PAGE. Blots were probed with anti-cyclin D1 (BD Biosciences, Le Pont de Claix, France), Rho-A (Santa Cruz Biotechnology), p-focal adhesion kinase (Y925) (p-FAK, Cell Signaling, Danvers, MA), and Patched-1 (Santa Cruz Biotechnology) antibodies. Monoclonal anti-ß-actin antibody (Sigma-Aldrich) was used at 1/2000 dilution to visualize protein gel loading. The membranes were then washed at least three times in Tris buffer solution containing 0.05% Tween and incubated for 1 hour at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). The protein-antibody complexes were detected by ECL plus (Amersham) according to the protocol of the manufacturer.

Migration assay

Eahy 926 cells were seeded and grown to confluence in cell culture medium. After 24 hours of serum starvation, cells were rinsed three times with serum-free medium and then, incubated with supplemented medium. The monolayer cell was wounded with a sterile pipet tip to make a gap as previously described (25). Then, detached cells were

removed by washing, and cells were culture in the absence or in the presence of MPs or MPs with cyclopamine. After 48 hours, three selected non-adjacent fields at the lesion border were acquired using a 10x phase objective on an inverted microscope (MOTIC AE21).

Immunochemical staining

After treatment, cultured cells were fixed with CELLFIX 1x (BD Biosciences) solution for 15 minutes at room temperature in culture dishes, permeabilized with 0.1%Triton X-100 in PBS, and then blocked with 5% non-fat milk in PBS for 1 hour at room temperature. The cells were treated with a rabbit polyclonal p-FAK (Y925) antibody, or mouse monoclonal anti-Rho A antibody in 5% non-fat milk in PBS for 1 hour at room temperature. After washing with PBS, cells were treated with Alexa 488-conjugated goat anti-rabbit antibody (Interchim, Montluçon, France) and Alexa488-conjugated goat anti-mouse (Interchim) in 5% non-fat milk in PBS for 1 hour. After washing with PBS, the cells were mounted and visualized with a fluorescent microscopy (Nikon Eclipse 2000-S).

Quantitative real time RT-PCR analysis

Eahy 926 cells were grown for 24 hours in the absence or the presence of 10 µg/mL MPs preincubated or not with cyclopamine. Cells were detached using trypsin and, after 2 subsequent steps of centrifugation at 500 *g* for 10 minutes, the pellet containing cells were frozen in liquid N_2 and used to investigate the expression of mRNA for 46 transcripts related to angiogenesis by real-time reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR analyses were carried out by Service Commun de Cytométrie et d'Analyses Nucléotidiques from Angers University, using a Chromo 4™

(Bio-Rad, Hercules, CA) and SYBR Green detection. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quantifications were realized according to the ΔCt method and the relative gene-expression levels were normalized using the geometric mean of three housekeeping genes as previously described (26).

Proliferation Assay

Effects of MPs on proliferation on Eahy 926 cells or HUVECs were analyzed by using CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Briefly, $10x10^3$ cells/well were seeded into 96-well plates and allowed to attach overnight, and then, cells were treated with MPs for 24 hours. After growth medium removal, dye binding solution was added into each microplate well and cells was incubate at 37°C for 30 minutes. The fluorescence levels were read on a fluorescent microplate reader (Sinergy HT Biotek) with filters for ~485 nm excitation and ~530 nm emission.

Adhesion assay

Evaluation of adherent cells was performed using crystal violet staining (27). For adhesion experiments $10x10^3$ cells/well were seeded into 96 well plates for 24 hours before addition of the stimuli. After 24 hours of incubation, the plate was shacked for 15 seconds. The supernatant with non-adherent cells was removed by three washes with washing buffer (0.1% BSA in medium without serum). Attached cells were fixed with 4% of paraformaldehyde for 15 min at room temperature. Cells were rinsed two times with washing buffer, stained with crystal violet (Sigma Aldrich) (1mg/mL in 2% of ethanol) for 10 minutes at room temperature and extensively washed with distilled water. After SDS 2% was added and incubated for 30 minutes at room temperature.

Absorbance was then evaluated using a microplate reader at 550 nm (Sinergy HT Biotek).

Statisticals analysis

Data are represented as mean ± SEM, *n* represents the number of experiments repeated at least in triplicate. Statistical analyses were performed by Mann-Whitney U-tests (non-parametric). $P < 0.05$ was considered to be statistically significant.

RESULTS

MPs^{Shh+} promote the formation of capillary-like structures

As shown in Figure 1, in the absence of treatment, cells failed to organize in capillary-like structures. After 24 hours of treatment with MPs^{Shh+}, endothelial Eahy cells or HUVECs reorganized and formed capillaries on Matrigel®. In order to determine whether Shh accounts for the effects evoked by MPs^{Shh+}, we examined the effect of Shh inhibition on MPs^{Shh+}-induced angiogenesis. Treatment of endothelial cells either with the selective Shh inhibitor, cyclopamine (30 µM), or siRNA to Shh receptor, Patched-1, resulted in an abolition of MPs^{Shh+} effects on capillary-like structure formation (Figure 1). It should be noted that cyclopamine alone or siRNA scrambled had no effect on endothelial cell angiogenesis. Together, these findings suggest that the effects of MPs^{Shh+} are directly mediated by the Shh cascade.

MPs^{Shh+} reduce both endothelial cell migration and proliferation

Migration of endothelial cells, which allows cells to disseminate from the pre-existing vessel to form new vessels, contributes to angiogenesis. We studied the effects of MPs^{Shh+} on endothelial cell migration using a model of wound healing. MPs^{Shh+} slightly reduced migration of endothelial Eahy cells, which was not affected by cyclopamine (Figure 2A). Similar results were obtained in HUVECs treated with MPs^{Shh+} (not shown).

Also the effect of MPs^{Shh+} on cell proliferation was investigated since this process represents a critical step in angiogenesis. Figure 2B and 2C illustrate the antiproliferative effect of MPs^{Shh+}. MPs^{Shh+} were able to reduce cell proliferation by ~20 and 30% for Eahy cells and HUVECs, respectively. In addition, cyclopamine treatment did not modify the inhibitory effect of MPs^{Shh+} on cell proliferation. These data indicate

that Shh cascade is not implicated in the MPs^{Shh+} effect on endothelial cell proliferation. We also assessed the effect of MPs^{Shh+} treatment on the expression of one of the major cyclin operative in G1/S transition by western blotting. Surprisingly, analysis revealed that incubation of endothelial cells with MPs^{Shh+} induced a significant increase of cyclin D1 expression, which was not inhibited by cyclopamine treatment (Figure 2D).

MPs^{Shh+} up-regulate proteins involved in cell adhesion through RhoA and Shh pathways

Adhesion assay using crystal violet shows that MPs^{Shh+} increased adhesion in both Eahy and HUVECs (Figure 3A and 3B). Although cyclopamine was not able to reduce the effects of MPs^{Shh+} on cell adhesion, inhibition of Rho-associated coiled coil-containing protein kinase (ROCK) by Y-27632 (10 μM) decreased MPs^{Shh+}-induced cell adhesion (Figure 3A). Specific transcripts for the intercellular adhesion molecule (ICAM)-1 measured by real-time quantitative RT-PCR were enhanced by MPs^{Shh+} treatment and this effect was abolished by cyclopamine (Figure 3C).

Then, the effect of Y-27632 on the expression and activation of proteins linked to cell adhesion was investigated. MPs^{Shh+} induced an increase in Rho A expression and FAK phosphorylation as evidenced by immunolabeling and western blot. These effects were inhibited not only by Y-27632 but also by cyclopamine (Figure 4A and 4B). These results suggest that proteins such as Rho A and FAK, which are involved in cell adhesion, are up-regulated by MPs^{Shh+} through both ROCK and Shh pathways, despite the existence of pathways not associated with Shh activation.

MPs^{Shh+} up-regulate pro-angiogenic factors through the activation of the Shh pathway

Angiogenic factors were analyzed by real-time quantitative RT-PCR using a panel of

different human angiogenic factor mRNA. Among the 46 transcript studied, those for pro-angiogenic factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) A and its receptor fms-like tyrosine kinase (FLT-1), interleukin (IL-)1β, and metalloproteinase (MMP)-1 were increased by MPs^{Shh+} treatment. These effects were partially inhibited by cyclopamine. In contrast, transforming growth factor (TGF)-β2 mRNA was reduced by MPs^{Shh+}, independently of the inhibition of the Shh pathway (Figure 5).

DISCUSSION

In the present study, we demonstrate that MPs generated from activated/apoptotic T cells harboring Shh differentially regulate the steps implicated in angiogenesis. Thus, MPs^{Shh+} increased capillary-like formation through the increase of cell adhesion, the up-regulation of proteins such as Rho A and FAK, and pro-angiogenic factors via the activation of the Shh pathway. In addition, the effects induced by MPs^{Shh+} on cell adhesion were dependent on ROCK pathway. However, MPs^{Shh+} reduced cell migration and proliferation through the mechanisms independent of the Shh pathway activation. These data suggest that MPs transfer a biological message carried by Shh that can account for a large number of events associated with angiogenesis.

In tumor-associated angiogenesis, angiogenic factors secreted by endothelial and tumor cells stimulate endothelial cells to degrade the vascular basal membrane and migrate into surrounding tissues promoting the proliferation of solid tumors (28). Here, we showed that MPs^{Shh+} are able to produce endothelial release of pro-angiogenic factors such as VEGF A, FLT-1, ICAM-1, MMP-1, IL-1β, and HGF, and these effects were Shh-dependent. Among these factors, the complex VEGF A/Flt-1 represents the major player in angiogenesis initiation by inducing endothelial NO production (29), in parallel to the increase of ICAM-1 expression (30). Also, MMPs are essential for angiogenesis due to their ability to degrade the components of the extracellular matrix and thus, MMPs participate in the remodeling of basement membranes (31). Moreover, IL-1ß induces VEGF A expression through the PI3-kinase pathway (32). Finally, HGF is identified as a member of angiogenic growth factors with a potent action on human endothelial cells (33). Interestingly, expression of TGF-ß2 was down-regulated by MPs^{Shh+}. TGF-ß acts as a tumor suppressor early in carcinogenesis, but then switches to a tumor promoter later by affecting both the cancer cells and the tumor

microenvironment (34). Both TGF-ß2 and Shh are involved in processes during embryonic development and cancer. Indeed, it has been recently shown that TGF-ß induces the expression of the Hh signaling molecules Gli1 and Gli2 in various cancer cell lines independently of the Hh receptor activation (35). The fact that MPs^{Shh+} reduced TGF-ß2 expression may favor angiogenesis. Regarding the effects of Shh on angiogenic factors, it has been reported that recombinant Shh is able to induce the expression of angiopoietin-1 and reduce angiopoietin-2 expression in embryonic fibroblast cells (36). By contrast, angiopoietin-1, angiopoietin-2 and VEGF are over-expressed by recombinant Shh treatment in human fibroblasts (3), whereas Shh-deficient mice present a down-regulation of mRNA expression of angiopoietin-1 but not of angiopoietin-2 and VEGF (37). Altogether, the present study shows that Shh carried by MPs acts on a large number of target genes that regulate angiogenesis at different phases.

In the present study, MPs^{Shh+} up-regulated Rho A expression and induced the phosphorylation of FAK which were reversed by both ROCK inhibitor and cyclopamine, suggesting a crosslink between ROCK and Shh pathways. It has been shown that the activities of Rho A and ROCK are responsible for several effects of Shh activation (38, 39). Paradoxically, MPs^{Shh+} increased endothelial cell adhesion by a mechanism sensitive to ROCK inhibitor but not cyclopamine. The lack of effect of cyclopamine in cell adhesion might be due to the complexity of this process. Thus, Shh pathway may be involved in the activation of certain (here, Rho A and p-FAK), but not all, proteins implicated in the adhesion process. Nevertheless, MPs^{Shh+} enhanced endothelial cell adhesion indicating a role of these MPs in angiogenesis.

Both cell migration and proliferation were inhibited by MPs^{Shh+} and, these effects were independent of Shh pathway, suggesting that although formation of capillary-like

structures is promoted by MPs^{Shh+}, these MPs are able to differentially regulate cell events leading to angiogenesis. Similar results have been reported for the angiopoietin-like 4, which is able to promote angiogenesis (40) and inhibit endothelial cell migration by an interaction with the extracellular matrix (41). Conversely with these results, expression of cyclin D1 was increased MPs^{Shh+} treatment. One possible explanation for this observation could be the fact that, although cyclin D1 expression is up-regulated, cell cycle could be arrested in G2/M phase, as described recently (42). Moreover, it should be noted that the nature of all molecular components of MPs^{Shh+} is not known. Proteomic studies have shown that MPs composition is complex, and dependent on the stimuli used for their generation as well as on the cell origin (43, 44). In the present study, we have engineered MPs harboring Shh but other components are not determined and, obviously, they can influence angiogenesis.

In summary, MPs carrying Shh regulate multiple pathways related to *in vitro* angiogenesis, mainly through the production of pro-angiogenic factors and up-regulation of proteins involved in cell adhesion. Expression of Shh correlates with the tumorigenesis of different types of cancer such as basal cell carcinoma, pancreatic and prostatic cancer, and gliomas (45-48). In addition, release of MPs from tumor or vascular cells is also related with tumorigenesis. Thus, targeting Shh pathway would represent a novel therapeutic tool that can regulate angiogenesis and in consequence, tumor development.

ACKNOWLEDGEMENTS

We thank L. Preisser and M.-H. Guilleux from Service Commun de Cytométrie et d'Analyses Nucléotidiques from Institut Fédératif de Recherche 132 (Univesité d'Angers) for their assistance in q-PCR quantification.

References

1. Bailey JM, Singh PK, Hollingsworth MA. (2007) Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, Notch, and bone morphogenic proteins. *J. Cell Biochem*., **102**, 829-839.

2. Kanda S, Mochizuki Y, Suematsu T, Miyata Y, Nomata K, Kanetake H. (2003) Sonic hedgehog induces capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase. *J. Biol. Chem*., **278**, 8244-8249.

3. Pola R, Ling LE, Silver M, Corbely MJ, Kearney M, Blake Pepinsky R, Shapiro R, Taylor FR, Baker DP, Asahara T, Isner JM. (2001) The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat. Med*., **7**, 706-711.

4. Riobó NA, Lu K, Ai X, Haines GM, Emerson CP Jr. (2006) Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. *Proc. Natl. Acad. Sci. U S A*, **103**, 4505-4510.

5. Fu JR, Liu WL, Zhou JF, Sun HY, Xu HZ, Luo L, Zhang H, Zhou YF. (2006) Sonic hedgehog protein promotes bone marrow-derived endothelial progenitor cell proliferation, migration and VEGF production via PI 3-kinase/Akt signaling pathways. *Acta Pharmacol. Sin*., **27**, 685-693.

6. Kusano KF, Pola R, Murayama T, Curry C, Kawamoto A, Iwakura A, Shintani S, Ii M, Asai J, Tkebuchava T, Thorne T, Takenaka H, Aikawa R, Goukassian D, von Samson P, Hamada H, Yoon YS, Silver M, Eaton E, Ma H, Heyd L, Kearney M, Munger W, Porter JA, Kishore R, Losordo DW. (2005) Sonic hedgehog myocardial gene therapy: tissue repair through transient reconstitution of embryonic signaling. *Nat. Med*., **11**, 1197-1204.

7. Asai J, Takenaka H, Kusano KF, Ii M, Luedemann C, Curry C, Eaton E, Iwakura A, Tsutsumi Y, Hamada H, Kishimoto S, Thorne T, Kishore R, Losordo DW. (2006) Topical sonic hedgehog gene therapy accelerates wound healing in diabetes by enhancing endothelial progenitor cell-mediated microvascular remodeling. *Circulation*, **113**, 2413-2424.

8. Taipale J, Beachy PA. (2001) The Hedgehog and Wnt signalling pathways in cancer. *Nature*, **411**, 349-354.

9. Pasca di Magliano M, Hebrok M. (2003) Hedgehog signalling in cancer formation and maintenance. *Nature Rev. Cancer,* **3**, 903-911.

10. Ruiz i Altaba A, Sánchez P, Dahmane N. (2002) Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nature Rev. Cancer,* **2**, 361-372.

11. Xie J, Aszterbaum M, Zhang X, Bonifas JM, Zachary C, Epstein E, McCorminck F. (2001) A role of PDGFRα in basal cell carcinoma proliferation. *Proc. Natl. Acad. Sci. U S A*, **98**, 9255-9259.

12. Zedan W, Robinson PA, Markham AF, High AS. (2001) Expression of the Sonic Hedgehog receptor "PATCHED" in basal cell carcinomas and odontogenic keratocysts. *J. Pathol*., **194**, 473-477.

13. Zwicker JI, Furie BC, Furie B. (2007) Cancer-associated thrombosis. *Crit. Rev. Oncol. Hematol*., **62**, 126-136.

14. Kanazawa S, Nomura S, Kuwana M, Muramatsu M, Yamaguchi K, Fukuhara S. (2003) Monocyte-derived microparticles may be a sign of vascular complication in patients with lung cancer. *Lung Cancer*, **39**, 145-149.

15. Kim HK, Song KS, Park YS, Kang YH, Lee YJ, Lee KR, Kim HK, Ryu KW, Bae JM, Kim S. (2003) Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. *Eur. J. Cancer*, **239**, 184-191.

16. Yu JL, Rak JW. (2004) Shedding of tissue factor (TF)-containing microparticles rather than alternatively spliced TF is the main source of TF activity released from human cancer cells. *J. Thromb. Haemost*., **2**, 2065-2067.

17. Angelucci A, D'Ascenzo S, Festuccia C, Gravina GL, Bologna M, Dolo V, Pavan A. (2000) Vesicle-associated urokinase plasminogen activator promotes invasion in prostate cancer cell lines. *Clin. Exp. Metastasis*, **18**, 163-170.

18. Millimaggi D, Mari M, D'Ascenzo S, Carosa E, Jannini EA, Zucker S, Carta G, Pavan A, Dolo V. (2007) Tumor vesicle-associated CD147 modulates the angiogenic capability of endothelial cells. *Neoplasia,* **9**, 349-357.

19. Janowska-Wieczorek A, Marquez-Curtis LA, Wysoczynski M, Ratajczak MZ. (2006) Enhancing effect of platelet-derived microvesicles on the invasive potential of breast cancer cells. *Transfusion*, **46**, 1199-1209.

20. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J, Ratajczak MZ. (2005) Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. *Int. J. Cancer*, **113**, 752-760.

21. Kalinkovich A, Tavor S, Avigdor A, Kahn J, Brill A, Petit I, Goichberg P, Tesio M, Netzer N, Naparstek E, Hardan I, Nagler A, Resnick I, Tsimanis A, Lapidot T. (2006) Functional CXCR4-expressing microparticles and SDF-1 correlate with circulating acute myelogenous leukemia cells. *Cancer Res*., **66**, 11013-11020.

22. Martínez MC, Larbret F, Zobairi F, Coulombe J, Debili N, Vainchenker W, Ruat M, Freyssinet JM. (2006) Transfer of differentiation signal by membrane microvesicles harbouring hedgehog morphogens. *Blood*, **108**, 3012-3020.

23. Agouni A, Motefai AH, Porro C, Carusio N, Favre J, Richard V, Henrion D, Martínez MC, Andriantsitohaina R. (2007) Sonic hedgehog carried by microparticles corrects endothelial injury through nitric oxide release. *FASEB J*., **21**, 2735-2741.

24. Favot L, Martin S, Keravis T, Andrianstitohaina R, and Lugnier C. (2003) Involvement of cyclin-dependent pathway in the inhibitory effect of delphinidin on angiogenesis. *Cardiovasc. Res.*, **59**, 479-487.

25. Gross I, Duluc I, Benameur T, Calon A, Martin E, Brabletz T, Kedinger M, Domon-Dell C, Freund JN. (2008) The intestine-specific homobox gene *Cdx2* decreases mobility and antagonizes dissemination of colon cancer cells. *Oncogene*, **27**, 107-115.

26. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.*, **3**, RESEARCH0034.

27. Qin G, Ii M, Silver M, Wecker A, Bord E, Ma H, Gavin M, Goukassian DA, Yoon YS, Papayannopoulou T, Ashara T, Kearney M, Thorne T, Curry C, Eaton L, Heyd L, Dinesh D, Kishore R, Zhu Y, Losordo DW. (2006) Functional disruption of alpha4 integrin mobilizes bone marrow-derived endothelial progenitors and auments ischemic neovascularization. *J. Exp. Med*., 203, 153-163.

28. Griffioen AW, Molema G. (2000) Angiogenesis: Potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol. Rev*., **52**, 237-268.

29. Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bicknell R. (1997) Nitric oxide lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J. Clin. Invest*., **99**, 2625-2634.

30. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. (2001) Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J. Biol. Chem*., **276**, 7614-7620.

31. Raffetto JD, Khalil RA. (2008) Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. *Biochem. Pharmacol*., 75, 346-359.

32. Solà-Villà D, Camacho M, Solà R, Soler M, Diaz JM, Vila L. (2006) IL-1beta induces VEGF, independently of PGE2 induction, mainly through the PI3-K/mTOR pathway in renal mesangial cells. *Kidney Int*., **70**, 1935-1941.

33. Morishita R, Aoki M, Hashiya N, Makino H, Yamasaki K, Azuma J, Sawa Y, Matsuda H, Kaneda Y, Ogihara T. (2004) Therapeutic angiogenesis using hepatocyte growth factor (HGF). *Curr. Gene Ther*., **4**, 199-206.

34. Gordon KJ, Blobe GC. (2008) Role of transforming growth factor-ß superfamily signaling pathways in human disease. *Biochim. Biophys. Acta*, **1782**, 197-228.

35. Dennler S, André J, Alexaki I, Li A, Magnaldo T, ten Dijke P, Wang XJ, Verrecchia F, Mauviel A. (2007) Induction of sonic hedgehog mediators by transforming growth-

beta: Smad3-dependent activation of Gli2 and Gli1 expression in vitro and in vivo. *Cancer Res*., **67**, 6981-6986.

36. Lee SW, Moskowitz MA, Sims JR. (2007) Sonic hedgehog inversely regulates the expression of angiopoietin-1 and angiopoietin-2 in fibroblasts. *Int. J. Mol. Med.*, **19**, 445-451.

37. van Tuyl M, Groenman F, Wang J, Kuliszewski M, Liu J, Tibboel D, Post M. (2007) Angiogenic factors stimulate tubular branching morphogenesis of sonic hedgehog-deficient lungs. *Dev. Biol*., 303, 514-526.

38. Kasai K, Takahashi M, Osumi N, Sinnarajah S, Takeo T, Ikeda H, Kehrl JH, Itoh G, Arnheiter H. (2004) The G12 family of heterotrimeric G proteins and Rho GTPase mediate Sonic hedgehog signaling. *Gene Cells*, **9**, 49-58.

39. Nishimaki H, Kasai K, Kozaki K, Takeo T, Ikeda H, Saga S, Nitta M, Itoh G. (2004) A role of activated Sonic hedgehog signaling for the cellular proliferation of oral squamous cell carcinoma cell line. *Biochem. Biophys. Res. Commun*., **314**, 313-320.

40. Le Jan S, Amy C, Cazes A, et al. Angiopoietin-like 4 is a proangiogenic factor produced during ischemia and in conventional renal cell carcinoma. Am J Pathol 2003;162:1521-8.

41. Cazes A, Galaup A, Chomel C, Bignon M, Bréchot N, Le Jan S, Weber H, Corvol P, Muller L, Germain S, Monnot C. (2006) Extracellular matrix-bound angiopoietin-like 4 inhibits endothelial cell adhesion, migration, and sprouting and alters actin cytoskeleton. *Circ. Res*., **99**, 1207-1215.

42. Wang JS, Wang CL, Wen JF, Wang YJ, Hu YB, Ren HZ. (2008) Lithium inhibits proliferation of human esophageal cancer cell line Eca-109 by inducing a G2/M cell cycle arrest. *World J. Gastroenterol*., **14**, 3982-3989.

43. Miguet L, Pacaud K, Felden C, Hugel B, Martínez MC, Freyssinet JM, Herbrecht R, Potier N, van Dorsselaer A, Mauvieux L. (2006) Proteomic analysis of malignant lymphocyte membrane microparticles using double ionization coverage optimization. *Proteomics*, **6**, 153-171.

44. Smalley DM, Root KE, Cho H, Ross MM, Ley K. (2007) Proteomic discovery of 21 proteins expressed in human plasma-derived but not platelet-derived microparticles. *Thromb. Haemost*., **97**, 67-80.

45. Becher OJ, Hambardzumyan D, Fomchenko EI, Momota H, Mainwaring L, Bleau AM, Katz AM, Edgar M, Kenney AM, Cordon-Cardo C, Blasberg RG, Holland ED. (2008) Gli activity correlates with tumor grade in platelet-derived growth factor-induced gliomas. *Cancer Res*., **268**, 2241-2249.

46. Marsh D, Dickinson S, Neill GW, Marshall JF, Hart IR, Thomas GJ. (2008) Alpha vbeta 6 integrin promotes the invasion of morphoiec basal cell carcinoma through stromal modulation. *Cancer Res*., **68**, 3295-3303.

47. Mimeault M, Johansson SL, Vankatraman G, Moore E, Henichart JP, Depreux P, Lin MF, Batra SK. (2007) Combined targeting of epidermal growth factor receptor and hedgehog signaling by gefitinib and cyclopamine cooperatively improves the cytotoxic effects of docetoxel on metastatic prostate cancer cells. *Mol. Cancer Ther*., **6**, 967-978.

48. Nakashima H, Nakamura M, Yamaguchi H, Yamanaka N, Akiyoshi T, Koga K, Yamaguchi K, Tsuneyoshi M, Tanaka M, Katano M. (2006) Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. *Cancer Res*., **66**, 7041-7049.

FIGURE LEGENDS

Fig. 1. Shh promotes *in vitro* tube formation of endothelial cells. Phase contrast micrographs showing that 10 µg/ml MPs carrying Sonic Hedgehog (Shh) induce network formation on Matrigel® in Eahy 926 cells. Silencing Shh pathway by either the pharmacological inhibitor cyclopamine (30 µM) or siRNA of the Shh receptor, Patched-1, prevented MP-induced capillary formation. However, siRNA scrambled had no effect on *in vitro* angiogenesis evoked by MPs. Reproducible data were obtained from four independent experiments.

Fig. 2. MPs^{Shh+} decrease cell migration and proliferation. (A) Ten µg/ml MPs^{Shh+} treatment decrease cell migration when compared to the control (CTL) conditions (in the absence of MPs^{Shh+}). Neither cyclopamine (30 µM) alone nor cyclopamine in the combination of MPs^{Shh+} modified the inhibition of migration induced by MPs^{Shh+}. Reproducible data were obtained from four independent experiments. (B) Anti-proliferative effects of MPs^{Shh+} were not inhibited by treatment with cyclopamine (Cycl). Results are means ± SEM from six independent experiments. *$P < 0.05$ *vs* in the absence of MPs^{Shh+}. (C) Immunoblots showing cyclin D1 expression in endothelial cells, ß-actin control is included. Data are representative of four separate blots.

Fig. 3. MPs^{Shh+} increase adhesion of endothelial cells. (A) Ten µg/ml MPs^{Shh+} enhance the number of endothelial cells that resulted positive to crystal violet staining. Treatment with the ROCK inhibitor (Y-27632, 10 µM) but not cyclopamine (Cycl, 30 µM) prevented the MPs^{Shh+}-induced increase of endothelial adhesion. Results are means ± SEM from six independent experiments. *$P < 0.05$ *vs* in the absence of MPs^{Shh+}. †$P < 0.05$ *vs* in the presence of MPs^{Shh+} alone. (B) Quantitative RT-PCR analysis was

conducted on total RNA from 6 independent Eahy cultures. ICAM-1 mRNA expression levels were enhanced by MPs^{Shh+} treatment. Cyclopamine partially reversed the MPs^{Shh+}-induced increase of ICAM-1 mRNA expression. *P < 0.05 *vs* in the absence of MPs^{Shh+}. #P < 0.05 *vs* in the presence of MPs^{Shh+} alone.

Fig. 4. MPs^{Shh+} increase expression of Rho A and phosphorylation of FAK. (A) Immunofluorescence staining of endothelial cells for Rho A and p-FAK showed up-regulation of Rho A expression and activation of FAK pathway after MPs^{Shh+} treatment. These effects were reversed by either cyclopamine (Cycl, 30 μM) or Y-27632 (10 μM) treatment. (B) This was confirmed by Western blot. ß-actin control is included. Data are representative of five separate blots, and the densitometry values are expressed in arbitrary units (A.U.) as mean ± SEM. **P<0.01 *vs* in the absence of MPs^{Shh+}. #P < 0.05 *vs* in the presence of MPs^{Shh+} alone.

Fig. 5. MPs^{Shh+} modify mRNA expression of angiogenic factors. Quantitative RT-PCR analysis was conducted on total RNA from 6 independent Eahy cultures. HGF, VEGF A, FLT1, IL 1β, and MMP 1 mRNA expression levels were enhanced by MPs^{Shh+} treatment. Cyclopamine partially reversed the MPs^{Shh+}-induced increase of VEGF A, IL 1β, and MMP 1 mRNA expressions. TGF-β2 mRNA expression was reduced by MPs^{Shh+} treatment. *P < 0.05 *vs* in the absence of MPs^{Shh+}.

Figure 1

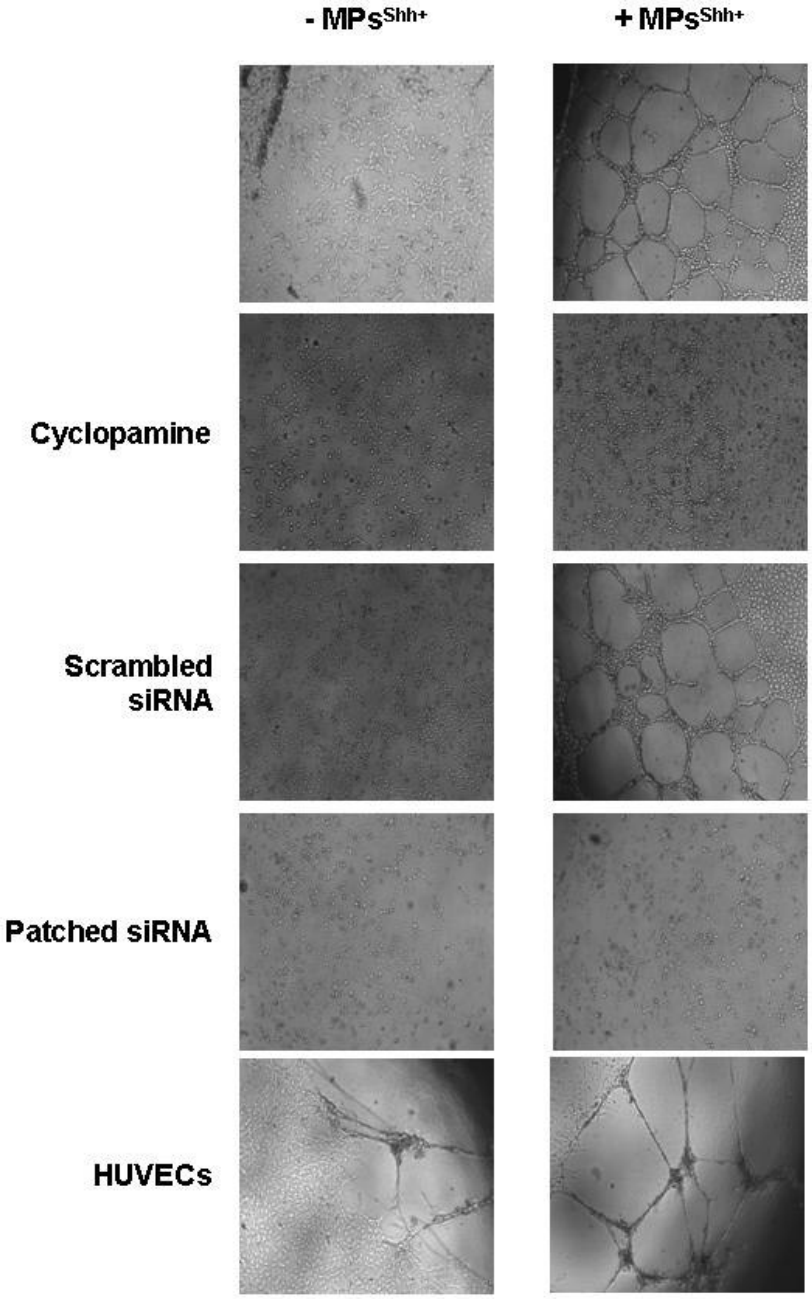

- MPs Shh+
+ MPs Shh+
Cyclopamine
Scrambled siRNA
Patched siRNA
HUVECs

Figure 2

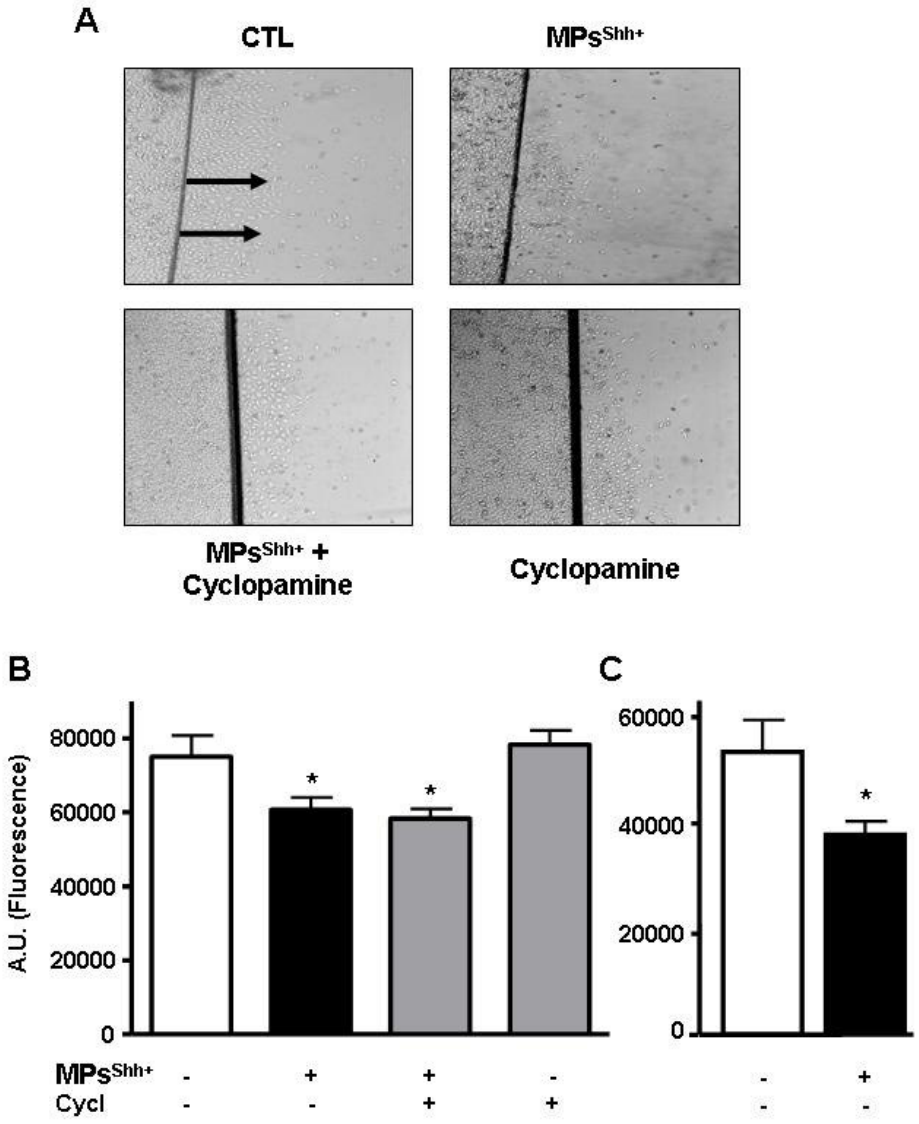

A
CTL
MPsShh+
MPsShh+ +
Cyclopamine
Cyclopamine
B
C
A.U. (Fluorescence)
80000
60000
40000
20000
0
*
*
MPsShh+ - + + -
Cycl - - + +
60000
40000
20000
0
*
- +
- -

Figure 3

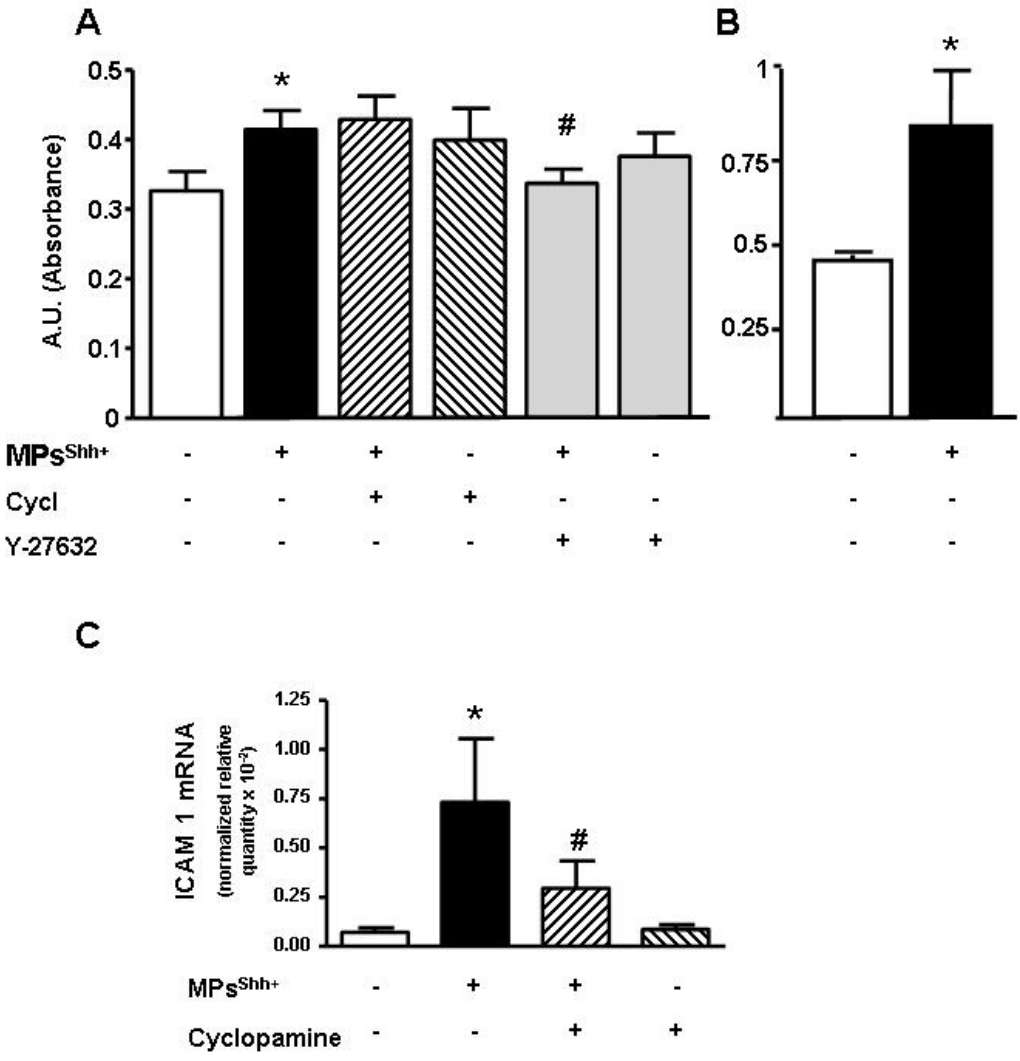

A
B
C
A.U. (Absorbance)
0.5
0.4
0.3
0.2
0.1
0
*
#
1
0.75
0.5
0.25
MPsShh+
Cycl
Y-27632
ICAM 1 mRNA
(normalized relative quantity x 10-2)
1.25
1.00
0.75
0.50
0.25
0.00
Cyclopamine

Figure 4

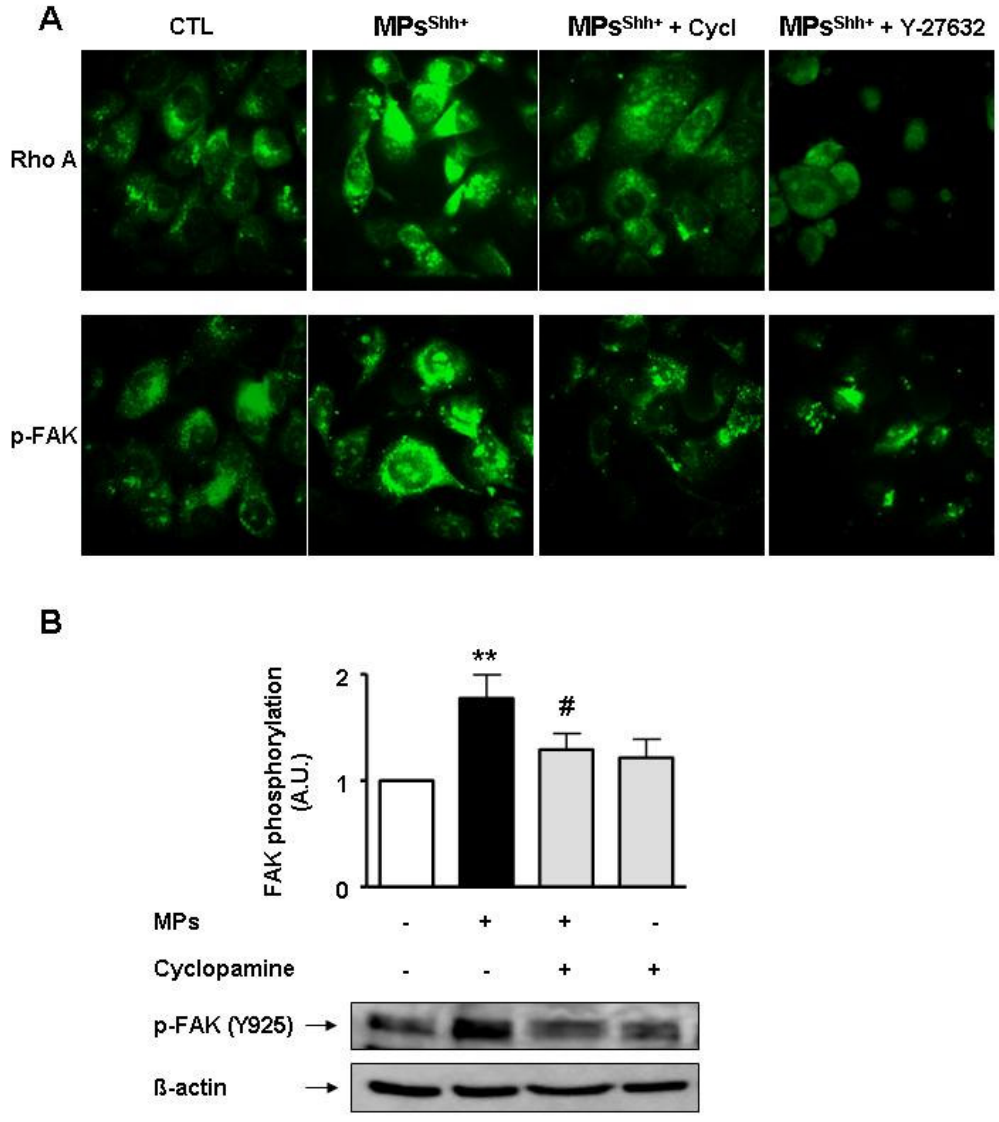
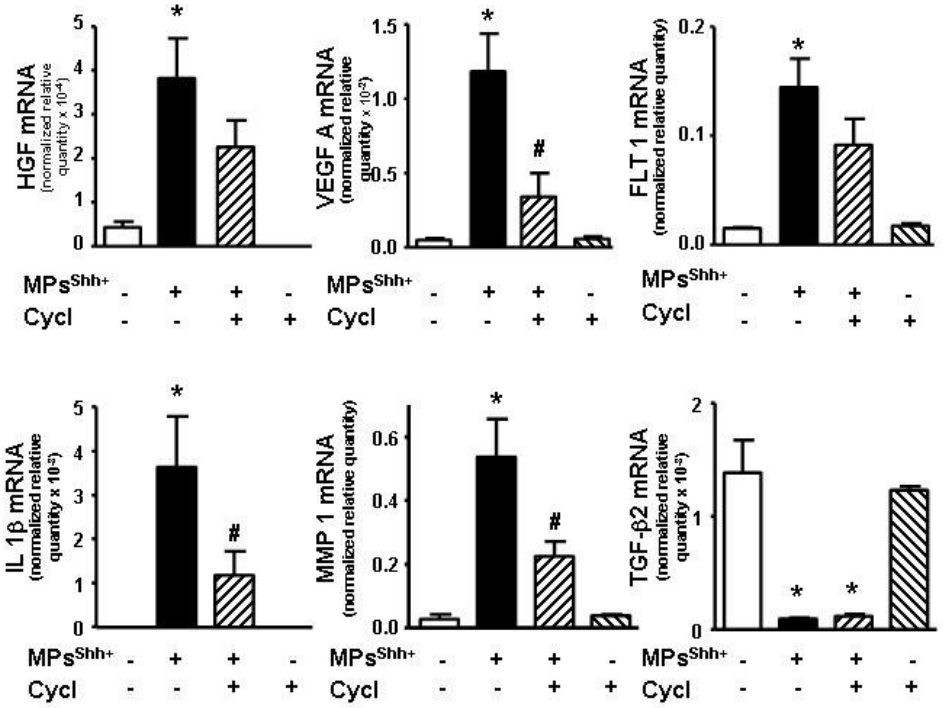

A
CTL
MPsShh+
MPsShh+ + Cycl
MPsShh+ + Y-27632
Rho A
p-FAK
B
FAK phosphorylation (A.U.)
2
1
0
**
#
MPs
Cyclopamine
p-FAK (Y925)
ß-actin

Figure 5


HGF mRNA
(normalized relative quantity x 10-4)
VEGF A mRNA
(normalized relative quantity x 10-5)
FLT 1 mRNA
(normalized relative quantity)
IL 1β mRNA
(normalized relative quantity x 10-5)
MMP 1 mRNA
(normalized relative quantity)
TGF-β2 mRNA
(normalized relative quantity x 10-5)
MPsShh+
Cycl
*
#

MANUSCRIPT II

In vivo pro-angiogenic effects exhibited by sonic hedgehog carried by microparticles

Microparticles (MPs) are vesicles released from plasma membrane during cell activation and apoptosis. Engineering human T lymphocytes undergoing activation and apoptosis generate MPs bearing morphogen Shh (MPs^{Shh+}), which are able to regulate *in vitro* angiogenesis. Here, we investigated the ability of MPs^{Shh+} to modulate neo-vascularization in a model of mouse hind limb ischemia.

Three groups of mice were treated *in vivo* for 21 days: (I) mice receiving vehicle (i.v.); (II) mice receiving MPs (i.v.) at 10 µg/ml of blood; and (III) mice receiving both MPs (i.v.) and cyclopamine (i.p.) 10 mg/kg, a pharmacological inhibitor of Hedgehog signalling.

Laser doppler analysis revealed that the recovery of the ischemic/normal blood flow ratio is higher in MPs-treated mice (1.4 fold) than in controls. In muscle, western blot analysis of the ratio between ischemic and non ischemic hind limbs showed that MPs enhance expression of eNOS (124%), its phosphorylation at activation site (Ser1177) (75%) and strongly decrease caveolin-1 expression (28%) compared to controls. However, MPs do not affect Akt expression and phosphorylation.

Moreover, quantitative RT-PCR displays that MPs treatment induces the augmentation of FGF5 levels and decrease those of Flt4, TSP-1, MMP-1 and MMP-2. In aorta, MPs increase activation of eNOS and Akt, and VEGF expression (87%, 231%, 55%, respectively). Moreover, NO production in response to MPs was significantly increased (51%) in mice aortas versus controls.

Silencing the effects of Shh by cyclopamine completely reversed the improvement of blood flow induced by MPs. Also, in muscle cyclopamine reverse the effects of MPs on eNOS expression and activation. In addition, inhibition of Shh signalling abolishes the effects of MPs on phospho-eNOS (Ser 1177) and VEGF expressions in aorta and these effects are associated with a decrease in NO production induced by MPs.

These findings suggest that MPs harbouring Shh may contribute to reparative neo-vascularization after ischemic injury by regulating NO pathway and the subsequent recuperation of blood flow.

Introduction

Microparticles (MPs) are small plasma membrane fragments shed by cells after blebbing due to activation and/or apoptosis. They play an important role in cell to cell communication because of their ability to act at distant site as well as locally, and to propagate the functional antigens of their parent cell (Martinez *et al* 2005). MPs are involved in pathologies associated with impaired angiogenesis which is a process induced to maintain sufficient oxygen supply. Neo-vascularization occurs not only in physiological events such as embryonic development, wound healing, organ regeneration and placentation (Hoeben *et al*. 2004), but also in pathological conditions such as tumour growth, diabetic retinopathy, atherosclerosis, rheumatoid arthritis, endometriosis and ischemic diseases. Angiogenesis proceeds by a carefully orchestrated series of events and is subjects to a complex control system, which consists of balance between pro-angiogenic and anti-angiogenic factors.

It was documented that MPs are implicated in modulation of different stages of angiogenesis, even if the data concerning the effects they elicit result contradictory. However, the different responses evoked by MPs are dependent on their cellular origin, stimulus of their formation and their concentration.

MPs shedding from ECs shed contain both active proteases, ready to promote matrix degradation, but also the machinery to generate them, presumably initiated by stimuli from environment. Thus, they induce proteolysis during cell migration and three-dimensional morphological organization during angiogenesis (Taraboletti *et al*. 2002). By contrast, another study showed that endothelial MPs are able to impair angiogenesis *in vitro* by affecting all parameters of the capillary network formation (Mezentsev *et al*. 2005). The different concentration of MPs used in these two studies

may explain the difference observed: low concentrations result pro-angiogenic, higher ones being inhibitory.

It has been shown that MPs released by apoptotic lymphocytes inhibited *in vitro* and *in vivo* angiogenesis, by enhancing ROS production, which leads suppression of vascular cell survival, proliferation and migration (Yang *et al*. 2008). Furthermore, the same MPs are able to decrease NO production via PI3K (Mostefai *et al*. 2008b).

On the other hand, MPs generated from human lymphocytes undergoing activation and apoptosis express morphogen Shh (MPs^{Shh+}) at their surface and induce cell differentiation (Martínez *et al*. 2006). Besides, these MPs have concomitant effect of increasing NO production directly by Shh and PI3K pathways and decreasing ROS production by a mechanism dependent on PI3K and ERK cascades (Agouni *et al*. 2007).

Moreover, we have observed that MPs^{Shh+} regulate multiple pathways related to *in vitro* angiogenesis, mainly through the production of pro-angiogenic factors and up-regulation of proteins involved in cell adhesion (see previous manuscript). Thereby, the different effects evoked by MPs from apoptotic and activated/apoptotic cells are probably due to different stimulation at their origin and also to the absence and presence, respectively, of Shh.

Beyond, Shh morphogen orchestrated several processes such as cell proliferation, differentiation and angiogenesis (Porro *et al*. 2009). Concerning angiogenesis, it has been described that activation of Shh cascade evokes formation of capillary-like structures *in vitro* (Kanda *et al*. 2003) and new vessel generation *in vivo* (Pola *et al*. 2001). Moreover the effects promoted by Shh affect modulation of VEGF and eNOS activities (Podlasek *et al*. 2005) and involve PI3K/Akt pathway which also

belongs to intracellular mechanism for endothelial NO release.

MPs^{Shh+} are able to differentially regulate cell events leading to angiogenesis. They increase capillary-like formation through the increase of cell adhesion, the up-regulation of proteins such as Rho A and FAK, and pro-angiogenic factors via the activation of the Shh pathway. Also, MPs^{Shh+} induce endothelial release of pro-angiogenic factors such as VEGF A, Flt1, ICAM-1, MMP-1, IL-1β, and HGF, and these effects were Shh-dependent (see previous articles).

In this study, we used a mouse hind limb ischemia model to investigate the efficacy of MPs^{Shh+} pro-angiogenic properties in neo-vascularization process with respect to recovery of blood flow, implication of pathways which regulate NO production and expression of key factors involved in angiogenesis control system. Understanding the mechanism evoked by MPs in response to ischemia is essential for development of new therapeutic strategies for ischemic cardiovascular diseases.

MATERIALS AND METHODS

MP production

The human lymphoid CEM T cell line (ATCC, Manassas, VA) was used for MP production. Cells were seeded at 10^6cells/ml and cultured in serum-free X-VIVO 15 medium (Lonza, Walkersville, MD). MPs were produced as described previously (Martinez et al., 2006; Agouni et al., 2007). Briefly, CEM cells were treated with phytohemagglutinin (5 µg/ml; Sigma, St. Louis, MO) for 72 hours, then with phorbol-12-myristate-13-acetate (20 ng/ml, Sigma Aldrich) and actinomycin D (0.5 µg/ml, Sigma Aldrich) for 24 hours. A supernatant was obtained by centrifugation at 750 *g* for 15 minutes, then at 1500 *g* for 5 minutes to remove cells and large debris, respectively. MPs from the supernatant were washed after three centrifugation steps (45 minutes at 14000 *g*) and recovered in 400 µl NaCl (0.9% w/v). Washing medium for the last supernatant was used as control. Determination of the amount of MPs was carried out by measuring MP-associated proteins, using the Bradford method, and BSA (Sigma Aldrich) for the standard curve.

Mouse Ischemic Hindlimb Model

Three groups of male Swiss mice (6-8 weeks old) were treated, each three days *in vivo* for 21 days: (I) mice receiving i.v. injection of vehicle; (II) mice receiving i.v. injection of MPs (10 µg/ml of blood); (III) mice receiving i.v. injection of MPs after 30 minutes of i.p. injection of cyclopamine (Biomol, 10mg/Kg). The animals were housed in a regulated environment with a constant ambient temperature of 24°C. They had free access to standard laboratory food and water.

Twenty four hours after the first injection, the femoral artery was occluded surgically using gaseaus anaesthesia (isoflurane, Nicolas Piramal (I) Limited). The ligature was performed on the left femoral artery proximal to the bifurcation to the saphenous and popliteal arteries. After 7 and 21 days of ligature, blood flow was measured as described below. The mice were then sacrificed and tissues were sampled for biochemical and histological analysis. The procedure followed in the care and euthanasia of the study was in accordance with the European Community standards on the care and use of laboratory animals.

Laser-Doppler Blood Flow (LDBF) Analysis

In order to provide a functional evidence of ischemia, laser doppler perfusion imaging was performed in anesthetized mice, as previously described (Tamarat *et al.* 2002). Animals were settled on a heating plate to maintaining a stable cutaneous temperature (35 ± 0.5 °C) throughout the experiments. Leg perfusion was then measured using a Laser Doppler flow probe (PF 408, Perimed). Blood flow was recorded during 5 minutes. At least 2 flow measurements were performed per leg. Blood flow perfusion was expressed as a ratio of left (ischemic) to right (non ischemic) leg.

Capillary density

Ischemic and non ischemic muscles were dissected and progressively frozen in isopentane solution cooled in liquid nitrogen. Sections (7 μm) were first incubated for 30 minutes in PBS containing 5% BSA at room temperature, and then, for 1 hour with rat anti-mouse CD31 antibody (BD Pharmigen, dilution 1:100) followed by an

incubation with goat anti-rat IgG fluorescein conjugate (Southern Biotech, Birmingham, AL, diluition 1:100) to identify capillaries. MRC-1024ES confocal equipement mounted on a Nikon Eclipse TE 300 inverted microscope was used for the optical sectioning of the tissue. Digital image recording was performed using the Laser Sharp Software. Green staining corresponded to FITC detection in sections. Capillary to section were calculated, and the results were then expressed according to ischemic to non ischemic ratios. Capillary number was calculated in at least four randomly chosen fields of a definite area for each animal.

Western blot analysis

In each experiment, the aorta and the skeletal muscle from both ischemic and non ischemic leg were removed and frozen in liquid nitrogen. Samples were homogenized and lysed. Proteins (80µg) were separated in a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. Membranes were then saturated at room temperature for 60 minutes in TBS-T buffer (200mM Tris base, 615mM NaCl pH7.8 and 1% tween20) containing 5% BSA. Membranes were incubated overnight at 4°C with one of the following primary monoclonal antibodies: anti-endothelial NOS (eNOS) (BD Biosciences, San Jose, CA), Phospho-eNOS (ser-1177) (Cell Signaling, Beverly, MA), caveolin-1 (BD Biosciences, San Jose, CA), Akt (Cell Signaling, Beverly, MA), Phospho-Akt (ser473) (Cell Signaling, Beverly, MA) and VEGF (R&D systems, Minneapolis, MN). To visualize protein gel loading, a polyclonal rabbit anti-human β-actin antibody (Sigma Aldrich) was used at 1/2000 dilution. The membranes were then washed at least three times in Tris buffer solution containing 0.05 Tween and incubated for 1 hour at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham, Piscataway, NJ). The protein-antibody

complexes were detected by ECL-Plus Chemiluminescence kit (Amersham) according to the protocol of manufacturer.

Quantitative real time RT-PCR analysis

In another set of experiments, the skeletal muscle from both ischemic and non ischemic legs were frozen in liquid nitrogen and used to investigate the expression of mRNA for 46 transcripts related to angiogenesis by real-time reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR analyses were carried out by Service Commun de Cytométrie et d'Analyses Nucléotidiques from Angers University, using a Chromo 4™ (Bio-Rad, Hercules, CA) and SYBR Green detection. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quantifications were realized according to the ΔCt method and the relative gene-expression levels were normalized using the geometric mean of three housekeeping genes as previously described (Vandesompele *et al*. 2002). Data were expressed as a ratio of ischemic on non ischemic gene-levels expression.

NO determination by electronic paramagnetic resonance (EPR)

After animals sacrifice, aorta and both ischemic and normal muscles were dissected and incubated for NO production for 30 min in Krebs–Hepes buffer containing: BSA (20.5 g/l), CaCl2 (3 mmol/l) and L-arginine (0.8 mmol/l). NaDETC (3.6 mg) and FeSO4.7H2O (2.25 mg) were separately dissolved under nitrogen gas bubbling in 10 ml volumes of ice-cold Krebs–Hepes buffer. These were rapidly mixed to obtain a pale yellow-brown opalescent colloid Fe $(DETC)_2$ solution (0.4 mmol/l), which was used immediately to incubate tissues for 45 min at 37 °C. Then, tissues were immediately frozen using liquid N_2. NO measurement was performed using a table-top

x-band spectrometer Miniscope (Magnettech, MS200, Berlin, Germany). Recording were made at 77°K using a Dewar flask. Instrument setting was 10 mW of microwave power, 1 mT of amplitude modulation frequency, 60 seconds of sweep time and 3 scans.

Statistical analysis

Data are represented as mean ± SEM, *n* represents the number of experiments repeated at least in triplicate. Statistical analyses were performed by Mann-Whitney U-tests (non-parametric). $P < 0.05$ was considered to be statistically significant.

RESULTS

Enhanced recovery of blood flow after induction of hindlimb ischemia in MPs^{Shh+}-treated mice.

In a previous study (Soleti *et al*. manuscript I), we have demonstrated the pro-angiogenic effects of MPs^{Shh+} *in vitro*. Here, to further corroborate the role of MPs^{Shh+} in postnatal angiogenesis, we used a mice model of hind limb ischemia. Just 24 hours before surgery and every 3 days, mice received intravenous injections into caudal vein of vehicle solution or MPs^{Shh+} at concentration of 10µg/ml of blood alone or in combination with cyclopamine by intra-peritoneal injections (10mg/kg). The blood flow in the ischemic (left) and non-ischemic (right) hind limbs was analyzed by an LDBF image analyser and expressed as pseudocolour images (Figure 1A). The surgery led to severe occlusion of the right femoral artery, and there was no significant difference in the degree of post-operative ischemia between groups (data not shown). Also, at day 7 after resection, there was no difference in blood flow in all groups. However, 21 days after surgery, ischemic-to-non-ischemic limb perfusion ratio was modified and became evident in response to distinct treatments (Figure 1B). In control mice (n=14) the recovery of blood flow was not significantly different at day 7 and 21. In MPs^{Shh+}-treated mice, the improvement of blood flow was significantly increased after 21 days from ligation compared to day 7 ($p<0.01$). Moreover, mice treated for 21 day with MPs^{Shh+} showed a significantly increase of blood flow ratio (1.4 fold) than control mice. Interestingly, at day 21, treatment of mice with cyclopamine reversed the enhancement of blood flow evoked by MPs^{Shh+} ($p<0.01$). In mice group receiving concomitant administration of MPs^{Shh+} and pharmacological inhibitor of Shh cascade, cyclopamine, a delay in blood flow recovery at day 21 compared to day 7 was observed. Together,

these findings suggest that MPs^{Shh+} are able to induce improvement of blood flow and that this effect is directly mediated by the Shh signalling, as illustrated by failed recuperation of blood flow in presence of Shh pathway inhibitor.

Also, as shown in figure 2, by targeting PECAM-1, an adhesion protein on EC, we evaluated capillary density. Preliminary results indicate that MPs^{Shh+} are able to induce enhancement of capillary number after 21 days of treatment. This hopeful result needs to be confirmed by increasing the number of experiments.

MPs^{Shh+} promote NO production and enhance VEGF expression in aorta

To determine whether MPs^{Shh+} effects are not restricted to ischemic area, but may target other vascular beds, we used aorta as control. Firstly, we evaluated NO production in aorta. Aortic rings with functional endothelium from mice treated with either vehicle, MPs^{Shh+} alone or together with cyclopamine, preincubated with $Fe(DETC)_2$, exhibited an EPR feature of signal derived from $NO\text{-}Fe(DETC)_2$ (data not shown). The quantitative measurement of the $NO\text{-}Fe(DETC)_2$ signal amplitude was reported in unit/mg weight of dried aorta (A/dW). As shown in figure 3A, MPs^{Shh+} enhanced significantly NO production in aorta compared to control ($p<0.05$). Moreover, treatment with cyclopamine slightly decreased this augmentation. Next, we investigated the pathway involved in MPs^{Shh+}-induced NO production (Figure 3B). MPs^{Shh+} treatment enhanced significantly phosphorylation of eNOS and Akt on their activator site (Ser1177 and Ser472, respectively), without affecting their expression. Interestingly, blockade of Shh cascade by cyclopamine, suppressed the MPs^{Shh+}-induced activation of eNOS, but not that of Akt. These differential effects elicited by cyclopamine suggest a direct involvement of Shh carried by MPs on eNOS activation. However, MPs^{Shh+} treatment, as well as the administration of cyclopamine, did not modify caveolin-1

expression. Moreover, because VEGF plays a role in NO regulation, and VEGF-induced angiogenesis is mediated by NO, we examined its expression. MPs^{Shh+} promoted significant increase of VEGF expression. We also found that this effect was abolished when cyclopamine was administered, indicating that Shh may act as coordinator of crosstalk between VEGF and NO.

MPs^{Shh+} enhance eNOS expression and concomitantly decrease that of caveolin-1 in skeletal muscles

Although experiences of NO production are not yet completed, first results are encouraging because of they reveal a modest increase of NO production ratio between ischemic and normal skeletal muscles following MPs^{Shh+} treatment, as well as a NO release decrease, when cyclopamine is used. After femoral artery ligation, administration of MPs^{Shh+} induced an increase in eNOS expression ($p<0.05$), although their activation was not significantly enhanced. Moreover, expression of caveolin-1, which plays a key role in negative regulation of eNOS, was decreased in muscles from MPs^{Shh+}-treated mice ($p<0.001$). By contrast, neither expression nor activation of Akt was influenced by MPs^{Shh+} administration. Also, VEGF expression was not altered by the same treatment. When Shh cascade was pharmacologically inhibited, the effect induced by MPs^{Shh+} on eNOS expression was completely reversed, as well as its activation; whereas caveolin-1 expression was not affected compared to MPs^{Shh+}-treated mice. Concerning VEGF, cyclopamine was able to decrease its expression (Figure 4). These data suggest that the effects evoked by MPs^{Shh+} on eNOS expression and activation may be related to Shh, whereas, the effect elicited by MPs^{Shh+} on caveolin-1 occurs by a Shh-independent mechanism. Although endogenous Shh controls VEGF basal expression, MPs^{Shh+} do not affect this pathway.

MPs^{Shh+} modulate gene expression of angiogenic factors in skeletal muscles

To investigate candidate targets of MPs^{Shh+} implicated in vessel formation process, we have evaluated the mRNA expression levels of 46 different angiogenic factors by real-time quantitative RT-PCR (see annexe). Among these, MPs^{Shh+} were able to enhance FGF5 mRNA expression and to decrease those of TSP1 and Flt4 probably by a Shh-dependent mechanism (Figure 5). On the other hand, MPs^{Shh+} treatment reduced level expression of MMPs, such as MMP-1 and MMP-2, probably in Shh-independent manner. The evaluation of transcripts is ongoing. Despite that, it is evident that MPs^{Shh+} can modulate the expression of different angiogenic factors.

DISCUSSION

The present study is the first report showing the stimulatory effects of MPs^{Shh+} on *in vivo* angiogenesis and the resultant blood flow recovery in an experimental ischemic hind limb ischemia. The effects evoked by MPs^{Shh+} involve NO pathway and differential modulation of angiogenic factor expressions. However, not all the events implicated on neovascularization and triggered by MPs^{Shh+} are modified in presence of Shh inhibitor, cyclopamine, indicating that other molecules carried by MPs play a role in this phenomenon. These evidences corroborate the hypothesis that MPs^{Shh+} are efficient bioeffectors able to transfer angiogenic message.

Our data demonstrate that administration of MPs^{Shh+} stimulated blood flow recovery in ischemic limbs after femoral artery ligation. This effect is mediated directly by Shh as illustrated by the abrogation of the capacity of MPs^{Shh+} in increasing perfusion in presence of the Shh antagonist, cyclopamine. Besides, the stimulation of blood flow recovery seems to be accompanied by an increase in capillary density in ischemic hind limb muscle suggesting that MPs^{Shh+}-induced stimulation of blood flow is due to neovessel formation.

Moreover, MPs^{Shh+} treatment was able to promote NO production in the aorta, a vascular bed far from ischemic area. The increase in NO release is associated with an enhanced of both eNOS and Akt activities, as reflected by increase in eNOS and Akt phosphorylation at their activator site. The effect triggered by MPs^{Shh+} treatment on eNOS activation is inhibited when Shh pathway is silenced by cyclopamine. In a previous study (Agouni *et al.* 2007), we have found that Shh bound to MPs directly produces NO release from endothelial cells via mechanisms sensitive to PI3K inhibitor. Moreover, it has been reported that Akt is important for Shh signalling (Riobò *et al.*

2006; Kanda *et al*. 2003). In this study we also found that MPs^{Shh+} increased expression of VEGF by a mechanism sensitive to cyclopamine. VEGF, in addition to be pivotal angiogenic factor, plays a role in NO regulation, although it is not completely elucidated whether it acts upstream or downstream of NO (Papapetropoulos *et al*. 1997; Parenti *et al*. 1998). Besides, exogenous Shh protein induces eNOS and VEGF expressions (Podlasek *et al*. 2005). Thus, our results also suggest that Shh may act as coordinator of crosstalk between VEGF and NO and that eNOS and VEGF display synergism in promoting NO production.

Regarding skeletal muscles, MPs^{Shh+} induced an increase in eNOS expression, although their phosphorylation in activator site was not significantly enhanced. Concomitantly they decreased caveolin-1 expression, which acts as negative regulator of eNOS (Ju *et al.* 1997). On the other hand, MPs^{Shh+} did not affect expression and activation of Akt. eNOS activation occurred by a Akt-independent mechanism, but it is also subject to regulation by other signalling pathways, which involved probably Ca^{2+} signals. These data may explain, at least in part, the augmentation of NO production observed in muscles of MPs^{Shh+}-treated mice. Cyclopamine administration completely reversed the effect on eNOS expression, as well as its activation, which may be involved in the blunting of the increase of NO production by MPs^{Shh+}; whereas caveolin-1 expression was not modify after the same treatment, suggesting that eNOS expression and activation may be related to Shh, while changes in caveolin-1 expression occurs independently from Shh pathway.

Moreover, our results suggest that MPs^{Shh+} can modulate new vessels formation either at posttranscriptional, and at transcriptional level. In fact, the analysis of transcripts of a large of genes involved in angiogenesis revealed that MPs^{Shh+} strongly

influence the angiogenic switch. For example, they enhance expression of FGF5, a potent mitogenic factor, which is widely expressed in embryonic but scarcely in adult tissue, in which is in large measure restricted to the central nervous system (Allerstorfer *et al*. 2008). MPs^{Shh+} decrease expression of TSP1, a protein well known to be antiangiogenic, (Rastinejad *et al*. 1989) which acts by inhibiting endothelial cell proliferation and migration (Sottile 2004). Other genes, such as those of Flt4, are not modified after treatment of MPs^{Shh+}. Although Flt4 receptor is essential in the formation of the primary cardiovascular network (Dumont *et al*. 1998), later to development, its expression become restricted mainly to lymphatic vessels (Kaipainem *et al*. 1995; Kukk *et al*. 1996) or in tumour blood vessels (Valtola *et al*. 1999). Moreover, MPs^{Shh+} administration decreases level expression of MMP-1 and MMP-2 that was sensitive to cyclopamine. These findings may be explained by the fact that MMPs activity is temporally regulated (Ghajar *et al.* 2008). In fact, there is a number of evidences that MMP-1 is present at the leading tip of invading cells (Seiki. 2003; Itoh and Seiki M. 2006) and at proteolytic zones of the leading edge of invading cells (Wolf *et al*. 2007). Moreover, MMP-1 has the ability to activate MMP-2 and thus, extends its degradive effect on extracellular matrix (Visse and Nagase 2003; Seiki 2003). The interaction between pericytes and the newly formed endothelial tubes is accompanied by silencing of MMPs activities (van Hinsbergh and Koolwijk 2008). All these regulated events may explain their diminished expression after MPs^{Shh+} treatment.

In conclusion, we provide evidence that MPs^{Shh+} are effective angiogenesis stimulators in post-ischemic condition, by promoting blood flow recovery, neovessels formation and acting on angiogenic switch. These data, collectively, suggest potential usefulness of MPs^{Shh+} for therapeutic angiogenesis in ischemic condition. Further

investigations aim to define the subtle mechanisms by which MPs^{Shh+} are able to modulate *in vivo* vessel formation are needed.

FIGURE LEGENDE

Figure 1. MPs^{Shh+}-treated mice display enhanced recovery after induction of hind limb ischemia. (A) Representative pseudocolour Doppler flow images at day 7 and 21 following ligation of the left femoral artery in mice receiving vehicle (n=14), MPs^{Shh+} alone (n=14), or with cyclopamine (n=9). (B) Histogram showing the quantification of the limb perfusion as a ratio of blood flow on the ischemic and non-ischemic sides (mean ± SEM). At day 7 after surgery, perfusion rates are not different between groups. At day 21 the recovery of blood flow ratio is greater in MPs^{Shh+}-treated mice (1.4 fold) than in mice receiving vehicle ($p<0.05$). Treatment with cyclopamine completely reverses the improvement of blood flow induced by MPs^{Shh+} ($p<0.01$).

Figure 2. MPs^{Shh+} treatment leads to capillary density enhancement. (A) Representative pictures of PECAM-1 immunostaining of capillaries in skeletal muscle of legs at day 21, from mice receiving vehicle (-MPs^{Shh+}) and MPs^{Shh+}. (B) Quantification of PECAM-1 stained capillary density in vehicle- and MPs^{Shh+}-treated mice, data are expressed as ratio of ischemic to non-ischemic leg and represent the mean of five sections from 2 animals.

Figure 3. NO production and VEGF expression are enhanced in aorta after MPs^{Shh+} administration. (A) NO production significantly increased (51%) in MPs^{Shh+}-treated mice (n=4) when compared with vehicle-treated mice (n=4). However, in mice receiving cyclopamine (n=3), NO production is decreased weakly. Values are expressed in units/mg weight of dried aorta as mean ± SEM (*$p<0.05$ *vs* in absence of MPs^{Shh+}). (B) MPs^{Shh+} increased activation of eNOS, Akt and VEGF expressions (87%, 231%, 55% respectively). Caveolin-1 expression is not significantly different in aortae from all groups. Pharmacological inhibition of Shh cascade abolished the effects of MPs^{Shh+} on

phospho-eNOS (Ser1177) and VEGF expressions. Data are representative of five separate blots, and the densitometry values are expressed in arbitrary units (A.U.) as mean ± SEM. *p<0.05, **p<0.01 *vs* in absence of MPs^{Shh+}; #p<0.05 *vs* in presence of MPs^{Shh+} alone.

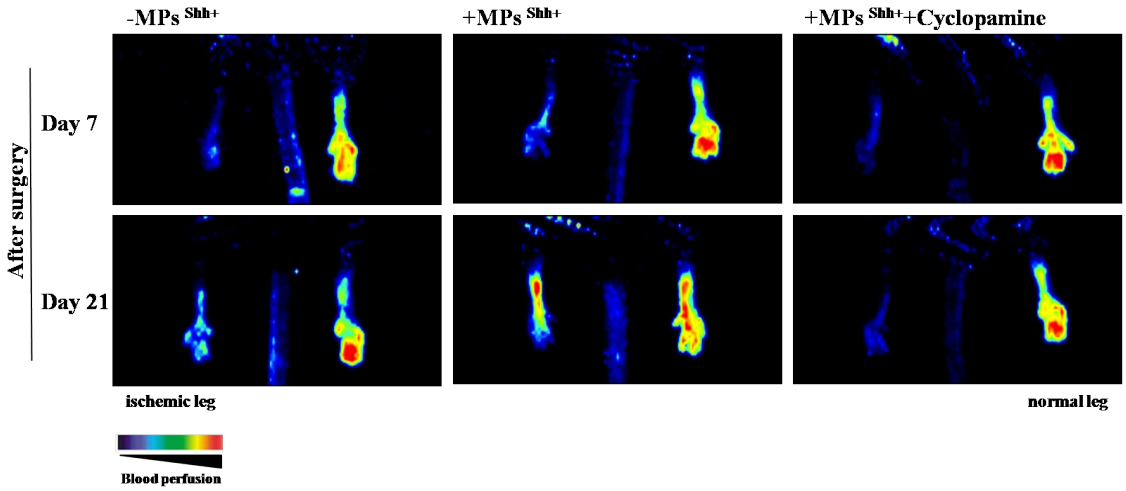
Figure 4. MPs^{Shh+} are able both to increase eNOS and decrease caveolin-1 expression in skeletal muscles. (A) Quantification of NO production in muscles after ischemia reveals a tendency of increasing NO levels in MPs^{Shh+}-treated mice, as well as its diminution after cyclopamine administration (in absence of MPs^{Shh+} n=1, in presence of MPs^{Shh+} alone n=2, and in combination with cyclopamine n=2). (B) Immunoblot analyses reveal that MPs^{Shh+} enhanced expression of eNOS (124%) and decrease that of caveolin-1 (28%) compared to vehicle-treated mice. However, MPs^{Shh+} treatment did not affect Akt expression and phosphorylation, as well as VEGF expression. Moreover, cyclopamine is able to inhibit the effect induced by MPs^{Shh+} on eNOS expression and also, influence VEGF expression. Furthermore, Shh inhibition did not alter level expression of caveolin-1 evoked by MPs^{Shh+}. Data are representative of five separate blots, and the densitometry values are expressed as mean ± SEM of ratio of ischemic to non-ischemic values. *p<0.05, ***p<0.001 *vs* in absence of MPs^{Shh+}, #p<0.05 *vs* in presence of MPs^{Shh+} alone.

Figure 5. MPs^{Shh+} take part in balance of angiogenic factors expression. Quantitative RT-PCR analysis was performed on ischemic and non-ischemic skeletal muscles from mice treated with vehicle, MPs^{Shh+} alone or together with cyclopamine. FGF5 expression results increased, whereas, TSP1, Flt4, MMP-1 and MMP-2 transcripts decrease after MPs^{Shh+} administration. Moreover, the effects elicited by MPs^{Shh+} on TSP1, Flt4 and FGF5 seem to be mediated by Shh. Data are expressed as

ischemic/normal ratio of normalized relative quantity (n=1-4).

Figure 1

A


-MPs Shh+
+MPs Shh+
+MPs Shh++Cyclopamine
After surgery
Day 7
Day 21
ischemic leg
normal leg
Blood perfusion

B

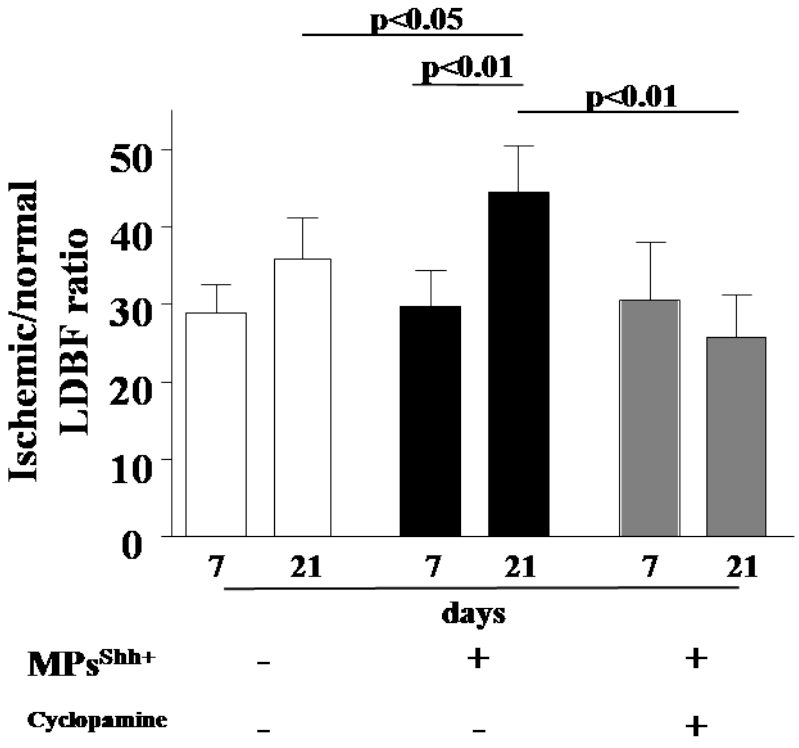
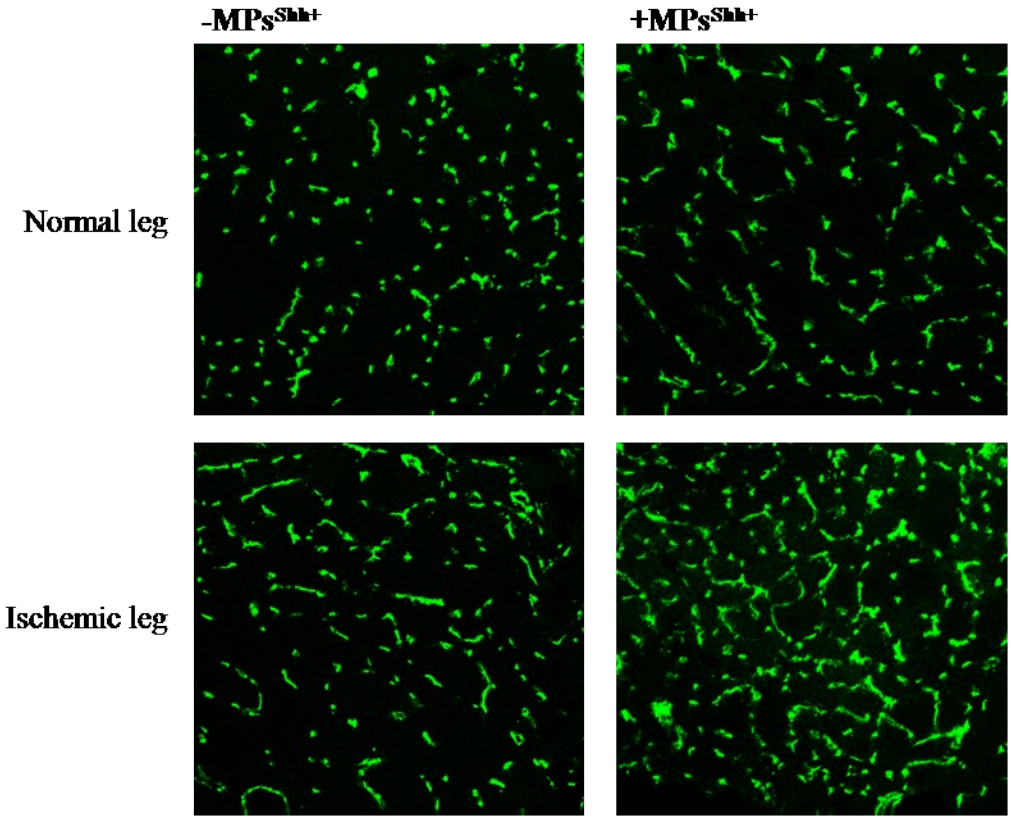

p<0.05
p<0.01
p<0.01
Ischemic/normal LDBF ratio
50
40
30
20
10
0
7
21
7
21
7
21
days
MPsShh+
-
+
+
Cyclopamine
-
-
+

Figure 2

A


-MPs^Shh+
+MPs^Shh+
Normal leg
Ischemic leg

B

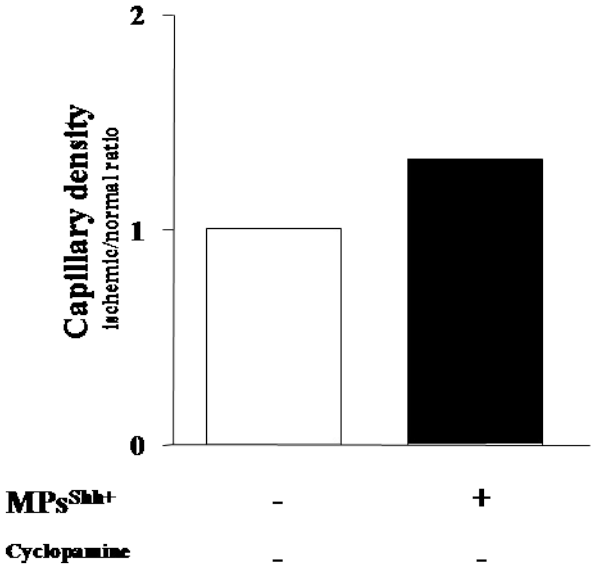
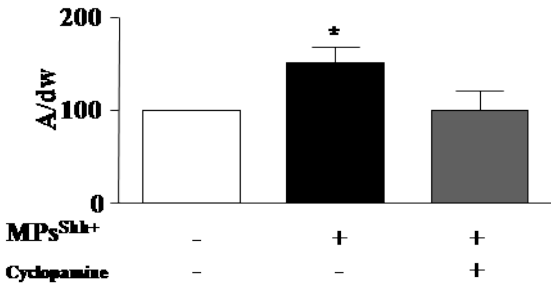

2
1
0
Capillary density
Ischemic/normal ratio
MPs^Shh+
-
+
Cyclopamine
-
-

Figure 3

A


200
100
0
A/dw
*
MPsShh+
Cyclopamine
–
+
+
–
–
+

B

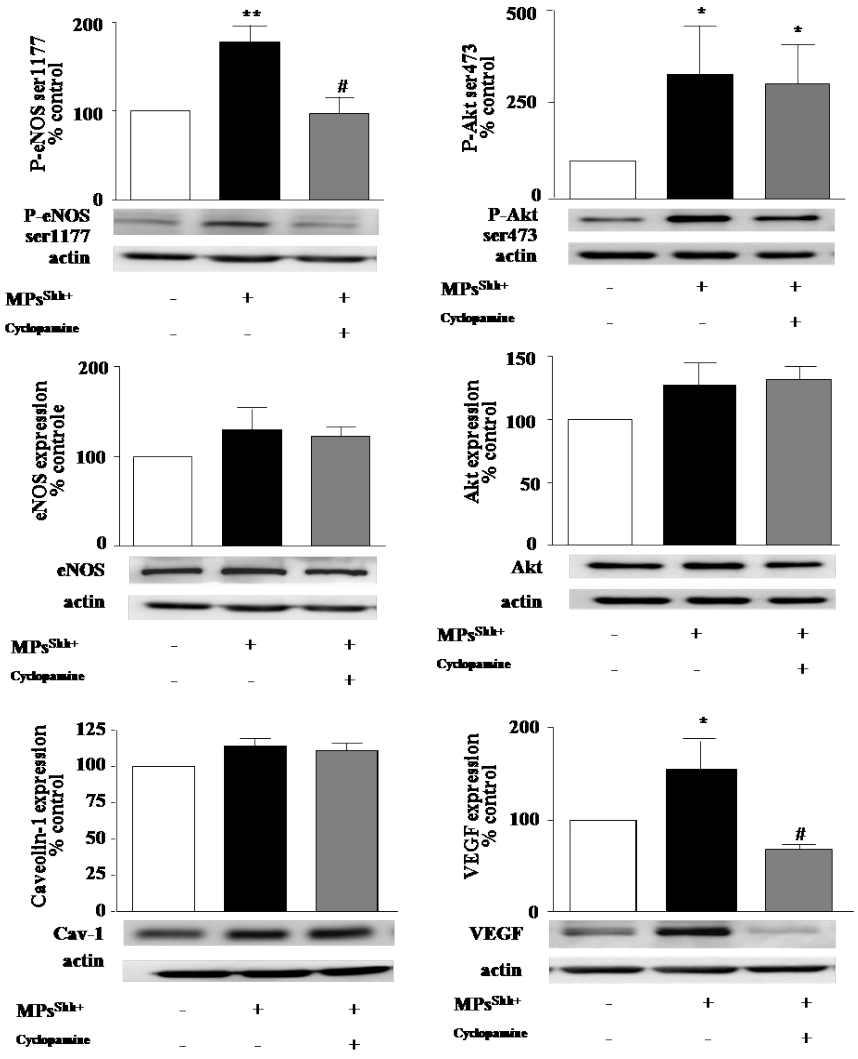
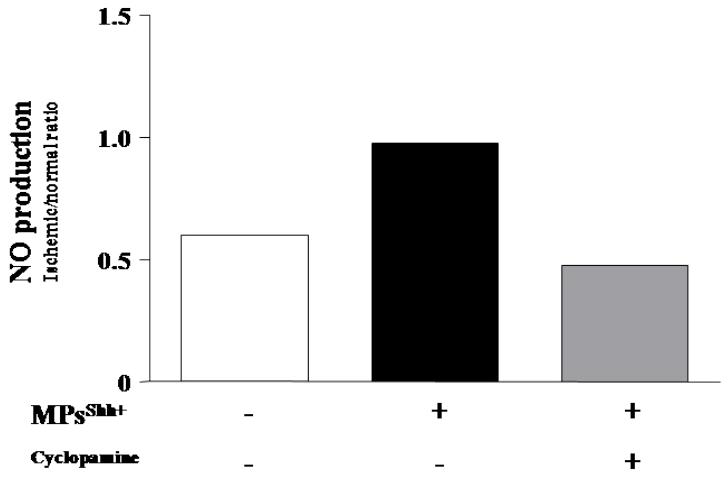

P-eNOS ser1177 % control
200
100
0
**
#
P-eNOS ser1177
actin
P-Akt ser473 % control
500
250
0
*
*
P-Akt ser473
actin
eNOS expression % controle
200
100
0
eNOS
actin
Akt expression % control
150
100
50
0
Akt
actin
Caveolin-1 expression % control
125
100
75
50
25
0
Cav-1
actin
VEGF expression % control
200
100
0
*
#
VEGF
actin
MPsShh+
Cyclopamine
–
+
+
–
–
+

Figure 4

A


NO production
Ischemic/normal ratio
1.5
1.0
0.5
0
MPs Shh+ - + +
Cyclopamine - - +

B

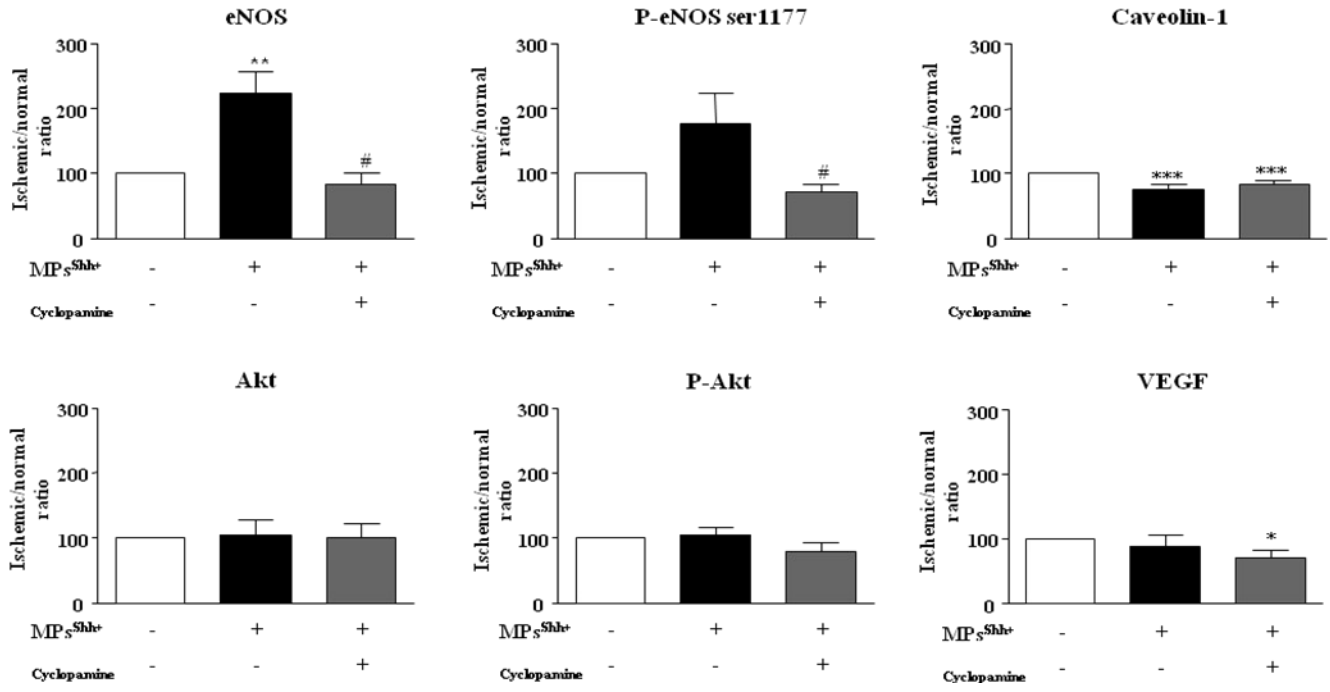

eNOS
P-eNOS ser1177
Caveolin-1
Akt
P-Akt
VEGF
Ischemic/normal ratio
300
200
100
0
**
#
***
*
MPs Shh+ - + +
Cyclopamine - - +

Figure 5

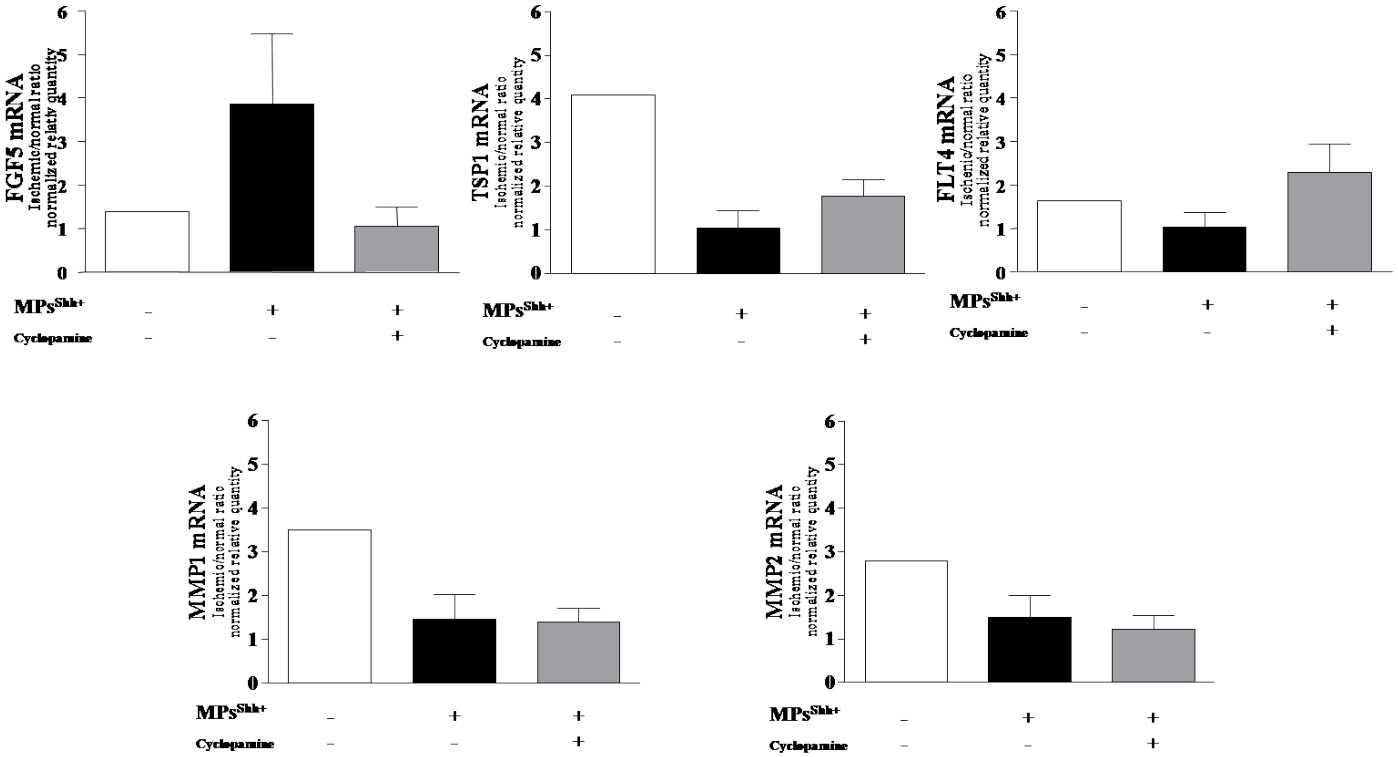
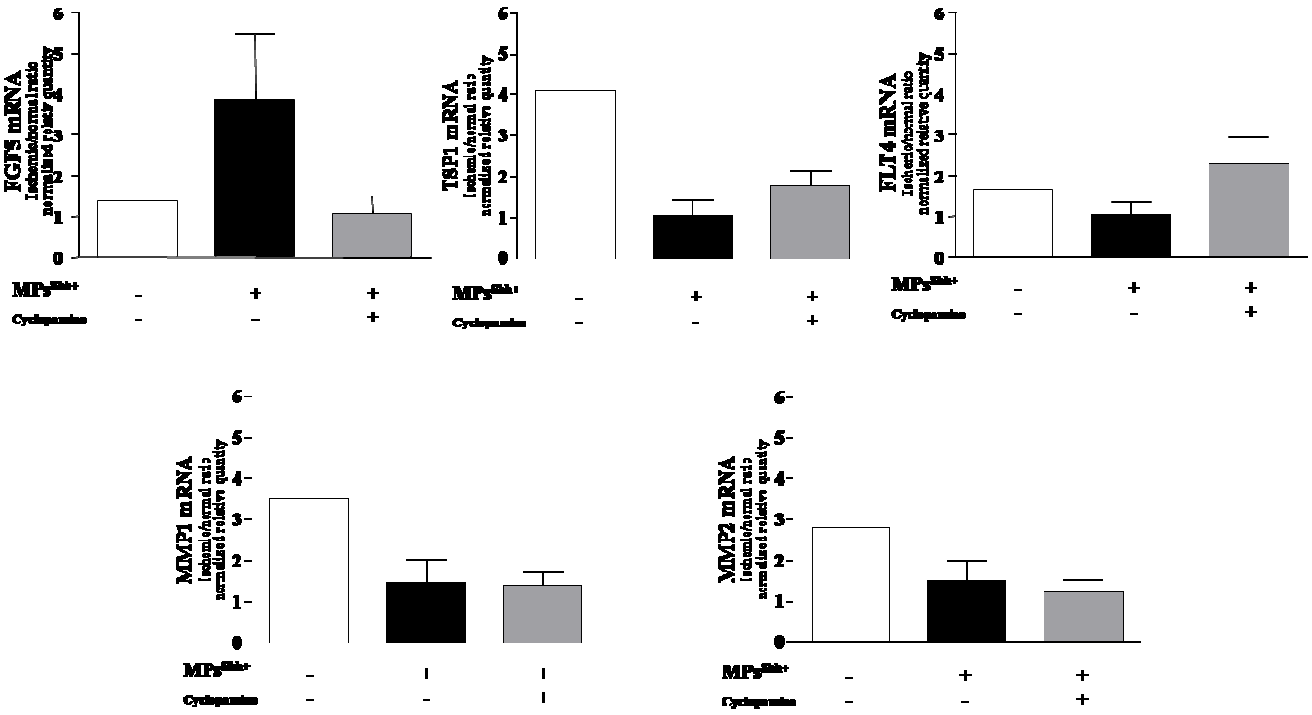

FGF5 mRNA
Ischemic/normal ratio
normalized relative quantity
TSP1 mRNA
Ischemic/normal ratio
normalized relative quantity
FLT4 mRNA
Ischemic/normal ratio
normalized relative quantity
MMP1 mRNA
Ischemic/normal ratio
normalized relative quantity
MMP2 mRNA
Ischemic/normal ratio
normalized relative quantity
0
1
2
3
4
5
6
MPs^Shh+
Cyclopamine
–
+

Figure 5


FGF5 mRNA
TSP1 mRNA
FLT4 mRNA
MMP1 mRNA
MMP2 mRNA
MPs
Cyclosporine

REVIEW

Sonic Hedgehog Pathway as a Target for Therapy in Angiogenesis-Related Diseases

Chiara Porro[1,2], Raffaella Soleti[1], Tarek Benameur[1], Angela Bruna Maffione[2], Ramaroson Andriantsitohaina[1], Maria Carmen Martinez[1,*]

[1]*CNRS UMR, 6214, INSERM, U771, Université d'Angers, Angers, France;* [2]*Department of Biomedical Sciences, Faculty of Medicine, University of Foggia, Italy*

Abstract: Hedgehog (Hh) proteins belong to a class of morphogens involved in many biological processes during embryonic development; they are relatively silent during normal adult life although they may be recruited postnatally in response to tissue injury. Three secreted proteins have been identified: Sonic hedgehog (Shh), Desert hedgehog and Indian hedgehog. The interaction of Hh ligand with its receptor Patched-1 triggers the activation of smoothened and initiates transduction events that lead to the regulation of transcriptional factors belonging to the Gli family. Hh pathway orchestrates both coronary development and adult coronary neovascularisation by controlling the expression of multiple pro-angiogenic genes and anti-apoptotic cytokines. Shh pathway enhances the recruitment of endothelial progenitor cells in addition to the mechanisms described for other Hh and concurs to its myocardial protection. In cerebral ischemia, Hh mimicking molecules has been reported to limit damages caused by vessel occlusion. Besides, Shh carried by microparticles corrects endothelial injury through nitric oxide release. Anomalous activations of Hh pathway are implicated in various types of tumours including medulloblastoma, carcinoma of esophagus, stomach, pancreas and colon. Hh can influence angiogenesis in both positive and negative manner and they may have implication for therapeutic strategies to treat either ischemic or cancer diseases.

INTRODUCTION

Hedgehog (Hh) family proteins are morphogens widely distributed throughout much of the animal kingdom being first identified in *Drosophila melanogaster* [1]. In vertebrates, genome duplication has given rise to multiple Hh genes. There are three mammalian Hh genes named *Sonic hedgehog* (*Shh*), after a popular video game character, *Desert hedgehog* (*Dhh*), after an Egyptian species of Hh (*Hemiechinus auritus*), and *Indian hedgehog* (*Ihh*), a Hh species endemic in Pakistan (*Hemiechinus micropus*) [2-5].

The core components of the Hh-family signalling pathway have been found highly conserved during the divergent evolution of insects and mammals, but Shh is the most well characterized human homologue [6, 7].

Hh morphogens can act as intracellular signals and are responsible for multiple cellular fate decisions [8]. Of considerable importance are the roles of Hh family in the development of various embryonic tissues, including brain, spinal cord, axial skeleton, limb, lung and gut [9-12], in the vascularisation [13] and in the maintenance of adult tissue homeostasis, tissue repair during chronic persistent inflammation, and carcinogenesis [14-19].

The present review summarizes the main molecular mechanisms of the Hh signalling as well as the consequences of Hh pathway activation in pathologies such as ischemic and cancer diseases. Finally, we propose helpful indications for the design of effective strategies in using molecules acting on Hh pathway.

*Address correspondence to this author at the CNRS UMR, 6214, Faculté de Médecine, Rue Haute de Reculée, Angers, F-49045 France; Tel: +33 2 41 73 58 57; Fax: +33 2 41 73 58 95;
E-mail: carmen.martinez@univ-angers.fr

1574-3624/08 $55.00+.00

Hh SIGNALLING PATHWAY

Despite extensive studies addressing the regulation and function of the pathway in different organs, the exact mechanisms by which Hh signals are transmitted and how they elicit diverse activities in a cell-specific manner remain obscure.

Newly synthesized Hh proteins undergo a series of post-translational processing reactions within the secretory pathway that result in the formation and cell surface presentation of the active species in signalling (Fig. **1**). Although elements of the reaction mechanisms employed are also represented in the metabolism of other proteins, Hh family members are the only examples of signalling proteins known to be covalently modified by cholesterol [20].

Hh is translated into an approximately 47 kDa precursor protein and, during their maturation, undergoes two lipophilic modifications. Following cleavage of an amino-terminal signal sequence upon entry into the secretory pathway, the Hh protein undergoes an autocatalytic processing reaction that involves intramolecular cleavage between Gly-Cys residues that form part of an absolutely conserved Gly-Cys-Phe tripeptide [12, 21-23].

A 19 kDa amino-terminal product (termed Hh-Np) is responsible for the signalling activity of Hh, whereas a 26 kDa carboxy-terminal product functions as a cholesterol transferase, covalently modifying the N-terminal product on its terminal amino acid [24, 25]. The amino-terminal product of this cleavage receives a covalent cholesteryl adduct [24]. Besides, the N-terminal product of Hh proteins is subjected to a second modification that is catalyzed by an acyltransferase encoded by *Skinny hedgehog* (*ski*). This additional modifying adduct is a fatty acid, usually palmitate, and is

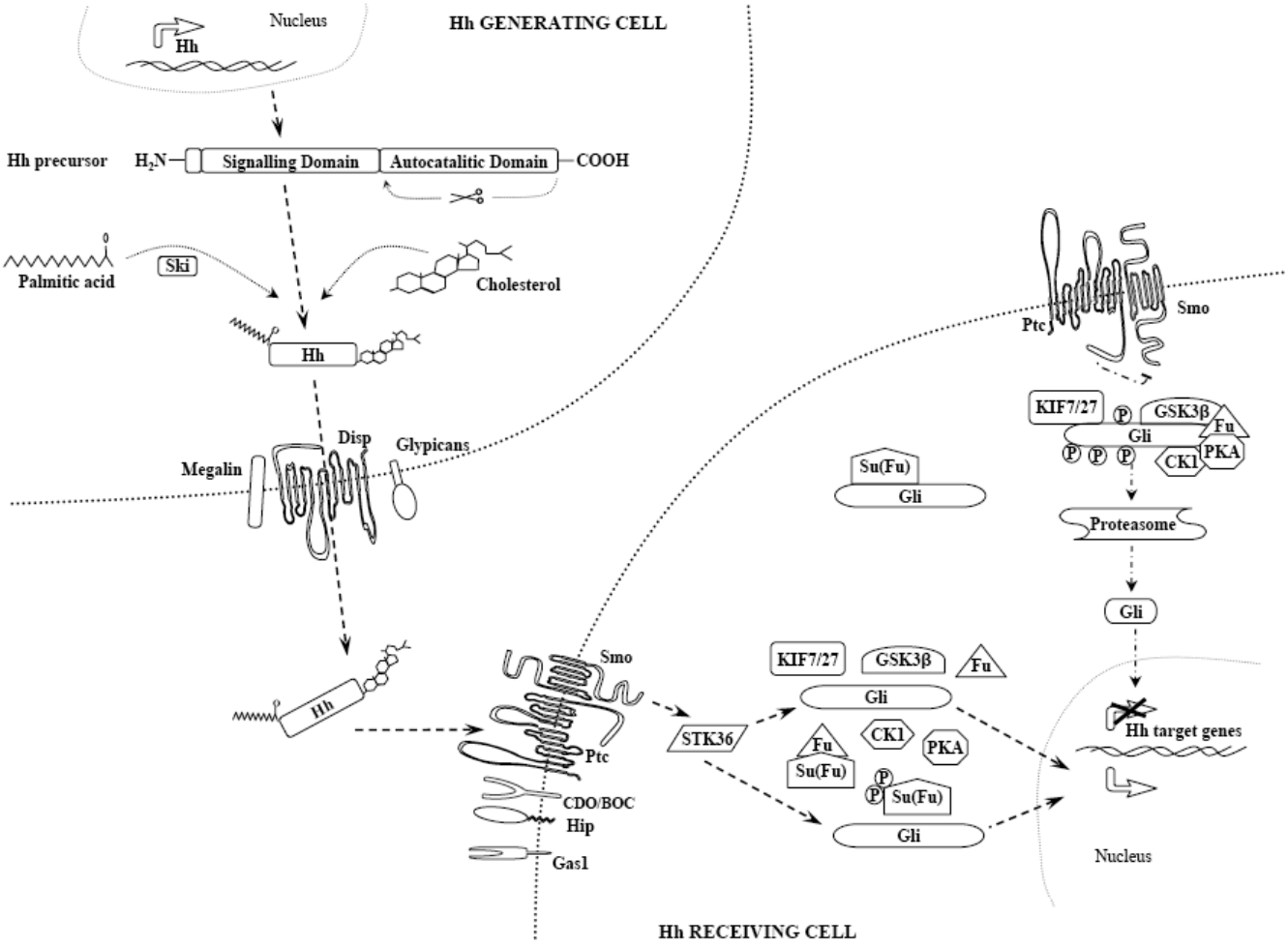


Fig. (1). Schematic diagram of Hh protein biogenesis and signalling pathway. Hh protein is synthesized as a 47 KDa precursor protein; its auto processing generate N-terminal domain to yield a 19KDa product, dually lipidate that mediates signalling. C-terminus receives cholesterol by 26 KDa C-terminal product of auto cleavage and N-terminus receives palmitic acid by Ski. Hh protein is then trafficked to cell surface and released by aid of Disp and other cellular activities such Megalin and Glipicans. Hh signalling regulates balance of its transcriptional factor target, Gli, from the repressor form to activator form. On receiving cells, in absence of Hh, Ptc blocks Smo transduction function. This inhibition favours the cytoplasmic retention and inactivation of Gli. Repressed Smo induces the assembly of complex of full-length Gli and SUFU and, also, of the cytoplasmic Gli degradation complex, in which Gli is phosphorylated initially by CKI and then by GSKβ and PKA. Hyperphosphorylated Gli and its subsequent polyubiquitination induce its processing by proteasome. Released truncated Gli enters the nucleus and represses the transcription of target genes. Hh-binding to complex of Ptc and CDO/BOC but also with other proteins, such Hip and Gas1, derepresses the activity of Smo, allowing the activation of STK36 serine/threonine kinase. STK36 inhibits the formation of Gli degradation complex and the phosphorylation of SUFU, promoting stabilization of full-length Gli and its accumulation into nucleus, where it can bind DNA and regulate the expression of its target genes.

found in an amide linkage with the amino-terminal cysteine that is exposed by signal sequence cleavage. Because this Cys residue is the first of a pentapeptide, which is widely conserved among species, there is a possibility that these residues and others nearby may constitute an important determinant for the palmitoylation reaction [20, 26]. These hydrophobic modifications have profound effects on the properties of Hh proteins, because they promote retention of Hh in the plasma membrane and, paradoxically, play a crucial role in the regulation of the range of Hh signalling in a tissue.

Conflicting reports have been published on the role of cholesterol moiety in Hh signalling. The cholesterol modification anchors Hh-Np to the membrane of the producing cells and therefore originally Hh-Np is considered mainly responsible for short-range signalling. However, subsequent studies have shown that Hh-Np is essential for the activity of Hh signalling, especially the long-range effects far from the producing cells [21, 27-29]. The palmitoylation is essential to both the activity of the Hh protein and the signal range [5, 30, 31].

Since most Hh proteins are anchored on the cell membrane attributing to lipid modification, various mechanisms have been proposed for Hh long-range effects, three of which are active diffusion through extracellular matrix, indirect transmission through secondary signal cascade and cy-

tonemes through cellular extensions [32-35]. Also, Zeng *et al*. [35] have shown the presence of two forms of soluble Shh-Np, far from producing source, namely monomer and multimer. In addition, they reported that the multimeric form cannot be observed if cholesterol modification is disrupted, leading to the hypothesis that Shh-Np embeds the lipid moieties in the hydrophobic surface to form the multimers and thereby diffuse freely to the distal compartment. Further studies have demonstrated that protein-protein and protein-lipid interactions are required for Shh-Np multimer formation [36, 37]. Another suggestion based in the ability of Hh to remain anchored on the plasma membrane has been recently reported. Martínez and co-workers [38] have shown that long-range Hh signal can be mediated also when Shh is carried by microparticles (MPs), small membrane fragments shed from blebbing plasma membrane of various cell types, such as platelets, T and B cells, monocytes, and endothelial cells during activation by agonists, shear stress or apoptosis [39]. MPs harbouring the morphogen Shh (MPs^{Shh+}), generated from engineering human T lymphocytes undergoing activation/apoptosis or from plasma from diabetic patients, are functionally active and induce intracellular responses in receiving cells probably due to ligand/receptor interaction [38].

Because of lipid modifications, specific cellular activities are required in signal-generating and in signal-receiving cells (Fig. **1**). These include Dispatched (Disp) and Exotosin (EXT, homologous to Drosophila tout-velu, Ttv). Dispatched is a 12-pass transmembrane-domain protein. Five adjacent transmembrane segments compose a sterol-sensing domain required for Hh release and are also representative of other members of proteins that include Patched (Ptc-1), a component of the Hh receptor [40-42]. By contrast, EXT regulates synthesis of proteoglycans and functions to allow trafficking of Hh. Hh is also affected by the glycoprotein Hh-interacting protein (Hip) to which Hh binds with high affinity. Hip is induced in receiving cells and acts in parallel to Ptc-1 as an attenuator of the Hh response. Hip is also a target gene in Hh signalling in a negative feedback regulating manner [43-46].

Moreover, Hh trafficking potentially involves interaction with other proteins such glypicans, megalin and growth arrest-specific gene 1 (Gas1). Glypicans such Dally and Dally-like proteins (Dlp) are substrate of Ttv and transfer Hh along the cell membrane [47]. Megalin and Gas1 are proteins that can bind Shh and can also modulate its activity [48]. Additional proteins function to receive the Hh signal: Patched, CDO (cell-adhesion-molecule related/down-regulated by oncogenes, also known as CDON), BOC (bioregional cell adhesion molecule-related/down-regulated by oncogenes (Cdon) binding protein) and Smoothened (Smo) [49].

Unusually for a signal receptor, Ptc-1 is not activated in response to ligand binding but rather is suppressed by its interaction with Hh. Two *Ptc* genes exist in vertebrates, *Ptc1* and *Ptc2*. These genes encode for 12-pass transmembrane receptors with two extracellular hydrophilic loops that mediate Hh binding [50, 51]. Smo is a 7-transembrane-domain protein homologous to G-protein-coupled receptors. It does not bind Hh directly but acts a focal point of Hh signal transduction [14, 15, 17, 19, 52-56]. In its unbound state, Ptc-1 represses the activity of Smo signal transducer, which is necessary for the transduction of the Hh signalling across the plasma membrane and into the cell. Therefore Hh activates Smo by binding to inactivating Ptc-1. Hh may induce Smo to change its conformation or to oligomerize. Recent findings provide evidence for constitutive Smo dimers or multidimers that adopts different intracellular conformation in response to Hh. It is interesting to note that Smo is more fully phosphorylated, it localizes more predominantly to the plasma membrane as opposed to internal vesicles and it is present at higher overall levels [17, 57].

Cyclopamine, which is isolated from the corn lily *Veratrum californicum*, and synthetic small molecules including CUR61414 and SANT1-4 have been identified as modulating Smo activity. It has been proposed that they act at level of the hydrophobic domain delineated by 7-putative transmembrane domains of Smo. Another class of molecules including SAG derived from a chlorothiophen moiety acts as Smo agonists when applied to Hh responding cells [58-61]. Also, it has been showed that vitamin D3 and pro-vitamin D3 bind to Smo with high affinity and inhibit its activity [62]. Moreover, sterol synthesis inhibitors reduce Shh target gene transcription and block Shh pathway-dependent proliferation probably acting on the pathway upstream or at the level of Smo [63].

Once Shh binds to Ptc-1, the activity of Smo is no longer inhibited and multimolecular network transduces Hh signal through activation and nuclear translocation of members of the glioma-associated oncogene (Gli) family of transcription factors (Gli1, Gli2 and Gli3). The process by which Gli is regulated consists in post-transcriptional transition and balance of Gli from the repressor form to activator form. The Gli proteins bind DNA in a sequence-specific manner through the last three fingers of 5-zinc-finger domain [64]. They have specialized functions and distinct temporal and spatial expression patterns. Gli1 can only act as an activator of transcription, whereas Gli2 and Gli3 can be processed to function as transcriptional activators or repressors [65, 66].

Gli proteins activity is controlled by two different complexes (Fig. **1**). The first is a multicomponent cytoplasmic complex which includes the kinesin-like proteins KIF7 and KIF27 [67], the serine/threonine kinase Fused (FU) [68], casein kinase I (CKI), glycogen synthase kinase-3ß (GSK3ß) and protein kinase A (PKA). Inactivated Smo induces the formation of the cytoplasmic Gli degradation complex, in which Gli members are phosphorylated initially by CKI, and subsequently by GSK3ß and PKA [69, 70]. Hyperphoshorylated Gli confers binding to ßTRCP, an ubiquitin F-box protein [71-73]. Subsequently polyubiquitinated Gli is processed by proteasome machinery to release its intact N-terminal moiety that enters the nucleus and represses the transcription of target genes. The second complex composed of full-length Gli and the suppressor of Fused (SUFU) [74] results in the cytoplasmic retention of Gli and then in its inactivation.

With regard to the second complex, Hh-binding to Ptc-1 receptor releases the Smo signal transducer from Ptc-1-dependent suppression. Then, Smo activates STK36 serine/threonine kinase to inhibit the assembly of Gli degradation complex allowing the stabilization of full-length Gli [75]. Activated STK36 also phosphorylates SUFU to promote accumu-

lation of full-length Gli into nucleus [76], where it can bind DNA and regulate the expression of its target genes.

To date, only a small number of Hh target genes have been characterized in detail. Ptc-1 gene is a target of its own repression, and its transcription is elevated upon stimulation with Hh. This up-regulation may antagonize further Hh stimulation, or may play a direct role in cell cycle control as Ptc-1 is reported to interact with cyclin B1 (Barnes *et al*., 2001). Gli1 and Hip are transcriptional targets of Hh signalling pathway implicated in the negative feedback mechanism of Hh cascade [77, 78]. Cell cycle regulators like cyclin D1 and D2, c-Myc, N-Myc and L-Myc, as well as Forkhead-box transcription factors, are reported to be upregulated by the Hh signalling [14, 17, 52, 67, 79, 80]. Other targets include HNF-3b [27] in the developing neural tube, SWiP-1 [81] and SFRP-2 [82] in somitic mesoderm, and angiopoietin 2 (Ang-2) in developing vasculature [83]. Other tissue-specific targets include members of the bone-marrow protein family in gastrointestinal tract [84], PAX family in motor neurons differentiation [85], SOX family in spinal cord [86], and TBX family in developing hind limb and forelimb [87].

Complexity of Hh cascade is also provided by its cross-talk with other pathways. Additional proteins have been identified as components of mammalian Hh signalling such as PI3K, Akt, PKCδ, MEK-1, IFT88, IFT172, SIL, Kif3a, MIM/BEG4, β-arrestin2, and Rab23 [88-94]. For example, Smo can activate non canonical signals, like PI3K and Akt. At the same times, Hh pathway is subjected to regulation by other signal cascades, like PKCδ/MEK-1 pathway. Hh signalling can be modulated by parallel activation of additional intracellular signalling routes, such as those triggered by epidermal growth factor or fibroblast growth factor [95, 96].

Recently, we reported that MPs^{Shh+} trigger changes in the expression and phosphorylation of enzymes related to the nitric oxide (NO) pathway. Shh carried by MPs promote, in endothelial cells, endothelial NO-synthase (eNOS) expression and module its activity as revealed by increasing of phosphorylation of activator site (Ser-1177) by a cyclopamine-sensitive mechanism [97].

The Hh family of proteins is powerful morphogen mediating embryonic development but several recent studies reveal its implication during adult life such in neovascularisation through an upregulation of multiple families of angiogenic growth factors (VEGF, Ang) [83].

EFFECTS OF Shh IN NEOVASCULARISATION

Postnatal neovascularisation, including both angiogenesis and vasculogenesis, is regulated by a complex interplay among various growth factors, circulating bone marrow-derived precursor/stem cells, endothelial and inflammatory cells [98, 99]. Whereas angiogenesis is defined as the formation of new blood vessels by way of sprouting of pre-existing mature endothelial cells [98], vasculogenesis is referred to the formation of the earliest vascular network *via* the differentiation of endothelial progenitor cells into endothelial cells [100].

In established blood vessels in mature organisms, endothelial cells remain in a quiescent, non-proliferate state until stimulation of angiogenesis occurs. The formation of new vessels can be considered as the result of several processes: (i) dissolution of the matrix underlying the endothelial cell layer; (ii) migration, adhesion and proliferation of endothelial cells; (iii) formation of a new three-dimensional tube, which then lengthens from its tip as circulation is re-established; and (iv), in larger vessels, vascular smooth muscle cells also migrate and adhere to the newly deposited matrix of the native vessel [101].

Angiogenic growth factors induce, promote and/or interfere with all these steps of angiogenesis [102-104]. The variety of growth factors plays significant role in cell proliferation, maturation and differentiation leading to the formation of mature blood vessels. These factors act as signalling molecules between cells, and bind to specific receptors on the surface of their target cells. The best known growth factors with proven angiogenic potency are the family of fibroblast growth factors (FGF) and VEGF [101], insuline-like growth factor-1 (IGF1), Ang, and hepatocyte growth factor (HGF) [105].

In 2001, Pola *et al* [83]. described for the first time the involvement of the Hh family of morphogens in angiogenesis. There are several features worthy of consideration in Hh signalling in angiogenesis. First, the physiological functions of Hh signalling are broader than other the so called specific angiogenesis factors, such as VEGF or Ang. Secondly, their role in embryogenesis is temporo-spatially determined by the developmental stage and context [106]. Thus, Hh signalling might be better considered as a coordinator of the cross-talk between angiogenesis and other developmental processes such as neurogenesis. Therefore, due to its pleiotropic function, targeting Hh signalling might affect both angiogenesis and morphogenesis. Thus, care must be taken when using such strategy.

Shh is involved in de novo vascularisation of certain embryonic tissues. Several studies have reported that Shh signalling derived from the notochord stimulates the formation of the aorta in the zebrafish embryo [107]. Vokes *et al*. [108] described essential roles for Shh signalling in dorsal aorta formation in avian and mouse embryos. Indeed, Shh is expressed in the avian endoderm and formation of the dorsal aorta is significantly inhibited in the endodermless or cyclopamine-treated avian embryo, as well as, in Smo-deficient mouse embryos in first embryonic days.

Besides, Shh signalling stimulates angiogenesis in various embryonic tissues at late developmental stages including its release from epicardial layer of the heart in mouse embryos [109]. Shh expression is triggered by FGF and resulting in upregulation of the expression of VEGF and Ang-2 in the myocardium. In mouse embryos, transgenic mice reveal that overexpression of Shh leads to hypervascularisation of the neuroectoderm [110]. In contrast, mutant zebrafishs with deficient Hh signalling have defects in circulation and vascularisation including the formation of a single axial vessel with no arterial markers [107, 111]. The latter is implicated in the development of coronary vessels in the subepicardial space and within the myocardium.

Shh signalling also plays a role in the developing lung vasculature. Indeed, Van Tuyl *et al*. [112] reported that the

expression of Ang-1 and its receptor Tie-2 are downregulated in the lungs of Shh null embryos. In this study the authors advance the hypothesis that defective angiogenesis results in impaired airway branching [112] and therefore mice lacking Shh function exhibit poor vascularisation of developing lung [113]. Of note is the fact that Shh can act synergistically with other growth factors such as FGF9 in pulmonary capillary network [114]. In this context, the mechanism involves mesenchymal expression of VEGF but not Ang-1.

Shh AND PATHOLOGIES ASSOCIATED WITH ISCHEMIA

Among Hh-regulated processes in adults, revascularization of ischemic tissue is of the outmost clinical importance [115]. Ischemic injury results in deprivation of oxygen and nutrients, and then the growth and viability of cells is reduced. The Hh-dependent revascularization probably reflects a physiological response to ischemic stress (hypoxia or inflammation). To date, the studies are conduced to understand Shh potentialities in the rescue of tissues that can be subjected to ischemic insults. Reports documents positive effects of this morphogen in different tissue such as myocardium [116], cornea and hindlimb [13], kidney [117] and brain [53, 118].

With regard to wound healing, Asai *et al*. [119] reported that endogenous Shh signalling is activated in a mouse wound model. Thus, the transfection of naked Shh-DNA promotes wound healing *via* vasculogenesis, i.e. recruitment of endothelial progenitor cells from the bone marrow. However, no clear evidence is shown for the involvement of activated endogenous Shh signalling in the wound healing process because pharmacological inhibitors of the downstream signalling pathway have not been used. Nevertheless, these data point to the involvement of Shh signalling in angiogenesis and vasculogenesis in response to skin injury.

It has been shown that administration of recombinant Shh protein induces angiogenesis in ischemic limbs and cornea of adult mice [83], indicating the involvement of Hh in postnatal vascular development. The same group has reported the induction of nerve vessels and restoration of nerve function in rat diabetic neuropathy after systemic injection of recombinant Shh protein, suggesting a therapeutic angiogenic potential of exogenous Shh. In these cases, VEGF expression was upregulated by Shh signalling in the cornea stromal cells and ‘‘nerve fibroblasts’’, respectively [120].

It is of interest to consider whether endogenous Shh signalling in postnatal vascular development is a physiological and/or repair process. Shh expression is upregulated in regenerating muscle in mouse ischemic legs [13]. Thus, administration of a Shh-neutralising antibody inhibited angiogenesis and VEGF upregulation in this model, implying a role for endogenous Shh signalling in ischemic angiogenesis. Similar findings have been reported in a study in which retinal angiogenesis is associated with upregulation of Shh signalling and VEGF by a mechanisms sensitive to cyclopamine [121].

A field of recent interest is the potential of modulating Shh signalling as a therapy for myocardial ischemia. We have provided data that Shh carried by MPs. are able to restore endothelial dysfunction in a model of mice coronary ischemia by a mechanism sensitive to cyclopamine.(see below) [97].

Conditional activation of Gli-2, which is downstream of Shh activation, resulted in increased coronary vessel density within the myocardium along with upregulation of VEGF in mouse adult heart [109]. Furthermore, Shh gene therapy restores myocardial function in a mouse model of acute or chronic myocardial infarction. Indeed, transfection of DNA encoding human Shh not only promotes neovascularisation *via*, at least in part, recruitment of endothelial progenitor cells, but also reduces cardiac fibrosis and apoptosis. Several angiogenic factors, such as VEGF and Ang, are upregulated by Shh in the cardiac fibroblasts [116]. Altogether, these data supports the view that Shh signalling during ischemia results in upregulation of VEGF, thereby promoting angiogenesis.

With regard to cerebral ischemia, a positive role for Hh-mimicking molecules has also been reported to limit the damage caused by artificial vessel occlusion in rats [53,118].

One can advanced two theories to explain the effects of morphogens in angiogenesis (Fig. **2**). Secreted Hh ligands could act directly on the endothelial cells to stimulate proliferation and vessel formation. Alternatively, the more commonly accepted theory is that Hh ligands act on support cells (pericytes and smooth muscle cells), the nature of which is still not fully elucidated, which produce vascular growth factors, such as VEGF to promote angiogenesis [122].

Supporting the first assumption, Geng and co-workers [123] suggest a direct interaction between Hh and endothelial cells. They have shown, in an *in vitro* single cell line that exogenous treatment with Shh stimulates proliferation of endothelial cells and blockade of the Hh pathway reduces this response. Besides, the group of Hochman [124] reports that endothelial cells derived from the mouse embryo yolk sac, as well as, embryonic mouse fibroblasts respond directly to Hh by transcriptional activation of multiple genes coding for proteins involved in migration and angiogenesis. They further point out that Hh signalling enhances *in vitro* wound healing *via* neuropilin-flavomonooxygenase pathway. In line with the first theory, Shh mediates *in vitro* migration and capillary formation of mature endothelial cells and putative endothelial progenitor cells from the bone marrow [119, 124, 125]. Altogether, these studies underscore endothelial cells as direct targets of Shh.

On the other hand, many reports have also shown indirect actions of Shh-mediated angiogenesis. Shh-dependent aorta formation in zebrafish, reported by Lawson *et al*. [107], is also mediated by expression of VEGF in the mesoderm. Shh treatment, *in vitro,* upregulates angiogenic genes, such as VEGF and Ang in NIH3T3 embryonic fibroblasts, as well as, in adult dermal fibroblasts [83, 126]. Thus, Shh promotes its action secondary to the release of proangiogenic factors.

Altogether, these findings support a dual mechanism of Shh-induced angiogenesis, namely, direct and indirect. Even though Shh directly interacts with endothelial cells, *in vitro,* this finding does not rule out the indirect interaction. It is possible that Hh peptides affect both the stroma and the endothelium.

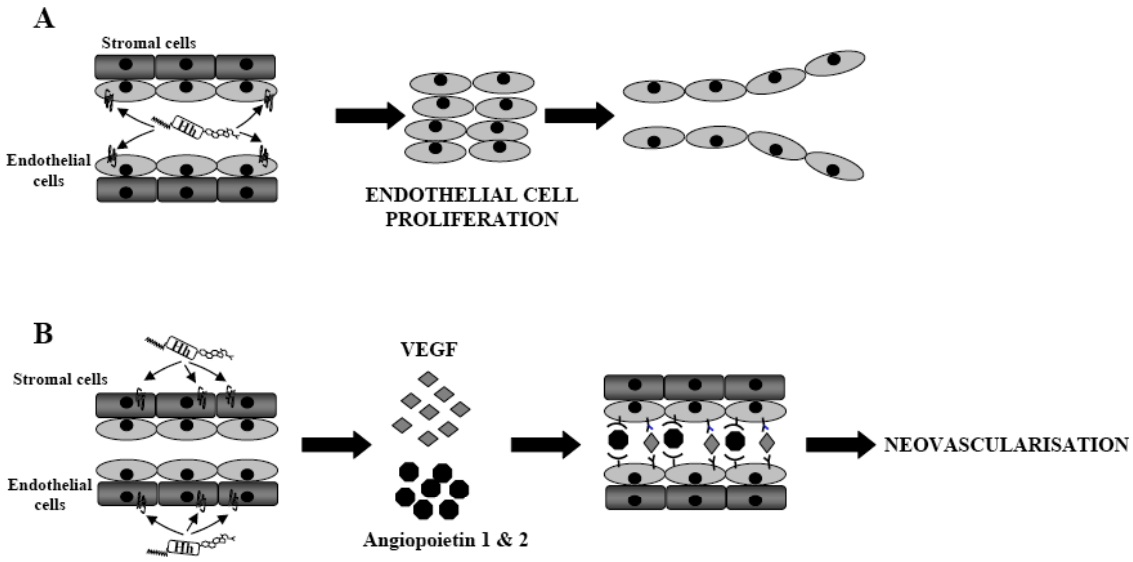


Fig. (2). Hh mechanism of action on angiogenesis. A, Direct mechanism: Hh directly stimulates endothelial cells to proliferate and to organize the new vessels formation. B, Indirect mechanism: Hh acts on stroma cells which respond by producing proangiogenetic molecules such as VEGF and Angiopoietin 1 and 2. Angiogenic molecules act on endothelial cells to induce the neovascularisation. The direct and indirect models are not exclusive; it is possible that Hh affects both the stromal and endothelial cells.

The exact Shh pathway involved in the reduction of ischemia is not fully understood, even if some cross-link between Hh pathway and proangiogenic molecules have been reported. Growing evidence reveals that among the proangiogenic factors that could be possible target of Shh pathway to reduce ischemia, hypoxia-inducible factors (HIF) [127], insulin-like growth factor (IGF)-2 [128], nitric oxide (NO) [129] and reactive oxygen species (ROS) [130] are the most representative.

It is important to note that the most powerful activator of angiogenesis is hypoxia. Under hypoxic conditions, hypoxia-induced mitogenic factor, promotes proliferation and migration of endothelial cells [105] and low levels of reactive oxygen species (ROS) (which are produced in response to growth factor, tissue ischemia/hypoxia or ischemic preconditioning) function as signalling molecules to mediate endothelial cell migration [131-133] and may contribute to angiogenesis *in vivo* [134, 135].

One of the main targets of hypoxia is HIFs. HIFs are transcription factors that activate pathways with the ability to increase the oxygen supply and promote adaptive responses to stress. Among the multiple targets of HIF genes are VEGF, erythropoietin, Ang, placental growth factor, and platelet-derived growth factor, indicating that HIF-1 functions as a master regulator of angiogenesis in ischemic tissues [136]. These angiogenic factors recruit subsets of proangiogenic hematopoietic cells along with endothelial progenitor cells. These, in turn, may also release new angiogenic factors contributing to the repair by paracrine effects. Indeed, hypoxic preconditioning of endothelial progenitor cells can increase their ability to repair ischemic limbs through the activation of the angiogenic program. [137].

There are some reports showing that HIF-1 and Shh with other factors can act in synergy to protect tissue against ischemic insults. Indeed in embryo rat heart, transient ischemia induces expression of HIF-1α, Shh, IGF-2, and VEGF genes, suggesting that these proteins may play a role in cardioprotective effect especially in cardiomyoblast. HIF-1α regulates some proteins adapted to hypoxia, including IGF-2 and VEGF; IGF-2 improves growth and proliferation; and Shh improves myogenesis in synergy with IGF .The exposure of cardiomyoblast cells to HIF-1α, and Shh inhibitors respectively, resulted in the cross-inhibition of each of the pathway. Moreover a complex cross-talk between these proteins has been proposed and could have important implications in the understanding of proliferation and angiogenesis following ischemia [127]. These results are also supported by a recent study in which the authors have highlighted the role of IGF-2 in Shh signalling and angiogenesis. They advance the hypothesis that IGF-2 may mediate the angiogenic effects of Shh, providing a critical link between Shh and VEGF [128].

The involvement of NO in ischemia-induced angiogenesis is supported by the studies performed by Luque Contreras and co-workers [129], which show that (i) NO levels are increased in the ischemic limb; (ii) pharmacological inhibition or gene disruption of endothelial NO-synthase decreases NO levels and inhibits ischemia-induced angiogenesis; (iii) supplementation of NO, by the use of exogenous sources, restores ischemia-induced angiogenesis; and (iv) cardiovascular diseases associated with decreased NO synthesis display an impairment in ischemia-induced angiogenesis.

There are some crucial reports showing the link between Shh and NO pathway. Indeed, Shh is involved in the regulation of NO release. NO-synthase (NOS) and VEGF are downstream target of exogenous Shh signalling suggesting that Shh can act as a modulator of the regulation of VEGF and NO production [138].

In an experimental model of kidney ischemia reperfusion, Ozturk [118] suggests that NO reduces the renal dysfunction

associated to this pathology and they suggest that NO can act as a trigger to induce Shh and HIF-1 activities.

On the course of liver ischemia and reperfusion in rats, inhibition of NO production upregulates Shh expression, in line with the hypothesis that Shh pathway is critical factor in the pathophysiology of inflammation in the liver injury induced by ischemia/reperfusion [139].

Recently, we have reported that MPs harbouring Shh are able to favour endothelial cell spreading and promote vascularisation in a model of mice hind limb ischemia by a mechanism sensitive to cyclopamine and trough the increase of NO production (unpublished data).

Finally, oxidative stress induced by reactive species including superoxide, hydrogen peroxide, hydroxyl anion, and peroxynitrite, are biologically active oxygen derivatives that are increasingly recognized to play major roles in vascular biology through redox signalling [140-142]. ROS have been suggested as important mediators of angiogenesis. ROS stimulate cell migration and proliferation in endothelial cells [143] and directly modulate VEGF expression and vascular smooth muscle cell proliferation [144]. Of interest, ROS was reported to stimulate post-ischemic revascularization at low concentrations but to inhibit it at high concentrations [145].

A recent study of our laboratory shown that MPs bearing Shh are able to restore endothelial dysfunction in a model of mice coronary arteries subjected to ischemia/reperfusion. Shh carried by MPs restores endothelial injury probably through their dual ability to increase NO and reduce ROS. The concomitant effect of NO and ROS productions might result in an enhancement of the bioavailability of generated NO by reducing oxidative stress and the subsequent scavenging of NO. In addition, the increase in NO release is associated with an enhancement of endothelial NO-synthase expression and activity in human endothelial cells, as reflected by both the increase in endothelial NO-synthase phosphorylation, and changes in the expression and phosphorylation of caveolin-1. Altogether, these results indicate that Shh harboured by MPs are able to modulate endothelial NO-synthase expression and activity and reduce oxidative stress in human endothelial cells, leading to a beneficial potential effect on the cardiovascular system [97].

Hh pathway and cancer

While Shh signalling is required for normal development, it has an equally important role in adulthood where mutations in the pathway give rise to a variety of tumours. Hh signalling targets include genes that are important for cell proliferation proto-oncogenes as well as growth factors. Deregulation of the Hh pathway is a characteristic feature of several pathologic states, including developmental syndromes with high predisposition to cancer [146, 147] (Table **1**). Indeed, Hh signalling has been implicated in the development of several human cancers, including medulloblastoma, digestive tract tumours and basal cell carcinoma (Fig. **3**).

Hh Signalling and Medulloblastoma

Medulloblastomas (MB) are the most common malignant brain tumours of childhood. A growing body of evidence indicates that MB can arise by transformation of granule neuron precursors. Shh pathway plays a critical role in cerebellar development and its aberrant expression has been identified in MB. The target genes for Shh signalling after receptor activation include the transcription factors Gli-1, N-Myc and Ptc-1. These transcription factors can act as tumour suppressors and negative regulators of the pathway. Promoter methylation of tumour suppressors plays a significant role in different stage of tumour formation by gene silencing. For example in a mouse model of MB in which one allele of Ptc-1 is genetically disabled along with the p53, the remaining allele is naturally silenced by methylation [148]. More recently, it is found that the Ptc-1 promoter is methylated in ovarian tumours, but not in basocellular carcinomas implying differential Ptc-1 methylation as a contributing factor in tumourigenesis, depending on tumour type [149]. Unexpectedly, no methylation has been detected in the promoter im-

Table 1. Main Alterations in Shh Signalling in Several Types of Cancer

Target Genes	Tumour Type	Mechanism(s)	References
Nhlh1/NSCL	Medulloblastoma	Nhlh1 activation by Gli-1	[151]
Gli-1	Esophageal cancer	Downregulation of Gli-1 expression	[153]
Ptc-1	Medulloblastoma Basal-cell carcinoma	Inactivation of Ptc-1 by methylation Mutation of Ptc-1	[148] [172]
Gli-1, Gli-2, TGFβ	Gastric carcinoma	Activation of Gli1 and Gli2 by TGFβ	[157]
Shh	Pancreatic carcinogenesis	Overexpression of Shh	[14, 161, 163]
Gli-1, Ptc-1	Colorectal cancer	Aberrant activation of Hh signaling (uncorrelated expression of *Gli-1* with *Ptc-1*)	[164]
Hh, ERK1/2	Cholangiocarcinoma	Simultaneous inhibition of Hh and ERK1/2 pathways	[175]
Smo, c-myc	Hepatocarcinoma	Tumorigenesis by overexpression of Smo and c-myc (activated Hh pathway)	[176]

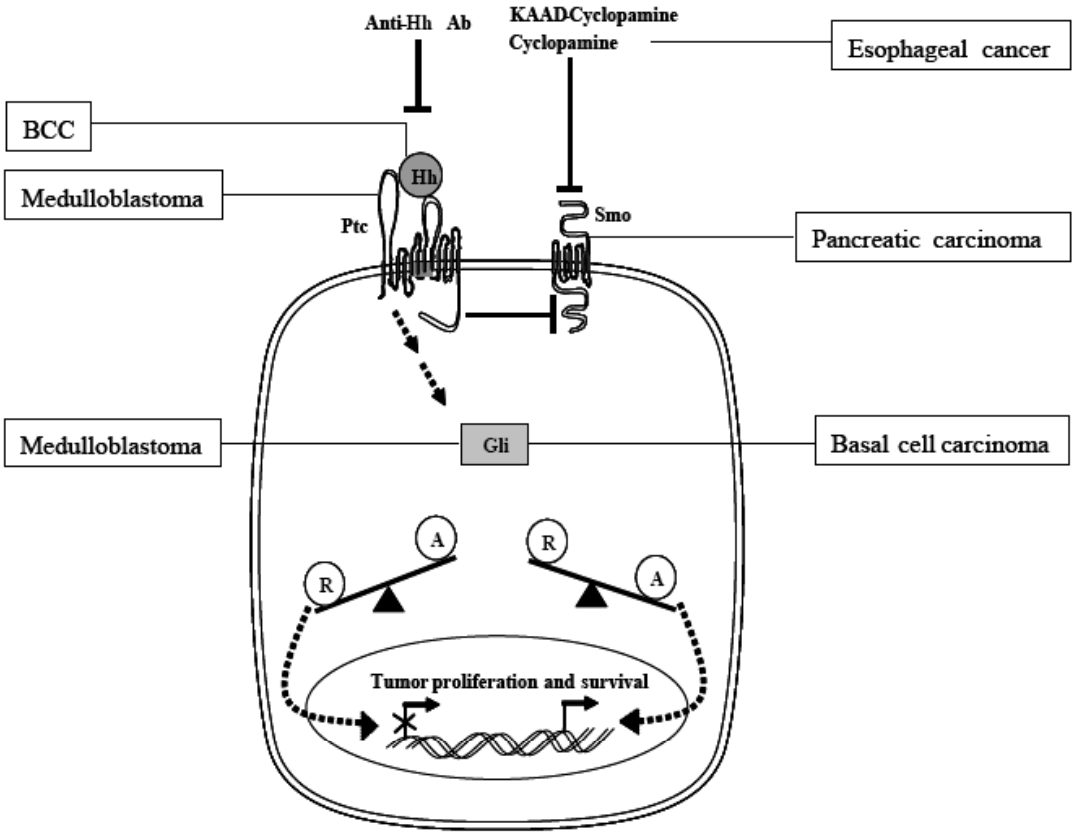


Fig. (3). Hh pathway and cancer. The Hh pathway can be blocked at different levels, and Hh inhibitors could serves as anti-cancer agent. Inhibition of ligand activity has been reported with neutralising antibodies against Hh. A specific Smo inhibitor, cyclopamine. KAAD-cyclopamine, is identified as therapeutic agent to inhibit growth of esophageal cancer cells. Other compounds that block Gli activity could be used to treat wide of variety of Hh-dependent tumours. *Ptc* mutations are associated with basal-cell carcinoma (BCC), as well as with medulloblastoma. Constitutively active forms of Smo are oncogenic and can function independently of ligand binding to Ptc, leading to BCC. Constitutively active forms of Smo are oncogenic and can function independently of ligand binding to Ptc, leading to BCC. An oncogenic form of Hh has been associated with BCC. Deregulation of Hh signalling has also been associated with pancreatic adenocarcinoma and oesophageal cancer. This highly stylized representation shows requirement for pathway activation in tumour formation and/or tumour cell growth, and the functional interactions between the various components. In unstimulated cells, the activity of the transmembrane protein Smo is suppressed by the Hh receptor Ptc and the majority of the Gli transcription factors are present in their repressive (R) form. In stimulated cells, binding of Hh to Ptc activates Smo, which in turn shifts the Gli balance in favour of the activator (A) forms, resulting in the transcription of Hh target genes. Little is known about the molecular mechanisms by which Hh signalling is upregulated in these tumours.

mediately upstream of Ptc-1B in either MB or control samples. Future directions include examination of distal regions of the Ptc-1B promoter as well as alternative exon variants, most notably the CpG island (genomic regions that contain a high frequency of CG dinucleotides), containing Ptc-1C promoter [150]. Identification of methylated genes that regulate pathways common to many cancers may provide key prognostic indicators and therapeutic targets.

Furthermore, insulinoma-associated 1 and nescient helix–loop–helix 1 (Nhlh1)/NSCL1 are identified as new Hh target genes that are overexpressed in MB. Through the identification of functional Gli binding sites on the promoters of mouse and human Nh1h1, Nhlh1 promoter is found to be bound and activated by Gli1 transcription factor. Remarkably, the expression of these genes is also upregulated in mouse and human Hh-dependent MBs, suggesting that they may be either a part of the Hh-induced tumorigenic process or a specific feature of Hh-dependent tumour cells [151].

Hh Signalling and Gastrointestinal Carcinogenesis

a- Function of the Hh Signalling in Esophageal Cancers

The Hh pathway plays a critical role in the development of the foregut. However, the role of the Hh pathway in primary esophageal cancers is not well understood.

Downregulation of Gli1 expression may be an important mechanism by which KAAD-cyclopamine, found to be a more powerful derivative of cyclopamine [152], inhibits growth and induces apoptosis in esophageal cancer cells. This finding suggests that targeted inhibition of the Hh signalling by KAAD-cyclopamine or Shh neutralizing antibodies may be effective in chemoprevention and treatment of esophageal cancers [153]. In addition, the Shh signalling pathway is extensively activated in esophageal cancer xenografts and residual tumours after chemoradiotherapy and the temporal kinetics of Hh signalling precedes increases in proliferation and tumour size during tumour regrowth. The

mechanism involved in the increase of proliferation of esophageal cancer cell lines is linked to an up-regulation of the G1-cyclin-Rb axis. Additionally, the same authors show that blocking Hh signalling enhances radiation cytotoxicity of esophageal cancer cells [154]. These results raise the question of what is a reliable marker of Hh pathway status in cells where the pathway is not manipulated. Identification of post-transcriptional modifications, such as processing, subcellular localization or phosphorylation of some Hh pathway members might predict more accurately the Hh pathway activity than expression levels. Clearly further studies are needed to understand the possible involvement of this pathway in esophageal cancer before it can be considered as a suitable therapeutic target.

b- Shh Signalling in Gastric Carcinoma

Recent studies have suggested that constitutive activation of the Hh pathway in cancers of the digestive tract may contribute to the growth and maintenance of cancer.

As mentioned previously, Hh proteins have long been known to act in the developing organism and to remain active in adult physiology (i.e., in the histostability of the gastrointestinal tract) [155]. Previous studies have reported that aberrant activation of Shh signalling through Ptc-1 is frequently detected in cases of advanced gastric adenocarcinoma and that activation of this pathway is associated with poorly differentiated gastric tumours [156].

Furthermore, interactions between Shh and TGF-β pathways have been described in gastric cancer. Shh and TGF-β family members are involved in numerous overlapping processes during embryonic development, hair cycle, and cancer [158]. For TGF-β, this growth factor is a pleiotropic cytokine that plays a critical role in the modulation of cell growth, differentiation, plasticity and migration. TGF-β receptors, which function as tumour suppressors in normal and preneoplastic tissues, acquire oncogenic functions during tumour progression. In addition, TGF-β receptors are mutated or expressed at substantially attenuated levels in a variety of human cancers and are correlated with the acquisition of resistance to growth suppression by TGF-β [158]. In addition, activation of MAP kinase and PI3K/Akt signalling cascades by TGF-β receptors can also potentially contribute to cell migration and invasion. TGF-β signalling is mediated by either ALK1 or ALK5. Despite the critical role that Hh signalling plays in the promotion of tumourigenesis, the molecular and cellular mechanisms behind Hh regulation in tumour metastatic behavior are unknown. Interestingly, Bertolino *et al*. [159] report that the Shh signalling pathway, acting through the TGF-β–ALK5 cascade, may selectively contribute to tumour cell motility and invasion in gastric cancer. In addition, they demonstrated that disruption of TGF-β–ALK5 signalling by anti-TGF-β neutralizing antibody or ALK5 kinase inhibitor results in suppression of Shh-mediated cell migration and invasion. These results indicate that Shh promotes motility and invasiveness of gastric cancer cells through TGF-β-mediated activation of the ALK5–Smad 3 pathway. Additionally, these findings are the first to suggest a role for Shh signalling in the metastatic potential of gastric cancer, thereby indicating potential therapeutic molecular targets to decrease metastasis [157].

Although activation of Hh signalling maintains proliferation of some gastric cancer cells, Van Den Brink *et al*. [155] find that treatment of mice with cyclopamine increased epithelial proliferation *in vivo* by 60–70%. Similarly, Fukaya *et al*. [160] show that treatment of primary cultures of normal mouse gastric epithelial cells with cyclopamine increase their proliferation. This may indicate a difference in the role of Hh signalling in cell cycle regulation between normal and cancer cells. More likely, one of the factors that may explain the difference observed in the response of gastric carcinoma cells and primary gastric epithelial cells *in vivo* and in culture is probably linked to the use of pure population of cultured cancers cells rather than a combination of cells occurring in *in vivo* situation. [5].

c-Hh Signalling in Initiation of Pancreatic Carcinogenesis

Shh is reported to play an essential role in the development of pancreatic cancer as well as pancreatic organogenesis [148]. Thayer *et al*. [161] show that Shh is overexpressed in human preneoplastic pancreatic lesions and reported that a mice model expressing Shh behind a pancreas specific promoter developed preneoplastic lesions. Shh has been detected in pancreatic cancers, and cyclopamine suppresses the growth of pancreatic cancer cells both *in vitro* and *in vivo*.

Recently, it is reported that NF-κB contributes to Hh pathway activation through up-regulation of Shh expression in pancreatic cancer cells [148]. In contrast, Hh signalling pathway and pancreatic cancer proliferation are suppressed with Ptc-1 antibodies, indicating that Hh pathway may represent a potential target in therapeutic against pancreatic cancers [163].

Recent evidence also indicates that deregulated signalling not only causes tumour formation, but it is also required for tumour maintenance, as transformed cells continue to depend on Hh activity for survival and growth. Analysis of 26 human pancreatic adenocarcinoma cell lines indicate that all cell lines express Hh target genes, and interestingly treatment with cyclopamine induces both apoptosis and loss of proliferation in 50% of the cell lines tested [14].

d- Hh Signalling in Colorectal Cancer

In contrast to other type of cancer, there is little evidence for activation of Hh signalling at later stages of colorectal carcinogenesis. Berman *et al*. [164], find that in contrast to proximal gastrointestinal cancer cells, colon cancer cells dos not depend on Hh signalling for their survival. The authors report that the lack of sensitivity of colorectal cancer cells to cyclopamine correlates with the absence of active Hh signalling in these cells. This corroborates with the findings that Hh signalling is low or absent in undifferentiated colorectal cancer cells. Furthermore, Berman *et al*. [164] do not detect any *Ptc-1* mRNA in colorectal cancer cells. However, *Gli1* expression correlates with *Ptc-1* expression in all cell lines examined except in the colorectal cancer cells. Correlation between *Ptc-1* and *Gli1* expression may be expected, as both are transcriptional targets of Hh signalling. Also, since *Smo* is expressed in colorectal cancer cells [165, 164] absence of *Ptc-1* expression would be expected to activate the pathway and would not result in low to absent activity of Hh signalling as observed by Berman *et al*. [164]. The loss of Hh sig-

nalling in colorectal carcinogenesis is not yet completely understood. It may be involved in the loss of epithelial differentiation that is a hallmark of adenomatous polyps. Chatel *et al*. [167] study seven colon cancer cell lines and find that none of the cell lines expressed all the key members of the Hh pathway. They provide evidence that Ptc-1 and Gli1 transcripts levels are not significantly altered in cyclopamine-treated colon cancer cells, in contrast with the cyclopamine-responsive pancreatic cells. These results support the view that the aberrant activation of the Hh signalling pathway is not common in colorectal cancer cell lines. In contrast, Qualtrough *et al*. [165] observe increased cell death in a variety of colonic epithelial cell lines treated with cyclopamine. Currently, there is no explanation for this difference, but the sensitivity of the viability of colorectal cancer cells to the effects of cyclopamine may depend on cell culture conditions. Further studies are needed to sort out these differences.

Shh in Basal-Cell Carcinoma

Basal-cell carcinomas (BCC), account for 90% of all skin cancers, represent the commonest human cancer in fair skinned populations, and they are clearly associated with constitutive activation of Shh signalling.

Mutations in the Shh receptor, Ptc-1, are found in familial [168, 169] and sporadic [170, 171] forms of BCC. In the developing epidermis, Shh signalling is required to maintain the balance between cell proliferation and differentiation. Ptc-1 plays an important tumour suppressor function in the mammalian epidermis. This conclusion is based on the fact that spontaneous allele of Ptc-1, Ptc-1mes, which encodes a mutant Ptc-1 protein lacking the last 220 amino acid residue, has a critical role in epidermal hyperplasia [172]. Indeed, Nieuwenhuis *et al*. [173] have bypassed the early embryonic lethality associated with the Ptc-1null mutation and demonstrate an important function of Ptc-1 in adult skin. The observation that Ptc-1mes/mes mice possess excess skin suggests a potential role for the CTD (carboxy-terminal domain) of Ptc-1 in skin development. A detailed analysis of epidermal development and homeostasis in Ptc-1mes/mes mice has shown that embryonic skin development appears normal. Interestingly, Ptc-1mes/mes mice display epidermal hyperplasia beginning at postnatal day 12. Importantly, an expansion of the epidermal stem cell compartment in Ptc-1mes/mes mice is described. These results suggest that the CTD of Ptc-1 is not required for epidermal development in embryonic skin, but is necessary for epidermal homeostasis in adult skin [173].

Hh in Cholangiocarcinoma (CCA) and Hepatocarcinogenesis

Hh pathway deregulation has been reported in CCA and hepatoblastoma cell lines [174]. Jinawath *et al*. [175] suggest that the Hh and ERK1/2 pathways are important for CCA cell proliferation, and simultaneous inhibition of the two pathways may lead to stronger decreases in cell growth and viability in a subset of CCA cases. Sicklick *et al*. (2006) [176] show that in hepatocarcinoma, overexpression of the Smo protooncogene, as well as an increase in the stoichiometric ratio of Smo to Ptc-1 mRNA levels, correlate with tumour size, can be used as a prognostic indicator in hepatocarcinoma. They also demonstrated that hepatocarcinoma cell lines (HepG2 and Hep3B) express Hh pathway components and activate Hh transcriptional targets. Hh pathway activation may occur as an early event during the evolution of hepatic neoplasia. These data support the hypothesis that Hh signalling is deregulated in human hepatocarcinogenesis. The authors report that overexpression and/or tumorigenic activation of the Smo protooncogene mediates c-myc overexpression which plays a critical role in hepatocarcinogenesis. The results also suggest that Smo is a prognostic factor in hepatocarcinoma tumourigenesis [176].

FUTURE DIRECTIONS

Data in the literature suggest that an increase of neovascularisation through the Shh pathway activation might have beneficial effects on pathologies associated with ischemic complications whereas in cancers, novel potential therapeutic strategies addresses against Shh pathway may lead to reduced tumour development.

Concerning neovascularisation, it has been recently reported that inhibition of endogenous Shh pathway may ameliorate cardiac function after myocardial ischemia. This effect is associated with reduced apoptosis and fibrosis and increased vascularisation.

Thus, a dual ability of Shh pathway in cardiac ischemia has been proposed by these authors [177]. On one hand, high exogenous level of Hh may favour tissue repair. On the other hand, endogenous Hh may act as a deleterious factor. These data may explain, at least in part, why exogenous therapy with recombinant Hh proteins or genetic therapy with adenovirus are effective in inducing neovascularisation of tissue repair. Furthermore, other potential therapies for example using MPs harbouring Shh seem promising, as shown in ischemic animal models and *in vitro*. However, clinical studies need to be performed in order to validate these approaches.

Also, the use and development of specific agonists may constitute another potential tool to activate Hh pathway. In this respect, effort of the pharmaceutical companies will be necessary in order to evolve towards a large spectrum of new drugs targeting, specifically endothelial cells and endothelial progenitors in order to favour their ability to promote angiogenesis.

Taking in consideration that in several type of tumours Hh signalling is upregulated, the use of cyclopamine or its derivates may be an interesting tool for decrease tumour formation. Parker and Inghram (2008) [178] have reviewed these aspects and they consider that the pathway downstream of Smo, such as the Gli transcription factors may be the targets of new drugs developed in order to limit cancer growth. In addition, it should be noted that the concomitant inhibition of pathways that can be implicated separately in several cancer development may increase the success of tumour therapy. For instance, a correlation between estrogen receptor alpha (ERα) and Shh expression is found in the breast cancer tissues. Moreover estrogen triggers Shh up-regulation and inhibition of ERα suppresses this effect, suggesting that ERα regulates the Hh pathway though Shh induction [179]. Con-

comitant inhibition of both pathways (ERα and Shh) may lead to a reduction of breast cancer development.

Taking together, one can advanced the prediction that our understanding of Hh pathway mechanisms and the participation of Hh pathway in angiogenesis related disease is a growing area of research. Together with progress in technologies such as cell and gene therapy and diagnostic, this will aid in applying our knowledge to clinical practice and will offer us a means to fight against ischemic and cancer diseases in the adult**.**

CONCLUSION

The Hh family of proteins is powerful morphogen mediating embryonic development as well as adult morphogenesis. The majority studies have focused on the role of Hh family members during embryogenesis, they have shown the morphogen implicated in the regulation of epithelial-mesenchymal interaction crucial to limb, lung, gut, hair follicle, and bone formation, including a possible role during vascularisation of certain embryonic tissues [115].

In addition to its role in patterning the developing embryo, several recent studies indicate that Hh signalling is involved in neovascularisation in part by upregulation of multiple families of angiogenic growth factors [83]. The innate Hh pathway is activated after myocardial ischemia and Shh gene therapy may have therapeutic potential under pathological conditions that need angiogenesis such as lower extremity ischemia, myocardial ischemia, and in cerebral ischemia.

Even if Shh therapy may have positive effects in angiogenesis-related diseases, there is a significant concern that the induction of angiogenesis may increase the risk of neoplastic diseases. Indeed, there are also data supporting this theory inasmuch upregulation of Shh signalling has been reported in human and animal models of basal cell carcinoma. This might suggest that transfection of Shh could be limited as a clinical strategy for ischemia, because of a potential risk for cancer induction.

However, it should be noted that reports regarding the carcinogenic effect of Shh have been conducted in transgenic or mutated mice and the observation that a mutation in Shh pathway is found in a percentage of patient with basal carcinoma. It is probable that the high dose and the long-term exposure of Shh resulting from the genetic models, as well as the deregulated signalling that occurs in the presence of the human mutation, are factors in the development of cancer. Thus, it is possible that the lower dose and the short-term exposure to unmutated Shh, would not generate a similar degree of risk, if any [119].

However Hh signalling is not upregulated in all forms of cancer. Therefore its role in angiogenesis may be limited to a group of neoplasm [123]. Moreover treatment with Hh antagonist, cyclopamine reduces tumour vascular density, alters vascular morphology, and reduces tumour blood vessel permeability, showing important application for cancer therapy.

Together these data provide evidence that angiogenesis-related diseases could be improved by modulating Hh pathway, but more investigations of the therapeutic potential of this new gene therapy approach are needed to better validate drugs acting on Hh pathway against these two killing diseases (i.e. ischemic and cancer diseases).

ACKNOWLEDGEMENTS

This work was supported by institutional grants from the Agence National pour la Recherche (ANR-07-PHYSIO-010-01).

REFERENCES

[1] Nüsslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. Nature 1980; 287: 795-801.

[2] Marigo V, Roberts DJ, Lee SM, *et al*. Cloning, expression, and chromosomal location of SHH and IHH: two human homologues of the Drosophila segment polarity gene hedgehog. Genomics1995; 28: 44-51.

[3] Katoh Y, Katoh M. Identification and characterization of rat Desert hedgehog and Indian hedgehog genes in silico. Int J Oncol 2005; 26: 545-9.

[4] Katoh Y, Katoh M. Comparative genomics on Sonic hedgehog orthologs. Oncol Rep 2005; 14: 1087-90.

[5] van den Brink GR. Hedgehog signaling in development and homeostasis of the gastrointestinal tract. Physiol Rev 2007; 1343-75.

[6] Hammerschmidt M, Brook A, McMahon AP. The world according to hedgehog. Trends Genet 1997; 13: 14-21.

[7] Ingram PW. Transducing Hedgehog: the story so far. EMBO J 1998; 17: 3505-11.

[8] Katoh Y, Katoh M. Hedgehog signaling pathway and gastrointestinal stem cell signaling network. Int J Mol Med 2006; 18:1019-23.

[9] Chiang C, Litingtung Y, Lee E, *et al*. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 1996; 383: 407-13.

[10] Litingtung Y, Lei L, Westphal H, Chiang C. Sonic hedgehog is essential to foregut development. Nat Genet 1998; 20: 58-61.

[11] Bhardwaj G, Murdoch B, Wu D, *et al*. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells *via* BMP regulation. Nat Immunol 2001; 2: 172-80.

[12] Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev 2001; 15: 3059-87.

[13] Pola R, Ling LE, Aprahamian TR, *et al*. Postnatal recapitulation of embryonic hedgehog pathway in response to skeletal muscle ischemia. Circulation 2003; 108: 479-85.

[14] Pasca di Magliano M, Hebrok M. Hedgehog signalling in cancer formation and maintenance. Nat Rev Cancer 2003; 3: 903-11.

[15] Bijlsma MF, Spek CA, Peppelenbosch MP. Hedgehog: an unusual signal transducer. Bioessays 2004; 26: 387-94.

[16] Lum L, Beachy PA. The Hedgehog response network: sensors, switches, and routers. Science 2004; 304: 1755-9.

[17] Hooper JF, Scott MP. Communicating with Hedgehogs. Nat Rev Mol Cell Biol 2005; 6: 306-17.

[18] Briscoe J, Therond P. Hedgehog signaling: from the Drosophila cuticle to anti-cancer drugs. Dev Cell 2005; 8: 143-51.

[19] Katoh Y, Katoh M. Hedgehog signaling in gastric cancer. Cancer Biol Ther 2005; 4: 1050-4.

[20] Mann RK, Beachy PA. Novel lipid modifications of secreted protein signals. Annu Rev Biochem 2004; 73:891-923.

[21] Porter JA, von Kessler DP, Ekker SC, *et al*. The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. Nature1995; 374: 363-6.

[22] Bumcrot DA, Takada R, McMahon AP. Proteolytic processing yields two secreted forms of Sonic hedgehog. Mol Cell Biol 1995; 15: 2294-303.

[23] Lee JJ, Ekker SC, von Kessler DP, Porter JA, Sun BI, Beachy PA. Autoproteolysis in hedgehog protein biogenesis. Science 1994; 266: 1528-37.

[24] Porter JA, Young KE, Beachy PA. Cholesterol modification of hedgehog signaling proteins in animal development. Science 1996; 274: 255-9.

[25] Porter JA, Ekker SC, Park WJ, *et al*. Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. Cell 1996; 86: 21-34.

[26] Chamoun Z, Mann RK, Nellen D, *et al*. Skinny Hedgehog, an acyltransferase required for palmitoylation and activity of the Hedgehog signal. Science 2001; 293: 2080-4.

[27] Roelink H, Porter JA, Chiang C, *et al*. Floor plate and motor neuron induction by different concentrations of the amino terminal cleavage product of Sonic hedgehog autoproteolysis. Cell 1995; 81: 445-55.

[28] Lewis PM, Dunn MP, McMahon JA, *et al*. Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. Cell 2001; 105: 599-612.

[29] Li Y, Zhang H, Litingtung Y, Chiang C. Cholesterol modification restricts the spread of Shh gradient in the limb bud. Proc Natl Acad Sci USA 2006; 103: 6548-53.

[30] Chen MH, Li YJ, Kawakami T, Xu SM, Chuang PT. Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. Genes Dev 2004; 18: 641-59.

[31] Ingham PW. Hedgehog Signaling: A Tale of Two Lipids. Science 2001; 294: 1879-81.

[32] Lee JJ, Von Kessler DP, Parks S, Beachy PA. Secretion and localized transcription suggest a role in positional signalling for products of the segmentation gene hedgehog. Cell 1992; 71: 33-50.

[33] Basler K, Struhl G. Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. Nature 1994; 368: 208-14.

[34] Struhl G, Barbash DA, Lawrence PA. Hedgehog acts by distinct gradient and signal relay mechanisms to organize cell type and cell polarity in the Drosophila abdomen. Development 1997; 124: 2155-65.

[35] Zeng X, Goetz JA, Suber LM, Scott WJ, Schreiner CM, Robbins DJ. A freely diffusible form of Sonic hedgehog mediates long-range signaling. Nature 2001; 411: 716-20.

[36] Hall TM, Porter JA, Beachy PA, Leahy DJ. A potential catalytic site revealed by the 1.7-A crystal structure of the amino-terminal signalling domain of Sonic hedgehog. Nature 1995; 378: 212-6.

[37] Goetz JA, Singh S, Suber LM, Kull FJ, Robbins DJ. A highly conserved amino-terminal region of sonic hedgehog is required for the formation of its freely diffusible multimeric form. J Biol Chem 2006; 281:4087-93.

[38] Martínez MC, Larbret F, Zobairi F, *et al*. Transfer of differentiation signal by membrane microvesicles harboring hedgehog morphogens. Blood 2006; 108: 3012-20.

[39] Martínez MC, Tesse A, Zobairi F, Andriantsitohaina R. Shed membrane microparticles from circulating and vascular cells in regulating vascular function. Am J Physiol 2005; 288: H1004-9.

[40] Burke R, Nellen D, Bellotto M, *et al*. Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. Cell 1999; 99: 803-15.

[41] Ma Y, Erkner A, Gong R, *et al*. Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. Cell 2002; 111: 63-75.

[42] Katoh Y, Katoh M. Identification and characterization of DISP3 gene in silico. Int J Oncol 2005; 26: 551-6.

[43] Mullor JL, Sánchez P, Altaba AR. Pathways and consequences: Hedgehog signaling in human disease.Trends Cell Biol 2002; 12: 562-9.

[44] Jeong J, McMahon AP. Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. Development 2005; 132: 143-54.

[45] Olsen CL, Hsu PP, Glienke J, Rubanyi GM, Brooks AR. Hedgehog-interacting protein is highly expressed in endothelial cells but down-regulated during angiogenesis and in several human tumors. BMC Cancer 2004; 4: 43.

[46] Ruiz-Gómez A, Molnar C, Holguín H, Mayor F Jr, de Celis JF. The cell biology of Smo signalling and its relationships with GPCRs. Biochim Biophys Acta 2007; 1768: 901-12.

[47] Han C, Belenkaya TY, Wang b, Lin X. Drosophila glypicans control the cell-to-cell movement of Hedgehog by a dynamin-independent process. Development 2004; 131: 601-11.

[48] Nusse R. Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface. Development 2003; 130: 5297-305.

[49] Tenzen T, Allen BL, Cole F, Kang JS, Krauss RS, McMahon AP. The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. Dev Cell 2006; 10: 647-56.

[50] Goodrich LV, Johnson RL, Milenkovic L, McMahon JA, Scott MP. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. Genes Dev 1996; 10: 301-12.

[51] Motoyama J, Takabatake T, Takeshima K, Hui C. Ptch2, a second mouse Patched gene is co-expressed with Sonic hedgehog. Nat Genet 1998; 18: 104-6.

[52] Lum L, Beachy PA. The Hedgehog response network: sensors, switches, and routers. Science 2004; 304: 1755-9.

[53] Briscoe J, Therond P. Hedgehog signaling: from the Drosophila cuticle to anti-cancer drugs. Dev Cell 2005; 8: 143-51.

[54] Johnson RL, Rothman AL, Xie J, *et al*. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science1996; 272: 1668-71.

[55] Carpenter D, Stone DM, Brush J, *et al*. Characterization of two patched receptors for the vertebrate hedgehog protein family. Proc Natl Acad Sci USA 1998; 95: 13630-4.

[56] Xie J, Murone M, Luoh SM, *et al*. Activating Smoothened mutations in sporadic basal-cell carcinoma. Nature 1998; 391: 90-2.

[57] Zhao Y, Tong C, Jiang J. Hedgehog regulates smoothened activity by inducing a conformational switch. Nature 2007; 450: 252-8.

[58] Chen JK, Taipale J, Cooper MK, Beachy PA. Inhibition of Hedgehog signaling by direct binding of cyclopamine to smoothened. Genes Dev 2002; 16: 2743-8.

[59] Chen JK, Taipale J, Young KE, Maiti T, Beachy PA. Small molecule modulation of smoothened activity. Proc Natl Acad Sci. USA 2002; 99: 14071-6.

[60] Williams JA, Guicherit OM, Zaharian BI, *et al*. Identification of a small molecule inhibitor of the hedgehog signaling pathway: effects on basal cell carcinoma-like lesions. Proc Natl Acad Sci USA 2003; 100: 4616-21.

[61] Frank-Kamenetsky M, Zhang XM, Bottega S, *et al*. Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. J Biol 2002; 1:10.

[62] Bijlsma MF, Spek CA, Zivkovic D, van de Water S, Rezaee F, Peppelenbosch MP. Repression of smoothened by patched dependent (pro-)vitamin D3 secretion. PLoS Biol 2006; 4: 1397-410.

[63] Corcoran RB, Scott MP. Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells. Proc Natl Acad Sci USA 2006; 103: 8408-13.

[64] Koebernick K, Pieler T. Gli-type zinc finger proteins as bipotential transducers of Hedgehog signaling. Differentiation 2002; 70: 69-76.

[65] Aza-Blanc P, Lin HY, Ruiz i Altaba A, Kornberg TB. Expression of the vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities. Development 2000; 127: 4293-301.

[66] Sasaki H, Nishizaki Y, Hui C, Nakafuku M, Kondoh H. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development 1999; 126: 3915-24.

[67] Katoh M, Katoh M. Human FOX gene family. Int J Oncol 2004; 25: 1495-500.

[68] Préat T, Thérond P, Lamour-Isnard C, *et al*. A putative serine/threonine protein kinase encoded by the segment-polarity fused gene of Drosophila. Nature 1990; 347: 87-9.

[69] Kinzler KW, Bigner SH, Bigner DD, *et al*. Identification of an amplified, highly expressed gene in a human glioma. Science1987; 236: 70-3.

[70] Price MA. CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. Genes Dev 2006; 20: 399-410.

[71] Jiang J, Struhl G. Regulation of the Hedgehog and Wingless signaling pathways by the F-box/WD40-repeat protein Slimb. Nature 1998; 391: 493-6.

[72] Koike J, Sagara N, Kirikoshi H, *et al*. Molecular cloning and characterization of the ßTRCP2 gene on chromosome 5q35.1. Biochem Biophys Res Commun 2000; 269: 103-9.

[73] Saitoh T, Katoh M. Expression profiles of ßTRCP1 and ßTRCP2, and mutation analysis of ßTRCP2 in gastric cancer. Int J Oncol 2001; 18: 959-64.
[74] Stegman MA, Vallance JE, Elangovan G, Sosinski J, Cheng Y, Robbins DJ. Identification of a tetrameric hedgehog signaling complex. J Biol Chem 2000; 275: 21809-12.
[75] Osterlund T, Everman DB, Betz RC, *et al*. The FU gene and its possible protein isoforms. BMC Genomics 2004; 5: 49.
[76] Taylor MD, Liu L, Raffel C, *et al*. Mutations in SUFU predispose to medulloblastoma. Nat Genet 2002; 31: 306-10.
[77] Chuang PT, McMahon AP. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. Nature 1999; 397: 617-21.
[78] Yoon JW, Kita Y, Frank DJ, *et al*. Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. J Biol Chem 2002; 277: 5548-55.
[79] Teh MT, Wong ST, Neill GW, Ghali LR, Philpott MP, Quinn AG. FOXM1 is a downstream target of Gli1 in basal cell carcinomas. Cancer Res 2002; 62: 4773-80.
[80] Laoukili J, Kooistra MR, Bras A, *et al*. FoxM1 is required for execution of the mitotic programme and chromosome stability. Nat Cell Biol 2005; 7: 126-36.
[81] Vasiliauskas D, Hancock S, Stern CD. SWiP-1: novel SOCS box containing WD-protein regulated by signalling centres and by Shh during development. Mech Dev 1999; 82: 79-94.
[82] Lee CS, Buttitta LA, May NR, Kispert A, Fan CM. SHH-N upregulates Sfrp2 to mediate its competitive interaction with WNT1 and WNT4 in the somitic mesoderm. Development 2000; 127: 109-18.
[83] Pola R, Ling LE, Silver M, *et al*. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. Nat Med 2001; 7: 706-11.
[84] Bitgood MJ, McMahon AP. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Dev Biol 1995; 172: 126-38.
[85] Ericson J, Morton S, Kawakami A, Henk Roelink H, Jessell TM. Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell 1996; 87: 661-73.
[86] Hargrave M, Karunaratne A, Cox L, Wood S, Koopman P, Yamada T. The HMG box transcription factor gene Sox14 marks a novel subset of ventral interneurons and is regulated by sonic hedgehog. Dev Biol 2000; 219: 142-53.
[87] Gibson-Brown JJ, Agulnik SI, Silver LM, Niswander L, Papaioannou VE. Involvement of T-box genes Tbx2-Tbx5 in vertebrate limb specification and development. Development 1998; 125: 2499-509.
[88] Huangfu D, Anderson KV. Cilia and Hedgehog responsiveness in the mouse. Proc Natl Acad Sci USA 2005; 102: 11325-30.
[89] Riobo NA, Lu K, Ai X, Haines GM, Emerson Jr CP. Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. Proc Natl Acad Sci USA 2006; 103: 4505-10.
[90] Riobo NA, Haines GM, Emerson Jr CP. Protein kinase C-delta and mitogen-activated protein/ extracellular signal-regulated kinase-1 control Gli activation in Hedgehog signaling. Cancer Res 2006; 66: 839-45.
[91] Izraeli S, Lowe LA, Bertness VL, *et al*. The SIL gene is required for mouse embryonic axial development and left-right specification. Nature 2001; 399: 691-4.
[92] Callahan CA, Ofstad T, Horng L, *et al*. MIM/BEG4, a Sonic hedgehog-responsive gene that potentiates Gli-dependent transcription. Genes Dev 2004; 18: 2724-9.
[93] Chen W, Ren XR, Nelson CD, *et al*. Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. Science 2004; 306: 2257-60.
[94] Eggenschwiler JT, Espinoza E, Anderson KV. Rab23 is an essential negative regulator of the mouse Sonic hedgehog signalling pathway. Nature 2001; 412: 194-8.
[95] Kessaris N, Jamen F, Rubin LL, Richardson WD. Cooperation between sonic hedgehog and fibroblast growth factor/MAPK signalling pathways in neocortical precursors. Development 2004; 131: 1289-98.
[96] Lupo G, Liu Y, Qiu R, *et al*. Dorsoventral patterning of the Xenopus eye: a collaboration of Retinoid, Hedgehog and FGF receptor signaling. Development 2005; 132: 1737-48.
[97] Agouni A, Mostefai HA, Porro C, *et al*. Sonic hedgehog carried by microparticles corrects endothelial injury through nitric oxide release. FASEB J 2007; 21, 2735-41.
[98] Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995; 1: 27-31.
[99] Isner JM, Asahara T. Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. J Clin Invest 1999; 103: 1231-6.
[100] Asahara T, Murohara T, Sullivan A, *et al*. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-7.
[101] Jacobs J. Combating cardiovascular disease with angiogenic therapy. Drug Discov Today
2007; 12: 1040-5.
[102] Losordo DW, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic diseases. Circulation 2004; 109, 2487-91.
[103] Ng YS, D'Amore PA. Therapeutic angiogenesis for cardiovascular disease. Curr Control Trials Cardiovas Med 2001; 2: 278-85.
[104] Post MJ, Laham R, Sellke FW, Simons M. Therapeutic angiogenesis in cardiology using protein formulations. Cardiovasc Res 2001; 49: 522-31.
[105] Tong Q, Zheng L, Li B, Wang D, *et al*. Hypoxia-induced mitogenic factor enhances angiogenesis by promoting proliferation and migration of endothelial cells. Exp Cell Res 2006; 312: 3559-69.
[106] Nagase T, Nagase M. Time windows of hedgehog signalling in craniofacial and vascular development: Analyses using a mouse whole embryo culture system. In: Grachevsky N Ed, Signal transduction research trends. Hauppauge, NOVA Science Publishers, 2007; 131-70.
[107] Lawson ND, Vogel AM, Weinstein BM. Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. Dev Cell 2002; 3: 127-36.
[108] Vokes SA, Yatskievych TA, Heimark RL, *et al*. Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. Development 2004; 131: 4371-80.
[109] Lavine KJ, White AC, Park C, *et al*. Fibroblast growth factor signals regulate a wave of Hedgehog activation that is essential for coronary vascular development. Genes Dev 2006; 20: 1651-66.
[110] Rowitch DH, S-Jacques B, Lee SM, Flax JD, Snyder EY, McMahon AP. Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. J Neurosci 1999; 19: 8954-65.
[111] Brown LA, Roadway AR, Schilling TF, *et al*. Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. Mech Dev 2000; 90: 237-52.
[112] Van Tuyl M, Groenman F, Wang J, *et al*. Angiogenic factors stimulate tubular branching morphogenesis of sonic hedgehog-deficient lungs. Dev Biol 2007; 303: 514-26.
[113] Pepicelli CV, Lewis PM, McMahon AP. Sonic hedgehog regulates branching morphogenesis in the mammalian lung. Curr Biol 1998; 8: 1083-6.
[114] White AC, Lavine KJ, Ornitz DM, *et al*. FGF9 and SHH regulate mesenchymal Vegfa expression and development of the pulmonary capillary network. Development 2007; 134: 3743-52.
[115] Bijlsma MF, Peppelenbosch MP, Spek CA. Hedgehog morphogen in cardiovascular disease. Circulation 2006; 114: 1985-91.
[116] Kusano KF, Pola R, Murayama T, *et al*. Sonic hedgehog myocardial gene therapy: tissue repair through transient reconstitution of embryonic signaling. Nat Med 2005; 11: 1197-204.
[117] Ozturk H, Tuncer MC, Ozturk H, Buyukbayram H. Nitric oxide regulates expression of sonic hedgehog and hypoxia-inducible factor-1alpha in an experimental model of kidney ischemia-reperfusion. Ren Fail 2007; 29: 249-56.
[118] Briscoe J. Graded Hedgehog signalling and the specification of neural cellfate. Presented at the 45th Karolinska Institutet Nobel Conference, Stockholm, Sweden, 2004.
[119] Asai J, Takenaka H, Kengo F, *et al*. Topical sonic hedgehog gene therapy accelerates wound healing in diabetes by enhancing endothelial progenitor cell-mediated microvascular remodelling. Circulation 2006; 113: 2413-24.
[120] Kusano KF, Allendoerfer KL, Munger W, *et al*. Sonic hedgehog induces arteriogenesis in diabetic vasa nervorum and restores function in diabetic neuropathy. Arterioscler Thromb Vasc Biol 2004; 24: 2102-7.

[121] Surace EM, Balaggan KS, *et al*. Inhibition of ocular neovascularisation by hedgehog blockade. Mol Ther 2006; 13: 573-9.
[122] Byrd KL, Grabel L. Hedgehog signaling in murine vasculogenesis and angiogenesis. Trends Cardiovasc Med 2004; 14: 308-313.
[123] Geng L, Cuneo KC, Cooper MK, *et al*. Hedgehog signaling in the murine melanoma microenvironment. Angiogenesis 2007; 10: 259-67.
[124] Hochman E, Castiel A, Jacob-Hirsch J, Amariglio N, Izraeli S. Molecular pathways regulating pro-migratory effects of Hedgehog signaling. J Biol Chem 2006; 281: 33860-70.
[125] Kanda S, Mochizuki Y, Suematsu T, Miyata Y, Nomata K, Kanetake H. Sonic hedgehog induces capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase. J Biol Chem 2003; 278: 8244-9.
[126] Lee SW, Moskowitz MA, Sims JR. Sonic hedgehog inversely regulates the expression of angiopoietin-1 and angiopoietin-2 in fibroblasts. Int J Mol Med 2007; 19: 445-51.
[127] Hwang JM, Weng YJ, Lin JA, *et al*. Hypoxia-induced compensatory effect as related to Shh and HIF-1alpha in ischemia embryo rat heart. Mol Cell Biochem 2008; 311: 179-87.
[128] Chao W, D'Amore PA. IGF2: Epigenetic regulation and role in development and disease. Cytokine Growth Factor Rev 2008; 19: 111-20.
[129] Luque Contreras D, Vargas Robles H, Romo E, Rios A, Escalante B. The role of nitric oxide in the post-ischemic revascularization process. Pharmacol Ther 2006; 112: 553-63.
[130] Mimeault M, Moore E, Moniaux N, *et al*. Cytotoxic effects induced by a combination of cyclopamine and gefitinib, the selective hedgehog and epidermal growth factor receptor signalling inhibitors, in prostate cancer cells. Int J Cancer 2006; 118: 1022-31.
[131] Stone JR, Collins T. The role of hydrogen peroxide in endothelial proliferative responses. Endothelium 2002; 9: 231-8.
[132] Ushio-Fukai M, Tang Y, Fukai T, *et al*. Novel role of gp91phox-containing NAD(P)H oxidase in vascular endothelial growth factor induced signaling and angiogenesis. Circ Res 2002; 91: 1160-7.
[133] Luczak K, Balcerczyk A, Soszynski M, Bartosz G. Low concentration of oxidant and nitric oxide donors stimulate proliferation of human endothelial cells in vitro. Cell Biol Int 2004; 28: 483- 6.
[134] Maulik N. Redox regulation of vascular angiogenesis. Antioxid Redox Signal 2002; 4: 783-4.
[135] Tanaka K, Weihrauch D, Kehl F, et al. Mechanism of preconditioning by isoflurane in rabbits: a direct role for reactive oxygen species. Anesthesiology 2002; 97: 1485- 90.
[136] Kelly BD, Hackett SF, Hirota K, *et al*. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circ Res 2003; 93: 1074-81.
[137] Akita T, Murohara T, Ikeda H *et al*. Hypoxic preconditioning augments efficacy of human endothelial progenitor cells for therapeutic neovascularization. Lab Invest 2003; 83: 65-73.
[138] Podlasek CA, Meroz CL, Korolis H, Tang Y, McKenna KE, McVary KT. Sonic hedgehog, the penis and erectile dysfunction: A review of sonic hedgehog signalling in the penis. Curr Pharma Des 2005. 11: 4011-27.
[139] Tuncer MC, Ozturk H, Buyukbayram H, Ozturk H. Interaction of L-arginine-methyl ester and Sonic hedgehog in liver ischemia-reperfusion injury in the rats. World J Gastroenterol 2007; 13: 3841-6.
[140] Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and *in vivo* monitoring of ROS. Circulation 2003; 108: 1912-6.
[141] Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part II: animal and human studies. Circulation 2003; 108: 2034-40.
[142] Li JM, Shah AM. Endothelial cell superoxide generation:regulation and relevance for cardiovascular pathophysiology. Am J Physiol Regul Integr Comp Physiol 2004; 287: R1014-30.
[143] Yasuda M, Ohzeki Y, Shimizu S. *et al*. Stimulation of *in vitro* angiogenesis by hydrogen peroxide and the relation with ETS-1 in endothelial cells. Life Sci 1999; 64: 249-58.
[144] Ruef J, Hu ZY, Yin LY, *et al*. Induction of vascular endothelial growth factor in balloon-injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. Circ Res 1997; 81: 24-33.
[145] Ebrahimian TG, Heymes C, You D. *et al*. NADPH oxidase-derived overproduction of reactive oxygen species impairs postischemic neovascularization in mice with type 1 diabetes. Am J Pathol 2006; 169: 71928.
[146] McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol 2003; 53: 1-114.
[147] Ruiz i Altaba A, Sanchez P, Dahmane N. Gli and hedgehog in cancer: tumours, embryos, and stem cells. Nat Rev Cancer 2002; 2: 361-72.
[148] Berman DM, Karhadkar SS, Hallahan AR, *et al*. Medulloblastoma growth inhibition by hedgehog pathway blockade. Science 2002; 297: 1559-61.
[149] Cretnik M, Musani V, Oreskovic S, *et al*. The Patched gene is epigenetically regulated in ovarian dermoids and fibromas,but not in basocellular carcinomas. Int J Mol Med 2007; 19: 875-83.
[150] Pritchard JI, Olson JM. Methylation of PTCH1, the Patched-1 gene, in a panel of primary medulloblastomas. Cancer Genet Cytogenet 2008; 180: 47-50.
[151] De Smaele E, Fragomeli C, Ferretti E,*et al*. An integrated approach identifies Nhlh1 and Insm1 as Sonic Hedgehog-regulated genes in developing cerebellum and medulloblastoma. Neoplasia 2008; 10: 89-98.
[152] Taipale J, Chen JK, Cooper MK, *et al*. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature 2000; 406: 1005-9.
[153] Ma X, Sheng T , Zhang Y, *et al*. Hedgehog signaling is activated in subsets of esophageal cancers. Inter J Cancer 2006; 118:139-48.
[154] Sims-Mourtada J, Izzo JG, Apisarnthanarax S, *et al*. Hedgehog: an attribute to tumor regrowth after chemoradiotherapy and a target to improve radiation response. Clin Cancer Res 2006; 6565: 12.
[155] Van Den Brink GR, Hardwick JC, Tytgat GN, *et al*. Sonic Hedgehog regulates gastric gland morphogenesis in man and mouse. Gastroenterology 2001; 121: 317-328.
[156] Yoo YA, Kang MH, Kim JS, *et al*. Sonic hedgehog signaling promotes motility and invasiveness of gastric cancer cells through TGF-b-mediated activation of the ALK5-Smad 3 pathway. Carcinogenesis 2008; 29: 480-490.
[157] Dennler S, André J, Alexaki I, *et al*. Induction of Sonic Hedgehog mediators by transforming growth factor-beta: Smad3-dependent activation of Gli2 and Gli1 expression *in vitro* and *in vivo*. Cancer Res 2007; 67: 6981-6.
[158] Gorska AE, Jensen RA, Shyr Y, *et al*. Transgenic mice expressing a dominant-negative mutant type II transforming growth factor-beta receptor exhibit impaired mammary development and enhanced mammary tumor formation. Am J Pathol 2003; 163: 1539-49.
[159] Bertolino P, Deckers M, Lebrin F, *et al*. Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. Chest 2005; 128: 585S-590S.
[160] Fukaya M, Isohata N, Ohta H, *et al*. Hedgehog signal activation in gastric pit cell and in diffuse-type gastric cancer. Gastroenterology 2006; 131: 14-29.
[161] Thayer SP, di Magliano MP, Heiser PW, *et al*. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature 2003; 425: 851-6.
[162] Hebrok M. Hedgehog signaling in pancreas development. Mech Dev 2003; 120: 45-57.
[163] Nakamura M, Kubo M, Yanai K, *et al*. Anti-patched-1 antibodies suppress hedgehog signaling pathway and pancreatic cancer proliferation. Anticancer Res 2007; 27: 3743-7.
[164] Berman DM, Karhadkar SS, Maitra A, *et al*. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature 2003; 425: 846-851.
[165] Qualtrough D, Buda A, Gaffield W, Williams AC, Paraskeva C. Hedgehog signalling in colorectal tumour cells: induction of apoptosis with cyclopamine treatment. Int J Cancer 2004; 110: 831-7.
[166] Zhu Y, James RM, Peter A, *et al*. Functional Smoothened is required for expression of GLI3 in colorectal carcinoma cells. Cancer Lett 2004; 207: 205-14.
[167] Chatel G, Ganeff C, Boussif N, *et al*. Hedgehog signaling pathway is inactive in colorectal cancer cell lines. Int J Cancer 2007; 121: 2622-7.
[168] Farndon PA, Del Mastro RG, Evans DG, Kilpatrick MW. Location of gene for Gorlin syndrome. Lancet 1992; 339: 581-2.

[169] Gailani MR, Bale SJ, Leffell DJ, *et al*. Developmental defects in Gorlin syndrome related to a putative tumor suppressor gene on chromosome 9. Cell 1992; 69: 111-7.

[170] Gailani MR, Stahle-Backdahl M, Leffell DJ, *et al*.The role of the human homologue of Drosophila patched in sporadic basal cell carcinomas. Nat Genet 1996; 14: 7-8.

[171] Wolter M, Reifenberger J, Sommer C, Ruzicka T, Reifenberger G. Mutations in the human homologue of the Drosophila segment polarity gene patched (PTCH) in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. Cancer Res 1997; 57: 2581-5.

[172] Makino S, Masuya H, Ishijima J, *et al*. A spontaneous mouse mutation, mesenchymal dysplasia (mes), is caused by a deletion of the most C-terminal cytoplasmic domain of patched (ptc). Dev Biol 2001; 239: 95-106.

[173] Nieuwenhuis E, Barnfield PC, Makino S, Hui CC. Epidermal hyperplasia and expansion of the interfollicular stem cell compartment in mutant mice with a C-terminal truncation of Patched1. Dev Biol 2007; 308: 547-60.

Received: July 22, 2008 Revised: August 19, 2008 Accepted: September 04, 2008

[174] Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. Nature 2004; 432: 324-31.

[175] Jinawath A, Akiyama Y, Sripa B, *et al*. Dual blockade of the Hedgehog and ERK1/2 pathways coordinately decreases proliferation and survival of cholangiocarcinoma cells. J Cancer Res Clin Oncol 2007; 133: 271-8.

[176] Sicklick JK, Li YX, Jayaraman A, *et al*. Dysregulation of the Hedgehog pathway in human hepatocarcinogenesis. Carcinogenesis 2006; 27: 748-57.

[177] Bijlsma MF, Leenders PJ, Janssen BJ, Peppelenbosch MP, Ten Cate H, Spek CA. Endogenous Hedgehog expression contributes to myocardial ischemia reperfusion-induced injury. Exp Biol Med (Maywood) 2008;233:989-96

[178] Ingram AL, Parker AR. A review of the diversity and evolution of photonic structures in butterflies, incorporating the work of John Huxley (The Natural History Museum, London from 1961 to 1990). Philos Trans R Soc Lond B Biol Sci 2008; 363: 2465-80.

[179] Koga K, Nakamura M, Nakashima H, *et al*. Novel link between estrogen receptor alpha and hedgehog pathway in breast cancer. Anticancer Res 2008; 8:731-40.

DISCUSSION and CONCLUSION

MPs, which are released from the plasma membrane blebs upon activation and/or apoptosis, reflect pathophysiologic condition of cells they stem from and possess a broad spectrum of biological activities, facilitating cell-cell interaction, inducing cell signalling, or even transferring molecules between different cell types.

Understanding the precise role of MPs in cardiovascular function may be helpful not only to increase the comprehension of pathophysiologic mechanisms, but also may have implication in treatment of different diseases.

Our study suggests new insight into the role of MPs^{Shh+} in vascular medicine and may have significant implication for therapy in disease associated with an impairment or excess of angiogenesis.

Angiogenesis is a highly regulated mechanism under control of fine balance of angiogenic stimulators and inhibitors. Deviation from such control often leads to or is associated with diseases. Vessels formation is a fundamental process required for the normal growth and development of tissues. It is a prerequisite for the development and differentiation of vascular tree, as well as, for a wide variety of fundamental physiological process (embryogenesis, tissue repair and regeneration, cyclical growth of endometrium, and placenta implantation). By contrast, angiogenesis occurs in tumours, which continuously stimulate vessel formation in order to grow.

The implication of MPs in the modulation of angiogenesis is described in the literature, but the effects elicited appear controversial, because of responses they generated depend on stimulation, activation status of origin cell and their concentration.

Our study examines the role of MPs, generated from T lymphocytes undergoing activation and apoptosis, bearing the morphogen Shh, in angiogenesis.

Previous studies have been shown the implication of Shh in angiogenesis using *in vitro* and *in vivo* models (Kanda *et al*. 2003; Pola *et al*. 2001) and its ability in modulate VEGF and eNOS activities (Podlasek *et al*. 2005). Moreover, we have demonstrated that MPs^{Shh+} generate modifications in expression and activation of enzymes related to NO pathway, directly mediated by Shh (Agouni *et al.* 2007).

Supported by these evidences, the first part of our study shows that MPs^{Shh+} are able to regulate multiple steps related to *in vitro* angiogenesis. Although EC migration and proliferation are inhibited by MPs^{Shh+} treatment, independently from Shh cascade, other phases of angiogenic process are activated (Fig. 6). Analogous results have been described about angiopoietin-like 4, which plays a role in angiogenic modulation. In fact, it acts as a pro-angiogenic factor in tumour and ischemic tissues (Le Jan *et al.* 2003), but its interaction with ECM-associated proteins negatively regulates their angiogenic capacities, inhibiting EC migration (Cazes *et al.* 2006). Moreover, other studies, which demonstrate Shh ability in stimulating angiogenesis, do not observe Shh-induced proliferation and migration of cultured ECs (Pola *et al*. 2001; Kanda *et al*. 2003). Besides, MPs^{Shh+} promote the formation of capillary-like structures by a mechanism sensitive to cyclopamine. Previous study has shown that Shh induces capillary morphogenesis through Patched/Smoothened system (Kanda *et al.* 2003). Moreover, EC adhesion and expression of proteins implicated in this step such as Rho A and phosphorylated FAK are enhanced in response to MPs^{Shh+} through both ROCK and Shh pathways.

Accordingly, Shh cascade is able to induce EPC adhesion activity (Asai *et al.* 2006). Besides, as shown by up-regulation of ICAM-1, HGF, VEGF A, Flt1, IL-1β and MMP-1 and down-regulation of TGFβ2 transcripts, MPs^{Shh+} modify expression levels of angiogenic factors (Fig. 6). VEGF binding to an appropriate receptor like Flt1, is a major inducer of neovessels formation, based on its ability to stimulate vasodilatation via endothelial NO production (Ziche *et al.* 1997). VEGF expression can be up-regulated by specific cytokines such as IL-1β, which it has been shown to be a pro-angiogenic factor (Voronov *et al.* 2003). Furthermore, in ECs, IL-1β can down-regulate the expression of Ang1 and this occurs concomitantly to an increase in VEGF expression. This mechanism results in a net gain of pro-angiogenic stimuli that overall may promote and potentiate neovascularization (Fan *et al*. 2004). Also, VEGF stimulates expression of ICAM-1 and induces adhesiveness. ECs exposed to mitogenic signals up-regulate ICAM-1 and then a marked and prolonged suppression occurs. It is unknown why ECs up-regulate ICAM-1 expression, but it may be related to the function of ICAM-1 as a receptor for ECM components (McCourt *et al* 1994). It is known that receptors for matrix interactions are induced during angiogenesis to prepare the cells for migration into the direction of the angiogenic stimulus. It is possible that ICAM-1 has a dual function: involvement in EC migration during early stages of cell activation, and suppression of leukocyte interaction during later stages (Griffioen 2008). Also, we have observed that ECs stimulated with MPs^{Shh+} display enhanced expression of MMP1, an important protease involved mainly in the early stage of vessel formation.

Sprouting angiogenesis is an invasive process that involves matrix-degrading proteases production, particularly MMPs, required for the degradation of the endothelial basement membrane, cell migration and generation of space in the matrix to allow ECs to form a proper lumen. MMPs exert additional, more subtle functions. They modulate the balance between pro- and anti-angiogenic factors by activation and modification of growth factors and chemokines, ectodomain shedding with accompanied receptor activation, shedding of cytokines from membrane-bound precursors, and generation of protein fragments that inhibit or activate angiogenesis. Furthermore, they participate in the recruitment of leukocytes and progenitor cells, which contribute to the onset and progression of angiogenesis (van Hinsbergh and Koolwijk 2008). Treatment of ECs with MPs^{Shh+} results in increased expression of HGF, which is another endothelial growth factor with potent angiogenic and mitogenic effects (Nakagami *et al*. 2001; Morishita *et al.* 2002), able to promote tube formation of ECs. MPs^{Shh+} down-regulate expression of TGF-β2. TGF-β plays an important role in controlling various endothelial functions, among which vascular development. Indeed, while the nascent endothelial tube requires VEGF for survival, the final stages of vessel formation depend upon TGF-β. In particular, TGF-β leads to vessel stabilization, because it inhibits EC proliferation and migration and induces pericyte/SMC muscle cell differentiation (Hirschi *et al*. 1999). In fact, in EC, TGF-β signalling can activate two distinct pathways, which have opposing effects on proliferation and migration. The balance of signalling between these cascades regulates EC biology through the activation (increased EC proliferation and migration) and maturation (decreased EC proliferation and migration) phases of angiogenesis (Goumans *et al*. 2003; Lamouille *et al.* 2002. David L *et al*. 2007). These opposing pathways likely explain the ability of TGF-β to mediate pro-angiogenic or

anti-angiogenic effects *in vitro* (Elliott and Blobe 2005).

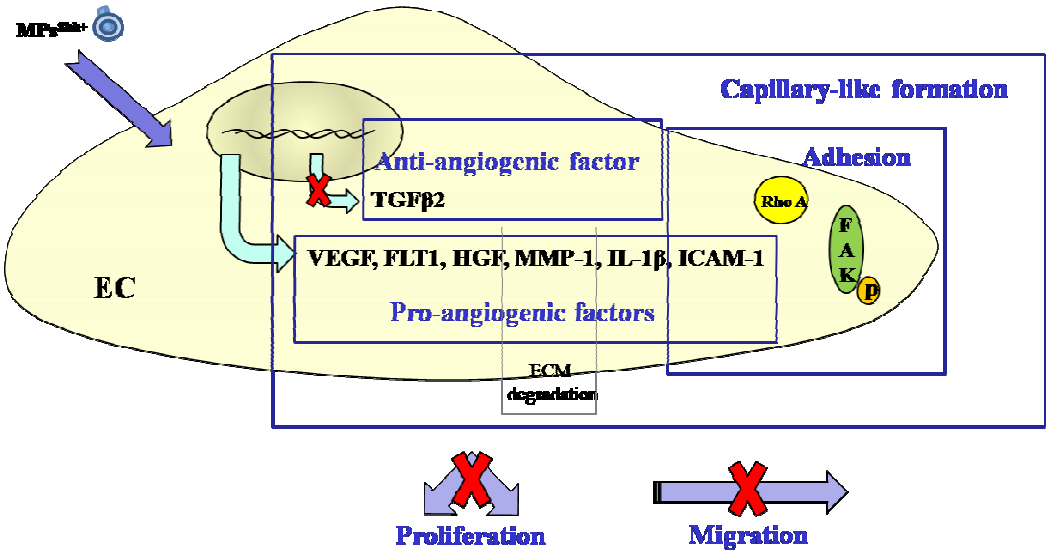


Fig. 6: Schematic representation of *in vitro* effects evoked by MPs^{Shh+}.

Collectively, our data demonstrated that MPs^{Shh+} acts at different phases of vessels formation. As consequence, we suggest that MPs^{Shh+} may contribute to generation of a vascular network in diseases associated with alterations of angiogenesis and therefore represent a new target in pathologies such as cancer. Expression of Shh correlates with the tumorigenesis of different types of cancer (Porro *et al*. 2009). In addition, release of MPs from tumor or vascular cells is also related with tumorigenesis. Thus, targeting Shh pathway and/or MPs production would represent a novel therapeutic tool that can regulate angiogenesis and in consequence, tumor development.

On the other hand, promoting angiogenesis is desirable in situations who vascularization is to be established or extended, such as after tissue or organ transplantation, or to stimulate establishment of collateral circulation in tissue infarction or to enhance in ischemic conditions, including cardiovascular and limb ischemia.

Accordingly, the second part of our study investigates the capacity of MPs^{Shh+} to induce neo-vascularization in a mouse hindlimb ischemia model. Our results show that MPs^{Shh+} promote blood flow recovery in ischemic limb by a mechanism sensitive to Shh antagonist, cyclopamine. Probably, stimulation of perfusion evoked by MPs^{Shh+} takes place in concert with an increasing in capillary formation. Moreover, in skeletal muscle, MPs^{Shh+} are able to enhance eNOS and decrease Cav-1 expressions, concomitantly. Binding of Cav-1 to reductase domain of eNOS compromises its activity, thereby inhibiting NO synthesis (Ghosh *et al.* 1998). Even if our study has not yet demonstrated the co-expression of these two proteins, it is possible that this interaction occurs. Alternatively, the enhancement in NO production observed, may be due to other Ca^{2+}-sensitive signalling pathways, because MPs^{Shh+} treatment is unable to modify Akt expression and phosphorylation, upstream of eNOS activation. Besides, our results suggest a MPs^{Shh+} modulation of angiogenesis factors at transcriptional levels. For example, they down-regulate expression of TSP1, which is the first naturally arising angiogenesis inhibitor to be described (Good *et al.* 1990; Iruela-Arispe *et al.* 1991; Dameron *et al.* 1994), as well as those of MMP-1 and MMP-2, which are mostly expressed during the early steps of angiogenesis, when degradation of ECM is essential to allow sprouting. MMP activity is an early event in the angiogenic response, and several findings suggest that this activity may directly influence EC behaviour. Some evidences suggest that MMPs may facilitate angiogenesis as well as function to generate angiogenesis inhibitors (Stetler-Stevenson 1999). Besides, administration of MPs^{Shh+} reduces expression of VEGFR, Flt4, which is involved in the development of blood vessels in the embryo (Dumont *et al.* 1998), but becomes restricted to the endothelia of lymphatic vessels in the adult (Kaipainem *et al.* 1995; Kukk *et al.* 1996) or in tumour blood vessels (Valtola *et al.* 1999). Moreover, transcript of FGF5, known

to stimulate cell growth and proliferation in multiple cell types, are enhanced after MPs^{Shh+} treatment. The most commonly cited effect of FGF-5 regards the heart who it promotes angiogenesis. In fact, several studies have shown that gene transfer of FGF-5 in the heart increases vessel formation and regional blood flow (Vatner 2005; Giordano *et al.* 1996; Li *et al*. 1999; Lewis *et al*. 1997).

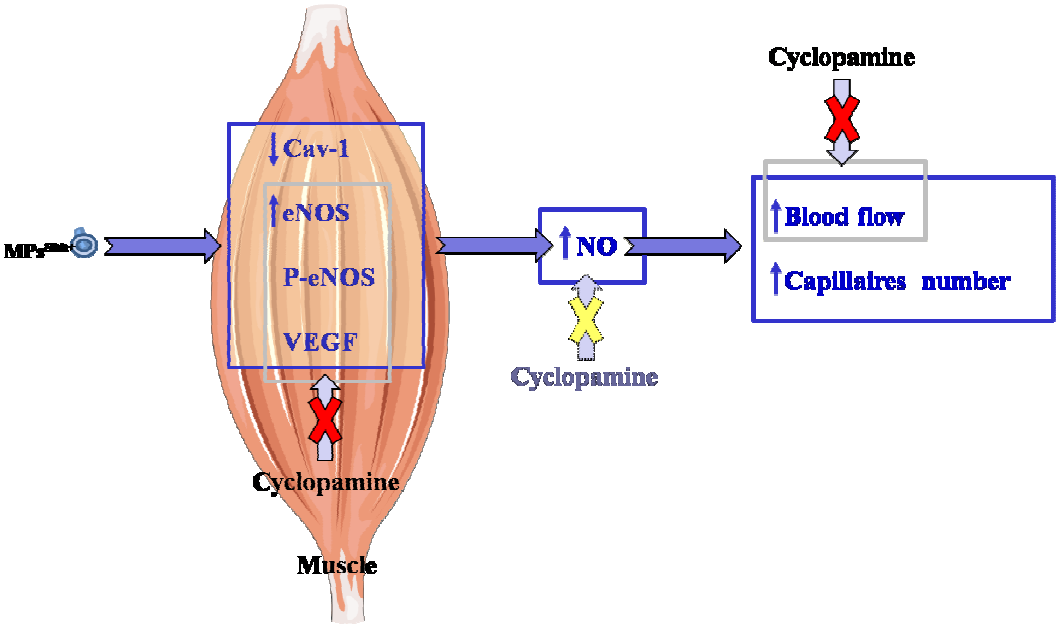


Fig. 7: Schematic representation of *in vivo* effects triggered by MPs^{Shh+} on skeletal muscle.

Screening the differential expression levels during the early and late phases of MPs^{Shh+} treatment may be helpful to further understand the modulation of genes involved in angiogenic process and to uncover a strategy by which attenuate, repress or maintain the same genes in order to re-address angiogenic switch. Moreover, identification of other antigens carried by MPs^{Shh+} by proteomic analysis, in order to determinate their composition and concentration, may help in better explication on their

effects. Further investigation on mechanisms evoked by MPs^{Shh+} on SMCs *in vitro*, and on *in vivo* angiogenesis such as the implication of EPCs and further examination to prevent risk of pathological angiogenesis far from interest area, are necessary to elaborate an efficacy strategy which envisage the application of MPs^{Shh+}; as well as knowledge of MPs half-life are needed to elaborate approaches to monitoring MPs and to maintain the benefit of MPs^{Shh+}.

Angiogenesis therapy with MPs^{Shh+} is attractive for several reasons: they are intuitively right to create a natural bypass around vessels, increase NO and reduce ROS production (Agouni *et al*. 2007), may represent an autologous therapy, may decrease risk of systemic toxicity, may allow the modulation of excessive or improved angiogenic response. Elucidation of MPs^{Shh+} composition, as well as, the underlying mechanisms involved in their effects will help us to develop additional interventional strategies for prevention and treatment of diseases associated to angiogenesis.

Furthermore, because of few studies have shown the effects of MPs on apoptosis, because of the paradoxical effects triggered by MPs depend on stimulus at their origin, and because of apoptosis is associated to ischemia and tumour angiogenesis, future study on MPs^{Shh+} could be addressed to elucidate their role in these processes.

Tumour growth is angiogenesis-dependent (Folkman 1971). In the absence of angiogenesis, tumour growth is restricted to a microscopic size and, under these conditions, tumours cells do not shed into the circulation. In non-angiogenic in situ tumours (Achilles *et al.* 2001) or in dormant micro-metastases (Holmgren *et al*. 1995; Gimbrone *et al.* 1972), tumour cell proliferation continues balanced by high rates of tumours cell apoptosis (Holmgren *et al.* 1995). High rates of human tumours cell

apoptosis can persist for as long as a tumours remains non-angiogenic (Udagawa *et al.* 2002). Also, ischemia is associated with apoptosis of ECs due to hypoxia and deficiency of survival growth factors (Lee *et al.* 2005; Hogg *et al*. 1999; Matsushita *et al*. 2000; Meeson *et al*. 1999).

Regarding the effects of MPs on apoptosis, it has been demonstrated that PMPs inhibit this process in human hematopoietic cells (Baj-Krzyworzeka *et al.* 2002) and in human ECs (Zhong *et al.* 2007), by contrast, induce apoptosis in human macrophages (Böing *et al.* 2008). Another study displays that EMPs increased the apoptosis rate in a dose-response manner: physiological concentration of EMPs such as those found in circulation of healthy subjects (10^3 and 10^4 EMPs/ml) do not affect apoptosis (Mezentsev *et al.* 2005). Moreover, MPs from apoptotic lymphocytes have no effect on ECs apoptosis (Yang *et al*. 2008), although they are able to activate pathways leading to an increase oxidative stress (Mostefai *et al*. 2008b; Yang *et al*. 2008). Apoptosis of ECs can critically disturb the integrity of the endothelial monolayer (Haimovitz-Friedman *et a*l 1997). Moreover, EC death may contribute to the initial endothelial injury. Enhanced production of ROS in the vascular endothelium can trigger apoptosis of ECs (Dimmeler *et al.* 1999; Hermann *et al.* 1997), whereas, endothelial-derived NO inhibits EC apoptosis.

Since MPs^{Shh+} posses these dual ability, favouring NO increasing, and reducing ROS production (Agouni *et al.* 2007), they could prevent EC apoptosis. Probe this hypothesis may be interesting to further understand their activity and to better address their potential employ and application in vascular therapy.

ANNEXE

Genes analyzed by qRT-PCR

TGFb1
TGFb2
FGF1
FGF2
FGF3
FGF5
FGF7
FGF8
FGF10
PEDF
ephrin b2
alk1
edg1
CD148
EPO
IGF1
a5 integrin
b3 integrin
TSP1
Vcam 1
E-seleetin
ANG1
ANG2
MT1MMP1
MMP2
angiogenin
HGF
PECAM 1
VE-cadherin
VEGFA
VEGFB
VEGFC
VEGFD
PDGFA
PDGFB
IL1b
IL6
TNFa
ICAM 1
GROa
GROb
ENA78
SDF1
MCP1
RANTES
FGFR1
FGFR2
CCR2
IL8RB
FLT1
KDR
FLT4
Tie1
Tie2
PDGFRb

REFERENCES

Achilles EG, Fernandez A, Allred EN, Kisker O, Udagawa T, Beecken WD, Flynn E, Folkman J. 2001. Heterogeneity of angiogenic activity in a human liposarcoma: a proposed mechanism for “no take” of human tumors in mice. *J Natl Cancer Inst*. 93: 1075-1081.

Agouni A, Lagrue-Lak-Hal AH, Ducluzeau PH, Mostefai HA, Draunet-Busson C, Leftheriotis G, Heymes C, Martinez MC, Andriantsitohaina R. 2008. Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome. *Am J Pathol*. doi: 10.2353/ajpath.2008.080228.

Agouni A, Mostefai HA, Porro C, Carusio N, Favre J, Richard V, Henrion D, Martínez MC, Andriantsitohaina R. 2007. Sonic hedgehog carried by microparticles corrects endothelial injury through nitric oxide release. *FASEB J*. 21: 2735-2741.

Allerstorfer S, Sonvilla G, Fischer H, Spiegl-Kreinecker S, Gauglhofer C, Setinek U, Czech T, Marosi C, Buchroithner J, Pichler J, Silye R, Mohr T, Holzmann K, Grasl-Kraupp B, Marian B, Grusch M, Fischer J, Micksche M, Berger W. 2008. FGF5 as an oncogenic factor in human glioblastoma multiforme: autocrine and paracrine activities. *Oncogene*. 27: 4180-4190.

Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, Rak J. 2008. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol*. 10: 619-624.

Angelucci A, D'Ascenzo S, Festuccia C, Gravina GL, Bologna M, Dolo V, Pavan A. 2000. Vesicle-associated urokinase plasminogen activator promotes invasion in prostate cancer cell lines. *Clin Exp Metastasis*. 18: 163-170.

Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, Yancopoulos GD, Isner JM. 1998. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. *Circ Res*. 83: 233-240.

Asahara T, Murohara T, Sullivan A, Silver M, Zee RVD, Li T, Witzenbichler B, Schattemen G, Isner JM. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 275: 964-967.

Asai J, Takenaka H, Kusano KF, Ii M, Luedemann C, Curry C, Eaton E, Iwakura A,

Tsutsumi Y, Hamada H, Kishimoto S, Thorne T, Kishore R, Losordo DW. 2006. Topical sonic hedgehog gene therapy accelerates wound healing in diabetes by enhancing endothelial progenitor cell-mediated microvascular remodeling. *Circulation* 113: 2413-2424.

Auerbach HR, Auerbach W. 1997. Profond effects on vascular development caused by perturbations of during organogenesis. *Am J Pathol*. 151: 1183-1186.

Aupeix K, Hugel B, Martin T, Bischoff P, Lill H, Pasquali JL, and Freyssinet JM. 1997. The significance of shed membrane particles during programmed cell death in vitro and in vivo in HIV-1 infection. *J Clin Invest*. 99: 1546-1554.

Ausprunk DH, Folkman J. 1977. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during angiogenesis. *Microvasc Res*. 14: 53-65.

Azevedo LC, Pedro MA, Laurindo FR. 2007. Circulating microparticles as therapeutic targets in cardiovascular diseases. Recent *Patents Cardiovasc Drug Discov*. 2: 41-51.

Baj-Krzyworzeka M, Majka M, Pratico D, Ratajczak J, Vilaire G, Kijowski J, Reca R, Janowska-Wieczorek A, Ratajczak MZ. 2002. Platelet-derived microparticles stimulate proliferation, survival, adhesion, and chemotaxis of hematopoietic cells. *Exp Hematol*. 30: 450-459.

Barry OP, Kazanietz MG, Pratico D, FitzGerald GA. 1999. Arachidonic acid in platelet microparticles up-regulates cyclooxygenase-2-dependent prostaglandin formation via a protein kinase C/mitogen-activated protein kinase dependent pathway. *J Biol Chem*. 274: 7545-7556.

Barry OP, Pratico D, Lawson JA, FitzGerald GA. 1997. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J Clin Invest*. 99: 2118-2127.

Bayless KJ, Salazar R, Davis GE. 2000. RGD-dependent vacuolation and lumen formation observed during endothelial cell morphogenesis in three-dimensional fibrin matrices involves the alpha(v)beta(3) and alpha(5)beta(1) integrins. *Am J Pathol*. 156: 1673-1683.

Bein K, Simons M. 2000. Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. *J Biol Chem.* 275: 32167-32173.

Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM. 2000. CXC chemokines in angiogenesis. *J Leukoc Biol*. 68: 1-8.

Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. 1982. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem*. 122: 429-436.

Bevers, EM, Comfurius P, Dekkers DW, Zwaal RF. 1999. Lipid translocation across the plasma membrane of mammalian cells. Biochim. *Biophys Acta*. 1439: 317-330.

Blair RJ, Meng H, Marchese MJ, Ren S, Schwartz LB, Tonnesen MG, Gruber BL. 1997. Human mast cells stimulate vascular tube formation. Tryptase is a novel, potent angiogenic factor. *J Clin Invest*. 99: 2691-2700.

Böing AN, Hau CM, Sturk A, Nieuwland R. 2008. Platelet microparticles contain active caspase 3. *Platelets*. 2008. 19: 96-103.

Bretscher MS. 1972. Asymmetric lipid bilayer structure for biological membranes. *Nature (New Biol.)*. 236: 11-12.

Brew K, Dinakarpandian D, Nagase H. 2000. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta.* 1477: 267-283.

Brill A, Dashevsky O, Rivo J, Gozal Y, Varon D. 2005. Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization. *Cardiovasc Res*. 67: 30-38.

Brisset AC, Terrisse AD, Dupouy D, Tellier L, Pech S, Navarro C, Sié P. 2003. Shedding of active tissue factor by aortic smooth muscle cells (SMCs) undergoing apoptosis. *Thromb Haemost*. 90: 511-518.

Brodsky SV, Zhang F, Nasjletti A, Goligorsky MS. 2004. Endothelium-derived microparticles impair endothelial function in vitro. *Am J Physiol Heart Circ Physiol*. 286: 1910-1915.

Carmeliet P, Ng YS, Nuyens D, Theilmeier G, Brusselmans K, Cornelissen I, Ehler E, Kakkar VV, Stalmans I, Mattot V, Perriard JC, Dewerchin M, Flameng W, Nagy A, Lupu F, Moons L, Collen D, D'Amore PA, Shima DT. 1999. Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Nat Med*. 5: 495-502.

Carmeliet P. 2000. Fibroblast growth factor-1 stimulates branching and survival of myocardial arteries: a goal for therapeutic angiogenesis? *Circ Res*. 87: 176-178.

Cazes A, Galaup A, Chomel C, Bignon M, Bréchot N, Le Jan S, Weber H, Corvol P, Muller L, Germain S, Monnot C. 2006. Extracellular matrix-bound angiopoietin-like 4 inhibits endothelial cell adhesion, migration, and sprouting and alters actin cytoskeleton. *Circ Res*. 99: 1207-1215.

Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF.2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat Cell Biol*. 3: 339-345.

Corada M, Mariotti M, Thurston G, Smith K, Kunkel R, Brockhaus M, Lampugnani MG, Martin-Padura I, Stoppacciaro A, Ruco L, McDonald DM, Ward PA, Dejana E. 1999. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proc Natl Acad Sci U S A*. 96: 9815-9820.

Dameron KM, Volpert OV, Tainsky MA, Bouck N. 1994. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science*. 265: 1582-1584.

David L, Mallet C, Vailhé B, Lamouille S, Feige JJ, Bailly S. 2007. Activin receptor-like kinase 1 inhibits human microvascular endothelial cell migration: potential roles for JNK and ERK. *J Cell Physiol*. 2007. 213: 484-489.

Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B, Camussi G. 2007. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood*. 110: 2440-2448.

Diamant M, Tushuizen ME, Sturk A, Nieuwland R. 2004. Cellular microparticles: new players in the field of vascular disease? *Eur J Clin Invest*. 34: 392-401.

Dimmeler S, Hermann C, Galle J, Zeiher AM. 1999. Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. *Arterioscler Thromb Vasc Biol*. 19: 656-664.

Djonov V, Schmid M, Tschanz SA, Burri PH. 2000. Intussusceptive angiogenesis: its role in embryonic vascular network formation. *Circ Res*. 86: 286-292.

Drake CJ, Cheresh DA, Little CD. 1995. An antagonist of integrin $a_v\beta_3$ prevents maturation of blood vessels during embryonic neovascularization. *J Cell Sci*. 108: 2655-2661.

Duan HF, Wu CT, Lu Y, Wang H, Liu HJ, Zhang QW, Jia XX, Lu ZZ, Wang LS. 2004 Sphingosine kinase activation regulates hepatocyte growth factor induced migration of endothelial cells. *Exp. Cell Res*. 298: 593-560.

Dulak J, Józkowicz A, Dembinska-Kiec A, Guevara I, Zdzienicka A, Zmudzinska-Grochot D, Florek I, Wójtowicz A, Szuba A, Cooke JP. 2000. Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 20: 659-666.

Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A, Breitman ML. 1994. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* 8: 1897-1909.

Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, BreitmanMand Alitalo K. 1998. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science.* 282: 946-949.

Dvorak HF. 1976. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med.* 315: 1650-1659.

Egginton S, Gerritsen M. 2003. Lumen formation: in vivo versus in vitro observations. *Microcirculation.* 10: 45-61.

Eliceiri BP, Cheresh DA. 1999b. The role of alphav integrins during angiogenesis: insights into potential mechanisms of action and clinical development. *J Clin Invest.*

103: 1227-1230.

Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA. 1999 a. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell.* 4: 915-924.

Elliott RL, Blobe GC. 2005. Role of transforming growth factor Beta in human cancer. *J Clin Oncol*. 23: 2078-2093.

Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. 1998. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ*. 5: 551-562.

Fadok VA, Bratton DL, Henson PM. 2001. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J. Clin. Invest*. 108: 957-962.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol*. 148: 2207-2216.

Fan F, Stoeltzing O, Liu W, McCarty MF, Jung YD, Reinmuth N, Ellis LM. 2004. Interleukin-1beta regulates angiopoietin-1 expression in human endothelial cells. *Cancer Res*. 64: 3186-3190.

Ferrara N, Alitalo K. 1999. Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med*. 5: 1359-1364.

Folkman J. 1971. Tumor angiogenesis: therapeutic implications. *New Engl J Med*. 285: 1182-1186.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*. 16: 4604-4613.

Fourcade O, Simon MF, Viodé C, Rugani N, Leballe F, Ragab A, Fournié B, Sarda L, Chap H. 1995. Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell*. 80: 919-927.

Fox JE. Shedding of adhesion receptors from the surface of activated platelets. 1994. *Blood Coagul Fibrinolysis.* 5: 291-304.

Freyssinet JM. 2003.Cellular microparticles: what are they bad or good for? *J Thromb Haemost.* 1: 1655-1662.

Fujimi S, Ogura H, Tanaka H, Koh T, Hosotsubo H, Nakamori Y, Kuwagata Y, Shimazu T, Sugimoto H. 2003. Increased production of leukocyte microparticles with enhanced expression of adhesion molecules from activated polymorphonuclear leukocytes in severely injured patients. *J Trauma*. 54: 114-119.

Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD. 2002. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Dev Cell*. 3: 411-423.

Gale NW, Yancopoulos GD. 1999. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev.* 13: 1055-1066.

Gendron RL, Adams LC, Paradis H. 2000. Tubedown-1, a novel acetyltransferase associated with blood vessel development. *Dev Dyn*. 218: 300-315.

Ghajar CM, George SC, Putnam AJ. 2008 Matrix metalloproteinase control of capillary morphogenesis. *Crit Rev Eukaryot Gene Expr*. 18: 251-278.

Ghosh S, Gachhui R, Crooks C, Wu C, Lisanti MP, Stuehr DJ. 1998. Interaction between caveolin-1 and the reductase domain of endothelial nitric-oxide synthase. Consequences for catalysis. *J Biol Chem*. 273: 22267-71.

Gilbert GE, Sims PJ, Wiedmer T, Furie B, Furie BC, Shattil SJ. 1991. Platelet-derived microparticles express high affinity receptors for factor VIII. *J Biol Chem*. 266: 17261-17268.

Gimbrone Jr MA, Leapman SB, Cotran RS, Folkman J. 1972. Tumor dormancy in vivo by prevention of neovascularization. *J Exp Med*. 136: 261-276.

Giordano FJ, Ping P, McKirnan MD, Nozaki S, DeMaria AN, Dillmann WH, Mathieu-

Costello O, Hammond HK. 1996. Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. *Nat Med.* 2: 534–539.

Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP. 1990. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. Natl. Acad. Sci. U. S. A*. 87: 6624-6628.

Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, Karlsson S, ten Dijke P. 2003. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol Cell*. 12: 817-828.

Griffioen AW, Molema G. 2000. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev*. 52: 237-268.

Griffioen AW. 2008. Anti-angiogenesis: making the tumor vulnerable to the immune system. *Cancer Immunol Immunother*. 57: 1553-1558.

Haimovitz-Friedman A, Cordon-Cardo C, Bayoumy S, Garzotto M, McLoughlin M, Gallily R, Edwards CK, Schuchman EH, Fuks Z, Kolesnick R. 1997. Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. *J Exp Med*. 186: 1831-1841.

Hellström M, Kalén M, Lindahl P, Abramsson A, Betsholtz C. 1999. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development*.126: 3047-3055.

Hermann C, Zeiher AM, Dimmeler S. 1997. Shear stress inhibits H2O2-induced apoptosis of human endothelial cells by modulation of the glutathione redox cycle and nitric oxide synthase. *Arterioscler Thromb Vasc Biol*. 17: 3588-3592.

Hirschi KK, Rohovsky SA, D'Amore PA. 1998. PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol*. 141: 805-814.

Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA.

2004. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev*. 56: 549-580.

Hoffman M, Monroe DM, Roberts HR. 1992. Coagulation factor IXa binding to activated platelets and platelet-derived microparticles: a flow cytometric study. Thromb Haemost. 68: 74-78.

Hogg N, Browning J, Howard T, Winterford C, Fitzpatrick D, Gobé G. 1999. Apoptosis in vascular endothelial cells caused by serum deprivation, oxidative stress and transforming growth factor-beta. *Endothelium*. 7: 35-49.

Holmgren L, O'Reilly MS, Folkman J. 1995. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med*. 1: 149-153.

Horstman LL, Jy W, Jimenez JJ, Ahn YS. 2004. Endothelial microparticles as markers of endothelial dysfunction. *Front Biosci*. 9: 1118-1135.

Huang LE, Willmore WG, Gu J, Goldberg MA, Bunn HF. 1999. Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide. Implications for oxygen sensing and signaling. *J Biol Chem*. 274: 9038-9044.

Huber J, Vales A, Mitulovic G, Blumer M, Schmid R, Witztum JL, Binder BR, Leitinger N. 2002. Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. *Arterioscler Thromb Vasc Biol*. 22: 101-107.

Hugel B, Martínez MC, Kunzelmann C, Freyssinet JM. 2005. Membrane microparticles: two sides of the coin. *Physiology (Bethesda)*. 20: 22-27.

Huynh-Do U, Stein E, Lane AA, Liu H, Cerretti DP, Daniel TO. 1999. Surface densities of ephrin-B1 determine EphB1-coupled activation of cell attachment through alphavbeta3 and alpha5beta1 integrins. *EMBO J*. 18: 2165-2173.

Ilan N, Mahooti S, Rimm DL, Madri JA. 1999. PECAM-1 (CD31) functions as a reservoir for and a modulator of tyrosine-phosphorylated beta-catenin. *J Cell Sci*. 112: 3005-3014.

Iruela-Arispe ML, Bornstein P, Sage H. 1991. Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 88: 5026-5030.

Itakura Sumi Y, Ogura H, Tanaka H, Koh T, Fujita K, Fujimi S, Nakamori Y, Shimazu T, Sugimoto H. 2003. Paradoxical cytoskeleton and microparticle formation changes in monocytes and polymorphonuclear leukocytes in severe systemic inflammatory response syndrome patients. *J Trauma.* 55: 1125-1132.

Itoh Y, Seiki M. 2006 MT1-MMP: a potent modifier of pericellular microenvironment. *J Cell Physiol*. 206: 1-8.

Jacobs J. 2007. Combating cardiovascular disease with angiogenic therapy. *Drug Discov Today*. 12: 1040-1045.

Janowska-Wieczorek A, Majka M, Kijowski J, Baj-Krzyworzeka M, Reca R, Turner AR, Ratajczak J, Emerson SG, Kowalska MA, Ratajczak MZ. 2001. Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment. *Blood*. 98: 3143-3149.

Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J, Ratajczak MZ. 2005. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. *Int J Cancer*. 113: 752-760.

Jones MK, Tsugawa K, Tarnawski AS, Baatar D.2004. Dual actions of nitric oxide on angiogenesis: possible roles of PKC, ERK, and AP-1. *Biochem Biophys Res Commun*. 318: 520-528.

Ju H, Zou R, Venema VJ, Venema RC. 1997. Direct interaction of endothelial nitric oxide synthase and caveolin-1 inhibits synthase activity *J Biol Chem.* 272: 18522-18525.

Jy W, Minagar A, Jimenez JJ, Sheremata WA, Mauro LM, Horstman LL, Bidot C, Ahn YS. 2004. Endothelial microparticles (EMP) bind and activate monocytes: elevated EMP-monocyte conjugates in multiple sclerosis. *Front Biosci*. 9: 3137-3144.

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M and Alitalo K. 1995. Expression of the fms-like tyrosine kinase 4 gene

becomes restricted to lymphatic endothelium during development. *Proc. Natl. Acad. Sci. USA*. 92: 3566-3570.

Kanazawa S, Nomura S, Kuwana M, Muramatsu M, Yamaguchi K, Fukuhara S. 2003. Monocyte-derived microparticles may be a sign of vascular complication in patients with lung cancer. *Lung Cancer*. 39: 145-149.

Kanda S, Hodgkin MN, Woodfield RJ, Wakelam MJ, Thomas G, Claesson-Welsh L. 1997. Phosphatidylinositol 3'-kinase-independent p70 S6 kinase activation by fibroblast growth factor receptor-1 is important for proliferation but not differentiation of endothelial cells. *J Biol Chem*. 272: 23347-23353.

Kanda S, Miyata Y, Kanetake H.2004. Role of focal adhesion formation in migration and morphogenesis of endothelial cells. *Cell Signal*. 16: 1273-1281

Kanda S, Mochizuki Y, Suematsu T, Miyata Y, Nomata K, Kanetake H. 2003. Sonic hedgehog induces capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase. *J Biol Chem*. 278: 8244-8249.

Kessaris N, Jamen F, Rubin LL, Richardson WD. 2004. Cooperation between sonic hedgehog and fibroblast growth factor/MAPK signalling pathways in neocortical precursors. *Development*. 131: 1289-1298.

Kim CW, Lee HM, Lee TH, Kang C, Kleinman HK, Gho YS. 2002. Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. *Cancer Res.* 62: 6312-6317.

Kim HK, Song KS, Chung JH, Lee KR, Lee SN. 2004. Platelet microparticles induce angiogenesis in vitro. *Br J Haematol*. 124: 376-384.

Kim HK, Song KS, Park YS, Kang YH, Lee YJ, Lee KR, Kim HK, Ryu KW, Bae JM, Kim S. 2003. Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. *Eur J Cancer*. 39: 184-91.

Kim I, Kim HG, Moon SO, Chae SW, So JN, Koh KN, Ahn BC, Koh GY. 2000. Angiopoietin-1 induces endothelial cell sprouting through the activation of focal

adhesion kinase and plasmin secretion. *Circ Res*. 86: 952-959.

Kimura H, Weisz A, Kurashima Y, Hashimoto K, Ogura T, D'Acquisto F, Addeo R, Makuuchi M, Esumi H. 2000. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood.* 95:189-197.

Klein S, Bikfalvi A, Birkenmeier TM, Giancotti FG, Rifkin DB. 1996. Integrin regulation by endogenous expression of 18-kDa fibroblast growth factor-2. *J Biol Chem.* 271: 22583-22590

Knudsen KA, Frankowski C, Johnson KR, Wheelock MJ. 1998. A role for cadherins in cellular signaling and differentiation. *J Cell Biochem Suppl.* 30-31:168-176.

Kobayashi S, Gando S, Morimoto Y, Nanzaki S, Kemmotsu O. 2001. Serial measurement of arterial lactate concentrations as a prognostic indicator in relation to the incidence of disseminated intravascular coagulation in patients with systemic inflammatory response syndrome. *Surg Today*. 31: 853-859.

Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V and Alitalo K. 1996. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development*. 122: 3829-3837.

Lacroix R, Sabatier F, Mialhe A, Basire A, Pannell R, Borghi H, Robert S, Lamy E, Plawinski L, Camoin-Jau L, Gurewich V, Angles-Cano E, Dignat-George F. 2007. Activation of plasminogen into plasmin at the surface of endothelial microparticles: a mechanism that modulates angiogenic properties of endothelial progenitor cells in vitro. *Blood*. 110: 2432-2439.

Lamouille S, Mallet C, Feige JJ, Bailly S. 2002. Activin receptor-like kinase 1 is implicated in the maturation phase of angiogenesis. *Blood.* 100: 4495-4501.

Lau YT, Ma WC. 1996. Nitric oxide inhibits migration of cultured endothelial cells. *Biochem Biophys Res Commun*. 221: 670-674.

Le Jan S, Amy C, Cazes A, Monnot C, Lamandé N, Favier J, Philippe J, Sibony M, Gasc JM, Corvol P, Germain S. 2003. Angiopoietin-like 4 is a proangiogenic factor produced during ischemia and in conventional renal cell carcinoma. *Am J Pathol*. 162:

1521-1528.

Lee CN, Cheng WF, Chang MC, Su YN, Chen CA, Hsieh FJ. 2005. Hypoxia-induced apoptosis in endothelial cells and embryonic stem cells. *Apoptosis*. 10: 887-894.

Lewis BS, Flugelman MY, Weisz A, Keren-Tal I, Schaper W. 1997. Angiogenesis by gene therapy: a new horizon for myocardial revascularization? *Cardiovasc Res.* 35: 490–497.

Li J, Hampton T, Morgan JP, Simons M. 1997. Stretch-induced VEGF expression in the heart. *J Clin Invest*. 100: 18-24.

Li K, Stewart DJ, Ward HJ. 1999. Technology evaluation: gene therapy (FGF-5), Vical. *Curr Opin Mol Ther.* 1: 260–265.

Lindahl P, Hellström M, Kalén M, Karlsson L, Pekny M, Pekna M, Soriano P, Betsholtz C. 1998. Paracrine PDGF-B/PDGF-Rbeta signaling controls mesangial cell development in kidney glomeruli. *Development*. 125: 3313-3322.

Lindahl P, Johansson BR, Levéen P, Betsholtz C. 1997. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science*. 277: 242-245.

Losordo DW, Dimmeler S. 2004. Therapeutic angiogenesis and vasculogenesis for ischemic diseases. *Circulation*. 109: 2487-2491.

Lubin B, Chiu D, Bastacky J, Roelofsen B, Van Deenen LL. 1981. Abnormalities in membrane phospholipid organization in sickled erythrocytes. *J Clin Invest*. 67: 1643-1649.

Lynch SF, Ludlam CA. 2007. Plasma microparticles and vascular disorders. *Br J Haematol*. 137: 36-48.

Mack M, Kleinschmidt A, Bruhl H, Klier C, Nelson PJ, Cihak J, Plachy J, Stangassinger M, Erfle V, Schlondorff D. 2000. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat Med*. 6: 769-775.

MacKenzie A, Wilson HL, Kiss-Toth E, Dower SK, North RA, and Surprenant A. 2001. Rapid secretion of interleukin-1ß by microvesicle shedding. *Immunity.* 15: 825-

835.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science*. 277: 55-60.

Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, Tedgui A. 2000. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation*. 101: 841-843.

Manganini M, Maier JA. 2000. Transforming growth factor beta2 inhibition of hepatocyte growth factor-induced endothelial proliferation and migration. *Oncogene*. 19: 124-133.

Martin S, Tesse A, Hugel B, Martínez MC, Morel O, Freyssinet JM, Andriantsitohaina R. 2004. Shed membrane particles from T lymphocytes impair endothelial function and regulate endothelial protein expression. *Circulation*. 109: 1653-1659.

Martínez MC, Larbret F, Zobairi F, Coulombe J, Debili N, Vainchenker W, Ruat M, Freyssinet JM. 2006. Transfer of differentiation signal by membrane microvesicles harboring hedgehog morphogens. *Blood*. 108: 3012-3020.

Martínez MC, Tesse A, Zobairi F, Andriantsitohaina R. 2005. Shed membrane microparticles from circulating and vascular cells in regulating vascular function. *Am J Physiol Heart Circ Physiol*. 288: 1004-1009.

Matsushita H, Morishita R, Nata T, Aoki M, Nakagami H, Taniyama Y, Yamamoto K, Higaki J, Yasufumi K, Ogihara T. 2000. Hypoxia-induced endothelial apoptosis through nuclear factor-kappaB (NF-kappaB)-mediated bcl-2 suppression: in vivo evidence of the importance of NF-kappaB in endothelial cell regulation. *Circ. Res*. 86: 974-981.

McColl BK, Stacker SA, Achen MG.2004. Molecular regulation of the VEGF family - inducers of angiogenesis and lymphangiogenesis. *APMIS*. 112: 463-480.

McCourt PA, Ek B, Forsberg N, Gustafson S. 1994. Intercellular adhesion molecule-1 is

a cell surface receptor for hyaluronan. *J Biol Chem*. 269: 30081-30084.

Meeson AP, Argilla M, Ko K, Witte L, Lang RA. 1999. VEGF deprivation-induced apoptosis is a component of programmed capillary regression. *Development*. 126: 1407-1415.

Mezentsev A, Merks RM, O'Riordan E, Chen J, Mendelev N, Goligorsky MS, Brodsky SV. 2005. Endothelial microparticles affect angiogenesis in vitro: role of oxidative stress. *Am J Physiol Heart Circ Physiol*. 289: 1106-1114.

Meziani F, Tesse A, David E, Martinez MC, Wangesteen R, Schneider F, Andriantsitohaina R. 2006. Shed membrane particles from preeclamptic women generate vascular wall inflammation and blunt vascular contractility. *Am J Pathol*. 169: 1473-1483.

Miguet L, Pacaud K, Felden C, Hugel B, Martinez MC, Freyssinet JM, Herbrecht R, Potier N, van Dorsselaer A, Mauvieux L. 2006. Proteomic analysis of malignant lymphocyte membrane microparticles using double ionization coverage optimization. *Proteomics*. 6: 153-171.

Milkiewicz M, Brown MD, Egginton S, Hudlicka O. 2001.Association between shear stress, angiogenesis, and VEGF in skeletal muscles in vivo. Microcirculation. 8: 229-241.

Millimaggi D, Mari M, D'Ascenzo S, Carosa E, Jannini EA, Zucker S, Carta G, Pavan A, Dolo V. 2007. Tumor vesicle-associated CD147 modulates the angiogenic capability of endothelial cells. *Neoplasia*. 9: 349-357.

Morishita R, Sakaki M, Yamamoto K, Iguchi S, Aoki M, Yamasaki K, Matsumoto K, Nakamura T, Lawn R, Ogihara T, Kaneda Y. 2002. Impairment of collateral formation in lipoprotein(a) transgenic mice: therapeutic angiogenesis induced by human hepatocyte growth factor gene. *Circulation*. 105: 1491-1496.

Morrissey JH. 2001. Tissue factor: an enzyme cofactor and a true receptor. *Thromb Haemost*. 86: 66-74.

Mostefai HA, Agouni A, Carusio N, Mastronardi ML, Heymes C, Henrion D, Andriantsitohaina R, Martinez MC. 2008b. Phosphatidylinositol 3-kinase and xanthine

oxidase regulate nitric oxide and reactive oxygen species productions by apoptotic lymphocyte microparticles in endothelial cells. *J Immunol*. 180: 5028-5035.

Mostefai HA, Andriantsitohaina R, Martínez MC. 2008a. Plasma membrane microparticles in angiogenesis: role in ischemic diseases and in cancer. *Physiol Res*. 57: 311-320.

Mostefai HA, Meziani F, Mastronardi ML, Agouni A, Heymes C, Sargentini C, Asfar P, Martinez MC, Andriantsitohaina R. 2008c. Circulating microparticles from septic shock patients exert protective role on vascular function. *Am J Respir Crit Care Med.* doi:10.1164/rccm.200712-1835OC.

Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM. 1998. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest*. 101: 2567-2578.

Nakagami H, Morishita R, Yamamoto K, Taniyama Y, Aoki M, Matsumoto K, Nakamura T, Kaneda Y, Horiuchi M, Ogihara T. 2001.Mitogenic and antiapoptotic actions of hepatocyte growth factor through ERK, STAT3, and AKT in endothelial cells. *Hypertension.* 37: 581-586.

Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. 2000. Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol.* 18: 1135-1149.

Ng YS, D'Amore PA. 2001. Therapeutic angiogenesis for cardiovascular disease. *Curr Control Trials Cardiovas Med*. 2: 278-285.

Nieuwland R, Berckmans RJ, McGregor S, Böing AN, Romijn FP, Westendorp RG, Hack CE, Sturk A. 2000. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood*. 95: 930-935.

Noden DM. 1989. Embryonic origins and assembly of blood vessels. *Am Rev Respir Dis*. 140: 1097-1103.

Nomura S, Kanazawa S, Fukuhara S. 2002. Effects of efonidipine on platelet and monocyte activation markers in hypertensive patients with and without type 2 diabetes

mellitus. *J Hum Hypertens*. 16: 539-547.

Nomura S, Suzuki M, Katsura K, Xie GL, Miyazaki Y, Miyake T, Kido H, Kagawa H, Fukuhara S. 1995. Platelet-derived microparticles may influence the development of atherosclerosis in diabetes mellitus. *Atherosclerosis*. 116: 235-240.

Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, and Kambayashi J. 2001. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. *Atherosclerosis.* 158: 277-287.

Ogura H, Kawasaki T, Tanaka H, Koh T, Tanaka R, Ozeki Y, Hosotsubo H, Kuwagata Y, Shimazu T, Sugimoto H. 2001. Activated platelets enhance microparticle formation and platelet-leukocyte interaction in severe trauma and sepsis. *J Trauma*. 50: 801-809.

Ogura H, Tanaka H, Koh T, Fujita K, Fujimi S, Nakamori Y, Hosotsubo H, Kuwagata Y, Shimazu T, Sugimoto H. 2004. Enhanced production of endothelial microparticles with increased binding to leukocytes in patients with severe systemic inflammatory response syndrome. *J Trauma*. 56: 823-830.

Omoto S, Nomura S, Shouzu A, Nishikawa M, Fukuhara S, Iwasaka T. 2002. Detection of monocyte-derived microparticles in patients with type II diabetes mellitus. *Diabetologia*. 45: 550-555.

Papapetropoulos A, García-Cardeña G, Madri JA, Sessa WC. 1997. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest.* 100: 3131-3139.

Pardanaud L, Dieterlen-Lièvre F. 1999. Manipulation of the angiopoietic/hemangiopoietic commitment in the avian embryo. *Development*. 126: 617-627.

Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, Ledda F, Ziche M. 1998. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. *J Biol Chem*. 273: 4220-4226.

Pepper MS, Mandriota SJ, Jeltsch M, Kumar V, Alitalo K. 1998. Vascular endothelial

growth factor (VEGF)-C synergizes with basic fibroblast growth factor and VEGF in the induction of angiogenesis in vitro and alters endothelial cell extracellular proteolytic activity. *J Cell Physiol*. 177: 439-452.

Pepper MS. 2001. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol*. 21: 1104-1117.

Pfister SL. 2004. Role of platelet microparticles in the production of thromboxane by rabbit pulmonary artery. *Hypertension*. 43: 428-433.

Podlasek CA, Meroz CL, Korolis H, Tang Y, McKenna KE, McVary KT. 2005. Sonic hedgehog, the penis and erectile dysfunction: a review of sonic hedgehog signaling in the penis. *Curr Pharm Des*. 11: 4011-4027.

Pola R, Ling LE, Silver M, Corbley MJ, Kearney M, Blake Pepinsky R, Shapiro R, Taylor FR, Baker DP, Asahara T, Isner JM. 2001. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med*. 7: 706-711.

Porro C, Soleti R, Benameur T, Maffione AB, Andriantsitohaina R, Martinez MC. 2009. Sonic hedgehog pathway as a target for therapy in angiogenesis-related diseases. *Current Signal Transduction Therapy.* In press.

Post MJ, Laham R, Sellke FW, Simons M. 2001. Therapeutic angiogenesis in cardiology using protein formulations. *Cardiovasc Res*. 49: 522-531.

Preston RA, Jy W, Jimenez JJ, Mauro LM, Horstman LL, Valle M, Aime G, Ahn YS. 2003. Effects of severe hypertension on endothelial and platelet microparticles. *Hypertension*. 41: 211-217.

Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J. 1995. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J*. 14: 5884-5891.

Rastinejad F, Polverini PJ, Bouck NP. 1989. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell.* 56: 345-355.

RayChaudhury A, Frischer H, Malik AB. 1996 Inhibition of endothelial cell

proliferation and bFGF-induced phenotypic modulation by nitric oxide. *J Cell Biochem.* 63: 125-134.

Redman CW, Sargent IL. 2007. Microparticles and immunomodulation in pregnancy and pre-eclampsia. *J Reprod Immunol*. 76: 61-67.

Reisinger K, Kaufmann R, Gille J. 2003. Increased Sp1 phosphorylation as a mechanism of hepatocyte growth factor (HGF/SF)-induced vascular endothelial growth factor (VEGF/VPF) transcription. *J Cell Sci*. 116: 225-238.

Ribatti D, Conconi MT, Nussdorfer GG. 2007. Nonclassic endogenous novel regulators of angiogenesis. *Pharmacol Rev*. 59: 185-205.

Riobó NA, Lu K, Ai X, Haines GM, Emerson CP Jr. 2006. Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. *Proc Natl Acad Sci U S A.* 103: 4505-4510.

Risau W. 1997. Mechanisms of angiogenesis. *Nature*. 386: 671-674.

Rozmyslowicz T, Majka M, Kijowski J, Murphy SL, Conover DO, Poncz M, Ratajczak J, Gaulton GN, and Ratajczak MZ. 2003. Platelet- and megakaryocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV. *AIDS.* 17: 33-42.

Sabatier F, Darmon P, Hugel B, Combes V, Sanmarco M, Velut JG, Arnoux D, Charpiot P, Freyssinet JM, Oliver C, Sampol J, Dignat-George F. 2002. Type 1 and type 2 diabetic patients display different patterns of cellular microparticles. *Diabetes*. 51: 2840-2845.

Sapet C, Simoncini S, Loriod B, Puthier D, Sampol J, Nguyen C, Dignat-George F, Anfosso F.2006. Thrombin-induced endothelial microparticle generation: identification of a novel pathway involving ROCK-II activation by caspase-2. *Blood.*108: 1868-1876

Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y. 1995. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature.* 376: 70-74.

Schecter AD, Spirn B, Rossikhina M, Giesen PL, Bogdanov V, Fallon JT, Fisher EA,

Schnapp LM, Nemerson Y, Taubman MB. 2000. Release of active tissue factor by human arterial smooth muscle cells. *Circ Res*. 87: 126-132.

Schiffenbauer YS, Abramovitch R, Meir G, Nevo N, Holzinger M, Itin A, Keshet E, Neeman M. 1997. Loss of ovarian function promotes angiogenesis in human ovarian carcinoma. *Proc Natl Acad Sci U S A*. 94: 13203-13208.

Schlegel RA, McEvoy L, Williamson P. 1985. Membrane phospholipid asymmetry and the adherence of loaded red blood cells. Bibl. *Haematol*. 51: 150-156.

Seiki M. 2003. Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. *Cancer Lett*. 194: 1-11.

Semenza GL. 2001. Regulation of hypoxia-induced angiogenesis: a chaperone escorts VEGF to the dance. *J Clin Invest*. 108: 39-40.

Sengupta S, Gherardi E, Sellers LA, Wood JM, Sasisekharan R, Fan TP. 2003. Hepatocyte growth factor/scatter factor can induce angiogenesis independently of vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol.* 23: 69-75.

Shah NM, Groves AK, Anderson DJ. 1996. Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. *Cell*. 85: 331-343.

Shima DT, Mailhos C. 2000. Vascular developmental biology: getting nervous. *Curr Opin Genet Dev*. 10: 536-542.

Sogawa K, Numayama-Tsuruta K, Ema M, Abe M, Abe H, Fujii-Kuriyama Y.1998. Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia. *Proc Natl Acad Sci U S A*. 95: 7368-7373.

Soriano AO, Jy W, Chirinos JA, Valdivia MA, Velasquez HS, Jimenez JJ, Horstman LL, Kett DH, Schein RM, Ahn YS. 2005. Levels of endothelial and platelet microparticles and their interactions with leukocytes negatively correlate with organ dysfunction and predict mortality in severe sepsis. *Crit Care Med*. 33: 2540-2546.

Sottile J. 2004. Regulation of angiogenesis by extracellular matrix. *Biochim. Biophys. Acta.* 1654: 13-22.

Stetler-Stevenson WG. 1999. Matrix metalloproteinases in angiogenesis: a moving

target for therapeutic intervention. *J Clin Invest*. 103: 1237-1241.

Sunderkötter C, Steinbrink K, Henseleit U, Bosse R, Schwarz A, Vestweber D, Sorg C. 1996. Activated T cells induce expression of E-selectin in vitro and in an antigen-dependent manner in vivo. *Eur J Immunol*. 26: 1571-1579.

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD. 1996. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell.* 87: 1171-1180.

Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, Sato TN, Yancopoulos GD. 1998. Increased vascularization in mice overexpressing angiopoietin-1. *Science.* 282: 468-471.

Tamarat R, Silvestre JS, Kubis N, Benessiano J, Duriez M, deGasparo M, Henrion D, Levy BI. 2002. Endothelial nitric oxide synthase lies downstream from angiotensin II-induced angiogenesis in ischemic hindlimb. *Hypertension*. 39: 830-835.

Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, Dolo V. 2002. Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *Am J Pathol*. 160: 673-680.

Tesse A, Martínez MC, Hugel B, Chalupsky K, Muller CD, Meziani F, Mitolo-Chieppa D, Freyssinet JM, Andriantsitohaina R. 2005. Upregulation of proinflammatory proteins through NF-kappaB pathway by shed membrane microparticles results in vascular hyporeactivity. *Arterioscler Thromb Vasc Biol*. 25: 2522-2527.

Tesse A, Meziani F, David E, Carusio N, Kremer H, Schneider F, Andriantsitohaina R. 2007. Microparticles from preeclamptic women induce vascular hyporeactivity in vessels from pregnant mice through an overproduction of NO. *Am J Physiol Heart Circ Physiol*. 293: 520-525.

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD. 2000. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med. 6: 460-463.

Thurston G. 2003. Role of Angiopoietins and Tie receptor tyrosine kinases in

angiogenesis and lymphangiogenesis. *Cell Tissue Res*. 314: 61-68.

Tong Q, Zheng L, Li B, Wang D, Huang C, Matuschak GM, Li D. 2006. Hypoxia-induced mitogenic factor enhances angiogenesis by promoting proliferation and migration of endothelial cells. *Exp Cell Res*. 312: 3559-3569.

Tunyogi-Csapo M, Koreny T, Vermes C, Galante JO, Jacobs JJ, Glant TT. 2007. Role of fibroblasts and fibroblast-derived growth factors in periprosthetic angiogenesis. *J Orthop Res*. 25: 1378-1388.

Udagawa T, Fernandez A, Achilles E-G, Folkman J, D'Amato RJ. 2002. Persistence of microscopic human cancers in mice: alterations in the angiogenic balance accompanies loss of dormancy. *FASEB J*. 16: 1361-1370.

Valtola R, Salven P, Heikkila P, Taipale J, Joensuu H, Rehn M, Pihlajaniemi T, Weich H, de Waal R and Alitalo K. 1999. VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am. J. Pathol*. 154: 1381-1390.

van Hinsbergh VW, Koolwijk P. 2008. Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. *Cardiovasc Res*. 2008. 78: 203-212.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 3: RESEARCH0034.

VanWijk MJ, Svedas E, Boer K, Nieuwland R, Vanbavel E, Kublickiene KR. 2002. Isolated microparticles, but not whole plasma, from women with preeclampsia impair endothelium-dependent relaxation in isolated myometrial arteries from healthy pregnant women. *Am J Obstet Gynecol*. 187: 1686-1693.

VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. 2003. Microparticles in cardiovascular diseases. *Cardiovasc Res*. 59: 277-287.

Vatner SF. 2005. FGF Induces Hypertrophy and Angiogenesis in Hibernating Myocardium. *Circ Res*. 96: 705-707.

Visse R, Nagase H. 2003 Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res*. 92: 827-839.

Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN. 2003. IL-1 is required for tumor invasiveness and angiogenesis. *Proc Natl Acad Sci U S A*. 100: 2645-2650.

Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z. 1999. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell*. 93: 411-422.

Wali, RK, Jaffe S, Kumar D, Kalra VK. 1988. Alterations in organization of phospholipids in erythrocytes as a factor in adherence to endothelial cells in diabetes mellitus. *Diabetes*. 37: 104-111.

Wali, RK, Jaffe S, Kumar D, Sorgente N, Kalra VK. 1987. Increased adherence of oxidant treated human and bovine erythrocytes to cultured endothelial cells. *J Cell Physiol*. 133: 25-36.

Weerheim AM, Kolb AM, Sturk A, Nieuwland R. 2002. Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography. *Anal Biochem.* 302: 191-198.

Wilkinson DG. 2000. Eph receptors and ephrins: regulators of guidance and assembly. *Int Rev Cytol*. 196: 177-244.

Wilson MJ, Richter-Lowney K, Daleke DL. 1993. Hyperglycemia induces a loss of phospholipid asymmetry in human erythrocytes. *Biochemistry*. 32: 11302-11310.

Wolf K, Wu YI, Liu Y, Geiger J, Tam E, Overall C, Stack MS, Friedl P. 2007. Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. *Nat Cell Biol*. 9: 893-904.

Yang C, Mwaikambo BR, Zhu T, Gagnon C, Lafleur J, Seshadri S, Lachapelle P, Lavoie JC, Chemtob S, Hardy P. 2008. Lymphocytic microparticles inhibit angiogenesis by stimulating oxidative stress and negatively regulating VEGF-induced pathways. *Am J Physiol Regul Integr Comp Physiol*. 294: 467-476.

Yu JL, Rak JW. 2004. Shedding of tissue factor (TF)-containing microparticles rather than alternatively spliced TF is the main source of TF activity released from human

cancer cells. *J Thromb Haemost*. 2: 2065-2067.

Yu Y, Sato JD. 1999. MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. *J Cell Physiol*. 178: 235-246.

Zachary I. 2003. VEGF signalling: integration and multi-tasking in endothelial cell biology. *Biochem Soc Trans*. 31: 1171-1177.

Zhang YW, Su Y, Volpert OV, Vande Woude GF. 2003. Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. *Proc Natl Acad Sci U S A*. 100: 12718-12723.

Zhang YW, Vande Woude GF. 2003. HGF/SF-met signaling in the control of branching morphogenesis and invasion. *J Cell Biochem*. 88: 408-417.

Zhong YJ, Chen BA, Huang CY, Li CP, Gao F, Fei F, Pei XP, Gao C, Ding JH, Sun YY, Cheng J, Wang J, Zhao G, Ma Y. 2007. Effects of platelet-derived membrane microparticles on the proliferation and apoptosis of human umbilical vein endothelial cells. *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 15: 858-861.

Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bicknell R. 1997. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest*. 99: 2625-2634.

Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, Geppetti P, Ledda F. 1994. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J Clin Invest*. 94: 2036-2044.

Zwaal RF, Schroit AJ. 1997. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood*. 1997 89: 1121-1132.

ROLE OF MEMBRANE MICROPARTICLES IN ANGIOGENESIS

Microparticles (MPs) are small vesicles released from the plasma membrane after cell stimulation or apoptosis. Recent studies show that MPs can be implicated in modulation of angiogenesis, an essential cellular process from cardiovascular system during physiological and pathological conditions. Moreover, engineered MPs generated from apoptotic/stimulated human lymphocytes harbour at their surface the morphogen Sonic Hedgehog (Shh) which also play a critical role in vessel formation.

Accordingly, the present study was aimed to examine how engineered human MPs bearing Shh (MPs^{Shh+}) are able to modulate the different steps implicated in angiogenesis process. In human endothelial cells (ECs), MPs^{Shh+} induce the formation of capillary-like structures. Moreover, MPs^{Shh+} are able to modulate EC proliferation, adhesion and mRNA levels of proangiogenic factors. Silencing of Shh receptor, by siRNA or Shh signalling by pharmacological inhibitor cyclopamine, strongly reverses tubulogenesis, and concomitantly decreases mRNA levels of pro-angiogenic factors. Furthermore, we investigate the effects of MPs^{Shh+} on post-ischemic neovascularization using mice model of femoral artery ligation. The recovery of ischemic/normal blood flow ratio is greater in MPs^{Shh+}-treated mice than in control. In muscle, MPs^{Shh+} enhance the expression of eNOS, decrease expression of caveolin-1 and modify expression of several angiogenic genes. Pharmacological inhibition of Shh pathway completely reverses the improvement of blood flow and the effects on eNOS expression.

Besides, in light of our results, we proposed the hypothesis that MPs^{Shh+} may represent a new therapeutic approach to treat pathologies associated with failed angiogenesis and a new target in pathologies associated with exaggeration of angiogenesis, such as cancer.

RÔLE DES MICROPARTICULES MEMBRANAIRES DANS L'ANGIOGENÈSE

Les microparticules (MPs) sont des fragments de la membrane plasmique libérés suite à une stimulation cellulaire ou apoptotique. Des études récentes ont montré que les MPs peuvent être impliquées dans la modulation de l'angiogenèse, un processus essentiel du système cardio-vasculaire pendant les états physiologiques et pathologiques. De plus, les MPs dérivées des cellules T humaines activées et apoptotiques sont porteuses du morphogène Sonic Hedgehog (Shh), lequel a aussi un rôle dans la formation des vaisseaux.

En conséquence, l'objectif de cette étude est d'élucider l'implication des MPs porteuses de Shh (MPs^{Shh+}) sur les différentes étapes associées à l'angiogenèse. Dans les cellules endothéliales, les MPs^{Shh+} provoquent la formation des structures capillaires. Également, les MPs^{Shh+} sont capables de moduler la prolifération, l'adhésion et le niveau d'expression des ARNm des facteurs pro-angiogéniques dans les cellules endothéliales. De plus, l'inhibition de l'expression du récepteur de Shh, ou l'utilisation de la cyclopamine, un inhibiteur pharmacologique de Shh, restitue la capacité des MPs^{Shh+} de former des capillaires et aussi diminue l'expression des facteurs pro-angiogéniques. Ensuite, nous avons évalué les effets des MPs^{Shh+} sur la neovascularization post-ischémique en utilisant le modèle in vivo de la ligature de l'artère fémorale chez la souris. Une meilleure récupération du flux sanguin est observée chez les souris traitées avec les MPs^{Shh+} par rapport aux témoins. Dans le muscle squelettique, les MPs^{Shh+} augmentent l'expression de la eNOS et diminuent celle de la caveoline-1. L'inhibition pharmacologique de la voie Shh prévient l'amélioration du flux sanguin et les effets induits sur l'expression de la eNOS.

L'ensemble des résultats suggère que les MPs^{Shh+} pourraient être utilisées comme de nouveaux outils thérapeutiques dans certaines pathologies caractérisées par une angiogenèse défaillante et elles pourraient représenter une nouvelle cible dans les pathologies associées à une angiogenèse exagérée, comment le cancer.