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Par **Lionel LEROUX**

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NOUVELLES STRATEGIES DE THERAPIE CELLULAIRE A VISEE PRO-ANGIOGENIQUE : IMPLICATIONS DU SYSTEME WNT/FRIZZLED.

Directeur de Thèse

Monsieur le Professeur Thierry COUFFINHAL

Membres du Jury

Monsieur le Professeur Hubert DE VERNEUIL	Président
Madame le Professeur Patricia LEMARCHAND	1er rapporteur
Monsieur le Professeur Bernard LEVY	2eme rapporteur
Monsieur le Professeur Thierry COUFFINHAL	Directeur de thèse

Madame et Messieurs les membres du jury

Vous nous faites l'honneur de juger ce travail, soyez en ici remerciés.

RESUME

La thérapie cellulaire suscite de grands espoirs dans le domaine cardiovasculaire. Cependant les premières études humaines sont décevantes. Parmi les explications avancées, citons un mauvais choix de cellules, une méthode de délivrance inadéquate, une préparation des cellules et des tissus hôtes insuffisante ou une trop grande mortalité cellulaire après injection.

Durant ce travail nous avons voulu explorer 3 pistes d'optimisation de la thérapie cellulaire à visée pro-angiogénique utilisant les cellules souches mésenchymateuses (MSC) et contribué à l'exploration des mécanismes mis en jeu, en particulier en explorant le rôle du système Wnt/Frizzled.

Nous avons tout d'abord étudié un système original de délivrance de cellules utilisant un « patch » musculaire cousu en regard d'un myocarde infarci de souris. Puis nous avons étudié l'effet d'une surexpression de sFRP1, inhibiteur de la voie Wnt, sur un modèle de matrice sous cutanée. Enfin, nous avons testé l'hypothèse qu'un préconditionnement hypoxique des cellules permettrait une meilleure survie cellulaire et améliorerait la réparation vasculaire et tissulaire après ischémie de patte chez la souris.

Nos résultats permettent notamment de montrer que les MSC ont des capacités d'invasion des tissus ischémiques et qu'elles se différencient en péricytes en formant un réseau tridimensionnel de soutien aux cellules endothéliales. Par ailleurs, via sFRP1, nous mettons en évidence un rôle du système Wnt/Fzd dans l'effet pro-angiogénique. Enfin, nous montrons l'intérêt du préconditionnement hypoxique dont les effets sont médiés par Wnt4.

L'ensemble de ces données permet d'envisager des voies d'optimisation de la thérapie cellulaire.

Some of the challenges facing stem-cell therapy for cardiac disease are which type of stem cell or progenitor cell is the best candidate for therapy, how to survive in the low oxygen environment of ischemic myocardium. Here we studied the potential of mesenchymal stem cells (MSCs) as vascular progenitor cells *in vitro* and *in vivo*, and we studied the effects of sFRP-1/Wnt signaling modulation or hypoxia on MSC properties.

First, we demonstrated the beneficial effect of MSC application on ischemic heart repair using an original surgical model (patch) to deliver stem cells. This study showed that the contribution of the MSCs in the mouse infarcted myocardium was beneficial either on the scar (increase in angiogenesis, in cell proliferation, reduction in ventricular remodeling) or on the trophicity of the patch.

Then we characterized the angiogenic properties of MSC *in vitro* and *in vivo*. Our data demonstrate that MSCs could be recruited and formed vascular structures around endothelial tubes. We showed *in vivo* that the surexpression of *sFRP-1* (regulating factor of the Wnt system) in MSCs increased their potential of pericyte-like cells correlated with an increased maturation of the vessels via an intracellular GSK-3 dependent pathway in MSCs.

Our next objective was to investigate the effects of hypoxia exposure on MSC before implantation for vascular and tissue regeneration in mice with hind limb ischemia. Our data suggest that hypoxic preconditioning has a critical role on MSC dynamic functions, shifting MSC location *in situ* to enhance ischemic tissue recovery, facilitating vascular cell mobilization and skeletal myoblast regeneration via a paracrine Wnt dependent mechanism.

Titre en Anglais :

Development of novel stem-cell therapies for cardiac diseases and critical hindlimb ischemia: involvement of the Wnt/Frizzled pathway

Mots clés en Français :

Thérapie cellulaire ; angiogenèse ; infarctus du myocarde ; artériopathie oblitérante des membres inférieurs ; cellules souches mésenchymateuses ; système Wnt/Frizzled ; Préconditionnement hypoxique.

Mots clés en Anglais :

Stem cell therapy; angiogenesis; myocardial infarction; hindlimb ischemia; mesenchymal stem cells; Wnt/Frizzled pathway; hypoxia preconditioning.

Laboratoire :

Unité Inserm U828 – Adaptation cardiovasculaire à l'ischémie

Avenue du Haut-Lévêque, 33600 Pessac, France

TABLE DES MATIERES

ABREVIATIONS

I) ABREVIATIONS

ALK	Activin receptor-Like Kinase
SMA	Smooth Muscle Actin
Ang	Angiopoïétine
APC	Adenomatous Poliposis Coli
ARN	Acide RiboNucléique
bFGF	basic Fibroblast Growth Factor
BMC	Bone Marrow Cell
BMP	Bone Morphogenic Proteins
BrdU	BromoDésoxyUridine
CaM/KII	Calcium/Calmoduline Kinase II
CD	Cluster of Differentiation
CE	Cellule Endothéliale
CFU	Colony-Forming Units
CML	Cellule Musculaire Lisse
CRD	Cystein Rich Domain
Dkk	Dickkopf
Dll	Delta-like ligand
Dsh / Dvl	Dishevelled
eNOS	endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cell
EPO	Erythropoïétine
ERK	Extracellular signal-Regulated Kinase
ESC	Embryonic Stem Cell
FEVG	Fraction d'éjection ventriculaire gauche
FGF	Fibrosblast Growth Factor
FGFR	Fibrosblast Growth Factor Receptor
Flk-1	Vascular Endothelial Growth Factor Receptor-2 (KDR)
Flt-1	Vascular Endothelial Growth Factor Receptor-1
Fzd	Frizzled
G-CSF	Granulocyte-Colony Stimulating Factor
GFP	Green Fluorescent Protein
Gli-1	Glioblastoma 1
GSK-3β	Glycogene Synthase Kinase 3β
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HRE	Hypoxia Response Elements
HSC	Hematopoïetic Stem Cell
ICAM-1	intracellular adhesion molecule-1

IEC	Inhibiteurs de l'Enzyme de Conversion
IGF-1	Insuline-like Growth Factor-1
IL	Inter Leukin
iNOS	inducible Nitric Oxide Synthase
IP3	Inositol triphosphate
iPS	induced Pluripotent Stem Cells
Jak	Janus kinase
JNK	Jun NH2-terminal Kinase
KDR	Vascular Endothelial Growth Factor Receptor-2 (Flk-1)
KO	Knock-Out
LEF	Lymphoid Enhancer-binding Factor
LiCl	Chlorure de Lithium
LRP5/6	Lipoprotein Receptor-related Protein 5/6
MAPK	Mitogen Activated Protein Kinases
MB	Membrane Basale
MCP-1	Monocyte Chemotactic Protein-1
MEC	Matrice Extracellulaire
MHC	Myosin Heavy Chain
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell
NAC	N-acétyl-Cystéine
NFAT	Nuclear Factor of T Cells)
NF-κB	Nuclear Factor-κB
NG2	neural-Glial antigen 2
NO	Monoxyde d'azote
Nrarp	Notch-regulated ankyrin repeat protein
NTR	Netrin-like domaine
OPG	Osteoprotegerin
PBMC	Péripheral Blood Mononuclear Cells
PCP	Planar Cell Polarity
PDE	Phosphodiesterase
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PI3k	PhosphoInositol-3 Kinase
PKG	Protein Kinase G
PLC	Phospholipase C
PlGF	Placental Growth Factor
PTH	Parathormone
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
Rspo	R-spondin

SDF-1	Stromal cell-Derived Factor-1
sFRP	secreted Frizzled Related Protein
Shh	Sonic hedgehog
siRNA	short interfering RNA
STAT	Signal Transducer and Activator of Transcription
TCF	T-Cell Factor
TGFβ	Transforming Growth Factor β
TNF-α	Tumor Necrosis Factor-α
VCAM-1	Vascular Endothelial Cell Adhesion Molecule-1
VE-cadhérine	Vascular Endothelial-cadhérine
VEGF	Vascular Endothelial Growth Factor
VEGFR1	Vascular Endothelial Growth Factor Receptor-1 (Flt-1)
VEGFR2	Vascular Endothelial Growth Factor Receptor-2 (KDR / Flk-1)
WIF	Wnt Inhibitory Factor

AVANT PROPOS

II) AVANT PROPOS

Les maladies cardiovasculaires sont encore aujourd'hui la première cause de morbi-mortalité dans nos pays industrialisés. L'athérosclérose en est le plus souvent à l'origine. Les organes le plus souvent touchés sont le cœur, le cerveau ou les muscles des membres inférieurs. En cas de sténose vasculaire, l'apport en oxygène aux tissus dépendants du vaisseau atteint devient insuffisant lorsque les besoins en oxygène augmentent comme lors d'un effort. Ce déséquilibre entre apports et besoins se traduit en général par des douleurs et limite les capacités d'effort des patients. Il s'agit de l'angine de poitrine lorsque l'atteinte est coronarienne et de la claudication artérielle intermittente des membres inférieurs lorsque ce sont les artères des jambes qui sont touchées. En cas d'occlusion vasculaire aiguë, l'anoxie entraînera douleur et mort cellulaire d'où une perte de fonction pour l'organe. L'infarctus du myocarde peut alors aboutir à une myocardiopathie dilatée ischémique avec insuffisance cardiaque et, au niveau des membres inférieurs, le stade avancé de l'artériopathie oblitérante se traduit par une nécrose distale avec perte de substance, dénommée gangrène. Bien sûr, la prévention cardiovasculaire, par la maitrise des facteurs de risque, et de nombreux moyens thérapeutiques bien standardisés, aussi bien médicaux (antiagrégants plaquettaires, inhibiteurs de l'enzyme de conversion, statines…), que plus agressifs (pontages aorto-coronariens, angioplastie) peuvent être mis en œuvre pour limiter les conséquences de l'atteinte vasculaire. Néanmoins, pour un grand nombre de malades l'arsenal thérapeutique actuel ne permet pas d'éviter les amputations, l'insuffisance cardiaque et finalement le décès prématuré.

De la capacité d'adaptation à l'ischémie des tissus, mais aussi de leurs possibilités de cicatrisation ou de régénération, dépend le pronostic de ces organes et donc des patients. Parmi les phénomènes impliqués, la faculté de développer de nouveaux vaisseaux pour former un réseau vasculaire collatéral est primordiale. Or en général, cette néo-angiogenèse naturelle est très insuffisante. D'où l'idée que la facilitation de l'apparition de nouveaux vaisseaux pouvait être une voie thérapeutique intéressante. Cependant le développement d'un système vasculaire est un phénomène biologique complexe faisant appel à la vasculogénèse, l'angiogenèse et l'artériogenèse. Parmi les méthodes envisagées pour

stimuler la néo-angiogenèse, l'introduction de cellules souches au sein des tissus atteints est apparue séduisante. Pourtant, les résultats issus des premières études cliniques sont globalement décevants. Plusieurs raisons peuvent être évoquées, comme un mauvais choix de cellules, une méthode de délivrance inadéquate, une très faible survie des cellules injectées, ou leur faible capacité à se différencier.

Plusieurs pistes, en vue d'améliorer les capacités angiogéniques des cellules souches injectées dans des tissus ischémiques, sont actuellement étudiées et c'est dans cet axe que s'est orienté mon travail de thèse. Du fait de leurs caractéristiques, nous avons choisi de travailler sur les cellules souches mésenchymateuses issues de la moelle osseuse. Tout d'abord, j'ai participé à une étude visant à améliorer la délivrance des cellules souches dans le muscle cardiaque lésé, dans un modèle murin d'infarctus du myocarde, en utilisant un « patch » musculaire contenant des cellules. Le laboratoire s'intéressant plus particulièrement au rôle du système Wnt/Frizzled dans le développement des vaisseaux, nous avons ensuite réalisé une étude portant sur l'implication d'un antagoniste sécrété de ce système, sFRP1, sur le rôle pro-angiogénique des cellules souches mésenchymateuses *in vitro* et *in vivo* dans un modèle d'implant sous-cutané chez la souris. Puis, notre hypothèse a été que si les cellules ne survivaient pas après injection, en grande partie du fait de la faible teneur en oxygène des tissus hôtes, un préconditionnement de ces cellules à l'hypoxie permettrait d'améliorer leur survie et donc leur efficacité. Ici encore, nous avons voulu savoir quelle était l'implication du système Wnt/Frizzled dans ces évènements.

INTRODUCTION

III) INTRODUCTION

A) THERAPIE CELLULAIRE A VISEE ANGIOGENIQUE

1) Développement d'un réseau vasculaire

(a) Généralités

Un réseau vasculaire se développe de façon normale durant l'embryogenèse et la croissance mais aussi, dans des situations pathologiques comme dans les tumeurs néoplasiques où la croissance vasculaire est un facteur d'expansion de la tumeur ; ou bien comme dans l'ischémie où les néo-vaisseaux participent à la cicatrisation de l'organe atteint. Cependant, dans cette dernière situation, la néo-vascularisation est en général insuffisante pour restaurer l'intégralité des fonctions de l'organe touché (Habib, Heibig et al. 1991). L'importance de la néo-angiogenèse post-ischémique est dépendante de très nombreux facteurs, tels que la nature de l'organe atteint, la sévérité de l'ischémie et sa vitesse d'installation mais aussi l'âge du patient ou ses facteurs de risque cardiovasculaires (Tamarat, Silvestre et al. 2004; You, Cochain et al. 2008). (Figure 1).

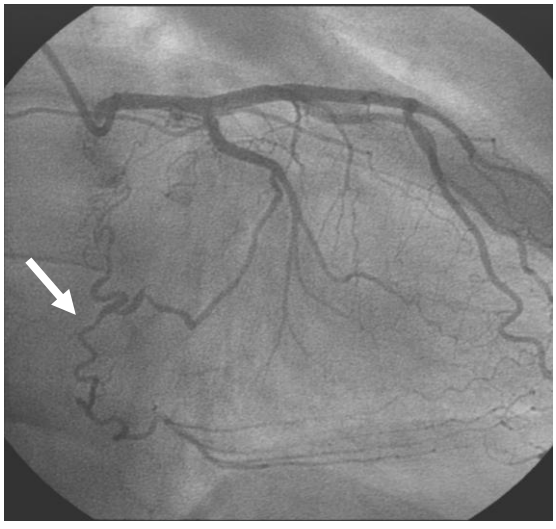

Figure 1. Coronarographie de l'artère coronaire gauche chez un patient dont l'artère coronaire droite est thrombosée. Il existe un réseau collatéral permettant de visualiser la coronaire droite distale, et donc de vasculariser la paroi inférieure du ventricule gauche.

Le développement d'un réseau vasculaire fonctionnel est un phénomène complexe faisant appel à plusieurs mécanismes hautement régulés (van Weel, van Tongeren et al. 2008) (Figure 2). La vasculogenèse est la formation d'un plexus capillaire primitif à partir de cellules souches. Les précurseurs endothéliaux, les angioblastes, semblent être les principaux intervenants. Ils sont caractérisés par des marqueurs de surface comme le CD44 (Schatteman, Hanlon et al. 2000) ou le CD133 (Yang, Zhang et al. 2004). Cependant d'autres cellules souches, progéniteurs non endothéliaux, portant des marqueurs CXCR4 et VEGFR1 ont été proposés comme participant à la vasculogenèse (Grunewald, Avraham et al. 2006).

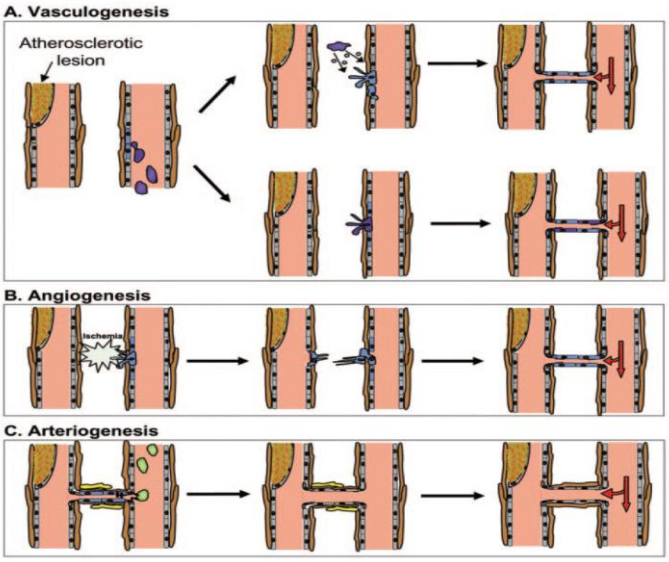

Figure 2. Les 3 étapes du développement d'un néo-vaisseau. (D'après Van Ostrom 2008).

Puis l'angiogenèse correspond au développement, à la croissance, au remodelage et à l'organisation de ce réseau primitif. Elle s'effectue par bourgeonnements capillaires à partir de vaisseaux préexistants (ou « sprouting ») et permet à ce réseau de devenir fonctionnel. Les mécanismes mis en jeu sont la dégradation de la matrice extracellulaire (MEC) et de la membrane basale (MB), la migration et la prolifération de cellules endothéliales (CE) et la formation de tubes endothéliaux avec une nouvelle membrane basale. Des péricytes, voire des cellules musculaires lisses (CML) viennent alors soutenir ces néo-vaisseaux.

Enfin l'artériogenèse est la phase de maturation et de stabilisation des vaisseaux par recrutement de péricytes et de cellules musculaires lisses.

Ces évènements, et plus particulièrement la vasculogenèse et l'artériogenèse, sont régulés par d'importants facteurs de croissance vasculaire comme le Vascular Endothelial Growth factor (VEGF), le basic Fibroblast Growth Factor (bFGF), le Transforming Growth Factor β (TGFβ) ou le Platelet Derived Growth Factor (PDGF), mais aussi par le monoxyde d'azote (NO), des facteurs régulant la perméabilité vasculaire comme les angiopoïetines (Ang-1 et Ang-2) ou des protéines de dégradation de la matrice extracellulaire, les métalloprotéinases (MMPs) (van Weel, van Tongeren et al. 2008). Si ces deux premières phases sont fortement induites par l'ischémie tissulaire, l'artériogenèse semble plus être sous la dépendance de forces de cisaillement ou « shear-stress » (Schaper 2009) et de phénomènes inflammatoires (Silvestre, Mallat et al. 2008), médiés par des protéines d'adhésion comme l'ICAM-1 (intracellular adhesion molecule-1) (Hoefer, van Royen et al. 2004) ou PECAM-1 (Chen, Rubin et al. 2010). Le PDGF (Lindahl, Hellstrom et al. 1998) et les angiopoïetines (Thomas and Augustin 2009) ont un rôle important dans ces phases de maturation vasculaire.

Les mécanismes moléculaires mis en jeu en cas d'hypoxie tissulaire sont assez bien connus (Sieveking and Ng 2009). Notamment l'activation de la voie dépendante du facteur de transcription HIF-1α (Hypoxia Inducible Factor 1α) (Semenza 2010). HIF-1α induit l'expression de facteurs de croissance vasculaire comme le VEGF-A et son récepteur. Le VEGF peut alors activer la voie de signalisation d'Akt, impliquée dans la survie et la prolifération cellulaire. L'angiopoïetine 2, le Stromal Cell Derived Factor-1 (SDF-1) et son récepteur (CXCR4), le TGF-β1 le PDGF BB ou l'érythropoïétine (EPO) sont aussi exprimés. Parallèlement, les NO-synthases entraînent l'apparition du NO et l'ensemble de ces modifications permet aux cellules endothéliales de passer d'un état quiescent à un état prolifératif (Rey and Semenza 2010).

(b) "Sprouting », « tip cell », « stalk cells »

De récents travaux permettent de mieux appréhender la phase d'angiogenèse au cours de laquelle le bourgeonnement endothélial ou « sprouting » entraîne la formation de nouvelles ramifications (« branching ») pour aboutir à un réseau tridimensionnel efficient.

L'initiation, puis l'orientation des bourgeons ne peut se faire au hasard et des mécanismes régulateurs doivent permettre la fusion des néo-vaisseaux pour constituer un réseau. A chaque bourgeonnement se trouvent deux types de cellule : la « tip cell » et les « stalk cells ». La « tip cell » est la cellule endothéliale, jusque là quiescente, sélectionnée dans le vaisseau préexistant, qui va être activée et servir de « locomotive » à la création de la nouvelle ramification. Celle-ci va présenter des filipodes et des lamellipodes, s'étendre, proliférer et migrer en allongeant la ramification. En arrière, les « stalk cells » stabilisent le néo-vaisseau alors que la lumière apparaît (Gerhardt, Golding et al. 2003) (Figure 3).

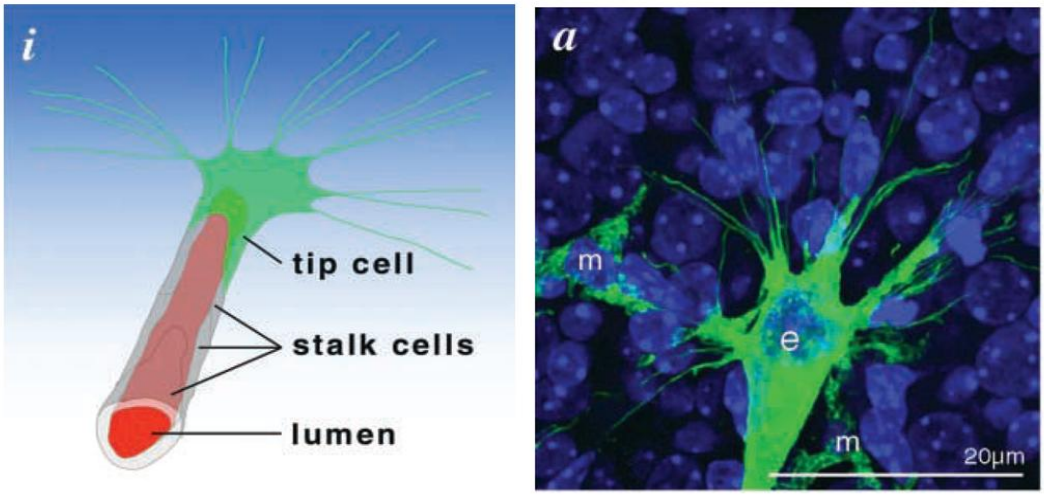

Figure 3. Le bourgeonnement endothélial. (D'après Gerhardt 2003)

Plusieurs études ont montré que le processus de sélection de la « tip cell » est induit par un gradient de concentration de VEGF (Figure 4). Une seule CE par site de « branching » doit être sélectionnée, les CE adjacentes devenant des « stalk cells » (Ruhrberg, Gerhardt et al. 2002; Gerhardt, Golding et al. 2003).

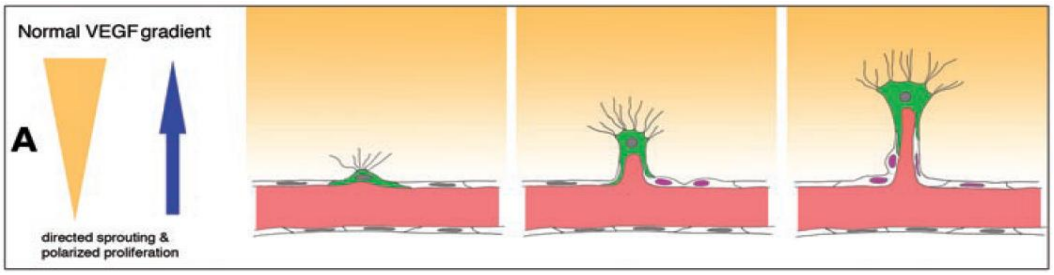

Figure 4. VEGF et "tip cell" (D'après Gerhardt 2008).

Les « stalk cells » se mettent alors à proliférer, également sous l'influence du VEGF, avec des divisions polarisées selon un axe perpendiculaire au néo-vaisseau, permettant ainsi son élongation (Zeng, Taylor et al. 2007).

Cette sélection « tip cell / stalk cells» est médiée par la « tip cell » elle-même. En effet, parmi les gènes préférentiellement exprimés par la « tip cell » on retrouve le Delta-like ligand 4 (Dll4), ligand du récepteur Notch (Shutter, Scully et al. 2000; Claxton and Fruttiger 2004). L'abolition de la signalisation Dll4/Notch aboutit à l'apparition d'un bourgeonnement anarchique avec plusieurs « tip cells » à chaque embranchement (Claxton and Fruttiger 2004). (Figure 5).

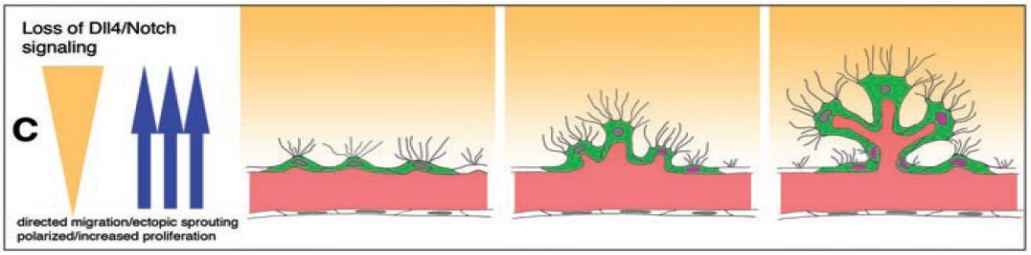

Figure 5. Signalisation Dll4/Notch et balance "tip cell / stlk cell". (D'après Gerhardt 2008).

Chez les mammifères, les récepteurs Notch 1,2,3 et 4 interagissent avec les ligands Jagged 1 et 2 et Dll 1, 3 et 4. L'activation de la voie de signalisation entraîne l'expression de gènes cibles dont la plupart exerce un contrôle négatif sur la voie tels que Hes1, Hes7 ou Nrarp (Notch-regulated ankyrin repeat protein) (Roca and Adams 2007). La voie est décrite comme antiprolifération et anti-angiogénique (Lobov, Renard et al. 2007) notamment en diminuant l'expression des récepteurs du VEGF (Jakobsson, Bentley et al. 2009). Des

données très récemment publiées permettent d'affirmer qu'il s'agit en fait d'un équilibre très finement régulé entre l'expression des récepteurs VEGFR1 et VEGFR2 qui aboutit à la sélection et la maintenance de la « tip cell » (Jakobsson, Franco et al. 2010). C'est donc par un effet juxtacrine qu'est régulée la balance « tip cell » / « stalk cells ». (Roca 2007; De Smet, Segura et al. 2009) (Figure 6). Quant à la formation des filipodes dans la CE « tip cell », elle est régulée par plusieurs éléments schématisés dans la figure ci-dessous (Figure 7).

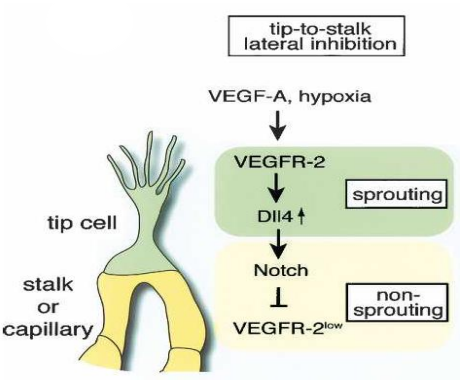

Figure 6. Effet juxtacrine de la voie Notch dans la régualtion de la balance "tip cell / stalk cell". (D'après Roca 2007).

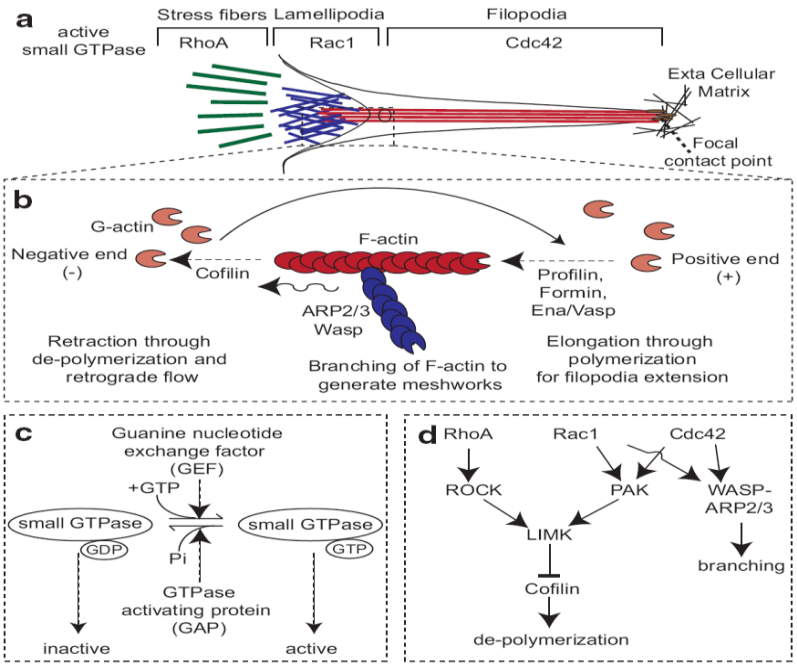

Figure 7. Régulation des filipodes de la CE "tip cell". Les filipodes sont représentés en rouge, les lamellipodes en bleu et les fibres de stress en vert. (D'après De Smet 2009).

Ces extensions cellulaires sont le lieu d'interactions étroites avec la matrice extracellulaire : apparition de points de contact focaux, dégradation via des MMP, influence de différents systèmes de guidance. Récemment, Fantin a montré dans un modèle de développement de vaisseaux dans le cerveau du poisson zèbre, que certains macrophages exprimant le récepteur des Angiopoïetines Tie2, étaient impliqués dans les jonctions entre les « tip cell » aboutissant aux fusions des néo-vaisseaux (Fantin, Vieira et al. 2010) (Figure 8).

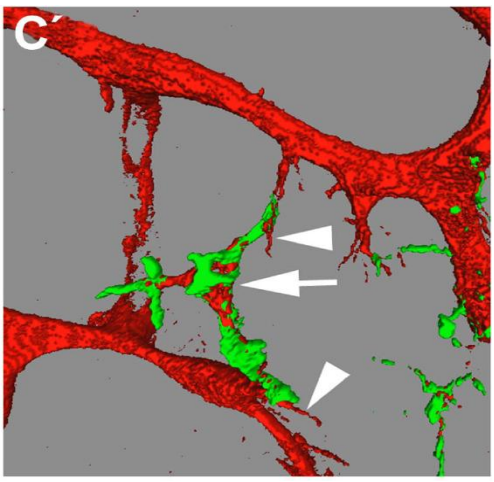

Figure 8. Rôle des macrophages (en vert) dans l'organisation du réseau de "tip cells" (en rouge). (D'après Fantin 2010).

Comme on peut donc le constater, le développement d'un réseau vasculaire est un phénomène complexe et très finement régulé faisant entre autre intervenir des notions de gradients spatio-temporels d'hypoxie, ou de facteurs de croissance vasculaire et d'interactions cellules-cellules et cellules-MEC.

(c) Rôle des cellules souches

L'ischémie tissulaire induit une mobilisation de cellules progénitrices, notamment endothéliales, à partir de la moelle osseuse, vers le sang circulant ; d'autres sources de progéniteurs existent comme le foie ou l'intestin (Aicher, Rentsch et al. 2007). Ces cellules souches se stabilisent alors préférentiellement dans les zones ischémiques. Ce phénomène est appelé « homing ». Là, elles vont pouvoir y exercer leurs effets pro-angiogéniques. La véritable contribution de ces cellules à la formation du réseau de vaisseaux collatéraux en réalité est inconnue et très probablement variable d'une situation à une autre (Vieyra, Jackson et al. 2005).

Ces cellules sont maintenues dans notre moelle osseuse à l'état pluripotent dans des niches qui ont la particularité d'être des zones où la pression partielle en oxygène est très faible (Chow, Wenning et al. 2001; Scadden 2006) (Figure 9).

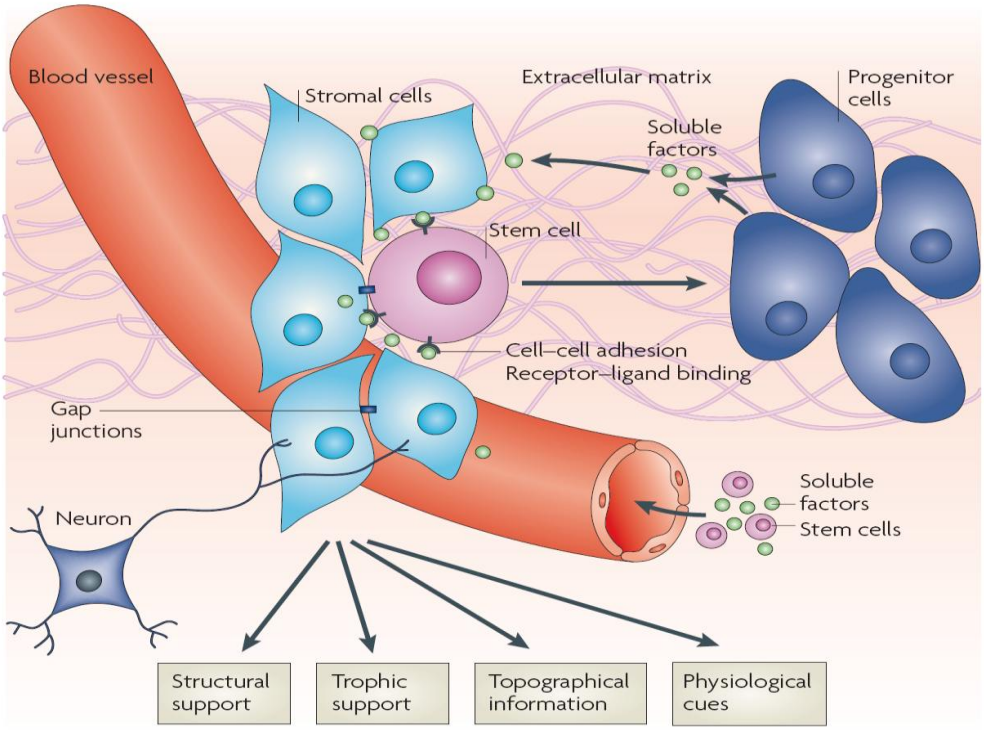

Figure 9. Le concept de niche médullaire. (D'après Jones 2008).

Lors d'une ischémie tissulaire, la première étape de leur mise en jeu est leur mobilisation c'est-à-dire le passage dans la circulation systémique depuis les niches médullaires. L'ischémie tissulaire induit l'expression de HIF et donc de VEGF-A. Ce facteur notamment, active, au niveau de la moelle, l'expression de MMP-9. Cette métalloprotéinase induit alors à son tour la libération du facteur soluble Kit ligand, cytokine facilitant la libération des précurseurs dans le sang circulant. De la même façon, le SDF-1 et son récepteur CXCR4 semblent jouer un rôle important dans la migration et la mobilisation des cellules souches (Lapidot, Dar et al. 2005). D'autres molécules sont impliquées dans la mobilisation des cellules progénitrices comme les angiopoïétines (Hattori, Dias et al. 2001), le Placental Growth Factor (PlGF), le NO (Smart and Riley 2008) ou l'érythropoïétine (EPO) (Bahlmann, De Groot et al. 2004)

Puis, ces cellules vont se concentrer dans les tissus atteints. Les mécanismes permettant la fixation préférentielle des cellules progénitrices circulantes dans les zones ischémiques ou homing, ne sont encore pas complètement élucidés. La première étape est une fixation de ces cellules sur l'endothélium des zones concernées. Un gradient d'hypoxie pourrait stabiliser la sous-unité HIF-1α et induire l'expression de SDF-1 au niveau des cellules endothéliales, favorisant l'adhésion des progéniteurs circulants. Les molécules d'adhésion E-selectin et P-selectin ainsi que l'ICAM-1 (Intracellular Adhesion Molecule-1) ou la VCAM-1 (Vascular endothelial Cell Adhesion Molecule-1) sont alors mises en jeu. Dans les zones désendothélialisées, les plaquettes pourraient interagir dans le même sens. La deuxième étape est la migration transendothéliale ou diapédèse, notamment sous la dépendance d'intégrines comme l'intégrine β2 et faisant intervenir des métalloprotéinases comme MMP-2 ou MMP-9 (Yagi, Soto-Gutierrez et al. 2010) (Figure 10).

Une fois dans les tissus ischémiés, les cellules progénitrices vasculaires vont participer à la néo-vascularisation, et en s'intégrant dans la structure des néo-vaisseaux, sous forme de cellules de soutien ou de nouvelles cellules endothéliales, et par un effet paracrine en produisant localement des facteurs de croissance pro-angiogéniques (Kovacic, Moore et al. 2008). Notamment des cellules issues de la moelle peuvent être retrouvées sous forme de péricytes, entourant des cellules endothéliales et exprimant les protéoglycanes NG2, spécifiques des péricytes, sans exprimer les marqueurs des CML (Rajantie, Ilmonen et al. 2004). Récemment, une origine adventitielle des cellules souches donnant les péricytes a également été évoquée (Corselli, Chen et al. 2010).

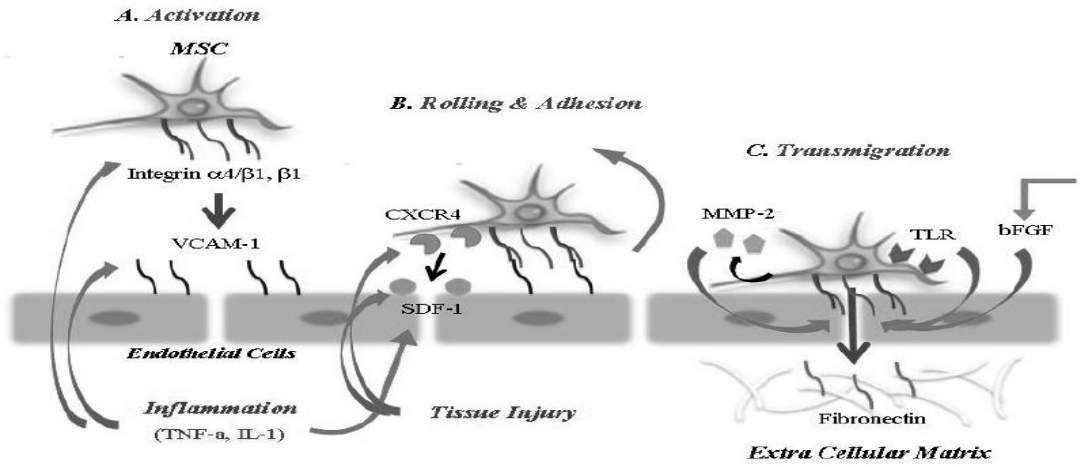

Figure 10. Mécanismes impliqué dans le "homing" des cellules souches mésenchymateuses (MSC). (D'après Yagi 2010)

L'ensemble de ces modifications ainsi que les phénomènes inflammatoires qui surviennent alors, aboutissent à la formation de néo-vaisseaux, constituant un réseau collatéral dont le rôle est primordial dans la réparation tissulaire.

2) Thérapie angiogénique : des facteurs de croissance aux cellules souches

Que ce soit au niveau du muscle cardiaque, dans une situation d'ischémie myocardique ou de post-infarctus, ou des muscles squelettiques des membres inférieurs, dans la maladie artérielle périphérique, l'idée de stimuler la croissance vasculaire en cas d'ischémie, pour en limiter les conséquences, a donné lieu au concept de thérapie angiogénique, au milieu des années 1970 (Folkman 1971). Lors des premières expérimentations, 10 ans plus tard (Isner, Vale et al. 2001), des facteurs de croissance vasculaire ont été injectés sous forme de protéines recombinantes ou surexprimés de façon génique après injection de vecteurs viraux ou de plasmides codant pour la protéine d'intérêt, seuls ou via un liposome. Puis ce sont des combinaisons de plusieurs facteurs qui

ont été utilisées. Malgré des premiers essais prometteurs menés chez l'homme dès 1994 et publiés entre 1996 et 2000 (Isner, Walsh et al. 1996; Baumgartner, Pieczek et al. 1998; Symes, Losordo et al. 1999) , la thérapie génique n'a pas satisfait, avec de plus larges études randomisées, les espoirs qu'elle avait générés (Lederman, Mendelsohn et al. 2002; Simons, Annex et al. 2002; Grines, Watkins et al. 2003; Henry, Annex et al. 2003; Rajagopalan, Mohler et al. 2003; Jones and Annex 2007). Plusieurs équipes travaillent toujours aujourd'hui, et avec des résultats encourageants (Kusumanto, van Weel et al. 2006; Kastrup 2010; Losordo 2010) sur le transfert de gènes codants pour des facteurs de croissance et tentent de répondre aux nombreuses questions soulevées par les études cliniques.

Parallèlement, des études animales visant à utiliser des cellules comme vecteurs de transfert de protéines recombinantes, mettaient en évidence des capacités pour ces cellules de se fixer durablement dans les tissus hôtes (Nabel, Plautz et al. 1989; Barr and Leiden 1991; Koh, Klug et al. 1993). Si bien qu'au début des années 1990, un nouveau concept reposant sur le transfert de cellules souches dans les tissus pathologiques avec pour but une restauration de l'organe atteint, a fait son apparition (Reinlib and Field 2000). Cependant de nombreuses questions se sont très vite posées : quel type de cellules humaines choisir, comment se les procurer, quel mode de culture, combien, quand, à quel moment et comment les injecter, comment répondre aux questions éthiques et de sécurité, ou encore, comment augmenter leur survie à l'ischémie ? Les mécanismes mis en jeu dans les améliorations constatées expérimentalement sont nombreux, intriqués et complexes à étudier : différenciation cellulaire, effets paracrines, effets immunomodulateurs, anti-apoptotiques et bien sûr, effets pro-angiogéniques (Silvestre 2009).

Il faut alors distinguer les deux situations différentes que sont la maladie artérielle périphérique et la maladie coronaire. En effet, après un évènement ischémique, et à condition que le flux sanguin soit au moins en partie rétabli, le muscle strié squelettique est capable de se régénérer ad integrum à partir de cellules satellites ou myoblastes constitutivement présentes dans le muscle, sous la membrane basale du sarcolemne (Mauro 1961; Ten Broek, Grefte et al. 2010). Les travaux portant sur la thérapie cellulaire dans l'ischémie de membre se focalisent donc sur le potentiel pro-angiogénique de ces techniques.

Pour ce qui est du muscle cardiaque, les choses sont différentes. Le muscle cardiaque est un tissu très différencié dont le taux de renouvellement tissulaire est très faible. En effet,

on estime que, dans un cœur normal, moins de 1% des cardiomyocytes sont renouvelés annuellement (Bergmann, Bhardwaj et al. 2009; Di Nardo, Forte et al. 2010). Et ce chiffre diminuant avec l'âge, c'est finalement moins de la moitié des cardiomyocytes qui sont renouvelés au cours de notre vie. De même, les capacités de réparation du myocarde après un évènement ischémique sont limitées. Le muscle détruit est en général remplacé par un tissu cicatriciel non contractile, ce qui a pour conséquence d'aboutir à l'insuffisance cardiaque. Si bien que nous pouvons distinguer deux objectifs distincts dans les travaux menés sur la thérapie cellulaire au niveau cardiaque : soit un but de régénération tissulaire avec pour objectif la synthèse de nouvelles cellules myocardiques fonctionnelles, soit un but pro-angiogénique. Cependant, rapidement après l'enthousiasme soulevé par les travaux d'Orlic, (Orlic, Kajstura et al. 2001), il est apparu difficile d'imaginer dans un avenir proche faire apparaître un nouveau tissu cardiaque fonctionnel dans un cœur infarci. Quant à l'effet pro-angiogénique, l'hypothèse initiale qui envisageait la différenciation des cellules souches en cellules endothéliales fonctionnelles, incorporées aux nouveaux vaisseaux (Takahashi, Kalka et al. 1999), a laissé place à des concepts plus complexes impliquant des cellules péri-endothéliales sécrétant des chimiokines et des facteurs de croissance (Ziegelhoeffer, Fernandez et al. 2004; Gnecchi, He et al. 2006).

3) Les cellules souches candidates pour un usage thérapeutique

(a) Définition des cellules souches

Les cellules souches peuvent être classées selon leurs capacités à s'auto-renouveler et à produire des cellules différenciées. Les cellules totipotentes peuvent donner tout type de cellule et donc assurer le développement d'un organisme entier. Les cellules pluripotentes peuvent se différencier en tout type de cellules de l'organisme sauf les cellules extra-embryonnaires comme le placenta. Ce sont par exemple les cellules souches embryonnaires (ESCs) (Thomson, Itskovitz-Eldor et al. 1998). Lorsqu'elles se spécialisent, elles donnent des cellules multipotentes spécifiquement dévolues à une fonction. Par exemple, les cellules souches hématopoïétiques sont des cellules multipotentes,

uniquement capables de se transformer en tous les éléments figurés du sang. Enfin les cellules souches unipotentes ne peuvent donner qu'un seul type de cellule. Leurs capacités d'auto-renouvellement sont plus limitées. Ce sont par exemple les précurseurs endothéliaux (EPC) ou les myoblastes (Figure 11).

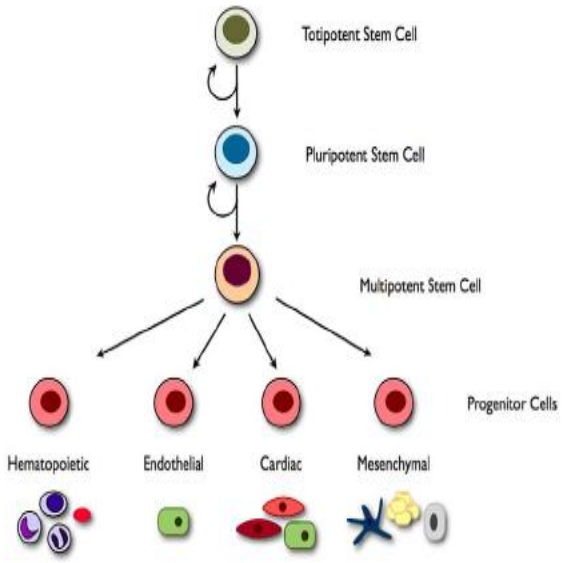

Figure 11. Illustration de la hiérarchie de la pluripotence des cellules souches. (D'après Sieveking 2009).

Par ailleurs, on distingue les cellules souches embryonnaires au fort potentiel d'auto-renouvellement des cellules souches adultes. Les cellules souches adultes, souvent issues de la moelle osseuse, peuvent être transplantées de manière autologue et ne posent pas les mêmes problèmes éthiques que les cellules souches embryonnaires en vue d'une utilisation en thérapeutique. Il s'agit soit d'un ensemble de différentes cellules appelées BMC (Bone Marrow Cells) soit de cellules sélectionnées selon la présence ou l'absence à leur surface de marqueurs spécifiques. Ce sont par exemple les précurseurs endothéliaux (EPC) ou les Cellules souches mésenchymateuses (MSC). Des cellules souches peuvent également être extraites du sang circulant (PBMC ou Péripheral Blood Mononuclear Cells) après simulation par le G-CSF (Granulocyte-Colony-Stimulating Factor), ou du sang de cordon ombilical (cellules souches fœtales) ou bien encore de tissus différenciés. En effet, des MSC peuvent être extraites du tissu adipeux (Planat-Benard, Silvestre et al. 2004) et des cellules souches

pluripotentes peuvent être induites à partir de fibroblastes cutanés, ce sont les iPS pour induced Pluripotent Stem cells (Takahashi and Yamanaka 2006). Plus récemment, des cellules avec des caractéristiques de cellules souches ont été isolées dans le myocarde (Beltrami, Barlucchi et al. 2003).

Toutes ces cellules sont de possibles candidates pour un usage thérapeutique mais seules les BMC, EPC, MSC et myoblastes ont, pour l'heure, donné lieu à des études cliniques (Figure 12). Nous nous proposons ici de résumer les caractéristiques de chacun de ces 4 types de cellules et envisagerons rapidement les études précliniques réalisées dans des modèles d'ischémie de membre ou de d'infarctus du myocarde.

Candidate cell	Potency	Role in angiogenesis	Human clinical trial
Endothelial progenitor cells (EPCs)	Monopotent	Paracrine and direct	Yes
Bone marrow-derived cells (BMCs)	Multipotent	Paracrine	Yes
Mesenchymal stem cells (MSCs)	Multipotent	Paracrine	Yes
Embryonic stem cells (ESCs)/induced pluripotent stem (iPS) cells	Pluripotent	Paracrine and direct	N/A
Adipose-derived stem cells (ADSCs)	Multipotent	Paracrine and direct	N/A
Cardiac stem/progenitor cells (CSCs/CPCs)	Multipotent	Paracrine and direct	N/A

Figure 12. Les cellules candidates pour une application humaine. (D'après Sieveking 2009).

(b) Les cellules de la moelle osseuse

Les cellules de la moelle osseuse, Bone Marrow Cells (BMC), représentent un ensemble hétérogène de cellules. Après aspiration médullaire au niveau de la crête iliaque, sous anesthésie locale pour l'homme, les cellules mononucléées de faible densité sont isolées sur gradient de Ficoll. 95% de ces cellules sont des cellules myéloïdes, les cellules souches représentant donc seulement moins de 5% de l'ensemble des cellules. Parmi ces dernières, des cellules souches hématopoïétiques (HSC), des précurseurs endothéliaux (EPC) et des cellules souches mésenchymateuses (MSC). Ne faisant aucun doute que les cellules souches médullaires participent de façon physiologique aux mécanismes de développement vasculaire, leur utilisation à visée thérapeutique a été testée précocement dans différents

modèles animaux puis chez l'humain. Pour ce faire les cellules sont soit extraites de la moelle, soit mobilisées de la moelle du patient par l'injection de G-CSF (Granulocyte-colony stimulating factor). De dénomination commune internationale filgrastim, cette cytokine est commercialisée en France sous le nom de Neupogen®. Cette technique nécessite chez l'homme l'injection quotidienne de G-CSF durant 4 à 6 jours. Pour une injection autologue intracoronaire ou directe dans le muscle cardiaque, il est possible de récupérer les cellules circulantes par aphérèse. Dans tous les cas, un des intérêts de l'utilisation des BMC réside dans l'absence de nécessité de culture cellulaire, les cellules étant utilisées de manière extemporanée. Les risques infectieux, toxiques ou de modifications épigénétiques s'en trouvent donc diminués.

Après Tomita en 1999 (Tomita, Li et al. 1999) et les résultats très encourageants d'Orlic en 2001 (Orlic, Kajstura et al. 2001; Orlic, Kajstura et al. 2001), un grand nombre d'études vont confirmer, dans des modèles d'infarcus comme d'ischémie de membre, le potentiel thérapeutique de ces BMC extraites directement de la moelle osseuse (Hamano, Li et al. 2000; Hamano, Li et al. 2001; Hamano, Li et al. 2002; Dowell, Rubart et al. 2003).

Les études conduites avec le G-CSF chez l'animal ont été marquées par une deuxième publication d'Orlic en 2001 (Orlic, Kajstura et al. 2001) qui, chez la souris traitée plusieurs jours avant l'infarctus par du G-CSF, retrouve un effet bénéfique sur l'ensemble des paramètres étudiés. D'autres études plus ou moins enthousiastes suivront. Chez le primate également prétraité, les résultats sont moins clairs (Norol, Merlet et al. 2003). Cependant chez la souris, l'effet semble exister même si l'injection de G-CSF est postérieure à la ligature coronaire (Ohtsuka, Takano et al. 2004). Toujours sur des modèles d'infarctus du myocarde, on peut encore citer les études de Fukuhara (Fukuhara, Tomita et al. 2004), de Deindl (Deindl, Zaruba et al. 2006) ou de Li (Li, Takemura et al. 2006). Pour ce qui est de l'ischémie de membre inférieur, les publications sont plus rares. Il s'agit des travaux de Grundmann chez le lapin et le cochon (Grundmann, Hoefer et al. 2006), de Lee chez le rat (Lee, Aoki et al. 2005) ou de Ieda chez la souris (Ieda, Fujita et al. 2007). Outre les mécanismes dus aux cellules souches elles-mêmes, un effet propre au G-CSF, via la voie Jak/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) (Harada, Qin et al. 2005; Kurdi and Booz 2009) ou la voie PI3K/Akt/eNOS (Takano, Qin et al. 2006), permettrait de préserver le myocarde d'un remodelage délétère. Cependant le G-CSF pourrait aussi avoir un rôle négatif

sur le « homing » des cellules souches dans le myocarde en agissant sur la voie SDF-1/CXCR-4 (Brunner, Engelmann et al. 2008) (Figure 13).

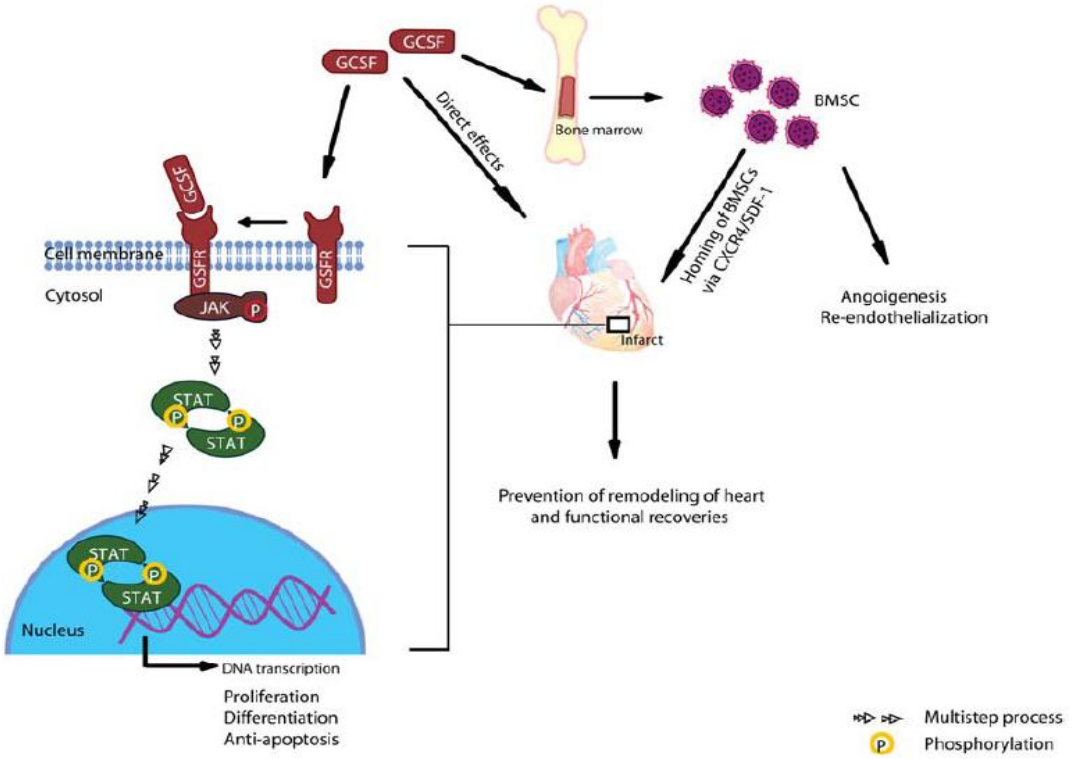

Figure 13. Rôle du G-CSF. (D'après Shim 2010).

(c) Les Précurseurs endothéliaux

Les précurseurs endothéliaux (EPCs), se caractérisent par la présence à leur surface des marqueurs CD34 et du récepteur au VEGF, VEGFR-2 (Asahara, Murohara et al. 1997). En culture, deux populations d'EPCs peuvent êtres distinguées : les « early EPCs » apparaissent après 4 à 7 jours de culture alors que les « late EPCs » ne surviennent qu'après 2 à 3 semaines (Lin, Weisdorf et al. 2000; Yoder, Mead et al. 2007). Les EPCs tardives se distinguent des précoces par l'absence à leur surface de marqueurs de cellules hématopoïétiques (CD14 et CD45), leur morphologie plus pavimenteuse et moins allongée et

leurs plus grandes capacités de prolifération. Même si la moelle osseuse est la principale source d'EPC, ces cellules ont pu être extraites de différents tissus comme le sang, le foie, la rate, l'intestin, les tissus adipeux ou les parois des vaisseaux (Ingram, Mead et al. 2005; Zengin, Chalajour et al. 2006; Aicher, Rentsch et al. 2007). Dans plusieurs études animales, les EPCs se sont montrées capables de participer à la ré-endothélialisation de vaisseaux lésés (Griese, Ehsan et al. 2003), d'améliorer la fonction endothéliale (Shi, Rafii et al. 1998; Wassmann, Werner et al. 2006), et d'améliorer la récupération musculaire après ischémie de membre inférieur ou infarctus du myocarde (Asahara, Murohara et al. 1997; Crosby, Kaminski et al. 2000; Kalka, Masuda et al. 2000; Kobayashi, Hamano et al. 2000; Kawamoto, Gwon et al. 2001; Orlic, Kajstura et al. 2001; Kawamoto, Tkebuchava et al. 2003; Rehman, Li et al. 2003; Ziegelhoeffer, Fernandez et al. 2004; Yoder, Mead et al. 2007; Timmermans, Plum et al. 2009). De récentes données suggèrent que les deux types d'EPCs early et late, agiraient de manière synergique mais avec des rôles différents, les tardives s'incorporant aux néo-vaisseaux et les précoces ayant un rôle paracrine (Yoon, Hur et al. 2005; Sieveking, Buckle et al. 2008). Séduisantes par leur mode d'action, les EPC présentent l'incovénient, pour une utilisation en clinique humaine, de nécessiter des étapes de culture cellulaire.

(d) Les cellules souches mésenchymateuses

Découvertes dans les années 1960 par Friedenstein (Friedenstein, Petrakova et al. 1968), et alors appelées colony-forming unit-fibroblasts (Perl, Weissler et al.), les cellules souches mésenchymateuses (MSC) sont habituellement localisées dans la moelle osseuse où elles servent de soutien aux cellules souches hématopoïétiques. Elles ne représentent qu'une faible proportion (0,01%) des cellules mononucléées issues de la moelle. Ce sont des cellules multipotentes capables de se différencier en plusieurs types de cellules : cellules musculaires squelettiques, ostéoblastes, chondrocytes ou adipocytes (Charbord; Pittenger, Mackay et al. 1999; Deans and Moseley 2000) (Perl, Weissler et al. 2010) (Figure 14).

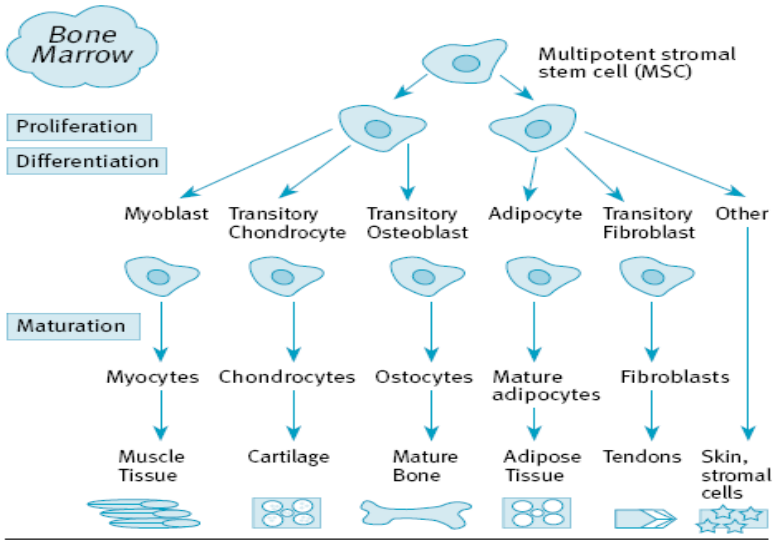

Figure 14. Differenciation des MSC. (D'après Perl 2010).

Elles ne possèdent pas à leur surface de marqueurs de cellules sanguines comme le CD45, CD34, CD11 ou le CD14. Par contre, il est convenu qu'elles expriment des marqueurs tels que CD105, CD44, CD90, STRO-1 ou des molécules d'ahésion telles que VCAM-1 ou ICAM-1. Cependant aucun de ces marqueurs n'est spécifique des MSC (Dominici, Le Blanc et al. 2006; Psaltis, Zannettino et al. 2008) (Figure 15).

Table 1. Surface antigen profile of MSCs [36]

Positive	Negative
Minimal immunophenotypic criteria as recommended by ICT	
CD105	CD34
CD73	CD45
CD90	CD14 or CD11b
	CD79α or CD19
	HLA-DR
Other antigens used to isolate MSCs by immunoselection	
STRO-1	Glycophorin A
CD49a/CD29	
CD106	
CD146	
NGFR	
PDGFR	
EGFR	
IGFR-1	

Abbreviations: EGFR, epidermal growth factor-receptor; ICT, International Society for Cellular Therapy; IGFR, insulin-like growth factor-receptor; MSC, mesenchymal stromal cell; NGFR, nerve growth factor-receptor; PDGFR, platelet-derived growth factor-receptor.

Figure 15. Profil d'expression des antigènes de surface des MSC. (D'après Psaltis 2008).

Si ces MSC sont habituellement extraites de la moelle osseuse, il a été montré qu'elles pouvaient provenir d'autres tissus, plus facilement accessibles, comme le sang de cordon ombilical ou les tissus adipeux sous-cutanés (van der Bogt, Schrepfer et al. 2009). Le principe d'isolement des MSC repose sur une séparation par gradient de densité et culture sur support plastique. En effet, contrairement aux cellules hématopoïétiques, les MSC sont adhérentes au support et donc facilement isolables. Dans des conditions appropriées, elles donnent alors naissance à des colonies (colony-forming units fibroblast [CFU-F] cells) qui, pour certaines auront de grandes capacités de prolifération (Gronthos, Zannettino et al. 2003). *In vitro*, différents stimuli extrinsèques, permettant de les orienter vers une lignée cellulaire, ont été identifiés. Elles sont aussi facilement transfectables.

De manière très intéressante, les MSC ne provoquent pas de réaction immunologique lorsqu'elles sont transplantées d'un organisme à un autre car ne portent pas de molécules de classe II du complexe majeur d'histo-compatibilité (Kawamoto, Tkebuchava et al. 2003) et une fois implantées dans les tissus, elles montrent un important rôle immuno-modulateur (Uccelli, Moretta et al. 2006). Cette donnée est fondamentale puisque permettant d'envisager une transplantation allogénique sans traitement immunosuppresseur préalable. Outre les intérêts pratiques évidents, ce type de transplantation permet de s'amender de la probable moindre efficacité des cellules souches des patients qui, par définition, présentent des pathologies et des facteurs de risque cardiovasculaires susceptibles d'altérer leurs fonctions régénératrices (Tamarat, Silvestre et al. 2004). Leur rôle immuno-modulateur ouvre certaines perspectives thérapeutiques intéressantes dans des pathologies inflammatoires mais n'est pour l'heure pas parfaitement expliqué (Aggarwal and Pittenger 2005). Une inhibition des lymphocytes T et B ainsi que des cellules Natural Killer a été constatée (Augello, Tasso et al. 2005) avec diminution de l'expression de certaines cytokines pro-inflammatoires comme IL-10 ou TGF-β (Tse, Pendleton et al. 2003) (Figure 16).

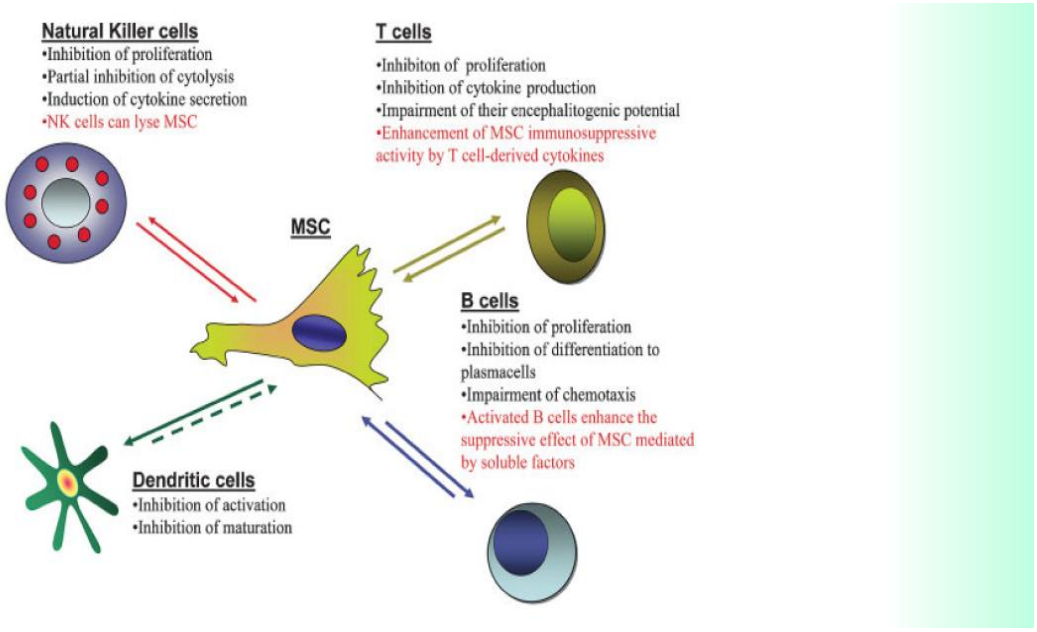

Figure 16. Rôle immunomodulateur des MSC. (D'après Uccelli 2006).

Par ailleurs, il a été montré que les MSC avaient la faculté de se localiser préférentiellement dans les zones tissulaires ischémiques ou inflammatoires (Wang, Shum-Tim et al. 2001; Barbash, Chouraqui et al. 2003; Chamberlain, Fox et al. 2007), et que leurs capacités de régénération tissulaire étaient importantes (Psaltis, Zannettino et al. 2008). En effet, certains auteurs ont montré que, chez différents animaux, l'apport de MSC après infarctus du myocarde diminuait la taille de la cicatrice, améliorait la densité capillaire et la fonction cardiaque (Barbash, Chouraqui et al. 2003; Saito, Kuang et al. 2003; Nagaya, Fujii et al. 2004; Hattan, Kawaguchi et al. 2005) (Toma, Pittenger et al. 2002; Amado, Saliaris et al. 2005; Valina, Pinkernell et al. 2007). De même, l'effet bénéfique apparaît nettement sur des modèles de cardiomyopathie dilatée (Nagaya, Kangawa et al. 2005; Silva, Litovsky et al. 2005; Ohnishi, Yanagawa et al. 2007). Plus récemment, ces cellules ont été envisagées pour restaurer des fonctions rythmiques (Plotnikov, Shlapakova et al. 2007) ou valvulaires (Knight, Booth et al. 2005; Vincentelli, Wautot et al. 2007). L'ensemble de ces expérimentations animales a permis d'exclure de fréquentes et/ou graves complications de l'injection de cellules souches et des MSC en particulier. Breitbach a cependant rapporté la formation de zones calcifiées encapsulées aux points d'injection (Breitbach, Bostani et al. 2007) et une grande attention est apportée aux potentiels déclenchements de troubles du rythme

ventriculaire (Pak, Qayyum et al. 2003; Chang, Tung et al. 2006; Fernandes, van Rijen et al. 2009; Ly and Nattel 2009)

Le mécanisme d'action de ces effets thérapeutiques est probablement principalement paracrine, en amplifiant les phénomènes naturels de réparation des tissus lésés. En effet, les MSC sécrètent différents facteurs pro-angiogéniques comme SDF-1, Hepatocyte Growth Factor (HGF), Insuline-like Growth Factor-1 (IGF-1), bFGF, VEGF, Ang-1 (Kinnaird, Stabile et al. 2004), Placental Growth Factor (PlGF), MCP-1, mais aussi des interleukines, IL-1, IL-6 ou TNF-α (Kamihata, Matsubara et al. 2001; Gnecchi, He et al. 2006; Sadat, Gehmert et al. 2007). Enfin, les MSC sont capables d'exprimer HIF-1α, facteur induit par l'hypoxie et à l'origine de l'expression notamment de VEGF (Dai, Xu et al. 2007; Potier, Ferreira et al. 2007). Outre la facilitation de l'apparition de néo-vaisseaux, ces phénomènes pourraient protéger les tissus existants (Xu, Uemura et al. 2007), voire amplifier les phénomènes d'auto-régénération de l'organe (Urbanek, Rota et al. 2005). Cependant il faut noter que plusieurs études ont mis en évidence pour ces cellules sous divers stimuli, naturels ou non, ou en conditions de co-culture, des capacités de différenciation en cellules endothéliales, cellules musculaires lisses ou péricytes (Davani, Marandin et al. 2003; Silva, Litovsky et al. 2005), mais aussi en cardiomyocytes. En effet, ces cellules peuvent acquérir des caractéristiques de cellules musculaires cardiaques avec expression de Troponine T, Myosin Heavy Chain (MHC) et Beta-actine. Les MSC peuvent alors avoir un automatisme de type nodal avec potentiels d'action de type ventriculaire et présentent entre elles des interactions spécifiques de type gap junction (Toma, Pittenger et al. 2002; Potapova, Plotnikov et al. 2004; Valiunas, Doronin et al. 2004; Minguell and Erices 2006). (Makino, Fukuda et al. 1999; Rangappa, Entwistle et al. 2003; Rangappa, Fen et al. 2003; Yoon, Min et al. 2005; Balana, Nicoletti et al. 2006). L'incorporation de ces cellules dans les nouvelles structures vasculaires ou en tant que néo-cardiomyocytes donne toujours lieu à débat. En tout état de cause, même si ce mécanisme existe, il reste probablement modeste dans les phénomènes de régénération observés (Kajstura, Rota et al. 2005). Enfin, un autre mécanisme potentiellement impliqué est la participation de ces cellules à la digestion des tissus cicatriciels et au remodelage de la matrice extracellulaire (Aupperle, Garbade et al. 2007).

Malgré la nécessité de culture, l'ensemble de ces données font des MSC les candidates idéales pour l'application d'une thérapie cellulaire (Chamberlain, Fox et al. 2007),

voire pour l'utilisation de ces cellules comme vecteurs d'une thérapie génique (Matsumoto, Omura et al. 2005).

(e) Les myoblastes

Chez l'homme, une simple biopsie au niveau du vaste externe du quadriceps permet d'obtenir en 3 semaines de culture, de 0.5 à 1 milliard de myoblastes. C'est en 1961 que Mauro démontre la présence et le rôle des cellules satellites, ou myoblastes, au sein du muscle strié squelettique normal (Mauro 1961). Outre leurs grandes capacités de prolifération en culture, ces cellules sont particulièrement résistantes aux conditions d'hypoxie (Csete, Walikonis et al. 2001). En 1993, Koh greffe avec succès des cellules d'une lignée de myoblastes (C2C12) sur des cœurs de souris (Koh, Klug et al. 1993). Initiées avec des cardiomyocytes fœtaux, puis continuées avec des myoblastes adultes, de nombreuses études sur des modèles d'infarctus du myocarde ont permis de conclure à une efficacité de ces cellules pour limiter la taille de l'infarctus et le remodelage et augmenter la contractilité myocardique (Dowell, Rubart et al. 2003). Notamment, Taylor en 1998 a été le premier à mettre en évidence le rôle de ces cellules sur un modèle d'infarctus par cryolésion chez le lapin (Taylor, Atkins et al. 1998). Puis, en 2001, Jain a montré leur intérêt pour prévenir le remodelage ventriculaire chez le rat (Jain, DerSimonian et al. 2001; Dowell, Rubart et al. 2003). S'il a été montré que la plupart de ces cellules mourraient rapidement après leur transplantation, un certain nombre persistent en formant des myotubes multinucléés mais sans connexion avec les cardiomyocytes hôtes (Reinecke, Poppa et al. 2002; Maurel, Azarnoush et al. 2005), les privant de tout couplage électromécanique avec le reste du myocarde. Cette limite demeurera importante malgré les efforts de certains auteurs qui ont, par exemple, tenté d'utiliser des myoblastes surexprimant la connexine 43 (Suzuki, Brand et al. 2001). Par contre très peu d'études sont disponibles pour ce qui concerne les membres inférieurs. Parmi les mécanismes mis en jeu l'augmentation de l'angiogenèse est possiblement primordiale dans ces observations (Menasche 2008).

A) LES ETUDES CLINIQUES CARDIOVASCULAIRES DE THERAPIE CELLULAIRE

1) Les études cliniques de thérapie cellulaire à visée cardiaque

(a) Dans l'infarctus du myocarde

Une méta-analyse des 13 premiers principaux essais menés chez l'homme en situation de post-infarctus avec des BMC a été publiée en 2008 par Martin-Rendon (Martin-Rendon, Brunskill et al. 2008). Concernant près de 900 patients, ce travail montre que la thérapie cellulaire, ainsi réalisée chez l'humain, ne permet pas de réduire, de manière statistiquement significative, la mortalité ni la morbidité cardiovasculaire. Seule la fraction d'éjection ventriculaire s'élève mais de façon très modeste : 3% (Figure 17).

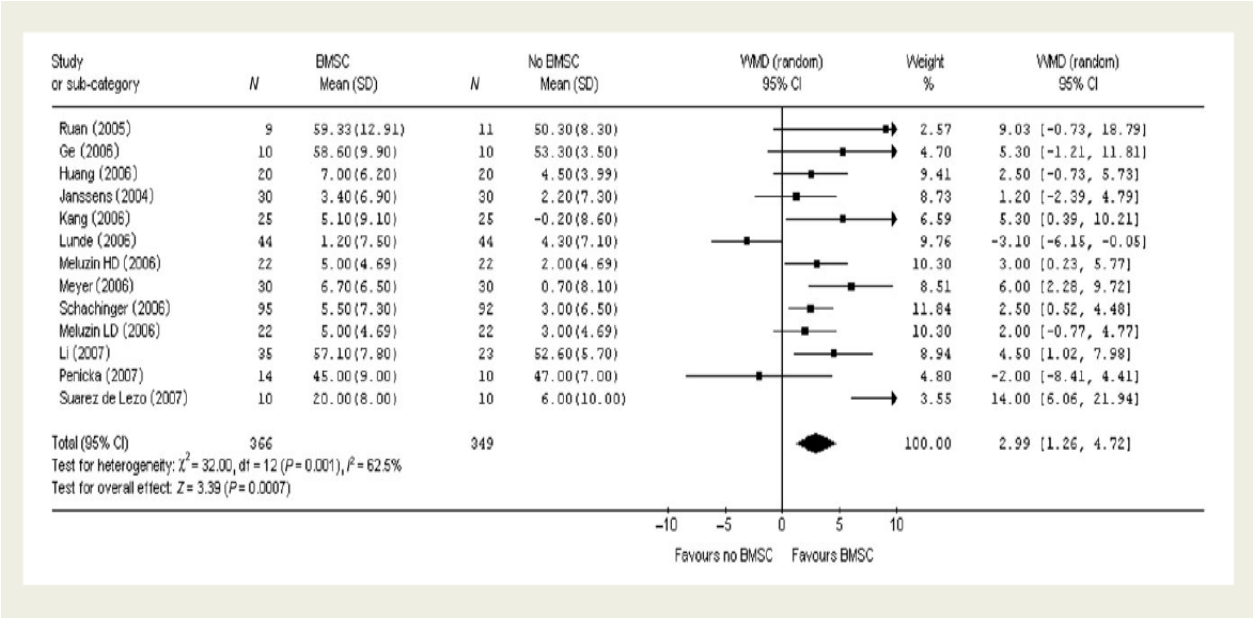

Figure 17. Méta-analyse de Martin-Rendon 2008: Fonction ventriculaire gauche.

(Wollert, Meyer et al. 2004; Karpov, Popov et al. 2005; Ruan, Pan et al. 2005; Ge, Li et al. 2006; Huang, Yao et al. 2006; Janssens, Dubois et al. 2006; Kang, Lee et al. 2006; Lunde, Solheim et al. 2006; Meluzin, Mayer et al. 2006; Meyer, Wollert et al. 2006; Schachinger, Erbs et al. 2006; Li, Zhang et al. 2007; Penicka, Horak et al. 2007; Suarez de Lezo, Herrera et al. 2007).

Si l'on considère seulement les études conduites par injection intra-coronaire de BMC après infarctus, la méta-analyse de Lipinski et al. (Lipinski, Biondi-Zoccai et al. 2007) permet de constater que les résultats regroupant 10 essais soit plus de 700 patients, sont là aussi très disparates et globalement faiblement positifs. La mortalité et la morbidité ne sont pas modifiées ; la diminution de la taille de l'infarctus et surtout l'augmentation de la fraction d'éjection ventriculaire (2,97%) sont d'autant plus marquées que la fraction d'éjection de départ est basse (<45%) et que les cellules sont injectées entre le 5eme et le 7eme jour après l'infarctus (Figure 18).

Study	Year	Design	Patients Enrolled (Patients at Follow-Up)	Cell Type	Follow-Up (Months)	Primary End Point	Imaging Modality for LVEF Assessment
Strauer et al. (10)	2002	Non-RCT	20 (20)	BMC	3	LVEF	LV angiography
Bartunek et al. (11)	2005	Non-RCT	35 (35)	BMC	4	Safety, LVEF	LV angiography, SPECT
Jannsens et al. (8)	2006	RCT	67 (66)	BMC	4	LVEF	Cardiac MRI
BOOST (7)	2006	RCT	60 (60)	BMC	18	LVEF, safety	Cardiac MRI
Zhan-Quan et al. (13)	2006	Non-RCT	70 (58)	PMC	6	LVEF, LV volumes, WMSI	Echocardiography
MAGIC CELL-3-DES (12)	2006	RCT	56 (50)	PMC	6	LVEF	Cardiac MRI
TCT-STAMI (15)	2006	RCT	20 (20)	BMC	6	LVEF	Echocardiography, SPECT
ASTAMI (2,4)	2006	RCT	100 (97)	BMC	6	LVEF, EDV, infarct size	SPECT, MRI, echo
REPAIR-AMI (5)	2006	RCT	204 (187)	BMC	12	LVEF	LV angiography
Meluzin et al. (16)	2006	RCT	66 (66)	BMC	3	Infarct zone systolic function	SPECT

BMC = bone marrow cells; EDV = end-diastolic volume; LV = left ventricular; LVEF = left ventricular ejection fraction; MRI = magnetic resonance imaging; PMC = peripheral mononuclear cells; RCT = randomized controlled trial; SPECT = single-photon emission computed tomography; WMSI = wall motion score index.

Figure 18. Méta-analyse de Lipinski 2007

(Strauer, Brehm et al. 2002; Wollert, Meyer et al. 2004; Bartunek, Vanderheyden et al. 2005; Ge, Li et al. 2006; Janssens, Dubois et al. 2006; Kang, Lee et al. 2006; Lunde, Solheim et al. 2006; Meluzin, Mayer et al. 2006; Meyer, Wollert et al. 2006; Schachinger, Erbs et al. 2006; Li, Zhang et al. 2007).

Citons également l'étude TOPCARE-AMI (Assmus, Schachinger et al. 2002; Schachinger, Assmus et al. 2004) qui a comparé, sur de petits effectifs, BMC et EPC, sans d'ailleurs mettre en évidence une différence. Un peu plus tard, en 2007, deux autres études ont été publiées mais sans apporter d'information supplémentaire (Penicka, Horak et al. 2007; Tatsumi, Ashihara et al. 2007).

Pour ce qui est de l'utilisation du G-CSF dans l'infarctus du myocarde, il a procuré certains espoirs après les travaux menés sur des modèles d'animaux d'Orlic et de Takahashi (Takahashi, Kalka et al. 1999; Orlic, Kajstura et al. 2001) si bien que des études humaines ont rapidement été conduites (Ripa, Jorgensen et al. 2006; Zohlnhofer, Ott et al. 2006). Mais elles se sont malheureusement avérées négatives ou faiblement positives (Abdel-Latif, Bolli et al. 2008). Ces études ont récemment fait l'objet de revues (Brunner, Engelmann et al. 2008; Ripa and Kastrup 2008; George 2010; Shim, Mehta et al. 2010) (Figure 19).

Table II. Clinical trials of stem cell mobilization by G-CSF therapy in AMI

Study	Trial Design	Groups	GCSF Dose	Timing	Follow up	Primary Endpoint	Change in EF vs Control (Group)
Valgimigli et al,[25] 2005	RC	PCI only (n = 10) GCSF (n = 10)	5 μg/kg × 4 d	PCI < 12 h post MI GCSF < 24 h post PCI	6 m	LVEF by SPECT	No change [+ 22% in GCSF]
Kuethe et al,[26] 2005	NR C	PCI only (n = 9) GCSF (n = 14)	5 μg/kg bid × 7 d	PCI < 8 h post MI GCSF 48 h post PCI	3 m	LVEF by RNV	Inc by 5% [+ 8% in GCSF]
Ince et al,[27] FIRSTLINE-AMI 2005	RC	PCI only (n = 25) GCSF (n = 25)	10 μg/kg × 6 d	PCI <5 h post MI GCSF 90 m post PCI	4 m	LVEF by Echo	Inc by 10% [+ 6% in GCSF]
Zohlnhofer et al,[28] REVIVAL-2 2006	RC	PCI only (n = 58) GCSF (n = 56)	10 μg/kg × 5 d	PCI <12 h post MI GCSF 5 d post PCI	5 m	LVEF by MRI, LVG	No change [+ 1% by MRI] [+ 1% by LVG]
Ripa et al,[29] STEMMI 2006	RC	PCI only (n = 39) GCSF (n = 39)	10 μg/kg × 6 d	PCI 4 h post MI GCSF 28 h post PCI	6 m	LVEF by Echo, MRI	No change [+ 6% by Echo] [+ 9% by MRI]
Engelmann et al,[30] G-CSF-STEMI 2006	RC	PCI only (n = 21) GCSF (n = 23)	10 μg/kg × 5 d	PCI 40 h post MI GCSF 35 h post PCI	3 m	LVEF by MRI	No change [+ 6% in GCSF]
Ellis et al,[31] 2006	RC	PCI only (n = 6) LD GCSF (n = 6) HD GCSF (n = 6)	LD: 5 μg/kg × 5 d HD: 10 μg/kg × 5 d	PCI <12 h post MI GCSF <30 h post PCI	30 d	LVEF by Echo	No change [+ 4% in LD GCSF] [+ 5% in HD GCSF]
Leone et al,[32] Rigenera Study 2007	RC	PCI only (n = 27) GCSF (n = 14)	10 μg/kg × 5 d	PCI <5 d post MI GCSF 5 d post PCI	6 m	LVEF by Echo	Inc by 5% [+ 5% in GCSF]
Takano et al,[33] 2007	RC	PCI only (n = 22) GCSF (n = 18)	2.5 μg/kg × 5 d	PCI <6 h post MI GCSF <24 h post PCI	6 m	LVEF by SPECT	Inc by 2% [+ 5% in GCSF]

Abbreviations: *bid*, twice daily; *C*, controlled; *d*, days; *HD*, high dose; *Inc*, increase; *LD*, low dose; *LVG*, left ventriculography; *m*, months; *n*, number; *NC*, noncontrolled; *NR*, nonrandomized; *R*, randomized.

Figure 19. Etudes menées avec le G-CSF dans l'infractus (D'après George 2010)

Par ailleurs, les essais « MAGIC » ou l'étude de Steinwender ont permis de tester l'association de l'utilisation du G-CSF avec l'injection intracoronaire de cellules, mais, là encore, les résultats étaient négatifs (Kang, Kim et al. 2004; Kang, Lee et al. 2006; Steinwender, Hofmann et al. 2006) (Figure 20).

Table III. Clinical trials of stem cell mobilization by G-CSF therapy + intracoronary injection of stem cells in AMI

Study	Trial Design	Groups	GCSF Dose	Cell Count (Characterization)	Timing	Follow up	Primary Endpoint	Change in EF vs Control (Group)
Kang et al,[34] MAGIC 2004	RC	PCI only (n = 10) GCSF (n = 10) GCSF + PBSC (n = 10)	10 μg/kg × 4d	PB: 1×10^9 [8% CD34+]	PCI <48 h post MI GCSF <24 h post PCI	6 m	LVEF by SPECT	No change [Inc vs GCSF] [+ 0% in GCSF] [+ 10% in PBSC]
Steinwender et al,[35] 2006	NR NC	GCSF (n = 20)	5 μg/kg bid × 4d	PB: 5×10^9 [0.1% CD34+]	PCI 6 h post MI GCSF 24 h post PCI	6 m	LVEF by LVG	No control [+ 8% in PBSC]
Kang et al,[36] MAGIC 3-DES 2006	RC	**AMI:** PCI only (n = 25) GCSF + PBSC (n = 25) **OMI:** PCI only (n = 16) GCSF + PBSC (n = 16)	10 μg/kg × 3d	PB: 2×10^9 [9% CD34+] [15% KDR+]	**AMI:** PCI 4 d post MI GCSF <24 h post PCI **OMI:** PCI 750 d post MI GCSF <24 h post PCI	6 m	LVEF by MRI	**AMI:** Inc by 5% [+ 5% in PBSC] **OMI:** No change [N/c in PBSC]

Abbreviations: bid, twice daily; *C*, controlled; *d*, days; *HD*, high dose; *LD*, low dose; *LVG*, left ventriculography; *m*, months; *n*, number; *NC*, noncontrolled; *n/c*, no change; *NR*, nonrandomized; *OMI*, old myocardial infarction; *R*, randomized.

Figure 20. Etudes menées avec l'association G-CSF + Cellules dans l'infarctus. (D'après George 2010)

Si l'on s'intéresse à présent aux études réalisées avec des MSC isolées, Hare (Hare, Traverse et al. 2009) a retrouvé des résultats encourageants chez l'homme (60 patients) après infarctus en perfusant des MSC humaines par voie veineuse lors d'un essai randomisé en double aveugle contre placébo. Dans une autre étude randomisée, Chen (Chen, Fang et al. 2004) a perfusé au 18eme jour après infarctus, des MSC par voie intracoronaire et a constaté, une amélioration de la fraction d'éjection ventriculaire gauche.

Sur le long terme, peu de données sont disponibles et elles sont globalement décevantes. Seule l'étude BOOST proposait jusque là des résultats à 18 mois avec disparition de la significativité de la différence sur la fraction d'éjection. Cependant, très récemment, les résultats à 2 ans de REPAIR-MI ont été publiés (Assmus, Rolf et al. 2010). Sur les 204 patients randomisés après survenue d'un infarctus revascularisé avec succès mais fraction d'éjection inférieure à 45% (103 placébo et 101 BMC), 3 seulement ont été perdus de vue. Il n'y a pas de différence entre les deux groupes en termes de survenue d'arythmie ventriculaire, de syncope, d'accident vasculaire cérébral (AVC), ou de cancers. Par contre sur un total de 11 décès, 8 ont concerné des patients du groupe contrôle, et le critère combiné associant décès et récurrence d'infarctus apparaît significativement amélioré par la thérapie cellulaire. Chez quelques patients, une étude en imagerie par résonnance magnétique (IRM) de la FEVG a seulement permis de retrouver une tendance positive pour la thérapie (+6,5%) (Figure 21). Il s'agit là, pour l'heure, de la seule étude permettant d'envisager un effet bénéfique minime à long terme de cette thérapie chez l'homme.

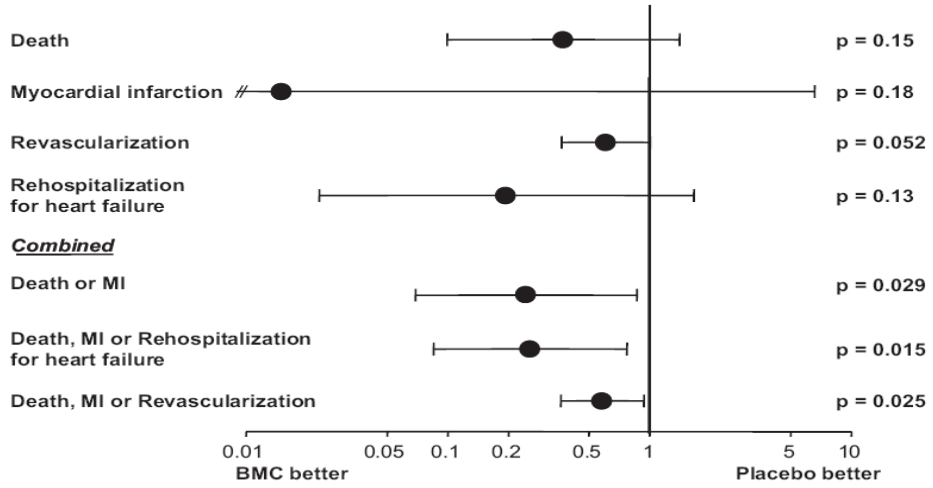

Figure 21. Résultats à 2 ans de l'étude REPAIR-MI. (D'après Assmus 2010).

(b) Dans la myocardiopathie dilatée chronique

Dans la myocardiopathie dilatée d'origine ischémique, la voie intracoronaire apparaît moins séduisante. Les études pilote de Fuchs, Perin ou Tse ont testé, sur quelques dizaines de patients porteurs d'une myocardiopathie ischémique chronique, la faisabilité de la voie endocardique avec des BMC autologues (Fuchs, Satler et al. 2003; Perin, Dohmann et al. 2003; Tse, Kwong et al. 2003). Par contre, c'est la voie directe, lors de la réalisation de pontages aorto-coronariens qui est utilisée par Menashe pour incorporer des myoblastes chez des patients également atteints d'une cardiomyopathie dilatée d'origine ischémique et nécessitant un pontage (Menasche, Hagege et al. 2003). En 2006, Beeres rapporte des effets modestes mais bénéfiques sur la FEVG de BMC injectées par voie endocardique, sur des patients atteints de myocardiopathie dilatée chronique (Beeres, Bax et al. 2006), résultats confirmés récemment par un essai randomisé contre placébo réalisé par la même équipe (van Ramshorst, Bax et al. 2009). Cependant, il n'y a que dans le registre TOPCARE-CHD (Assmus, Fischer-Rasokat et al. 2007), qu'une différence statistiquement significative en terme de mortalité, a pu être mise en évidence dans le groupe de patients recevant les plus

fortes quantités de cellules. Un tableau résumant ces premiers essais a été publié en 2008 dans une revue de Richard Burt (Burt, Loh et al. 2008) (Figure 22).

Table 4. Clinical Trials of Stem Cell Therapy for Chronic Myocardial Ischemia and/or Heart Failure With ≥20 Patients

Source	Trial Type/Name	No. of Patients	Follow-up, mo	Stem Cell Route	Stem Cell Source	LVEF Outcome; Comment
Assmus et al,[87] 2007	TOPCARE-CHD	121	19	Intracoronary	Bone marrow	Improved mortality in high-order CFUs injected
Losordo et al,[97] 2007	Randomized	24	12	Intramyocardial	CD34	Not examined
Manginas et al,[96] 2007	Unblinded	24	28	Intracoronary	CD133, CD34	Improved LVEF and left ventricular volumes
Stamm et al,[99] 2007	Unblinded	40	6	Intramyocardial	CD133	Improved LVEF
Assmus et al,[86] 2006	TOPCARE-CHD Randomized	75	3	Intracoronary	Bone marrow/ CPCs	Improved with bone marrow
Beeres et al,[93] 2006	Single group	25	12	Intramyocardial	Bone marrow	Improved LVEF, CCS angina score, perfusion
Chen et al,[95] 2006	Unblinded	45	12	Intracoronary	Mesenchymal	Improved ischemia, NYHA class, and LVEF
Fuchs et al,[92] 2006	Single group	27	12	Intramyocardial	CD34	Improved CCS angina score
Gao et al,[94] 2006	Unblinded	28	3	Intracoronary	Bone marrow	Improved LVEF, improvement in CHF
Hendrikx et al,[91] 2006	Randomized	20	4	Intramyocardial	Bone marrow	NS
Mocini et al,[98] 2006	CABG + cells or CABG alone	36	12	Intramyocardial	Bone marrow	Improved LVEF and wall motion
Erbs et al,[84] 2005	Randomized	26	3	Intracoronary	CPCs	Improved
Patel et al,[90] 2005	Randomized	20	6	Intramyocardial	CD34	Improved
Strauer et al,[85] 2005	IACT[a]	36	3	Intracoronary	Bone marrow	Improved
Perin et al,[88] 2004	Sequential enrollment; treatment or control	20	12	Intramyocardial	Bone marrow	NS
Perin et al,[89] 2003	Single group	21	4	Intramyocardial	Bone marrow	Improved

Abbreviations: CABG, coronary artery bypass graft; CCS, Canadian Cardiovascular Society; CFU, colony-forming unit; CHF, congestive heart failure; CPC, circulating progenitor cell; IACT, Intracoronary Autologous Bone Marrow Cell Transplantation in Chronic Coronary Artery Disease; LVEF, left ventricular ejection fraction; NS, not significant; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association; TOPCARE-CHD, Transplantation of Progenitor Cells and Recovery of Left Ventricular Function in Patients With Chronic Ischemic Heart Disease.

[a]Control group refused treatment with bone marrow-derived cells.

Figure 22. Etudes cliniques dans la cardiomyopathie dilatée. (D'après Burt 2008).

(c) Etudes cliniques à visée cardiaque : limites et perspectives

Cependant, bien que prometteuses, les résultats de ces études de petites tailles ne nous permettent pas d'entrevoir prochainement l'émergence d'une thérapie cellulaire en pratique médicale courante. A ce jour, environ 2000 patients seulement sur la planète ont bénéficié d'un traitement cardiaque par injection de cellules souches. Nous devrons donc attendre de plus larges essais pour connaître le rôle réel de ces nouvelles thérapeutiques. Plusieurs raisons peuvent être avancées pour expliquer ces résultats disparates et globalement décevants, mais surtout des réponses à diverses questions doivent maintenant être amenées (Lasala and Minguell 2009; Lemarchand 2009; Chavakis, Koyanagi et al. 2010; Kumar and Caplice 2010): quelles sont les meilleures cellules, comment faut-il les isoler et les

préparer à l'injection, comment doit-on préparer le tissu receveur, quelle quantité doit être délivrée, avec quelle technique et à quel moment ? Doit-on renouveler les injections ? Pour être interprétables, les études à venir devront être réalisées en double aveugle avec un groupe contrôle sous placébo et les méthodes de suivi et d'imagerie devront être validées.

Les premières études réalisées chez l'homme ont surtout permis de démontrer la faisabilité dans de bonnes conditions de sécurité de la transplantation de cellules souches. Il semble notamment que les troubles du rythme graves ne soient pas plus à craindre, sauf peut-être avec les myoblastes (Fernandes, Amirault et al. 2006; Ly and Nattel 2009).

Actuellement, un certain nombre d'études sont en cours et pourraient apporter quelques réponses. Sur le site clinicaltrial.gov (http://www.clinicaltrial.gov) on ne répertorie pas moins d'une centaine d'études dont 3 concernent des équipes qui recrutent des patients en France (Lemarchand 2010; Lipiecki 2010; Roncalli 2010). Par ailleurs, on peut noter les études de phase II TRACIA (Pena-Duque 2010) et REGENERATE-AMI (Marthur 2010). L'essai SWISS-AMI quant à lui va permettre de comparer l'injection intracoronaire précoce (5 à 7 jour) de l'injection plus tardive (3 à 4 semaines) (Corti 2010; Surder, Schwitter et al. 2010). Pour ce qui est des MSC, de premières réponses pourrait venir de l'étude PROMETHEUS (Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery), actuellement en cours. Il s'agit ici d'injecter directement dans le muscle cardiaque, durant une chirurgie de pontages aorto-coronariens, des MSC autologues, chez des patients souffrant de myocardiopathie dilatée d'origine ischémique (Hare 2010). A noter l'apparition d'études utilisant des cellules allogéniques comme l'étude pilote POSEIDON (Gerstenblith 2010), qui a pour but de vérifier l'innocuité de l'injection de MSC allogéniques en comparaison avec des MSC autologues. Remarquons, à ce sujet, l'arrivée de compagnies privées proposant des cellules standardisées. C'est ainsi qu'une étude propose l'utilisation de MSC allogéniques dans l'infarctus du myocarde (Perin 2010), sponsorisée par une compagnie privée détentrice des cellules MSC standardisées Revascor®, Angioblast Systems Inc. (http://www.angioblast.com). De telles cellules sont aussi par exemple proposées par Osiris Therapeutics Inc. (http://www.osiris.com) sous le nom de Provacel® (Hare 2010) et des myoblastes sont produits par Bioheart Inc. (http:www.//bioheartinc.com) appelés MyoCell® (BioheartInc 2010). Enfin, deux protocoles ALCADIA et CADUCEUS, vérifieront la faisabilité d'utiliser des cellules souches autologues d'origine cardiaque (Marban 2010; Matsubara 2010). Une liste de ces études en cours est proposée en annexe page 156.

2) Les études cliniques dans l'artériopathie oblitérante des membres inférieurs. Limites et perspectives.

La plupart des études consistant à injecter des cellules souches dans les muscles des membres inférieurs ischémiques ont utilisé la voie directe intramusculaire. Cependant la voie artérielle a également été testée (Lawall, Bramlage et al. 2010).

En 2002, Tateishi-Yuyama publie l'étude TACT, un premier travail mené chez une cinquantaine de patients et montrant l'intérêt possible de l'injection de cellules autologues de moelle osseuse dans les muscles ischémiques de membres inférieurs d'artériopathes (Tateishi-Yuyama, Matsubara et al. 2002). Puis vint l'étude TAM-PAD (Transplant of Autlogous Mononuclear Bone Marrow Cells in Peripheral arterial Disease) où les deux voies d'administration ont été utilisées (Bartsch, Brehm et al. 2007). De nombreux essais suivront mais malheureusement souvent sans groupe contrôle et sur de petits effectifs. Les résultats restent donc d'interprétation difficile, mais dans tous les cas, l'amélioration semble transitoire et ne concerne au mieux que deux tiers des patients (Tableau I).

A noter la particularité de l'étude pilote BONMOT-1, récemment publiée, (Amann, Luedemann et al. 2009) où le principe a été d'injecter les cellules, des BMC autologues, le long de l'artère occluse et non pas dans le muscle ischémique pour faciliter l'apparition des collatérales dans cette zone. Les injections, profondes et espacées de 1 cm étaient réalisées en commençant 5 cm au dessus de l'occlusion artérielle. Cette théorie est en cours de vérification dans BONMOT-CLI, étude en double aveugle contre placébo (Amann 2010).

Tableau I : Premières études humaines de thérapie cellulaire concernant l'artériopathie oblitérante des membres inférieurs.

Etudes	Nbre de Patients	Groupe Contrôle	type cellulaire	Voie d'admin	bénéfice
Tateishi, 2002	45	Oui	BMC	IM	Oui
Esato, 2002	8	Non	BMC	IM	NS
Higashi, 2004	7	Non	BMC	IM	NS
Miyamoto, 2004	12	Non	BMC	IM	+ / -
Saigawa, 2004	8	Non	BMC	IM	NS
Huang, 2005	28	Oui	G-CSF-m PBMC	IM	Oui
Lenk, 2005	7	Non	G-CSF-m PBMC	IA	NS
Tateno, 2006	29	Non	G-CSF-m PBMC	IM	+ / -
Bartsch, 2006	8	Non	BMC	IA+IM	NS
Durdu, 2006	26	Non	BMC	IM	+ / -
Miyamoto, 2006	8	Non	BMC	IM	NS
Kawamura, 2006	92	Non	G-CSF-m PBMC	IM	NS
Bartsch, 2007	25	Oui	BMC	IA+IM	Oui
Kajiguchi, 2007	7	Non	BMC	IM	NS
Saito, 2007	14	Non	BMC	IM	+ / -
Huang, 2007	74	Non	BMC	IM	+ / -
Huang, 2007	76	Non	G-CSF-m PBMC	IM	NS
Hernandez, 2007	12	Non	BMC	IM	NS
Gu, 2008	32	Non	BMC	IM vs IA	NS
Chochola, 2008	24	Non	BMC	IA	+ / -
Wester, 2008	8	Non	BMC	IM	NS
Van Tongeren, 2008	27	Non	BMC	IA+IM vs IM	NS
De Vriese, 2008	16	Non	BMC	IM	+ / -
Amann, 2009	51	Non	BMC	IM	+ / -

(Esato, Hamano et al. 2002; Tateishi-Yuyama, Matsubara et al. 2002; Higashi, Kimura et al. 2004; Miyamoto, Yasutake et al. 2004; Saigawa, Kato et al. 2004; Huang, Li et al. 2005; Lenk, Adams et al. 2005; Bartsch, Brehm et al. 2006; Durdu, Akar et al. 2006; Kawamura, Horie et al. 2006; Miyamoto, Nishigami et al. 2006; Tateno, Minamino et al. 2006; Bartsch, Brehm et al. 2007; Hernandez, Cortina et al. 2007; Huang, Yang et al. 2007; Kajiguchi, Kondo et al. 2007; Saito, Sasaki et al. 2007; Chochola, Pytlik et al. 2008; De Vriese, Billiet et al. 2008; Gu, Zhang et al. 2008; Van Tongeren, Hamming et al. 2008; Wester, Jorgensen et al. 2008; Amann, Luedemann et al. 2009).

Pour ce qui est du long terme, les données sont rares. Les résultats du suivi à 3 ans de la population de TACT ont été publiés en 2008 (Matoba, Tatsumi et al. 2008). Cependant, même si l'évolution semble favorable au moins durant les deux premières années, l'absence de groupe contrôle rend l'interprétation délicate.

Parmi les études à venir, on répertorie une quarantaine d'études actuellement en cours sur le site clinicaltrial.gov, dont une est menée par une équipe française (Pignon 2010). Outre BONMOT-CLI cité plus haut, on retiendra également JUVENTAS (Sprengers 2010; Sprengers, Moll et al. 2010) où les auteurs répèteront 3 fois à 3 semaines d'intervalle, les injections intra-artérielles de BMC. Un total de 150 patients est espéré avec un groupe contrôle et un suivi de 6 mois est prévu. Enfin, une étude chinoise projette d'utiliser des cellules souches mésenchymateuses autologues (Chen 2010). Une liste de ces études en cours est proposée en annexe page 182.

B) VOIES D'AMELIORATION DES THERAPIES CELLULAIRES

1) Généralités

Comme on peut le constater, que ce soit pour le cœur ou l'ischémie des membres inférieurs, les résultats obtenus chez l'animal ne sont pas vraiment reproduits lorsqu'il s'agit de s'adresser à des patients. Des questions fondamentales sont pour l'instant sans réponse. Le site idéal, le mode d'injection et l'intérêt de répéter les greffes sont en cours d'évaluation. Plusieurs autres voies d'amélioration sont envisageables : quel est le meilleur choix de cellules ou d'association de cellules, les modes d'injection actuels sont-ils adaptés à cette thérapie, les cellules ne doivent-elles pas absolument être préparées *ex vivo* avant leur transfert ? En effet, il est maintenant communément admis que 70 à 95% des cellules, cultivées à 20% d'oxygène, disparaissent dans les 48 premières heures après leur implantation dans le tissu pathologique (Davis, Schroeder et al. 2007). Un effort sur la survie des cellules en situation d'hypoxie paraît donc être une priorité pour envisager une optimisation de ce type de thérapie. A noter que, pour ce qui est de l'infarctus, nous n'avons pas retrouvé d'étude chez le petit animal, faisant état d'une thérapie cellulaire sur un modèle d'infarctus reperfusé. Ce modèle aurait l'intérêt de mimer au plus près la réalité de la pratique courante chez l'homme et permettrait de tester l'hypothèse d'une meilleure survie des cellules en situation d'infarctus reperfusé par rapport à un infractus non reperfusé.

2) Choix de cellules

Du fait d'un accès relativement aisé, ce sont les cellules autologues de la moelle osseuse qui sont le plus souvent utilisées. Le fait d'utiliser des cellules autologues permet certes de s'amender des problèmes de rejet des cellules mais oblige à utiliser les cellules d'un sujet, par définition, malade. Or, on sait maintenant que les cellules souches sont d'autant plus compétentes qu'elles sont issues d'un organisme jeune sans maladie ni facteurs de risque cardiovasculaires (Silvestre 2008; Dimmeler 2010). Par ailleurs, les

contraintes de temps, empêchent en général de sélectionner et faire proliférer les cellules souches qui ont un réel pouvoir thérapeutique. Hormis pour les pathologies chroniques, les cliniciens en sont donc réduits à utiliser un ensemble hétérogène de cellules dont seulement certaines sont réellement efficaces pour le but recherché. Enfin, les ponctions réitérées de moelle osseuse étant irréalisables, cette technique ne permet pas d'envisager des injections répétées. Or, et c'est là une des questions fondamentales à laquelle quelques auteurs tentent de répondre, il y a fort à parier, que, comme tout traitement de fond, celui de la pathologie ischémique par des cellules souches, doit être, sinon continu, au moins répété dans le temps. Seul un transfert allogénique à partir d'une banque de cellules souches permettrait à la fois de garantir la sélection et la qualité des cellules, de standardiser leur nombre, de les prétraiter *ex vivo* avant implantation, pour en accentuer la survie et les effets après transfert, et de permettre de répéter les injections. Les cellules seraient disponibles en permanence (« off-the-shelf ») et pourraient bénéficier aux patients même en cas d'urgence. Les inconvénients majeurs sont représentés par le coût et les risques inhérents à la culture cellulaires (infectieux, génétiques, organisationnels, règlementaires).

Du fait de leur effet immuno-modulateur et de leur potentielle efficacité thérapeutique, seules les cellules souches mésenchymateuses permettent actuellement d'envisager une telle banque de cellules.

3) Mode de délivrance des cellules souches

L'efficacité d'une thérapie cellulaire à but pro-angiogénique dépend également en grande partie de la capacité des cellules introduites dans l'organisme ou l'organe défaillant de se localiser de manière adéquate pour optimiser les effets bénéfiques et limiter les risques d'effets néfastes comme le développement de tumeur par exemple. Les modes de délivrance des cellules sont la voie vasculaire, veineuse ou artérielle, et la voie tissulaire directe. La voie veineuse présente le handicap de projeter les cellules dans le filtre vasculaire pulmonaire alors que la voie artérielle nécessite un abord plus invasif. Pour ce qui est des coronaires, les essais cliniques ont souvent été réalisés par cathétérisme coronaire sélectif

puis utilisation d'un ballon « over-the-wire » permettant d'injecter les cellules en aval d'un ballon gonflé, diminuant ainsi le lavage durant la transplantation. La voie tissulaire présente, quant à elle, l'avantage de localiser les cellules dans des zones qui sont justement peu ou pas vascularisées, mais en contrepartie, les expose à un transfert brutal dans un environnement pauvre en oxygène ce qui ne favorise pas leur survie (Smart and Riley 2008). Des injections itératives pourraient alors être envisagées, mais ce qui est possible pour les membres inférieurs, ne l'est pas pour le cœur, dont l'accès direct est plus complexe, soit chirurgical soit transendocardique grâce à un abord percutané mais avec nécessité de techniques spécifiques (Perin, Silva et al. 2002; Perin, Dohmann et al. 2003; Barbash, Leor et al. 2004; Gavira, Perez-Ilzarbe et al. 2006) (Figure 23).

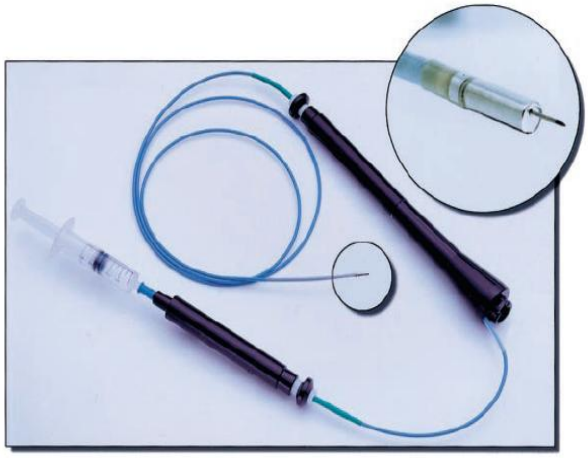

Figure 23. Système d'injection Biosense (D'après Perin 2003)

Il faut noter que quelques craintes étaient apparues avec la voie intracoronaire ; des phénomènes d'obstruction vasculaire ayant pour conséquence l'apparition de no-reflow ou de micro-infarctus avaient été constatés chez l'animal (Vulliet, Greeley et al. 2004). Cependant, la tolérance semble excellente chez l'homme.

Ces questions ont donné lieu à plusieurs travaux comme ceux de George (George, Goldberg et al. 2008) ou de Thompson (Thompson, Nasseri et al. 2003) qui injectent de façon intra-myocardique les cellules en passant par le sinus veineux coronaire (Figure 24).

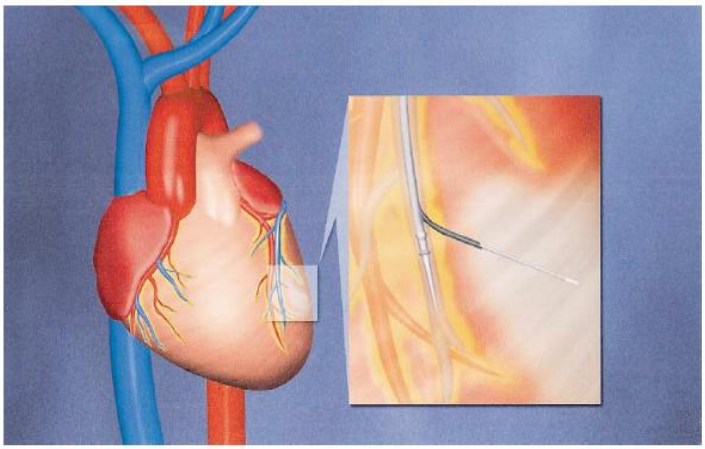

Figure 24. Système d'injection intramyocardique via le système veineux cardiaque (D'après Thompson 2003)

Kaye, quant à lui, (Kaye, Preovolos et al. 2007) propose, après transfert des cellules dans le réseau coronaire, un système de capture, oxygénation et recirculation du sang issu du sinus veineux coronaire (Figure 25).

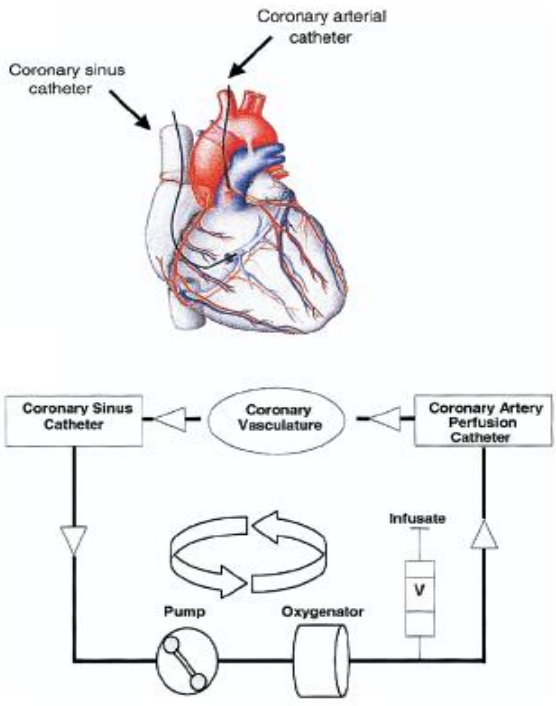

Figure 25. Système de capture, oxygénation et recirculation du sang issu du sinus veineux coronaire (D'après Kaye 2007)

Dans l'étude MYSTAR, les auteurs ont testé avec succès la combinaison et de la voie intracoronaire et intra myocardique percutanée guidée par le système NOGA (Gyongyosi, Lang et al. 2009; Charwat, Lang et al. 2010). D'autres approches sont en cours d'évaluation comme l'utilisation de l'IRM (Dick, Guttman et al. 2003; Corti, Badimon et al. 2005) ou d'un système de guidance par champ magnétique (Stéréotaxis®) (Perin, Silva et al. 2007) notamment.

Cependant aucun de ces modes de délivrance ne propose de système de réservoir de cellules, lieu où elles seraient maintenues vivantes et à partir duquel elles pourraient alors migrer progressivement vers le tissu ischémique. C'est pour cette raison que le laboratoire a proposé l'utilisation d'un « patch » musculaire contenant des cellules souches et appliqué sur la zone du myocarde préalablement infarci (Barandon, Couffinhal et al. 2004). Cette technique, alliant thérapie cellulaire et tissulaire, a été mise au point chez la souris, en utilisant des BMC. Des souris C57/Bl6 étaient réopérées 30 jours après la réalisation d'un infarctus par cryolésion et un « patch » de muscle abdominal était cousu sur la zone infarci après avoir été recouvert de $5X10^6$ BMC provenant de souris ROSA26 exprimant de façon constitutive la β-galactosidase (Figure 26).

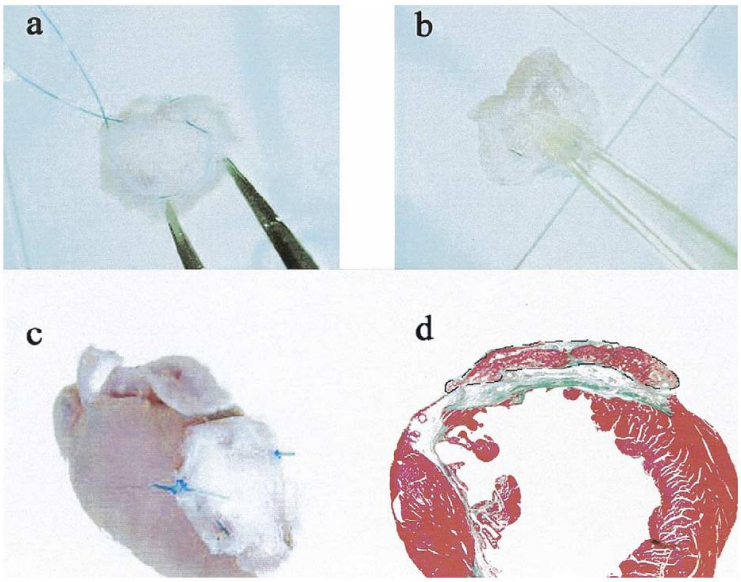

Figure 26. a: Confection du patch musculaire; b: mise en place des cellules; c: patch cousu sur le coeur de la souris en regard de la zone infarcie; d: coupe histologique transversale du ventricule gauche avec un "patch".

Il a été montré dans ce système que 2 semaines plus tard, 8% des cellules avaient migré vers la zone infarcie. Dans le groupe de souris traitées, le remodelage cardiaque, la fonction ventriculaire et la densité capillaire étaient augmentés par rapport au groupe contrôle. J'ai donc alors participé à un travail visant à étudier le devenir de MSC ou d'EPC ainsi transférés et à déterminer si la présence surajoutée de BMC pouvait améliorer les résultats. Ce travail est reporté dans la partie « résultats », page 103 du présent document.

4) Modifications *ex vivo* des cellules souches avant implantation

La préparation des cellules avant implantation est également une voie de recherche très active dans le but d'améliorer les capacités thérapeutiques des cellules. Il peut s'agir de faire exprimer par les cellules, par transfection, différents facteurs, ou bien de les prétraiter avec divers stimuli : exposition à des facteurs de croissance (Pasha, Wang et al. 2008), à la mélatonine (Mias, Trouche et al. 2008), des agents chimiques (Kubo, Li et al. 2007), des médicaments (Wisel, Khan et al. 2009) ou des conditions d'hypoxie (Li, Hamano et al. 2002; Hu, Yu et al. 2008; Kubo, Li et al. 2008; Rosova, Dao et al. 2008; Wang, Chen et al. 2008; Wang, He et al. 2009). Ce dernier mode de prétraitement, dit préconditionnement hypoxique, fait l'objet d'un développement spécifique à partir de la page 78 du présent document.

Un des premiers travaux intéressant a été celui de Mangi qui a montré que des MSC qui sur-exprimaient la protéine Akt étaient plus résistantes à l'apoptose que les MSC contrôles (Mangi, Noiseux et al. 2003; Gnecchi, He et al. 2005). Cette donnée a récemment été vérifiée sur un modèle d'infarctus porcin (Yu, Shen et al. 2010). D'autres facteurs tels que SDF-1, BCl-2, IGF-1 ou VEGF ont été surexprimés dans des MSC et ont montré leur efficacité angiogénique ou tissulaire (Matsumoto, Omura et al. 2005; Kutschka, Kofidis et al. 2006; Li, Ma et al. 2007; Zhang, Mal et al. 2007; Haider, Jiang et al. 2008)

D'autres études ont utilisé des EPC modifiées pour surexprimer le VEGF (Iwaguro, Yamaguchi et al. 2002) ou inhiber la Glycogene synthase kinase 3β (GSK-3β) (Choi, Hur et al. 2004).

5) Autres voies d'amélioration de l'efficacité thérapeutique

D'autres voies semblent intéressantes dans le but d'améliorer l'efficacité de la thérapie cellulaire.

En effet, certaines molécules habituellement utilisées en cardiologie semblent potentialiser l'effet de la thérapie cellulaire. C'est le cas des statines (Assmus, Urbich et al. 2003) ou des inhibiteurs de l'enzyme de conversion (IEC) (Silvestre, Bergaya et al. 2001; Min, Zhu et al. 2004). Par ailleurs, outre le G-CSF, d'autres molécules pourraient s'avérer intéressantes pour mobiliser les cellules souches de la moelle. C'est le cas de l'érythropoïétine (EPO) (Westenbrink, Lipsic et al. 2007) ou de la Parathormorne (PTH) (Zaruba, Huber et al. 2008)

La co-administration de cellules avec un cocktail contenant des facteurs favorisant la survie a récemment été utilisée avec un certain succès (Laflamme, Chen et al. 2007).

Le tissu receveur peut également être le lieu d'une action facilitatrice préalable à l'implantation des cellules souches (Dimmeler and Leri 2008). C'est ainsi que l'intérêt de préparer le tissu hôte avec par exemple du SDF-1 (Segers, Tokunou et al. 2007) ou de l'IGF-1 (Davis, Hsieh et al. 2006), trappés dans des nanofibres, peut s'avérer intéressant. Enfin, de manière plus étonnante, l'application d'ondes de choc à basse énergie stimulerait le tissu hôte ce qui faciliterait le homing des cellules souches (Aicher, Heeschen et al. 2006).

C) IMPLICATION DU SYSTEME WNT/FZD

1) Présentation du système Wnt/Fzd

(a) Généralités

Le système Wnt/Frizzled (Wnt/Fzd) est un système hautement conservé dans l'évolution, consistant en une voie de signalisation intracellulaire mettant en jeu des récepteurs à 7 domaines transmembranaires Frizzled (Fzd) et leurs ligands Wnt, petites glycoprotéines sécrétées. Ce système contrôle divers processus physiologiques comme le renouvellement des cellules souches (Nusse 2008), l'adhésion cellulaire (Lilien and Balsamo 2005), la différenciation, la polarité, la prolifération cellulaire et le développement tissulaire (Peifer and Polakis 2000; Eisenberg and Eisenberg 2006). Mais il est également impliqué en pathologie, notamment dans le développement de cancers (Reya and Clevers 2005), l'ostéoporose ou les maladies cardiovasculaires. La synthèse de l'ensemble des connaissances sur ce système est accessible sur un site spécifiquement créé et mis à jour par l'équipe de R. Nusse (Stanford University) : « The Wnt Homepage » dont l'adresse est : http://www.stanford.edu/~rnusse/wntwindow.html.

Le système Wnt/Fzd murin comprend 19 membres de la famille des Wnt et 10 membres de la famille Frizzled (Fzd). Les récepteurs Fzd ont une partie N-terminale extracellulaire comportant un domaine riche en cystéines (ou CRD pour Cystein Rich Domain) suivie d'une région hydrophile (Schulte and Bryja 2007) (Figure 27).

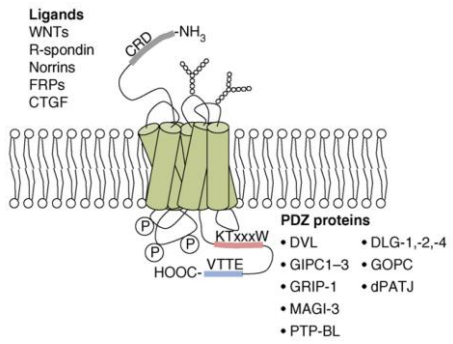

Figure 27. Proteine Fzd. (D'après Schulte 2007)

Ce domaine CRD permet la liaison aux Wnt mais aussi à des protéines d'autres familles comme celle des sFRP (soluble Frizzled related proteins) (Bafico, Gazit et al. 1999; Rodriguez, Esteve et al. 2005), des R-spondines (Nam, Turcotte et al. 2006), ou des ligands Norrins (Smallwood, Williams et al. 2007). Enfin, les récepteurs Fzd peuvent se dimériser entre eux via leurs domaines CRD (Dann, Hsieh et al. 2001). En C-terminal des récepteurs Fzd se trouve un domaine de liaison intracellulaire PDZ permettant l'interaction avec d'autres protéines à domaine PDZ comme la protéine centrale Dishevelled (Dsh ou Dvl) (Chen, ten Berge et al. 2003; Wong, Bourdelas et al. 2003). Les récepteurs Fzd sont localisés à la surface des cellules mais peuvent aussi être internalisés (Dubois, Lecourtois et al. 2001; Chen, ten Berge et al. 2003). Cette localisation, parfois asymétrique des Fzd est à la base de la polarité cellulaire chez la drosophile via la voie non canonique de la polarité planaire ou PCP (Planar Cell Polarity) (Strutt 2001; Montcouquiol, Crenshaw et al. 2006). Dans les tissus, l'expression des Fzd est large et dynamique selon les stimuli. On distingue, en effet, plusieurs voies de signalisation activables dans ce système (Rao and Kuhl 2010), habituellement dénommées voie canonique et voies non canoniques. La voie canonique est dépendante de la β-caténine. Les voies non canoniques sont la voie de la polarité planaire (PCP) et la voie calcium dépendante (Wnt/Ca^{2+}) (Ling, Nurcombe et al. 2009) (Figure 28).

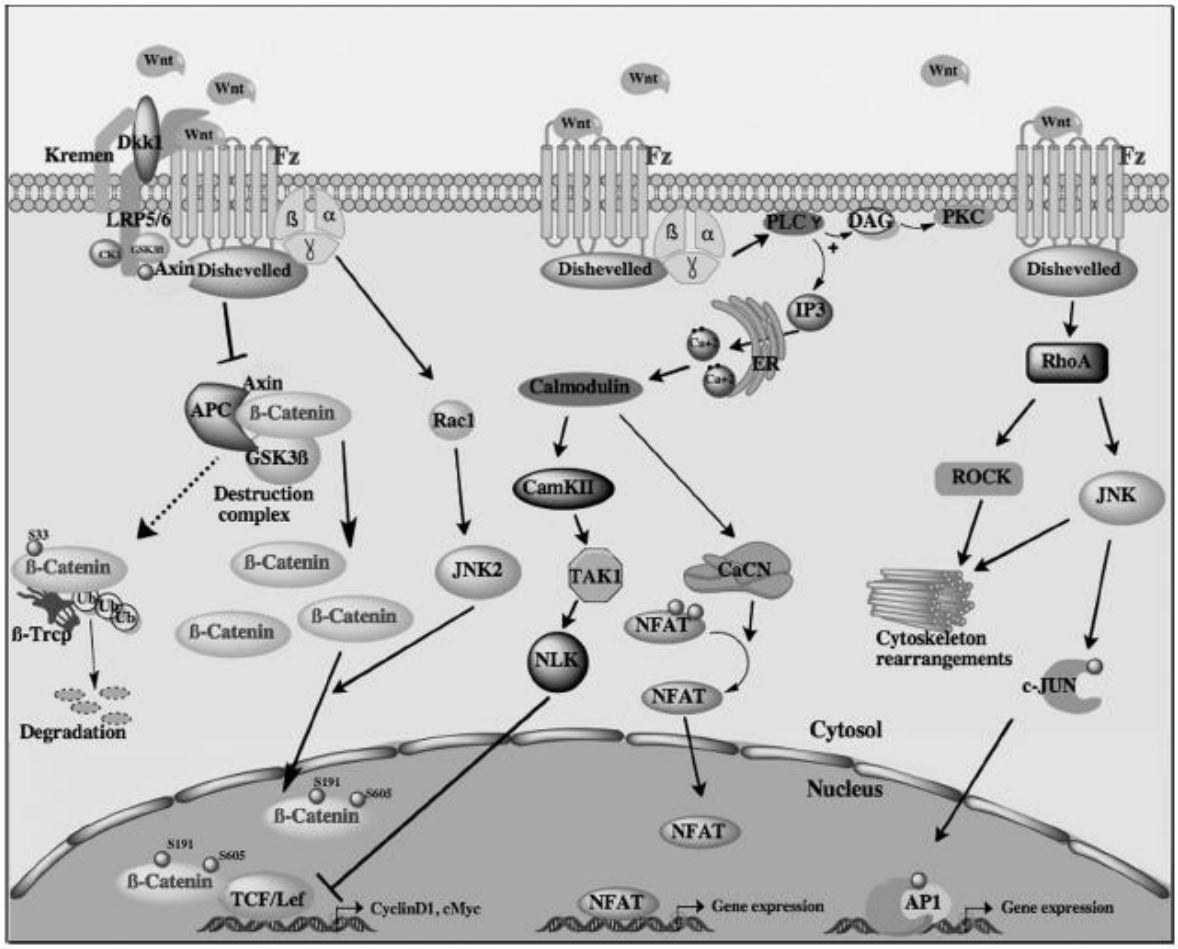

Figure 28. Les voies de signalisation Wnt/Fzd. De gauche à droite: la voie Wnt/β-caténine, la voie Wnt/Ca^{2+} et la voie de la PCP. (D'après Rao 2010)

Basé sur des essais biologiques, les facteurs Wnt ont été subdivisés en deux groupes : le groupe 1 comprenant Wnt 1, 3, 3a, 7a activerait la voie canonique alors que le groupe 2 comprenant Wnt 2, 4, 5a, 5b, 6, 7b, 11 activerait les voies non canoniques. Mais plusieurs membres du système Wnt/Fzd possèdent un profil d'expression spatio-temporel et pourraient interagir de façon spécifique, permettant une régulation fine de la réponse initiée par l'activation du système (Rao and Kuhl 2010). L'activation du récepteur Fzd par un ligand Wnt ou une dimérisation recrute et active la protéine intracellulaire Dvl (Boutros, Mihaly et al. 2000; Veeman, Axelrod et al. 2003), protéine cytoplasmique essentielle puisque participant à l'activation des 3 voies de signalisation. Des co-récepteurs transmembranaires comme le LRP5/6 (Lipoprotein receptor-related protein 5/6) peuvent également jouer un

rôle important dans l'activation de la voie canonique par les Wnt (Tamai, Semenov et al. 2000; Cadigan and Liu 2006). La protéine Dvl (Gao and Chen 2009), plaque tournante du système, va alors activer un ensemble de protéines spécifiques qui lui sont associées permettant l'activation de telle ou telle voie. Chez les mammifères, il existe 3 homologues de Dvl (1, 2 et 3).

Outre son implication au sein de la voie Wnt/Fzd, il faut garder en mémoire que la β-caténine est une protéine multifonctionnelle puisqu'elle est retrouvée dans les jonctions adhérentes intercellulaires, fondamentales pour l'adhésion des cellules entre elles. Elle y interagit avec le cytosquelette via les Cadhérines et son rôle y est hautement régulé par des phénomènes de phosphorylation. Si bien qu'une relation entre la signalisation Wnt/Fzd et l'adhésion cellulaire médiée par les Cadhérines est maintenant suspectée (Heuberger and Birchmeier 2010) (Figure 29).

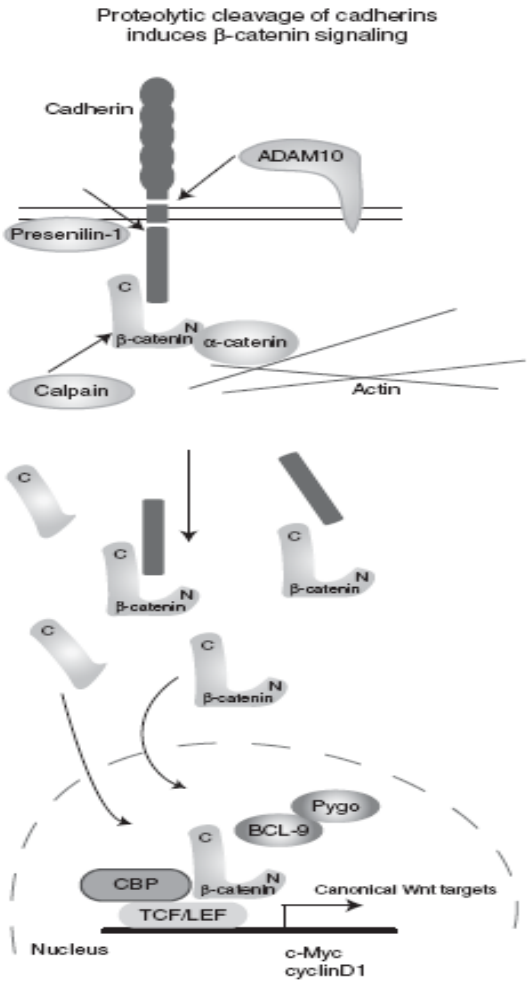

Figure 29. β-caténine: une protéine multifonctionnelle. (D'après Heuberger 2010).

(b) La voie canonique

En l'absence de ligand Wnt, la β-caténine cytosolique est phosphorylée par la GSK-3β (Glycogene synthase kinase 3β) ce qui permet sa dégradation par le protéasome. La GSK-3β est une sérine/thréonine protéine kinase qui agit au sein d'un complexe comprenant également l'Adenomatous Poliposis Coli (APC) et l'axine. Le niveau de β-caténine cytosolique et nucléaire reste donc bas dans cette situation. En présence d'une activation de la voie, la protéine Dvl est activée par phosphorylation ce qui a pour conséquence de recruter à la membrane et de dégrader l'axine, ainsi que d'inhiber la GSK-3β. Le complexe de la GSK-3β devient donc incapable de phosphoryler la β-caténine. Celle-ci, n'étant plus dégradée par le protéasome s'accumule dans le cytoplasme et peut être transloquée dans le noyau. Là, elle va interagir avec des facteurs de transcription LEF/TCF (Lymphoid Enhancer-binding Factor / T-Cell Factor) à qui elle sert de co-facteur, d'où l'activation de l'expression de gènes cibles (Nelson and Nusse 2004; Tolwinski and Wieschaus 2004; Gordon and Nusse 2006). Les gènes cibles de la voie canonique sont divers et spécifiques du contexte cellulaire (Logan and Nusse 2004; Vlad, Rohrs et al. 2008; MacDonald, Tamai et al. 2009) (Gordon and Nusse 2006) (Figure 30). Parmi ceux-ci, on note cyclin D1, c-myc, E-Cadherin, plasminogen activator uPA, OPG (osteoprotegerin) ou des métalloprotéases comme MMP7 et MMP26.

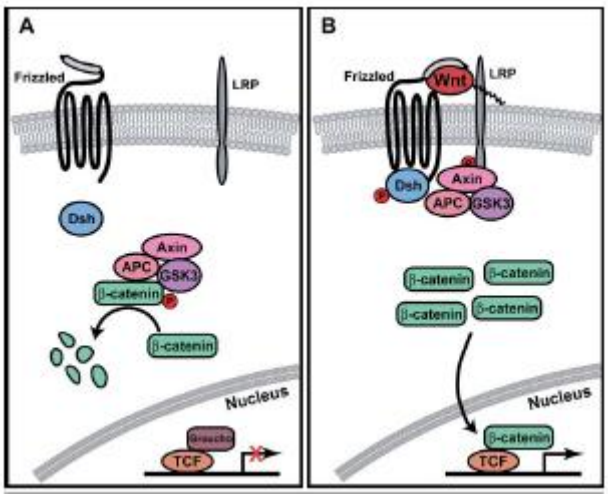

Figure 30. Voie canonique. (D'après Gordon 2006)

(c) Les voies dites non-canoniques

Ces voies, indépendantes de la β-caténine, sont la voie de la polarité planaire, ou voie PCP ou encore voie Wnt/JNK, et la voie calcium dépendante (Wnt/Ca^{2+}). Ces voies contrôleraient les mouvements cellulaires, ce que l'on englobe sous le terme de « convergence extension » (Wallingford, Fraser et al. 2002; Veeman, Axelrod et al. 2003).

La voie de la PCP met en jeu la régulation du cytosquelette d'actine pour une organisation polarisée des structures et une migration dirigée. Cette voie semble fonctionner indépendamment de la transcription. Ainsi, par le biais d'interactions spécifiques avec certains récepteurs Fzd et d'autres co-récepteurs, les membres de la classe Wnt5a sont susceptibles de médier ces effets par l'activation de la voie PCP (Moon, Campbell et al. 1993; Du, Purcell et al. 1995). L'implication de petites GTPases Rho, Rac, cdc42 par la protéine Dvl a également été démontrée au sein de cette voie (Wallingford and Habas 2005). L'activation de Rho conduit ainsi à l'activation de la Rho-associated kinase (ROCK) (Winter, Wang et al. 2001; Marlow, Topczewski et al. 2002) et de la myosine (Weiser, Pyati et al. 2007). L'activation de Rac induit plutôt l'activité de la kinase C-Jun (JNK) (Habas, Dawid et al. 2003), mais les facteurs en aval dans la signalisation non canonique restent encore peu connus. Dans les 2 cas, les éléments du cytosquelette, tel que l'actine et les microtubules sont modulés, amenant à un réarrangement du cytosquelette. Leur implication dans une régulation transcriptionnelle de gènes de la voie non canonique n'est pas encore établie (Habas, Dawid et al. 2003) (Figure 31).

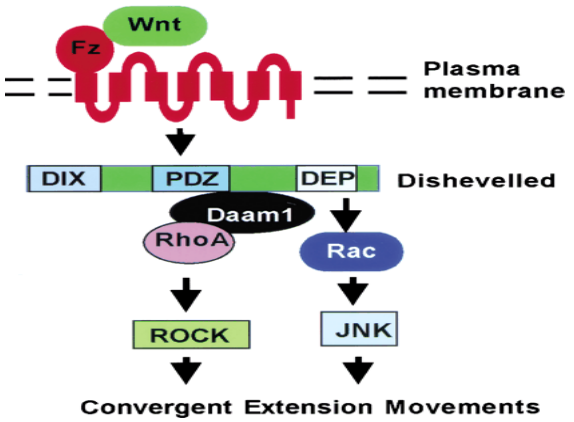

Figure 31. Voie PCP. (D'après Habas 2003)

Dans la voie non canonique Wnt/Ca^{2+} la signalisation Wnt/Fzd induit un relargage de Ca^{2+} intracellulaire à travers les petites protéines G hétéro-trimériques. L'activation de Dvl par ces protéines G induit d'une part, l'activation de l'inositol-4,5-biphosphate (IP3) par la phopholipase C (PLC) et d'autre part, l'inhibition de la protéine kinase G (PKG) par la phophodiesterase PDE. L'IP3 et la PKG inhibée créent un relargage de Ca^{2+} dans la cellule. En retour, ce calcium intracellulaire active 3 enzymes : la calcium/calmoduline kinase II (CaM/KII), la phosphatase calcineurine et la protéine kinase C (PKC) (Slusarski, Corces et al. 1997; Kuhl, Sheldahl et al. 2000; Sheldahl, Slusarski et al. 2003; Komiya and Habas 2008) (Figure 32). L'élévation du niveau de calcium aboutit également à l'accumulation du facteur de transcription NFAT (Nuclear Fcator of T Cells) dans le noyau d'où l'activation de la transcription de gènes cibles (van Es, Barker et al. 2003). Les conséquences de ces activations ont été étudiées au niveau de l'embryogénèse du xénope et il apparaît que cette voie module et la voie canonique et la voie de la PCP.

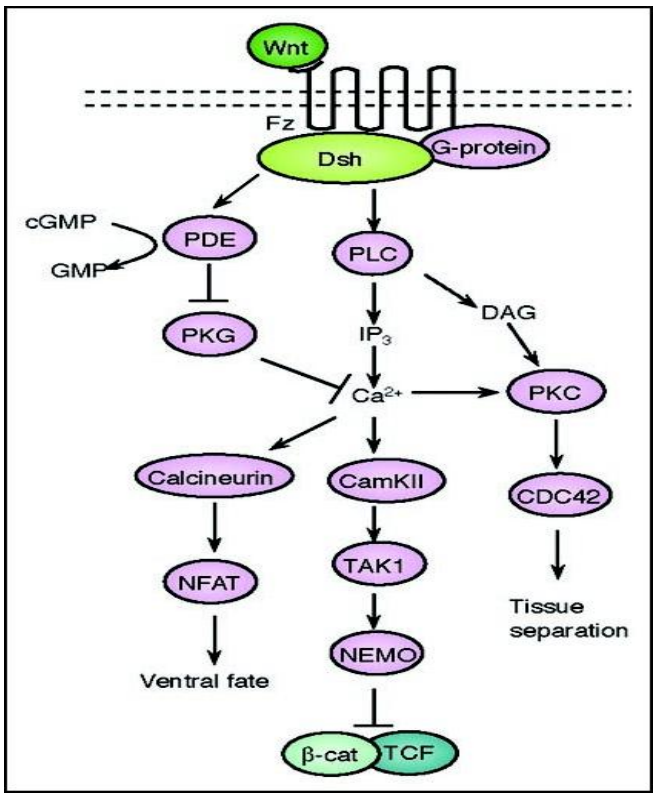

Figure 32. Voie Wnt/calcium. (D'après Komiya 2008)

(d) Les modulateurs extérieurs

Les voies de signalisation Wnt/Fzd sont contrôlées par un grand nombre de modulateurs extracellulaires qui se lient soit sur les Wnt directement, soit sur les récepteurs de Wnt et agissent en tant qu'agonistes ou antagonistes. Ces régulateurs sécrétés jouent des rôles majeurs dans l'embryogénèse et sont largement impliqués dans certains processus physiologiques et pathologiques (Jones and Jomary 2002; Kawano and Kypta 2003; MacDonald, Tamai et al. 2009).

(i) sFRP

Les sFRP sont de puissants régulateurs extracellulaires du système Wnt. Ils peuvent lier les facteurs Wnt et agir ainsi en tant que modulateurs de la signalisation Wnt (Rattner, Hsieh et al. 1997). Le génome humain comporte 5 gènes *sFRP* dont les orthologues ont été identifiés chez plusieurs vertébrés, et il en existe en tout 8 membres dans cette famille. La nomenclature actuelle s'accorde pour utiliser les termes sFRP1, 2, 3 (ou sFrzB), 4, 5. Les sFRP possèdent un domaine CRD (Cystein Rich Domain) et un domaine NTR (Netrin-like). Il apparaît que les sFRP se lient de façon spécifique à certains Wnt pour empêcher la liaison à leurs récepteurs. Cependant un mécanisme d'action alternatif serait la dimérisation des domaines CRD des Fzd et des sFRP empêchant la liaison des Wnt à leurs récepteurs (Bafico, Gazit et al. 1999; Dann, Hsieh et al. 2001) (Figure 33).

Mais d'autres études laissent supposer que les sFRP peuvent se lier avec des éléments d'autres voies comme RANKL, un membre de la famille des TNF, impliqué dans la formation des ostéoclastes. La régulation de l'expression des sFRP est encore incomplètement connue. Par exemple, il a été montré que le gène sFRP1 était un gène cible de voie Sonic hedgehog (Shh). En effet l'effecteur de cette voie Gli1 (glioblastoma 1) active l'expression de sFRP1 par liaison au promoteur de sFRP1 (He, Sheng et al. 2006). Pourtant dans des cellules mésenchymateuses, Ingram montre une répression de sFRP1 par activation de la voie Shh (Ingram, Wicking et al. 2002).

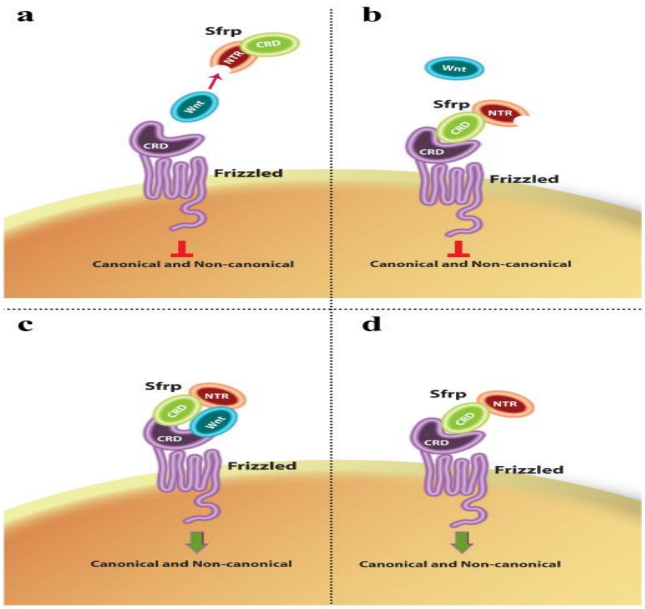

Figure 33. Modes d'action des sFRP. (Lopez-Rios 2008)

Durant ces dernières années, le laboratoire s'est plus particulièrement intéressé à sFRP1 ou FrzA et son rôle potentiel dans le développement des vaisseaux après un évènement ischémique. Durant le développement embryonnaire, les sFRP agissent comme modulateurs des évènements apoptotiques (Nusse 2005), mais plus tard, ils sont impliqués dans des pathologies comme les cancers, l'ostéoporose ou l'infarctus du myocarde. Dans de nombreux cancers, les sFRP sont décrits comme anti-tumoraux et anti-métastatiques. En effet, lors de l'apparition et le développement de la tumeur, la suppression de la régulation de la voie Wnt/β-caténine du fait de la diminution de l'expression de sFRP1 est un facteur de croissance tumorale et donc de mauvais pronostic (Veeck, Niederacher et al. 2006; Dahl, Wiesmann et al. 2007). Par contre, au niveau des os, sFRP1 pourrait être responsable de l'ostéoporose, qu'elle soit secondaire à l'utilisation de corticoïdes, post-ménopausique ou tout simplement sénile (Wang, Lin et al. 2005; Sims, Shephard et al. 2008; Ohnaka, Yamamoto et al. 2009). Si bien qu'aujourd'hui, sFRP1 est une cible thérapeutique pour combattre l'ostéoporose (Bodine, Stauffer et al. 2009). En 1999, le laboratoire a montré que sFRP1 était exprimé à l'état basal dans l'endothélium d'aorte bovine et qu'il diminuait la prolifération des CE *in vitro* (Duplaa, Jaspard et al. 1999). Puis Dufourcq a mis en évidence l'expression de sFRP1 dans les vaisseaux en formation et montré, sur certains modèles, un

effet pro-angiogénique de la protéine (Dufourcq, Couffinhal et al. 2002). D'ailleurs, en 2003 sur un modèle d'infarctus du myocarde chez des souris surexprimant sFRP1, Barandon retrouve des infarctus de plus petite taille contenant des vaisseaux plus nombreux et plus souvent muscularisés (Barandon, Couffinhal et al. 2003). Sur un modèle d'ischémie de patte, nous avons montré que l'effet de sFRP1 résultait en deux phases successives : durant la première période, une diminution de prolifération des CE induit un retard de cicatrisation, puis une deuxième période pro-angiogénique permet, à la fin du processus de cicatrisation, de retrouver plus de vaisseaux, là encore, plus souvent muscularisés (Ezan, Leroux et al. 2004).

(ii) Autres modulateurs

Mais d'autres inhibiteurs ont été mis à jour. WIF (Wnt Inhibitory Factor) est également un antagoniste et de la voie canonique et non canonique (Bovolenta, Esteve et al. 2008). Quant à la Sclérostine (gène *SOST*) ou aux membres de la famille Dickkopf (Dkk) ils interviendraient en inhibant l'activité des co-récepteurs LRP5/6 et en favorisant leur internalisation pour dégradation (Krishnan, Bryant et al. 2006). En particulier *Dkk1* étant lui-même un gène cible de la signalisation Wnt/β-caténine, il agirait en rétro-contrôle négatif de la voie canonique, et son utilisation pour inhiber cette voie activerait dans certaines études la voie PCP (Caneparo, Huang et al. 2007).

A l'opposé, les protéines Norrin et R-spondin (Rspo) sont deux familles agonistes de la signalisation Wnt/β-caténine. Rspo3 serait même indispensable pour la vasculogenèse et l'angiogenèse chez l'embryon, via une expression Wnt/β-caténine-dépendante du VEGF (Kazanskaya, Ohkawara et al. 2008). De la même façon, l'interaction Norrin – Fzd4 a été reliée à la vasculogenèse (Xu, Wang et al. 2004)

Enfin, il a été récemment décrit que le recrutement de Dvl par le récepteur Fzd était dépendant du pH local (Simons, Gault et al. 2009; Cruciat, Ohkawara et al. 2010).

2) Wnt/Fzd et développement vasculaire

Le système Wnt/Fzd joue un rôle clef dans le développement des néo-vaisseaux (Parmalee and Kitajewski 2008; Zerlin, Julius et al. 2008; Dejana 2010). Ces données sont soutenues principalement par l'analyse de souris transgéniques délétées pour les membres Wnt ou Fzd. Par ailleurs, le laboratoire s'intéresse depuis plusieurs années à la relation Wnt/Fzd et néo-angiogenèse.

(a) Expression du système Wnt/Fzd dans les cellules vasculaires

In vitro et/ou *in vivo*, il a été montré que les cellules endothéliales (CE) pouvaient exprimer Wnt2, 2b, 3, 4, 5a, 5b, 7a, 8a, 9a, 9b, 10b et 11 (Wright, Aikawa et al. 1999; Maretto, Cordenonsi et al. 2003; Masckauchan, Shawber et al. 2005; Goodwin, Sullivan et al. 2006; Matsumoto, Miki et al. 2008). *In vivo*, par exemple, les vaisseaux du placenta expriment Wnt2 (Monkley, Delaney et al. 1996) et ceux du sac vitellin de la souris expriment Wnt5a et Wnt10b (Ishikawa, Tamai et al. 2001).

Les récepteurs Fzd et les co-récepteurs LRP5/6 sont pour la plupart exprimés au niveau des cellules vasculaires *in vitro* (Masckauchan, Shawber et al. 2005) ou dans une grande variété de tissus *in vivo* : Fzd2 est exprimé dans l'aorte de l'embryon de la souris et du rat et Fzd5 a été détecté dans les vaisseaux du placenta. Enfin, Fzd4 est retrouvé dans les vaisseaux de l'utérus durant l'implantation (Hayashi, Erikson et al. 2009), au niveau des vaisseaux ovariens, et rétiniens (Masckauchan and Kitajewski 2006).

Par ailleurs, sFRP1 a été retrouvé dans l'intima et la média des aortes de souris adultes (Jaspard, Couffinhal et al. 2000), mais d'autres modulateurs de la voie peuvent être présents dans les CE comme sFRP3, Dkk1 ou Dkk3 (Goodwin, Sullivan et al. 2006).

Dans le modèle d'infarctus du myocarde, l'équipe de Blankensteijn avait montré dès 1997, une régulation positive de Fzd2 durant les deux premières semaines, autour, puis au centre de la zone nécrotique (Blankesteijn, Essers-Janssen et al. 1997). Plus récemment, au laboratoire, Barandon a, en outre, montré une régulation positive de Fzd1, 5, 10 et Wnt 10B,

alors que Wnt7b était régulé à la baisse dans un modèle d'infarctus chez la souris (Barandon, Couffinhal et al. 2003).

(b) Implications génétiques du système Wnt/Fzd dans la formation des vaisseaux

Pour ce qui est des Wnt, l'invalidation des gènes Wnt chez la souris entraîne en général des malformations précoces et létales. Cependant les souris invalidées pour Wnt2 présentent des anomalies de vascularisation placentaire dues à une altération du réseau capillaire fœtal dans le placenta (Monkley, Delaney et al. 1996). Ces défauts sont liés à une augmentation de l'apoptose ou une diminution de la prolifération des CE. L'invalidation de Wnt4 induit un défaut de formation des vaisseaux gonadiques (Jeays-Ward, Hoyle et al. 2003), de même que sa surexpression inhibe la migration des CE au niveau des testicules (Jordan, Shen et al. 2003). Quant à Wnt7b, son invalidation est létale par défaut de vascularisation pulmonaire touchant plus spécifiquement les CML (Shu, Jiang et al. 2002).

En ce qui concerne les récepteurs Fzd, il a été montré que Fzd5 était essentiel pour l'angiogenèse placentaire et vitelline. Chez les souris invalidées pour Fzd5, une réduction de prolifération de CE empêche le développement du placenta et entraîne donc une mort in utero d'embryons qui eux, par contre, ont une vascularisation normale. (Ishikawa, Tamai et al. 2001). Cette donnée suggère qu'il existerait pour les récepteurs Fzd, une redondance fonctionnelle pour la formation des vaisseaux chez l'embryon. Cependant, plus tard, les souris Fzd5KO présentent une persistance anormale de la vascularisation fœtale au niveau de l'œil (Liu and Nathans 2008). Les souris Fzd4KO sont également viable mais non fertiles et de taille réduite. Elles présentent des défauts de développement au niveau du cerveau, de l'oreille interne, de l'œil et de l'œsophage et des défauts des structures vasculaires au niveau du corps lutéal (Hsieh, Boerboom et al. 2005). Au niveau de l'œil, ces souris présentent des malformations du réseau vasculaire (Xu, Wang et al. 2004), reproduisant une maladie génétique humaine, la rétinopathie exsudative familiale (Kondo, Hayashi et al. 2003).

(c) Activité des voies Wnt/Fzd dans les cellules vasculaires

L'implication de la voie Wnt/Fzd est communément retrouvée dans le développement des vaisseaux, que ce soit durant l'embryogenèse ou en en situation pathologique, alors qu'elle semble quasi inexistante dans un réseau vasculaire mature.

D'un point de vue fonctionnel, *in vitro*, les CE répondent aux ligands Wnt via la voie canonique. Par exemple la transfection de Wnt1 dans les HUVEC augmente leur prolifération via une stabilisation de la β-caténine (Wright, Aikawa et al. 1999). L'expression ectopique de Wnt1, Wnt3a, Wnt5a ou β-caténine stimule également la prolifération des HUVEC (Masckauchan, Shawber et al. 2005; Masckauchan, Agalliu et al. 2006). Le Chlorure de Lithium, inhibiteur non spécifique de GSK-3β mime une activation de la voie canonique et aboutit lui aussi à un effet pro-prolifératif. Quant à sFRP1, il réduit la prolifération des CE (Duplaa, Jaspard et al. 1999) et y modifie l'organisation des fibres de stress d'actine via GSK-3β (voie canonique) et Rac1 (voie non canonique) (Dufourcq, Leroux et al. 2008).

In vivo, au niveau de la barrière hémato-encéphalique (BBB), la voie Wnt/β-caténine est mise en jeu dans le développement et la stabilisation du réseau vasculaire (Liebner, Corada et al. 2008; Stenman, Rajagopal et al. 2008). Un rôle de la voie PCP a aussi été montré dans l'angiogenèse où l'inhibition de la voie entraîne des défauts dans la croissance, la polarité et la migration des CE (Cirone, Lin et al. 2008).

En pathologie, l'accumulation de β-caténine, indicateur d'une signalisation canonique est retrouvée dans les CE des néo-vaisseaux après infarctus du myocarde chez le rat (Blankesteijn, van Gijn et al. 2000). En 2003, Barandon a démontré *in vivo*, au laboratoire, que la surexpression de sFRP1 diminuait l'accumulation de la β-caténine dans les CE des néo-vaisseaux après infarctus du myocarde chez la souris (Barandon, Couffinhal et al. 2003). La voie est aussi mise en jeu dans les CE des néo-vaisseaux de tumeurs cérébrales (Goodwin and D'Amore 2002)

(d) Relations entre les voie Wnt/Fzd et celles des facteurs de croissance vasculaire

Parmi les nombreux gènes cibles de la voie Wnt/Fzd, certains sont impliqués dans le développement vasculaire au premier rang desquels le VEGF (Zhang, Gaspard et al. 2001) et l'interleukine-8 (IL-8) (Levy, Neuveut et al. 2002). Mais il faut aussi citer MMP-1, induite via la voie non canonique (Masckauchan, Agalliu et al. 2006).

Enfin, la voie Wnt/Fzd interagit avec d'autres voies communément impliquées dans la formation des vaisseaux. C'est le cas de la voie du bFGF et la voie du TGFβ.

Dans la grande famille des FGF (18 membres), le basic FGF (bFGF ou FGF2) a été le premier facteur angiogénique mis en évidence (Shing, Folkman et al. 1984). Exprimé de façon ubiquitaire, il possède un site de liaison aux héparanes sulfates ce qui le lie avec une forte affinité à la plupart des cellules et dans la matrice extracellulaire. Les héparanases libèrent donc le stock de bFGF et il induit ses diverses fonctions en se liant à un de ses récepteurs tyrosine kinase FGFR. La liaison bFGF et FGFR active de multiples voies de signalisation avec pour conséquence un effet pro-angiogénique. Différentes études indiquent que bFGF et VEGF agissent de concert dans l'angiogenèse (Presta, Dell'Era et al. 2005) et que c'est en synergie avec le PDGF-BB que bFGF favorise l'artériogenèse (Lu, Xu et al. 2007; Nissen, Cao et al. 2007). De la même façon, bFGF interagit avec la voie Wnt/Fzd (Dailey, Ambrosetti et al. 2005) (Figure 34).

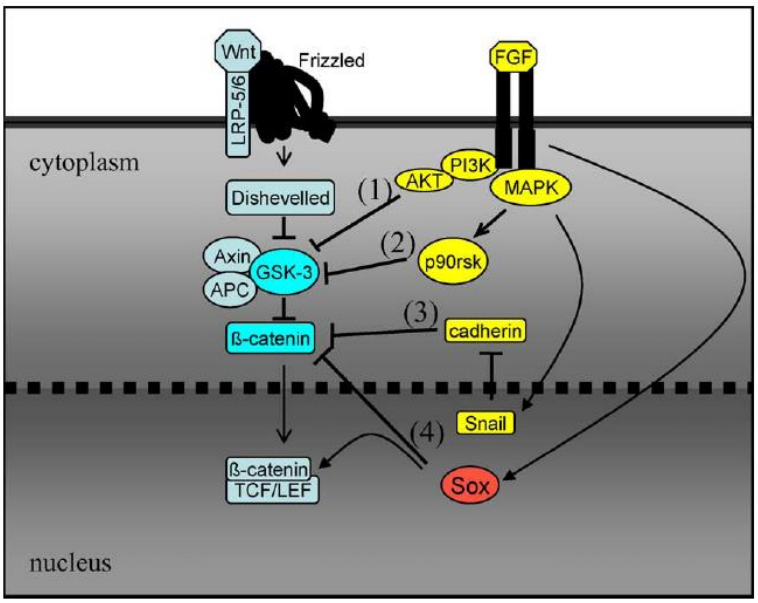

Figure 34. Interactions Wnt/FGF. (D'après Dailey 2005)

En effet, GSK-3β est une cible commune aux deux voies. L'activation de PI3K/Akt par un traitement de CE humaines par du bFGF inactive GSK-3β et induit la translocation de la β-caténine dans le noyau (Holnthoner, Pillinger et al. 2002). D'autre part, l'induction des facteurs de transcription Sox par le bFGF peut réprimer l'activité transcriptionnelle β-caténine-TCF/LEF, ce qui a été montré sur la formation de l'axe embryonnaire du Xénope (Zorn, Barish et al. 1999).

Pour ce qui est de la famille des TGF-β, elle comprend une trentaine de membres dont les TGF-β1, 2 et 3 un certain nombre de BMP (Bone Morphogenic Proteins). La transduction du signal après stimulation des récepteurs aux TGF-β est principalement assurée par la phosphorylation d'une famille de protéines cytoplasmiques, les Smads (Massague and Wotton 2000), par l'intermédiaire d'Activin receptor-Like Kinases : ALK1 et ALK5. Mais d'autres voies de signalisation peuvent être activées selon le contexte, et pourraient participer à l'angiogenèse. Ainsi, il a été montré que le TGF-β pouvait activer les voies des MAP Kinases (p38, JNK, ERK1/2), de la PI3K, de RhoA et de la phosphatase A2 (PP2A/P70s6K), par l'intermédiaire d'ALK5 (Derynck and Zhang 2003). TGF-β aurait donc un effet biphasique sur l'angiogenèse (Goumans, Lebrin et al. 2003). Ainsi via ALK1, TGF-β stimulerait la prolifération et la migration des CE alors qu'avec ALK5, il favoriserait la

stabilisation des vaisseaux. Par ailleurs, TGF-β est un régulateur de la matrice extracellulaire (MEC) (Verrecchia and Mauviel 2002). Grâce à toutes ces études, l'implication de TGF-β dans l'angio- et l'artériogenèse est de mieux en mieux comprise (Gaengel, Genove et al. 2009). Des interactions entre les voies Wnt/Fzd et TGF-β ont été mises en évidence dans des modèles de développement embryonnaire chez le xénope ou le poulet. Chez la souris, Han a montré que Smad7 pouvait promouvoir la dégradation de la β-caténine par une interaction directe, réduisant ainsi l'activité de la voie Wnt/Fzd (Han, Li et al. 2006). Plus récemment, BMP2 s'est montré capable d'activer les voies canoniques et non canoniques de Wnt/Fzd dans des CE (de Jesus Perez, Alastalo et al. 2009). D'un coté il inhibe la GSK-3β, d'où une accumulation de β-caténine, favorisant survie et prolifération cellulaire ; d'un autre coté, il recrute Dvl et promeut les GTPases RhoA et Rac1, nécessaires à la mobilité des cellules.

(e) Voie Wnt/Fzd et balance « tip cell » / « stalk cells »

Bien que les souris délétées pour le gène *Lef1* ne présentent pas moins de « tip cells », laissant supposer que la voie Wnt/β-caténine n'intervient pas dans l'induction ou la fonction des « tip cells », ce système pourrait tout de même indirectement intervenir dans cette phase de l'angiogenèse. Comme nous l'avons vu, la balance « tip cell » / « stalk cells » est régulé par le système Dll4/Notch (Franco, Liebner et al. 2009). Un des gènes cibles de cette voie, Nrarp, semble mimer une activation de la voie Wnt/β-caténine par une action directe sur Lef1. En effet, dans les CE dépourvue de Nrarp, le niveau de Cyclin D1 est abaissé et la prolifération cellulaire ralentie. Ces données suggèrent un rôle de la voie de signalisation Wnt/Fzd dans la prolifération des CE au moment du « sprouting » (Phng, Potente et al. 2009). Par ailleurs, Jagged-1, un des ligands de Notch, est exprimé dans les « stalk cells », mais aurait un rôle opposé à Dll4 au cours de l'angiogenèse. La sélection et le maintien de la « tip cell » pourrait résulter d'un équilibre entre Dll4 et Jagged-1 sur le récepteur Notch (Benedito, Roca et al. 2009; Suchting and Eichmann 2009). Or Jagged-1 est aussi une cible transcriptionnelle de la voie Wnt/Fzd canonique dans les follicules pileux et le cancer colorectal (Estrach, Ambler et al. 2006; Rodilla, Villanueva et al. 2009). La voie pourrait donc participer à la détermination de la « tip cell ». Enfin, il a été montré que le VEGF-A est lui aussi un gène cible de la voie Wnt (Zhang, Gaspard et al. 2001).

3) Système Wnt/Fzd et cellules souches

(a) Concept de niche

Dans la moelle osseuse, les cellules souches hématopoïétiques sont localisées dans des entités anatomiques appelées « niches », qui présentent plusieurs caractéristiques permettant un équilibre entre prolifération, auto-renouvellement, différenciation des cellules filles et adaptation aux besoins de l'organisme. Un déséquilibre pourrait aboutir, soit à une prolifération anarchique, soit à une capacité insuffisante à fournir de nouvelles cellules (Moore and Lemischka 2006). Cet équilibre est régulé par de nombreux et redondants systèmes qui font intervenir, entre autre, la matrice extracellulaire, les ostéoblastes et ostéoclastes et les cellules stromales de soutien retrouvées à proximité du sinus vasculaire (Jones and Wagers 2008). Par ailleurs l'activité physique (Dimmeler 2010) et le stress adrénergique interviennent également dans la mobilisation des cellules souches (Spiegel, Shivtiel et al. 2007).

De telles niches ont été identifiées dans différents tissus chez le rongeur: Intestin grêle, peau, cerveau ou testicules (Doetsch, Caille et al. 1999; Pinto, Gregorieff et al. 2003; Silva-Vargas, Lo Celso et al. 2005; Barker, van Es et al. 2007; Yoshida, Sukeno et al. 2007). Dans la moelle osseuse, les concentrations en calcium (Adams, Chabner et al. 2006) et en oxygène (Ito, Hirao et al. 2004), ou les systèmes Notch, Hedgehog et Wnt/Fzd semblent fondamentaux dans la régulation des propriétés des cellules souches. A noter que l'équilibre ostéoblaste/ostéoclaste paraît finement régulé par un couple RANKL/OPG où la cytokine RANKL, ligand du NF-κB, active des ostéoclastes et où l'OPG (Osteoprotegerin) limite son action. Le ratio RANKL/OPG régule donc la masse osseuse. Les ostéoblastes jouant un rôle important dans l'homéostasie de la niche (Porter and Calvi 2008), un déséquilibre en faveur de RANKL entraîne une mobilisation accrue des cellules immatures (Kollet, Dar et al. 2006).

(b) Wnt/Fzd et cellules souches

Il est acquis que la voie Wnt/Fzd est impliquée dans l'auto-renouvellement et la maintenance d'un état indifférencié des cellules souches, notamment grâce à sa voie canonique. Les MSC expriment de nombreux éléments de la voie Wnt/Fzd : Wnt2, 4, 5a, 11et 16 ainsi que les Fzd2, 3, 4, 5 et 6 et leurs co-récepteurs (Etheridge, Spencer et al. 2004). Les Wnt pourraient agir par voie auto et paracrine. L'application exogène de Wnt3a sur des cultures de MSC diminue l'apoptose et augmente l'auto-renouvellement (Boland, Perkins et al. 2004; Cho, Kim et al. 2006). Cet effet pro-prolifératif est médié par une régulation positive de Cyclin D1 et c-Myc (Baek, Kioussi et al. 2003). En outre, une sur-expression de LRP5 se traduit aussi par une prolifération accrue des MSC en culture. Par contre Wnt5a, en activant des voies non canoniques annule l'effet de Wnt3a (Baksh, Boland et al. 2007; Baksh and Tuan 2007). Mais des régulations très fines interviennent. Par exemple l'effet de Wnt5a est dépendant du support dans lequel les cellules sont cultivées, anti-prolifératif sur des MSC maintenues en suspension mais pro-prolifératif lorsque les cellules adhèrent au support plastique (Baksh and Tuan 2007). De la même façon, il a été montré que sur des MSC humaines, une faible activation de la voie canonique était pro-proliférative, alors qu'une forte activation avait plutôt tendance à les entraîner vers une différenciation (de Boer, Siddappa et al. 2004; De Boer, Wang et al. 2004). D'autre part, dans les conditions particulières de culture en hypoxie, Wnt3a promouvoit alors plutôt l'apoptose des MSC ou des cardiomyocytes. Cet effet est diminué par sFRP2 (Mirotsou, Zhang et al. 2007; Alfaro, Vincent et al. 2010). La densité cellulaire de la culture sur laquelle sont appliqués les stimuli ou inhibiteurs des voies Wnt/Fzd, semblent également jouer un rôle important et sur la prolifération (Gregory, Singh et al. 2003) et sur la différenciation. Ces effets sont dépendants de RhoA (McBeath, Pirone et al. 2004; Nelson, Pirone et al. 2004). Pour ce qui est de la différenciation ostéoblastique des MSC, par exemple, les voies canoniques et non canoniques sont impliquées. Boland a montré que durant cette phase, plusieurs éléments de la voie sont sur-exprimés comme Wnt11, Fzd6 ou sFRP2 alors que l'expression de Wnt9a et Fzd7 diminue (Boland, Perkins et al. 2004). Ces données sont concordantes avec l'idée qu'une stimulation modérée de la voie canonique maintiendrait les MSC dans un état indifférencié alors que l'activation des voies non canoniques les orienterait vers un

phénotype d'ostéoblaste. Il n'est donc pas étonnant que des souris délétées pour sFRP1, inhibiteur de la voie canonique, présentent une augmentation de leur masse osseuse (Bodine, Zhao et al. 2004). Mais d'autres voies de différenciation des MSC sont dépendantes du système Wnt/Fzd comme la chondrogenèse ou la myogenèse. Par ailleurs, le système Wnt/Fzd interagit avec d'autres voies de signalisation dont le rôle dans la différenciation des cellules souches est établi. Comme pour la formation des vaisseaux, c'est le cas de la voie TGF-β. TGF-β1 participe en effet au contrôle de la progression des MSC vers un phénotype de chondrocyte par activation des MAP kinases qui régulent les adhésions intercellulaires dépendantes des N-Cadhérines. Or, comme nous l'avons vu, le niveau cytosolique de la β-caténine, constituant des plaques adhérentes, est dépendant de la voie Wnt. TGF-β activerait la voie canonique via Wnt7a (Tuli, Tuli et al. 2003) ou Smad3 (Jian, Shen et al. 2006). Enfin, la voie Notch, très impliquée dans le développement de l'appareil musculo-squelettique (Buas, Kabak et al. 2009) jouerait de concert avec la voie Wnt pour activer la différenciation des cellules vers un phénotype musculaire, la première inhibant la seconde en maintenant active la GSK-3β (Brack, Conboy et al. 2008)

A noter, par ailleurs, que les effets antiapoptotiques des MSC ont été en partie attribués à IGF2 dont l'expression est régulée par la PI3 kinase d'une manière dépendante de sFRP2 (Mirotsou, Zhang et al. 2007; Gehmert, Sadat et al. 2008)

(c) Wnt/Fzd et thérapie cellulaire

En 2003, Mangi (Mangi, Noiseux et al. 2003) formulait l'hypothèse que des MSC prétraitées pour surexprimer la protéine Akt, auraient une meilleure résistance à l'apoptose et seraient plus efficaces du point de vue thérapeutique. Et en effet, il a montré tout d'abord que des conditions de culture hypoxiques provoquaient l'augmentation de l'expression spontanée d'Akt. Puis, il a mis en évidence une grande résistance à l'hypoxie des MSC surexprimant Akt, et a obtenu des résultats spectaculaires avec ces cellules sur un modèle d'infarctus chez le rat (Figure 35).

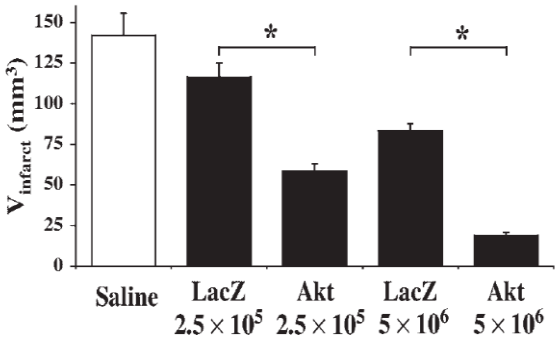

Figure 35. Différences de volume myocardique infarci selon le traitement reçu. (Mangi 2003)

La même équipe, avec Mirotsou (Mirotsou, Zhang et al. 2007) montre que l'effet observé avec les Akt-MSC est médié par sFRP2, protéine sécrétée, modulatrice du système Wnt/Fzd. L'utilisation d'un siRNA dirigé contre sFRP2 diminuait d'ailleurs considérablement le bénéfice du prétraitement des MSC.

C'est aussi à sFRP2 que s'est intéressé Alfaro (Alfaro, Pagni et al. 2008; Alfaro, Vincent et al. 2010) . Il a montré, également sur un modèle d'infarctus murin, que des MSC surexprimant sFRP2 entrainaient des infarctus de plus petit taille, avec une densité capillaire augmentée.

D) HYPOXIE ET THERAPIE CELLULAIRE

1) Niveaux d'oxygène dans l'organisme et mort cellulaire

Dans le domaine de la thérapie cellulaire en cardiovasculaire, il est admis que la très faible teneur en oxygène des tissus hôtes est en grande partie responsable de la mort de plus de 90% des cellules transférées dans les 24 premières heures. Cependant, les teneurs en oxygène de nos tissus sont physiologiquement basses. En effet, si l'air que nous respirons contient 21% d'oxygène, notre sang est à 12% et la moyenne de nos tissus aux alentours de 3%. Les cellules souches de la moelle osseuse sont même localisées dans des niches dont la teneur en oxygène se situe aux alentours de 1%. Un certain nombre de dispositifs disponibles en recherche permettent d'obtenir des mesures tissulaires de la concentration en oxygène *in vivo*. Il s'agit par exemple de l'électrode de Clarke, ou de l'EPR spectroscopie (Troitzsch, Moosdorf et al. 2009; Ahmad and Kuppusamy 2010). Notamment, c'est cette dernière méthode, non invasive et permettant des mesures répétées, qui a été récemment utilisée *in vivo* chez des rongeurs par Kahn (Khan, Kutala et al. 2008; Khan, Kwiatkowski et al. 2010). Il montre que la pression partielle en oxygène, la pO_2 au niveau de la paroi antérieure du myocarde passe de 20 à 2 mmHg lors de la ligature de l'interventriculaire antérieure. En cas de reperfusion 30 minutes plus tard, une hyperoxygénation est observée pendant 48h, correspondant à un défaut d'utilisation de l'oxygène. En cas d'infarctus non reperfusé, la mesure à 15 jours retrouve des valeurs de l'ordre de 3 à 4 mmHg. Cette valeur très basse correspond à 0,5% d'O2, et représente un environnement anoxique, et donc létal, pour les cellules qui y seront transférées. Malgré tout, et toujours grâce à l'EPR spectroscopie, au niveau de la paroi antérieur du ventricule gauche de rats infarcis, Kahn montre qu'une thérapie cellulaire par MSC permet d'accroître la valeur de la pO2 de 3 à 10 mmHg. Cependant, s'il soumet ces animaux à des séances d'hyperoxygénation par caisson hyperbare, les mesures de la pO2 passent transitoirement de 20 à 30 mmHg sur cœurs sains, et de 3 à 15 mmHg sur cœurs infarcis. Dans le groupe de rat infarcis traités par MSC et soumis aux séances d'hyperoxygénation la valeur obtenue n'est plus de 10 mais de 16 mmHg, laissant envisager un effet bénéfique de l'hyperoxygénation sur l'efficacité de la thérapie, éventuellement par un accroissement de la survie des cellules après transplantation (Khan, Meduru et al. 2009).

Ces expériences démontrent qu'un lien étroit existe entre le niveau d'oxygène du tissu dans lequel les cellules sont délivrées et leur efficacité. Elles mettent donc en exergue une grande différence en fonction que l'on s'adresse à un tissu reperfusé (infarctus du myocarde traité en urgence par angioplastie) ou non, comme dans l'infarctus non reperfusé, l'ischémie de membre inférieur ou la cardiomyopathie ischémique chronique.

2) Hypoxie et niches

L'étude de la localisation des cellules souches hématopoïétiques dans les niches permet de penser qu'elles se trouvent dans un environnement pauvre en oxygène. En 2001, Chow a montré par une modélisation mathématique que la concentration en oxygène au niveau de ces cellules est inférieure à 1% (Chow, Wenning et al. 2001). Plus précisément, il semble qu'un gradient de pression partielle en oxygène existe au sein des niches, s'étendant de 6% à proximité du sinus vasculaire à moins de 1% au niveau des cellules souches hématopoïétiques (Harrison, Rameshwar et al. 2002). Ces conditions d'hypoxie participent à l'environnement favorable pour la stabilité des cellules souches (Kubota, Takubo et al. 2008; Eliasson, Rehn et al. 2010). L'oxygène ne doit alors plus être considéré comme un simple substrat mais également comme une molécule de signalisation.

Les mécanismes de cette signalisation ne sont pour l'heure pas entièrement élucidés. Parmi ceux-ci, la voie de HIF-1 et la régulation des ROS (Réactive Oxygen Species) responsables du stress oxydatif, peuvent être évoqués (Figure 36).

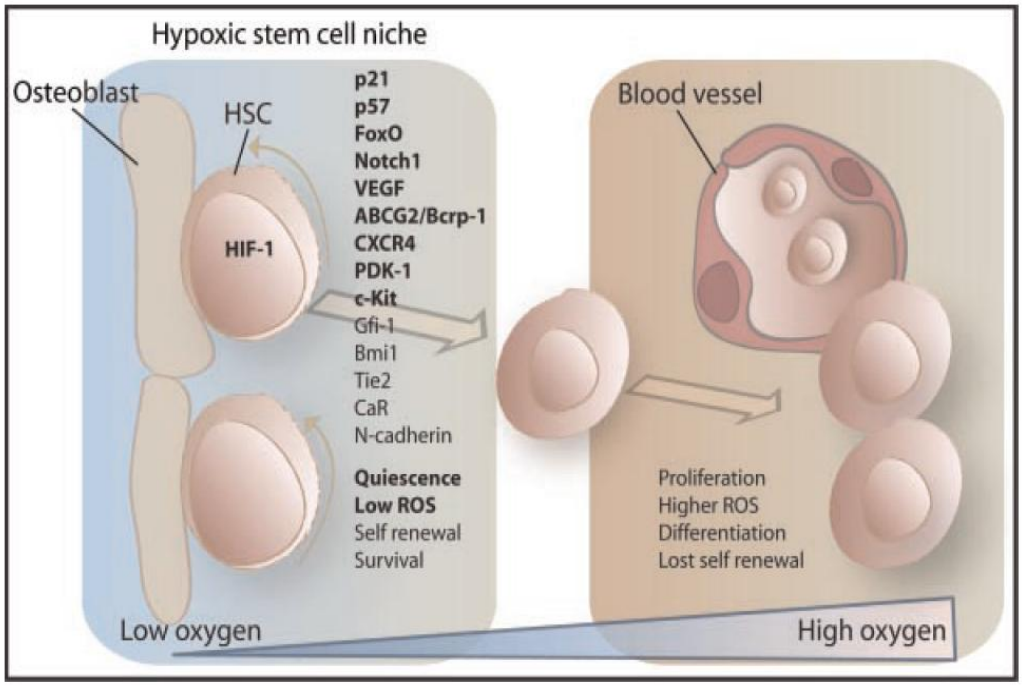

Figure 36. Rôle de HIF-1 dans les niches. HSC: Hematopïetic Stem Cell. (D'après Eliasson 2010)

Plus d'une centaine de gènes cibles de HIF-1 ont été identifiés. Ils régulent des mécanismes comme le métabolisme énergétique, le transport du glucose et la glycolyse, l'angiogenèse ou l'érythropoïèse. Parmi ceux-ci, VEGF-A et son récepteur flt-1 auraient un rôle sur la maintenance des cellules souches soit direct soit via d'autres éléments activés par VEGF, comme FGF-2, IGF-2, PDGF ou Notch-1 (Eliasson and Jonsson 2010). D'autre part, HIF-1 pourrait intervenir sur la prolifération cellulaire en agissant sur le cycle cellulaire via c-Myc (Koshiji, Kageyama et al. 2004). Enfin, HIF-1 régulerait le « homing » des cellules souches via notamment SDF-1 et son récepteur CXCR4 (Hattori, Heissig et al. 2001).

Par ailleurs, il a été montré que les ROS avaient un effet délétère sur la moelle, effet qui pouvait être contrecarré par un antioxydant, le N-acétyl-Cystéine (NAC) (Ito, Hirao et al. 2004) et que, par ailleurs, les cellules souches hématopoïétiques étaient pauvres en ROS intracellulaires (Jang and Sharkis 2007). Pour autant les mécanismes concernés pourraient, là encore, impliquer HIF via des éléments mis en jeu indirectement (Simon 2006) ou directement dans le métabolisme des mitochondries (Benita, Kikuchi et al. 2009) (Figure 37).

L'ensemble de ces données suggère un effet protecteur de la faible concentration en oxygène sur les cellules souches médullaires.

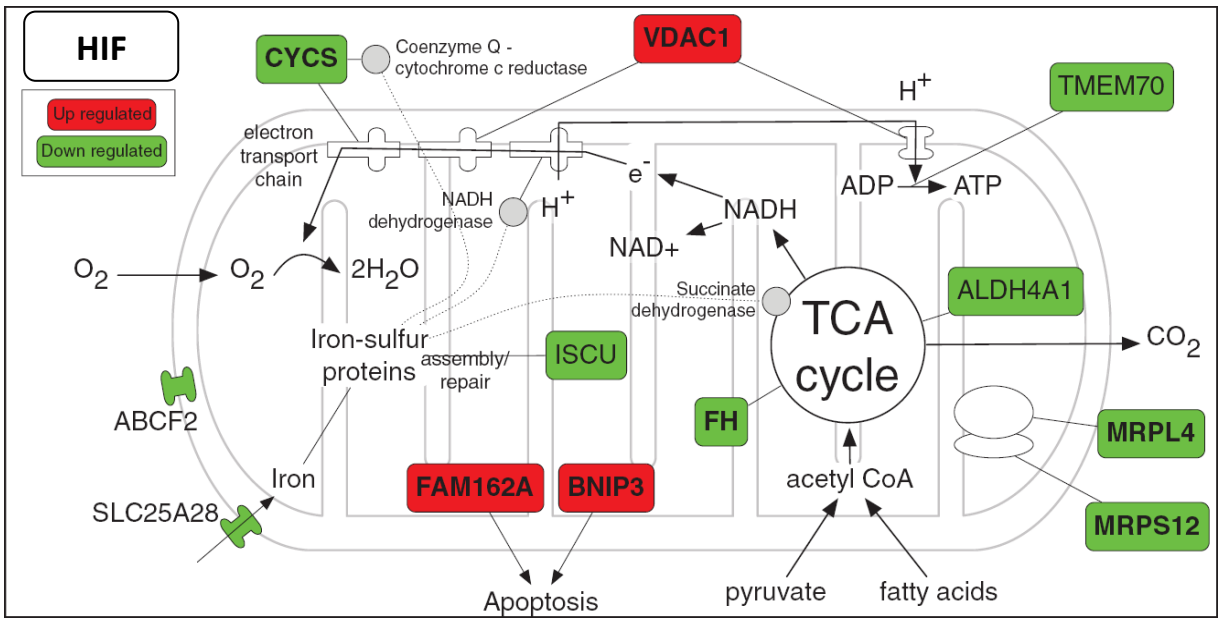

Figure 37. Effets de HIF dans les mitochondries: en rouge les éléments "up-régulés" par HIF et en vert, les éléments "down-régulés" par HIF (D'après Benita 2009).

3) Principe et rôle du préconditionnement hypoxique sur les propriétés des cellules souches

(a) Généralités

La culture de cellules en condition d'hypoxie est connue et étudiée depuis de nombreuses décennies. Les premières publications sur le sujet remontent à 1958 (Cooper, Burt et al. 1958) puis en 1977 (Packer and Fuehr 1977). La culture en hypoxie a tout d'abord été appliquée aux cellules souches hématopoïétiques (Murphy and Lord 1973; Maeda, Hotta et al. 1986; Broxmeyer, Cooper et al. 1990; Cipolleschi, Rovida et al. 2000; Ivanovic, Bartolozzi et al. 2000; Ivanovic, Dello Sbarba et al. 2000). Ces études ont montré que des conditions de culture à 5% ou 10% d'O2 permettaient d'améliorer la formation des colonies. Par ailleurs, la production de mégacaryocytes est augmentée à 5% d'O2 alors que c'est la

maturation et la formation des plaquettes qui est stimulée à 20% d'O2 (Mostafa, Miller et al. 2000). Des concentrations plus basses, de l'ordre de 1% diminuent la formation des colonies érythrocytaires mais préservent la maintenance des cellules souches hématopoïétiques (Rich and Kubanek 1982; Cipolleschi, Dello Sbarba et al. 1993; Ivanovic, Belloc et al. 2002). Si les premières expériences ont consisté à exposer des souris vivantes à de basses pressions atmosphériques pendant quelques heures ou quelques jours, les chambres de culture à hypoxie basée sur le déplacement de l'O2 par de l'azote (N2), sont rapidement apparues. La concentration de CO2 y reste de 5%.

Fort de l'expérience acquise en hématologie, certains auteurs se sont donc intéressés à l'analyse *in vitro* des mécanismes mis en jeu lorsque l'on soumet les cellules souches à un faible régime en oxygène.

Pour pouvoir travailler à l'échelle de la cellule isolée, Silverman a mis au point une chambre à hypoxie sans couvercle. La première expérience avec une telle chambre a été publiée par Stern en 1988 (Stern, Silverman et al. 1988). Ce dernier avait montré qu'avec un flux laminaire d'Argon, les cellules en culture pouvaient être entièrement et rapidement privées d'oxygène. Sur des cardiomyocytes isolés, privés d'oxygène et stimulés, il a alors montrait que ceux-ci continuaient de battre pendant plusieurs minutes. Mais ce n'est que 10 ans plus tard que, des travaux menés par la même équipe, laissent supposer un effet bénéfique du préconditionnement hypoxique sur des cardiomyocytes de rat (Silverman, Wei et al. 1997). Silverman montre que 48h de culture à 1% d'O2 ne modifie pas l'aspect ou la viabilité des cellules. Par contre les phases de contraction et de relaxation des cellules sont ralenties mais redeviennent normales lors de la ré-oxygénation. Surtout, comparées aux cellules cultivées en normoxie, ces cellules préconditionnées survivent mieux lorsqu'elles sont à nouveau soumises à une privation d'oxygène.

Depuis, de nombreuses études ont porté sur les capacités de prolifération et de différenciation des cellules souches, cultivés à faibles niveaux d'oxygène en chambres à hypoxie classiques (Figure 38).

Figure 38. Chambre à hypoxie à l'Unité U828: La chambre est installée dans l'incubateur à cellules. Elle est reliée par cables au boitier de réglage, à l'extérieur, où s'affiche le niveau d'O2 demandé et le niveau mesuré, en pourcentage. La bouteille d'azote est reliée au boitier.

Ces chambres permettent de régler le pourcentage d'O2 dans l'atmosphère de la chambre et donc, par exemple pour un taux de 1,5%, d'appauvrir rapidement en oxygène le milieu de culture des cellules. Après 15 à 20 heures de culture, la phase de plateau est obtenue. Quant au pH, il ne s'abaisse qu'en présence de cellules et au-delà de la trentième heure (Lavrentieva, Majore et al. 2010) (Figure 39).

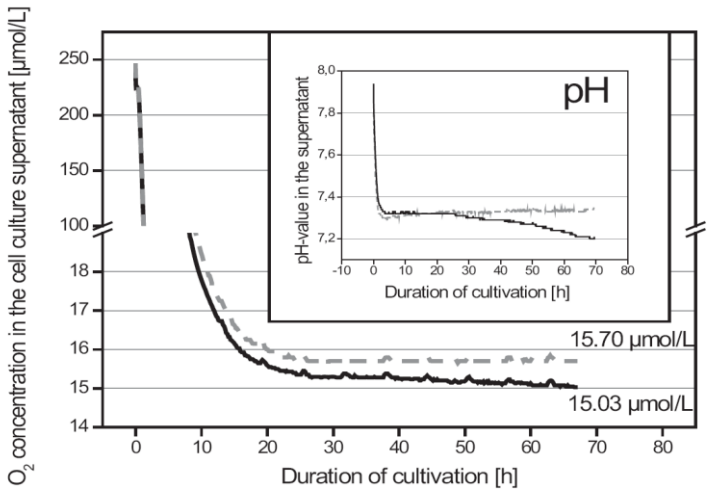

Figure 39. Evolution de la concentration en O2 et du PH (encadré) du mileu de culture en chambre à hypoxie. En Noir: avec cellules. En Gris: sans cellules. (D'après Lavrentieva 2010).

(b) Effet de l'hypoxie sur la prolifération des cellules souches

Outre les cardiomyocytes de rat, un effet positif sur la prolifération a aussi été montré pour des cellules comme le péricytes de boeuf (Brighton, Lorich et al. 1992), les fibroblastes humains (Balin, Fisher et al. 1984) les cellules souches multipotentes dérivées du système nerveux central du rat (Studer, Csete et al. 2000) ou les myoblastes issus du muscle squelettique du rat (Csete, Walikonis et al. 2001).

Pour les MSC en particulier, les études *in vitro* ont montré, pour la plupart, que des conditions de culture entre 1% et 5% d'O2 permettaient d'augmenter leur capacité de prolifération (Lennon, Edmison et al. 2001; D'Ippolito, Diabira et al. 2006; Grayson, Zhao et al. 2006; Ren, Cao et al. 2006; Fehrer, Brunauer et al. 2007; Grayson, Zhao et al. 2007; Hung, Pochampally et al. 2007; Martin-Rendon, Hale et al. 2007; Carrancio, Lopez-Holgado et al. 2008; Rosova, Dao et al. 2008).(Figure 40)

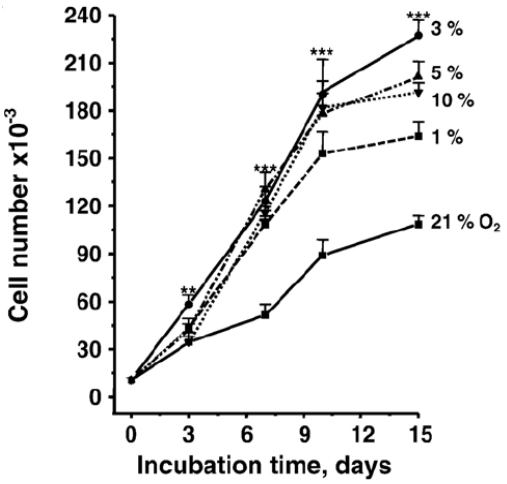

Figure 40. Prolifération des MSC sous différentes concentration en oxygène. (D'après D'Ippolito 2006)

La plupart de ces études viennent d'être compilées dans une revue de Ruud Das (Das, Jahr et al. 2010) (Figure 41).

Table 1. Effects of Hypoxia in Proliferation

O_2 tension (%)	Cell type	Serum type/ concentration	Duration	Proliferation	Author	Reference
1	Human MSCs	17% FBS	10 days	Decreased rate of proliferation	Hung	65
1–3	Human MSCs from BMA	Serum-free	16 h	No effect of hypoxia on cell viability	Rosova	52
1, 3, 5, and 10	MIAMI	2% FBS for expansion	3, 7, 10, and 15 days	Increase, highest at 3% pO_2	D'Ippolito	61
1 and 50	BMMC (Cambrex, East Rutherford, NJ)	N/A (MSCGM [Cambrex])	24 h	1.61-fold increase in viable cell number	Martin-Rendon	64
2	Human MSCs	10% FBS	Up to 6 weeks	30-fold increase	Grayson	60
2	Human MSCs	10% FBS	Up to 30 days	Increase after initial lag	Grayson	42
5	MNC from BMA	10% fetal calf seaum (FCS) or platelet lysate	P1–4 to 90% confluency	Decreased time to reach confluency	Carrancio	62
5	Rat MSCs	10% FBS	First passage	Mean increase in cell number of 41.2%	Lennon	20
8	Murine MSCs	10% FBS	7–8 days	2.8-fold increase	Ren	63

Figure 41. (D'après Das 2010)

(c) Effet de l'hypoxie sur la différenciation des cellules souches

Pour ce qui est de leur différenciation, les résultats sont plus discordants : Si certains auteurs affirment que l'hypoxie permet de maintenir les MSC dans un état indifférencié, plusieurs études ont montré que de faibles concentrations en oxygène orientaient la différenciation des MSC vers un phénotype adipocyte-like, voire diminuait leur plasticité (Fink, Abildtrup et al. 2004; Potier, Ferreira et al. 2007), alors que d'autres ont trouvé que l'hypoxie entrainait une différenciation des MSC vers un phénotype chondroblastique (Robins, Akeno et al. 2005) ou endothélial (Annabi, Lee et al. 2003).

Il semble qu'il y ait deux valeurs seuil : une valeur seuil aux alentours de 1,5% en dessous de laquelle la prolifération est à nouveau diminuée et une autre valeur seuil, aux alentours de 3% d'O2 au dessus de laquelle la différenciation est à nouveau favorisée (Holzwarth, Vaegler et al. 2010; Lavrentieva 2010; Rasmussen, Frobert et al. 2010).

(d) Effets de l'hypoxie sur l'expression de facteurs

L'expression de ces facteurs participe à l'effet positif des cellules sur la réparation tissulaire après ischémie et notamment par un mécanisme paracrine comme le laissent supposer les études menées en utilisant le surnageant des cellules préconditionnées en hypoxie :

- ✓ En culture tout d'abord, Gnecchi (Gnecchi, He et al. 2005) montre l'effet anti-apoptotique des surnageants sur une culture de cardiomyocytes de rat soumis à l'hypoxie : si le surnageant de MSC cultivées dans des conditions standards a peu d'effet, celui des MSC préconditionnées par 12 heures d'hypoxie à 0,5%, protège les cardiomyocytes de l'apoptose. Cet effet est encore plus marqué si ces MSC sont prétraitées pour surexprimer la protéine Akt. Citons également l'étude de Hung (Hung, Pochampally et al. 2007) qui a montré que le surnageant de MSC cultivées 48h à 1% d'oxygène, permettait de protéger de l'apoptose des cellules endothéliales soumises à l'hypoxie et d'accélérer la formation des tubes endothéliaux.
- ✓ Sur l'animal ensuite, Gnecchi (Gnecchi, He et al. 2005) montre l'effet positif de l'injection intramyocardique après infarctus chez le rat, de surnageants de MSC soumises à l'hypoxie. Puis, par exemple, Takahashi (Takahashi, Li et al. 2006), sur un modèle d'infractus également chez le rat, a retrouvé une très nette régression de la taille de l'infarctus par l'injection du surnageant de BMC, qui est corrélé au préconditionnement hypoxique des BMC 24h à 1% (Figure 42).

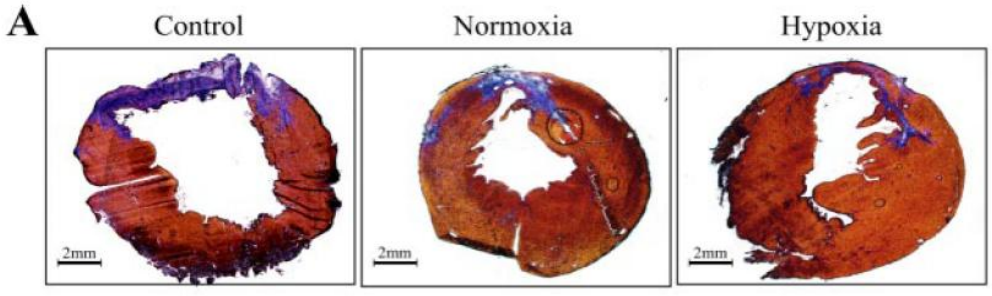

Figure 42. Réduction de la taille de l'infarctus par le surnageant de BMC préconditionnées en hypoxie (D'après Takahashi 2006).

Plus récemment, Nguyen (Nguyen, Maltais et al. 2010) sur un modèle d'infarctus chez le cochon, montre que le surnageant de MSC soumises à 16 heures d'hypoxie à moins de 1% d'O2, injecté par voie intracoronaire, permet de limiter la taille de l'infarctus.

D'un point de vue mécanistique, plusieurs études (Mangi, Noiseux et al. 2003; Kinnaird, Stabile et al. 2004; Rehman, Traktuev et al. 2004; Takahashi, Li et al. 2006; Martin-Rendon, Hale et al. 2007; Ohnishi, Yasuda et al. 2007; Thangarajah, Vial et al. 2009) se sont focalisées sur le profil transcriptionnel des MSC soumises à des conditions de cultures hypoxiques, et retrouvent un phénotype :

- ✓ pro-angiogénique, les cellules surexprimant HIF-1α, VEGF, bFGF, Flk-1, Ang-1, PDGF, IGF-1, TGF-β, EPO ou iNOS,
- ✓ anti-apoptotique, avec l'augmentation de l'expression d'Akt, Bcl-2 ou Bcl-xL,
- ✓ anti-oxydant via notamment l'Heme oxygenase-1,
- ✓ anti-fibrosant et anti-inflammatoire. En effet dans des conditions de cultures normales, le sérum conditionné par des MSC diminue la prolifération des fibroblastes et l'expression du collagène I et III (Ohnishi, Yasuda et al. 2007). Par ailleurs, l'expression de cytokines telles que TNF-α ou IL1-β sont diminuées dans un modèle d'infarctus de rat transplanté avec des MSC (Guo, Lin et al. 2007). En culture dans des conditions d'hypoxie (4% d'O2), Li retrouve dans le surnageant des MSC l'IL10, cytokine anti-inflammatoire, alors qu'il n'y retrouve ni TNF-α ni IL1-β (Li, Wei et al. 2010).
- ✓ Enfin, des facteurs d'interaction avec l'environnement cellulaire et notamment SDF-1, impliqué dans le « homing », sont également surexprimés par les MSC cultivées en hypoxie.

4) Applications *in vivo*

Dans le domaine des pathologies cardiovasculaires, *in vivo*, Li sera le premier, dès 2002, à utiliser un préconditionnement hypoxique de cellules souches sur un modèle

d'ischémie de membre inférieur chez le rat (Li, Hamano et al. 2002). Des MSC collectées dans la moelle osseuse étaient exposées à 24h de culture à 2% d'oxygène avant d'être injectées dans le muscle de pattes de rat immédiatement après la chirurgie. Il montre alors que le VEGF est fortement exprimé par les cellules cultivées en hypoxie mais aussi dans les muscles ayant reçu les cellules préconditionnées par rapport à ceux ayant reçu des cellules cultivées dans des conditions standards (Figure 43). La densité capillaire y est d'ailleurs plus importante et la récupération du flux plus rapide.

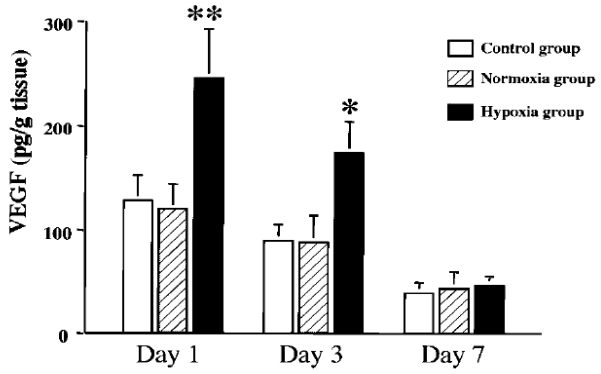

Figure 43. Evaluation du VEGF dans le muscle. (D'après Li 2002).

En 2003, Akita (Akita, Murohara et al. 2003) utilise un modèle d'ischémie de patte chez le rat. Il injecte 3 jours après la chirurgie des EPC qu'il a préalablement préconditionnées en hypoxie et retrouve alors une cicatrisation plus rapide. L'année suivante, Rehman (Rehman, Traktuev et al. 2004) préconditionne 72h à 1% d'O2 des MSC humaines issues du tissu adipeux. Il retrouve également une augmentation de plusieurs facteurs de croissance et injecte ses cellules dans le muscle tibial antérieur 24h après réalisation d'une ischémie chez la souris nude. Même si les groupes sont limités à 3 animaux, il montre une accélération de la réparation tissulaire avec les cellules préconditionnées comparées à celles cultivées en normoxie.

Puis, pendant que nous réalisions nos expérience au laboratoire, de nouvelles études sont venue éclairées ce champs d'application.

En 2006, Uemura (Uemura, Xu et al. 2006) utilise le préconditionnement hypoxique (4 heures à 0%) pour préparer des BMC à l'injection intraventriculaire immédiatement après réalisation d'un infarctus chez la souris. *In vitro*, il montre que ce traitement entraîne une surexpression du VEGF, du bFGF de l'IGF et du SDF. Par ailleurs la voie Akt est très clairement mise en jeu (Figure 44).

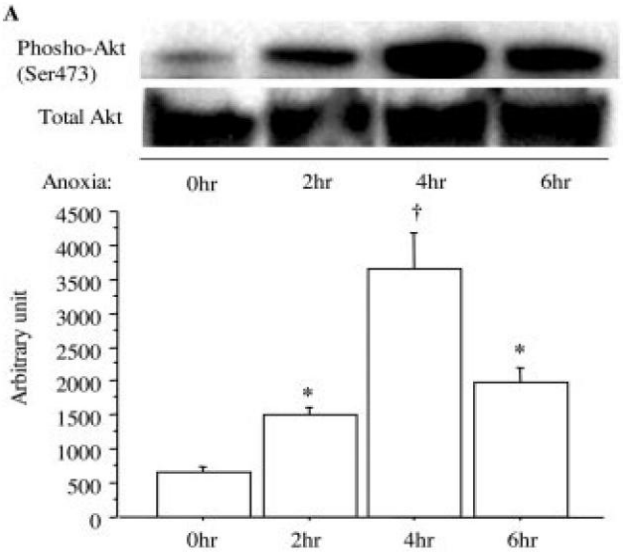

Figure 44. Mise en jeu de la voie Akt dans des BMC soumises à l'hypoxie. (D'après Uemura 2006).

Il formule alors l'hypothèse que ce préconditionnement permet de préserver de l'apoptose les cellules environnantes, pouvant ainsi réduire la taille de l'infarctus. Cette hypothèse se vérifie d'abord sur un modèle de co-culture de cardiomyocytes avec des BMC. C'est la raison pour laquelle il choisit un modèle animal où l'injection des cellules est très précoce par rapport à l'infarctus. Il montre alors que les cellules se retrouvent en petit nombre, préférentiellement au niveau des zones bordantes de l'infarctus. La densité capillaire est augmentée dans les groupes d'animaux ayant reçu des cellules par rapport à celle retrouvée après injection de liquide de culture seul. Cependant il n'y a pas de différence selon le préconditionnement des cellules. Une analyse des coupes histologiques en TUNEL vient confirmer l'effet protecteur des cellules préconditionnées, montrant alors

moins de cardiomyocytes en apoptose (Figure 45). L'ensemble aboutit à une diminution de la taille de l'infarctus et du remodelage ventriculaire gauche.

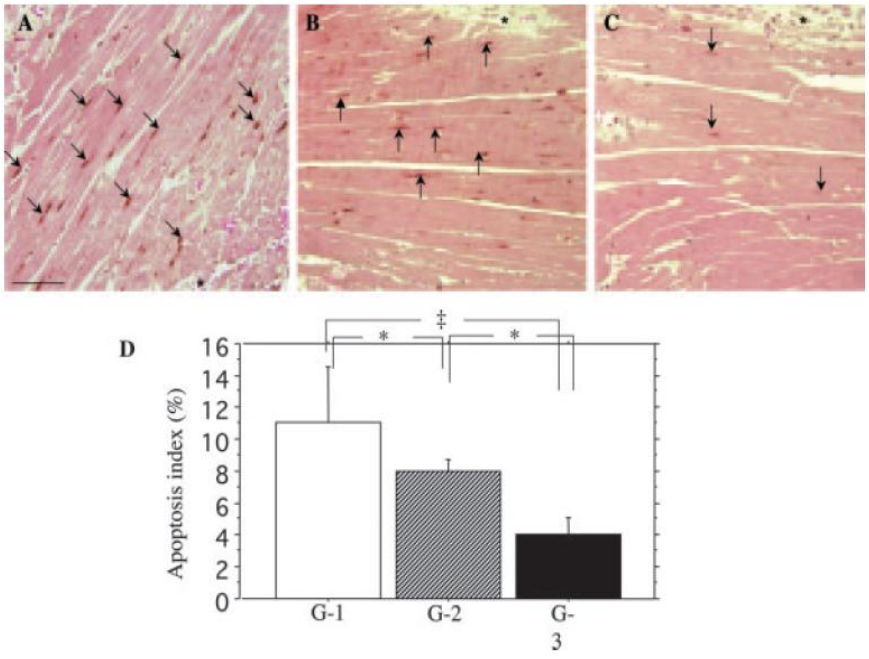

Figure 45. Evaluation de l'apoptose par méthode TUNEL. L'injection de BMC préconditionnées en hypoxie prévient de l'apoptose les cardiomyocytes *in vivo*. A (G-1): groupe sans cellules, B (G-2): groupe BMC normoxiques, C (G-3): groupe BMC hypoxiques. (D'après Uemura 2006)

Pour Niagara, en 2007 (Niagara, Haider et al. 2007), l'idée était de transférer le principe du préconditionnement ischémique efficace sur les cardiomyocytes, à la thérapie cellulaire en utilisant des myoblastes squelettiques. Bien que les mécanismes mis en jeu semblent diffrérents, Kim (Kim, Haider et al. 2009), a montré que la survie de MSC transplantés, là aussi, dans des infarctus myocardiques chez le rat était d'autant meilleure que les cellules subissaient un plus grand nombre de cycles d'hypoxie/réoxygénation. D'autre part, le prétraitement de MSC par le Diazoxide, actif sur le canal potassique mitochondrial sensible à l'ATP (mitoKATP channel) (Pasdois, Beauvoit et al. 2008) permet, en mimant le préconditionnement ischémique, d'améliorer la survie cellulaire via le NF-κB. Ces données issues de la culture cellulaire sont vérifiées in vivo sur un modèle d'infarctus chez le rat (Afzal, Haider et al. 2010).

En février 2008, Kubo publie son travail consistant à tester l'efficacité du préconditionnement hypoxique (24h à 2%) sur un modèle murin d'ischémie de membre inférieur (Kubo, Li et al. 2008). Il a utilisé des cellules souches (BMC) issues du sang de souris après mobilisation par du G-CSF. *In vitro* après hypoxie, ses cellules expriment significativement plus de molécules protectrices du stress oxydatif : Autocrine motility factor, Hexokinase-2, Heme oxygenase-1, IL-1β, iNOS et VEGF. Et en effet, les BMC ainsi préconditionnées survivent mieux à un stress oxydatif mimé par LY-83583. Il en déduit que le préconditionnement doit permettre une meilleure survie des cellules après transplantation, notamment par un mécanisme de protection contre le stress oxydatif. Chez l'animal, il montre alors que les cellules survivent mieux à 3 jours, et que la récupération est plus rapide, tant en doppler tissulaire qu'en histologie (Figure 46) et (Figure 47).

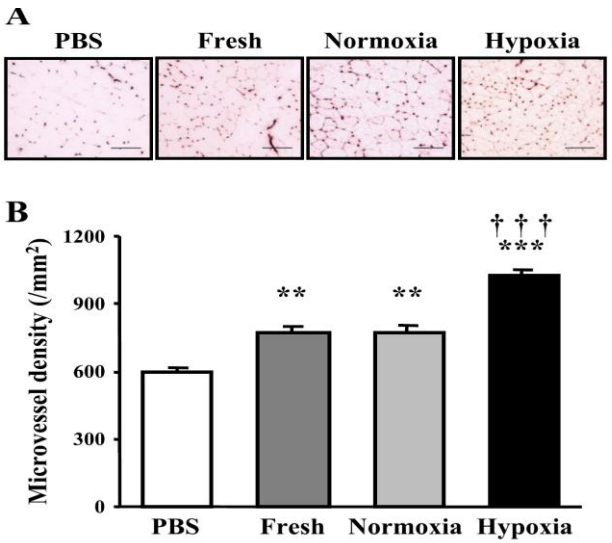

Figure 46. Densité capillaire évaluée en histologie après marquage au CD31. (D'après Kubo 2008)

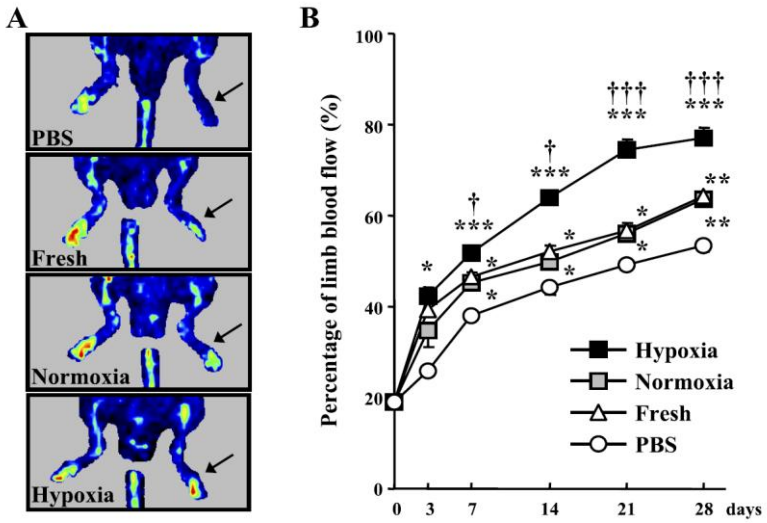

Figure 47. Evolution du flux sanguin mesuré en Doppler laser. (D'après Kubo 2008)

Puis Hu, en avril 2008, propose de préconditionner en hypoxie (24h à 0,5%) des MSC de souris transgéniques, surexprimant la GFP, avant de les transplanter sur un modèle d'infarctus chez le rat, directement dans le myocarde, 30 minutes après ligature coronaire (Hu, Yu et al. 2008). *In vitro* tout d'abord, il montre en cas d'hypoxie, une surexpression de HIF-1α, VEGF et son récepteur Flk-1, Ang-1 et EPO, mais aussi NF-κB, Bcl-2 et Bcl-xL. En contrepartie, la caspase-3 apparaît diminuée. Chez l'animal, il observe alors que les cellules survivent mieux (à 3 jours) et retrouve des infarctus de plus petite taille. Les mécanismes mis en jeu sont là encore une diminution de l'apoptose et un effet proangiogénique avec augmentation de la densité capillaire et de la muscularisation vasculaire.

Ce sont également des MSC, mais de rat cette fois, que Wang va préconditioner (10 minutes à 0%) avant de les injecter dans le myocarde de rat, une semaine après réalisation d'un infarctus antérieur (Wang, He et al. 2009). Il confirme les résultats de Hu et montre la persistance de cellules transplantées à 3 semaines (Figure 48).

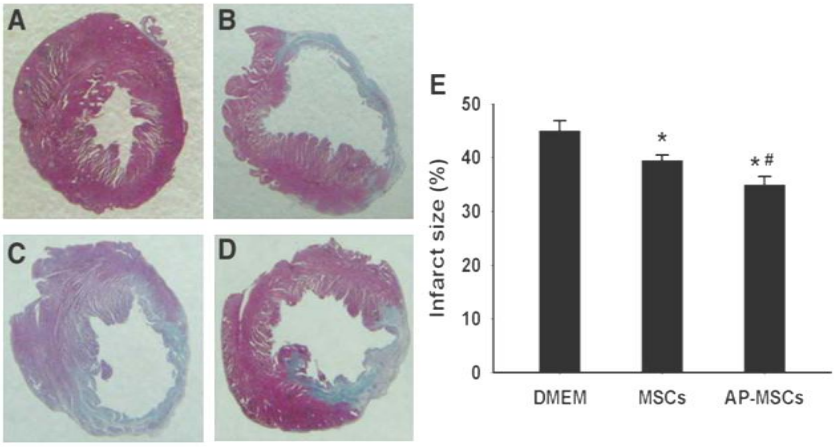

Figure 48. Réduction de la taille de l'infarctus: A:coeur normal, B: après infarctus, sans cellules, C: après infractus avec cellules normoxiques, D: après infarctus avec cellules hypoxiques. (D'après Wang 2009)

Toujours en 2009, Tang (Tang, Zhu et al. 2009) montre que des progéniteurs d'origine cardiaque soumis à l'hypoxie expriment fortement SDF-1 et CXCR4. Injectés de manière intraveineuse après réalisation d'un infarctus chez la souris, ces cellules, lorsqu'elles sont préconditionnées par 6 heures d'hypoxie, présentent un meilleur « homing » dans la zone ischémique, et favorisent donc d'autant mieux la régénération myocardique.

Enfin, la même année, Rosova préconditionne aussi des MSC de souris, 24h à 1% d'oxygène. Comme d'autres auteurs, il retrouve *in vitro* une mise en jeu de la voie Akt, mais aussi de cMet, récepteur de HGF (Hépatocyte growth factor) (Rosova, Dao et al. 2008). Sur un modèle murin d'ischémie de membre inférieur, c'est la voie intra-artérielle à 24h de la chirurgie qui sera utilisée pour transférer les cellules. Dans les muscles ischémiques traités par des cellules préconditionnées, il retrouve significativement plus d'HGF. La récupération du flux sanguin est améliorée lorsque l'on fait subir aux cellules un passage en chambre à hypoxie.

E) HYPOXIE ET SYSTEME WNT/FZD

Peu d'études se sont jusque là intéressées à l'évolution de la voie de la signalisation Wnt/Fzd en condition d'hypoxie. En 2003, Mangi (Mangi, Noiseux et al. 2003) montrait que des conditions de culture hypoxiques provoquaient l'augmentation de l'expression spontanée d'Akt dans des MSC. En 2007, la même équipe, avec Mirotsou (Mirotsou, Zhang et al. 2007) montre que l'effet bénéfique de la surexpression d'Akt par des MSC prétraitées pour surexprimer la protéine (Akt-MSC), est médiée par sFRP2. Pour expliquer l'effet anti-apoptotique de sFRP2, des cardiomyocytes ont été cultivés en hypoxie pendant 24 heures. Les auteurs montrent que l'expression de Wnt3a est alors fortement augmentée. Or Wnt3a sur les cardiomyocytes en hypoxie induirait une activité caspase3, qui est inhibée par l'addition de sFRP2 dans le milieu de culture. D'ailleurs, dans ces conditions particulières, sFRP2, en général considéré comme un inhibiteur de la voie canonique, s'est montré capable de mimer l'activation de la voie en conduisant à une accumulation intracellulaire de β-caténine dans ces cardiomyocytes en hypoxie.

Puis, cette année, Nguyen (Nguyen, Maltais et al. 2010) vient de retrouver une augmentation de sFRP1 et de sFRP4 dans le surnageant de MSC d'origine porcine cultivées en hypoxie.

Enfin, très récemment, la relation entre le faible niveau en oxygène (1,5%) dans lequel sont cultivées des cellules souches embryonnaires murines et le système Wnt/Fzd a été mise en exergue par l'équipe de Simon (Kaufman 2010; Mazumdar, O'Brien et al. 2010). Celui-ci montre tout d'abord que la voie Wnt/β-caténine est activée dans les ES cultivées en hypoxie comparée aux ES cultivées à 20% d'O2. Des gènes cibles du système Wnt sont d'ailleurs surexprimés comme *Axin2* ou *Dkk-4*, ainsi que *Lef-1* et *Tcf-1* et cet effet semble GSK-3β dépendant. Par ailleurs, ces constatations disparaissent lorsqu'ils utilisent des ES délétées pour le HIF-1α, laissant penser que l'activation du système Wnt est sous la dépendance de HIF. En fait, les auteurs montrent la présence, sur la séquence génomique de *Lef-1* et *Tcf-1*, de plusieurs séquences HRE (Hypoxia Response Elements) et montrent que HIF régule directement les quantités cellulaires de LEF/TCF dans ces cellules. Par l'utilisation de cellules surexprimant LEF-1, ils mettent en évidence une activation de la voie Wnt/β-

caténine, concluant que HIF active la voie canonique par surexpression de LEF-1 en agissant directement sur le génome. Enfin, cet effet disparaît lorsque les cellules entrent en différenciation neuronale, suggérant alors des modifications épigénétiques empêchant l'accès de HIF aux gènes *Lef-1* et *Tcf-1* (Figure 49).

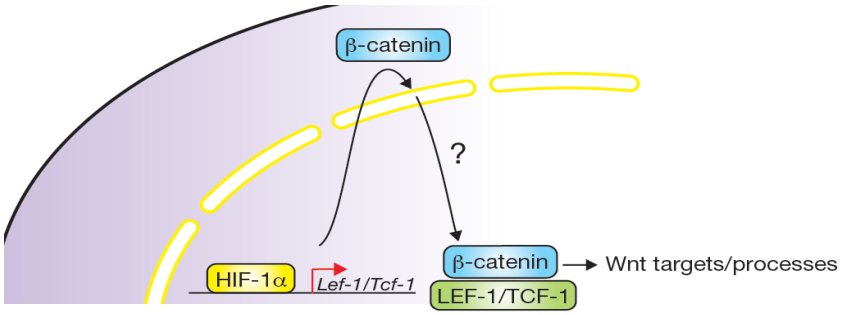

Figure 49. Action de HIF sur la voie Wnt (D'après Mazumdar 2010 et Kaufman 2010)

Les auteurs montrent ensuite que ce système est actif *in vivo.* Dans des niches de cellules souches situées dans la région de l'hippocampe de cerveaux adultes, ils mettent en évidence une faible teneur en oxygène correspondant à des zones d'activité de la voie Wnt/β-caténine dépendante de HIF.

TRAVAUX ET RESULTATS

IV) TRAVAUX ET RESULTATS

A) ETUDE DE L'INTERET D'UN NOUVEAU SYSTEME DE DELIVRANCE DES CELLULES SOUCHES POUR AMELIORER L'EFFICACITE DE LA THERAPIE CELLULAIRE

1) Introduction

Parmi les questions concernant la thérapie cellulaire, celle de la technique d'implantation reste primordiale. Cette technique doit permettre d'amener suffisamment de cellules au bon endroit et au bon moment, sans altérer leurs capacités thérapeutiques. Quelque soit la voie utilisée, peu d'auteurs proposent actuellement de renouveler cette implantation dans le temps ou d'implanter un réservoir de cellules permettant une délivrance étalée dans le temps de la thérapeutique.

Par ailleurs, des techniques de cardiomyoplastie tissulaire par utilisation d'un « patch » musculaire issu du grand dorsal a été testé chez l'homme au début des années 1990, avant d'être finalement abandonnée (Hagege, Desnos et al. 1995; Hagege, Desnos et al. 1996). Cette thérapie tissulaire avait pour but, non pas de régénérer le muscle cardiaque, mais de suppléer à sa déficience contractile. Malgré de nombreuses améliorations, utilisant des greffons pédiculés, la stimulation électrique ou des facteurs de croissance, le muscle grand dorsal, ainsi utilisé, évolue inexorablement vers une fibrose irréversible.

Cependant ce « patch » autologue au contact du muscle cardiaque pouvait être envisagé comme vecteur de cellules. Celles-ci seraient alors incorporées dans une matrice riche en collagène polymérisant à 37°C puis déposées sur le « patch » musculaire. Enfin ce dernier serait appliqué en regard de la zone infarcie du ventricule. C'est cette hypothèse qui a été testée au laboratoire par L. Barandon, peu avant mon arrivée, sur un modèle murin d'infarctus par cryolésion. (Barandon, Couffinhal et al. 2004). Des souris C57/Bl6 étaient réopérées 30 jours après réalisation d'un infarctus par cryolésion et un « patch » de muscle abdominal était cousu sur la zone infarci après avoir été recouvert de $5X10^6$ BMC provenant de souris ROSA26 exprimant de façon constitutive la β-galactosidase. Il a alors montré dans

ce système que 2 semaines plus tard, environ 8% des cellules avaient été capables de migrer vers la zone infarcie. Dans le groupe de souris traitées, le remodelage cardiaque, la fonction ventriculaire et la densité capillaire étaient augmentés par rapport au groupe contrôle. Les effets bénéfiques et du « patch » et de la thérapie cellulaire s'additionnant dans ce modèle (Figure 50).

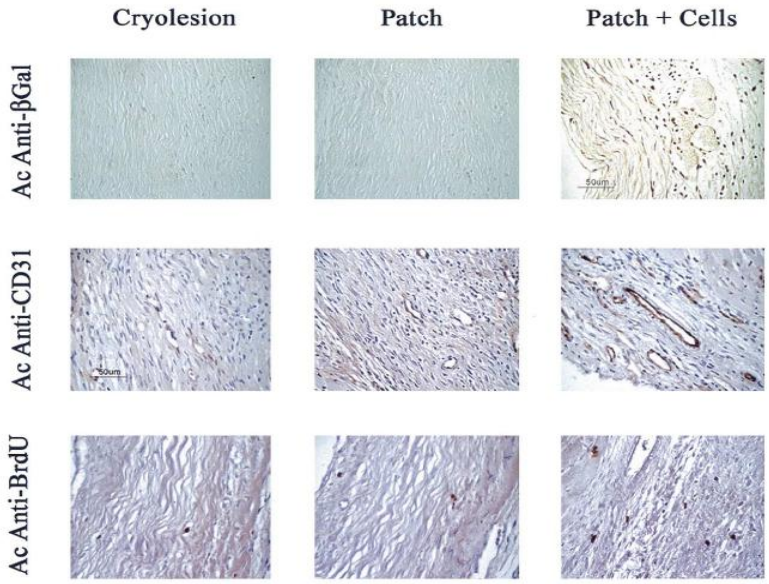

Figure 50. Evaluation histologique de la présence des cellules transférées, de la densité capillaire et de la prolifération cellulaire, dans la cicatrice (D'après Barandon 2004).

2) Hypothèse et but du travail

J'ai alors participé à un travail visant à étudier le devenir de MSC ou d'EPC ainsi transférées et à déterminer si la présence surajoutée de BMC pouvait améliorer les résultats. En effet, les résultats obtenus avec les BMC étaient encourageant, mais 15 jours après la mise en place du « patch », plus de 90% des cellules mouraient ou n'étaient pas transférées. Nous avons alors formulé l'hypothèse que l'utilisation de ce système de délivrance était valable pour le transfert de cellules souches sélectionnées, comme les cellules souches mésenchymateuses (MSC) ou les précurseurs endothéliaux (EPC), et que cette présélection permettrait d'améliorer l'effet thérapeutique. Par ailleurs, partant du principe que, dans la moelle osseuse, les cellules souches cohabitent, nous avons également voulu déterminer

quel était le rôle de l'ajout de cellules souches mononucléées médullaire (BMC) sur notre système.

3) Résultats

L'ensemble des résultats est détaillé dans l'article suivant « **Epicardial deposition of endothelial progenitor and mesenchymal stem cells in a coated muscle patch after myocardial infarction in a murine model** ». Des MSC et des EPC ont été transfectées avec un lentivirus leur permettant d'exprimer la GFP (Green Fluorescent Protein). Des souris C57/Bl6 ont subi un infarctus du ventricule gauche par cryolésion. Trente jours plus tard elles étaient réparties en 5 groupes et réopérées : 11 animaux contrôle recevaient un « patch » sans cellules, et 4 groupes de 24 animaux recevaient un « patch » avec soit $5X10^5$ GFP/MSC, soit $5X10^5$ GFP/EPC, soit une association $5X10^5$ GFP/MSC + $5X10^6$ BMC, soit une association $5X10^5$ GFP/EPC + $5X10^6$ BMC. La mortalité attendue de 40%, a été la même dans tous les groupes. L'analyse à 15 jours a permis de montrer que si les MSC étaient capables de migrer vers la zone bordante de l'infarctus, ce n'était pas le cas pour les EPC. Par ailleurs, l'adjonction de BMC (non détectables) améliorait de manière significative le nombre de MSC capables de migrer dans la paroi du ventricule. Pour autant, la densité capillaire ou l'épaisseur de la cicatrice, plus élevées que dans le groupe contrôle, étaient comparable entre les 4 groupes traités.

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Epicardial deposition of endothelial progenitor and mesenchymal stem cells in a coated muscle patch after myocardial infarction in a murine model☆

Nicolas Derval [a,b,c], Laurent Barandon [a,b,c,*], Pascale Dufourcq [c], Lionel Leroux [a,b,c], Jean-Marie Daniel Lamazière [c], Danièle Daret [c], Thierry Couffinhal [a,b,c], Cécile Duplàa [c]

[a] Cardiovascular Surgery and Cardiology Department, Hôpital Haut-Lévêque, Pessac 33604, France
[b] Medical University of Victor Segalen Bordeaux 2, Bordeaux, France
[c] Inserm U828, 33600 Pessac, France

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Abstract

Objectives: To assess, using an *in vivo* engraftment strategy combining bone marrow cell (BMC) transplantation and tissue cardiomyoplasty, the functional outcome of distinct vascular progenitor cell therapy (endothelial progenitor (EPC) and mesenchymal stem (MSC) cells) at distance of myocardium infarction (MI). The study was also designed to test whether scaffold mixing progenitors with unfractionated BMC could improve progenitor recruitment in the damaged myocardium. **Methods:** To track engrafted progenitor cells *in vivo*, cultured murine MSC and EPC were transduced with eGFP lentiviruses. Thirty days after cryogenical induction of MI, C57BL/6J mice were randomized to receive muscle patch placement coated or not (control group), labeled EPC or MSC mixed to the ration of 1:10, or not with unfractionated BMC. Two weeks after transplantation, cardiac function was recorded and heart sections were examined to detect GFP-labeled progenitor cells and analyze cell differentiation. **Results:** This study showed that either type of mono cell therapy improved angiogenesis and cell survival in the scar but only MSC exhibited the capacity to invade the scar. We found no evidence of myocardial or vascular regeneration from progenitor cells. Engraftment of the progenitors/unfractionated BMC mix increased repopulation and thickness of the scar. **Conclusion:** Combined therapy with unfractionated BMC and expanded MSC appeared thus promising for scar repopulation.

Keywords: Mesenchymal stem cell; Bone marrow cells; Progenitor cells; Tissue regeneration; Engraftment; Angiogenesis

1. Introduction

Despite improvements in medical and surgical therapy [1], ischemic heart disease remains the leading cause of heart failure in Western countries. In recent years, there has been a growing enthusiasm for the use of blood and marrow-derived cell-based therapies to protect, repair or regenerate damaged myocardium [2,3]. Conceptually, cell therapy for cardiovascular disease has evolved from the idea that cardiac progenitor or stem cells could regenerate injured myocardium by *de novo* myogenesis to a broader premise that cell therapy may help facilitate tissue repair. Such complementary aspects might include improved cardiomyocyte survival (limited apoptosis), tissue oxygenation (angiogenesis) and improvement in recovery of cellular and tissue function (positive remodeling).

We have previously developed a 3D scaffold method for cardiac tissue engineering in a mouse model of myocardial infarction, in which a muscle patch containing bone marrow cell (BMC) embedded into a 3D collagen matrix is maintained vis-à-vis the myocardial infarct area. In this model, we showed that BMC were able to migrate from the muscle patch into the infarct zone, to increase capillary density and restore cardiac function. The beneficial effect of this cell therapy on ischemic myocardium was likely due to a paracrine release of pro-angiogenic factors and cytokines, thereby facilitating cell survival, cell recruitment and angiogenesis [4].

Bone marrow-derived subpopulations, i.e., endothelial progenitor cells (EPC) or mesenchymal stromal cells (MSC), have shown promise in regenerative medicine [5,6]. MSC have indeed demonstrated their ability to regenerate vessels

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* Corresponding author. Address: Cardiovascular Surgery Department, Haut-Lévêque Hospital, Avenue de Magellan, 33600 Pessac, France.
Tel.: +33 5 57 89 19 79; fax: +33 5 56 36 89 79.
E-mail address: laurent.barandon@chu-bordeaux.fr (L. Barandon).

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and damaged myocardium in several experimental studies [7–9]. EPC are present in the systemic circulation, increase in response to tissue ischemia, home to and incorporate into sites of neovascularization [10]. Moreover, mobilization and recruitment of EPC contributes to vasculogenesis *in vivo* [11]. EPC and MSC appear to be attractive cells in therapy because of their role as source of cytokines and growth factors but also because of their supposed cardiomyocyte and endothelial transdifferentiation potential [12,13]. As stem cells reside in highly regulated microenvironments called niches [14,15], a recent scenario for stem cell therapy envisioned the use of a mixture of bone marrow cells with enriched purified stem cells to recreate a niche in the treated tissue.

In view of these findings, we sought to assess the fate and outcome of purified and expanded EPC and MSC on vascular and cardiac regeneration into the infarct myocardium following engraftment in a 3D collagen patch model. In addition, as environment has a crucial role in the commitment of stem cells, we compared the functional benefit of mixing unfractionated BMC with either purified progenitor cell populations on angiogenesis and myogenesis within the ischemic heart.

2. Materials and methods

2.1. Isolation and preparation of stem cells

2.1.1. Bone marrow cells (BMC)

Bone marrow cells were harvested by flushing the tibiae and femurs of C57BL/6 mice (Charles River Laboratories) with DMEM, and further purified in Ficoll Paque (Amersham Biosciences, Orsay, France).

2.1.2. Endothelial progenitor cells (EPC)

1×10^6/ml mononuclear cells without any further cell subpopulation enrichment procedure were plated on different culture dishes coated with fibronectin (Sigma) in endothelial basal medium (EBM2) (Clonetics Inc., San Diego, California, USA) supplemented with endothelial growth medium Single Quots and 20% FCS, and incubated at 37 °C in a 5% CO_2 humidified environment. After 24 h, unattached cells and debris were removed by washing with medium. Culture medium was changed every 5 days, and cells were characterized and used after 12 days of the primary culture.

2.1.3. Mesenchymal stem cells (MSC)

Mononuclear cells were resuspended and seeded at a density of 1×10^6 cells/cm^2 in petri dishes and cultured in McCoy's medium (Invitrogen) with 10% BFS and 10% horse serum. Five to seven days later the nonadherent hematopoietic cells were discarded. The adherent bone MSCs were maintained in a humidified incubator at 37 °C with 5% CO_2 in the absence of any exogenous growth factor or anchoring materials such as fibronectin or collagen. The attached cells grew and developed colonies in 15–30 days, when the colonies were subcloned and re-seeded. Five selected clones were passed more than eight times prior to their characterization and further use. These clones displayed all the same phenotypic characteristics.

2.2. Phenotype characterization of EPC and MSC

To detect the uptake of 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine-labeled acetylated LDL (DiI-acLDL), cells were incubated with DiI-acLDL (2.5 μg/ml) (molecular probe) at 37 °C for 1 h.

MSC and EPC were characterized using immunostaining. Cells from cultures were rinsed twice with PBS and fixed immediately with 2% PFA for 10 min, permeabilized (0.2% Triton X-100, 2 min) and saturated with 5% of BSA (Sigma). Endothelial or EPC markers were used: CD31 (Pharmingen), CD34 (Immunotech Inc.), and VE-cadherin (Santa Cruz Biotechnology Inc.), leukocytes markers: CD45 (30-F11, Pharmingen), CD4 (W3/25, Serotec), CD3 (Becton Dickinson), mesenchymal stem cell markers Sca1 (D7, Becton Dickinson), cKit (Santa Cruz), Thy1 (sc9163, Santa Cruz), Endoglin (sc18893, Santa Cruz), yoyo-1 (Molecular Probes) for nucleus staining. Biotinylated secondary antibodies used were: anti-rabbit IgG or anti-mouse IgG (Amersham), or anti-goat IgG (Jackson ImmunoResearch Laboratories Inc). Streptavidin–FITC complex or Alexa 568 was used for immunofluorescence analysis (Molecular Probes). All primary and secondary antibodies were diluted in PBS plus 1% BSA.

Total RNAs were prepared from cultured cells in guanidinium thiocyanate buffer, and reverse transcription-polymerase chain reactions (RT-PCR) were carried out as previously described [16]. Negative controls without RT were prepared in parallel for each RNA sample. The list of primers used to characterize each cell type is shown in Table 1.

2.3. Transduction of stem cells with lentivirus

Cultured EPC or MSC were transduced with SIN eGFP lentiviral vector from Moreau-Gaudry laboratory by replacing the medium with fresh medium containing viral supernatant and incubating for 3 h at 37 °C. Cells were harvested 48 h after the infection and seeded as described above.

Table 1
List of primers used for EPC characterization

Gene	Sense (5′–3′)	Antisense (5′–3′)
VE-cadherin	CCTGTAGGGAAAGAGTCCATTGTG	ACTTGACCGTGATGTTGGCG
Flk-1	GCCTACCTCACCTGTTTCCTGTATG	TTACTTCTGGTTCCTCCAATGGG
Flt-1	TACGAAAAGTCCGTGTCCTCGC	AGCCACCACCAATGTGCTAACC
Angiopoietin-1	GCACGAAGGATGCTGATAACGAC	TGATTTTGTCCCGCAGTGTAGAAC
Angiopoietin-2	GCACAAAGGATTCGGACAATGAC	GTTCTTCTGGTAGTGTAGGCAGGC
Tie-1	CGAAACTGCGATGACGAAGTG	CAGAGGTGAGAAGGTTCCAAAGTC
Tie-2	AGTGCGTTGGATGGCAATCG	TGATTTTGTCCCGCAGTGTAGAAC
α-Actin	GGAGGAAGAGGATGCGGCA	GAAGCTGTGCTATGTTGCTCTA

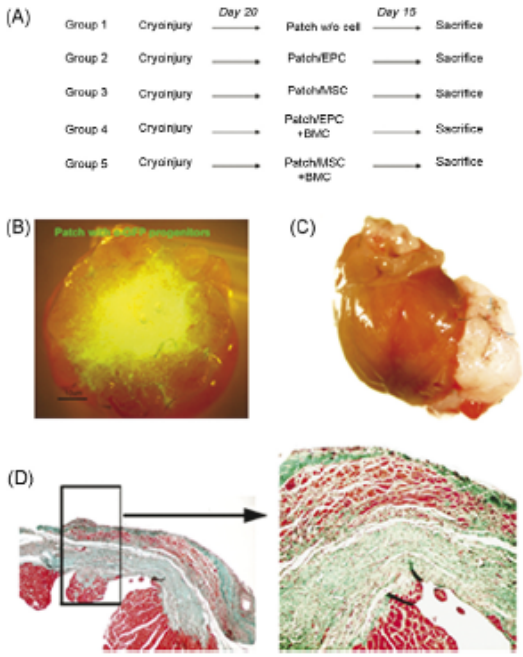

Fig. 1. Epicardial deposition of stem cells coated on an abdominal muscle patch. (A) Study design. (B) Thirty days after MI induction by cryoinjury, an abdominal muscle patch was dissected and then cupped by means of a purse suture to adapt its shape to the LV geometry and to form a cell tank in which stem cells were deposited. (C) The coated muscle patch is shown wrapped around the infarcted zone 15 days after patch implantation. (D) Masson's trichrome staining at respectively magnification ×4 and ×10.

Transduction was repeated for each EPC culture. MSC were transduced once and retained the fluorescence during the following passages (Fig. 1C). BMC were not GFP-transduced and were directly seeded in the patch, unlabeled.

2.4. Mice muscle patch model

All mice used were male C57BL/6J (Charles River), 9–12 weeks old and weighing 26–30 g. All mice received humane care in compliance with the European Convention on Animal Care (L358-86/609/EEC). Myocardial infarction was induced by cryoinjury as previously described [4].

2.4.1. Study treatments

Mice were randomly assigned to five treatment groups (Fig. 1A). One group underwent sham treatment (i.e., rethoracotomy and immediate closure) 30 days after MI induction. The other groups received rethoracotomy and placement of a muscle patch coated with EPC or MSC ± BMC adjunction 30 days after MI induction (Fig. 1A). All groups were also injected with BrdU 24 h before sacrifice in order to measure the rate of cell proliferation. All mice that survived for 15 days were sacrificed.

2.4.2. Isolation and placement of muscle patches

The muscle patches were obtained as previously described [4]. Briefly, 30 days after MI, a 1 cm × 1 cm patch of abdominal muscle was harvested and cupped so as to fit the left ventricular geometry and form a tank in which cells were deposited and secured (Fig. 1C and D).

2.4.3. Deposition of stem cells on muscle patch

We used 5×10^6 BMC, 5×10^5 eGFP/MSC and 5×10^5 eGFP/EPC in their corresponding groups. These stem cells were mixed into a growth factor-deprived liquid matrix rich in collagen type 1 (200 μl, 200 mg/ml, Becton Dickinson) [4]. This mixture was then deposited within the muscle patch and the coated muscle patch was positioned on the exposed epicardium directly above the MI zone.

2.5. Hemodynamic analysis

Hemodynamic analysis was performed as previously described using a 1.4F Millar transducer (Millar Instruments, Houston, TX) [4].

2.6. Histological analysis

2.6.1. Morphometric analysis

Infarct size was determined in a minimum of six mice in each treatment group as described previously [17].

2.6.2. Immunochemistry and confocal analysis

Mice were sacrificed by lethal injection of potassium chloride. Their hearts were fixed by pressure perfusion with a 4% paraformaldehyde solution and cut perpendicularly to the longitudinal axis of the LV in the middle of the patch. These two sections were embedded in OCT compound and stored at −80 °C. To detect cell repopulation in the scar, tissue sections were incubated with popo (popo™-3 iodide, Molecular Probes). To detect angiogenic activity, endothelial cells were stained with CD31 antibody (Pharmingen). To determine the extent of transplanted cell proliferation, specimens were immunostained with a BrdU antibody (Harlan). A minimum of 30 random digital photographs was taken at ×40 magnification for each mouse specimen analyzed. Positively stained eGFP cells or CD31-positive capillaries and BrdU positive cells were manually counted by a blinded observer with the help of Sigma Scan Plot software.

To evaluate cell differentiation, a CD31 antibody (BMA) was used for endothelial cell detection, a smooth muscle α-actin antibody for smooth muscle cell detection, and an anti-α-cardiac sarcomeric actin (Abcam, 5C5) for myocyte detection. Slides were analyzed under a confocal microscope searching for eGFP/rhodamine double staining.

The number of transplanted cells, capillary density, and cell proliferation per square millimeter were determined and recorded.

2.7. Statistical analysis

All data were expressed as mean ± SD. All analyses were performed using appropriate software (Statview 5.1). Comparisons of continuous variables between two groups were made using one-way ANOVA and, when a statistically significant difference was observed, a two-sided paired *t* test. A value of $p < 0.05$ was considered statistically significant.

3. Results

Recent evidence has shown that it is possible to improve cardiac function or perfusion or to decrease the loss of viable myocardium and with bone marrow-derived progenitor cells. To identify a source of progenitors capable of restoring damaged cardiac tissue, we engrafted EPC and MSC embedded in a muscular patch placed above the cardiac ischemic area.

3.1. Phenotype of EPC and MSCs

We used both EPC and MSC to test their vascular or cardiogenic potential *in vitro* and *in vivo*. First, the expression of genes of primitive and fully differentiated endothelial and smooth muscle cells was analyzed in both cell lines.

The surface markers of EPC were determined by immunostaining and flow cytometry analyses as previously reported. EPC were co-stained with acetylated-LDL (Fig. 2A). EPC expressed VEGF Receptor 2, CD31 and CD34 (data not shown). Real-time PCR amplification showed that EPC featured expression of genes reflective of endothelial lineages (VE-cadherin, Flk-1, Flt-1, angiopoietin-1 and 2, Tie-1 and 2) (Fig. 2A). MSC expressed moderate to high level of Sca1, cKit, Thy1 while Endoglin, CD31, CD34, CD3 and CD45 were negative (data not shown and Fig. 2A). To track engrafted cells *in vivo*, both EPC and MSC were infected at a low level with a lentiviral vector expressing GFP that did not alter *in vitro* growth rate and viability. Almost 100% of the cells expressed GFP after a single round of infection.

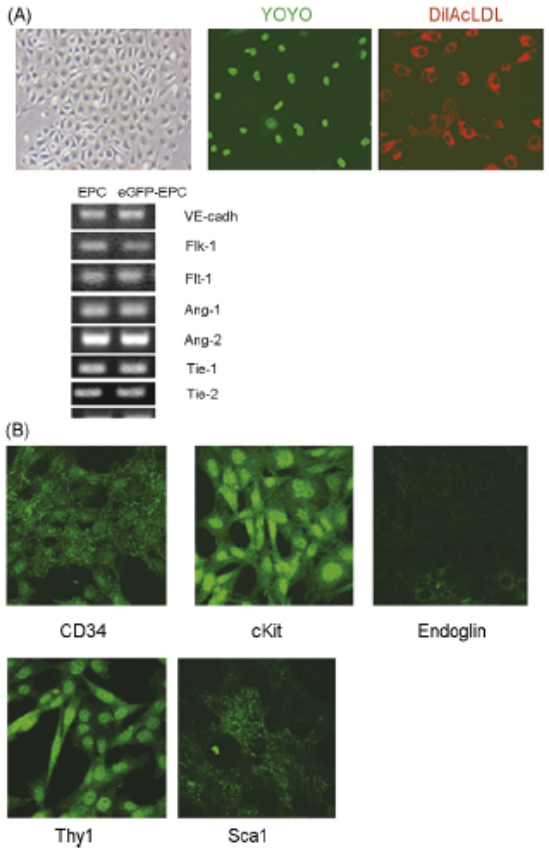

Fig. 2. Progenitor cell characterization. (A) EPC in culture. The majority of cultured EPC were cobblestone-like EC and are able to incorporate the DiI-acLDL. RT-PCR confirmed that mouse EPC expressed endothelial cell lineage markers before and after infection with eGFP-lentivirus (eGFP-EPC). The eGFP labeling did not modify the transcriptional profile of EPC. (B) Immunostaining of MSC showed that MSC expressed CD34, cKit, endoglin, Thy1 and sca1.

3.2. Myocardial infarction and mortality

Of the 119 C57BL/6 mice that underwent MI induction by cryoinjury, 12 mice died during the first 24 h (10%). Thirty days after MI, 96 C57BL/6 mice received progenitor cells embedded in the muscular patch. For this purpose, mice were randomized to five groups: the first group received 5×10^5 eGFP-labeled EPC ($n = 24$), the second group, 5×10^5 eGFP-MSC ($n = 24$), the third group, a mix of 5×10^5 eGFP-labeled EPC and 5×10^6 unlabeled BMC ($n = 24$) and the fourth group, a combination of 5×10^5 eGFP-labeled MSC and 5×10^6 unlabeled BMC ($n = 24$). The last group was the control (operation with uncoated patch) performed on 11 animals (Fig. 1). After coated-patch implantation, the early mortality rate was approximately around 40%, consistent with our previous observation. The mortality did not differ significantly between the four groups.

3.3. MSC but not EPC can migrate from the implanted patch to the infarcted area

We then investigated the capacity of the progenitors to penetrate and migrate into the ischemic and scar area. In MI hearts covered with a patch containing MSC, 46.6 ± 3.1 eGFP-positive MSC/mm^2 were detected in the MI area, representing about 1.5% of the total cells of the scar. Most of eGFP-positive cells were detected in the ischemic border zone while none were found in uninjured zones. In contrast, only sporadic eGFP-positive EPC were detected in the MI area in patch plus EPC-treated mice (Table 2 and Fig. 3). These findings suggested that, in this model of epicardial cell deposition, only MSC but not EPC could be successfully mobilized to migrate to the MI area.

3.4. Epicardial deposition of MSC or EPC increased angiogenesis and cell survival in the scar

As formation of new blood vessels within the provisional ventricle wound matrix is known to be essential for normal healing, frozen heart sections were immunostained with anti BrdU and CD31 antibodies to evaluate respectively cell proliferation and capillary formation as markers of myocardial recovery in the different groups. Examination of the sections from mice grafted with patch plus either EPC or MSC showed a significant increase of the proliferation and of the capillary number as compared to sections from control mice (Table 2, $p < 0.01$). The proliferation and capillary density were similar in sections from both EPC- and MSC-treated mice (Table 2, p = NS).

Table 2
Cellular composition of myocardial infarction scar

	Treatment group				
	Sham ($n = 11$)	MSC ($n = 14$)	EPC ($n = 14$)	MSC + BMC ($n = 16$)	EPC + BMC ($n = 14$)
eGFP positive cells/mm^2	0	$46.6 \pm 3.1^{*}$	0.8 ± 3.1	$286 \pm 11.5^{**}$	0.1 ± 2.5
Total number of cells/mm^2	3119 ± 376	$3441 \pm 478^{*}$	3178 ± 539	$4018 \pm 353^{*}$	$3995 \pm 502^{*}$
BrdU positive cells/mm^2	47 ± 13	$141 \pm 17^{*}$	$153 \pm 24^{*}$	$160 \pm 56^{*}$	$152 \pm 45^{*}$
Capillary density/mm^2	119 ± 21	$482 \pm 39^{*}$	$490 \pm 26^{*}$	$486 \pm 142^{*}$	$502 \pm 131^{*}$
Scar thickness (mm)	0.30 ± 0.13	0.37 ± 0.14	0.35 ± 0.13	$0.53 \pm 0.17^{*}$	$0.51 \pm 0.12^{*}$
Infarct size (% LV necrosis)	27 ± 5	26 ± 2	25 ± 3	27 ± 5	26 ± 5

Values are expressed as mean ± SD.
* $p < 0.01$ as compared to control group.
** $p < 0.01$ as compared to MSC or EPC alone-treated group.

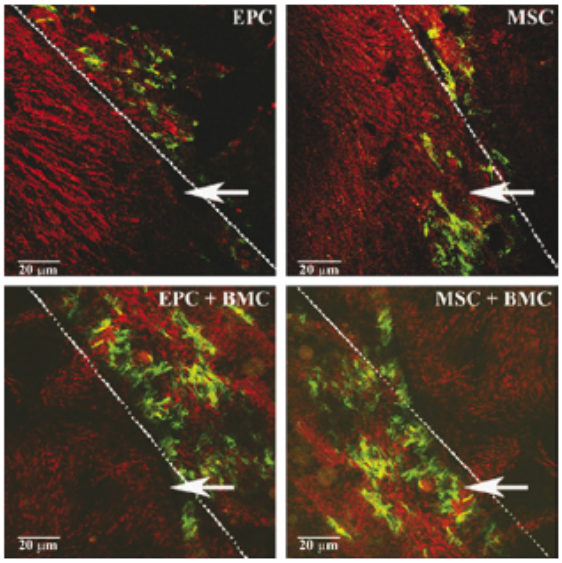

Fig. 3. Stem cell migration to the scar. Transplanted stem cells that have migrated into the scar are detected by eGFP analysis under fluorescent confocal microscopy. The white line represents the border zone between the infarct scar and the coated muscle patch. Arrows represent cell migration from the patch to the scar. In EPC or in EPC + BMC coated muscle patch, only sporadic eGFP-positive cells were found in the scar. Better results were found in the MSC-coated muscle patch group. In the MSC + BMC coated muscle patch group, the number of eGFP-positive cells was significantly higher in the scar, $p < 0.01$.

3.5. Mobilization of progenitor cells did not contribute to the regeneration of vascular endothelium, smooth muscle or cardiac tissue

Here, we analyzed whether mobilized progenitor cells can differentiate *in situ* to form capillary network or to regenerate cardiomyocytes using immunofluorescent techniques. In the myocardial area, a few of eGFP-positive MSC cells (less than one per MI heart) were positive for CD31 or α-actin staining using confocal microscopy analysis (Fig. 4), suggesting that transdifferentiation or fusion between transplanted and resident cells were very rare events. Moreover, no transdifferentiation into cardiomyocytes was observed as eGFP-positive MSC cells remained negative for α-sarcomeric actin (Fig. 4).

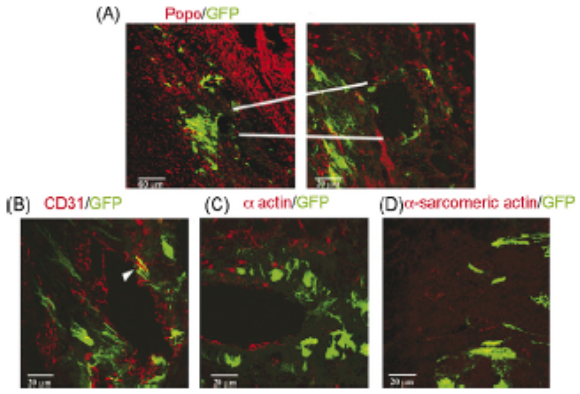

Fig. 4. Differentiation of MSC *in vivo*. (A) Low and high magnification of scar tissue after cell nucleus staining with popo™-3 (red) revealed engraftment of GFP-positive MSC in a repopulated scar. Immunostaining (red) of cryosection from MSC-engrafted myocardium using an anti CD31 (B), an anti-α-actin (C) and anti-α-sarcomeric actin antibodies (D). Images were acquired in confocal microscopy. GFP expression in vascular structures colocalized only with CD31 in a rare event. GFP and CD31 co-staining was evidenced by the yellow staining pattern (arrow) (B).

3.6. Mixing total BMC with MSC increased MSC numbers in the scar, modified myocardial infarction scar tissue but did not promote MSC transdifferentiation

To determine the role of the environment on progenitor behavior, we tested whether a mixture of total BMC either with EPC or MSC could be instrumental to trigger progenitor cells to migrate and achieve tissue repair in the infarct scar.

An eGFP-MSC mix with BMC adjunction led to a higher quantity of MSC in the scar as compared with eGFP-MSC alone ($p < 0.01$; Table 2). A mean of 286 ± 11.5 eGFP-positive MSC/mm^2 was detected in the necrotic area, representing 7% of the cells into MI area, in the group MSC plus BMC (Table 2, Fig. 3). In contrast, BMC adjunction to EPC in the patch did not induce EPC migration in the scar. We next tested whether BMC could provide a microenvironment that would contribute to MSC commitment. We failed to detect any progenitor cell that co-expressed eGFP and either endothelial or cardiac markers indicating that BMC environment did not favor MSC incorporation into neovascular structure or into cardiac tissue.

Noteworthy, BMC adjunction modified infarct scar tissue since the total cell number in the scar was significantly higher

in the BMC plus EPC or MSC groups than in the progenitor cells alone groups (Table 2). One of the consequences was a significant increase in scar thickness, probably caused by an increase in cell density due to BMC invasion. However, cell proliferation and capillary density were not significantly different between the progenitors groups with or without BMC (Table 2).

3.7. MSC and EPC engraftment improved cardiac function

Infarct size as measured by the percentage of left ventricle necrosis was similar in all groups, control, and EPC or MSC-patch with or without BMC. This was not surprising as the cryolesion-induced MI was done 30 days before cell therapy was applied. Cardiac function as measured by dp/dt was significantly increased in all groups compared to the control group. However no significant differences were found between the four progenitor groups (Table 3).

4. Discussion

The main new findings of the study are: (1) at 2 weeks after deposition of either progenitor cells, only MSC but not EPC are able to migrate to the ischemic zone, (2) both EPC and MSC have the capacity to enhance capillary formation and cell survival in the scar but do not give rise to vascular or myocardial cells (3) combined progenitor cell with unfractionated BMC therapy results in an enhancement of MSC migration to the scar and favors scar repopulation.

4.1. Background and muscle patch cell delivery

In a precedent study, we showed the feasibility of improving cardiac function and remodeling irreversibly damaged myocardial tissue after MI by embedding BMCs in a collagen-rich matrix, applying the matrix directly to the damaged epicardium. The intramyocardial injection technique is limited by (1) the extensive mechanical trauma to cells (and consequent apoptosis) that can be caused by the syringes and needles that must be used, (2) the limited total number of cells in solution that can be injected, and (3) and the inhospitability of the scar environment to transplanted cells. Thus, in developing our epicardial deposition strategy, we have tried to offset these disadvantages by making it possible to (1) use unrestricted numbers of transplanted cells; (2) deposit cells in a trauma-free way that avoids the use of inappropriate devices and avoids immunogenic reactions; (3) place transplanted cells in a favorable environment (i.e., collagen) conducive to their survival, proliferation, and migration; and (4) use a homologous muscle patch as a reservoir in which to allow transplanted cells in the matrix to form a stronger, more adherent bond with the patch itself. We demonstrated that this combined strategy could increase capillary density, improve cardiac function and increase scar thickness. The effect on scar thickness could be related to an important cell migration, cell survival and proliferation rate. Moreover, we had found that autologous abdominal muscle patches could become easily integrated into, and in some cases even merge with, the epicardium. These advantages were offset somewhat by the tendency of the abdominal muscle patches to become thinner over time because of ischemic complications; however, this thinning was significantly less severe when the patch was coated with BMCs, presumably owing to the evident tropic and degradation-inhibiting effects of the BMCs. We were not able to demonstrate any inflammatory response in the different sites of fixation. This data showed that the paracrine effect, increasing capillary density in the scar, could not be related to a secretion of growth factor by the postoperative inflammatory response [4].

4.2. Progenitor cell differentiation

Our goal was to define the optimal cell type for myocardial revascularization in this epicardial deposition model. It has been demonstrated that, in some culture conditions, progenitor cells are able to transdifferentiate into vascular cells or cardiomyocytes [18,19]. We isolated and cultured MSC and EPC that presented the same characteristics and properties as previously reported [20,21]. To date, EPC and MSC have been used for cardiac regeneration [2,9,22]. Functional efficacy of such progenitor cells has been well documented, even if there are some controversies in their *in vivo* and *in vitro* differentiation abilities [23,24]. However, with more than 50 mice carefully examined, we found no evidence of vascular or myocardial regeneration from immunolabeled injected progenitor cells.

4.3. Modification of infarct scar

3D engraftment of EPC and MSC has improved cardiac function although scar area was not reduced by any of the cell types. Indeed, we chose a delayed timing of cell delivery to make the protocol more clinically relevant. In a clinical perspective, such a deleterious environment remains a major step for rationalizing the progenitor use. It is also possible that this delay of 1 month after MI onset for progenitor engraftment could be a limit in the repair benefit. Nevertheless, MSC, but not EPC, were able to migrate to the infarct scar but to a limited extent (1% of the scar cellularity), limiting their functional potential in situ.

Table 3
Comparison of the groups on cardiac function, hemodynamic study as measured by dp/dt max

	Sham	Control	MSC	EPC	MSC + BMC	EPC + BMC
Heart rate (bpm/min)	413 ± 23	387 ± 28	361 ± 91	356 ± 73	381 ± 86	372 ± 84
dp/dt max	11263 ± 302	6115 ± 257	7655 ± 214*	7519 ± 351*	7891 ± 265*	7628 ± 311*

Values are mean ± SD.
* $p < 0.01$, compared with control group.

However, independent of their migration capacities, both progenitor cells induced modifications of the infarcted myocardial tissue (increased in capillary density and cell proliferation) as compared to control mice. As we did not detect any transdifferentiation into endothelial or smooth muscle cells or cardiomyocytes, the angiogenic effect and cell proliferation in the scar were probably induced via a paracrine effect of the progenitors. *In vitro* characterization has shown that both MSC and EPC were able to secrete growth factors, angiogenic factors and some cytokines (laboratory unpublished data) [13,25].

4.4. Bone marrow cell environment benefit

We investigated whether the adjunction of BMC could enhance EPC or MSC numbers in the infarct area. Mix of MSC with unfractionated BMC gave rise to larger MSC fraction in the scar than that with MSC alone. EPC did not migrate in the scar even if BMC were added. This suggests that BMC create a specific and favorable environment that facilitate and/or preserve MSC repopulation into the scar. This further emphasizes that BMC environment is crucial to ensure a proper MSC engraftment or migration to the scar but may not allow their commitment toward a vascular or myogenic phenotype. We should however point out that the increase in MSC population did not account for a better capillary formation or cell proliferation.

5. Conclusion

This study suggests that a combined strategy of either unfractionated or purified progenitor cell therapy with cardiomyoplasty increases capillary density, cell proliferation and repopulation into the scar even at distance of an MI event. Expanded MSC kept the capacities to invade and participate in the scar repopulation but are not directed to form vascular or cardiac cells. Our study suggests mixing BMC with MSC is critical for the success of MSC engraftment and is promising as to the use of MSC in cell therapies.

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4) Discussion

Ce travail a permis de montrer qu'une technique de délivrance de cellules par juxtaposition d'un gel de collagène inclus dans un « patch » musculaire était efficiente sur le muscle cardiaque infarci et permettait d'ajouter les bénéfices du « patch » avec ceux de la thérapie cellulaire. Ce système permet de déposer de manière atraumatique un grand nombre de cellules, ce qui n'est pas le cas lors de l'injection directe dans les tissus ; de conserver les cellules dans un environnement plus favorable que le tissus nécrosé, permettant une meilleure survie et donc une mise à disposition prolongée des cellules souches aux tissus nécrosés ; et de montrer que l'adjonction de BMC améliorait les capacités de migration des MSC.

En effet, contrairement aux EPC, les MSC ont montré d'étonnantes capacités de migration dans les tissus, et en particulier dans les zones bordantes. Cette caractéristique sous-entend un équipement enzymatique adéquat et permet d'envisager une nouvelle méthode de délivrance des cellules où celles-ci seraient déposées, autant de fois que nécessaire, de manière peu invasive, dans un système de réservoir, fixé en regard de la zone nécrosée. Car, comme nous l'avons déjà fait remarqué, aucune étude jusqu'alors n'a permis de tester l'hypothèse que la thérapie cellulaire, sinon continue, devrait être répétée. Ces études sont cependant aujourd'hui en cours. Lors de nos expérimentations toutefois, la cardiomyoplastie ayant été abandonnée, une perspective alors séduisante était d'envisager de combiner la thérapie cellulaire avec la mise en place de filet de contention ventriculaire de type Corcap® de chez ACORN (Figure 51). Ce système implanté par sternotomie chez des patients souffrant de cardiomyopathie dilatée devait permettre de limiter la dilatation ventriculaire à long terme (Walsh 2005). Malheureusement, les premiers résultats d'études randomisées se sont avérés décevants (Starling and Jessup 2004; Starling, Jessup et al. 2007) et la plupart des équipes ont abandonné cette technique.

Figure 51. Filet Corcap (ACORN)

5) Conclusion

Cette étude a donc montré l'efficience d'un système original de délivrance prolongé des cellules souches au myocarde infarci, a confirmé les capacités de migration des MSC et a montré que l'amélioration de l'association avec des BMC avait un effet positif, probablement en améliorant la survie des cellules souches.

B) INTERET D'UN PRECONDITIONNEMENT DES MSC INDUISANT UNE SUREXPRESSION DE SFRP1 SUR L'EFFET PRO-ANGIOGENIQUE DE CES CELLULES

1) Introduction

Comme nous l'avons vu, le système Wnt/Frizzled (Wnt/Fzd) est une voie de signalisation hautement conservée mettant en jeu des récepteurs à 7 domaines transmembranaires Frizzled (Fzd) et leurs ligands Wnt, petites glycoprotéines sécrétées. Ce système contrôle divers processus physiologiques comme le renouvellement des cellules souches (Nusse 2008), l'adhésion cellulaire (Lilien and Balsamo 2005), la différenciation, la polarité, la prolifération cellulaire et le développement tissulaire (Peifer and Polakis 2000; Eisenberg and Eisenberg 2006). Mais il est également impliqué en pathologie, notamment dans le développement de cancers (Reya and Clevers 2005), l'ostéoporose ou les maladies cardiovasculaires (Rao and Kuhl 2010).

Certains inhibiteurs de la voie comme Dickkopf (Dkk) (Aicher, Kollet et al. 2008; Smadja, d'Audigier et al. 2010), sFRP2 (Mirotsou, Zhang et al. 2007; Alfaro, Vincent et al. 2010) ou sFRP1 (Nguyen, Maltais et al. 2010) ont récemment été mis en jeu dans les phénomènes d'angiogenèse. C'est d'ailleurs à sFRP1 que le laboratoire s'est plus particulièrement intéressé ces dernières années et notamment à son rôle dans le développement des vaisseaux après un évènement ischémique. En 1999, le laboratoire a montré que sFRP1 était exprimé à l'état basal dans l'endothélium et qu'il modulait la prolifération des CE (Duplaa, Jaspard et al. 1999). Puis Dufourcq a mis en évidence un effet pro-angiogénique de la protéine (Dufourcq, Couffinhal et al. 2002). En 2003 sur un modèle d'infarctus du myocarde chez des souris surexprimant sFRP1, Barandon retrouve des infarctus de plus petites tailles contenant des vaisseaux plus nombreux et plus souvent musculariséés (Barandon, Couffinhal et al. 2003). Sur un modèle d'ischémie de patte, nous avons montré que l'effet de sFRP1 résultait en deux phases successives : une première phase d'inhibition de la prolifération des CE, puis une seconde phase pro-angiogénique. A la fin du

processus de cicatrisation, les vaisseaux étaient, là encore, significativement plus musculariés (Ezan, Leroux et al. 2004).

2) Hypothèse et but du travail

Nous avons formulé l'hypothèse que le système Wnt/Fzd était impliqué dans l'effet pro-angiogénique des cellules souches mésenchymateuses. Pour étudier cette implication, nous avons utilisé un modèle de matrice injectée en sous-cutanés (Matrigel) chez des souris immunodéficientes. Ces matrices sont progressivement colonisées par des néo-vaisseaux de la souris hôte, constituant un modèle de néo-angiogenèse. Nous avons voulu tout d'abord montrer que la présence de MSC au sein de ces matrices avait un effet proangiogénique. Puis pour étudier l'implication du système Wnt/Fzd dans cet effet, nous avons utilisé des MSC transfectées pour qu'elles surexpriment sFRP1.

3) Résultats

L'ensemble des résultats est détaillé dans l'article suivant : « **Secreted Frizzled-Related Protein-1 Enhances Mesenchymal Stem Cell Function in Angiogenesis and Contributes to Neovessel Maturation** ». Des MSC murines ont été transfectées par un lentivirus de manière à exprimer la GFP. Les matrices ont consisté en 0,25 ml de Matrigel contenant 5µg/ml de FGF2. Elles ont été injectées en sous-cutané au niveau de l'abdomen de souris Nude, soit sans cellule, soit après incorporation de $0{,}5X10^6$ de GFP-MSC. Les animaux ont été sacrifiés à 5, 9, 13 et 90 jours. Les résultats montrent que la présence des MSC accélère la colonisation vasculaire de la matrice. En réalité, tout se passe comme si les MSC commençaient à former un réseau tubulaire, observable dès le $5^{ème}$ jour. Puis au $13^{ème}$ jour, le fait que plus de la moitié des néo-vaisseaux soient bordés de cellules exprimant la GFP et le protéoglycane NG2, spécifique des péricytes, laisse supposer que les MSC recrutent les CE le long de ce réseau et s'y différencient en péricytes. Les MSC participent donc à la formation d'un nouveau réseau vasculaire en organisant et stabilisant les néo-vaisseaux.

Puis des GFP-MSC ont été transfectées pour exprimer soit sFRP1 (sFRP1-MSC), soit la β-galactosidase (βgal-MSC) en contrôle, et utilisées de la même façon. Ainsi, nous avons montré qu'avec les sFRP1-MSC, les matrices, à J13, avaient une densité vasculaire accrue et que les vaisseaux y étaient plus souvent matures comparées aux matrices contenant des βgal-MSC. Par ailleurs, ces néo-vaisseaux étaient plus souvent bordés de cellules positives pour la GFP, laissant supposer que sFRP1 amplifie l'effet de recrutement des CE par les MSC. Cet effet semble dépendant de la GSK-3β. Le PDGF-BB pourrait jouer un rôle primordial.

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TRANSLATIONAL AND CLINICAL RESEARCH

Secreted Frizzled-Related Protein-1 Enhances Mesenchymal Stem Cell Function in Angiogenesis and Contributes to Neovessel Maturation

PASCALE DUFOURCQ,[a,b] BETTY DESCAMPS,[a] NANCY FERREIRA TOJAIS,[a] LIONEL LEROUX,[a,c] PIERRE OSES,[a,c] DANIÉLE DARET,[a] CATHERINE MOREAU,[a] JEAN-MARIE DANIEL LAMAZIÈRE,[a] THIERRY COUFFINHAL,[a,c] CÉCILE DUPLÀA[a]

[a]Institut National de la Santé et de la Recherche Médicale U828, Université Victor Ségalen, Bordeaux2, Pessac, France; [b]Biochemistry Laboratory, UFR Sciences Pharmaceutiques, Université Victor Ségalen, Bordeaux, France; [c]Department of Cardiology, Pole Cardiothoracique, Hôpital Haut Lévêque, Pessac, France

Key Words. Angiogenesis • Cellular therapy • Pericytes • Mesenchymal stem cell

ABSTRACT

Mesenchymal stem cell (MSC) transplantation offers a great angiogenic opportunity in vascular regenerative medicine. The canonical Wnt/β-catenin signaling pathway has been demonstrated to play an essential role in stem cell fate. Recently, genetic studies have implicated the Wnt/Frizzled (Fz) molecular pathway, namely Wnt7B and Fz4, in blood growth regulation. Here, we investigated whether MSC could be required in shaping a functional vasculature and whether secreted Frizzled-related protein-1 (sFRP1), a modulator of the Wnt/Fz pathway, could modify MSC capacities, endowing MSC to increase vessel maturation. In the engraftment model, we show that murine bone marrow-derived MSC induced a beneficial vascular effect through a direct cellular contribution to vascular cells. MSC quickly organized into primitive immature vessel tubes connected to host circulation; this organization preceded host endothelial cell (EC) and smooth muscle cell (SMC) recruitment to later form mature neovessel. MSC sustained neovessel organization and maturation. We report here that sFRP1 forced expression enhanced MSC surrounding neovessel, which was correlated with an increase in vessel maturation and functionality. In vitro, sFRP1 strongly increased platelet-derived growth factor-BB (PDGF-BB) expression in MSC and enhanced β-catenin-dependent cell-cell contacts between MSC themselves and EC or SMC. In vivo, sFRP1 increased their functional integration around neovessels and vessel maturation through a glycogen synthase kinase 3 beta (GSK3β)-dependent pathway. sFRP1-overexpressing MSC compared with control MSC were well elongated and in a closer contact with the vascular wall, conditions required to achieve an organized mature vessel wall. We propose that genetically modifying MSC to overexpress sFRP1 may be potentially effective in promoting therapeutic angiogenesis/arteriogenesis processes. STEM CELLS *2008;26:2991–3001*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Cell therapy and therapeutic angiogenesis are expected to be used in combination with conventional therapies in patients with ischemic cardiovascular diseases. Current approaches imply that mesenchymal stem cells (MSC) may provide angiogenic benefits [1–3]. MSC can be isolated from bone marrow, harbor expansion potential, and can differentiate not only into mesenchymal cells, such as osteoblasts and chondrocytes [4], but also into nonmesenchymal cells, such as vascular cells [5–7]. They have also been exploited in tissue-engineered vascular autografts and heart valves [8, 9]. Under conditions of stress, endocrine signals, released from injured tissue, act to mobilize multipotent MSC from the bone marrow. MSC are then recruited to the damage site for repair and angiogenesis [10]. These features make MSC an attractive therapeutic tool.

Current research indicates that MSC may provide an angiogenic benefit because of their potential to be recruited during arteriogenesis and vascular repair [6, 11], but the mechanism by which these cells enhance neo-vascularization remains unclear. Various studies have shown that bone marrow-derived cells incorporate into newly formed capillaries [1]; however, the absolute number of incorporated and differentiated vascular progenitors varies greatly [12, 13]. Other authors speculate that paracrine effects are responsible for the proangiogenic effect of MSC [14–17]. The difficulty in interpretation of their role could be linked to the diversity of mesenchymal cell types recruited in situ and to the elusive nature of the paracrine signals that are exchanged between MSC and vascular cells.

A major role was attributed to bone marrow-recruited pericytes [18–20] in microvascular stability and function [21]. Previous studies have demonstrated the role of these cells in distinct vessel formation steps, such as elongation, vascular remodeling,

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Correspondence: Pascale Dufourcq, Ph.D., Inserm U828, Avenue du Haut Lévèque, 33600 Pessac, France. Telephone: 33557891972; Fax: 3356368979; e-mail: pascale.dufourcq@biophar.u-bordeaux2.fr Received April 14, 2008; accepted for publication August 12, 2008; first published online in STEM CELLS *EXPRESS* August 28, 2008. 1066-5099/2008/$30.00/0 doi: 10.1634/stemcells.2008-0372

and maturation of blood vessels [22–24]. Furthermore, pericyte density was shown to have an impact on vessel morphology in response to vascular endothelial growth factor (VEGF) [25]. In this regard, we hypothesized that MSC, which express immature pericyte markers [26], could facilitate the anchoring and the maintenance of vascular cells into stable vascular structures. Further development of this concept requires further insight into the mechanisms of MSC maintenance on neovessels. The canonical Wnt/β-catenin signaling pathway has been found to play an essential role in stem cell fate [27–29]. We have previously demonstrated that secreted Frizzled-related protein-1 (sFRP1), a modulator of Wnt/Frizzled (Fz) pathway, has a role in blood vessel formation [30]. Recently, we showed that sFRP1 acts at a later stage, inducing vessel formation in a hind limb ischemia model, and that sFRP1 is able to activate a glycogen synthase kinase 3 beta (GSK3β)-dependent pathway involving Rac1 in endothelial cells [31].

Here, we performed a variety of studies to determine whether MSC could be required in shaping a functional vasculature and whether MSC genetically modified to overexpress sFRP1 would have an enhanced angiogenesis/arteriogenesis capacity compared with control MSC. In a model of xenograft, we assessed the ability of MSC to stimulate angiogenesis. Our study demonstrates that implanted MSC form a primitive vessel-like network that precedes endothelial cell (EC) recruitment. The cells did not differentiate in EC but were consistently recruited as mural cells. We then analyzed the role of sFRP1 on the MSC functional vascular plasticity and showed that forced expression of sFRP1 enhanced MSC organization and differentiation and that this step was a determinant for vessel maturation and stability.

Materials and Methods

Harvest and Culture Expansion of Murine MSC

MSC were isolated and cultured as previously described [32, 33]. Briefly, mouse bone marrow mononuclear cells purified by Ficoll Paque (Amersham Biosciences, Orsay, France, http://www.amersham.com) were resuspended and seeded at a density of 1×10^6 cells per cm^2 and cultured in McCoy's medium with 10% BFS and 10% horse serum (Invitrogen). Five to 7 days later, the nonadherent hematopoietic cells were discarded. The attached cells grew and developed colonies in 15–30 days. Colonies were then subcloned and reseeded in McCoy's medium with 20% serum. Five selected clones were amplified more than eight times prior to their characterization and further use. These clones displayed all the same phenotypic characteristics.

In Vitro MSC Characterization

Immunostaining. Immunostaining was performed as previously described [33, 34]. Cells were fixed with 2% paraformaldehyde (PFA) for 10 minutes and permeabilized with 0.2% Triton X-100 for 2 minutes. After saturation in 5% bovine serum albumin for 1 hour, cells were incubated with primary antibodies against CD31, CD45 (BD Pharmingen, San Diego, http://www.bdbiosciences.com), VE-cadherin, cKit, Thy1, Endoglin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), CD34, CD4 (AbD Serotec, Raleigh, NC, http://www.ab-direct.com), CD3, Sca-1 (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com), nerve glial antigen 2 (NG-2) (Chemicon, Temecula, CA, http://www.chemicon.com), and α-smooth muscle actin (SMA; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). Biotinylated secondary antibodies anti-rabbit IgG, anti-mouse IgG (Amersham Biosciences), and anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) were incubated for 1 hour followed by either streptavidin-fluorescein isothiocyanate or Alexa Fluor 568 complex (Molecular Probes, Eugene, OR, http://probes.invitrogen.com).

Real-Time Polymerase Chain Reaction Analysis. Total RNA was isolated using Tri Reagent (Euromedex, Souffelweyersheim, France, http://www.euromedex.com) and DNase (Promega, Madison, WI, http://www.promega.com) treatment according to the manufacturers' instructions. One microgram of total RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Promega) as previously described [31]. Polymerase chain reaction (PCR) was done using IQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com). An MJ Research Opticon (Bio-Rad Laboratories) and the following parameters were used for real-time PCR: 95°C for 5 minutes followed by 35 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 15 seconds. All experiments were done in triplicate. Negative controls without reverse transcription (RT) were prepared in parallel for each RNA sample. Primer sequences were as described in supplemental online Table 1. Glyceraldehyde-3-phosphate dehydrogenase was used as endogenous reference, and normalizations were calculated using the $\Delta\Delta C_T$ method. To compare expression patterns in MSC with those of commonly expressed in EC or smooth muscle cells (SMC), RNA was extracted from confluent EC line MS1 or mouse SMC from media aorta. MSC transcripts are expressed as a percentage of remaining gene expression method (supplemental online Table 2).

MSC Transduction with Lentivirus

Cultured MSC were transduced with lentivirus encoding enhanced green fluorescent protein (eGFP) (pMND-eGFP lentivirus from the Trono laboratory) by replacing the medium with fresh medium containing viral supernatant and incubating for 3 hours at 37°C. Transgene stability was verified after each passage and remaining stable throughout the passages.

Recombinant Adenovirus Infection

Green fluorescent protein (GFP)-MSC were infected at a multiplicity of infection of 100 for 2 hours with adenoviral vectors expressing either β-galactosidase (Adβgal), sFRP1 (AdsFrzA) [31], or catalytically inactive forms of GSK3β (Ad GSK3β-KM; kindly provided by Dr. K. Walsh) [35]. Recombinant adenoviral stocks were generated and provided by the vector core of the University Hospital of Nantes (Nantes, France) [36].

Overexpression was verified by Western blot analysis. MSC were lysed for 5 minutes in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM aprotinin, 1 mM AEBSF, 1 mM benzamidine, 1 mM orthovanadate). Fifty milligrams of protein, quantified by bicinchoninic acid assay (Pierce, Rockford, IL, http://www.piercenet.com), was separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, http://www.millipore.com). Membranes were incubated with antibodies against α-tubulin (Sigma-Aldrich), hemaglutinin A (HA) (12CA5; Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com), sFRP1 (sc7425; Santa Cruz Biotechnology), and β-galactosidase (AB1211; Chemicon), which were revealed with enhanced chemiluminescence (Amersham Biosciences). Relative protein levels were quantified using Scion software (Scion Corporation, Frederick, MD, http://www.scioncorp.com) on scanned films. Modified MSC were used in the Matrigel (BD Biosciences, San Diego, http://www.bdbiosciences.com) assay 2 days after adenovirus infection.

Murine Matrigel Assay and Cell Transplantation

Matrigel (0.25 ml) was mixed with fibroblast growth factor 2 (FGF2; 5 μg/ml; Sigma-Aldrich) at 4°C and injected subcutaneously into the abdomen of 8-week-old male nude mice (RAG2/γ; 10 animals per group) [37]. Mice were injected with cold Matrigel alone or Matrigel mixed with GFP-MSC (0.5×10^6). To stain the functional microvasculature, some mice were perfused with biotin-conjugated lectin (0.5 mg/ml; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) 10 minutes before the sacrifice. The animals were sacrificed with an overdose of sodium pentobarbital 5, 9, and 13 days and 3 months after the injection. This study was conducted in accordance with both institutional guidelines and

those in force in the European Community for experimental animal use (L358-86/609/EEC).

Tissue Processing, Histology, and Microscopy

Plugs were fixed in 4% PFA and embedded in paraffin. Five-micrometer paraffin-embedded sections cut transversely from the mouse plug and abdominal muscle. The following antibodies were used: CD31 (BMA Biomedicals, Augts, Switzerland, http://www.bma.ch), SMA (Sigma-Aldrich), NG-2 (Chemicon), smooth muscle-myosin heavy chain (SM-MHC; Biomedical Technologies Inc.), and GFP (Invitrogen). For biotinylated lectin staining, sections were incubated for 1 hour with streptavidin-conjugated Alexa Fluor dye. Capillary densities and functional vessels were examined by counting the number of capillaries stained with anti-CD31 or with lectin, respectively. The percentage of mature vessels (arterioles) was quantified after double staining for CD31 and SM-MHC. The number of MSC recruited around neovessel and into arterioles was assessed after double staining for CD31 and GFP or SM-MHC and GFP, respectively. A minimum of 30 randomized pictures were recorded at a magnification of ×20 for each animal at each time point. Positive cells were counted on captured images, and the data were analyzed with Sigma Plot software (Sigma Plot, Integral Software, Paris, http://www.sigmaplot.com). For confocal microscopy, sections were analyzed with a Nikon laser scanning confocal microscope using multichannel scanning (Nikon PCM 2000), ×60/1.4 ApoPlan oil immersion objective and EZ200 software (Nikon, Tokyo, http://www.nikon.com). Single *xy* scans had an optical slice thickness of 0.4 μm. Three-dimensional projections were digitally reconstituted from stacks of confocal optical slices by Imaris software (Bitplane, Zurich, Switzerland, http://www.bitplane.com).

Analysis of Wnt/Fz Pathway in MSC In Vitro

RT-PCR. Total RNA was isolated from confluent MSC as described above, and RT-PCR was performed as described previously [31], using primer sequences listed in supplemental online Table 1.

Reporter Gene Assay. Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions [31]. For TOPFLASH reporter assays, MSC were transfected in 24-well plates at 2×10^5 cells per cm^2 with Super8XTOPFLASH luciferase reporter plasmid (375 ng/well) containing LEF/TCF consensus binding sites (Randal T. Moon laboratory). pRL-CMV (Promega) was cotransfected (90 ng per well) to normalize samples for transfection efficiency (Dual luciferase kit; Promega). After 24 hours, MSC were treated either with LiCl (10 nM; Sigma-Aldrich) or with conditioned media obtained from CHO cells transfected with a plasmid harboring *WNT1* (kindly provided by Randal T. Moon). MSC were harvested 24 hours later, and luciferase activity was determined with a Turner Designs luminometer (Turner Designs, Sunnyvale, CA, http://www.turnerdesigns.com). Data are means ± SD of triplicate well measurements for one representative experiment.

sFRP1 Activation In Vitro

MSC were incubated with or without recombinant bovine (rb) sFRP1 (10 nmol/l) in MSC medium. Total RNA was extracted after 4 days, and quantification of MSC expression was realized by quantitative RT-PCR as described above. Normalizations and fold changes were calculated using the $\Delta\Delta C_T$ method. For protein expression, cells were lysed and Western blot analysis was performed using antibody anti-α-actin and anti-α-tubulin as describe above.

For coculture experiments, rat SMC, isolated from rat aorta, and MS1 (a mouse pancreatic EC line; American Type Culture Collection, Manassas, VA, http://www.atcc.org) were cultured in Dulbecco's modified Eagle's medium with 5% BFS. The mixed cells (1:1), GFP-MSC plus MS1 or SMC, were cultured on chamber slides (Labtek-II system; Nalge Nunc International, Rochester, NY, http://www.nalgenunc.com) and incubated or not with rb sFRP1. Forty-eight hours later, the cells were fixed with 2% PFA for 10 minutes and permeabilized with 0.2% Triton X-100 for 2 minutes. To investigate cell-cell junctions, staining for β-catenin was performed with antibody anti-β-catenin (Sigma-Aldrich) followed by second antibody conjugated with Alexa 568 (Molecular Probes). Finally, cells were mounted in Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories). The chamber slides were analyzed with a Zeiss microscope (Axio Observer Z) using a ×40 or ×63/1.4 ApoPlan oil immersion objective and AxioVision software (Carl Zeiss, Jena, Germany, http://www.zeiss.com) or with a confocal microscope (Nikon PCM 2000).

Statistical Analysis

Results are expressed as mean ± SD. All analyses were performed with appropriate software (Statview 5-1; Abacus, Grand Rapids, MI, http://www.abacuspub.com). Comparison of continuous variables between two groups was performed by a one-way analysis of variance and subsequently, if statistical significance was observed, by a two-sided paired *t* test. A value of $p < .05$ was considered significant.

RESULTS

Characterization of Cultured MSC

Immunofluorescence labeling and RT-PCR analyses were performed to characterize murine MSC (supplemental online Fig. 1; supplemental online Table 2). As we described previously [33], immunolabeling experiments showed that MSC express Thy1, Sca1, and cKit (supplemental online Table 2). At the protein level, MSC expressed α-actin and NG-2 pericyte markers (supplemental online Fig. 1). By quantitative RT-PCR, we compared gene expression in MSC with that in murine SMC. MSC expressed 132% of α-actin, 3% of Sm22, and 331% of platelet-derived growth factor receptor beta (PDGF-Rβ) compared with SMC (supplemental online Table 2). MSC did not express endothelial cell markers, such as CD31, VE-Cadherin, Tie2, or Flk, and expressed low levels of Flt1 and CD34. Moreover, MSC were devoid of the hematopoietic markers (CD45, CD3) (supplemental online Table 2). This pattern was stable on expanded MSC from 8 to 25 passages.

Angiogenic Effect of MSC in a Xenograft Model

To investigate the functional consequence of the presence of MSC on angiogenesis, we used a xenograft model in which MSC were embedded in a Matrigel matrix and injected subcutaneously into nude mice. We compared the kinetics of host vessel invasion into the Matrigel when MSC were transplanted or not. To track the MSC in vivo, they were transduced with a lentiviral vector harboring the gene encoding green fluorescent protein (GFP-MSC). GFP+ MSC were consistently detected throughout the course of the kinetics (Fig. 1) until 3 months after Matrigel implantation (supplemental online Fig. 3), demonstrating that MSC engraftment was stable for the long term.

We showed that MSC accelerated host vessel growth into the Matrigel. Five days after in vivo injection, control plug (i.e., Matrigel alone) was avascular (Fig. 1A, 1B), whereas we consistently observed a high number of CD31-positive endothelial cells at the periphery of the plug containing the Matrigel plus MSC (Fig. 1C, 1D). At day 9 (D9), in Matrigel alone (Fig. 1E, 1F), sparse blood vessels began to be detected at the periphery of the plug, but the major part of the plug remained avascular. In contrast, in the MSC-Matrigel implant, a strong angiogenic response occurred throughout the plug (Fig. 1G, 1H). At 13 days, in Matrigel alone and in Matrigel containing MSC (Fig. 1I–1L), CD31-positive neovessels were detected homogeneously throughout the plugs. Finally, we looked at the vessel maturation after double staining for CD31 and SM-MHC, a marker of differentiated SMC. The maturation of neovessels was delayed, as no SM-MHC-positive cells were detected before D9. At D13 we observed that approximately 20% of the

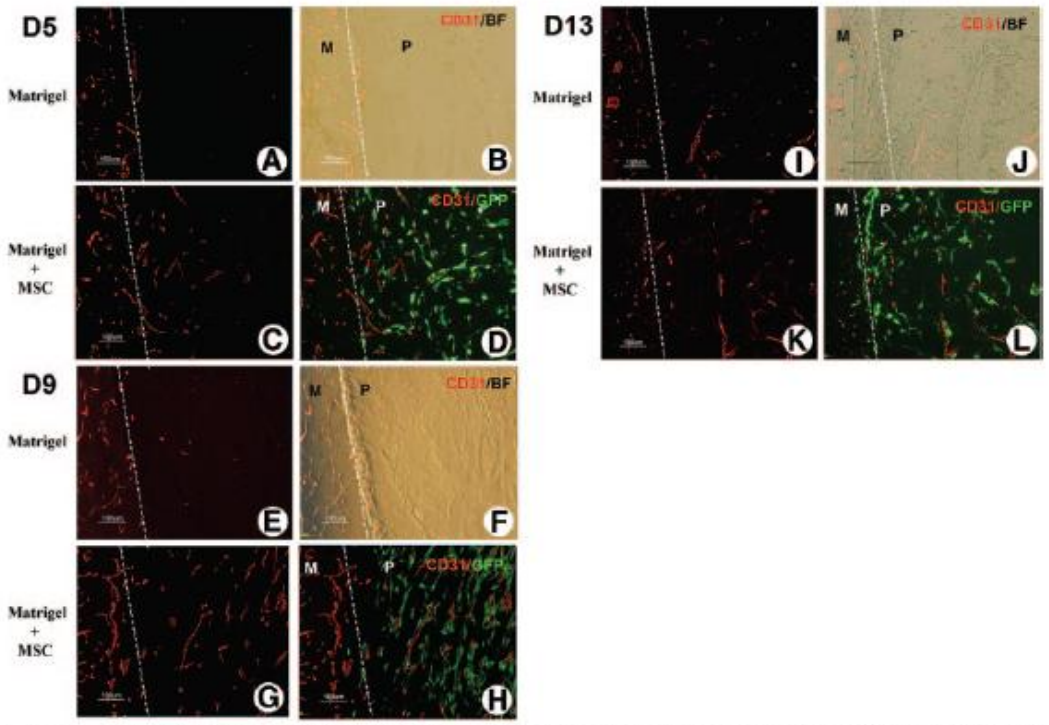

Figure 1. A strong angiogenic response was induced by MSC in a murine Matrigel plug assay. Matrigel alone or Matrigel with GFP-MSC Ps was extracted at days 5 (**A–D**), 9 (**E–H**), and 13 (**I–L**) and analyzed by immunostaining for CD31 (red). MSC were labeled with anti-GFP antibody (green). To localize plug structure and abdominal muscle in the Matrigel group, BF was merged with CD31 (CD31/BF). Dotted lines show borders between abdominal muscle and injected plug. Abbreviations: BF, bright-field; GFP, green fluorescent protein; M, muscle; MSC, mesenchymal stem cell; P, plug.

vessels contained SM-MHC-positive mural cells, revealing a late recruitment of SMC.

MSC Localization and Differentiation

GFP distribution in the Matrigel plug revealed that MSC formed primitive vessel-like tubes at D5 (Fig. 2Aa, 2Ab). MSC were aggregated into a network of elongated multicellular tubes containing red blood cells, evidenced by alizarin staining (Fig. 2Ab). These primitive MSC forming tube structures were observed even in CD31-negative areas in the middle of the plug (Fig. 2Ac, 2Ad). At this time point, immature CD34+ EC were not observed in the lumen of these primitives vascular structures (supplemental online Fig. 2). However, GFP+ cells expressed low levels of CD34. At day 13, as the angiogenic process progressed, in more than half of the vessels, CD31 was associated with GFP, suggesting a large recruitment of CD31-positive cells in tube-forming MSC (Fig. 2B).

To analyze the phenotype of MSC in vivo, multichannel laser scanning confocal microscopy, after labeling for EC (CD31), mural cells (NG-2 and SMA), or differentiated SMC (SM-MHC), was performed (Fig. 2C). GFP-positive MSC formed clusters that were in close contact with the EC. However, no double-labeled CD31/GFP-positive cells were observed on day 5, 9, or 13 (Fig. 2Ca). MSC did not differentiate into EC, but the shape, localization, and morphology of the GFP-positive cells suggested that they behaved as pericytes. Indeed, all the GFP-positive MSC in the plug expressed both SMA and NG-2 (the latter is reported to be a marker of less mature pericytes) but remained SM-MHC-negative (Fig. 2Cb–2Cd). We evaluated the late MSC contribution in Matrigel plugs obtained 3 months post-transplantation. At 3 months, the MSC phenotype remained stable (CD31−, SM-MHC−, SMA+) and MSC were localized as perivascular cells (supplemental online Fig. 3). It is interesting to note that 3 months after transplantation, we observed that only a small percentage (0.05%) of vessels coexpressed CD31 and GFP, suggesting that under some conditions, MSC have the ability to differentiate into EC. This differentiation was very rare and could not have accounted for the strong MSC-induced angiogenic response.

The sequence of these events led to the suggestion that MSC could form primitive vessel-like structures connected to host blood circulation. Next, these immature MSC tubes recruit first host EC and later mural cells to form mature multilayer vessels throughout the plug. MSC participate in neovessel formation and organization as mural cells stabilizing the neovessel.

Increased sFRP1 Expression in MSC Promotes Vessel Maturation In Vivo

Among many molecular players involved in stem cell regulation, the Wnt pathway is one of the core sets of conserved signaling pathways that regulate many aspects of stem cell fate. To determine a possible contribution for the Wnt/Frizzled pathway in the promotion of the MSC-enhanced angiogenesis, we first evaluated the expression of several components of this pathway in MSC. RT-PCR analysis showed that MSC expressed Fz1–Fz6 and sFRP1, a regulator of the Wnt pathway (Fig. 3A). We demonstrated using a β-catenin reporter TOP-Flash assay that the Wnt canonical signaling is active in MSC. Treatment with either LiCl, a Wnt mimetic, or Wnt1-containing conditioned medium strongly induced luciferase activities in MSC (Fig. 3B).

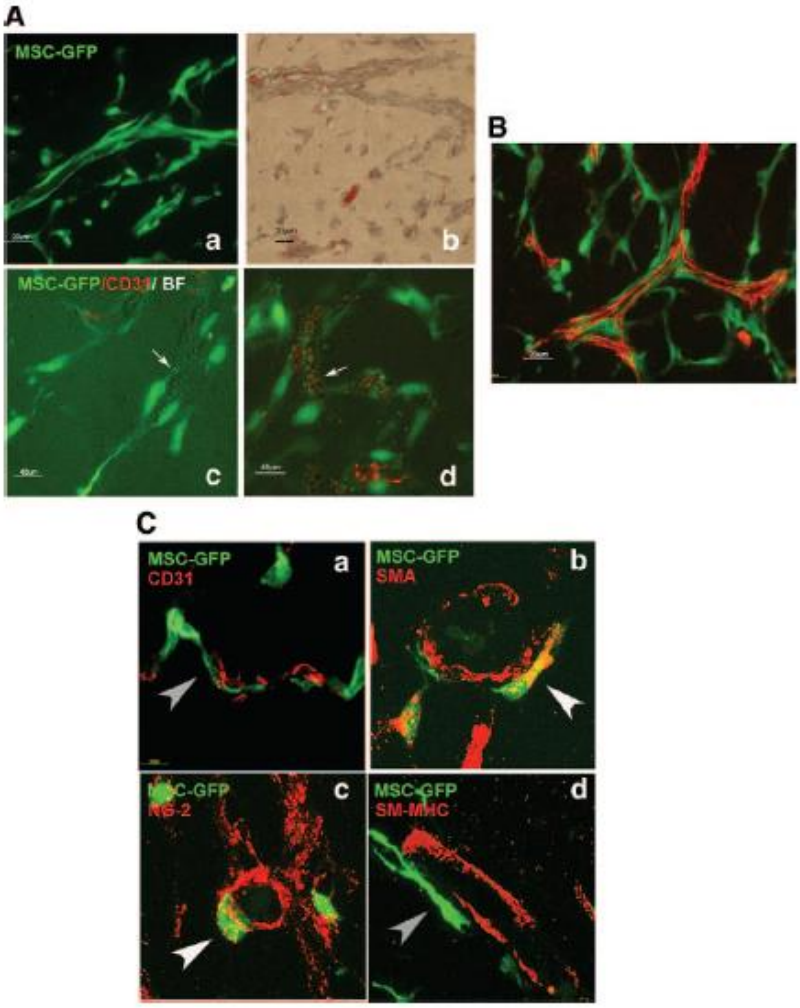

Figure 2. MSC were organized into primitive tube structures and were recruited as pericytes around neovessels. **(A):** At day 5, GFP-MSC (green) were organized in the plug and formed primitive tubular structures **(Aa, Ab)**. Alizarin staining (labeling of red blood cells) showed that these tubes were filled with red blood cells **(Ab)**, and alizarin staining was observed in the plug even in the CD31-negative zone. Vessel-like structures were analyzed after GFP staining (green), CD31 staining (red), and BF observations **(Ac, Ad)**. Arrows in **(Ac)** and **(Ad)** indicate GFP-positive/CD31-negative tubes containing red blood cells. **(B):** Double immunofluorescence analysis after GFP and CD31 staining of plug sections containing GFP-MSC at days 13. **(C):** Representative confocal micrographs of plug sections containing GFP-MSC after double staining for GFP (green) and for CD31 **(Ca)**, SMA **(Cb)**, NG-2 **(Cc)**, or SM-MHC **(Cd)** (red) at a magnification of ×63. *z*-Stack confocal images showed that GFP colocalized with neither CD31 nor SM-MHC labeling. In contrast, GFP-positive MSC were SMA- and NG-2-positive. MSC staining with GFP is indicated by arrowheads. Abbreviations: BF, bright-field; GFP, green fluorescent protein; MSC, mesenchymal stem cell; SMA, α-smooth muscle actin; SM-MHC, smooth muscle-myosin heavy chain.

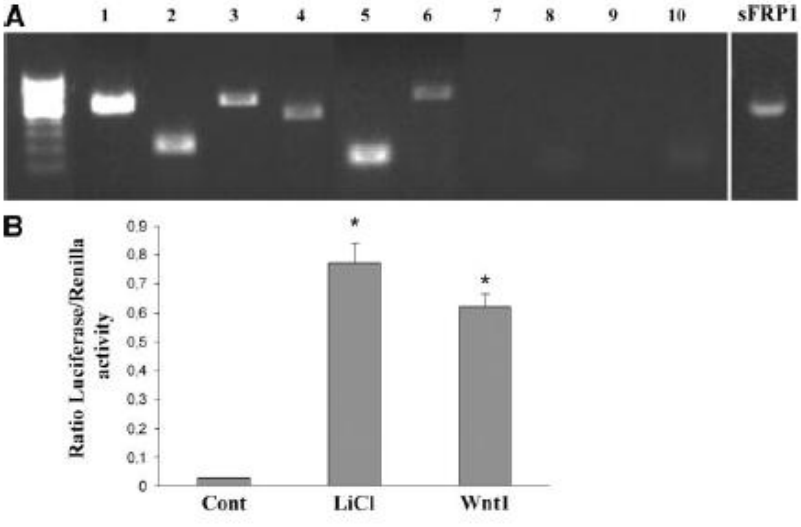

Figure 3. Expression of the components of the Wnt/Fz pathway by mesenchymal stem cells (MSC). **(A):** Reverse transcription-polymerase chain reaction analysis shows Frizzled receptor and sFRP1 expression in confluent MSC. **(B):** MSC were transfected with Top-Flash along with pRL-CMV and treated with Cont medium, Wnt1-conditioned medium, or LiCl. At 24 hours post-transfection, luciferase assay was performed as described in Materials and Methods, and the relative levels of luciferase activity were normalized to levels of luciferase activity of the *Renilla* Cont plasmid. Data represent the mean ± SD of three experiments. Abbreviations: Cont, control; sFRP1, secreted Frizzled-related protein-1.

In this study, given the previously reported role of sFRP1 in angiogenesis, we examined whether sFRP1 could act instructively in MSC to modify neovessel formation. GFP-MSC were transduced with adenoviruses harboring genes allowing expression of *sFRP1* or *LacZ* with more than 90% efficiency (data not shown). The increase in sFRP1 was on average 10 times higher

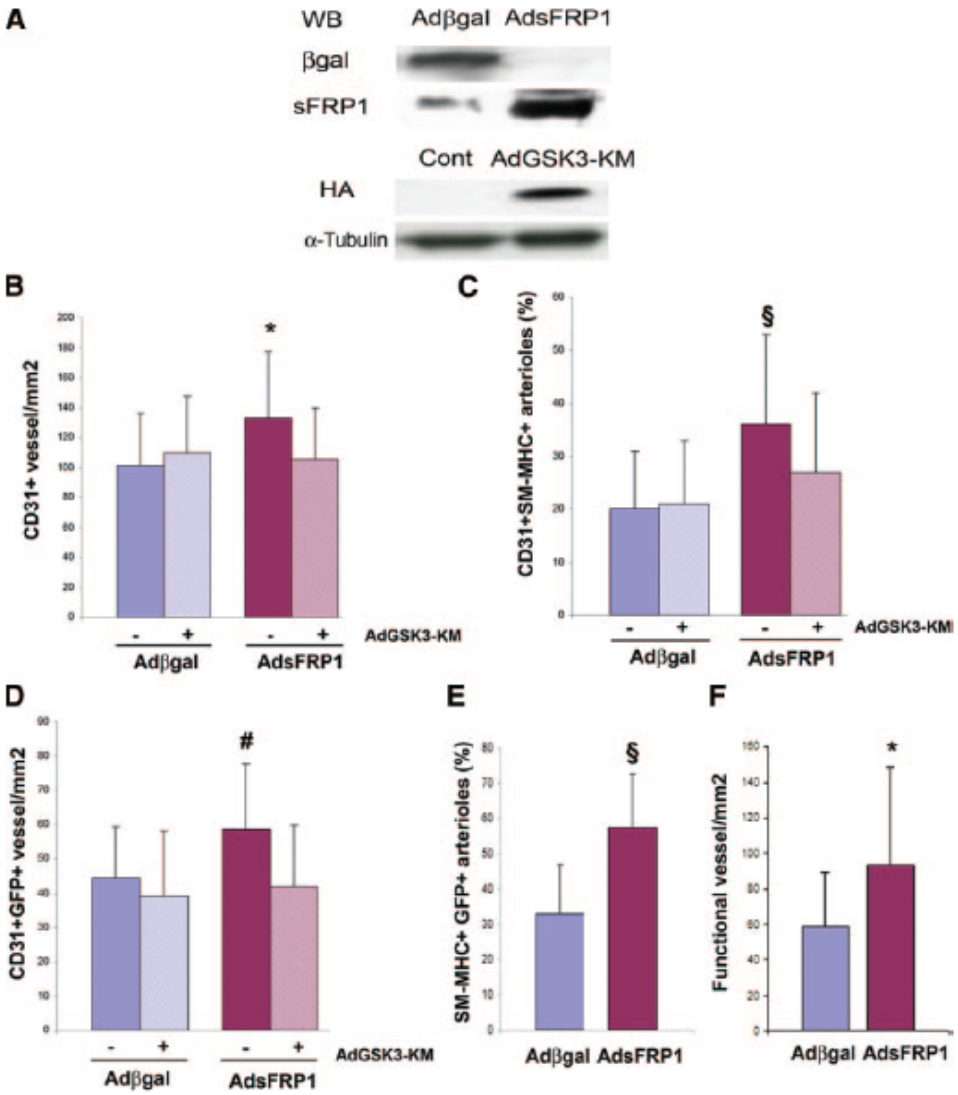

Figure 4. Overexpression of sFRP1 improved vessel density and arteriole formation. (**A**): Mesenchymal stem cells (MSC) were infected with Ad to overexpress negative Cont gene *LacZ* (Adβgal), *sFRP1* (AdsFRP1), or HA-tagged inactivated forms of GSK3β (AdGSK3β-KM) as evaluated by Western blot using anti-βgal, anti-sFRP1, and anti-HA antibodies, respectively. Effects of MSC overexpressing sFRP1 were evaluated at day 13 on capillary density (number of CD31 vessels per mm^2) (**B**), the percentage of arterioles (CD31+SM-MHC+ arterioles per total vessels in each condition) (**C**), GFP-MSC recruitment around CD31+ neovessels (number of CD31+GFP+ vessels per mm^2) (**D**), GFP-MSC recruitment into arterioles (percentage of SM-MHC+GFP+ arterioles) (**E**), and functional vessels (lectin+ vessels per mm^2) (**F**); *, $p < .05$; #, $p < .001$; §, $p < .0001$; $n = 30$. An average of five independent plugs per group were analyzed, and experiments were repeated twice, with two independent sets of MSC. Abbreviations: βgal, β-galactosidase; Ad, adenovirus; Cont, control; GFP, green fluorescent protein; sFRP1, secreted Frizzled-related protein-1; SM-MHC, smooth muscle-myosin heavy chain; WB, Western blot.

in AdsFRP1-MSC than in AdLacZ-MSC (Fig. 4A). We assessed the angiogenic effect of sFRP1 overexpression in vivo by injecting AdsFRP1-MSC or AdLacZ-MSC with Matrigel. At day 13, we observed that injection of sFRP1-MSC resulted in a 31% increase in neovessel number in the plug compared with control MSC (133 ± 45 vs. 101 ± 35 CD31+ vessels per mm^2; $p < .01$; Fig. 4B). This increase in vessel density was associated with a major increase in vessel maturation, evaluated by double staining for CD31 and SM-MHC. In the control group, CD31+SM-MHC+ arterioles represented 20% ± 11% of total vessels, whereas in AdsFRP1-MSC, 36% ± 17% of vessel were arterioles ($p < .0001$; Fig. 4C). To determine MSC incorporation into vascular structure, CD31+GFP+ and SM-MHC+GFP+ structures were quantified. As observed in Figure 4D, the number of capillaries covered with GFP-MSC were significantly increased in the sFRP1-MSC group (59 ± 19 vs. 44 ± 15 CD31/GFP-positive vessels per mm^2; $p < .005$; Fig. 4D). Notably, sFRP1 overexpression resulted in a greater increase of SM-MHC+GFP+ arterioles compared with control (57.5% ± 15% vs. 33% ± 14%; $p < .0001$; Fig. 4E). To study whether sFRP1 increased blood perfusion in the plug, lectin was then injected before sacrifice (Fig. 3F). Overexpression of sFRP1 significantly increased functional vessels, as assessed by the increase of lectin-positive structures in AdsFRP1-MSC plug compared with Adsβgal-MSC (93 ± 55 vs. 59 ± 30 lectin+/mm^2; $p < .01$). These findings demonstrate that MSC geneti-

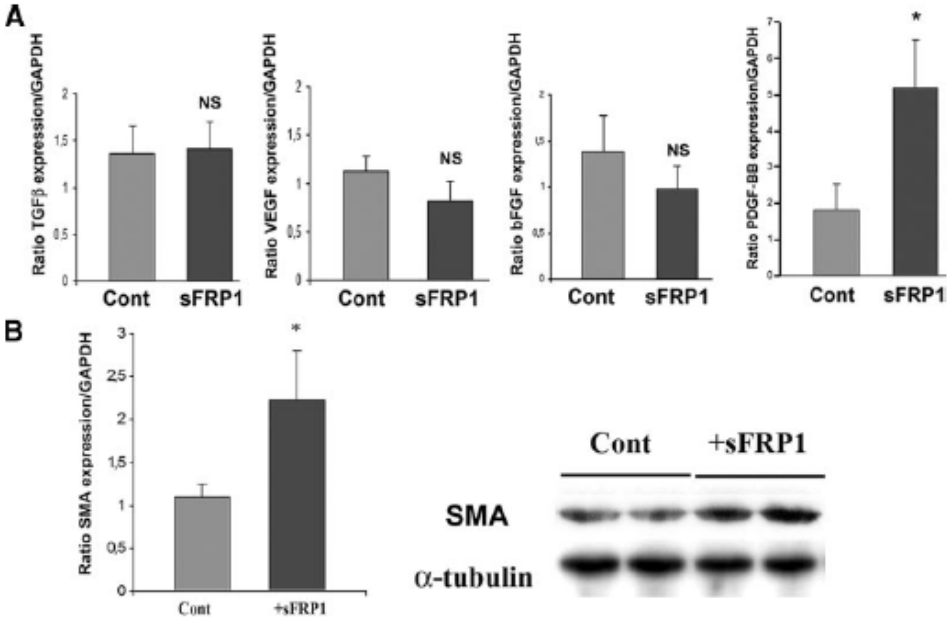

Figure 5. Growth factor expressions by mesenchymal stem cells (MSC) under sFRP1 activation. (**A**): MSC were treated with either Cont medium or with recombinant bovine (rb) sFRP1 for 4 days. Expression of TGFβ, VEGF, FGF2, and PDGF-B was determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) in MSC under sFRP1 treatment. Vertical axes show relative expression normalized to *GAPDH*. $n = 3$; *, $p < .001$. (**B**): SMA expression was analyzed in MSC treated or not treated with rb sFRP1, either by quantitative RT-PCR or by Western blot using an anti-SMA antibody. rb sFRP1 treatment induced an increase in SMA expression in MSC; *, $p < .001$; $n = 3$. Illustrated blot is representative of five experiments. Abbreviations: bFGF, basic fibroblast growth factor; Cont, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, not significant; PDGF, platelet-derived growth factor; sFRP1, secreted Frizzled-related protein-1; SMA, α-smooth muscle actin; TGFβ, transforming growth factor; VEGF, vascular endothelial growth factor.

cally enhanced to overexpress sFRP1 have an angiogenic/arteriogenic potency superior to that of control MSC.

sFRP1-Induced Vessel Maturation Is GSK3β-Dependent

As we have previously demonstrated that GSK3β is a downstream target of sFRP1 in EC [31], we tested in vivo the relevance of the GSK3β-dependent signaling cascade activation by sFRP1 in MSC. MSC were coinfected with or without adenovirus encoding the inactivated form of GSK3β (AdGSK3-KM) (Fig. 4A) [38]. This overexpression of GSK3-KM in sFRP1-MSC inhibited sFRP1-induced angiogenic effects (Fig. 4B–4D), demonstrating that exogenous sFRP1 instructs MSC in following a GSK3β-dependent pathway to mediate vascular cell recruitment. It should be noted that inhibition of GSK3β kinase in MSC did not alter their basal angiogenic effect, suggesting that endogenous sFRP1 expression in MSC is not required for the initial MSC-induced angiogenic step.

sFRP1 Increased PDGF-BB and SMA Expression in Cultured MSC

MSC expressed major angiogenic factors such as VEGF, FGF2, transforming growth factor β (TGFβ), PDGF-BB, and angiopoietin 2 (supplemental online Table 2). As published reports have demonstrated a crucial role of PDGF-BB in vessel maturation [39], we analyzed by quantitative PCR whether sFRP1 could modulate PDGF-B expression in MSC. In vitro, treatment of MSC with rb sFRP1 for 4 days resulted in an increase of ~5-fold in PDGF-B transcripts compared with control untreated MSC (Fig. 5; $n = 3$; $p < .001$). PDGF-B induction by sFRP1 was not observed in cultured EC (data not shown). In contrast, no consistent expression changes of TGFβ, FGF2, and VEGF levels were observed in response to sFRP1 activation by real-time quantitative PCR (Fig. 5). We analyzed the impact of sFRP1 on α-actin expression (SMA), a mural cell marker. sFRP1 treatment induced a twofold increase of SMA mRNA levels by quantitative PCR in MSC compared with the control (Fig. 5B; $n = 3$; $p < .001$). The same increase was observed at the protein level in Western blots (Fig. 5B). However, under sFRP1 activation, MSC did not acquire differentiated SMC markers, such as SM-MHC (data not shown).

sFRP1 Reinforces Homotypic and Heterotypic Cell-Cell Junctions

Treatment of cultured MSC with recombinant sFRP1 resulted in remarkable morphological changes in vitro (Fig. 6A); MSC exhibited an elongated, polygonal shape with reorganized stress fibers (data not shown). Confocal microscopy revealed a relocalization of β-catenin under sFRP1 treatment into cell-cell junctions. β-Catenin expression clearly delimitated cell-cell junctions in sFRP1-treated cells compared with the control, where β-catenin was observed more sparsely and appeared patchy at the cell membrane (Fig. 6A).

To better understand the crosstalk between MSC and vascular cells, in vitro coculture experiments were performed. Immunofluorescence analysis revealed that MSC were able to engage adhesion contact structures with SMC and EC, as evidenced by a strong expression of β-catenin at cell-cell junctions between MSC and EC, or MSC and SMC (Fig. 6Ba, 6Bb). When the cells were treated with sFRP1, an increase in β-catenin labeling was observed in MSC-EC (Fig. 6Bc) and MSC-SMC (Fig. 6Bd) coculture compared with control, suggesting that sFRP1 could increase MSC junctions with other cells.

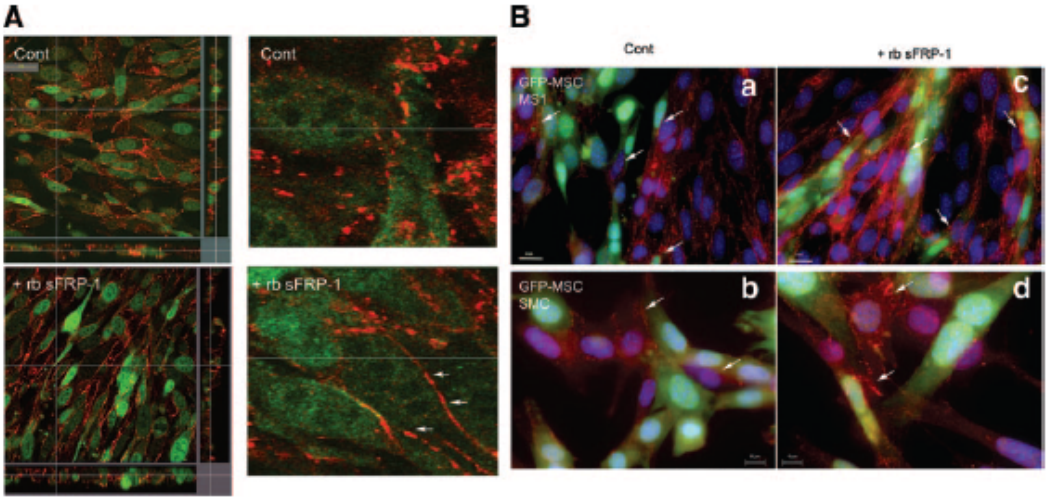

Figure 6. sFRP1 modified MSC morphology and increased β-catenin staining in homotypic and heterotypic cell-cell junction. **(A):** MSC were cultured in the presence of rb sFRP1 for 3 days, fixed, and stained with anti-β-catenin antibody (red). Images of MSC expressing GFP fluorescence (green) were analyzed by confocal microscopy. Left panels show *z*-stack confocal images (magnification, ×63); right panels show merged images, zoomed to area of interest. rb sFRP1 promoted the reorganization of MSC with a more longitudinal arrangement than Cont conditions and a relocalization of β-catenin labeling all along MSC membranes in cell-cell junctions (white arrows). Images of MSC with or without sFRP1 treatment are representative of three experiments. **(B):** MSC were cocultured with endothelial cells or with SMC for 48 hours, with (**Bc, Bd**) or without (**Ba, Bb**) rb sFRP1. Cells were fixed and stained for GFP (localization of MSC, green), β-catenin (red), and nuclei (blue). MSC interacted with both cell types through β-catenin cell-cell junctions. sFRP1 treatment increased β-catenin staining at cell-cell junctions in the coculture. Arrows indicate cell-cell junctions. Abbreviations: Cont, control; GFP, green fluorescent protein; MSC, mesenchymal stem cell; rb, recombinant bovine; sFRP-1, secreted Frizzled-related protein-1; SMC, smooth muscle cell.

sFRP1 Improved Vessel Organization

These data suggested that sFRP1 is involved in the MSC acquisition of a mature SMA-positive phenotype, an elongated morphology, stress fiber architecture, and an increase of β-catenin-dependent cell-cell junctions, which correlates with in vivo observations. We observed a rearrangement of MSC in the vessel wall under sFRP1 conditions. sFRP1-MSC were more elongated and aligned along the vessel wall compared with control MSC (Fig. 7A, 7B). Together, these data indicate that sFRP1 is required for formation of highly organized MSC joined around the neovessel wall.

We then speculated that the enhancement of endogenous sFRP1 signaling in MSC may modify MSC interaction with mural cells, reinforcing neovessel stabilization in vivo. GFP/SM-MHC double staining was analyzed by confocal analysis, and the three-dimensional images were reconstructed with Imaris software. We observed that MSC were in close contact with mural cells in sFRP1 group (i.e., less than 1.5 μm distance between MSC and SMC, on average), whereas control MSC were constantly in loose association (Fig. 7A–7C). Thus, a MSC source of sFRP1 appeared to be required for the establishment of a tight association of MSC with mural cells, which is crucial for the mature vessel persistence.

Discussion

In this study, we demonstrated that transplantation of MSC increased the angiogenic response in a Matrigel assay, and we propose that MSC contribute to the angiogenic processes by their participation as perimural effector cells. Genetic MSC modifications by a modulator of the Wnt/Fz pathway, sFRP1, enhanced their association to mural cells and increased neovessel maturation and stabilization.

The Matrigel model is an efficient angiogenic model that has been extensively used to study angiogenic events [1, 40]. In our study, 5 days after Matrigel injection, MSC were organized in primitive vessel-like structures, containing red blood cells with branches in connection with host-circulation. They induced a rapid recruitment of host CD31+ cells compared with control. After 13 days, more than 40% of CD31-positive blood vessels in the plug were covered with MSC. Earlier studies have shown that MSC transplantation has a beneficial therapeutic role in experimental models of ischemic disease [41, 42]; however, the governing molecular mechanism still remains unclear. Some groups have reported that secretion of a large number of potent mediators would potentially be more relevant to MSC angiogenic properties than transdifferentiation of MSC into EC [43]. All these previous divergent results could be ascribed to distinct sources of MSC [44]. In our transplantation model, MSC were very rarely integrated in the EC layer but were constantly observed as perivascular cells. Forming a coating around the EC sheet, MSC expressed the pericyte and SMC markers NG-2 and SMA, respectively. These results are in agreement with a recent study that demonstrated that human mesenchymal stem cells (hMSC) express SMC markers in vitro [26]. These findings prompted us to hypothesize that MSC could behave as pericyte-like cells that initiate and allow the establishment of mature vessels. In vivo hMSC coimplanted with EC differentiated into and functioned as perivascular cells, and several studies have shown that pericytes play an early role in the development of neovessels [21, 26, 45, 46]. These observations support recent evidence of immature pericyte tubes without endothelial cells in human fetal brain [47]. Moreover, MSC express high levels of angiogenic factors, such as VEGF, FGF2, TGFβ, and MMP2/9, which may be involved to a certain extent in MSC angiogenic-induced responses.

Insights into mechanisms that reinforce the recruitment of MSC around vessels may have important implications for the development of angiogenic cell therapy to stabilize neovessels. Notably, the degree of pericyte coverage influences vessel response to VEGF [48, 49]. We have previously demonstrated the

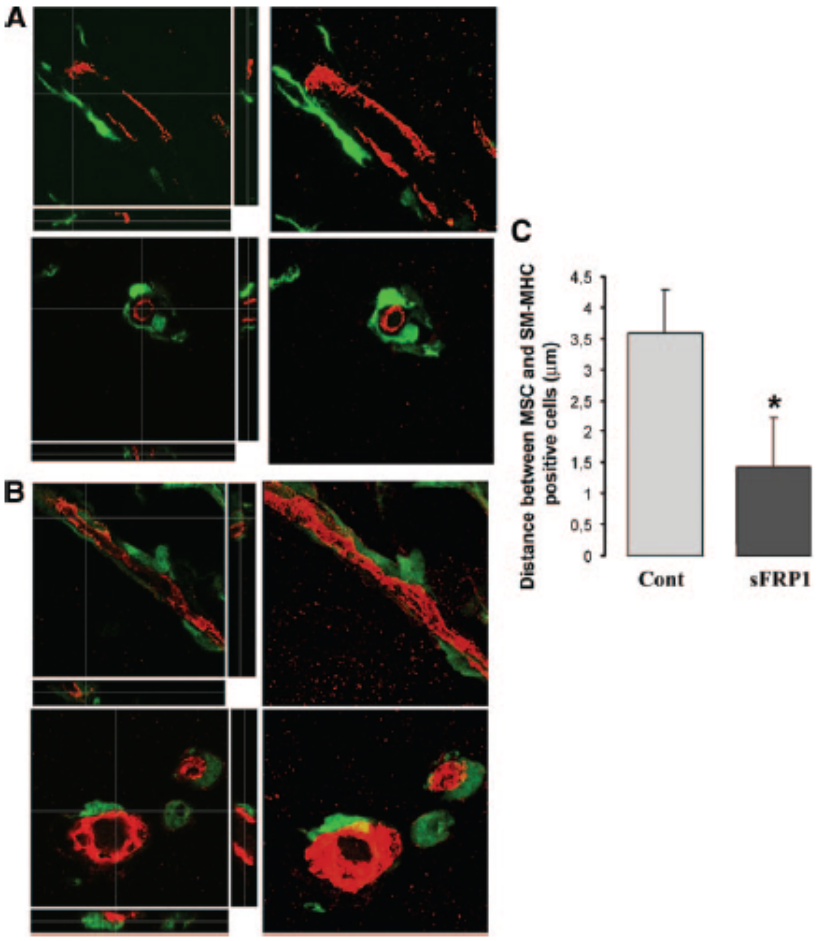

Figure 7. sFRP1 improved vessel organization. Confocal images of neovessels in plug containing either GFP-MSC infected with Cont adenoviral vector expressing β-galactosidase (**A**) or GFP-MSC infected with adenoviral vector expressing sFRP1 (**B**). Double immunolabeling for SM-MHC (red) labeled differentiated smooth muscle cells (SMC) and GFP (green) labeled MSC. In (**A**) and (**B**), left panels show *z*-stack confocal images, and right panels show three-dimensional reconstruction images obtained with Imaris software. In (**A**) and (**B**), right panels show close-ups of longitudinal and cross-sectional views of neovessels. Under sFRP1 overexpression conditions, MSC encircling neovessels were in closer contact with SMC compared with the Cont condition. (**C**): Histogram illustrates distance analysis between GFP-positive MSC and SM-MHC-positive SMC in neovessels under sFRP1 overexpression versus Cont condition in neovessels. *, $p < .001$. Abbreviations: Cont, control; MSC, mesenchymal stem cell; sFRP1, secreted Frizzled-related protein-1; SM-MHC, smooth muscle-myosin heavy chain.

importance of sFRP1, a modulator of the Wnt/Fz pathway, in neovessel formation in different angiogenic models [31, 34]. Recent data have demonstrated that Wnt signaling regulates basic stem cell features, such as self-renewal of intestinal epithelial stem cells [50] and hematopoietic stem cells [51]. In another study, Wnt3A-conditioned medium was shown to promote human MSC proliferation and invasion [28]. In addition, we have demonstrated here that murine MSC are equipped with many frizzled receptors and can respond to Wnt canonical activation. In this study, murine MSC engineered to overexpress sFRP1 enhanced angiogenic and arteriogenic MSC capacity. It appeared that sFRP1 had a critical role in favoring MSC association with neovessels. The functional importance of mural cell coverage by the pericytes has been established by the demonstration that pericyte depletion/genetic modification strategies can block neo-vascularization [25, 40, 52–54]. A key feature was the demonstration of their crucial role in neovessel stability and integrity [25]. In accordance with these studies, we showed that enhancement of MSC recruitment into arterioles under sFRP1 activation correlated with an increase in vessel maturation and functionality. Moreover, blockade of GSK3β activity in MSC abolished sFRP1-induced neovessel formation and maturation, demonstrating that GSK3β is a key relay for exogenous sFRP1 upstream signal to promote MSC angiogenic properties.

We demonstrate here that forced expression of sFRP1 is instrumental for an efficient MSC integration around vessels and induces mature vessels in Matrigel plug by affecting the organization of MSC around the vascular wall. sFRP1-MSC were elongated into the vessel wall. These observations were in line with the finding that in vitro, treatment of MSC with sFRP1 modified MSC morphology. Under control conditions, MSC contact neighboring mesenchymal cells only focally, whereas under sFRP1 activation, MSC are closely adjoined by β-catenin cell-cell junction enhancement that maintains the integrity of MSC layer [55]. We further analyzed whether in vitro MSC could engage cell-cell junctions with mural cells. To model the vascular cell-MSC communication induced by sFRP1, coculture experiments were designed and revealed the ability of MSC to communicate with both EC and SMC by β-catenin-dependent cell-cell junctions. These data suggest that sFRP1 promoted vessel stability and organization by increasing homotypic and heterotypic β-catenin cell-cell junctions. Interestingly, recombinant sFRP1 increased the SMA expression of MSC, suggesting that sFRP1 could reinforce MSC differentiation toward a pericyte-like phenotype. Together, these observations could account for a strengthened role of sFRP1 in primitive MSC pericyte-like tube organization.

sFRP1 activation did not modify VEGF, FGF2, or TGFβ expression in MSC and did not induce any MSC transdifferentiation in EC or SMC. It is worth noting that sFRP1 induced an enhancement of expression of PDGF-BB that may be functionally important during blood vessel formation. The PDGF-BB pathway is a key pathway involved in blood vessel maturation during embryonic [52] and postnatal [56] development and in tumor vessel formation [25]. It is tempting to speculate that PDGF-BB induction by sFRP1 could partici-

pate in pericyte/SMC recruitment, leading to enhancement of blood vessel stabilization by mural cells.

SUMMARY

Our study reveals a novel structural role of MSC in the angiogenesis process. MSC are able to form primitive vascular networks that precede endothelial cell recruitment and could function as perivascular cells. We demonstrate that genetic modification of MSC with the sFRP1 gene before transplantation could be a potent strategy for regenerative medicine of ischemic tissue. sFRP1 increases MSC homotypic and heterotypic cell-cell junctions in a manner correlated with neovessel maturation and stabilization.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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4) Discussion

Cette étude montre que les MSC induisent un recrutement plus rapide des CE au niveau de la matrice injectée en sous-cutané chez la souris, en prenant un phénotype de péricyte. La surexpression de sFRP1 par les MSC provoque *in vitro* une augmentation de l'expression de PDGF-BB et favorise leur différenciation en péricytes. *In vivo*, les MSC sont alors mieux recrutées au niveau des néo-vaisseaux, plus allongées, elles présentent un meilleur contact avec le mur vasculaire. Cet effet est GSK-3β dépendant.

Le modèle utilisé ici a l'intérêt de permettre l'étude *in vivo* de la formation de nouveaux vaisseaux en s'affranchissant de diverses interférences existant dans les modèles pathologiques : tissus nécrotiques, prolifération tissulaire. Cette étude met en évidence le rôle de précurseurs des péricytes des MSC vis-à-vis du développement des néo-vaisseaux et le rôle princeps du PDGF-BB dans les effets observés.

Or les péricytes ne sont l'objet d'un intérêt croissant que depuis quelques années. Il s'agit de cellules périvasculaires aux multiples fonctions, interagissant avec les cellules endothéliales. Ce sont des éléments importants de la régulation de l'homéostasie microvasculaire (Armulik, Abramsson et al. 2005; von Tell, Armulik et al. 2006; Feng, Mantesso et al. 2010) et plusieurs études ont montré leur rôle dans la formation des néo-vaisseaux (Ozerdem and Stallcup 2003; Tigges, Hyer et al. 2008).

Sur ce point, notre étude est à rapprocher de celle de Tigges publiée la même année (Tigges, Hyer et al. 2008). En utilisant exactement la même matrice, mais dépourvue de cellules, Tigges montre que l'invasion vasculaire d'un « plug » de Matrigel peut être divisée en deux phases : durant les 5 premiers jours, la matrice est colonisée par des péricytes dont l'origine est médullaire dans la moitié des cas et des macrophages. Ces cellules forment un réseau primitif très comparable à celui que nous avons pu observer dans notre étude. Sur ce réseau, des cellules endothéliales provenant des vaisseaux environnants vont venir s'installer durant les 5 jours suivant pour aboutir à un réseau capillaire fonctionnel (Figure 52).

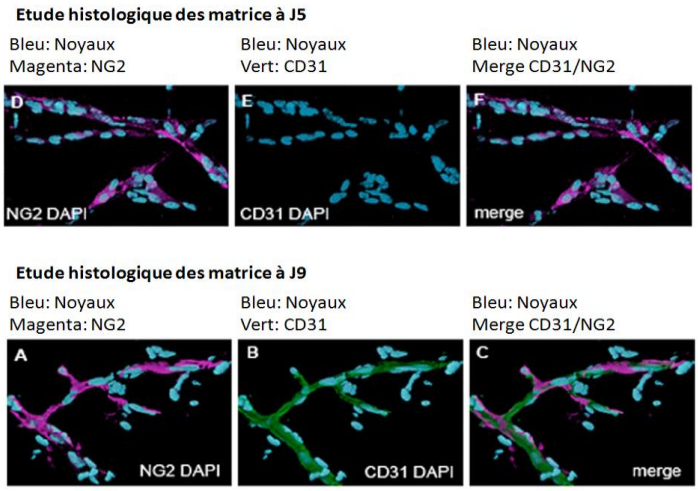

Figure 52. Aspect histologique des matrices de Matrigel à J5 et J9. (D'après Tigges 2008)

Si bien qu'aujourd'hui certains auteurs suggèrent que les péricytes sont à l'origine des MSC dans différents organes (Corselli, Chen et al. 2010; Feng, Mantesso et al. 2010) (Figure 53).

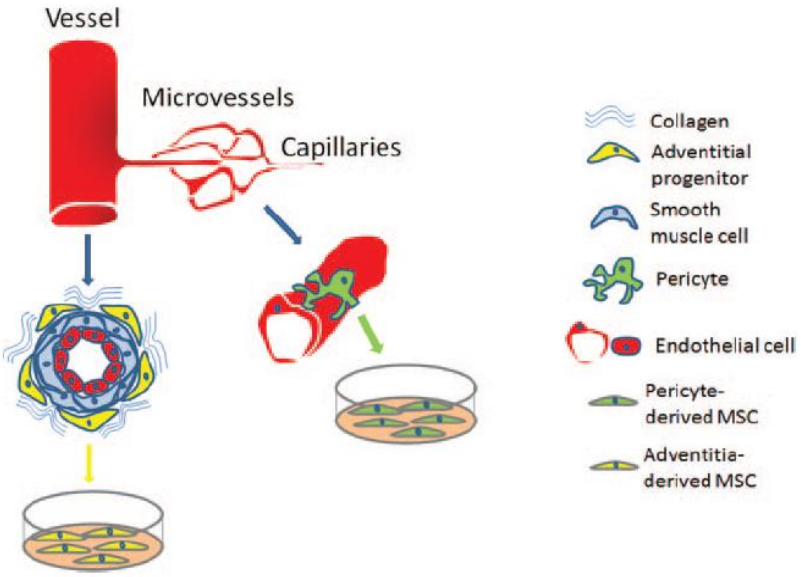

Figure 53. Origine péricytaire des MSC. (D'après Corselli 2010)

Par ailleurs cette étude confirme l'implication du système Wnt/Fzd dans le rôle pro-angiogénique des MSC avec, lors de la surexpression de sFRP1, une augmentation de l'expression de PDGF-BB. Or ce facteur est connu notamment pour favoriser le recrutement des cellules vasculaires et la stabilisation des néo-vaisseaux (Andrae, Gallini et al. 2008). Ces données sont donc concordantes avec de premières constatations réalisées au laboratoire où la surexpression de sFRP1 dans des modèles d'infarctus ou d'ischémie de patte amenait à une muscularisation plus fréquente des néo-vaisseaux.

5) Conclusion

L'ensemble de ces éléments laisse donc penser que la thérapie cellulaire par les MSC pourrait accélérer la phase initiale de la formation des néo-vaisseaux en formant un réseau de péricytes et que le système Wnt/Fzd interviendrait en favorisant les interactions entre ces cellules murales et les cellules endothéliales.

C) INTERET DU PRECONDITIONNEMENT HYPOXIQUE DES MSC EN VUE D'AMELIORER LEUR EFFET PRO-ANGIOGENIQUE SUR UN MODELE MURIN D'ISCHEMIE DE MEMBRE INFERIEUR

1) Introduction

Dans la moelle osseuse, les cellules souches sont maintenues dans des niches dont une des particularités est la très faible teneur en oxygène. Pourtant, dans le cadre de la thérapie celulaire, les cellules prélevées sont cultivées à 20% d'oxygène puis réinjectées dans un milieu hypoxique voire anoxique, pour y avoir un effet thérapeutique pro-angiogénique. Or il est établi que la grande majorité de ces cellules meurent dans les premières heures après leur transplantation. Par ailleurs, plusieurs travaux suggèrent que la culture en milieu pauvre en oxygène des cellules souches, comme les MSC, exerce un effet pro-prolifératif et entraîne l'expression de facteurs anti-apoptotiques et pro-angiogéniques. Par ailleurs, nous avons déjà vu l'implication du système Wnt/Fzd dans les mécanismes de l'angiogenèse et le rôle pro-angiogénique des MSC.

2) Hypothèse et but du travail

Nous avons donc formulé l'hypothèse que préconditionner des MSC par un séjour de 24 heures en chambre à hypoxie à 1% d'oxygène avant transplantation dans un tissu ischémique, permettrait d'améliorer la survie des cellules, et donc l'efficacité de la thérapie. Nous avons également évoqué une implication du système Wnt/Fzd dans les mécanismes déclenchés par ce préconditionnement hypoxique. Pour ce faire, nous avons utilisé un modèle d'ischémie de patte de la souris par ligature et résection de l'artère fémorale gauche. Les cellules ont été injectées directement dans le muscle de la cuisse et le muscle tibial antérieur 48h après la chirurgie. Les cellules préconditionnées sont appelées HypMSC et les cellules cultivées en normoxie NormMSC.

3) Résultats

L'ensemble des résultats est détaillé dans l'article suivant : « **Hypoxia Preconditioned Mesenchymal Stem Cells Improve Vascular and Skeletal Muscle Fiber Regeneration After Ischemia Through a Wnt4-dependant Pathway** ». Nous montrons que le préconditionnement hypoxique des MSC favorise la survie et la prolifération des cellules transplantées et améliore la régénération vasculaire et musculaire de la patte ischémique. Par ailleurs, les MSC préconditionnées (HypMSC) se localisent plus souvent dans des zones hypoxiques et y joueraient un rôle de détersion de débris cellulaire nécrotique en participant à la phagocytose des fibres musculaires. A J15, les HypMSC avaient plus souvent tendance à former un réseau de soutien aux cellules endothéliales des néo-vaisseaux en prenant le phénotype de péricytes. D'autre part, Wnt4 apparaît spécifiquement et fortement surexprimé dans les muscles ayant reçu des HypMSC, et l'utilisation d'un SiRNA dirigé contre *Wnt4* dans les MSC, fait disparaître l'effet bénéfique du préconditionnement. Ces données suggèrent que Wnt4, sécrété par les MSC en conditions d'hypoxie, est un médiateur important de la régénération tissulaire après ischémie.

Hypoxia Preconditioned Mesenchymal Stem Cells Improve Vascular and Skeletal Muscle Fiber Regeneration After Ischemia Through a Wnt4-dependent Pathway

Lionel Leroux[1,2], Betty Descamps[1], Nancy F Tojais[1], Benjamin Séguy[2], Pierre Oses[2], Catherine Moreau[1], Danièle Daret[1], Zoran Ivanovic[3], Jean-Michel Boiron[3], Jean-Marie D Lamazière[1], Pascale Dufourcq[1,4], Thierry Couffinhal[1,2] and Cécile Duplàa[1]

[1]Inserm U828, Université Victor Ségalen, Pessac, France; [2]Département de Cardiologie, Pôle Cardiothoracique, Hôpital Haut Lévêque, Pessac, France; [3]Etablissement Français du Sang (EFS), Hôpital Pellegrin, Bordeaux, France; [4]Laboratoire de biochimie, UFR Sciences Pharmaceutiques, Université Victor Ségalen, Bordeaux, France

Mesenchymal stem cells (MSC) are multipotent postnatal stem cells, involved in the treatment of ischemic vascular diseases. We investigate the ability of MSC, exposed to short-term hypoxic conditions, to participate in vascular and tissue regeneration in an *in vivo* model of hindlimb ischemia. Transplantation of hypoxic preconditioned murine MSC (HypMSC) enhanced skeletal muscle regeneration at day 7, improved blood flow and vascular formation compared to injected nonpreconditioned MSC (NormMSC). These observed effects were correlated with an increase in HypMSC engraftment and a putative role in necrotic skeletal muscle fiber clearance. Moreover, HypMSC transplantation resulted in a large increase in Wnt4 (wingless-related MMTV integration site 4) expression and we demonstrate its functional significance on MSC proliferation and migration, endothelial cell (EC) migration, as well as myoblast differentiation. Furthermore, suppression of Wnt4 expression in HypMSC, abrogated the hypoxia-induced vascular regenerative properties of these cells in the mouse hindlimb ischemia model. Our data suggest that hypoxic preconditioning plays a critical role in the functional capabilities of MSC, shifting MSC location *in situ* to enhance ischemic tissue recovery, facilitating vascular cell mobilization, and skeletal muscle fiber regeneration via a paracrine Wnt-dependent mechanism.

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INTRODUCTION

Bone marrow mesenchymal stem cells (MSC) can be isolated from bone marrow-derived mononuclear cells, expanded *in vitro* and have been shown to possess functional multilineage differentiation capacity.[1] MSC have been shown to differentiate into pericytes,[2] vascular endothelial cells (EC),[3,4] and cardiomyocytes,[5] and their transplantation has improved recovery from ischemia in rat hindlimb and swine dilated cardiomyopathy models.[6] Their precocious role in the formation and the maintenance of neovessels has been studied extensively.[2,7] Such reports have led to the hypothesis that MSC may be used in the treatment of ischemic cardiovascular pathologies. One major limitation demonstrated in both animal and human experiments has been the inability to maintain a large number of cells after grafting into hypoxic sites. In the present study, we employed a strategy of low oxygen (O_2)-preconditioning of MSC before tissue implantation. We hypothesized that creating this hypoxic environment might mimic the stem cell niche microenvironment *in vivo* which others have hypothesized to likely create a hypoxic cavity.[8] We chose to study 1% O_2 concentration because it has been reported as the level found *in vivo* in bone marrow stem cell niche areas.[9] Moreover, data from animal studies have demonstrated a role of hypoxic gradients in stem cell trafficking in ischemic tissue.[10] On the molecular level, temporary oxygen deprivation is known to regulate gene transcription and differentiation of stem cells.[11,12]

Several reports suggest that the Wnt (wingless-related MMTV integration site) pathway plays a role in maintaining hematopoietic stem cell self-renewal and pluripotency.[13,14] The Wnt family of genes encodes over 20 cysteine-rich secreted glycoproteins to regulate canonical and noncanonical signaling pathways.[15] The canonical Wnt pathway regulates gene expression due to the stabilization and nuclear localization of J3 catenin. We have previously demonstrated that MSC have a structural role in forming a precocious network and facilitating neovessel recruitment in an angiogenic model and reported that overexpression of a soluble modulator of Wnt functions, secreted frizzled-related protein 1 (sFRP-1), in MSC, enhanced their angiogenic capacities.[2] Experimental manipulation of a pluripotent mouse embryonal carcinoma stem cell line via another secreted Wnt modulator, sFRP-2, identified the Wnt pathway in cardiomyogenic differentiation.[16] Wnt3a activation modulated proliferation and differentiation of human MSC

Correspondence: Cécile Duplàa, Inserm U828, Université Victor Ségalen, Avenue du Haut Lévêque, Pessac 33600, France. *E-mail:* cecile.duplaa@inserm.fr

and murine embryonic mesenchymal cells.[17,18] Recently, another study demonstrated a role of the Wnt signaling pathway in the acquisition of the invasion property of human MSC.[19] These data have been particularly important to help elucidate the molecular mechanism that enables MSC to contribute to tissue regeneration under ischemic conditions.

In the present study, we compare the ability, of hypoxic preconditioned murine MSC (HypMSC) and nonpreconditioned MSC (NormMSC), to engraft into ischemic tissue and assessed the ability of these cells to participate in skeletal tissue and vascular regeneration. A subacute murine limb ischemia model was used to mimic the clinical setting of critical limb ischemia in patients. Our results demonstrate that HypMSC have a greater capacity for engraftment in ischemic tissue compared to NormMSC. HypMSC also improved skeletal muscle regeneration and induced more neoangiogenesis, and elicited significant improvement in blood flow as compared to NormMSC. Localization of HypMSC compared to NormMSC suggests that HypMSC participate in the remodeling of the stromal environment via structural interactions, first in encircling necrotic myocytes and then shaping a tight network around EC along growing neovessels. This preferential localization led us to speculate that MSC could functionally interact via paracrine effectors with myocytes and EC. Our data demonstrates that MSC express Wnt4 and *in vivo* Wnt4 transcripts were significantly increased in ischemic muscles engrafted with HypMSC whereas in muscle engrafted with NormMSC no significant increase was detected. We tested the biological activity of Wnt4 *in vitro* and found that it could stimulate EC migration, myogenic differentiation, and MSC proliferation but had no effect on EC proliferation. Importantly, inhibition of Wnt4 expression in MSC abrogated HypMSC-induced vascular regeneration. Our data provides strong evidence that Wnt signaling could be an important factor in the mechanism by which MSC contribute to tissue regeneration, as well as vessel formation and stabilization. As such, hypoxia preconditioning of MSC could be an attractive therapeutic approach for the treatment of ischemic vascular diseases.

RESULTS

Vascular and muscle regeneration of the ischemic hindlimb was improved after transplantation of HypMSC versus NormMSC

We examined the comparative effectiveness of transplanted murine HypMSC versus NormMSC for vascular and ischemic muscle regeneration after femoral ligation in C57BL/6J mice. Three groups were set up: a control sham group receiving saline injection, a NormMSC group receiving by intramuscular injection 0.5×10^6 MSC cultured in normoxia (20% O_2) and a HypMSC group transplanted with 0.5×10^6 MSC pretreated during 36 hours in a reduced oxygen tension (1% O_2).

HypMSC-enhanced vascular response. Blood flow recovery in the limb of HypMSC-treated animals was significantly improved when compared with sham-treated animals or NormMSC-treated animals (Figure 1a,b).

The therapeutic action of HypMSC was also evident at the angiogenic level, as evidenced by quantification of only viable muscle, excluding angiogenesis involved in an inflammatory response to granulation tissue. At day 15, there was an increase in $CD31^+$ capillary number in HypMSC-treated muscles compared respectively with that in sham-treated control ($P < 10^{-5}$) and in NormMSC groups ($P < 10^{-3}$) at day 15 (Figure 1c–f). These results suggest a potent role of HypMSC in new-vessel formation. Consistent with improved limb perfusion and capillary density, micro scanner (microcomputed tomography) analysis of the whole arterial network revealed the global impact of HypMSC on vessel formation in the hindlimb after ischemia. Images showed a greater number of vessels in HypMSC-treated hindlimb compared to NormMSC or sham-treated injected hindlimb at day 8 after the insult (Figure 1g–k). We calculated a twofold increase in the vessel number (number of vessels/μm^3, $P < 0.01$) and a 1.7-fold increase in their connectivity and branching ($P < 0.01$) in the HypMSC-treated hindlimb compared with sham-treated control animals whereas NormMSC-treated animals were at an intermediate level ($P < 0.05$) (Figure 1j–k).

HypMSC-enhanced muscle regeneration. Histological evaluation of muscle tissue, performed to analyze the impact of HypMSC transplantation on the repair process, revealed that administration of either HypMSC or NorMSC accelerated the repair process. At day 7, almost no overt necrotic area was found in either of the MSC-treated groups, whereas necrotic muscle fibers were markedly observed in sham-treated muscles (25% out of total muscle surface). However, HypMSC engraftment had a significant beneficial effect on muscle regeneration when compared with NormMSC-treated group. Less than 10% of the histological area in the ischemic muscle were still actively regenerating in HypMSC-treated tissue whereas in the NormMSC-treated group, the process was delayed with over 21% of the total muscle surface was still actively regenerating (Figure 1l–q).

Hypoxic pretreatment modified MSC engraftment in ischemic hindlimb

MSC were infected with a lentivirus encoding for the *GFP* gene [green fluorescent protein (GFP)-MSC] and expression was followed as a marker of viable GFP^+ MSC.[2] HypMSC robustly engrafted the adductor and tibial muscles in large patches, as revealed by direct fluorescence at day 7 (**Supplementary Figure S1a**) or anti-GFP immunohistochemistry at day 14 (**Supplementary Figure S1b**).

Hypoxic treatment improved MSC survival capacities in vivo. Real-time reverse transcription-PCR was performed using total RNA obtained from HypMSC- and NormMSC-injected muscles at day 7 after injury to determine the percentage of remaining GFP expression, and demonstrated that hypoxic pretreatment contributes to effective enhancement of MSC recruitment *in vivo*. In the HypMSC group, there was a significant increase in MSC engraftment at day 7 (150% of initially engrafted HypMSC) whereas in NormMSC group, the total number of MSC was not maintained after transplantation (63% of initially engrafted NormMSC) (Figure 2a). At day 7, caspase 3 labeling showed apoptotic muscle fibers surrounding by surviving Hyp and Norm GFP-MSC (Figure 2b). Double bromodeoxyuridine/GFP labeling revealed

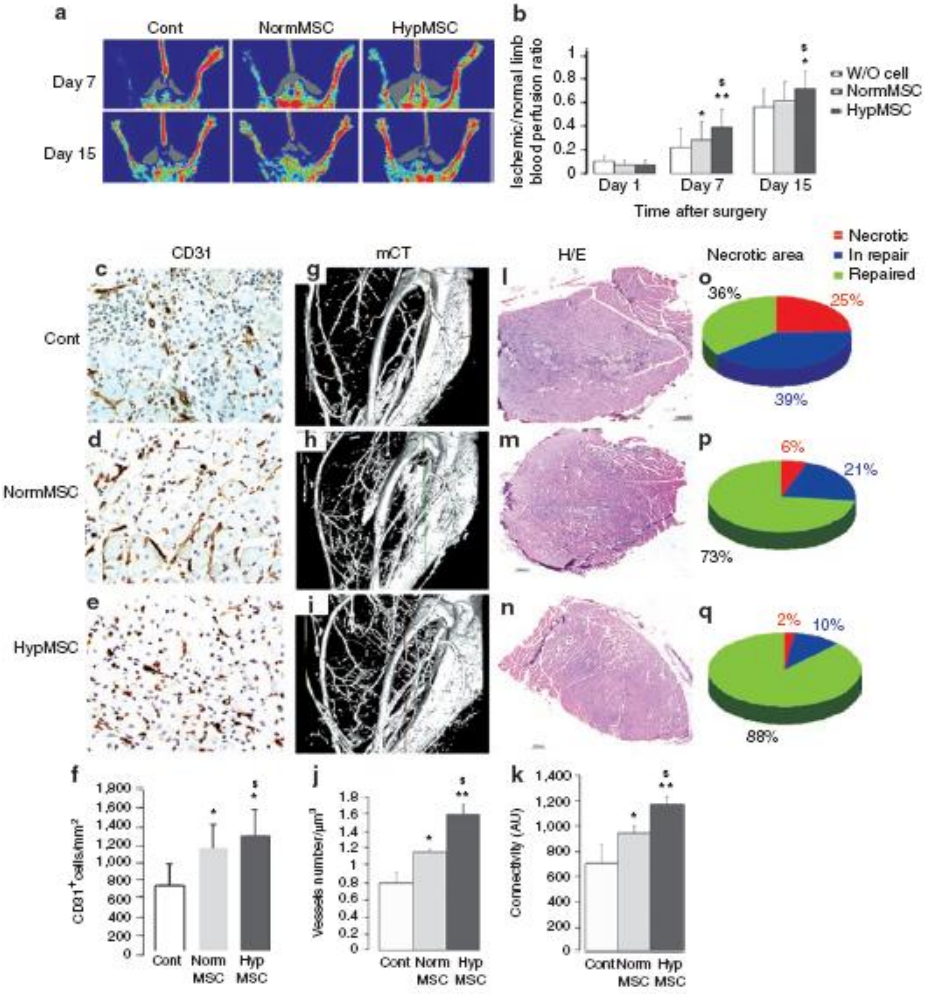

Figure 1 Augmented vascular regeneration by intramuscular transplantation of MSC in a hindlimb ischemia model. (**a**) Images of representative LPDI in sham mice (control W/O cell) and in HypMSC or NorMSC transplanted groups at day 7 ($n = 21$) and day 15 ($n = 15$) after ischemia. (**b**) Quantitative analysis of hindlimb blood perfusion by calculating the ischemic/normal limb perfusion ratios after the induction of hindlimb ischemia. Blood perfusion of the ischemic hindlimb markedly increased in the HypMSC and NormMSC group 1 week after transplantation. LPDI index in the HypMSC group was the highest among the three groups at day 7 and day 15. Data are means ± SEM; $^*P < 0.05$ and $^*P < 0.01$ versus control; $^{\$}P < 0.05$ versus NormMSC. (**c–f**) Vessel numbers were calculated after CD31 immunolabeling in the control, NormMSC, and HypMSC groups. $^*P < 0.00001$ versus control; $^{\$}P < 0.001$ versus NormMSC. (**g–i**) Reconstructed micro-CT images of control, NormMSC, or HypMSC-injected hindlimb 7 days after ischemia in age- and gender-matched mice (tm^3 resolution). Note marked increase in (**j**) vessel number and in (**k**) connectivity in HypMSC-treated mice versus NormMSC-treated mice and control mice. Data are % ± SEM; $^*P < 0.05$ and $^{**}P < 0.001$ versus control; $^{\$}P < 0.05$ versus NormMSC. $n = 6$ in each group. Repaired surface measurement of control and MSC group ($n = 6$ in each group) in hindlimb ischemic mice. (**l–q**) Representative repaired surface and necrotic area were quantified as ratio of necrotic or in repair or repaired surface out of total muscle area. cont., control; HypMSC, hypoxic preconditioned murine MSC; LPDI, laser doppler perfusion imaging; MSC, mesenchymal stem cells; NormMSC, nonpreconditioned MSC; W/O, without.

that bromodeoxyuridine incorporation rate was significantly increased in HypMSC group compared to NormMSC group (16.3 ± 8.1 versus 13.1 ± 6.6 bromodeoxyuridine/GFP + cells/mm² $P < 0.05$) (Figure 2c–d).

HypMSC were widespread in hypoxic zones and may play a macrophage-like function. HypMSC appeared to have a different pattern of engraftment as compared to NormMSC. To better understand this, we evaluated the scattering of MSC in hypoxic zones using the 2-nitroimidazole hypoxia marker, pimonidazole. Hypoxic sections of muscle produced a mottled staining with a characteristic hypoxic brown center. At day 7, a large number of GFP^+ HypMSC were evenly distributed in deep hypoxic zones around necrotic myocytes whereas GFP^+ NormMSC were consistently detected at the periphery of ischemic zones (Figure 3a and **Supplementary Figure S2**). The distribution of MSC around

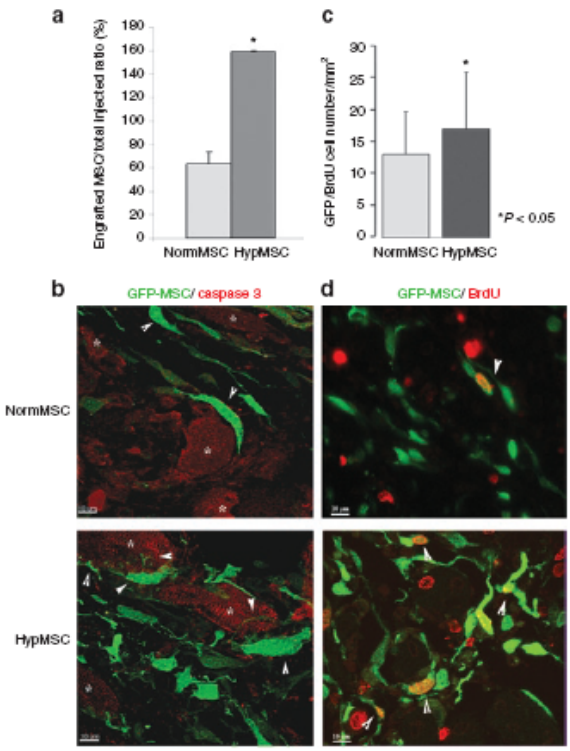

Figure 2 Engraftment, survival, and proliferation of MSC in ischemic hindlimb tissues. (**a**) Engraftment of GFP-MSC in muscle. The levels of GFP expression was determined by performing real-time RT-PCR. The percentage of engrafted MSC was assessed by the ratio of GFP level at day 7 with that of GFP level at D0 (initial MSC graft) ($n = 4$ in NormMSC and HypMSC groups). The histogram showed a higher persistence of MSC in the muscle at day 7 after injury in HypMSC-treated groups compared to NormMSC-treated group. *$P < 0.05$, $n = 10$. After 7 days of ischemia, (**b**) survival assay of MSC and (**c–d**) induction of MSC proliferation were assessed using respectively a double GFP (green)/caspase 3 (red) and a double GFP (green)/BrdU (red) immunofluorescent-labeling performed on NormMSC and HypMSC transplanted tissue sections. (**b**) Representative photomicrographs at day 7 after ischemia showed MSC (arrowhead) surrounding caspase 3 positive myocytes (white star). (**c**) The levels of double GFP/BrdU NormMSC and HypMSC (white arrowheads) were quantified and reported in histogram graphs. (**d**) Representative photomicrographs of ischemic hindlimb sections at day 7 after ischemia revealed a higher level of proliferating GFP-MSC in HypMSC-injected tissues compared to that in NormMSC-injected tissues. *$P < 0.05$, $n = 10$. BrdU, bromodeoxyuridine; GFP, green fluorescent protein; HypMSC, hypoxic preconditioned murine MSC; MSC, mesenchymal stem cells; NormMSC, nonpreconditioned MSC; RT, reverse transcription.

necrotic myocytes suggested a potential macrophage-like, phagocytic function for these cells. GFP immunofluorescent staining revealed that MSC harbor a phenotype with long filopodial protrusions that can encircle and penetrate into dead myocytes (Figure 3b–d). To evaluate the phagocytic capacity of MSC in regards to myocytes, *in vitro* experiments were conducted. Fluorescent microscopy examination revealed that GFP-MSC cocultured with necrotic cell bodies of PKH-labeled murine myoblasts (red) were able to robustly engulf part of the myoblast cell membranes (Figure 3f,g). GFP-MSC cocultured with non-necrotic PKH-labeled murine myoblasts (red) did not have this property (Figure 3e). At day 15, HypMSC appeared to be preferentially associated with capillaries and microvessels, behaving as perivascular cells shaping along the whole length of the vessel, covering growing CD31-forming tube-like structures, as mural cells (**Supplementary Figure S3**).

HypMSC paracrine Wnt-dependent effect on angiogenesis and muscle regeneration

The effects of temporary hypoxia on Wnt factor expression were also investigated. Several studies have demonstrated a role of the Wnt pathway in vascular development[20] as well as stem cell proliferation and maintenance.[13] We hypothesized that MSC express Wnt and use the Wnt pathway as a mechanism for activation of muscle regeneration processes. Screening by reverse transcription-PCR for the expression of different *Wnt* transcripts revealed that MSC express *Wnt4*, *5A*, *5B*, and *11*. *In vivo*, *Wnt4* transcripts were significantly increased in HypMSC-injected muscles (ratio *Wnt4/P0*: 15.6 ± 5.2, $P = 0.01$ versus control tissues) whereas NormMSC implantation did not significantly increase Wnt4 transcripts (ratio *Wnt4/P0*: 3.8 ± 1.8, P = NS versus control tissues) (Figure 4). Wnt5A, 5B, and 11 expressions were not significantly modified in either HypMSC- and NormMSC-injected muscles compared with control muscle at day 7 after ischemia. Corroborating evidence was found *in vitro* where a specific strong upregulation of *Wnt4* was detected after exposing MSC to hypoxia for 36 hours compared to normoxia-treated MSC (Wnt4/P0: 4.78 ± 1.8, $P = 0.01$ in comparison to control) (**Supplementary Figure S4**). In parallel, we report preconditioning effects on angiogenic growth factor expressions by HypMSC (**Supplementary Figure S4a–c**).

Functional validation of HypMSC secreted factors on cell functions

To further elucidate the functional paracrine and autocrine role of HypMSC secreted factors in vascular response and muscle regeneration enhancement, we studied the effect of *ex vivo* incubation of the different cellular components namely EC, muscle fibers, and MSC with HypMSC and Wnt4 conditioned medium (HypCM and Wnt4 CM, respectively).

HypMSC secreted factors and Wnt4 played a role on EC migration but not on EC proliferation. Transwell filters revealed that HypCM and Wnt4 CM induced significantly more migration of EC than did respectively CM from NormMSC (NormCM) and control CM ($P < 0.05$). Moreover, addition of a secreted Wnt antagonist, recombinant bovine sFRP-1, blocked the HypCM-induced migration suggesting that Wnt-dependent factors are the major active factors present in HypCM. (Figure 5a and **Supplementary Figure S5**). It is worth noting that recombinant bovine sFRP-1 enhanced migration of both NormCM-treated EC and control EC, which is in agreement with previous reports.[21] Interestingly, HypCM had no effect on EAHy926 proliferation compared with CM from NormMSC (NorCM) (data not shown). Taken together, these results support a role of HypCM and Wnt4 on EC migration.

Wnt4-induced skeletal myoblast differentiation. To explore the potential role of Wnt4 on skeletal muscle differentiation,

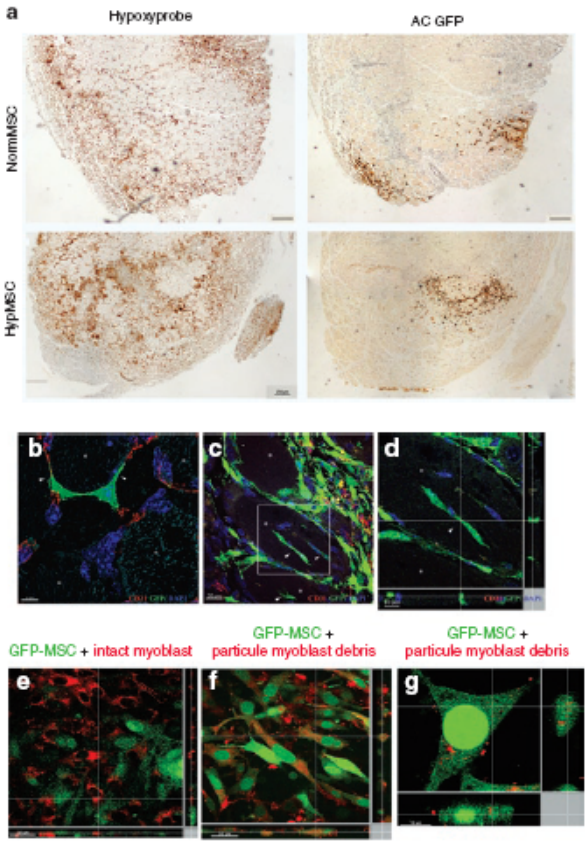

Figure 3 HypMSC engraftment sites could assist MSC in their macrophagic-like role. (**a**) At day 4, hindlimb muscles transplanted with either NormMSC or HypMSC have large hypoxic zones as revealed using pimonidazole binding (Hypoxyprobe). Immunostaining of GFP-MSC with an antibody directed against GFP (AC anti-GFP) showed localization of NormMSC at the peripheral front of hypoxia although HypMSC displayed a scattered distribution inside hypoxia zone. MSC macrophagic activity. (**b**) Immunofluorescent analysis for GFP expression (green) with CD31 (red) on hindlimb tissue section at day 4, revealed that during muscle regeneration, GFP^+ MSC (green) surrounded necrotic muscle fiber, harboring a spindle shape with long filopodial extensions. (**c**) Arrow heads point to MSC extensions, which enter through necrotic skeletal muscle fibers (**d**) close-up of the boxed region in the larger image. White stars indicate necrotic muscle fiber. Cell nuclei are stained with DAPI (blue). *In vitro* (**e**) GFP^+ MSC were mixed with intact PKH-labeled skeletal muscle fibers or (**f**,**g**) in contact with PKH-labeled skeletal muscle fiber particulate debris. In presence of muscle fiber membrane debris, MSC have the capacity to phagocyte particles. (**d**–**g**) The cartoons at right and bottom end side represent perpendicular views of the confocal acquired images. (**f**,**g**) White lines show myocyte red particulate debris in GFP-MSC cytoplasm. AC,; GFP, green fluorescent protein; HypMSC, hypoxic preconditioned murine MSC; MSC, mesenchymal stem cells.

intact primary murine skeletal myoblasts were cultured in the presence of Wnt4 CM for 16 hours and compared with myoblasts treated with control CM. The results revealed that the number of myogenin-expressing cells was significantly increased in Wnt4-treated myoblast culture compared to control condition ($P < 0.01$) (Figure 5b).

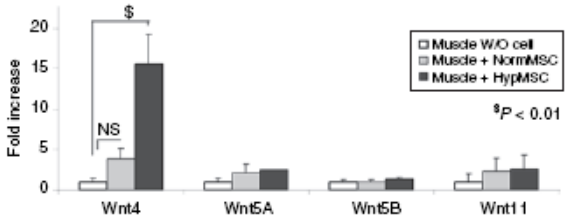

Figure 4 Effects of HypMSC transplantation on Wnt gene expressions. *Wnt4*, *5A*, *5B*, and *11* expressions in hindlimb ischemic muscle were analyzed by performing real-time PCR. Hindlimb muscles transplanted with NormMSC (gray bars) or HypMSC (black bars) or without cells (sham group, white bars) were extracted at day 7 after ischemia. Values are means ± SD; $n = 3$; the assays were carried out in triplicate. $^*P < 0.05$, $^{\$}P < 0.01$ versus control. HypMSC, hypoxic preconditioned murine MSC; NormMSC, nonpreconditioned MSC.

Autocrine role of Wnt4 promoted MSC proliferation, migration and Wnt canonical ß-catenin pathway activation. We then tested the hypothesis that enhancement of HypMSC engraftment could be due in part to *Wnt4* upregulation. The role of Wnt4 as a potent inducer of MSC migration was demonstrated (Figure 5c). In addition, data revealed an effect of Wnt4 on the number of MSC but only under hypoxic conditions (Figure 5d). Moreover, Wnt4 was able to activate the canonical Wnt pathway as shown by T-cell factor-dependent transcriptional activity measured under hypoxic conditions; transfection of MSC with a plasmid coding for Wnt4 provoked a 15-fold increase in luciferase activity (Figure 5e). No activation was seen by using the corresponding control reporter with mutated T-cell factor binding sites. Inhibition of Wnt signaling using recombinant bovine sFRP-1 also effectively suppressed TopFlash luciferase reporter activities in response to Wnt4. Our data are consistent with the interpretation that Wnt4 signals are transduced via the canonical branch of the Wnt pathway and reveal that MSC respond to Wnt4 stimuli in a hypoxic environment.

Suppression of Wnt4 expression in HypMSC prevented the vascular regenerative response induced by hypoxia pretreatment. An *in vivo* model of *Wnt4* suppression was employed to demonstrate the importance of Wnt4 as a key paracrine factor mediating proangiogenic effects of HypMSCs. As such, we treated HypMSC with two small interfering RNA against *Wnt4* (Figure 6a) and transplanted them into the mouse ischemic hindlimb model. We showed that siControl-treated HypMSC compared to siControl-treated NormMSC enhanced significantly the blood flow ($P < 0.05$) (Figure 6b), and vascular density ($P < 0.05$) and connectivity ($P < 0.05$) (Figure 6c–d). Treatment of HypMSC with small interfering RNA against *Wnt4* abrogated the difference between HypMSC and NormMSC groups; either blood flow or vascular density and connectivity were not significantly different between siWnt4-treated HypMSC and siControl-treated NormMSC groups.

DISCUSSION

The influence of temporary hypoxic preconditioning of MSC on the stimulation of angiogenesis and muscle regeneration was investigated and the data revealed that *ex vivo* hypoxic preconditioning of MSC plays a unique role in the enhancement of vessel

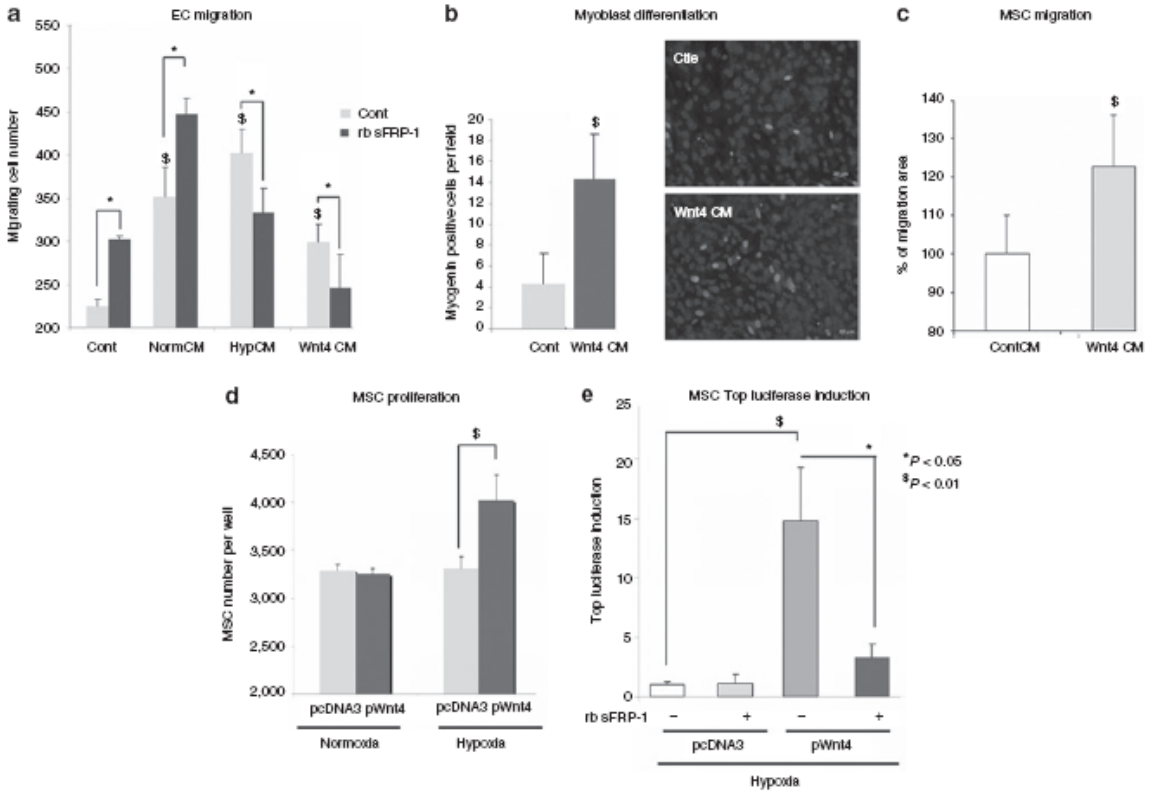

Figure 5 Effect of HypCM and Wnt4 CM on cell properties. (**a**) Endothelial cell chemotactic migration was quantified using transwell filters. Fluorescent-labeled EAHy926 migrated in the presence in the lower chamber of either NormCM, HypCM, and Wnt4CM with or without rb sFRP-1 in the lower chamber. Migrated EC were then lysed and quantified on a fluorimeter. All the CM induced EAHy926 migration. $^{*}P < 0.05$, $^{\$}P < 0.01$ versus control. rbsFRP-1 induced migration in NormCM assays and blocked HypCM and Wnt4CM induced EAHy926 migration ($^{*}P < 0.05$). (**b**) Muscle fibers were cultured either in control CM (cont. CM) or in Wnt4 CM during 48 hours. Increase expression of myogenin assessed an effect of Wnt4 on myoblast differentiation. The numbers of myogenin-positive nuclei were reported on the graph. $^{\$}P < 0.01$. (**c**) The effect of Wnt4 on MSC migration was quantified after in an *in vitro* wound assay. Confluent MSC were wounded and placed during 16 hours in presence of either control CM (cont. CM) or Wnt4 CM. Analysis of Wnt4 effect on wound closure was measured as area of migration (μm²) on phase contrast microscopy images. $^{\$}P < 0.01$. (**d**) MSC proliferation of MSC was influenced by Wnt4 overexpression (pWnt4 versus pCDNA3, $^{\$}P < 0.01$) in hypoxia but not in normoxia. MSC were maintained during 24 hours either in normoxia or in hypoxia after transfection before counting. Values correspond to total cell numbers per well. (**e**) Wnt4 activates the TopFlash reporter in MSC. Cotransfection of MSC with TopFlash or FopFlash reporter and Wnt4 resulted in more than a 15-fold increase in TopFlash over FopFlash activity compared with MSC transfected with an empty vector (pcDNA3) in hypoxia condition ($^{\$}P < 0.01$). Adding of rb sFRP-1 blocked either Wnt4 overexpression induced TopFlash over FopFlash activity ($^{*}P < 0.05$). The experiments were conducted in hypoxia. The error bars indicate on SD from the mean of triplicate samples. EC, endothelial cell; HypCM,; MSC, mesenchymal stem cells; rb, recombinant bovine; sFRP-1, secreted frizzled-related protein 1.

formation and repair of the necrotic zone after a hindlimb ischemia via a Wnt-dependent pathway.

We hypothesized that the 1% oxygen atmosphere in which MSC were maintained could condition them to survive in a hypoxic environment *in vivo* as reported *in vitro*.[22,23] Oxygen tension in bone marrow compartments has been reported to be very low as in injured tissue, suggesting that tissue hypoxia may be a fundamental mechanism governing stem cell recruitment and retention.[10] As a result, we demonstrated that hypoxic pretreatment can more effectively overcome the hurdle of cell death after implantation than normoxic conditioning, promoting MSC survival, and proliferation *in situ*. HypMSC engrafted robustly in ischemic tissue and their proliferation index in ischemic sites was superior to that of NormMSC. The decline MSC number that has been previously described could be due to cytokine secretion and tissue remodeling[24] or immune rejection by the host as they expressed GFP a well-known immunogen.[25]

MSC have been successively administered as a treatment in both animal studies[6,24,26] and human clinical trials.[27,28] Studies showed their possible contribution to vascular structures and skeletal muscle *in vivo*. For example, injection of MSC in a rat model of dilated cardiomyopathy revealed that some of the MSC were differentiated into cardiomyocytes.[6] The application of MSC improves wound healing regeneration through differentiation.[24] Recent studies suggest that monocytes under activation could express EC markers and form tube-like structures.[29] The setting of hypoxic preconditioning in this study did not enhance cell differentiation into EC, as HypMSC kept their pericyte-like properties and behaved as

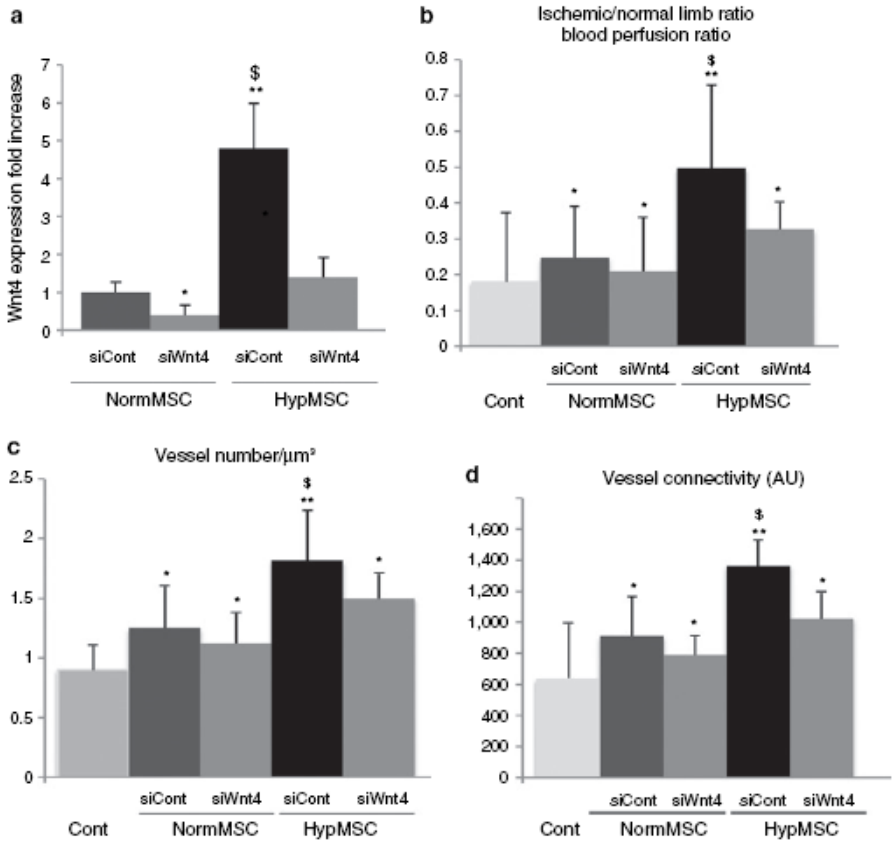

Figure 6 Effect of Wnt4 knockdown on NormMSC or HypMSC treated with siControl (siCont) or with siWnt4. (**a**) On Wnt4 expression *in vitro* followed by performing real-time PCR. (**b–d**) On vascular effect induced by MSC: quantitative analysis of hindlimb blood perfusion by calculating the ischemic/normal limb perfusion ratios after the induction of (**b**) ischemia, and microCT quantification of (**c**) vessel number and (**d**) connectivity at D7 after injury in mice transplanted either with NormMSC or HypMSC treated with siControl (siCont) or with siWnt4 or not transplanted (cont.). Data are % ± SEM; *$P < 0.05$ and **$P < 0.001$ versus control. $^{\$}P < 0.05$ versus NormMSC. $N = 9$ in each group. cont. control; HypMSC, hypoxic preconditioned murine MSC; microCT, micro-computed tomography; MSC, mesenchymal stem cells; NormMSC, nonpreconditioned MSC.

perivascular cells as reported.[2,30] Their tissue localization led us to hypothesize a potential macrophage-like role in which they participate in phagocytosis of necrotic muscle fibers. Our data indicates that MSC have the ability to participate in the phagocytic process of muscle fibers *in vitro* and to encircle and send extensions into necrotic muscle fibers *in vivo*, which could indicate that these cells participate in clearing skeletal muscle cellular debris. This process has similarities with previously published data which demonstrated that macrophages reorganize their actin cytoskeleton to form phagocytic lamellipodia structures to engulf apoptotic cells.[31] Moreover, at early time points, MSC appear to play a role in neovessel formation encircling EC in capillaries and microvessels and participating in their stabilization as previously described.[32,33] Our results support the notion that MSC play a cooperative role in both myocyte regeneration and vascular formation by structural and functional interaction with both myocytes and EC.

As MSC have been shown to secrete a large quantity of angiogenic factors,[34] we investigated the potential involvement of paracrine and/or endocrine factors produced by MSC. Previous reports have demonstrated that conditioned medium from MSC can promote EC migration, differentiation, and survival.[35] Our data indicate that conditioned medium from HypMSC is more effective than conditioned medium from NormMSC in inducing EC migration as well as MSC proliferation and migration. As the Wnt signaling pathway has been shown to play a role in stem cell fate,[13] muscle regeneration,[36] vascular formation,[37] and invasion capacity of MSC,[19] we hypothesized that this pathway may be involved in the mechanism involved in this process. Our study identified that Wnt4 was specifically overexpressed *in vitro* in conditioned media from HypMSC and that, *in vivo*, injection of HypMSC into the ischemic hindlimb strongly induced Wnt4 expression. We also demonstrate that Wnt4 exerts an effect on myoblast differentiation, *in vitro*. We have established a role of Wnt4 as a potent mediator in vascular regeneration as it affects EC migration. It is worth noting that Wnt4 conditioned medium could affect MSC migration and proliferation but only under hypoxic conditions through a canonical J3 catenin-dependent Wnt signaling. Thus, it is likely that, *in situ*, residual tissue hypoxia confers responsiveness of MSC to environmental signals. Moreover, Wnt4 signaling was shown to stimulate myogenic proliferation and differentiation during chick

development[38] and satellite cell proliferation in skeletal muscle in myostatin-knockout mice.[39] Finally, we demonstrate here that, knockdown of Wnt4 expression resulted in inhibition of the beneficial impact of HypMSC on vascular regeneration compared with that induced by NormMSC. Collectively, these results strongly support the hypothesis that Wnt4 secreted by MSC is an important factor in mediating tissue regeneration in the setting of ischemia.

In conclusion, we have demonstrated that hypoxic preconditioning of MSC before transplantation could be a potent strategy in overcoming the current limitations in the field of regenerative medicine. This study suggests that many of the cellular functions associated with the ability of MSC to regenerate ischemic tissue are often not expressed constitutively by MSC but rather only transiently. MSC respond to contextual signals according to their microenvironment.

MATERIALS AND METHODS

MSC[2,26] were treated for 36 hours at 1% O_2 in a hypoxia chamber (HypMSC) (C21 Proox chamber; BioSpherix, Lacona, New York) or maintained at 20% O_2 (NormMSC) before being injected into a mouse ischemic hindlimb. Mice were randomly assigned to three treatment groups. One group underwent sham treatment (*i.e.*, aperture and saline injection) (control group). The other groups received injection of either 0.5×10^6 NormMSC or HypMSC. Detailed description of materials, animal procedures, MSC preparation, hindlimb ischemia model, tissue collection and preparation for biochemical assays, laser doppler perfusion imaging, microcomputed tomography analysis, immunohistochemistry, macrophagic assays, and *in vitro* cell functional assays can be found in the **Supplementary Materials and Methods**.

SUPPLEMENTARY MATERIAL

Figure S1. MSC detection.

Figure S2. Double immunolabeling of GFP and CD31 in ischemic tissues at day 7 and day 15 after injury transplanted with either NormMSC or HypMSC.

Figure S3. MSC harbored a pericyte-like phenotype after implantation in ischemic muscle at day 7.

Figure S4. Effect of HypMSC transplantation on growth factor gene expressions and induction of growth factor and Wnt factors in HypMSC vs NormMSC.

Figure S5. Effect of HypCM or Wnt4 CM on EC wound migration.

Materials and Methods.

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Cell cultures and transfection

- Harvest and culture expansion of murine MSC

Bone marrow cells were harvested by flushing the tibiae and femurs of C57Bl/6J mice (Charles River Laboratories) with DMEM and further purified in Ficoll Paque (Amersham Biosciences, Orsay, France). MSC were characterized using immunostaining as previously described (Derval, Barandon et al. 2008; Dufourcq, Leroux et al. 2008).

MSC culture: we harvested bone marrow cells from femurs from 5 mice. Mouse bone marrow mononuclear cells were purified by Ficoll Paque and seeded. Five to seven days later, the non adherent hematopoietic cells were discarded. The attached cells grew and developed colonies in 15–30 days. Colonies were then sub cloned and then were passed more than 8 times prior to their characterization and further use. MSC have been used between passage 8 to 16 in our experiments. It has been previously shown that MSC phenotype varied during the first passages. After 8 passages, MSC phenotype remains stable.

MSC phenotype : MSC express Thy1, Sca1, and cKit and NG2 markers. By quantitative RT-PCR, we showed that MSC expressed a actin, and PDGFR b. MSC remained negatives for smooth muscle cell differentiation marker (smMHC negative) and endothelial cell markers (VE cadherin, CD31, Tie2 and Flk negatives). At long term, we showed that the phenotype of MSC was stable at 3 months after transplantation in a plug assay (Dufourcq, Descamps et al. 2008). We have run immunolabelling, FACS and RT-PCR analysis for the same markers after MSC hypoxia preconditioning. Under low oxygen concentration, MSC kept their pericyte markers and did not express any endothelial cell marker (Figure S3).

Experiment: for each conducted experiment, we have unfrozen MSC batches. 5 distinct MSC clones have been used in this study. MSC were seeded, and expanded in culture prior to injection. We controlled MSC populations for above markers by RT-PCR and FACS

analyzer.

Cultured MSC were transduced with pMND-eGFP lentivirus from Trono laboratory (Lausanne, Switzerland) by replacing the medium with fresh medium containing viral supernatant and incubating for 3h at 37°C. Cells were harvested 48 h after the infection and seeded as described here above. MSC were transduced once and retained the fluorescence during the following passages.

For hypoxia treatment, $25x10^3$ cells/cm^2 were seeded onto plastic petri dishes two days prior to experiments. Medium was changed to Optimem (GibcoBrl) and cells were either treated for 24 to 48 hours at 1% O_2 in a hypoxia chamber (HypMSC) (C21 Proox chamber, BioSpherix, New York) or maintained for 24 to 48 hours at 20% O_2 (NormMSC). Hypoxic conditioned media (HypCM) or normoxic conditioned media (NormCM) were obtained immediately after removal respectively either from the hypoxia chamber or from the regular incubator. We have verified that hypoxia treatment did not affect MSC viability using FACS analysis (data non shown).

- *EAhy926 and myoblast culture and transfection*

EAhy926 cell line and myoblasts (from Dr S. Germain, Inserm U833, France) were cultured in Dubelcco's modified essential medium (DMEM, Invitrogen) with 10% FCS supplemented with L-glutamin and vitamin/gluthation (Invitrogen). In all experiments, Wnt pathway blockade was done with 10 nmol/L estimated rb sFRP-1 protein (Dufourcq, Leroux et al. 2008) or with Dkk1 (R&D).Transfections were performed using Lipofectamine (Invitrogen) according to the manufacturer's protocol. The plasmid encoding the human Wnt4 (pWnt4) was kindly provided respectively by Dr Tsutomu Nohno (University of Hyogo, Hyogo, Japan).

- *Preparation of Wnt conditioned media:*

Serum free media (SFM) conditioned by CHO cells transiently transfected with the control plasmid pcDNA3.2 (cont CM) or with the plasmid containing human Wnt4 (Wnt4 CM) were assayed for their effects on the proliferation or the migration of MSC, myoblasts and EAHy926.

Mouse model of unilateral hind limb ischemia

Unilateral hind limb ischemia was operatively induced as previously described with the excision of the femoral and saphenous arteries (Ezan, Leroux et al. 2004). This study was conducted in accordance with both institutional guidelines and those in force in the European community for experimental animal use (L358-86/609/EEC).

Mice were randomly assigned to three treatment groups. One group underwent sham treatment (i.e., aperture and saline injection) (control group). The other groups received injection of either 0.5 x10^6 NormMSC or HypMSC. Cell injections were performed 2 days after hindlimb ischemia by direct intramuscular injection into the ischemic thigh and anterior tibialis muscle in multi sites with a 27-gauge needle. Each animal received an intraperitoneal injection of bromodeoxyuridine (BrdU Amersdham, UK) (30 mg/kg) 24 hours prior to sacrifice in order to measure the rate of cell proliferation. Laser Doppler perfusion imaging (Moor Instrument, Wilmington, DE) was used to record serial blood flow measurements over the course of 15 days post operatively as previously described (Ezan, Leroux et al. 2004). A group of mice were injected with pimonidazole (HypoxyProbe, Chemicon) (1.5 mg/ 20g intraperitoneously) 1 hour before sacrificing to investigate the hypoxic zones.

Tissue preparation: animals were sacrificed at different time points with an overdose of sodium pentobarbital. For immunohistochemistry, anterior tibialis muscles were fixed in paraformaldehyde (PFA 4%) and embedded in paraffin. For protein or RNA extraction, tissue samples were rinsed in PBS to remove excess blood, snap frozen in liquid nitrogen, and stored at -80°C until used.

For microCT scanning: the vasculature was imaged with a high-resolution micro-CT imaging system (GE eXplore Locus SP), set to a 0.016-mm effective detector pixel size. The apparatus used for imaging the vessels was the eXplore Locus Micro-CT scanner from General Electric Healthcare® with spatial resolutions of 36 to 7 µm, and used with the Scan Control® and Reconstruction Utility® programs. Data were acquired in an axial mode covering a single hindlimb as previously described(Tirziu, Moodie et al. 2005). The quantification parameters were obtained using the Microview ABA® program. We then delineated and digitally deleted the bones of the hind limb and used the density of our contrast medium as a reference for the program.

For each muscle, necrosis and regeneration measurements were done on cross-sections chosen at regular intervals spanning a 2 mm muscle segment. Entire cross-sections were evaluated on low power field images and zones were measured using Sigma scan pro software.

All parameters were expressed as % positive area per cross-sectional muscle area. Necrotic zones showed signs of massive tissue degeneration. Myofibers with pale cytoplasm and loss of peripheral nuclei were defined as necrotic as previously described (Shireman, Contreras-Shannon et al. 2007); zones in repair were defined by the presence of multinucleated skeletal muscle; repaired muscles after ischemia were characterized by the smaller size of the fibers and the presence of centrally located nuclei defined by the presence of skeletal fibers with a central nucleus; they are typically found in the middle of muscle area in repair (Fig1 Q-R) (Aranguren, McCue et al. 2008).

Immunohistochemistry tissue preparation.

Five-micron, paraffin-embedded sections were cut transversely from the mouse muscle. The following antibodies were used against CD-31 (BMA biomedical), SMA (Sigma), NG2 (Chemicon), GFP (Invitrogen), and BrdU (Harlan SERA-LAB, England) caspase 3 (Promega). Capillary and arteriole densities were recorded respectively as the number of CD31 positive capillaries and SMA-positive arterioles per mm^2. Double BrdU/GFP positive blood vessels were quantified, and results were expressed as the percentage of double stained/total GFP positive cells in each condition. A minimum of 30 randomly chosen pictures were recorded at 20X magnification under a microscope for each animal at each time point with a camera connected to a PC. Positive cells were manually counted on captured images and the data were analyzed with the help of Sigma Plot software. All analysis was performed by an investigator blinded to treatment groups.

Bound pimonidazole was detected in paraformaldehyde-fixed, paraffin embedded tissues with a biotin-streptavidin-peroxidase indirect immunostaining method (Samoszuk, Walter et al. 2004). Immunodetection was carried out using a Ventana Discovery automat for Pimonidazole and GFP detection on paraffin sections. Briefly, slides were deparaffinized and treated with protease 1 (Ventana) for 30 min before incubating with antibodies at 37°C

for 30 min. Slides were then incubated with corresponding biotinylated secondary antibodies before staining with DAB using DaBMap kit (Ventana).

Quantitative RT-PCR

Total RNA from MSC and anterior tibialis muscle was isolated with Tri reagent (Euromedex) and DNAse (Promega) according to the manufacturer's protocol. RNA from each sample was reverse transcribed as described (Dufourcq, Leroux et al. 2008). PCR was done using IQ SYBR Green supermix (Bio-Rad). An MJ Research Opticon and the following parameters were used for real time PCR: 95°C for 5 min followed by 35 cycles of 95°C for 15s, 60°C for 20s and 72°C for 15s. All experiments were done in triplicate and differences in cDNA imput were "compensated" by normalization to expression of P0. Primers uses were for VEGF forward 5'- CTCCAGGGCTTCATCGTTA -3', reverse 5'- CAGAAGGAGAGCAGAAGTCC -3'; TGFb forward 5'- GCTAATGGTGGACCGCAACAAC -3', reverse 5'- CACTGCTTCCCGAATGTCTGAC -3'; bFGF forward 5'- CCAAGCAGAAGAGAGAGGAGTTGTG -3', reverse 5'- TGCCCAGTTCGTTTCAGTGC -3'; Angiopoietin 1 forward 5'- GCACGAAGGATGCTGATAACGAC -3', reverse 5'- TGATTTTGTCCCGCAGTGTAGAAC -3'; Angiopoietin 2 forward 5'- GCACAAAGGATTCGGACAATGAC -3', reverse 5'- GTTCTTCTGGTAGTGTAGGCAGGC -3'; PDGFB forward 5'- TCTTCCTTCCTCTCTGCTGCTACC -3', reverse 5'- CCCCATCTTCATCTACGGAGTCTC -3'; Wnt4 forward 5'- TTCACAACAACGAGGCTGGCAG -3', reverse 5'- CACCGTCAAACTTCTCCTTTAGCG -3'; Wnt5A forward 5'- GGCATCAAGGAATGCCAGTA -3', reverse 5'- GTACGTGAAGGCCGTCTCTC-3'; Wnt5B forward 5'- CTTCCTGAAAATGGAGCCAGC -3', reverse 5'- TTGGTCCCCAGTCAAAACCC-3'; Wnt11 forward 5'- GTAGGGCCTTCGCTGACAT -3', reverse 5'- CGATGGTGTGACTGATGGTG -3'. The results were analyzed using the comparative C_t method.

Quantification of MSC in vivo :

To determine the percentage of GFP positive MSC in the muscle, the percent of GFP remaining gene expression was assessed with real time RT-PCR using the comparative C_T method ($\Delta\Delta C_T$). The sample is assessed with two assays one that measures the RNA level of the GFP and one that of P0 as an endogenous calibrator. We first harvested the muscles 10

hours after cell graft in the ischemic hind limb. After RNA extraction, GFP transcripts were quantified by PCR to determine the number of GFP positive cells in the muscle (number of engrafted MSC). We found that 5% of NorMSC and 4.5% HypMSC injected were viable at this early timepoint. We then compared the number of viable MSC at day 7 after injury to the number of MSC initially engrafted (i.e. viable) at 10 hours post injection and results are expressed as a ratio of GFP expression (n=3 animals per time per condition). Before, a linear GFP expression curve has been established: 2 000, 10 000, 50 000 and 250 000 MSC were injected in the tibialis (n=3 per conditions). Muscle were harvested, total RNA were extracted and subjected to qRT-PCR for GFP transcript detection. A linear correlation was observed between the fluorescence threshold cycle number (Ct) and the starting quantity of GFP-MSC injected in the muscle suggesting that this method was applicable to quantify MSC in muscle samples harvested at different time point after GFP-MSC injection.

Functional assays

Test MSC ability to phagocyte MSC debris in culture: myoblasts were labeled with PKH dye according to the manufacturers' recommendations (PKH linker kit, Sigma). To obtain myoblast particulate debris, myoblasts in suspension were submitted to 3 rounds of frozen-thawed cycles. GFP-MSC were seeded at a density of 40 000 cells/cm^2 in 2 cm^2 labtek. At confluence, MSC were overlayed with either 125 000 intact or PKH-labeled myoblast particulate debris. After 16 hours, cell cocultures were fixed in PFA 4% for 10 min and observed with a confocal microscope.

Cell proliferation assays: MSC or myoblasts were seeded into 24-well plates at $25x10^3$ cells/well and incubated overnight to allow adhesion with their respectively appropriate medium. Cells were then either transfected with control plasmid or plasmid coding for Wnt4 or incubated with conditioned media. After 48 hours, total cells were harvested and counted in each well.

Cell migration assays: cell chemotactic migration was carried out with 8 nm-pore size in 12-well Transwell migration chamber (Costar). Membranes were coated at room temperature for one hour with 0.1 % gelatin. Loading of EAhy926 and MSC for fluorescence was accomplished by incubation of cell suspensions in a 10mM solution of the cell permeant, the AM ester of BCECF. EAhy926 and MSC were then plated at 50 000 cells and 100 000

cells/well respectively, for one hour in serum-free medium (SFM) with 0.1% of BSA. After one hour, medium was removed and migration was activated with conditioned media in the lower chamber. After 16h, cells from the upper surface were removed with cotton swab, and migrated cells were lysed in Tris 100mM, PH 8.0, Triton X100 0.1%. Quantification of the fluorescent signal was measured using a fluorimeter with a fluorescent signal set at 530 nm. Migrated cell number was calculated starting from a calculated linear curve. Each experiment was performed in triplicate and migration was expressed as the % of migrating cells compared to control conditions (0.1% BSA).

Migration was also evaluated in the scratch wound assay. MSC or EAHy926, grown to a confluent monolayer in 1% coated gelatin were starved in SFM containing 0.1% FBS. A central scratch was created by scraping cells away with a p200 pipette tip. After removal of debris by washing the wells with PBS, cells were incubated in presence of SFM conditioned medium from cultured MSC or from Wnt4 conditioned media in different oxygen tensions. For each image, migration areas were measured using the Act1 software (Nikon). Each condition was run in triplicate and the assay was repeated three times.

Cell luciferase assay: for top flash assay, MSC were transfected in 24-well plates at 2,5 x 10^5 cell/cm^2 density with super8XTopFLASH or FopFLASH luciferase reporter plasmids (375 ng/well) containing LEF/TCF consensus binding sites (Randal T. Moon laboratory). pRL-CMV (Promega) was cotransfected (90 ng/well) to normalize samples for transfection efficiency (Dual luciferase kit, Promega). Transfections were performed with Lipofectamine 2000 (Invitrogen) reagents according to the manufacturers' recommendations. After 24 hours, the medium was replaced with SFM conditioned medium from MSC cultured in different oxygen tensions. Cells were harvested 24 hours later and luciferase activity was determined with a Turner Designs luminometer. The data are presented as the means SD of triplicate well measurements for one representative experiment.

Quantification of differentiation

Myoblasts were incubated during 48 hours with Wnt4 CM or with control CM, washed with PBS, fixed in PFA 4% and immunostained with an antibody against myogenin (SantaCruz). Myogenin-positive cell number were counted per field (n=10 fields per condition in triplicate).

Western Blotting

After cells were rinsed with cold PBS, they were lysed for 5 min in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 1mM aprotinin, 1mM AEBSF, 1mM benzamidin, 1mM orthovanadate). 50 µg of protein, quantified by BCA bicinchoninic acid assay (Pierce) were separated by SDS/PAGE and were transferred onto PVDF membranes (Millipore). The membrane was incubated with the polyclonal antibody against VEGF (Santa Cruz) was used. Binding of antibodies to the blots was detected using a chemiluminescence ECL Western-blotting system. Relative protein levels were quantified using Scion software (Scion Corporation) on scanned films.

Immunofluorescence and confocal microscopy

To evaluate *in vivo* GFP-MSC localization and differentiation and for *in vitro* MSC macrophage activity, tissue samples were analyzed with with a confocal microscope (Olympus FV 1000). using a 60x/1.4 ApoPlan oil immersion objective. Single XY scans had an optical slice thickness of 0.4mm. Visualisation was obtained after digitized image multichannel deconvolution by Autodeblur (Media Cybernetics Inc., MD 20910 USA) and three-dimensional projections were digitally reconstituted from stacks of confocal optical slices by Imaris software (Bitplane) (Bitplane AG, Zurich, Swizerland).

Suppression of Wnt4 by siRNA:

GFP-MSC were transfected with 30 nM of mixed siRNA for Wnt4 or with 30 nM siRNA negative control (siCtl) using Interferin (Polyplus, Ozyme) diluted in serum medium. The oligonucleotides used were two HP validated predesigned siRNAs by Qiagen against the following target sequences for Wnt4 gene (accession number: NM-009523) (5'–CCGGGAGGCGGCCTTTGTATA-3' and 5'-CCGGGCACTCATGAATCTTCA-3'). Scrambled SiCtl were synthetized by Eurogentec. Then, siRNA transfected GFP-MSC were either maintained in normoxia condition or in hypoxia condition before being injected. We have verified that HypMSC treated with mixed siRNA against Wnt4 had a 70 % decrease in *Wnt4* mRNA

expression after 48 hours of exposure to siRNA (data non shown). To analyse the impact of the knock down of Wnt4 in HypMSC, three groups of mice (n=9/group) were constituted: one group transplanted with HypMSC treated with siRNA against *Wnt4* (siWnt4-HypMSC), another group with HypMSC treated with control siRNA (siCtl-HypMSC) and the last group with NormMSC treated with control siRNA (siCtl-NormMSC). Seven days after the surgery, perfusion using LPDI vascular density and connectivity using microCT analysis on serial tissue sections were performed All results were analyzed by an investigator blinded to the treatment groups.

Statistical methods:

All analyses were performed using appropriate software (Statview 5.1). Comparisons of continuous variables between two groups were made using one-way ANOVA and, if a statistically significant difference was observed, a two-sided paired t test. For small samples sizes comparison, a Wilcoxon and Mann–Whitney U tests were used, A value of $P < 0.05$ was considered statistically significant.

SUPPLEMENTAL RESULTS

HypMSC did not differentiate into endothelial cells but instead played a role as mural cells

MSC have been shown to drive and participate in the angiogenic process (Abramsson, Lindblom et al. 2003). It has been shown that they provide a supporting frameworks amenable to easy penetration of endothelial cells (EC) leading to neo vascularization (Kalluri and Zeisberg 2006; Dufourcq, Descamps et al. 2008). Detailed localization has revealed MSC intercalated between muscle fibers. In the current study, at day 7 after ischemia, HypMSC were recruited, into highly reparative zones, primarily surrounding necrotic myocytes. At day 15, HypMSC appeared to be preferentially associated with capillaries and microvessels, behaving as perivascular cells shaping along the whole length of the vessel, covering growing CD31- forming tube-like structures, as mural cells (Figure S2). GFP Hyp- or Norm-MSC kept their pericyte-like phenotype, expressing the *in vivo* neural-glial antigen 2 (NG2) marker but did not adopt an endothelial phenotype as we did not detect any GFP/CD31 double-labeled cell in reparative areas (**Figure S3a**). Coverage of EC with mural cells renders vessels more mature, tight and stable (Dufourcq, Descamps et al. 2008). Double labeling for CD31 and GFP revealed that MSC are closely associated to EC, consistent with functional interactions (**Figure S3b**).

HypMSC paracrine effect on angiogenesis and muscle regeneration

The role of HypMSC implantation demonstrated in muscle perfusion and regeneration prompted us to examine whether or not these effects were linked at least in part to the production of trophic factors. Indeed, 7 days after surgery, the presence of HypMSC led to a significant up-regulation of vascular endothelial growth factor (VEGF) in ischemic muscles compared with sham-treated control tissues (VEGF/P0 ratio: 10.6 ± 3.3, $P<0.01$ in HypMSC group vs control group). NormMSC implantation had a moderate effect on VEGF expression (VEGF/P0 ratio: 1.6 ± 0.9, $P<0.05$ in NormMSC group vs control group). In both groups, MSC implantation resulted similarly in a moderate decrease in transforming growth factor beta 1 (TFG-β1), increase in angiopoietine 1 (Ang1) and no significant modulation of basic fibroblast growth factor (bFGF), Ang2 and platelet derived growth factor B (PDGFB) compared to the control group (**Figure S4a**). *In vitro*, we confirmed a moderate

up-regulation of VEGF and TFG-β1 transcripts in HypMSC vs NormMSC (fold 1.5 and 2 respectively, NS and P<0.05) (*Figure S4b*)**.** Western blot analysis confirmed the increase of VEGF expression after hypoxia treatment in MSC (**Figure S4c**). These data suggest that HypMSC implantation results in a further increase in secretion of VEGF which exerts an effect on muscles undergoing repair, and is required for growth of vessels after injury and for survival mechanisms.

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4) Discussion

Cette étude confirme sur un modèle murin d'ischémie de patte que le préconditionnement hypoxique est un moyen simple et efficace de prétraiter les cellules souches avant transplantation en milieu ischémique en vue d'améliorer l'effet thérapeutique. Comme nous l'avons vu, les cellules souches *in vivo* sont maintenues à l'état quiescent dans des niches où la pression partielle en oxygène est faible. Les cultiver à 20% d'O2 puis les soumettre à l'hypoxie entraîne toute une série d'évènements. HIF-1α, alors exprimé, semble être un élément clef des phénomènes constatés. Or très récemment, un lien direct entre HIF-1α et le système Wnt/Fzd a été mis en évidence par l'équipe de Simon (Mazumdar, O'Brien et al. 2010).

Dans notre expérience, l'effet semble médié par le ligand Wnt4. Outre son rôle dans le déterminisme sexuel (Biason-Lauber and Konrad 2008) et le développement rénal (Kispert, Vainio et al. 1998), Wnt4 a été impliqué dans le développement musculaire chez le poulet (Takata, Terada et al. 2007) et dans la prolifération des cellules musculaires satellites chez la souris (Steelman, Recknor et al. 2006). Nous montrons en outre ici que dans des conditions d'hypoxie, Wnt4 affecte la prolifération et la migration des MSC via une signalisation Wnt/β-caténine dépendante. Par ailleurs Wnt4 favorise la différenciation des myoblastes *in vitro*. Toutes ces données sont concordantes pour affirmer une implication importante du système Wnt/Fzd dans les phénomènes observés.

Par ailleurs, même s'il est indéniable que les molécules sécrétées aient à elles seules un effet, comme le démontre quelques expériences utilisant des surnageants de cultures cellulaires, nous confirmons ici, que le rôle thérapeutique des MSC est plus complexe qu'un seul vecteur de facteurs anti-apoptotiques ou pro-angiogéniques. Une fois transférées les cellules survivantes participeraient en fait à chaque phase de la régénération tissulaire et vasculaire: protection de l'apoptose des myocytes survivants par des facteurs anti-apoptotiques, dégradation des débris cellulaires des myocytes nécrosés avec une activité phagocytiques, sécrétion de facteurs pro-angiogénique, organisation en réseau de péricytes dont les rôles dans la formation des néo-vaisseaux est important, stabilisation et maturation de ces vaisseaux et enfin facilitation de la différenciation des myoblastes. Cependant la condition sine qua non pour permettre aux MSC d'exercer l'ensemble des ces effets

thérapeutiques est bien leur survie initiale après transplantation. Or si plusieurs systèmes de prétraitement de cellules souches ont montré un effet sur leur survie, seul le préconditionnement hypoxique peut prétendre à une activation simultanée de l'ensemble des mécanismes réparateurs.

5) Conclusion

Cette étude amène de nouvelles connaissances sur le comportement des cellules souches en hypoxie et leur rôle thérapeutique, et ouvre une voie vers un préconditionnement des MSC potentiellement applicable dans un contexte de thérapie cellulaire chez l'homme.

CONCLUSION ET PERSPECTIVES

V) CONCLUSION ET PERSPECTIVES

A) CONCLUSION

Le concept de la thérapie cellulaire dans le domaine cardiovasculaire est séduisant à bien des égards. Cependant, comme le démontre l'analyse des premières études cliniques humaines, ces techniques manquent pour l'heure de maturité pour envisager très prochainement une application en pratique courante.

Au cours de ma thèse, je me suis attaché à explorer des voies d'amélioration de l'effet bénéfique de cette thérapie cellulaire. Du fait de leurs caractéristiques nous avons fait le choix de travailler sur les cellules souches mésenchymateuses, modèle cellulaire permettant d'envisager une banque de cellules en permanence disponible pour un transfert allogénique dans de brefs délais.

J'ai d'abord participé à une étude visant à tester un système original de délivrance des cellules souches au myocarde infarci, Puis, j'ai participé à une étude visant à mieux comprendre les mécanismes de l'effet pro-angiogénique des MSC et notamment à déterminer si le système Wnt/Fzd était impliqué dans ces phénomènes. Nous avons ainsi mis en exergue le rôle de « précurseur de péricytes » des MSC injectées et avons montré que le système Wnt/Fzd était impliqué, sFRP1 représentant une cible thérapeutique potentielle. Enfin, en étudiant le rôle du préconditionnement hypoxique des cellules souches mésenchymateuses dans un modèle d'ischémie de patte chez la souris, nous avons confirmé dans un système plus complexe, les relations entre MSC et péricytes au cours de la réparation vasculaire et tissulaire. Nous avons par ailleurs montré que ces cellules étaient capables de phagocytose et mis en évidence le bénéfice important du préconditionnement hypoxique en termes de survie des cellules et de cicatrisation post ischémique. Du point de vue mécanistique, l'implication du système Wnt/Fzd semble être supportée par Wnt4. La réalisation pratique du préconditionnement hypoxique étant simple à effectuer, une utilisation en pratique courante peut être envisagée.

De nombreuses questions resteront probablement encore longtemps sans réponse claire, mais nos travaux offrent de nouvelles perspectives dans l'exploration par exemple du

rôle du système Wnt/Fzd, des péricytes ou du préconditionnement hypoxique dans ces thérapeutiques, avec toujours pour but, l'amélioration de l'efficacité.

B) PERSPECTIVES

Forts de ces résultats, et en vue d'un transfert de la technique chez l'homme, nous développons à présent un modèle de cardiomyopathie dilatée chronique d'origine ischémique chez le cochon, pour y tester la faisabilité et l'efficacité du préconditionnement hypoxique des cellules souches mésenchymateuses. Pour ce faire, nous avons structuré une équipe multidisciplinaire. L'ensemble des interventions est réalisé à l'animalerie dédiée aux animaux de type ovins et porcins, située sur le site de Xavier Arnozan, CHU de Bordeaux. Cette structure est intégrée au sein de la Pateforme Technologique d'Innovation Biomédicale (PTIB-Pr Dos Santos) et en relation avec l'Unité INSERM 828 (Pr Couffinhal, Dr Duplàa). Le Pr Barandon, chirurgien cardio-thoracique est chargé des interventions. L'anesthésie est prise en charge par le Dr Calderon. Les Dr Jaussaud et Réant réalisent les explorations échocardiographiques et cette équipe est soutenue par le plateau des techniques histologiques et immuno-histologiques de l'Inserm U828 avec le Dr Daret. La culture et le préconditionnement des cellules est assuré par le laboratoire Inserm U828, en étroite collaboration avec le centre bordelais des Etablissements Français de Sang (EFS), situé sur le site Pellegrin, CHU de Bordeaux, sous la responsabilité des Dr Ivanovic et Boiron. Les cellules sont désignées comme suit : HypMSC pour les MSC préconditionnées par 24h de chambre à hypoxie à 1% d'O2 et NormMSC pour les cellules non préconditionnées.

La méthode utilisée est le positionnement chirurgical d'un constricteur améroïde sur le pédicule circonflexe de cochons âgés de 3 à 4 mois pesant entre 20 et 25 Kg, de race YORK (Figure 54).

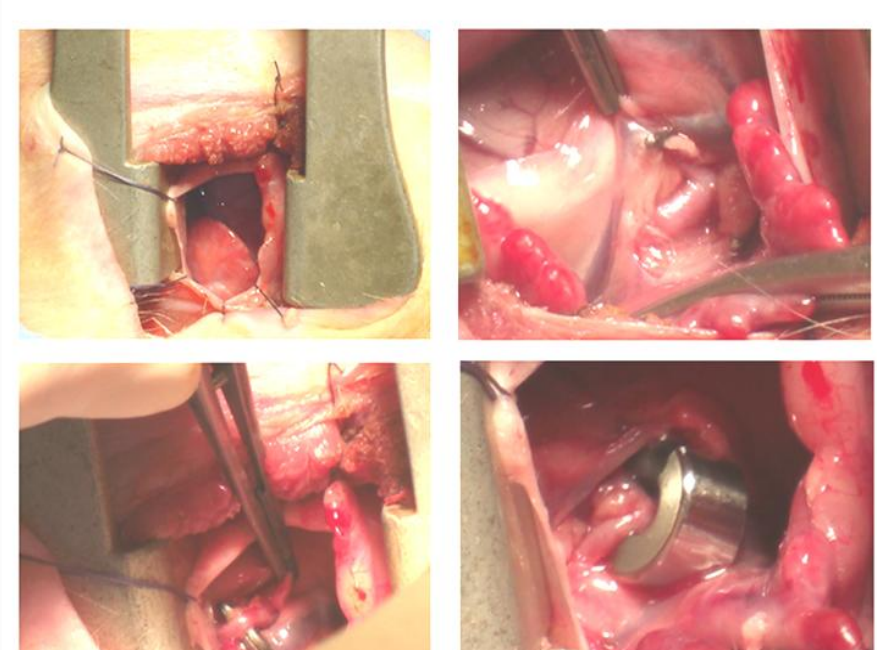

Figure 54. Exemple d'une vue chirurgicale après pericardotomie et mise en place du constricteur améroïde autour de l'artère circonflexe proximale.

Quatre semaines plus tard, après une évaluation échographique de la zone ischémique, le cochon est ré-opéré pour injection intramusculaire directe multifocale de $1X10^6$ cellules par Kg de poids. L'animal est sacrifié à 1 mois après une échographie finale.

Les résultats préliminaires concernent les 27 premiers animaux. Parmi ceux-ci, 9 sont décédés dans le premier mois (33%). Quatre ont été inclus dans le groupe contrôle, 8 dans le groupe NormMSC et 6 dans le groupe HypMSC. Alors que la FEVG ne semble pas améliorée de manière significative après injection dans le groupe contrôle ou NormMSC, elle apparaît significativement améliorée dans le groupe HypMSC. La dilatation ventriculaire gauche semble moins importante à 2 mois dans le groupe HypMSC sans qu'il n'existe de différence statistiquement significative avec les autres groupes (Figure 55).

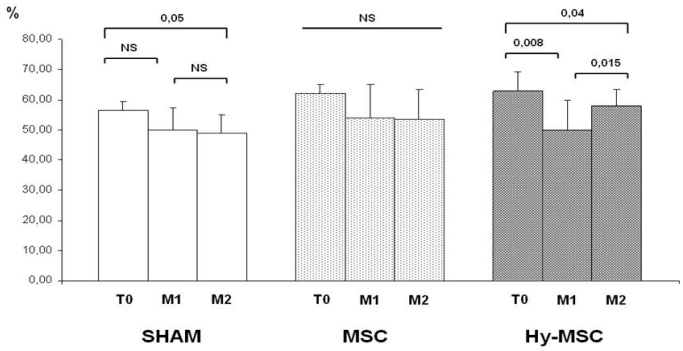

Figure 55. Evolution de la FEVG dans les différents groupes d'animaux.

Par ailleurs, l'étude histologique retrouve une augmentation significative de la densité capillaire chez les animaux ayant reçu des cellules préconditionnées (Figure 56).

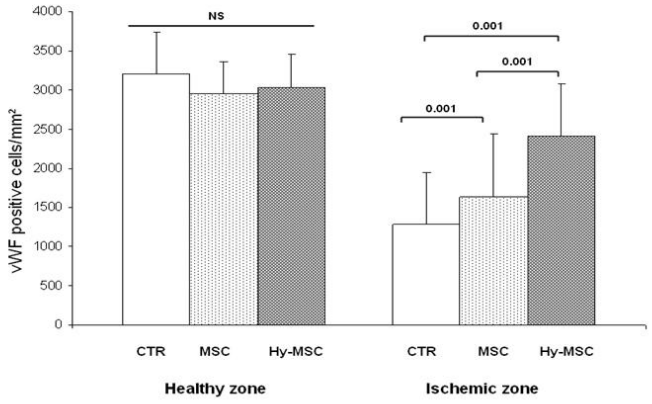

Figure 56. La densité capillaire est significativement plus élevée lorsque l'animal a reçu des MSC préconditionnées.

Ces résultats préliminaires encourageants laissent supposer que l'effet du préconditionnement hypoxique retrouvé dans un modèle murin (ischémie de patte) pourrait être transférable chez le gros animal (infarctus). De nombreux obstacles restent cependant encore à franchir avant d'envisager une application humaine du concept du préconditionnement hypoxique de MSC dans le cadre d'une thérapie cellulaire.

ANNEXE

VI) ANNEXE

Au premier septembre 2010, sur le site internet www.clinicaltrial.gov, la recherche par mots clefs associant « stem cell » et « heart » ou « stem cell » et « limb » a permis d'établir les listes ci-dessous de plus de 100 études en cours concernant la thérapie cellulaire à visée cardiaque chez l'homme et de près de 40 études pour ce qui est des membres inférieurs atteints d'ischémie.

A) LISTE DES ETUDES HUMAINES DE THERAPIE CELLULAIRE CARDIAQUE EN COURS

1 CArdiosphere-Derived aUtologous Stem CElls to Reverse ventricULar dySfunction

Conditions: Myocardial Infarction; Ventricular Dysfunction; Congestive Heart Failure; Heart Failure
Interventions: Other: Observation (Control Group); Biological: Autologous stem cell infusion
Phase: Phase I
Number Enrolled: 30
Study Design:
Allocation: Randomized; Control: Dose Comparison; Endpoint Classification: Safety Study; Intervention Model: Par allel Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00893360
Acronym: CADUCEUS
Outcome Measures: The primary objective is to demonstrate the safety of autologous cardiosphere-derived stem cells administered by intra-coronary infusion in patients with ischemic left ventricular dysfunction and a recent myocardial infarction.; The secondary objective is to demonstrate the efficacy of autologous cardiosphere-derived cells administered by intra-coronary infusion in patients with ischemic left ventricular dysfunction and a recent myocardial infarction.

2 Combined CABG and Stem-Cell Transplantation for Heart Failure

Conditions: Heart Failure; Myocardial Infarction; Coronary Artery Disease
Interventions:
Procedure: Coronary bypass operation; Procedure: Bone marrow aspiration (crista iliaca); Biological: Intramyocardial mesenchymal stem cell transplantation; Biological: Intramyocardial injection of autologous serum
Phase: Phase II
Number Enrolled: 60
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Efficacy Study; Intervention Model: Fac torial Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treat ment
NCT ID: NCT00418418
Outcome Measures: Does a bone marrow transplantation therapy increase the ejection fraction of the heart measured with MRI, when compared with placebo treatment?; Does a bone marrow transplantation therapy increase any cardiac function parameter measured by a an echocardiography, MRI or PET ischemia area, when compared with no treatment group?;

3 Stem Cells and Resynchronization Cardiac

Condition: Heart Failure
Interventions:
Procedure: Pacing and Stem Cells Implantations.; Procedure: Pacing and Stem Cells Implantation
Phase: Phase I
Number Enrolled: 30
Study Design:
Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group Assignment; Masking: Single Blind (Investigator); Primary Purpose: Treatment
NCT ID: NCT00800657
Acronym: RCT and SCT
Outcome Measures: Ejection Fraction(EF)-(Eco- Sympson):It is the comparison of these measurements between late pre and post operative. NYHA: Comparison of these measurements between late pre and post operative.; Ejection Fraction (Echo/Sympson) and functional class(NYHA).

4 AutoLogous Human CArdiac-Derived Stem Cell to Treat Ischemic cArdiomyopathy (ALCADIA)

Conditions: Congestive Heart Failure; Ischemic Cardiomyopathy; Ventricular Dysfunction
Intervention: Biological: Autologous hCSC intramyocardial injection
Phase: Phase I
Number Enrolled: 6
Study Design: Allocation: Non-Randomized; Control: Historical Control; Endpoint Classification: Safety Study; Intervention Model: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00981006
Acronym: ALCADIA
Outcome Measures: The primary objective is to evaluate the safety of autologous cardiac-derived stem cells administered by intra-myocardial injection with the controlled release of bFGF in severe refractory heart failure patients with chronic ischemic cardiomyopathy.; The secondary objective is to demonstrate the safety of autologous cardiac-derived stem cells administered by intra-myocardial injection with the controlled release of bFGF in severe refractory heart failure patients with chronic ischemic cardiomyopathy.

5 Cardiac Stem Cell Infusion in Patients With Ischemic CardiOmyopathy (SCIPIO)

Conditions: Coronary Artery Disease; Congestive Heart Failure
Intervention: Procedure: Intracoronary Injection (cardiac stem cell therapy)
Phase: Phase I
Number Enrolled: 40
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00474461
Acronym: SCIPIO
Outcome Measure: Monitoring adverse outcomes, death, sustained/symptomatic ventricular tachycardia, infection, bleeding, MI, stroke, peripheral embolism in the hospital after drug administration, in the first month after injection, and serially afterwards.

6 Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery (PROMETHEUS)

Conditions: Stem Cell Transplantation; Ventricular Dysfunction, Left
Interventions: Genetic: Lower dose mesenchymal stem cell (MSC) injection; Genetic: Placebo; Genetic: Higher dose MSC injection
Phases: Phase I / Phase II
Number Enrolled: 45
Study Design:
Allocation: Randomized; Control: Dose Comparison; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Double Blind (Subject, Investigator); Primary Purpose: Treatment
NCT ID: NCT00587990

Acronym: PROMETHEUS

Outcome Measures: Incidence of serious adverse events (SAEs), defined as the 6-month post-CABG surgery SAE proportion of patients experiencing sustained ventricular arrhythmias, ectopic tissue formation, or sudden unexpected death; MRI and echocardiographic measures of Infarct Scar Size (ISS) and left regional and global ventricular function;

7 IMPACT-CABG Trial: IMPlantation of Autologous CD133+ sTem Cells in Patients Undergoing CABG

Conditions: Myocardial Infarct; Heart Failure

Intervention: Procedure: Injection of stem cells at time of coronary artery bypass grafting

Phase: Phase II

Number Enrolled: 20

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatment

NCT ID: NCT01033617

Acronym: IMPACT-CABG

Outcome Measures: Freedom from Major Adverse Cardiac Event: cardiac death, myocardial infarct, repeat coronary bypass grafting or percutaneous intervention of bypassed artery.; Freedom from major arrhythmia: sustained ventricular tachycardia or survived sudden death.; Regional myocardial perfusion and function assessed by magnetic resonance scans.

8 Bone Marrow Derived Adult Stem Cells for Chronic Heart Failure

Condition: Chronic Ischaemic Heart Failure

Interventions: Drug: Granulocyte-

colony stimulating factor; Procedure: Percutaneous intracoronary injection; Procedure: Percutaneous intramyocardial inj ection

Phases: Phase II / Phase III

Number Enrolled: 165

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Investigator, Outcomes Assessor); Primary Purpose: Treatment

NCT ID: NCT00747708

Acronym: REGEN-IHD

Outcome Measures: Change in global left ventricular ejection

fraction; Change in quality of life; Change in regional wall motion score index; Occurence of major adverse cardiac even t

9 Safety and Efficacy of Autologous, Intracoronary Stem Cell Injections in Total Coronary Artery Occlusions

Condition: Coronary Occlusion

Intervention: Procedure: catheter-based intracoronary injection

Phase: Phase I

Number Enrolled: 10

Study Design: Allocation: Non-

Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group As signment; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00365326

Outcome Measures: Assess the safety and feasibility of performing autologous AC133+ selected bone marrow-derived stem cell intra-coronary infusion and determine whether any benefit is achieved from the infusion of stem cells by non-invasive cardiac assessment.; Improvement in ETT, reduction in ischemic area, viability improvement (nuclear stress imaging), improvement in angina (Seattle Angina Questionnaire), major adverse cardiac events assessment, echocardiogram assessment of left %EF and regional wall motion.

10 Paracrine Mechanisms of Bone Marrow Stem Cell Signalling in Chronic Heart Failure

Condition: Heart Failure

Intervention: Other: Surgery

Number Enrolled: 125
Study Design: Observational Model: Cohort; Time Perspective: Prospective
NCT ID: NCT01086787
Acronym: BM-CHF
Outcome Measures: Paracrine properties of bone marrow cells from patients with heart failure; To study preconditioning of different bone marrow stem cells with different cytokines; To study in detail the temporal and spatial expression of paracrine factors in bone marrow stem cells in vitro, and to study the differences between bone marrow stem cells from patients with and without chronic heart failure.

11 Intramyocardial Injection of Autologous Aldehyde Dehydrogenase-Bright Stem Cells for Therapeutic Angiogenesis

Condition: Coronary Artery Disease
Interventions: Procedure: stem cell injection under electromechanical guidance; Other: Placebo patients receive plasma injections.
Phase: Phase I
Number Enrolled: 20
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Crossover Assignment; Masking: Double Blind (Subject, Caregiver, Investigator); Primary Purpose: Treatment
NCT ID: NCT00314366
Outcome Measures: Safety of aldehyde dehydrogenase bright stem cells versus the control group; Efficacy of aldehyde dehydrogenase bright stem cells in improving myocardial ischemia and consequently cardiac contractile function

12 By Pass Surgery With Stem Cell Therapy in Chronic Ischemic Cardiopathy

Condition: Ischemic Heart Disease
Intervention: Procedure: Autologous bone marrow derived stem cells myocardial transplantation
Phase: Phase II
Number Enrolled: 30
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Parallel Assignment; Masking: Single Blind (Subject)
NCT ID: NCT00690209
Outcome Measures: Evolution of left ventricular volumes and contractility Evolution of left ventricular volumes and contractility; Functional status

13 Effectiveness of Stem Cell Treatment for Adults With Ischemic Cardiomyopathy (The FOCUS Study)

Conditions:
Chronic Ischemic Heart Disease; Left Ventricular Dysfunction; Angina; Ischemic Cardiomyopathy
Interventions: Biological: Adult stem cells; Biological: Placebo
Phase: Phase II
Number Enrolled: 87
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatment
NCT ID: NCT00824005
Outcome Measures: Change in maximal oxygen consumption (MVO2); Change in left ventricular end systolic volume (LVESV); Change in reversible defect size; Regional wall motion by MRI (in eligible patients); Regional blood flow improvement by MRI (in eligible patients); Regional wall motion by echocardiography; Clinical improvements.

14 Combination Stem Cell (MESENDO) Therapy for Utilization and Rescue of Infarcted Myocardium

Conditions:
Coronary Artery Disease; Coronary Arteriosclerosis; Coronary Atherosclerosis; Coronary Disease
Intervention: Biological: MESENDO
Phase: Phase I

Number Enrolled: 20
Study Design:
Allocation: Randomized; Control: Historical Control; Endpoint Classification: Safety/Efficacy Study; Intervention M odel: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00548613
Outcome Measure: Safety

15 Intramyocardial Transplantation of Bone Marrow Stem Cells in Addition to Coronary Artery Bypass Graft (CABG) Surgery

Conditions: Myocardial Ischemia; Coronary Artery Disease
Interventions: Drug: CD133+ autologous bone marrow stem cell; Drug: Placebo
Phase: Phase III
Number Enrolled: 142
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Efficacy Study; Intervention Model: Par allel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatm ent
NCT ID: NCT00950274
Acronym: PERFECT
Outcome Measures: Left ventricular ejection fraction at rest, measured by MRI; Change in LVEF as assessed by MRI and echocardiography; Regional contractility in the AOI / Change in LV dimensions (left ventricular end systolic diameter [LVESD], left ventricular end diastolic diameter [LVEDD]) as assessed by echocardiography; Physical exercise capacity determined by 6 minute walk test; NYHA and CCS class; MACE .

16 Autologous Progenitor Stem Cell Therapy for Congestive Heart Failure Patients Undergoing Coronary Artery Bypass Grafting (CABG) Surgery

Condition: Heart Failure, Congestive
Intervention: Procedure: Injection of Bone Marrow Progenitor Cells into the Heart
Phase: Phase I
Number Enrolled: 75
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety Study; Intervention Model: Fact orial Assignment; Masking: Double-Blind; Primary Purpose: Treatment
NCT ID: NCT00130377
Outcome Measures: Heart function change 6 months post-implant; Toxicity; Optimal dose of BMPCs

17 Bone Marrow Derived Adult Stem Cells for Acute Anterior Myocardial Infarction

Condition: Acute AMI
Interventions: Other: Bone marrow derived progenitor cells or placebo infusion; Other: Placebo infusion
Phases: Phase II / Phase III
Number Enrolled: 102
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator); Primary Purpose: Treatment
NCT ID: NCT00765453
Acronym: REGEN-AMI
Outcome Measures: Longitudinal change in left ventricular function (ejection fraction) as measured by cardiac MRI.; Longitudinal change in left ventricular function (ejection fraction) Reduction of left ventricular end systolic volume, Change in regional wall motion in infarct area and Change in infarct mass as measured by cardiac MRI; Change in global ejection fraction as measured by LV angiography and improvement of myocardial contractility, assessed by contrast echocardiogram(wall motion score index).; Major adverse cardiac events (MACE).

18 Use of Adult Autologous Stem Cells in Treating People 2 to 3 Weeks After Having a Heart Attack (The Late TIME Study)

Condition: Left Ventricular Dysfunction
Interventions: Biological: Adult stem cells; Biological: Placebo
Phase: Phase II

Number Enrolled: 87

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatment

NCT ID: NCT00684060

Outcome Measures: Left ventricular ejection fraction (global and regional); Combined endpoint: first of death, reinfarction, repeat revascularization, and hospitalization for heart
failure; Left ventricular mass; End diastolic volume; End systolic volume; Infarct size

19 The Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis Pilot Study (The POSEIDON-Pilot Study)

Condition: Stem Cell Transplantation

Interventions: Biological: Auto-hMSCs; Biological: Allo-hMSCs

Phases: Phase I / Phase II

Number Enrolled: 30

Study Design:

Allocation: Randomized; Intervention Model: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatm ent

NCT ID: NCT01087996

Outcome Measures: Incidence of TE-SAE define as composite of death, non-fatal MI, stroke, hospitalization for worsening heart failure, cardiac perforation, pericardial tamponade, ventricular arrhythmias >15 sec. or with hemodynamic compromise or atrial fibrillation; CT and Echocardiographic-derived measures of left ventricular function.

20 Stem Cell Therapy as Adjunct to Revascularization

Conditions: Coronary Arteriosclerosis; Coronary Artery Bypass Graft; Myocardial Revascularization

Interventions: Procedure: Autologous stem cell therapy; Procedure: CABG; Procedure: CMRI

Phase: Phase I

Number Enrolled: 15

Study Design: Allocation: Non-
Randomized; Control: Historical Control; Endpoint Classification: Safety Study; Intervention Model: Single Group Assign ment; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00463853

Acronym: STAR

Outcome Measures: The primary outcome measure will be safety as measured by the incidence of postoperative serious adverse events (SAE) and adverse events
(AE).; Ejection fraction measured by CMRI; Echo; Myocardial perfusion measured by gadolinium CMRI; Exercise toleran ce testing

21 Combination Stem Cell Therapy for the Treatment of Severe Coronary Ischemia

Conditions:

Coronary Ischemia; Coronary Disease; Coronary Artery Disease; Coronary Atherosclerosis; Coronary Arteriosclero sis

Intervention: Biological: MESENDO

Phase: Phase I

Number Enrolled: 10

Study Design:

Allocation: Randomized; Control: Historical Control; Endpoint Classification: Safety/Efficacy Study; Intervention M odel: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00643981

Acronym: CI

Outcome Measures: Safety as measured by laboratory assessments, ecg, temperature and holter monitor; Efficacy as measured by SPECT scan and 2-D Echo

22 Autologous Stem Cells for Cardiac Angiogenesis

Condition: Ischemic Cardiomyopathy

Intervention: Device: Intramyocardial Injection of stem cells via Noga Mapping

Phase: Phase I

Number Enrolled: 30

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Crossover Assignment; Masking: Single Blind (Subject); Primary Purpose: Treatment

NCT ID: NCT00203203

Outcome Measures: Safety of autologous-bone-marrow injections in endstage ischemic cardiomyopathy patients; To assess the efficacy of autologous bone marrow cells in improving cardiac contractile function and functional outcome in endstage ischemic cardiomyopathy patients. The efficacy will be primarily assessed on the basis of the treadmillMVO2

23 Safety and Efficacy Study of Stem Cell Transplantation to Treat Dilated Cardiomyopathy

Condition: Dilated Cardiomyopathy

Interventions: Biological: CD34+ autologous stem cell transplantation; Drug: Bone Marrow Stimulation

Phase: Phase II

Number Enrolled: 50

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Single Blind (Outcomes Assessor); Primary Purpose: Treatment

NCT ID: NCT00629018

Outcome Measures: Heart failure mortality; Changes in exercise capacity; Changes in electrophysiologic properties of ventricular myocardium; Changes in plasma inflammatory markers; Changes in left ventricular function

24 Use of Adult Autologous Stem Cells in Treating People Who Have Had a Heart Attack (The TIME Study)

Condition: Left Ventricular Dysfunction

Interventions: Biological: Adult stem cells; Biological: Placebo

Phase: Phase II

Number Enrolled: 120

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatment

NCT ID: NCT00684021

Outcome Measures: Left ventricular ejection fraction (global and regional); Combined endpoint: first of death, reinfarction, repeat revascularization, or hospitalization for heart
failure; Left ventricular mass; End diastolic volume; End systolic volume; Infarct size

25 Stem Cell Study for Subjects With Congestive Heart Failure

Conditions: Myocardial Ischemia; Congestive Heart Failure; Cardiovascular Disease

Intervention: Biological: Intramyocardial injection of autologous CD34-positive cells (stem cells)

Phase: Phase I

Number Enrolled: 10

Study Design: Allocation: Non-
Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignm ent; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00620048

Outcome Measures: Safety of intramyocardial administration of CD34-positive cells; Effects of intramyocardial injections of autologous CD34-positive cells on clinical outcomes.

26 Bone Marrow Stem Cell Infusion Following a Heart Attack

Condition: Acute Myocardial Infarction

Intervention: Drug: Autologous, Unfractionated Bone Marrow Mononuclear Cells

Phase: Phase I

Number Enrolled: 60

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo

del: Crossover Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpos e: Treatment

NCT ID: NCT00268307

Outcome Measures: Safety as measured by holter monitor, laboratory assessments, and cardiac MRI; Improvement of left ventricular function as assessed by serial measurements of infarct size and LV function by cardiac MRI in the active treatment group versus placebo.

27 Autologous Progenitor Stem Cell Therapy for Congestive Heart Failure

Condition: Heart Failure, Congestive

Intervention: Procedure: Injection of bone marrow cells in the heart

Phase: Phase I

Number Enrolled: 10

Study Design: Allocation: Non-Randomized; Control: Active Control; Endpoint Classification: Safety Study; Intervention Model: Single Group Assignme nt; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00128258

Outcome Measures: Heart function at 3 months after implant; Toxicity

29 Phase II Combination Stem Cell Therapy for the Treatment of Severe Coronary Ischemia(CI)

Conditions:

Heart Disease; Blocked Arteries; Coronary Ischemia; Coronary Disease; Coronary Artery Disease; Coronary Ather osclerosis

Interventions: Biological: MESENDO; Other: placebo

Phase: Phase II

Number Enrolled: 60

Study Design:

Allocation: Randomized; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group Assignme nt; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00790764

Outcome Measures: Safety as measured by laboratory assessments, ecg and temperature.; Efficacy as measured by SPECT scan, MUGA scan and 2D Echogradiogram

30 Congestive Heart Failure Surgical Treatmment With Autologous Stem Cell Therapy

Condition: Congestive Heart Failure

Intervention: Procedure: intramiocardiac autograft of autologous stem cells

Phase: Phase II

Study Design:

Control: Historical Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group Assign ment; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00480961

Outcome Measures: Primary target: determine the safety of introducing CD 34+ autologous progenitor cells (centrally grafted or mobilized in peripheral blood) in the myocardium of ischemic cardiac disease patients.; Secondary target: determine clinical effects of grafted cells on remodeling pathology.

31 Stem Cell Study for Patients With Heart Failure

Conditions: Myocardial Ischemia; Congestive Heart Failure; Cardiovascular Disease

Intervention: Biological: Autologous Stem Cells

Phase: Phase II

Number Enrolled: 30

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Investigator); Primary Purpose: Treatment

NCT ID: NCT00346177

Outcome Measure: The purpose of this study is to determine if cell therapy with your own cells (autologous cells) delivered with a catheter to regions of the heart with poor blood flow will be safe and if it will improve your ejection fraction and heart failure symptoms.

32 Endocardial Stem Cells Approach Efficacy

Condition: Ischemic Heart Failure
Intervention: Procedure: Autologous bone marrow mononuclear stem cells or peripheral blood stem cells
Phase: Phase III
Number Enrolled: 250
Study Design:
Allocation: Randomized; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Single Blind (Subject); Primary Purpose: Treatment
NCT ID: NCT00841958
Acronym: ESCAPE
Outcome Measure: Null Hypothesis (Ho): There is no survival benefit in the stem cells group compared to the control group (isolate MED therapy). H0: $\Theta \leq 1$ Alternative Hypothesis (Ha): There is a survival benefit in the stem cells group. HA: $\Theta > 1$

33 Myocardial Regeneration and Angiogenesis in Myocardial Infarction With G-CSF and Intra-Coronary Stem Cell Infusion-3-DES

Condition: Myocardial Infarction
Interventions: Drug: G-CSF (Dong-A pharmaceutical, Seoul, Korea); Procedure: collection of mobilized peripheral blood stem cells; Procedure: Intracoronary infusion of mobilized cells
Phase: Phase II
Number Enrolled: 96
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00291629
Outcome Measures: the change in left ventricular ejection fraction (LVEF), which was measured by cardiac MRI; changes in left ventricular volume measured by echocardiography and MRI, myocardial perfusion by coronary flow reserve, and the development of major adverse cardiac events

34 Stem Cell Therapy to Improve Myocardial Function in Patients Undergoing Coronary Artery Bypass Grafting (CABG)

Conditions: Coronary Disease; Myocardial Infarction
Intervention: Procedure: Bone Marrow Stem Cell Therapy combined CABG
Phases: Phase I / Phase II
Number Enrolled: 50
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Investigator); Primary Purpose: Treatment
NCT ID: NCT00395811
Outcome Measure: Changes in left ventricular ejection fraction from baseline to 6 months' follow-up

35 Intra-coronary Infusion of Bone Marrow Derived Autologous CD34+ Selected Cells in Patients With Acute Myocardial Infarction

Conditions: Myocardial Infarction; Coronary Artery Disease
Intervention: Procedure: Intra-coronary infusion of an autologous bone marrow derived CD34+ stem cell product
Phase: Phase I
Number Enrolled: 40
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Factorial Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00313339
Acronym: AMR-1
Outcome Measures: Cardiac function at 3 and 6 months follow-up will be compared to baseline measures obtained the day(s) before CD34+ cell product infusion.; Perfusion of the infarct region at 6 months follow-up will be compared to baseline measures obtained the day(s) before CD34+ cell product infusion; An assessment will be performed

to determine if a correlation exist between clinical outcome and cell content (CD34+) and/or in vitro colony growth (CFU-GM, CFU-GEMM, BFU-E) CXCR-4 mobility and CXCR-4 and/or VEGF surface antigen expression.

36 Stem Cell Study for Patients With Heart Disease

Conditions:
Chest Pain; Chronic Myocardial Ischemia; Coronary Artery Disease; Angina; Myocardial Infarction
Intervention: Genetic: Autologous peripheral blood CD34 positive cell therapy
Phases: Phase I / Phase II
Number Enrolled: 10
Study Design: Allocation: Non-Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group As signment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00221182
Outcome Measures: Efficacy: Severity of myocardial perfusion abnormality by sestamibi SPECT stress myocardial scintigraphy; Safety: Severe adverse events within 4 weeks after cell therapy; CCSAS (Canada Cardiovascular Society Anginal Score); NYHA (New York Heart Association) classification; Exercise tolerance by treadmill; Left ventricular function by ca rdiac MRI

37 Randomized Clinical Trial of Adipose-Derived Stem Cells in the Treatment of Pts With ST-Elevation Myocardial Infarction

Conditions:
Myocardial Infarction; Coronary Arteriosclerosis; Cardiovascular Disease; Coronary Disease
Interventions: Drug: Injection of ADRC's; Other: Injection of Placebo
Phase: Phase I
Number Enrolled: 48
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety Study; Intervention Model: Paral lel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatmen t
NCT ID: NCT00442806
Outcome Measures: Safety - Determined by Major Adverse Cardiac and Cerebral Events (MACCE); Feasibility - Assessment of cardiac function via functional and imaging studies including MRI, SPECT, and Echocardiography

38 Stem Cell Therapy for Vasculogenesis in Patients With Severe Myocardial Ischemia

Conditions: Myocardial Ischemia; Coronary Heart Disease
Intervention: Drug: stem cell
Phases: Phase I / Phase II
Number Enrolled: 40
Study Design: Allocation: Non-Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group A ssignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00260338
Outcome Measures: Improvement in myocardial perfusion measured by SPECT; Safety; Improvement in myocardial perfusion and function measured by PET and MR; Exercise time; Clinical angina status

39 Effect of Intramyocardial Injection of Mesenchymal Precursor Cells on Heart Function in People Receiving an LVAD

Condition: Heart Failure
Intervention: Biological: Revascor
Phase: Phase II
Number Enrolled: 80
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator); Primary Purpose: Treatment
NCT ID: NCT00927784

Outcome Measures: Incidence of infectious myocarditis, myocardial rupture, neoplasm, hypersensitivity reaction, or immune sensitization; The secondary endpoints include echocardiographic assessments, 6 minute walk, ability to tolerate wean from LVAD support, duration of ability to tolerate wean from LVAD support, and neuronal function.

40 Safety Study of Adult Mesenchymal Stem Cells (MSC) to Treat Acute Myocardial Infarction

Condition: Myocardial Infarction
Intervention: Drug: Provacel
Phase: Phase I
Number Enrolled: 48
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety Study; Intervention Model: Paral lel Assignment; Masking: Double-Blind; Primary Purpose: Treatment
NCT ID: NCT00114452
Outcome Measure: Comparison of treatment adverse event rates between the 0.5, 1.6 and 5.0 million mesenchymal stem cells per kilogram dose cohorts and placebo groups.

41 Randomized Evaluation of Intracoronary Transplantation of Bone Marrow Stem Cells in Myocardial Infarction

Condition: Myocardial Infarction
Intervention: Procedure: Intracoronary Transplantation of Bone Marrow Cells
Phase: Phase I
Number Enrolled: 30
Study Design:
Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00874354
Acronym: REVITALIZE
Outcome Measures: Safety and feasibility of intracoronary administration of autologous bone marrow-derived mononuclear cells; Improvement of global left ventricular ejection fraction by cardiac MRI and echocardiography after 4 months.; Freedom from Major Adverse Cardiac Event (MACE)

42 Intracoronary Stem Cell Therapy in Patients With Acute Myocardial Infarction (SCAMI)

Conditions: Acute Myocardial Infarction; Coronary Artery Disease
Interventions: Other: autologous stem cells; Other: placebo suspension
Phase: Phase II
Number Enrolled: 40
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Efficacy Study; Intervention Model: Par allel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatm ent
NCT ID: NCT00669227
Acronym: SCAMI
Outcome Measures: difference in left ventricular ejection fraction measured by magnetic resonance imaging at baseline and 6 months follow-up; left ventricular ejection fraction measured by magnetic resonance imaging; left ventricular enddiastolic volume measured by magnetic resonance imaging; left ventricular endsystolic volume measured by magnetic resonance imaging; major adverse cardiac events

43 Effects of Intracoronary Progenitor Cell Therapy on Coronary Flow Reserve After Acute MI

Conditions: Coronary Artery Disease; Acute Myocardial Infarction
Interventions: Biological: autologous bone marrow-derived progenitor cells; Biological: placebo medium
Phases: Phase I / Phase II
Number Enrolled: 100
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Efficacy Study; Intervention Model: Par

allel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatm ent

NCT ID: NCT00711542

Acronym: REPAIR-ACS

Outcome Measures: Improvement of coronary flow reserve in the infarct vessel; Improvement of relative coronary flow reserve; Improvement of global and regional left ventricular ejection fraction; Major adverse cardiac events (death, MI, rehospitalization for heart failure, revascularization)

44 Cellwave Study: Combined Extracorporal Shock Wave Therapy and Intracoronary Cell Therapy in Chronic Ischemic Myocardium

Condition: Congestive Heart Failure

Intervention: Procedure: intracoronay stem cell therapy

Phases: Phase I / Phase II

Number Enrolled: 100

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Investigator); Primary Purpose: Treatment

NCT ID: NCT00326989

Outcome Measures: Improvement in global ejection fraction on LV angiography; Global or regional wall motion at 4 months and 1 year; NYHA Class; NT BNP levels; MACE; Life quality

45 The Transendocardial Autologous Cells (hMSC or hBMC) in Ischemic Heart Failure Trial (TAC-HFT)

Conditions: Stem Cell Transplantation; Ventricular Dysfunction, Left

Interventions: Biological: Autologous human mesenchymal cells (hMSCs); Biological: Autologous human bone marrow cells (hBMCs); Biological: Placebo

Phases: Phase I / Phase II

Number Enrolled: 60

Study Design:

Allocation: Randomized; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Double Blind (Subject, Investigator); Primary Purpose: Treatment

NCT ID: NCT00768066

Acronym: TAC-HFT

Outcome Measures: Incidence of TE-SAE define as composite of death, non-fatal MI, stroke, hospitalization for worsening heart failure, cardiac perforation, pericardial tamponade, ventricular arrhythmias >15 sec. or with hemodynamic compromise or atrial fibrillation; MRI and echocardiographic-derived measures of left ventricular function

46 Myocardial Regeneration and Angiogenesis in Myocardial Infarction With G-CSF and Intra-Coronary Stem Cell Infusion

Condition: Myocardial Infarction

Interventions: Drug: Drug: G-CSF (Dong-A pharmaceutical, Seoul, Korea); Procedure: collection of mobilized peripheral blood stem cells; Procedure: Intracoronary infusion of mobilized cells

Phase: Phase II

Number Enrolled: 20

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00307879

Outcome Measures: the change in left ventricular ejection fraction, measured by SPECT, echocardiography; changes in left ventricular volume by SPECT, echocardiography; the development of major adverse cardiac events

47 A Phase II Dose-escalation Study to Assess the Feasibility and Safety of Transendocardial Delivery of Three Different Doses of Allogeneic Mesenchymal Precursor Cells (MPCs)in Subjects With Heart Failure

Condition: Heart Failure

Interventions: Biological: Mesenchymal Precursor Cells (MPCs); Procedure: standard-of-care treatment with mock mapping and injection procedures.

Phase: Phase II

Number Enrolled: 60

Study Design:

Allocation: Randomized; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Factorial Assignment; Masking: Single Blind (Subject); Primary Purpose: Treatment

NCT ID: NCT00721045

Outcome Measures: The primary objective of this study is to evaluate the feasibility and safety of transendocardial injection using mapping Catheter with the Left Ventricular Injection Catheter of 25 M, 75 M, and 150 M allogeneic MPCs in subjects with heart failure.; The secondary objectives are to explore functional efficacy for subsequent study design.

48 Stem Cell Mobilization by G-CSF Post Myocardial Infarction to Promote Myocyte Repair

Condition: Myocardial Infarction

Intervention: Drug: Granulocyte Colony Stimulating Factor

Phases: Phase II / Phase III

Number Enrolled: 86

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Single Group Assignment; Masking: Double-Blind; Primary Purpose: Treatment

NCT ID: NCT00394498

Outcome Measures:

6 month Left ventricular ejection fraction; 6 week left ventricular ejection fraction; 6 week myocardial FDG-PET uptake; 6 week myocardial Ammonia-
PET perfusion; 6 week/month left ventricular diastolic volume; 6 week/month left ventricular systolic volume

49 SWiss Multicenter Intracoronary Stem Cells Study in Acute Myocardial Infarction (SWISS-AMI)

Condition: Acute Myocardial Infarction

Intervention: Procedure: intracoronary bone marrow cells infusion

Phase: Phase II

Number Enrolled: 150

Study Design:

Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00355186

Outcome Measures: Change in global left ventricular ejection fraction (LVEF) at 4 months relative to baseline measured by quantitative MRI; Change in LVEF at MRI at 12 months; Change in regional left ventricular wall motion and thickness at 4 and 12 months; Change in infarct size at 4 and 12 months as assessed by "delayed enhancement" technique by MRI; Analysis of the myocardial infarct size.

50 Autologous Bone Marrow Derived Stem Cells for Acute Myocardial Infarction

Condition: Myocardial Infarction

Interventions: Biological: MNC; Biological: AC 133; Biological: Control

Phase: Phase III

Number Enrolled: 105

Study Design:

Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Parallel Assignment; Masking: Single Blind (Outcomes Assessor); Primary Purpose: Treatment

NCT ID: NCT01167751

Outcome Measures: Left ventricular ejection fraction at rest, measured by Dobutamine Stress Echocardiography; Regional contractility in the AOI / Change in LV dimensions (left ventricular end systolic diameter [LVESD], left ventricular end diastolic diameter [LVEDD]) as assessed by
echocardiography; changes in LVM index, LVEDV, LVESV

51 Stem Cell Study for Patients With Heart Disease

Conditions:
Chest Pain; Myocardial Ischemia; Heart Disease; Coronary Arterial Disease (CAD); Angina
Intervention: Genetic: Cell Therapy - Autologous CD34 Positive Cells
Phase: Phase I
Number Enrolled: 24
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Masking: Double -Blind; Primary Purpose: Treatment
NCT ID: NCT00081913

52 Stem Cell Therapy in Patients With Severe Heart Failure & Undergoing Left Ventricular Assist Device Placement

Condition: Heart Failure
Intervention: Other: Intramyocardial Delivery of Bone Marrow Derived Mononuclear Cells
Phase: Phase I
Number Enrolled: 24
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety Study; Intervention Model: Paral lel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatmen t
NCT ID: NCT00869024
Outcome Measures: Safety of cell delivery; Improvement in myocardial viability by PET scan; Combined end points of death and re-hospitalization; Hemodynamic parameters at baseline and with weaning of LVAD; Improvement in left ventricular ejection fraction; Histological assessment:

53 Cell Therapy for Coronary Heart Disease

Condition: Coronary Artery Disease
Intervention: Drug: intracoronary infusion of progenitor cells
Phase: Phase II
Number Enrolled: 75
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Crossover Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00289822
Outcome Measures: Change in global left ventricular function (measured by quantitative left ventricular angiography); Quantitative parameters of regional left ventricular function of the target area; changes in left ventricular volumes; functional status as assessed by NYHA classification; event-free survival after 4 months follow-up

54 Effect of Nitric Oxide Donor on Endothelial Progenitor Cells in Patients With Coronary Artery Disease

Condition: Coronary Artery Disease
Intervention: Drug: nitroglycerin
Phase: Phase II
Number Enrolled: 30
Study Design: Endpoint Classification: Safety/Efficacy Study; Primary Purpose: Treatment
NCT ID: NCT00090558

55 Mesenchymal Stem Cells and Myocardial Ischemia

Conditions: Chronic Myocardial Ischemia; Left Ventricular Dysfunction
Intervention: Genetic: Mesenchymal stem cells
Phases: Phase I / Phase II
Number Enrolled: 10
Study Design:
Control: Uncontrolled; Endpoint Classification: Safety Study; Intervention Model: Single Group Assignment; Maski ng: Open Label; Primary Purpose: Treatment

NCT ID: NCT01076920
Acronym: MESAMI
Outcome Measures: Evaluate safety and feasibility of transendocardial injection using the NogaStar XP Mapping Catheter with the MyostarTM Left Ventricular Injection Catheter of autologous MSC in subjects with chronic myocardial ischemia and left ventricular dysfunction; Change from baseline in SF-36, VO2max, 6 min walk test, and the NYHA, CCS Classifications Effect related to cardiac function

56 A Randomized Clinical Trial of Adipose-Derived Stem Cells in Treatment of Non Revascularizable Ischemic Myocardium

Conditions:
Ischemic Heart Disease; Coronary Arteriosclerosis; Cardiovascular Disease; Coronary Disease; Coronary Artery Dis ease
Interventions: Other: Direct injection of ADRCs into the Left Ventricle; Other: Direct injection of placebo into the Left Ventricle
Phase: Phase I
Number Enrolled: 36
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety Study; Intervention Model: Paral lel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatmen t
NCT ID: NCT00426868
Outcome Measures: Safety - Determined by Major Adverse Cardiac and Cerebral Events (MACCE); Feasibility - Assessment of cardiac function using a variety of functional and imaging studies including MRI, SPECT and Echocardiography

57 Safety Study of Allogeneic Mesenchymal Precursor Cells (MPCs) in Subjects With Recent Acute Myocardial Infarction

Condition: Myocardial Infarction
Interventions: Genetic: Allogeneic Mesenchymal Precursor Cells (MPCs); Procedure: Standard-of-care treatment with NOGA® mapping and staged injections.
Phases: Phase I / Phase II
Number Enrolled: 25
Study Design:
Allocation: Randomized; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Single Blind (Subject); Primary Purpose: Treatment
NCT ID: NCT00555828
Outcome Measures: Evaluate the safety and feasibility of transendocardial injection using the Cordis Biosense NogaStarTM Mapping Catheter with the Biosense MyostarTM Left Ventricular Injection Catheter of allogeneic mesenchymal precursor cells (MPCs) in subjects with AMI.; Explore efficacy for subsequent study design and dose related tolerance: •Effect related to cardiac function.•Change from baseline in SF-36, KCCQ, SAQ, and the NYHA Classification.•Follow-up safety through Day 360 • Dose selection for future stu

58 To Assess Safety and Efficacy of Myoblast Implantation Into Myocardium Post Myocardial Infarction

Condition: Congestive Heart Failure
Interventions: Biological: MyoCell; Procedure: Hypothermosol
Phases: Phase II / Phase III
Number Enrolled: 390
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator); Primary Purpose: Treatment
NCT ID: NCT00526253
Acronym: MARVEL
Outcome Measures: 6-minute walk test; Quality of Life Questionnaire; Hospitalization occurrences

59 Autologous Mesenchymal Stromal Cell Therapy in Heart Failure

Condition: Congestive Heart Failure

Interventions: Biological: Mesenchymal stromal cell; Biological: Saline
Phases: Phase I / Phase II
Number Enrolled: 60
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatment
NCT ID: NCT00644410
Outcome Measure: Clinical and ejection fraction improvements

60 Safety and Efficacy of Bone Marrow Cell Transplantation in Humans Myocardial Infarction

Condition: Acute Myocardial Infarction
Intervention: Procedure: cell therapy, bone marrow derived stem cell
Phases: Phase I / Phase II
Number Enrolled: 50
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00437710
Acronym: CARDIAC
Outcome Measures:
Mortality; Mortality and Morbidity; Left ventricular function; Left ventricular remodeling; Heart rate variability; Baroreflex sensitivity; Stress induced myocardial ischemia; Cell dose response

61 Autologous Transplantation of Bone Marrow Mononuclear Stem-Cells for Dilated Cardiomyopathy

Condition: Dilated Cardiomyopathy
Intervention: Procedure: intramyocardial bone marrow stem cells implantation
Phases: Phase II / Phase III
Number Enrolled: 30
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00743639
Acronym: SDILCM
Outcome Measure: Increase of the ejection function of the left ventricle

62 Progenitor Cell Therapy in Dilative Cardiomyopathy

Conditions: Heart Failure, Congestive; Cardiomyopathy, Dilated; Stem Cell Transplantation
Intervention: Procedure: intracoronary infusion of BMC
Phases: Phase I / Phase II
Number Enrolled: 30
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00284713
Outcome Measure: LV function (Ejection fraction within 3 months) Simpson

63 Stem Cell Therapy in Chronic Ischemic Heart Failure

Conditions: Myocardial Ischemia; Heart Failure, Congestive
Intervention: Procedure: Bone marrow transplantation
Phase: Phase II
Number Enrolled: 35
Study Design: Allocation: Non-Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group As signment; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00235417
Outcome Measure: Changes in left ventricular ejection fraction from baseline to 12 months' follow-up

64 REPAIR-AMI: Intracoronary Progenitor Cells in Acute Myocardial Infarction (AMI)

Condition: Myocardial Infarction
Intervention: Drug: Intracoronary infusion of enriched bone marrow-derived progenitor cells
Phase: Phase III
Number Enrolled: 200
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Efficacy Study; Intervention Model: Par allel Assignment; Masking: Double-Blind; Primary Purpose: Treatment
NCT ID: NCT00279175
Outcome Measures: Change in global left ventricular function in quantitative LV angiography after 4 months.; Primary endpoint in patients without restenosis.; Improvement of regional wall motion in infarct area; Reduction of LV end-
systolic volume; Major adverse cardiac events (MACE); Rehospitalization due to heart failure.; NYHA status after 12 mo nths.

65 Treatment of Myocardial Infarction With Bone Marrow Derived Stem Cells

Condition: Acute Myocardial Infarction
Intervention: Procedure: Coronary catherization and stem cell infusion
Phase: Phase I
Number Enrolled: 10
Study Design: Allocation: Non-
Randomized; Control: Historical Control; Endpoint Classification: Safety Study; Intervention Model: Single Group Assign ment; Masking: Open Label
NCT ID: NCT00275977
Outcome Measures: Safety; Change in left ventricular funtion at 4 months followup using contrast enhanced echocardiography

66 Bone Marrow Transplant to Induce Tolerance in Heart Transplant Recipients

Condition: Patients in Need of a Cardiac Transplant.
Intervention: Procedure: Stem cell transplant
Phases: Phase I / Phase II
Number Enrolled: 30
Study Design: Allocation: Non-
Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group As signment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00497757
Outcome Measures: Bone marrow engraftment/chimerism; Tolerance induction

67 MAGIC Cell-5-Combicytokine Trial

Condition: Acute Myocardial Infarction
Intervention: Drug: G-CSF with/without darbepoetin, peripheral blood stem cell infusion
Phases: Phase II / Phase III
Number Enrolled: 116
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Parallel Assignment; Masking: Double-Blind; Primary Purpose: Treatment
NCT ID: NCT00501917
Acronym: MAGIC Cell-5
Outcome Measures: change of left ventricular ejection fraction measured by cardiac
MRI; wall motion score index exercise capacity BNP

68 Prochymal® (Human Adult Stem Cells) Intravenous Infusion Following Acute Myocardial Infarction (AMI)

Condition: Myocardial Infarction
Interventions: Drug: Prochymal®; Drug: Placebo
Phase: Phase II
Number Enrolled: 220
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment.
NCT ID: NCT00877903
Outcome Measures:
Left ventricular end systolic volume (ESV); Left ventricular ejection fraction (LVEF); Infarct size; Major adverse card iovascular events (MACE)

69 Safety and Efficacy of Autologous Endothelial Progenitor Cell CD 133 for Therapeutic Angiogenesis

Conditions: Coronary Artery Disease; Refractory Angina
Interventions: Biological: Stem cell (Endothelial progenitor cell CD 133); Biological: Placebo
Phases: Phase I / Phase II
Number Enrolled: 30
Study Design:
Allocation: Randomized; Control: Placebo Control; point Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator); Primary Purpose: Treatment
NCT ID: NCT00694642
Acronym: PROGENITOR
Outcome Measures: Major adverse events; The change in the myocardial perfusion defect at baseline versus follow-up measured by Magnetic Resonance and Single Photon Emission Computed Tomography (SPECT)

70 Bone Marrow Derived AC 133+ and Mono-Nuclear Cells (MNC) Implantation in Myocardial Infarction (MI) Patient

Condition: Myocardial Infarction
Interventions: Biological: AC133; Biological: MNC; Biological: CONTROL
Phases: Phase II / Phase III
Number Enrolled: 100
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Parallel Assignment; Masking: Single Blind (Outcomes Assessor); Primary Purpose: Treatment
NCT ID: NCT01187654
Outcome Measures: Increase from baseline in ejection fraction; Decrease LVESV/LVEDV/LVM index; Decrease the number of Non Viable segments from baseline

71 Vescell(TM) for the Treatment of Patients With Severe Anginal Syndrome With or Without Heart Failure

Condition: Angina Pectoris
Intervention: Procedure: Intracoronary administration of autologous ACPs
Phases: Phase I / Phase II
Number Enrolled: 10
Study Design: Allocation: Non-Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group As signment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00416663
Outcome Measures: Safety of the procedure as manifested in the post treatment observation and; tests.; Changes from baseline to 1, 3 and 6 months in the CCS.; Changes from baseline to 1, 3 and 6 months of modified Bruce exercise test.; Changes from baseline to 6 months of exercise-induced ischemia on Sestamibi scan.; Changes from baseline to 6 months of %LVEF

72 CD133+ Cell Therapy for Refractory Coronary Heart Disease

Condition: Coronary Artery Disease
Intervention: Procedure: Intracoronary Infusion of CD133+ Cells

Phases: Phase I / Phase II
Number Enrolled: 10
Study Design: Allocation: Non-Randomized; Endpoint Classification: Safety Study; Intervention Model: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT01049867
Outcome Measures: Increased regional and global myocardial contractility measured by low-dose dobutamine echocardiography/MRI and increased myocardial perfusion measured by adenosine nuclear stress testing.; Improvement in the heart failure functional class measured by NYHA and CCS classification tests.

73 Stem Cells in Myocardial Infarction

Condition: Acute Myocardial Infarction
Intervention: Drug: Granulocyte Colony Stimulating Factor G-CSF (Neupogen®)
Phase: Phase II
Number Enrolled: 78
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Double-Blind; Primary Purpose: Educational/Counseling/Training
NCT ID: NCT00135928
Outcome Measures: The pre-specified primary endpoint is change in regional systolic wall thickening from day 1 to day 180 evaluated with cardiac magnetic resonance imaging (MRI); Change in ejection fraction, end-systolic and end-diastolic volumes, regional myocardial perfusion, and infarct size by MRI; Change in regional myocardial function by tissue Doppler echocardiography

74 ACPs Combined With CABG in Patients With CHF

Condition: Congestive Heart Failure
Interventions: Procedure: Angiogenic Cell Precursors(ACPs) or Vescell TM; Biological: Angiogenic Cell Precusors
Phase: Phase I
Number Enrolled: 5
Study Design: Allocation: Non-Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00523224
Outcome Measures: Evaluation criteria; Safety : no.& duration of adverse event & serious adverse event; Efficacy : EF , NYHA; change from baseline to 1 & 3 months of NYHA, 6-minute walking test; Change from baseline to 3 months of QoL(SF-36); Efficacy:%EF,NYHA; change from baseline to 3 months of % LVEF by Echocardiography & C-MRI; change from baseline to 3 months of % infracted scar area on C-MRI; change from baseline to 3 months of QoL(SF-36)

75 Strengthening Transplantation Effects of Bone Marrow Mononuclear Cells With Atorvastatin in Myocardial Infarction

Conditions:
Myocardial Infarction; Stem Cell Transplantation; Angioplasty, Transluminal, Percutaneous Coronary
Intervention: Drug: Atorvastatin and mononuclear cells transplantation
Phase: Phase II
Number Enrolled: 100
Study Design:
Allocation: Randomized; Control: Dose Comparison; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Factorial Assignment; Masking: Double Blind (Subject, Caregiver, Investigator); Primary Purpose: Treatment
NCT ID: NCT00979758
Acronym: STEM-AMI
Outcome Measure: Changes in left ventricular ejection fraction from baseline to 12 months' follow-up

76 Alster Stem Cells - Intramyocardial Stem Therapy

Conditions: Acute Myocardial Infarction; Early Left Ventricular Dysfunction
Interventions: Procedure: Percutaneous Coronary Intervention; Procedure: BMNC therapy
Phase: Phase II

Number Enrolled: 40

Study Design: Allocation: Non-Randomized; Control: Active Control; Endpoint Classification: Efficacy Study; Intervention Model: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00939042

Outcome Measures: Assessment of the effect of a direct intramyocardial injection of bone marrow mononuclear cells (BMNC) on heart function (LVEF).; Comparison of th eexplanatory power of echocardiography, endocardial left ventricular electromechanical mapping (LVEMM) with NOGA and cardiac MRI as an endpoint for myocardial regeneration.; Collection of first evidence on the best type and time points for the determination of myocardial regeneration by NOGA and other parameters.

77 EPC by Intracoronary Injection in Patients With Chronic Stable Angina

Condition: Coronary Artery Disease

Intervention: Device: VesCell (TM)-Autologous EPCs/Angiogenic Cell Precursors

Phase: Phase II

Number Enrolled: 24

Study Design: Allocation: Non-Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group As signment; Masking: Open Label; Primary Purpose: Treatment

NCT ID: NCT00384514

Outcome Measures: Safety : no.& duration of adverse event & serious adverse event; Efficacy :change from baseline to 1,3,6 months of CCS, 6-minute walking test; Efficacy : change from baseline to 3 & 6 months of Sesta-mibi scan; : change from baseline to 3 & 6 months of symptom-limited exercise time,exercise-induced ischemia & METs on Sesta-mibi scan

78 Cell Therapy in Myocardial Infarction

Condition: Acute Myocardial Infarction

Interventions: Procedure: Catheter based Stem cells delivery; Other: Autologous Bone Marrow Mononuclear Cells Transplantation

Phase: Phase III

Number Enrolled: 300

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Efficacy Study; Intervention Model: Par allel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatm ent

NCT ID: NCT00350766

Acronym: EMRTCC

Outcome Measures: Global Left Ventricular Ejection Fraction change; Death; Acute myocardial infarction, stroke and hospital admission due to cardiovascular cause; Reintervention of the AMI related artery and of the non-related artery; Regional wall motion, wall thickening, and volume of late contrast enhancement.

79 Bone Marrow-Derived Stem Cell Transfer in Acute Myocardial Infarctions

Condition: Myocardial Infarction

Intervention: Procedure: bone marrow-derived stem cell transfer

Phase: Phase II

Number Enrolled: 68

Study Design:

Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Efficacy Study; Intervention Model: Sin gle Group Assignment; Masking: Double-Blind; Primary Purpose: Educational/Counseling/Training

NCT ID: NCT00264316

Outcome Measures: increase in global LV ejection fraction fraction; evaluation by magnetic resonance (MRI) after 4 months; change in infarct size and regional LV function; evaluation by magnetic resonance (MRI) after 4 months; change in myocardial perfusion and oxidative metabolism; investigated using serial 1-[11C]acetate positron emission tomography after 4 months

80 Autologous Transplantation of Bone Marrow Mononuclear Stem-Cells by Mini-Thoracotomy

Condition: Dilated Cardiomyopathy

Intervention: Procedure: intramyocardial bone marrow stem cells implantation
Phases: Phase I / Phase II
Number Enrolled: 6
Study Design: Allocation: Non-Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group As signment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00615394
Acronym: STEMDILCARD
Outcome Measure: safety: morbidity/mortality

81 Stem Cell Therapy to Improve Myocardial Function in Patients With Acute Myocardial Infarction

Condition: Myocardial Infarction
Intervention: Procedure: Autologous bone marrow-derived stem cells
Number Enrolled: 200
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00316381
Outcome Measures: Left ventricular ejection fraction and volumes measured by echocardiography; Left ventricular ejection fraction and volumes measured by angiography; Safety; Left ventricular function in dobutamine stress test; Coronary flow reserve by adenosine MRI test

82 Bone Marrow Cell Transplantation to Improve Heart Function in Individuals With End-Stage Heart Failure

Condition: Heart Failure, Congestive
Interventions: Biological: Intramyocardial injection of bone marrow mononuclear cells; Biological: Intramyocardial injection of CD34+ selected bone marrow mononuclear cells; Device: LVAD alone
Phase: Phase II
Number Enrolled: 75
Study Design:
Allocation: Randomized; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00383630
Outcome Measures: Functional status; Echocardiographic assessments of myocardial size and function by transthoracic echocardiography with the LVAD at full support, and as tolerated at 1, 5, 10, and 15 minutes following initiation of hand
pumping; Six Minute Walk;Incidence of serious adverse events; Neovascularization and cardiomyocyte regeneration; Ab ility to tolerate wean from LVAD support for 30 minutes;

83 Myocardial Stem Cell Administration After Acute Myocardial Infarction (MYSTAR) Study

Condition: Myocardial Infarction
Intervention: Procedure: Bone marrow-derived stem cells implantation
Phase: Phase II
Number Enrolled: 116
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Parallel Assignment; Masking: Single Blind (Outcomes Assessor); Primary Purpose: Treatment
NCT ID: NCT00384982
Acronym: MYSTAR
Outcome Measures: Changes in resting myocardial perfusion defect size by gated SPECT scintigraphy 3-6 months after the percutaneous intracoronary or combined bone marrow-derived stem cells therapy.; Changes in global left ventricular ejection fraction by gated SPECT scintigraphy 3-6 months after the percutaneous intracoronary or combined bone marrow-derived stem cells therapy.; The feasibility of the bone marrow-derived stem cells delivery modes, determined by the rates of acute and subacute complications.

84 Intracoronary Autologous Stem Cell Transplantation in ST Elevation Myocardial Infarction: TRACIA STUDY.

Condition: Acute Myocardial Infarction
Intervention: Genetic: Stem Cell Transplantation
Phases: Phase II / Phase III
Number Enrolled: 80
Study Design:
Allocation: Randomized; Endpoint Classification: Efficacy Study; Intervention Model: Parallel Assignment; Masking : Single Blind (Outcomes Assessor); Primary Purpose: Treatment
NCT ID: NCT00725738
Acronym: TRACIA
Outcome Measures: Evaluate the mean LVEF increase by magnetic resonance imaging (MRI) at 6 months of follow up between the stem cell group and the control group.; Evaluate the left ventricular end diastolic volume (LVEDV) and left ventricular end systolic volume (LVESV)with image magnetic resonance(IMR) at 6 months follow up between stem cell and control group.; Evaluate the oxygen consumption during treadmill stress test (MVO2) by expired gases analysis and the incidence of MACE at 6 months follow up between stem cell and control group.

85 ACT34-CMI -- Adult Autologous CD34+ Stem Cells

Condition: Myocardial Ischemia
Intervention: Device: stem cell injection
Phase: Phase II
Number Enrolled: 150
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double-Blind; Primary Purpose: Treatment
NCT ID: NCT00300053
Outcome Measure: Frequency of angina episodes

86 Bypass Surgery and CD133 Marrow Cell Injection for Treatment of Ischemic Heart Failure

Conditions: Coronary Artery Disease With Need for Bypass
Surgery; Myocardial Ischemia, Angina Pectoris; Congestive Heart Failure; Previous Myocardial Infarction
Intervention: Procedure: Intramyocardial injection of autologous CD133+ marrow cells
Phases: Phase II / Phase III
Number Enrolled: 60
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Efficacy Study; Intervention Model: Par allel Assignment; Masking: Double-Blind; Primary Purpose: Treatment
NCT ID: NCT00462774
Acronym: Cardio133
Outcome Measures: Left ventricular ejection fraction at rest, measured six months postoperatively, measured by MRI; Change in LVEF compared with preoperatively and early postoperatively; Regional contractility in the AOI; Physical exercise capacity determined by 6 minute walk
test; Perfusion in the AOI; Change in LV dimensions; NYHA and CCS class; Minnesota Living with Heart Failure Score; D eath, myocardial infarction, or need for reintervention during follow-up

87 Cell Therapy In Dilated Cardiomyopathy

Condition: Dilated Cardiomyopathy
Intervention: Procedure: Stem cell
Phase: Phase III
Number Enrolled: 300
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mo del: Parallel Assignment; Masking: Double Blind (Subject, Investigator); Primary Purpose: Treatment
NCT ID: NCT00333827
Outcome Measures: increase of the ejection fraction of the left ventricle; Death by any cause; Maximum oxygen consumption difference, as measured by ergoespirometry, at six and twelve months in relation to

baseline; Difference in life quality as estimated by Minnesota living with Heart Failure Questionnaire; Difference in NYHA functional class; Percent number of patients that reached an absolute increase of 5% in ejection fraction

88 Long Term Follow-up of Autologous Bone Marrow Mononuclear Cells Therapy in STEMI

Condition: Myocardial Infarction
Interventions: Procedure: saline infusion; Procedure: autologous bone marrow mononuclear cells infusion
Phases: Phase I / Phase II
Number Enrolled: 37
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00626145
Outcome Measures: Left Ventricular Ejection Fraction(LVEF); in-stent restenosis; cardiac shock; myocardial viability of the infarcted area; end-diastolic Volume/end-systolic Volume(EDV/ESV); wall motion score index(WMSI); cumulative MACE(including cardiac death, non-fetal myocardial infarction and target lesion revascularization)

89 Allogeneic Multipotent Stromal Cell Treatment for Acute Kidney Injury Following Cardiac Surgery

Condition: Kidney Tubular Necrosis, Acute
Interventions: Biological: Multipotent Stromal Cells; Biological: Administration of MSC
Phase: Phase I
Number Enrolled: 15
Study Design: Allocation: Non-Randomized; Intervention Model: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00733876
Outcome Measure: Absence of MSC-specific Adverse or Serious Adverse Events

90 Ex Vivo Cultured Bone Marrow Derived Allogenic MSCs in AMI

Condition: Myocardial Infarction
Interventions: Drug: Stem cell; Drug: Plasmalyte A
Phases: Phase I / Phase II
Number Enrolled: 20
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator); Primary Purpose: Treatment
NCT ID: NCT00883727
Outcome Measures: AE and ECG parameters; Regional myocardial perfusion and infarct size

91 The Enhanced Angiogenic Cell Therapy - Acute Myocardial Infarction Trial

Condition: Acute Myocardial Infarction
Interventions: Biological: Autologous EMMCs; Biological: Plasma-Lyte A
Phase: Phase II
Number Enrolled: 100
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator); Primary Purpose: Treatment
NCT ID: NCT00936819
Acronym: ENACT-AMI
Outcome Measures: To assess changes from baseline in global LVEF, regional wall motion, wall thickening and infarct volume by cardiac MRI; To assess changes from baseline of Echocardiographic LVEF and ventricular volumes; To assess time to clinical worsening (death, hospitalization for angina or reinfarction); To assess any changes from baseline in quality of life (SF-36v2 and DASI)

92 BONAMI (BOne Marrow in Acute Myocardial Infarction)

Condition: Acute and Severe Myocardial Infarction
Intervention: Procedure: Intracoronary injection of autologous bone marrow mononuclear cells
Phase: Phase II
Number Enrolled: 100
Study Design:
Allocation: Randomized; Control: Active Control; Intervention Model: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00200707
Outcome Measures: Change in Myocardial viability evaluated by thallium scintigraphy (3 months and 1 year); Left ventricle ejection fraction (LVEF) evaluated by radionuclide ventriculography, (3 and 12 months) and by echography (1, 3, 6, and 12 months). Segmental EF and myocardial viability evaluated by MRI (3 months); Correlation with biological parameters (hematopoietic stem cell number, etc.) at the time of cell injection

93 ACT34-CMI -- Adult Autologous CD34+ Stem Cells (Follow-Up Study)

Condition: Myocardial Ischemia
Number Enrolled: 150
Study Type: Observational
Study Design: Observational Model: Case Control; Time Perspective: Prospective
NCT ID: NCT00545610
Outcome Measures: Long-term safety of intramyocardial injections of Auto-CD34+ cells relative to placebo for reducing the number of angina episodes in subjects with refractory chronic myocardial ischemia over a 12-month follow-up period.; Long-term efficacy of Auto-CD34+ cells relative to placebo for reducing the number of angina episodes in subjects with refractory chronic myocardial ischemia.

94 A Trial Using CD133 Enriched Bone Marrow Cells Following Primary Angioplasty for Acute Myocardial Infarction

Condition: Acute Myocardial Infarction
Interventions: Other: CD133+ infusion; Other: placebo infusion
Number Enrolled: 60
Study Design:
Allocation: Randomized; Intervention Model: Parallel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatment
NCT ID: NCT00529932
Acronym: SELECT-AMI
Outcome Measures: PRIMARY SAFETY ENDPOINT Comparison of progression in coronary atherosclerosis burden proximal and distal to the stented segment of the infarct-related artery in treated and control groups.; PRIMARY EFFICACY ENDPOINT Comparison of changes in myocardial thickening in non-viable akinetic / hypokinetic LV wall segments as determined by cardiac magnetic resonance imaging (cMRI) in treated and control groups.

95 Trial of Hematopoietic Stem Cells in Acute Myocardial Infarction

Condition: Reperfused Acute Myocardial Infarction
Interventions: Other: Granulocite Colony Stimulating Factor treatment (G-CSF); Other: Bone marrow mononuclear cells
Phase: Phase II
Number Enrolled: 120
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Single Blind (Outcomes Assessor); Primary Purpose: Treatment
NCT ID: NCT00984178
Acronym: TECAM2
Outcome Measures: The change in left ventricular ejection fraction and left ventricular end-systolic volume relative to baseline measured by magnetic resonance; The change in left ventricle end-dyastolyc volume, segment contractility, wall thickness and intravascular ultrasound reendothelization relative to baseline measured by magnetic resonance and other imaging techniques.

96 Bone Marrow Stem Cell Mobilisation Therapy for Acute Myocardial Infarction (AMI)(REVIVAL-2)

Condition: Myocardial Infarction
Interventions: Drug: G-CSF Granulocyte-Colony Stimulating Factor; Other: Placebo
Phase: Phase IV
Number Enrolled: 114
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Efficacy Study; Intervention Model: Par allel Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatm ent
NCT ID: NCT00126100
Outcome Measures: Reduction of infarct size (measured by Tc-sestamibi scintigraphy); Left ventricular ejection fraction; Incidence of angiographic restenosis

97 Autologous Stem Cell Transplantation in Acute Myocardial Infarction

Condition: Acute Anterior Wall Myocardial Infarction
Intervention: Genetic: Intracoronary aotologous stem cell transplantation
Phase: Phase II
Number Enrolled: 100
Study Design:
Allocation: Randomized; Control: Active Control; Endpoint Classification: Safety/Efficacy Study; Intervention Mode l: Parallel Assignment; Masking: Single Blind; Primary Purpose: Treatment
NCT ID: NCT00199823
Outcome Measures: whether intracoronary mBMC transplantation improve LVEF after AMI assessed by ECG-gated SPECT.; To test whether mBMC treatment improve exercise capacity assessed by bicycle ergometry; To test whether mBMC treatment improve quality of life assessed by the SF 36 formula

98 Intracoronary Stem Cells in Large Myocardial Infarction

Condition: Myocardial Infarction
Intervention: Procedure: Intracoronary infusion of autologous bone-marrow derived stem cells
Phase: Phase II
Study Design:
Allocation: Randomized; Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00389545

99 Intracoronary Infusion of Autologous Bone Marrow Cells for Treatment of Idiopathic Dilated Cardiomyopathy

Condition: Dilated Cardiomyopathy
Intervention: Procedure: Intracoronary infusion of autologous bone marrow cells
Phase: Phase II
Number Enrolled: 30
Study Design: Allocation: Non-Randomized; Control: Uncontrolled; Endpoint Classification: Efficacy Study; Intervention Model: Single Group Assignme nt; Masking: Open Label; Primary Purpose: Treatment
NCT ID: NCT00629096
Outcome Measures: Improvement of left ventricular function; Functional status

100 Bone Marrow Cells in Myocardial Infarction

Condition: Acute Myocardial Infarction
Intervention: Procedure: intracoronary injection of bone marrow cells
Phases: Phase II / Phase III
Number Enrolled: 100

Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Parallel Assignment; Masking: Double-Blind; Primary Purpose: Treatment
NCT ID: NCT00363324
Outcome Measures:
Arrhythmia risk variables; left ventricular function; restenosis assessed by IVUS; exercise tolerance

101 The Effect of Granulocyte Colony Stimulating Factor (G-CSF) on Myocardial Function After Acute Anterior Myocardial Infarction, a Prospective Double Blind Randomized Placebo Controlled Study

Condition: Myocardial Infarction
Interventions: Drug: G-CSF; Drug: placebo infusion of normal saline
Number Enrolled: 20
Study Design:
Allocation: Randomized; Control: Placebo Control; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group Assignment; Masking: Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor); Primary Purpose: Treatment
NCT ID: NCT00756756
Outcome Measures:
Ejection fraction; Diastolic function(Tei index); Drug complication; Mortality; New revascularization and MACE

B) LISTE DES ETUDES HUMAINES DE THERAPIE CELLULAIRE MEMBRES INF. EN COURS

1 Mesenchymal Stem Cells in Critical Limb Ischemia

Condition: Critical Limb Ischemia
Interventions: Drug: mesenchymal stem cells; Drug: Plasmalyte A
Phases: Phase I / Phase II
Number Enrolled: 20
NCT ID: NCT00883870
Outcome Measures: AE and symptomatic relief; Increase in transcutaneous partial oxygen pressure (TcPO2) and Ankle brachial pressure index (ABPI) - measured by Doppler

2 Hematopoietic Stem Cell Transplantation for the Treatment of Limb Ischemia and Diabetic Neuropathy

Conditions: Limb Ischemia; Diabetic Neuropathy
Intervention: Biological: Hematopoietic stem cell transplantation
Number Enrolled: 20
NCT ID: NCT00730561
Outcome Measure: Change in nerve conduction velocity

3 Induced Wound Healing by Application of Expanded Bone Marrow Stem Cells in Diabetic Patients With Critical Limb Ischemia

Condition: Diabetic Foot
Interventions: Biological: tissue repair cells (TRC); Biological: bone marrow stem cells (BMC)
Phase: Phase II
Number Enrolled: 30
NCT ID: NCT01065337
Outcome Measures: The patient is alive, the patient has not undergone any major amputation, complete primary wound healing has been achieved, no ipsilateral relapse has occurred; Rate major amputations Rate of patients with complete ulcer healing Rate of treatment related complications Improvement of ankle brachial index (ABI) Improvement of transcutaneous oxygen partial pressure (TcPO2) Improvement of local perfusion

4 A Clinical Trial on Diabetic Foot Using Peripheral Blood Derived Stem Cells for Treating Critical Limb Ischemia

Conditions: Diabetic Foot; Critical Limb Ischemia; Leg Ulcers
Interventions: Procedure: will receive G-CSF and peripheral blood derived mononuclear cells; Drug: G-CSF; Drug: Standard Therapy
Phases: Phase I / Phase II
Number Enrolled: 36
NCT ID: NCT00922389
Outcome Measures:
Adverse events and laboratory parameters; Trans Cutaneous partial pressure of Oxygen: TCpO2

5 Combination Stem Cell Therapy for the Treatment of Severe Leg Ischemia

Conditions: Critical Limb Ischemia; Peripheral Vascular Disease
Intervention: Biological: Mesendo
Phase: Phase I
Number Enrolled: 10
NCT ID: NCT00518401

Acronym: mesendo
Outcome Measure: Safety

6 Phase II Combination Stem Cell Therapy for the Treatment of Severe Leg Ischemia

Conditions:
Critical Limb Ischemia; Severe Leg Ischemia; Peripheral Artery Disease; Peripheral Vascular Disease
Interventions: Biological: MESENDO; Biological: Placebo
Phase: Phase II
Number Enrolled: 30
NCT ID: NCT00721006
Acronym: MESENDO
Outcome Measures: Enhancement of vessel formation accessed by Nuclear Perfusion Scan in critical limb ischemia.; Changes in resting leg pain identified by a Visual Analog Scale and patient safety

7 Autologous Bone Marrow Stem Cell Transplantation for Critical, Limb-threatening Ischemia

Conditions:
Peripheral Vascular Disease; Diabetic Foot; Peripheral Arterial Occlusive Disease; Leg Ulcer; Gangrene; Ischemia
Interventions: Procedure: Autologous bone marrow cell concentrate transplantation; Biological: saline injection
Phases: Phase II / Phase III
Number Enrolled: 90
NCT ID: NCT00434616
Acronym: BONMOT
Outcome Measures: Major amputation of the index limb or persisting, unchanged critical limb ischemia; Wound healing (wound size, wound stage); Pain and analgesics use; Rutherford grade and stage; Walking dis tance (treadmill) if possible; Quality of life (EQ-5D Questionnaire); Transcutaneous oxygen pressure (TcpO2), ABI, absolute ankle perfusion pressure; Collateral artery number as judged by contrast angiography after 3 months; Rate and extent of minor (below the ankle) amputations; survival without amputation

8 Efficacy and Safety of Umbilical Cord Blood Injection for Critical Limb Ischemia

Condition: Critical Limb Ischemia
Intervention: Biological: Cord blood stem cell injection
Phase: Phase I
Number Enrolled: 25
NCT ID: NCT01019681
Outcome Measures: Ankle brachial index (ABI), a 15% increase will be considered improvement; Healing of ischemic ulcers; Decreased pain level as reported by the patient; SF-36 quality of life (QOL); Walking Impairment Questionnaire; Increase in pain free ambulation time on treadmill by more than 25%; Increase in four meter walk or six minute walk by more than 25%

9 Intra-Arterial Stem Cell Therapy for Patients With Chronic Limb Ischemia (CLI)

Conditions:
Peripheral Vascular Diseases; Arterial Occlusive Diseases; Leg Ulcer; Gangrene; Ischemia
Interventions: Procedure: Bone marrow punction; Procedure: BM-MNC infusion; Procedure: Placebo infusion
Phases: Phase I / Phase II
Number Enrolled: 110
NCT ID: NCT00371371
Acronym: JUVENTAS
Outcome Measures:
major amputation; minor amputation; number and extent of leg ulcers; resolvement of rest pain; improvement of ankle-brachial index (ABI); improvement transcutaneous oxygen pressure (TcpO2); changes in quality of life; changes in clinical status (Rutherford classification)

10 Injection of Autologous CD34-Positive Cells for Critical Limb Ischemia

Conditions: Peripheral Artery Disease; Peripheral Vascular Disease; Critical Limb Ischemia
Intervention: Biological: Autologous Stem Cells (CD34+)
Phases: Phase I / Phase II
Number Enrolled: 75
NCT ID: NCT00616980
Acronym: ACT34-CLI
Outcome Measures: Safety of Intramuscular Injection of CD34-positive cells; Change in Rest Pain; Ulcer Healing; Functional Improvement; Limb Salvage; Disease Severity; Assessment of Co-morbidity

11 Safety Study of Adult Stem Cells to Treat Patients With Severe Leg Artery Disease

Conditions: Critical Limb Ischemia; Arterial Occlusive Disease; Vascular Diseases
Intervention: Biological: autologous CD133+ cells
Phase: Phase I
Number Enrolled: 24
NCT ID: NCT00913900
Acronym: SCRIPT-CLI
Outcome Measures: Death or amputation; Vascular hemodynamics and function

12 Stem Cell Study for Patients With Leg Ulcer/Gangrene

Conditions: Leg Pain; Ulcer; Gangrene; Ischemia; Peripheral Vascular Diseases
Intervention: Genetic: Autologous peripheral blood CD34 positive cell therapy
Phases: Phase I / Phase II
Number Enrolled: 15
NCT ID: NCT00221143
Outcome Measures: Major amputation; Limb ischemia

13 Safety Study of Using Stem Cells to Stimulate Development of New Blood Vessels in Peripheral Vascular Disease

Condition: Peripheral Vascular Diseases
Intervention: Drug: adult stem cells
Phase: Phase I
Number Enrolled: 20
NCT ID: NCT00113243
Outcome Measures: Adverse events recorded in the 12 week study period; Serious Adverse events recorded for one year; Changes in limb perfusion after treatment with stem cells will be assessed with arteriography, blood pressure recordings, oxygen measurements, and wound healing

14 Treatment of Severe Limb Ischemia With Autologous Bonemarrow Derived Mononuclear Cells

Condition: Ischemia
Intervention: Procedure: Stem cell implantation
Phase: Phase I
Number Enrolled: 10
NCT ID: NCT00442143
Acronym: DANCELL-CLI
Outcome Measures:
Transcutaneous oxygen pressure; 1st toe blood pressure (strain gauge); Ankle blood pressure; Wound healing; Pain; amputation; Infection

15 Stem Cell Mobilization by G-CSF to Treat Severe Peripheral Artery Disease

Condition: Peripheral Vascular Diseases
Interventions: Drug: G-CSF; Drug: Placebo; Drug: Aspirin; Drug: Clopidogrel
Phase: Phase III
Number Enrolled: 60
NCT ID: NCT00797056

Acronym: STEMPAD
Outcome Measures: Major limb amputation rate at one year; Toe pressure; Ankle-brachial index; Ulcer healing and improvement in rest pain

16 Autologous Bone Marrow For Lower Extremity Ischemia Treating

Condition: Lower Extremity Ischemia
Interventions: Procedure: Bone marrow aspiration, injection of cells; Procedure: Bone marrow aspiration , injection of isolated CD 133+ cells; Procedure: Bone marrow aspiration, injection of saline
Phase: Phase II
Number Enrolled: 42
NCT ID: NCT00753025
Outcome Measure: Increasing of painless walking distance

17 Autologous Bone Marrow Mononuclear Cell Implantation for Moderate to Severe Peripheral Arterial Disease

Condition: Peripheral Arterial Disease
Intervention: Procedure: Bone marrow mononuclear cell implantation
Number Enrolled: 36
NCT ID: NCT00919516
Outcome Measures:
Major limb amputation; Improved ABI measurements; Relief of rest pain; Ulceration healing

18 ALD-301 for Critical Limb Ischemia, Randomized Trial

Conditions: Critical Limb Ischemia; Peripheral Arterial Disease; Peripheral Vascular Disease
Intervention: Procedure: ALDH-br bone marrow cells vs. mononuclear bone marrow cells
Phases: Phase I / Phase II
Number Enrolled: 20
NCT ID: NCT00392509
Acronym: CLI-001
Outcome Measures: adverse events; ankle-brachial systolic pressure index; transcutaneous oxygen value (mm Torr); quality of life (questionnaires); size of lower e xtremity ulcer(s); peripheral nerve conduction exam; level of pain at rest (questionnaire); limb clinical status

19 Autologous Transplantation of Bone Marrow Mononuclear Cell (BM-MNC) With and Without Granulocyte-Colony Stimulation Factor (G-CSF) for Treatment of Chronic Lower Limb Ischemic Patients

Conditions: Peripheral Vascular Diseases; Ischemia
Interventions: Biological: BM-MNC; Biological: BM-MNC and G-CSF
Phases: Phase I / Phase II
Number Enrolled: 50
NCT ID: NCT00677404
Outcome Measures:
Major amputation; Minor amputation; Number and extent of leg ulcers; Resolvement of rest pain; Improvement of ankle-brachial index (ABI); Improvement of pain free walking distances (PFWD)

20 Stem Cells for Treating Critical Ischemia

Conditions: Critical Limb Ischemia; Ischemic Ulcers
Intervention: Procedure: stem cells transplant
Phase: Phase I
Number Enrolled: 10
NCT ID: NCT00488020
Outcome Measures: Suppress pain and heal ischemic ulcers; improve quality of life

21 Novel Therapy of PAD by Combined Transplantation of BMCs

Condition: Peripheral Arterial Disease
Intervention: Procedure: intraarterial and intramuscular injection of BMCs
Phase: Phase I
Number Enrolled: 100
NCT ID: NCT00411840
Outcome Measures: ABI, walking distance, capillary-venous oxygen saturation, venous occlusion plethysmography; feasibility

22 Autologous Transplantation of Bone Marrow Mesenchymal Stem Cells on Diabetic Foot

Conditions: Autologous Transplantation; Diabetic Foot
Intervention: Biological: Autologous transplantation of mesenchymal stem cells
Phases: Phase II / Phase III
Number Enrolled: 80
NCT ID: NCT00955669
Outcome Measure: Patients in both group received the same conventional treatment. Meanwhile, mesenchymal stem cells were separated by density gradient centrifugation and cultured in the medium with autologous serum.After three-weeks adherent culture in vitro, mesenchymal

23 Safety and Feasibility Study of Autologous Bone Marrow Cell Transplantation in Patients With Peripheral Arterial Occlusive Disease (PAOD)

Condition: Arterial Occlusive Diseases [C14.907.137]
Interventions: Procedure: intraarterial stem cell therapy; Other: Stem cells
Phases: Phase I / Phase II
Number Enrolled: 40
NCT ID: NCT00282646
Outcome Measures: Ankle brachial index; Ulcer size; Pain; Walking distance; TCO2

24 Autologous Bone Marrow Derived Mononuclear Cells in Treating Diabetic Patients With Critical Limb Ischemia

Conditions: Peripheral Vascular Diseases; Diabetic Foot
Intervention: Procedure: Autologous Bone Marrow Mononuclear Cells
Phases: Phase I / Phase II
Number Enrolled: 20
NCT ID: NCT00872326
Outcome Measures: Angiographic evaluation of angiogenesis and vasculogenesis at target limb; Ankle-Brachial pressure index

25 ACPs in Severe PAD/CLI by Direct Intramuscular Injection

Conditions: Peripheral Arterial Disease; Critical Limb Ischemia
Intervention: Procedure: Angiogenic Cell Precursors (ACPs) or Vescell TM
Phase: Phase I
Number Enrolled: 6
NCT ID: NCT00523731
Outcome Measures: Safety; Evaluate the safety of ACPs intramuscular injection; Efficacy; Attenuate CLI patients symptoms as; Rest pain; Pain-free walking distance; Ulcer size; Gangrene dimension and intensity; Obtain evidence for improvement of tissue perfusion due to ACPs injection; Reduction of CLI patients hospitalization time.; Decrease CLI patient amputation rate.

26 Feasability Study of Autologous Bone Marrow Aspirate Concentrate for Treatment of CLI

Condition: Arterial Occlusive Diseases
Intervention: Device: centrifuge, laboratory, tabletop (SmartPReP2 BMAC System)
Phases: Phase I / Phase II
Number Enrolled: 60

NCT ID: NCT00595257
Outcome Measures: avoid amputation; measurement of hemodynamic response

27 Autologous Bone Marrow Cell Treatment in Peripheral Atherosclerosis

Condition: Peripheral Vascular Diseases
Intervention: Genetic: Autologous bone marrow cells
Phase: Phase I
Number Enrolled: 20
NCT ID: NCT00306085
Outcome Measures: Clinical and instrumental indexes of functional impairment of peripheral atherosclerosis.; Angiographic and laser doppler measurements after six months from treatment.

28 Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Mobilization of Progenitor Cells in Peripheral Arterial Disease

Condition: Peripheral Arterial Disease
Interventions: Drug: Granulocyte-Macrophage Colony Stimulating Factor; Drug: Placebo
Phase: Phase II
Number Enrolled: 180
NCT ID: NCT01041417
Acronym: GPAD-2
Outcome Measures: To investigate whether GM-CSF will improve symptoms of claudication in patients with PAD, measured objectively as improvement in treadmill exercise tolerance.; Change in exercise tolerance will be due to improvement in collateral blood flow measured by ankle-brachial index, transcutaneous oxygen concentration, and calf blood flow measured by MRI, and/or improvement in endothelial dysfunction.

29 Bone Marrow Autograft in Limb Ischemia

Conditions: Peripheral Vascular Diseases; Limb Ischemia
Intervention: Procedure: Bone marrow harvest
Phase: Phase III
Number Enrolled: 110
NCT ID: NCT00904501
Acronym: BALI
Outcome Measures:
Major amputation rate and mortality; Clinical symptoms and haemodynamical parameters

30 Cell Therapy in Chronic Limb Ischemia

Condition: Peripheral Vascular Diseases
Interventions: Procedure: BM-MNC preparation; Procedure: PB-MNC preparation
Phases: Phase I / Phase II
Number Enrolled: 40
NCT ID: NCT00533104
Outcome Measures: Survival without major amputation; clinical symptoms and haemodynamic parameters

31 Intraarterial Infusion of Autologous Marrow Derived Mononuclear Cells in Diabetic Patients With Chronic Critical Limb Ischemia

Conditions: Arterial Occlusive Disease; Diabetic Foot
Intervention: Other: Intraarterial infusion of autologous bone marrow cells
Phases: Phase I / Phase II
Number Enrolled: 60
NCT ID: NCT00987363
Outcome Measures:
Number of adverse events; Arteriographic, AngioRNM and AngioTC changes; Clinically objective improvement in the ischemic limb

32 A Prospective, Open, Non-randomized Phase I/II Study of Therapeutic Angiogenesis in Diabetic Patients With Critic Ischemia of Lower Limbs While Administering Positive CD133 Mobilized With G-CSF

Condition: Diabetic Patients With Critic Ischemia in Lower Limbs Who Are Administered With CD133+ Cells Mobilized by G-CSF
Intervention: Biological: CD133+ cells transplant
Phases: Phase I / Phase II
Number Enrolled: 20
NCT ID: NCT00765050
Outcome Measures: analyze the safety and efficacy of CD133+ cells, obtained from peripheral blood in the treatment of diabetic patients with critic ischemia in lower limbs.; To determine the safety of the intramuscular administration of CD133+ cells that have been mobilized from peripheral blood; To determine the CD133+ capacity to increase the re-vascularization at lower limbs in diabetic patients with critic ischemia in the lower limbs; To evaluate the patient global health condition using the notified results of the SF-36 questionnaires completed by patients

33 Use of Vascular Repair Cells (VRC) in Patients With Peripheral Arterial Disease to Treat Critical Limb Ischemia

Condition: Peripheral Arterial Disease
Interventions:
Biological: autologous bone marrow cells; Biological: electrolyte solution (without cells)
Phase: Phase II
Number Enrolled: 150
NCT ID: NCT00468000
Acronym: RESTORE-CLI
Outcome Measures: Safety of TRCs in patients with CLI(key safety parameters include vital signs, physical exams, laboratory results, assessment of aspiration and injection sites, adverse events, major amputations, wounds presence(size and grading using the Wagner scale).

34 Autologous Bone Marrow-Derived Mononuclear Cells for Therapeutic Arteriogenesis in Patients With Limb Ischemia

Conditions: Intermittent Claudication; Peripheral Vascular Diseases
Interventions: Biological: bone marrow derived mononuclear cells; Biological: placebo
Phases: Phase II / Phase III
Number Enrolled: 108
NCT ID: NCT00539266
Acronym: ABC
Outcome Measures: Limb salvage/wound healing at t=6 months; Pain free walking distance; quality of life (RAND-36), pain Scores (Brief Pain Inventory), tcO2 (wrist/ankle ratio) ABI Collateral artery scores (angiogram) at t=6 months, Limb salvage/wound healing at t= 3 and 12 months, Pain free walking distance at t=3 and 12 months,

35 Study for Safety and Efficiency of Therapeutic Angiogenesis for Patients With Limb Ischemia by Transplantation of Human Cord Blood Mononuclear Cell

Condition: Ischemia
Intervention: Other: Study for safety and efficiency of therapeutic angiogenesis for patients with limb ischemia by transplantation of human cord blood mononuclear cell
Phases: Phase I / Phase II
NCT ID: NCT00518934

36 Safety of Intramuscular Injection of Allogeneic PLX-PAD Cells for the Treatment of Critical Limb Ischemia

Conditions: Peripheral Artery Disease; Peripheral Vascular Disease; Critical Limb Ischemia
Intervention: Biological: PLX-PAD IM injection
Phase: Phase I
Number Enrolled: 15
NCT ID: NCT00919958

Outcome Measures: Adverse events, Safety laboratory values and ECG findings; Immunological reaction; Tumorigenesis

37 Safety of Intramuscular Injections (IM) of Allogeneic PLX-PAD Cells for the Treatment of Critical Limb Ischemia (CLI)

Conditions: Peripheral Artery Disease; Peripheral Vascular Disease; Critical Limb Ischemia
Intervention: Biological: PLX-PAD
Phase: Phase I
Number Enrolled: 12
NCT ID: NCT00951210
Outcome Measures: Adverse events; Amputation incidence, Death incidence & Rehospitalization incidence; Immunological reaction

38 Safety Study of MultiGeneAngio in Patients With Chronic Critical Limb Ischemia

Conditions: Peripheral Arterial Disease; Chronic Critical Limb Ischemia
Intervention: Biological: MultiGeneAngio
Phases: Phase I / Phase II
Number Enrolled: 18
NCT ID: NCT00956332
Outcome Measures: The safety of MultiGeneAngio will be assessed by monitoring adverse events; Improvement in critical limb ischemia symptoms

39 Study of Autologous Bone Marrow Concentrate for the Treatment of Critical Limb Ischemia (CLI)

Condition: Ischemia
Interventions:
Device: Harvest Smartprep2 BMAC System; Device: SmartPReP2 BMAC System; Biological: placebo
Number Enrolled: 48
NCT ID: NCT00498069
Outcome Measure: To be determined by data from this feasibility study

PUBLICATIONS

ET COMMUNICATIONS

VII) PUBLICATIONS ET COMMUNICATIONS

A) PUBLICATIONS

1 - **Barandon L, Couffinhal T, Ezan J, Dufourcq P, Costet P, Alzieu P, Leroux L, Moreau C, Dare D, Duplaa C**. Reduction of infarct size and prevention of cardiac rupture in transgenic mice overexpressing FrzA. *Circulation. 2003 Nov 4;108(18):2282-9*

2 - **Barandon L, Leroux L, Dufourcq P, Plagnol P, Deville C, Duplaa C, Couffinhal T.** Gene therapy for chronic peripheral arterial disease: what role for the vascular surgeon? *Ann Vasc Surg. 2004 Nov;18(6):758-65.*

3 - **Ezan J, Leroux L, Barandon L, Dufourcq P, Jaspard B, Moreau C, Allieres C, Daret D, Couffinhal T, Duplaa C**. FrzA/sFRP-1, a secreted antagonist of the Wnt-Frizzled pathway, controls vascular cell proliferation in vitro and in vivo. *Cardiovasc Res. 2004 Sep 1;63(4):731-8.*

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B) COMMUNICATIONS ORALES ET POSTERS

1) Communications orales

1 **- AHA Orlando 2003 (American Heart Association). L Leroux, T Couffinhal, U Deutch, P Costet, L Barandon, P Alzieu, D Daret, C Duplàa.** Endothelial cell-specific overexpression of FrzA, a secreted antagonist of the Wnt-Frizzled pathway, induces a dual effect on neovascularization after ischemia. *Circulation Oct 2003 (suppl); 108(17): IV-298 - 1418 Abstract*

2 **- SFC (Société Française de Cardiologie) Journées Nationales, Lyon 2005. L Leroux, A.L Menguin, G Laplace, E. Gerbaud, C Jaïs, J.N. Labèque, P. Dos Santos, P. Coste**. Suivi clinique à 9,5 ans de 195 patients revascularisés par athérectomie directionnelle coronarienne. *Arch Mal Cœur et Vaisseaux 2005 ; 98(6) :8. Abstract*

3 - **GRRC (Groupement de recherche et de réflexion en cardiologie), Tours 2007. L. Leroux, P. Dufourcq, C. Moreau, T. Couffinhal, C. Duplàa.** Recruitment of mesenchymal stem cells in a model of hindlimb ischemia : role of hypoxia conditioning. *Arch Mal Cœur et Vaisseaux 2007 ; 100(4) :340. Abstract*

2) Communications affichées

1 - **GRRC 2002 (Groupe de Réflexion et de recherche en Cardiologie): L Leroux, T Traissac, V Pradeau, V Bernard, J Bonnet, T Couffinhal**. Nouveaux critères angiographiques de collatéralité coronarienne et application à l'étude de la néovascularisation au cours du viellissement.. *Arch Mal coeur Vaiss. 2002 Avril; 95(4) :336,4-6 Abstract.*

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3) Participations à communications

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3 - **GRRC 2002. L Barandon, T Couffinhal, D Daret, P Alzieu, C Allières , L Leroux, P Dufourcq, C Duplàa**. Augmentation de l'angiogenèse, réduction de la taille des infarctus et amélioration de la fonction cardiaque chez les souris transgéniques surexprimant FrzA.. *Arch Mal coeur Vaiss. 2002 Avril; 95(4) :335,4-3 Abstract.*

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5 - **GRRC 2002. P Dufourcq, T Couffinhal, J Ezan, L Barandon, D Daret, C Moreau, P Alzieu, L Leroux, C Duplàa**. FrzA un nouveau facteur angiogénique. *Arch Mal coeur Vaiss. 2002 Avril; 95(4) :335,4-10 Abstract.*

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15 - International Congress on Coronary Artery Disease 2007. **L Barandon, N Derval, L Leroux, D Daret, C Duplàa, T Couffinhal.** Epicardial deposition of unpurified bone marrow cells, endothelial progenitor cells or mesenchymatous cells in a coated patch after myocardial infarction. *Coronary Artery Disease. 2007 Oct;6(1) :3, 10 abstract.*

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Nouvelles stratégies de thérapie cellulaire à visée pro-angiogénique : Implication du système Wnt/Frizzled

La thérapie cellulaire suscite de grands espoirs dans le domaine cardiovasculaire. Cependant les premières études humaines sont décevantes. Parmi les explications avancées, citons un mauvais choix de cellules, une méthode de délivrance inadéquate, une préparation des cellules et des tissus hôtes insuffisante ou une trop grande mortalité cellulaire après injection.

Durant ce travail nous avons voulu explorer 3 pistes d'optimisation de la thérapie cellulaire à visée pro-angiogénique utilisant les cellules souches mésenchymateuses (MSC) et contribué à l'exploration des mécanismes mis en jeu, en particulier en explorant le rôle du système Wnt/Frizzled.

Nous avons tout d'abord étudié un système original de délivrance de cellules utilisant un « patch » musculaire cousu en regard d'un myocarde infarci de souris. Puis nous avons étudié l'effet d'une surexpression de sFRP1, inhibiteur de la voie Wnt, sur un modèle de matrice sous cutanée. Enfin, nous avons testé l'hypothèse qu'un préconditionnement hypoxique des cellules permettrait une meilleure survie cellulaire et améliorerait la réparation vasculaire et tissulaire après ischémie de patte chez la souris.

Nos résultats permettent notamment de montrer que les MSC ont des capacités d'invasion des tissus ischémiques et qu'elles se différencient en péricytes en formant un réseau tridimensionnel de soutien aux cellules endothéliales. Par ailleurs, via sFRP1, nous mettons en évidence un rôle du système Wnt/Fzd dans l'effet pro-angiogénique. Enfin, nous montrons l'intérêt du préconditionnement hypoxique dont les effets sont médiés par Wnt4.

L'ensemble de ces données permet d'envisager des voies d'optimisation de la thérapie cellulaire.

Development of novel stem-cell therapies for cardiac diseases and critical hindlimb ischemia: involvement of the Wnt/Frizzled pathway

Some of the challenges facing stem-cell therapy for cardiac disease are which type of stem cell or progenitor cell is the best candidate for therapy, how to survive in the low oxygen environment of ischemic myocardium. Here we studied the potential of mesenchymal stem cells (MSCs) as vascular progenitor cells *in vitro* and *in vivo*, and we studied the effects of sFRP-1/Wnt signaling modulation or hypoxia on MSC properties.

First, we demonstrated the beneficial effect of MSC application on ischemic heart repair using an original surgical model (patch) to deliver stem cells. This study showed that the contribution of the MSCs in the mouse infarcted myocardium was beneficial either on the scar (increase in angiogenesis, in cell proliferation, reduction in ventricular remodeling) or on the trophicity of the patch.

Then we characterized the angiogenic properties of MSC *in vitro* and *in vivo*. Our data demonstrate that MSCs could be recruited and formed vascular structures around endothelial tubes. We showed *in vivo* that the surexpression of *sFRP-1* (regulating factor of the Wnt system) in MSCs increased their potential of pericyte-like cells correlated with an increased maturation of the vessels via an intracellular GSK-3 dependent pathway in MSCs.

Our next objective was to investigate the effects of hypoxia exposure on MSC before implantation for vascular and tissue regeneration in mice with hind limb ischemia. Our data suggest that hypoxic preconditioning has a critical role on MSC dynamic functions, shifting MSC location *in situ* to enhance ischemic tissue recovery, facilitating vascular cell mobilization and skeletal myoblast regeneration via a paracrine Wnt dependent mechanism.

Mots clés en Français : Thérapie cellulaire ; angiogenèse ; infarctus du myocarde ; artériopathie oblitérante des membres inférieurs ; cellules souches mésenchymateuses ; système Wnt/Frizzled ; Préconditionnement hypoxique.

Mots clés en Anglais : Stem cell therapy; angiogenesis; myocardial infarction; hindlimb ischemia; mesenchymal stem cells; Wnt/Frizzled pathway; hypoxia preconditioning.

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Laboratoire : Unité Inserm U828 – Adaptation cardiovasculaire à l'ischémie
Avenue du Haut-Lévêque, 33600 Pessac, France