

PhD Thesis

Early Signaling Signature during Stem Cell-Associated Neo-Vasculogenesis Using Proteomic Profiling

submitted by

Rokhsareh Rohban

for the Academic Degree of a

Doctor of Philosophy (Ph.D.)

in Molecular Medicine

at the

Medical University of Graz

Stem Cell Research Unit

Division of Hematology and Stem Cell Transplantation,

Department of Internal Medicine

under the supervision of

Prof. Dr. Dirk Strunk

2014

Quotation

Watch your **thoughts**, for they become **words**.

Watch your **words**, for they become **actions**.

Watch your **actions**, for they become **habits**.

Watch your **habits**, for they become **your character**.

And watch **your character**, for it becomes **your DESTINY**.

What we think, we become.

Margaret Thatcher

from (The Iron Lady, director: Phyllida Lloyd, 2011)

Declaration

I, Rokhsareh Rohban, hereby declare that this dissertation is my own original work and that all of the contributors to this work have been acknowledged by name in the acknowledgments and all respective sections of this thesis. All referenced work has been acknowledged in the text. I declare that all work I have done follows the guidelines of “Good Scientific Practice”.

Graz, spring 2014

Rokhsareh Rohban

Acknowledgements

The printed pages of this thesis withhold far more than the culmination of years of study and scientific practice. These pages also mirror the relationships with many distinguished and inspiring people I have met during my doctoral studies. The list is long; however, I appreciate each contribution to my development as a true human and scientist:

To Professor Dr. Peter Holzer, dean of the PhD program, for his great understanding, generous support and big heart, without whom I could not have survived the process.

To my reviewers, Professor Dr. Akos Heinemann, for being a patient dissertation mentor and precise reviewer to me, and Professor Dr. Jürgen Becker who reviewed my thesis despite his restricted time and tight schedule.

To my PI Prof. Dr. Dirk Strunk, and my former colleagues during my doctoral studies: Dr. Nathalie Etchart, PD. Dr. Katharina Schallmoser, Dr. Dr. Andreas Reinisch, Prof. Dr. Eva Rohde, Dr. Nicole Hofmann, Mag. Margareta Frühwirth, Mag. Anna Ortner, Mag. Birgit Feilhauer, Daniela Thaler, Claudia Url and Monica Farrell, whose manners helped me to gain even more fortitude.

To my thesis committee members Prof. Dr. Irmgard Lippe and Prof. Dr. Gerhard Lanzer, for their constructive criticism, time and attention during my progress reports.

To my professors, in particular Prof. Dr. Heinz Sill, Prof. Dr. Andrea Olschewski and Prof. Dr. Anđelko Hrzenjak for their encouraging words, immeasurable backing and consultation.

To Prof. Dr. Thomas Pieber for creating a safe and encouraging space where I could home to, retrieve my enthusiasm and use my scientific expertise while achieving invaluable new experiences.

To my new colleagues to whom I owe my creativity and productivity in a peaceful working place: Alexandra Harger MSc, Katrin Kaineder MSc, Jasmin Hauzenberger MSc, Mina Bashir MSc, Barbara Prietl MSc, Dr. Selma Mautner and Dr. Thomas Birngruber.

To my outstanding network of supportive, generous and inspiring friends for persuading me throughout the process: Nathalie Etchart, Michaela Dacheva, Jing Fang, Solmaz Etemad, Fabian Ferder, Sepideh Mostofi, Ruben Charchoghlyan, Marjan Parvizi, Daniela Kleinschek and Forough Etemad whose blessings are always with me.

To Dr. Vera Fengler for correcting and proof-reading of the abstract of my dissertation in German language.

To Ehsan Bonyadi Rad for assisting me in doing the very last experiment before submitting my manuscript.

To Dr. Ruben Charchoghlyan who always had time for short, yet inspiring coffee breaks when it seemed impossible to go any further.

To my invaluable supportive, gracious, forgiving, loving and caring family without whom I could not have gone through this milestone: my parents, Farzaneh Sarshar and Jalal Rohban, for making me interested in science since I was a child, for their love, support and understanding during the long years of my education, for teaching me to benefit from tough situations to boost my inner strength and never be afraid to stand up for myself.

My siblings Sara and Mohammad Hossein, for being uniquely supportive and discerning, and letting me benefit from their beautiful minds through our off and on scientific discussions and inspiring brainstorming sessions.

And to Behzad Rahimpour, my dearest friend, greatest critic, cheerleader, voice of reason, counselor and spouse, for the past 14 years of being a kindred spirit to me.

Finally, to the most compassionate, the most merciful, my bosom God, for being there during all the ups and downs of my life, for being the reason behind my courage, persistence and enthusiasm. "You are the reason I am, you are all my reasons. Thank you."*

* A beautiful mind, Ron Howard, 2001

TABLE OF CONTENTS

1. ZUSAMMENFASSUNG	9
2. ABSTRACT	12
3. ABBREVIATIONS	14
4. INTRODUCTION	17
4.1. CELL THERAPY USING STEM/PROGENITOR CELLS	
4.1.1. DEFINITION AND HISTORY OF CELL THERAPY	
4.1.2. EMBRYONIC STEM CELLS AND CELL THERAPY	
4.1.3. ADULT STEM CELLS AND CELL THERAPY	
4.2. PROTEOMIC PROFILING	
4.2.1. PROTEIN MICROARRAY TECHNOLOGY	
4.2.2. ANTIBODY MEDIATED DETECTION OF SIGNALING MOLECULES	
4.3. VASCULARIZATION	
4.3.1. ANGIOGENESIS AND VASCULOGENESIS: DEFINITIONS AND DIFFERENCES	
4.3.2. NEO-VASCULOGENESIS: ROLE OF ADULT STEM CELLS IN VESSEL FORMATION	
4.3.3. MOLECULAR REGULATION OF NEO-VASCULOGENESIS	
4.3.4. SIGNALING PATHWAYS INVOLVED IN VESSEL FORMATION	
4.4. SIGNALING PATHWAYS	
4.4.1. PROTEIN KINASE SIGNALING PATHWAYS	
4.4.1.1. MAPK SIGNALING PATHWAY	
4.4.1.2. ERK SIGNALING PATHWAY	
4.4.1.3. ERBB SIGNALING PATHWAY	
4.4.1.4. FOCAL ADHESION KINASE PATHWAY	
4.4.2. DEATH ASSOCIATED SIGNALING PATHWAYS	
4.5. APOPTOSIS	
4.5.1. NON-APOPTOTIC ROLE OF CASPASES IN DEVELOPMENTAL BIOLOGY	
4.6. GOAL OF THIS THESIS	
5. MATERIALS AND METHODS	78
5.1. MATERIALS	
5.2. EXPERIMENTAL DEVICES	
5.3. EXPERIMENTAL PROCEDURES	
5.3.1. PREPARATION OF ECFC AND MSPC CULTURE MEDIUM	
5.3.1.1. POOLED HUMAN PLATELET LYSATE (PHPL)	
5.3.2. ECFC AND MSPC ISOLATION AND EXPANSION	
5.3.2.1. FLOW CYTOMETRIC CHARACTERIZATION OF ECFC AND MSPC	
5.3.3. ANIMAL EXPERIMENTS	
5.3.4. VESSEL FORMATION <i>IN VIVO</i>	
5.3.5. PROTEOMIC PROFILING: DETECTION OF SIGNALING MOLECULES	
5.3.5.1. SAMPLE PREPARATION	
5.3.5.2. ANTIBODY-MEDIATED DETECTION OF SIGNALING MOLECULES	
5.3.5.3. DATA ANALYSIS	
5.3.5.4. TARGET CONFIRMATION: WESTERN BLOT ANALYSIS	
5.3.6. <i>IN VIVO</i> SIGNALING MOLECULE INHIBITION IN MSPC AND ECFC	
5.3.7. <i>IN VITRO</i> CAPILLARY-LIKE STRUCTURE FORMATION	

5.3.8.	HISTOLOGICAL EXPERIMENTS AND ANALYSIS	
5.3.8.1.	HEMATOXYLIN-EOSIN STAINING (H&E)	
5.3.8.2.	IMMUNE HISTOCHEMISTRY STAINING	
5.3.8.3.	MOVAT'S PENTACHROME STAINING	
5.3.9.	TUNNEL ASSAY	
5.3.10.	STATISTICS	

6. RESULTS	101
-------------------	------------

6.1.	CONTRIBUTION OF ECFC AND MSPC IN NEO-VASCULOGENESIS	
6.2.	EXPRESSION OF SIGNALING MOLECULES UPON CO-TRANSPLANTATION	
6.3.	BLOCKING CASPASE SIGNALING LEADS TO IMPAIRED ECFC NETWORK FORMATION <i>IN VITRO</i>	
6.4.	BLOCKING CASPASE SIGNALING RESULTS IN DIMINISHED NEO-VASCULOGENESIS <i>IN VIVO</i>	

7. DISCUSSION	112
----------------------	------------

8. CONCLUSION	134
----------------------	------------

9. FIGURES	135
-------------------	------------

10. REFERENCES	182
-----------------------	------------

1. ZUSAMMENFASSUNG

The abstract in German language was corrected for language style and grammar by Dr. Vera Fengler, Biobank Medical University of Graz.

De-novo-Vasculogenese kann durch Ko-Transplantation von Perizyten, ihren mesenchymalen Stammzellen/Vorläuferzellen (MSPC), Endothelzellen oder endothelialen koloniebildenden Progenitorzellen (ECFC) induziert werden. Dabei sind die genauen Mechanismen der *In-vivo*-Vasculogenese noch immer ungeklärt, wodurch die Entwicklung von neuartigen therapeutischen Interventionen sowie deren regenerative Anwendung erschwert wird. In dieser Studie wurde eine neuartige Strategie zur Entschlüsselung der molekularen Determinanten der Neo-Vasculogenese durch Proteom-Profiling der frühen Signalsignatur *in vivo* entwickelt. Dazu verwendete ich MSPC und ECFC gemischt mit Matrigel entweder einzeln oder in einem Gemisch mit einem Verhältnis von 1:5 für subkutane Transplantationen in immundefiziente Mäuse des Stammes NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ; (NSG). Die Implantate wurden 24h nach der Transplantation explantiert und für das Proteom-Profiling verwendet, wobei der Proteinanteil der Transplantate mit dem KAM 1.3 Antikörper-Mikroarray (www.kinexus.ca) vermessen wurde. Dabei wurden über 800 Signal- und Phosphoproteine getestet. Der Grad der Gefäßbildung und die Stabilität der Transplantate wurde nach 2, 8 und 24 Wochen parallel an entsprechenden Explantanten durch histologische Untersuchungen überprüft. Signifikant exprimierte Proteine im Antikörper Microarray wurden ausgewählt und *in vitro* Toxizitäts- und Lebensfähigkeitsassays sowie *in vivo* zur Modulation von therapeutischer neo-Vaskulogenese verwendet.

Die Ergebnisse zeigen, dass die Ko-Transplantation von ECFC und MSPC am effizientesten für die Bildung stabil perfundierter menschlicher Gefäße war. Reine ECFC-Explantate zeigten vasculäre Strukturbildung nur nach Transplantation einer höheren Zellzahl und später im zeitlichen Verlauf nach der Transplantation. Eine frühe Chondrogenese konnte in reinen MSPC Explantaten erst nach 8 Wochen festgestellt werden.

Die Microarray-Datenanalyse ergab eine signifikante Veränderung von mehreren Proteinen, einschließlich (1) jener an den Apoptose-Signalwegen beteiligten Caspasen, Death protein-6 (DAXX) und p53, (2) Human epidermal growth factor receptor 2 (HER2/ ERB), mitogen activated protein kinase (MAPK), mammalian target of rapamycin (mTOR) und transforming growth factor- beta (TGF- β), (3) focal adhesion protein kinase (FAK), vascular endothelial growth factor (VEGF), JAK-STAT und Wnt. Die Beteiligung von ausgewählten Kandidaten der Caspase-Kaskade in der durch Stamm/Vorläuferzellen vermittelten Neo-Vasculogenese wurden *in vitro* und *in vivo* mittels Caspase-Blockierungsstrategien weiter untersucht, welche eine verminderte Bildung von kapillarartigen Netzstrukturen *in vitro* und eine signifikante Abnahme der Gefäßneubildung *in vivo* ergaben.

Ko-Transplantation von ECFC und MSPC mit dem definierten Mischungsverhältnis von 5:1 oder von reinen ECFC mit einer höheren Zellzahl war für Gefäßneubildung *in vivo* notwendig. Das Proteom-Profil deckte bestimmte und teilweise überlappende Signalnetzwerke auf, die in dem komplexen Prozess der vaskulären Regeneration beteiligt sind. Es konnte gezeigt werden, dass die pharmakologische Blockade von Caspasen, die signifikant hochreguliert sind, und Caspase-4 als ein ausgewähltes Beispiel für das frühe Vasculogenese-Proteom-Profil die ECFC Netzwerkbildung *in vitro* und die Vasculogenese *in vivo* reduziert. Somit spielen

Caspasen eine entscheidende Rolle bei der Initiierung der Bildung von gefäßartigen Netzwerken *in vitro* wie auch bei der neo-Vaskulogenese durch Stamm/Vorläuferzellen *in vivo*. Die Analyse des Proteom-Profiles legte eine Signalsignatur offen, die gezielt eingesetzt werden kann, um die neo-Vaskulogenese *in vivo* zu modulieren und dadurch zu effizienteren Methoden des Tissue-Engineering zu gelangen.

2. ABSTRACT

De novo vessel formation (neo-vasculogenesis) can be induced by co-transplantation of pericytes or their mesenchymal stem/progenitor cells (MSPC) with endothelial cells or endothelial colony-forming progenitor cells (ECFC). The precise mechanisms regulating neo-vasculogenesis *in vivo* are still a matter of debate which hampers the development of strategies for therapeutic intervention referred to as 'regenerative medicine'. Here we employed a novel strategy for unraveling the molecular determinants of neo-vasculogenesis by proteomic profiling of the early signaling signature *in vivo*. We applied MSPC and ECFC for subcutaneous transplantation in matrix composites either alone or mixed at a ratio of 1:5 into immune deficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ; NSG mice. Implants were harvested 24h after transplantation for proteomic profiling and the protein content of the transplants was subjected to KAM 1.3 antibody microarray (www.kinexus.ca) testing over 800 signaling and phospho-proteins. The state of vessel formation and stability of the transplants were verified in parallel by histological follow-up of corresponding explants harvested 2, 8 and 24 weeks after transplantation. Significantly expressed targets were pre-selected from antibody microarray data based on differential display and were used for *in vitro* toxicity and viability assays as well as *in vivo* modulation of induced neo-vasculogenesis.

Results confirmed that co-transplantation of ECFC with MSPC was most efficient at forming stable-perfused human vessels. "ECFC only" explants showed vascular structure formation only after transplantation of higher cell numbers and later in the time course after transplantation. Early chondrogenesis state was detected in "MSPC only" explants after 8 weeks.

Analysis of protein microarray data revealed significant alteration of components including (1) caspases, death protein-6 (DAXX) and p53 usually involved in death-associated signaling pathways, (2) epidermal growth factor receptor 2 (Her2/ERB), mitogen activated protein kinase (MAPK), mammalian target of rapamycin (mTOR) and transforming growth factor- beta (TGF- β), (3) focal adhesion protein kinase (FAK), vascular endothelial growth factor (VEGF), JAK-STAT and Wnt. Involvement of selected candidates from the caspase cascade in stem/progenitor cell-mediated neo-vasculogenesis was further assessed using *in vitro* and *in vivo* caspase blocking strategies which led to diminished capillary-like network structure formation *in vitro* as well as significant decrease in vessel formation *in vivo*.

Co-transplantation of ECFC and MSPC with the defined 5:1 ratio or sole ECFC with a higher cell dosage was essential for vessel generation *in vivo*. Proteomic profiling unraveled independent and partially overlapping signaling networks involved in the complex process of vascular regeneration. Pharmacological blockade of caspases as significantly up regulated proteins and caspase-4 as one selected target in the proteomic profile of early vasculogenesis was shown to hamper endothelial network formation *in vitro* and vasculogenesis *in vivo*.

Proteomic profiling unraveled a signaling signature which can be targeted to modulate neo-vasculogenesis *in vivo* and may lead to more efficient tissue engineering methods.

3. ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
BBB	Blood brain barrier
BM	Bone marrow
CAC	Circulating angiogenic cell
CDKs	Cyclin-dependent kinases
CFU-ECs	Colony forming unit of endothelial progenitor cells, renamed as CFU-Hill
CM	Cardiomyocytes
CRT	Cell replacement therapy
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
EC	Endothelial cell
ECFC	Endothelial colony-forming cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGM	Endothelial growth medium
eIF-4E	Eukaryotic initiation factor 4E
EPC	Endothelial progenitor cell
ER	Endoplasmic reticulum
ERB	Epidermal growth factor receptor 2 (HER 2) also known as ERB/Neu
ERK	Extracellular signal regulator kinase
FBS	Fetal bovine serum
FACS	Fluorescence-activated cell sorting / scanning
FADD	FAS associated death domain
FAK	Focal adhesion kinase
FSC	Forward scatter
GvHD	Graft versus host disease
GPCR	G protein coupled receptor
Grb2	Growth factor receptor bound protein-2
H&E	Hematoxylin and eosin
HB-EGF	Heparin-binding epidermal growth factor
HIF-2 α	Hypoxia-inducible factor-2 α
HSP90	Heat shock protein 90
HSPC	Hematopoietic stem and progenitor cell
HUVEC	Human umbilical vein endothelial cell
IGF	Insulin-like growth factor
I κ B	Inhibitory κ B

IL	Interleukin
IMG	Intussusceptive microvascular growth
ITS	Inter-vascular tissue structure
MAPK	Mitogen-activated protein kinase
MEK	MAP/ERK kinase
MNC	Mononuclear cell
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
MSCA-1	Mesenchymal stem cell antigen-1
MSC	Multipotent mesenchymal stromal / stem cell
MSPC	Mesenchymal stem / progenitor cell
mTOR	Mammalian target of rapamycin
NAADP	Nicotinic acid adenine dinucleotide phosphate
NF-KB	Nuclear factor-KB
NSG mouse	NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Wjl} /SzJ; Non-obese diabetic (NOD) severe combined immuno-deficient (Scid, mutation in human homologue <i>PRKDC</i> gene) IL-2 receptor γ knock-out mouse (lack T-, B-, NK-, and dendritic cells)
PB	Peripheral blood
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PTMs	Post-translational modifications
PLC	Phospholipase C
pHPL	Pooled human platelet lysate
PI	Propidium iodine
PI3K	Phosphatidylinositol 3-kinase
PIGF	Placental growth factor
RPPA	Reverse phase protein microarray
RACK1	Receptor of activated protein kinase C
RT	Room temperature
RTK	Receptor tyrosine kinase
SC	Stem cell
SDF-1	Stromal derived factor-1
SMA	Smooth muscle actin
SMC	Smooth muscle cells
SSC	Side scatter
TAD	Trans-activation domains
TCA	Tri-carboxylic acid (cycle)
TEM	TIE-2 expressing monocyte
TGF	Transforming growth factor

Treg	Regulatory T cell
TNF- α	Tumor necrosis factor- α
UC	Umbilical cord
UCB	Umbilical cord blood
UCMSC	Umbilical cord matrix stem cell
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1
VEGFR-2	Vascular endothelial growth factor receptor-2
vWF	Von Willebrand factor
WAT	White adipose tissue

4. INTRODUCTION

4.1. Cell therapy using stem/progenitor cells

4.1.1. Definition and history of cell therapy

Cell therapy is defined as “the use of cells to treat diseases”. In its two general categories, either cells are obtained from an individual and transferred back to the same individual upon required treatments and expansions, or cells derived from donors are harvested directly or cultured in large scale and kept in cell banks until further use for manipulation and/or therapeutic interventions. The earlier is named “Autologous” and the latter is referred to as “Allogeneic” cell therapy (Kessler et al., 1993). The allogeneic cell therapy approach is based on using cell types that ideally do not cause an immunological reaction upon being introduced into the host, and therefore these cells have the capacity of being transplanted into numerous patients using the same expanded cell lot. Nevertheless, the risk of immune rejection in the cell transplantation procedure may hamper efficacy of allogeneic cell therapy (Malliaras et al., 2012, Chimenti et al., 2010).

The cell therapy procedure was first investigated by Charles-Edward Brown-Séquard (1817–1894) hypothesizing that cells could have an increased life cycle when co-cultured with animal testicle debris. Niehans (1882–1971) further assessed this hypothesis using calf embryo cells (Rengachary et al., 2008).

In the mid-20th century, HLA antigens were recognized to be present on the cell surface (Cann et al., 2002, Charron, 2009, Starzl et al., 2000) that clarified uprising obstacles in cell transplantation; accordingly, successful bone marrow transplantation (Thomas, 1999, Storb and Thomas, 1983) launched modern cell

therapy (Kemeny and MacAry, 2007). HLA antigens as surface markers that cause immune reactions directed against different HLA patterns raised an interest in allogeneic bone marrow transplantation. These antigens are responsible for transplant rejection if they do not match between the donated tissue and the host's immune system; therefore, bone marrow transplantation has initially been restricted to those patients with siblings genotypically identical for HLA (Starzl, 2000).

Umbilical cord blood-derived stem cells were used for cell transplantation in disorders such as anemia and leukemia (Wagner and Gluckman, 2010). In a previous study human umbilical cord blood has been used as a replacement for bone marrow to obtain stem/progenitor cells for hematopoietic regenerative purposes. In this study, Broxmeyer et al. observed that removal of erythrocytes and/or granulocytes before freezing and washing steps of the thawed cells prior to plating causes a huge loss in the progenitor cell fraction. Nevertheless, human umbilical cord blood has been shown to be a potent source of cells for autologous as well as allogeneic hematopoietic system regeneration (Broxmeyer et al., 1989).

Development of embryonic stem cells with the potential to differentiate in to all three cell germ layers and lineages such as osteoblasts (Yamashita et al., 2010) and cardiomyocytes (Kumar et al., 2005, Gepstein, 2002) under defined conditions led to a progress in regenerative medicine and novel therapeutic strategies that allow replacement of damaged malfunctioning cells with the *in vitro* generated cells preserving the same cellular function. However, this aspect of regenerative medicine has a drawback due to ethical considerations about embryonic stem cell usage in research (Lo and Parham, 2009). In 2006, Yamanaka et al. produced

induced pluripotent stem cells (iPSC) using mouse embryonic and adult fibroblast cells introducing four transcription factors SOX2, OCT 3/4, KIF4 and c-myc to the cells (Takahashi and Yamanaka, 2006). Furthermore, they generated iPSC from human somatic fibroblasts and established reprogramming strategies to convert differentiated human adult cells into a pluripotent state. In-Hyun Park and colleagues from George Q. Daley's group were able to generate iPS cells from adult, neonatal and fetal primary cells of human including skin fibroblasts (Park et al., 2008). Consequently, patient- and disease-specific stem cell generation was developed as a crucial step towards modern regenerative medicine and cell therapy (Takahashi et al., 2007). For instance, Maehr and colleagues in Melton's group were able to generate type-1 diabetes-specific iPS cells from patients by reprogramming their fibroblasts with three transcription factors (OCT4, SOX2, KLF4) with the potential of differentiation into insulin-producing cells that could be used to treat type-1 diabetes (Maehr et al., 2009). In 2012, John B. Gurdon and Shinya Yamanaka were jointly awarded the Nobel Prize in Physiology or Medicine for discovery of the path through which differentiated cells can be reprogrammed to become pluripotent.

Stem and progenitor cells derived from variety of tissues such as umbilical cord and umbilical cord blood, bone marrow, epithelium and white adipose tissue have been characterized, expanded and applied for transplantation procedures in which allogeneic adult stem cells give rise to committed cells such as osteocytes, adipocytes and chondrocytes as well as functional vessels in the process of vasculogenesis (Friedenstein et al., 1966, Friedenstein et al., 1974, Cairo and Wagner, 1997, Rafii and Lyden, 2003, Reinisch et al., 2009, Hofmann et al., 2012a).

4.1.2. Embryonic stem cells (ESC) and cell therapy

Pluripotent, inner blastocyst cell mass-derived cells are defined as embryonic stem cells (ESC) that can proliferate without limitation, possess the potential of self-renewal and are able to differentiate into different cell types derived from all the three germ layers (Thomson et al., 2011). These characteristics together with ESC capability to differentiate into adult cells led scientists to speculate that ESC might be useful for allogeneic cell therapeutic research as well as clinical trials for diseases such as spinal cord injury, cardiovascular disorders and diabetes (Mays et al., 2007, Newman et al., 2009).

4.1.3. Adult stem cells and cell therapy

In the recent decade, somatic stem cells have become attractive tools for cell therapy due to their proliferation and differentiation potential as well as established isolation and propagation protocols promoting a high standard of purity and functionality of the cells when applied *in vivo*.

Bone marrow aspirate is rich in hematopoietic and non-hematopoietic stem cells, endothelial progenitor cells (EPC) derived from embryonic hemangioblasts as well as mesenchymal stem and progenitor cells. The latter has been shown to participate in hematopoiesis or bone marrow regeneration (Bryder et al., 2006, Asahara and Kawamoto, 2004). It also has the potential to give rise to “pericytes”, the perivascular cells on the outer layer of vessels supporting the stability of capillaries and directing the blood flow (Bergers and Song, 2005). Hematopoietic stem cells (HSC) derived from bone marrow and circulating EPC have been shown to play a crucial role in de-novo vessel formation; they possess the

potential to differentiate into non-hematopoietic cells upon injury and to rehabilitate the immune system upon transplantation. This indicates the importance of bone marrow cellular components as a source of adult stem cells for developing therapeutic strategies and regenerative medicine (Mimeault et al., 2007).

HSC were first isolated from bone marrow (Till and Mc, 1961). All the hematopoietic cell lineages including leukocytes, erythrocytes and thrombocytes can be derived from HSC in the bone marrow niche. These tissue-specific stem cells (Spangrude et al., 1988) could divide asymmetrically, thus possess the capability of self-renewal maintaining their immature, stemness status. Research on HSC led to isolation of a novel HSC hierarchy derived from umbilical cord blood expressing CD34 and CD90 but lacking CD45RA as surface markers (Majeti et al., 2007). Earlier contradictory results, however, show that CD34+, CD7+, CD45RA+ hematopoietic progenitor cells derived from umbilical cord blood can give rise to committed functional dendritic cells upon differentiation (Canque et al., 2000).

Phenotypic characterization of undifferentiated human HSC shows diversity in CD34 (Majdic et al., 1994, Civin et al., 1984), CD38 and Kit expression but positive expression signal for Thy-1 CD90 (Murray et al., 1995), CD133 and vascular endothelial growth factor receptor-2 (VEGFR- 2) (Bryder et al., 2006, Wilson and Trumpp, 2006). However, as these surface markers are not exclusively expressed on HSC, characterization of these cells requires multi-marker assessment.

HSC and HSPC can be distinguished by different patterns of surface marker expression. HSC show a positive signal for CD150 while lack the expression of CD48 and CD244. HSPC as multi-potent hematopoietic cells that lack the self-renewal capacity, do not express CD48 and CD150 but express CD244 as surface

marker (Kiel et al., 2005). Quiescent HSC home into either the endosteal bone to be in a close interaction with osteoblasts and supporting cells (e.g. MSPC) and participate in various cellular interactions such as adhesion and migration, or into bone marrow microvasculature sinuses to interact with endothelial cells (Kiel et al., 2005, Wilson and Trumpp, 2006, Arai and Suda, 2007). A various number of cytokines and growth factors regulate the stemness or commitment state of bone marrow derived HSC and their ability to migrate from endosteal bone to the vascular surface. That facilitates their segregation into the bone marrow vasculature, and consequently, into the peripheral blood under specific physiological alerts and injuries (Mimeault and Batra, 2006, Wilson and Trumpp, 2006, Moore and Lemischka, 2006, Bhattacharya et al., 2006). However, as indicated by a previous study in mouse models, only a few number of HSC will survive to maintain the bone marrow niche and substitute the damaged cells/tissues due to their short life-time in peripheral blood (Wright et al., 2001). However, it has been shown that HSC can also reside in human sinusoidal endothelium in spleen as well as in skin, gastrointestinal tube, neural tissue, lung and liver (Mimeault and Batra, 2006, Kiel et al., 2005, Bhattacharya et al., 2006). This and a previous finding of the presence of a bone marrow-derived CXCR4⁺ subpopulation of stem cells that express specific markers for non-hematopoietic, non-quiescent stem cells led us to the concept that HSC can substitute the damaged cells and participate in the “repair process” by differentiating into functional cells (Kucia et al., 2006).

HSC are considered as tools for autologous and allogeneic transplantation in case of immune, autoimmune and hematopoietic cell lineage deficiencies by improving the immune function, thus reducing the risk of post-transplantation infections

(Bryder et al., 2006, Mimeault and Batra, 2006, Mimeault et al., 2007). In case of high-dose irradiation or chemotherapy, HSC transplantation is a treatment procedure to reconstitute the immune and hematopoietic system using HSC derived from bone marrow aspirates and/or peripheral blood of healthy donors (Ringden, 2007). Some chemical agents including granulocyte colony stimulating-factor and AMD 3100 (plerixafor) facilitate the mobilization of HSC from bone marrow into the peripheral circulation, and thus can be used to increase the number of HSC that can be isolated from peripheral blood and stored for cell therapy (Mimeault et al., 2007). Other cell isolation strategies including a combination of specific cell marker detection and immune magnetic methods to absorb and purify immature HSC from bone marrow aspirates or peripheral blood have been also established, and the ability of injected HSC to home into bone marrow and develop mature hematopoietic cell lineage has been shown (Bryder et al., 2006, Burger et al., 2003). During migration, HSC have been found to express CXCR4, the receptor for the chemokine SDF-1, and $\alpha_4\beta_1$ -integrin. In bone marrow stromal microenvironment, osteoblasts and endothelial cells release SDF-1 while CXCR4 is expressed by hematopoietic progenitor cells, thus supporting successful bone marrow engraftment *in vivo* (Burger et al., 2003, De Clercq, 2005).

HSC transplantation can be carried out using whole body irradiation or broad spectrum chemotherapeutics prior to transplantation to deplete the damaged and non-functional resident HSC within the bone marrow and create empty niches for the newly transplanted functional HSC (Small et al., 2007). However, the efficacy of this transplantation protocol compared to other methods such as CD4⁺ T cell depletion (to allow for B cell regeneration and a successful HSC engraftment) (Bhattacharya et al., 2006) has yet to be studied. The transplantation strategies

focusing on replacement of non-functional and damaged host HSC with healthy normal transplanted HSC can be applied in case of deficiencies such as autoimmune diseases, aplastic anemia, Crohn's disease, multiple myeloma, Hodgkin's and non-Hodgkin's lymphomas (Barfield et al., 2007, Sora et al., 2006, Brittan and Wright, 2002, Small et al., 2007, Ringden, 2007).

Multi-potent endothelial progenitor cells (EPC) and mesenchymal stem and progenitor cells (MSPC) are additional key tools for stem cell therapy. These cells are localized in bone marrow stroma as well as vascular inner- and outer layer and perivascular niches, and are capable of forming mature endothelial cells and mesenchymal cell lineages such as osteoblasts, chondrocytes, adipocytes and myoblasts (Bryder et al., 2006, Mimeault and Batra, 2006, Ringden, 2007). EPC derived from bone marrow, inner vascular wall, umbilical cord and umbilical cord blood as well as circulating EPC are of great importance for clinical trials as well as cell therapy procedures. Being capable of migrating through the circulation and differentiating into committed endothelial cells, EPC are crucial mediators promoting angiogenesis and de-novo vasculogenesis as well as endothelium repair in case of vascular damage (Asahara and Kawamoto, 2004, Friedrich et al., 2006, Schatteman et al., 2007, Zengin et al., 2006). It has been previously revealed that SDF-1 can be expressed by activated thrombocytes within blood flow, which is responsible for EPC recruitment to artery structures *in vivo* (Stellos and Gawaz, 2007). This shows the potential of EPC to participate in vascular repair of damaged peripheral tissues. As indicated in a previous study, isolation and transplantation of a human EPC sub-population (negative for CD34 and CD14, positive for CD133 and VEGFR2) in nude mice with damaged artery resulted in a repaired endothelial layer and wound healing caused by the injected

EPC sub-population (Friedrich et al., 2006). In addition, bone marrow-derived MSC within perivascular niche has been shown to form bone marrow stroma, bone, cartilage, adipose tissue and myocytes *in vivo* (Asahara and Kawamoto, 2004, Mimeault and Batra, 2006, Bernardo et al., 2007).

Being first isolated in 1966 from bone marrow, MSC are non-hematopoietic cells that recruit hematopoietic host cells when forming bone *in vivo* (Friedenstein, Il & Petrakova 1966, Friedenstein 1968, Friedenstein, Gorskaja & Kulagina 1976). These cells are characterized by their adherence capability (e.g. to plastic), they differentiate into all three lineages (osteogenic, chondrogenic and adipogenic), and express CD105, CD73, CD90 (cell-surface markers), but lack the expression of CD14, CD19, CD34, CD45 and HLA-DR (Dominici et al. 2006). MSC have been isolated and purified not only from bone marrow where they contribute with HSC to form the niche, but also from various tissues such as umbilical cord (Romanov, Svintsitskaya & Smirnov 2003, Sarugaser et al. 2005, Lu et al. 2006, Reinisch, Strunk 2009) and umbilical cord blood (Erices, Conget & Minguell 2000, Bieback et al. 2004, Kogler et al. 2004, Reinisch et al. 2007), white adipose tissue (Gronthos et al. 2001, Zuk et al. 2002, Kern et al. 2006), placenta (Miao et al. 2006) as well as the amniotic membrane (In 't Anker et al. 2003). The capacity of MSC to differentiate into more than three cell lineages and develop teratomas - a preserved tumor that contains normal three germ layer-tissue and organ parts - is a reason to consider them as multi-potent progenitor cells suitable for regenerative stem cell therapy.

In addition, MSC could serve as pericytes (Prockop 1997, Pittenger et al. 1999) wrapping around blood vessels to support their structure and stability (Bergers and

Song, 2005). MSC home to the outer wall of the micro-vessels and arteries in many organs including spleen, liver, kidney, lung, pancreas and brain (da Silva Meirelles et al., 2006, Zengin et al., 2006). This lead us to speculate that both bone marrow- and vascular wall-derived MSC as well as white adipose tissue-, umbilical cord blood- and amniotic fluid-derived MSC could act as cell source for stem cell therapy to treat various disorders such as osteoporosis, arthritis and vessel regeneration after injury (Bernardo et al., 2007, Chan et al., 2007, Yamada et al., 2007, Wolbank et al., 2007). MSC may also be induced to differentiate into functional neurons, corneal epithelial cells and cardiomyocytes under specific pretreatments *ex vivo* and *in vivo* that broaden the capacity of these cells in therapeutic interventions (Mimeault and Batra, 2006, Griffiths et al., 2005, Ma et al., 2006, Chang et al., 2006, Bobis et al., 2006, Mimeault et al., 2007). In the past years, MSC have been used to treat disorders such as multiple sclerosis (MS) and Crohn's disease (Uccelli, Mancardi & Chiesa 2008) as well as graft-versus-host disease (GvHD) (Le Blanc et al. 2004) due to their broad differentiation capacity and potential of hematopoietic cell recruitment (Delorme B et al., 2006, Horwitz et al., 2005, Dominici et al., 2006).

Moreover, in a previous study umbilical cord matrix stem cells derived from human umbilical cord Wharton's jelly were aimed to treat neurodegenerative disorders such as Parkinson's disease by transplantation into the brain of non-immune deficient, hemiparkinsonian rats (Weiss et al., 2006). Interestingly, phenotypic characterization of the umbilical cord matrix stem cells revealed a similar surface marker expression pattern to mesenchymal stem and progenitor cells (positive for CD10, CD13, CD29, CD44, CD90, and negative for CD14, CD33, CD56, CD31, CD34, CD45 and HLA-DR). The transplantation resulted in a significant reduction

of rotator behavior as a symptom for Parkinson's disease, thus suggesting an additional therapeutic role for umbilical cord matrix stem cells/MSPC in treating central nervous disorders (Weiss et al., 2006).

4.2. Proteomic profiling

4.2.1. Protein microarray technology

Studying the structure and function of proteins in large scale is defined as proteomics (Anderson and Anderson, 1998, Blackstock and Weir, 1999). Enzymes as a major group of functional proteins are the key mediators of signaling pathways leading to different cellular interactions including proliferation, differentiation, migration as well as metabolic alterations. In order to detect protein expression and/or to analyze protein functions in complex probes, proteomic profiling is carried out that can reveal the expression levels of specific proteins in cells, tissues or cell-containing matrices (Adam et al., 2002, Patterson, 2003). Many proteins and enzymes, however, are subjected to post-translational modifications such as ubiquitination and phosphorylation that may alter or regulate protein function (Kobe and Kemp, 1999). The latter is common in enzymes and structural proteins involved in cell signaling. Phosphorylation takes place when one or more phosphate groups are added to particular amino acids (e.g. serine and threonine) within the protein structure (Olsen et al., 2006). This modification is mediated by serine/threonine kinases. Since protein phosphorylation is a common post-translational modification that regulates protein activity, many proteomic experiments are designated to reveal phosphorylated proteins (e.g. signaling mediators) in the cellular content (Zhang et al., 2009). Compared to genomics methods, proteomics has advantages and drawbacks. The total amount of the

produced mRNAs within the cell may not be a true representative of the total correlated protein amount due to common early mRNA degradation steps. Thus proteomics may give rise to a more reliable level of protein expression (Gygi et al., 1999). Moreover, post-translational modifications may alter protein activity; for instance, some enzymes are not functional before being phosphorylated and become activated upon phosphorylation. This is not detectable in the genomics approach; however the post-translational alterations are detectable using proteomics techniques such as phosphor-proteomics and glycol-proteomics that are used to study post-translational modifications. In addition, different splicing patterns of a unique transcript may result in more than one protein that may be functional in form of complexes with other proteins or RNA molecules. These all confirm the reliability of proteomics compared to genomics when studying protein-based structures and their function. Nevertheless, any proteomics study can be complex due to variability of proteomic systems in different cells and under different conditions affecting splicing, translation and post-translational modification procedures, and thus may give rise to a different proteomic profile (Belle et al., 2006). Proteomic experiments have been shown to possess a low rank of reproducibility. Variations seem to occur from one lab to another or from one set of proteomic experiments to another using the same living system. Therefore, it is usually recommended to confirm the proteomic data with another supportive experiment (e.g. immunoblotting).

In order to identify particular proteins in a proteomic experiment, post-translational modification-specific antibodies can be used to identify the expressed proteins that have undergone the specific modification of interest (Pelech, 2004, Zhang et al., 2009).

Most of the proteins are functional more efficiently in collaboration with other proteins (e.g. in signaling pathways). Proteomic studies can unravel the unique interactive components within the signaling cascade. Various methods have been used to investigate protein-protein interactions such as immunoaffinity chromatography, mass spectrometry and protein microarray technology. Proteomic profiling has an impact in the identification of new therapeutic targets for diseases associated with protein expression levels as well as development of personalized treatment regimens which account for individual differences in specific enzymatic system. The latter is the novel forum of drug discovery that could be carried out using proteomic and genomic profiling technologies (Vaidyanathan, 2012).

Having been introduced in 1983 by Chang et.al. (Chang, 1983), protein microarray technology is one method to investigate protein expression patterns as well as tracking protein-protein interactions in living organisms (Melton, 2004). The technique is based on incubating fluorescent dye-labeled protein lysates with an antibody-coated microarray chip. Consequently, dye-labeled protein lysates are bound to the right antibody epitope and the un-bound proteins are removed using washing steps. A reaction between the protein molecules and specific antibodies results in a fluorescent signal emission that is detectable using a laser scanner (www.kinexus.ca & www.functiongenomics.org.uk).

Protein microarray technology is used because of its rapidity, sensitivity and economical design that allows using small amounts of sample and reagents (Mitchell, 2002).

4.2.2. Antibody mediated detection of signaling molecules

Most of the protein microarray experiments are carried out in form of capture array, target protein array and reverse-phase protein microarray (RPPA).

Capture array (analytical protein microarray) is a suitable method to understand the proteomic profile of cellular functions through protein activation and expression (Zhu and Snyder, 2003). In this method, a library of antibodies that might be specific in detecting a unique post-translational modification is used. The antibodies are used as capturing molecules because of their ability to bind specifically to a particular protein. The chip is loaded with a complex protein sample such as cell lysate. Target proteins are bound to the capture antibody and detected with a second specific antibody. This method can give information about protein expression levels as well as the protein's affinity and specificity to bind to specific antibodies. When comparing different cell/protein lysates, this technique can serve as a semi-quantitative method to give an insight into different levels of expression upon different pretreatments or cellular conditions (Pelech, 2004, Zhu and Snyder, 2003).

Target protein array is performed using immobilized purified protein molecules when studying the protein functionality. Protein-protein, protein-DNA and protein-small molecule (chemical inhibitor) interactions can be studied using this method. The difference between target and capture protein array is that the target protein arrays are covering the full-length of the functional protein domains, thus suitable for biomedical studies and drug discovery researches.

Reverse phase protein microarray (RPPA) is designated to study complex samples such as tissue lysates. Various tissues are being subjected to cell lysis and protein fractioning procedures and spotted on the substrate, which is then

incubated with the specific antibodies against the proteins of interest. The antibodies are detected with fluorescence assays. This method is suitable for detection of the protein in its intact and/or abnormal altered state. Malignant alterations in post-translational modification of the proteins leading to disease development could also be determined using RPPA technology (Liotta et al., 2003).

Protein array technology could be used for diagnostic and research purposes (Irish et al., 2006, Zhang et al., 2009). The antigens and antibodies or other protein molecules can be monitored in blood and serum samples. That can give an insight into the state of disease and efficacy of small molecule drugs (Schweitzer et al., 2003, Alhamdani et al., 2009).

In the research area, proteomics can be used to monitor and quantify protein expression in lysate of specific cell types, protein-protein interactions as well as enzymatic (e.g. kinases) and related receptor complexes. Despite the considerable impact of proteomic technologies in diagnostics, treatment and research, some drawbacks of this method are yet to be solved. For instance, a protein chip requires more preparation and experimental steps compared to a DNA chip. Quantification of the captured proteins in proteomic experiment and extraction of the identified proteins from the chip for further analysis are some of the current challenges (Fung et al., 2001).

Antibody mediated detection of signaling molecules can be used in biomedical research, where signaling events related to a specific cellular function is studied in order to understand their linkage to normal and disorder-related situations (Pelech, 2004, Irish et al., 2006, Liotta et al., 2003). This experiment reveals the activated

(e.g. phosphorylated) proteins involved in various signaling pathways and quantifies the level of the expression of signaling proteins necessary for mapping the cellular signaling system (Pelech, 2004). The role of the signaling pathways such as focal adhesion, MAPK, HER2(ERB), mTOR, EGF, TGF- β , VEGF, JAK-STAT, Wnt and death-associated signaling pathways in cell migration, proliferation, adherence and differentiation could be investigated based on the expression level of the crucial proteins involved in signaling cascades of importance. Functional protein-protein interaction involved in EGF signaling has been studied using mass spectrometry-based proteomics method (Blagoev et al., 2003). This study revealed that in EGF signaling cascade, many signaling molecules including histon H3 and glycosylphosphatidylinositol-bound CD59 form complexes with plectin and cytokeratin networks to emit the signal. Moreover, cytoskeletal keratin 7, 8, 17 and 18 were shown to be in collaboration with EGFR complexes in the EGF signaling processes (Blagoev et al., 2003).

Since alterations in signaling events have been shown to allow for a higher rate of proliferation and survival amongst carcinogenic cells, single cell proteomics is a promising method for mapping normal and cancer cell signaling networks (Irish et al., 2006). A previous study on signaling networks of normal and cancer cells revealed that up regulation of cellular ligands such as PDGFR, EGFR and ERBB2 in cancer cells compared to that of normal cells results in a higher expression of intracellular molecules such as STAT 3/5, ERK, PI3K and AKT that conduct the cells to a more proliferative state. Down regulation of tumor necrosis factor proteins, I κ B and NF- κ B as well as p53 signaling cascade increases the evasion of cancer cells from being trapped by the immune system. Moreover, up regulation of WNT1 and EGFR receptor results in higher expression of β -catenin and Erk

that facilitate cellular invasion to the normal tissues through metastasis. The latter can be increased in the cell via up regulation of VEGFR1 receptor, thus sustaining angiogenesis capacity particularly in solid tumors (Irish et al., 2006). Unraveling the signaling profile of the cancer cells can help to create novel therapeutic strategies for blocking the crucial signaling molecules, druggable by chemical blockers and/or specific siRNA.

4.3. Vascularization

4.3.1. Angiogenesis and Vasculogenesis: Definitions and Differences

In the living organisms, two physiological mechanisms are responsible for blood vessel formation and maintenance. The physiological phenomenon that occurs during growth and development through which new blood vessels and capillaries are sprouting from the pre-existing ones is defined as angiogenesis (Risau and Flamme, 1995). The term angiogenesis was first used to describe the sprouting of endothelial cells from already existing vasculature structures. Later, that was spread to the development and remodeling of the primitive capillary structures to a more complex vascular network (Carmeliet, 2000). New vessels in the adults form through angiogenesis. Since it occurs also during tumor growth and transition from benign to malignant state, many anti-cancer drugs are designated to block angiogenesis (Griffioen and Molema, 2000).

During embryo development, however, the first vessels form through vasculogenesis (*de novo* vessel formation) from mesodermal derived hemangioblasts (Patan, 2000). Hemangioblast is a common progenitor for

endothelial and hematopoietic cells. The inner cell mass of the hemangioblast in yolk sac gives rise to hematopoietic precursors and the outer cell layer forms the endothelial cells. Thus, the first blood vessels in a developing embryo are generated through vasculogenesis and the vessel networks are expanded and remodeled through angiogenesis processes (Risau and Flamme, 1995, Flamme et al., 1997, Patan, 2000). In the process of vascularization, mesodermal-derived hemangioblasts differentiate in situ to generate the vessels; however, angiogenesis occurs upon endothelial cell proliferation and migration resulting in endothelial tubular network formation. In addition, during angiogenesis, intussusceptive microvascular growth (IMG) happens to generate inter-vascular tissue structures (ITSs) inside the vessel lumens or to create loops in the wall of large vessels, all resulting in vascular network expansion. Molecular components that are involved in the regulation of cellular commitment during vessel formation include VEGFR2, bFGF and TGF- β receptor 1 and 2 (Wang and Keiser, 1998).

During vascular maturation, mural cells also known as pericytes prohibit endothelial cell proliferation and migration, thus stabilize vessel structure and stimulate the development of extracellular matrix (ECM) to keep the endothelium-lined vessels from reaping apart (Carmeliet, 2000, Bergers and Song, 2005). Many molecular pathways have been shown to modulate vessel maturation. VEGFR1/2 is responsible for up regulation of PDGF- β to recruit mural cells and pericytes and provide stability to the vessels. PDGFB/PDGFR- β and Ang2/Tie2 allow for cellular proliferation and facilitate mural cell interaction with the endothelial cell line. The latter also provides vascular polarity and contributes to ECM development (Jain, 2003b).

4.3.2. Neo-Vasculogenesis: Role of Adult Stem Cells in Vessel Formation

Neo-vasculogenesis is regarded as a mechanism causing new vessel formation that may occur after birth or during tissue injuries to regenerate vascular structures. Revascularization of damaged tissues to restore their function is crucial in the healing process and requires recruitment of endothelial cells and proliferation, migration and differentiation of endothelial cell progenitors to form the endothelial cell lined-vessels prior to stabilizing and vessel maturation steps. Endothelial progenitors have already been isolated and characterized using different methods (Prater et al. 2007, Hirschi, Ingram & Yoder 2008, Critser, Yoder 2010, Strunk 2011). As a part of the cellular circulating fraction, endothelial progenitor cells were isolated from peripheral blood and used for revascularization purposes. The CD34 positive mononuclear cells (MNC) were isolated from peripheral blood and cultured on fibronectin-coated surface *ex vivo* to achieve the non-adherent, spindle-shaped endothelial progenitor cells (EPC). These cells were further characterized with respect to surface marker expression and were shown to express typical endothelial cell markers such as CD34, CD31, VEGFR2, and Tie-2, but also CD14, CD45, and CD115, which are known as hematopoietic cell markers. Applying CD34-positive fraction to ischemia mouse models unraveled the contribution of these cells to vascular regeneration (Asahara et al. 1997). Later in a similar set of experiments, colonies were isolated from the non-adherent phase of MNC culture that expressed CD31, CD105, CD144, CD146, von Willebrand factor (vWF), and VEGF-receptor 2 (VEGF-R2) as typical endothelial cell characteristics, thereafter named “colony forming unit endothelial cells (CFU-EC)” (Hill et al. 2003). Phenotypical characterization of these cells, however, unraveled

the existence of a hematopoietic fraction (Case et al. 2007, Timmermans et al. 2007, Yoder et al. 2007). Moreover, CFU-EC were not formed in the absence of CD14 positive monocytes or CD2 positive T cells suggesting a role for these immune cells to release angiogenic growth factors and hence supporting tubular network formation *in vitro* as reported previously (Rohde et al. 2007). The CFU are hence no longer considered as endothelial progenitor cells and were re-termed as CFU-Hill (Hirschi, Ingram & Yoder 2008), with reference to the authors of this assay protocol (Hill et al., 2003). Culturing MNC using endothelial growth medium (EGM-2) enriched with EGF, VEGF, IGF, bFGF, cortisone, ascorbic acid and fetal bovine serum (FBS) (Vasa et al. 2001a, Vasa et al. 2001b, Vasa et al. 2001c) lead to development of adherent cells named as “circulating angiogenic cells” (CAC). These colonies possess low proliferation capacity and express some hematopoietic cell markers like CD14, CD45, and CD115: therefore, they cannot be considered as endothelial progenitor cells as they show common characteristics for endothelial cells and HSPC (Yoder et al. 2007, Rohde et al. 2007). Isolation and characterization of endothelial colony forming cells (ECFC) was performed using MNC cultures on coated plates and by purifying the adherent cells that possess the morphological characteristics of endothelial cells as well as specific endothelial surface markers like CD31, CD105, CD144, CD146, vWF, and VEGF-R2, but lack the hematopoietic surface markers such as CD45, CD14 and CD115 (Ingram et al. 2004, Ingram et al. 2005, Yoder et al. 2007). ECFC were further characterized based on their proliferation capacity (Ingram et al. 2004, Ingram et al. 2005) as well as ability to develop functional and stable vascular structures when transplanted in a mouse model subcutaneously. Co-transplantation of ECFC with supportive stromal cells such as MSPC also allows

for a robust vascularization at an early time point (approximately after two weeks) (Au et al. 2008, Melero-Martin et al. 2008, Reinisch et al. 2009, Traktuev et al. 2009, Koike et al. 2004). MSPC serve as pericytes, those perivascular cells that are localized on the outer wall of the vessels and provide stability and hemodynamics to blood vasculatures (Bergers and Song, 2005).

A protocol for isolation and serum-free propagation of ECFC from peripheral blood and umbilical cord blood samples was established (Reinisch et al. 2009, Hofmann, Reinisch & Strunk 2009, Hofmann, Reinisch & Strunk 2012) giving rise to a highly proliferative, genomically stable ECFC population with specific ECFC surface marker profile. The isolated and expanded ECFC showed a robust potential for vessel formation *in vivo* as well as endothelial network formation *ex vivo*. Co-transplantation of these ECFC colonies with bone marrow-derived MSPC using extracellular matrix (*in vitro* angiogenesis assay kit, Millipore) gave rise to stable functional vessels *in vivo* one to two weeks post implantation (Reinisch et al. 2009, Hofmann, Reinisch & Strunk 2009, Hofmann, Reinisch & Strunk 2012). A recent study revealed the invasion of host vasculature into the implanted extracellular matrix (ECM) *in vivo* when the injected endothelial cells and MSPC within the ECM were forming capillary like structures. This led to vascular network expansion and connection to the host blood circulation as well as vascular maturation by recruiting host pericytes to support vessel stability (Cheng et al. 2011). Compared to previous studies, the time point for observing micro-vessels within the ECM plugs was delayed in this study, which might be due to differences in the transplantation potentials of endothelial and mesenchymal progenitor cells (Melero-Martin et al. 2008, Traktuev et al. 2009, Reinisch et al. 2009, Hofmann et al. 2012). Further investigations are required to unravel the precise mechanism of

neo-vasculogenesis and exact function of mesenchymal progenitor cells during tissue revascularization.

4.3.3. Molecular Regulation of Neo-Vasculogenesis

Molecular mediators governing vessel formation and maturation can be grouped into three categories: I) molecules that mediate mural-endothelial and endothelial-endothelial cell interaction; II) molecules involved in cell-matrix interaction; and III) signaling pathways, ligands and receptors (Jain, 2003b).

I) Endothelial cells are connected through functional proteins such as vascular endothelial cadherins (VE-cadherins) and claudins to form the inner layer of micro-vessels on an endothelial cell-lined basement. VE-cadherin is an important mediator for endothelial-endothelial cell junctions, whereas neural cadherin (N-cadherin) mediates mostly the EC-mural cell junction in the process of vessel maturation (Dejana et al., 2009). The cellular communication between supporting stromal cells can also be promoted through N-cadherin molecules. Gap junction component connexins (Cx37, Cx40 and Cx43) promote communication between endothelial cells and perivascular cells. Endothelial/leukocyte surface marker CD31 has a role in angiogenesis processes as well as in providing permeability to endothelial-endothelial cell junctions (Carmeliet and Jain, 2011). Tight junction molecules such as occludin, claudin, zonula occludens molecules (ZO-1, 2, 3) as well as the endothelial surface molecule CD148 responsible for forming tight junctions in blood brain barrier (BBB) and retinal micro-vessels regulate endothelial-mural cell interaction in these tissues. Mechanical forces such as growth and expansion of the vascular network serves as a stimulator to many of

the cellular and molecular interactions in the process of angiogenesis and vessel maturation (Jain, 2003b). How the mechanical parameters regulate the cellular junctions resulting in concise vessel formation pattern is yet to be studied.

II) The matrix serves as a pool enriched in different growth factors and enzymes contributing to vessel formation and development. Studies on integrins have provided information about the effect of various extracellular matrix components on the survival and migration of endothelial cells. It has been previously shown that receptor $\alpha 5 \beta 1$ of fibronectin, collagen I as well as collagen receptors $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$ are involved in promoting angiogenesis and inhibiting endothelial cell apoptosis, whereas thrombospondin 1 and 2 (Tsp1 and Tsp2) have been shown to block vessel formation through integrins and proteases (Lawler, 2000). Paradoxically, some studies reported that suppressing the genes encoding $\alpha \beta 3$ and $\alpha \beta 5$ (integrins binding fibronectin, fibrinogen, endostatin and von Willebrand factor) does not inhibit vessel formation (Hynes, 2002b, Stupack and Cheresch, 2002). Proteases released from endothelial cell and mural cells cleave the matrix and plasma proteins into components that mediate endothelial cell apoptosis (e.g. angiostatin that results from plasminogen cleavage) whereas protease inhibitors in the matrix sustain vessel stability by preventing cleavage of matrix components (Jain, 2003b). Further studies are required to unravel the precise role of cell-matrix interactions in vessel formation and stability processes.

4.3.4. Signaling Pathways Involved in Vessel Formation

In a healthy individual, endothelial progenitor cells are protected by molecular signaling mediators such as VEGF, NOTCH, fibroblast growth factor and

angiopoietin-1 (ANG-1) and thus may remain quiescent for a long time. VEGF family mediators such as VEGF-A play an important role not only during vasculogenesis initiation by signaling through VEGF receptor-2 (FLK-1), but also during vessel maturation that ultimately results in arteriogenesis (Ferrara, 2009, Nagy et al., 2007). Neuroligins (NRP1 and NRP2) serve as independent VEGF receptors that increase VEGFR-2 activity. VEGFR-2 deficiency and severe decrease in VEGF expression hampers vascular development (Carmeliet, 2003). Mutations and polymorphism in the gene encoding VEGFR2 result in vascular tumor formation as well as abnormal and/or pathological angiogenesis patterns (Jain et al., 2009).

In perfused vessels, an endothelial tip cell is activated by VEGF-C, a ligand for VEGFR-2 and VEGFR-3, to direct the vessel growth in the presence of VEGF receptors and NOTCH ligands like DLL4 and JAGGED1. Up regulation of DLL4 and activation of NOTCH signaling in stalk cells result in VEGFR-2 down regulation and cause the stalk cell to be less responsive to VEGF, thus ensuring a guiding role for the tip cell in the process of vascular development (Phng and Gerhardt, 2009, Tvorogov et al., 2010). Paracrine VEGF secreted from cancer cells, myeloid cells or pericytes promote vessel branching whereas vasculature homeostasis is sustained by autocrine VEGF secretion (Stockmann et al., 2008, Lee et al., 2007). VEGFR-3 signaling plays a crucial role in prenatal venous-derived angiogenesis as well as lymphatic vessel remodeling from the pre-existing ones. A study in Zebra fish revealed that the vessel formation through sprouting of venous endothelial cells is prohibited by VEGF-2 whereas VEGF-3 facilitates the sprouting of the venous-fated endothelial cells resulting in vein development (Herbert et al., 2009).

VEGF-B is another VEGF family member that shows angiogenic potential only in some tissues such as heart facilitating cardiac vessel development without additional effects on permeability of the vessels (Bry et al., 2010).

VEGFR-1 – also known as FLT-1 – possesses weak tyrosine kinase activity but can trap extra amounts of free VEGF to maintain VEGFR-2 activity to a normal state. VEGFR-1 blockade and/or deficiency lead to vessel overgrowth (Fischer et al., 2008). In contrast, endothelial and stromal VEGFR-1 signaling cascade has been shown to promote pathological vessel formation by providing a higher growth rate for VEGFR-1 positive cancer cells and by increasing matrix metalloproteinase 9 (MMP-9) expression in endothelial cells at metastatic state (Schwartz et al., 2010).

PDGF signaling has been shown to play role in angiogenesis. Endothelial cells release PDGF-B to stabilize the endothelial cell-lined structure. PDGF-B attracts pericytes that express PDGF receptor β (Hellberg et al., 2010). A study on PDGF-B knockout mice revealed that these animals develop more of fragile and leaky vessels as a result of deficiency in pericyte adherence. PDGFR- β hypomorph mice, however, fail to attract sufficient numbers of pericytes around brain capillaries resulting in blood brain barrier (BBB) deficiency as well as neurodegenerative disorders caused by toxin penetration (Quaegebeur et al., 2010). PDGFR blockade decreases pericyte attachment and keeps the vessels from maturation thus abolishing tumor growth (Bergers et al., 2003), while abnormal tumor vessel growth and decrease in size of tumors have been found in pericyte- and proteoglycan NG2-deficient mice (McCarty et al., 2007).

The importance of TGF- β signaling in vessel formation was revealed upon a study indicating that mutations in endoglin coding gene *ENG* or activin receptors like

kinase receptor of TGF- β family might cause hemorrhagic telangiectasia, a hereditary vascular malformation disease (ten Dijke and Arthur, 2007). Further studies confirmed that inhibition of TGF- β receptors ALK-1, ALK-5 (also known as TGFR-1) and ENG (TGFR-2) led to impaired arteriovenous formation as has been detected in hemorrhagic telangiectasia patients (Pardali et al., 2010, ten Dijke and Arthur, 2007). As the inhibitory role of ENG and ALK-1 in tumor angiogenesis has been worked through, several TGF- β inhibitors are in early-stage clinical trials.

FGF signaling plays a role in vessel formation through bFGF that was amongst the first angiogenic mediators that were characterized. bFGF and FGF1 possess angiogenic and arteriogenic properties, whereas FGF9 promotes vessel formation during bone regeneration. In cardiac cells, FGF signaling stimulates the secretion of hedgehog, ANG-2 and VEGF-B, thus give rise to vascularization. FGF is essential for the integration of vessels, whereas FGFR signaling blockade leads to vessel regression (Bergers and Hanahan, 2008). Suppression of the genes encoding FGF in mice did not cause vascular deficiencies, therefore, its therapeutic properties are under question and needs further investigations (Beenken and Mohammadi, 2009).

The human ANG signaling family consists of TIE-1 and TIE-2 receptors as well as ANG-1, ANG-2 and ANG-4 ligands. ANG-1 serves as TIE-2 agonist and ANG-2 serves as ANG-1 competitive antagonist. The exact role of TIE-1 is unclear, however, some speculate that this receptor acts as a regulator for TIE-2 (Augustin et al., 2009). Mural and tumor cells both express ANG-1, whereas angiogenic tip cells are responsible for ANG-2 secretion. ANG-1 allows vessel maturation by recruiting mural cells to the outer wall of the vessels. Endothelial cells in the vessel base line release ANG-2 that serves as an antagonist to ANG-1 and TIE-2

signaling causing an increase in stromal cell detachment and vascular leakage as well as endothelial sprouting (Augustin et al., 2009). In a previous study, a deficiency in the gene encoding TIE-2 in mice resulted in vascular regression. ANG-2 molecules derived from tumors possess a role in recruiting TIE-2 expressing monocytes (TEM) thus promotes angiogenesis (De Palma et al., 2005).

It has been illustrated in the vessel-branching model that the tip cells of the vessel migrate while stalk cells proliferate. This might be a result of NOTCH signaling in this model (Phng and Gerhardt, 2009). VEGFR-2 is activated in response to VEGF and cause expression of DLL4 in the tip cells. Consequently, DLL4 activates NOTCH in the stalk cells, that suppress VEGFR-2 while up regulates VEGFR-1 resulting in less sprouting and branching but more vessel formation (Phng and Gerhardt, 2009). JAGGED1 is another NOTCH ligand that is mainly expressed by stalk cells and contributes to DLL4 in order to select the tip cell (Benedito et al., 2009). However, as NOTCH signaling itself activates its inhibitor NOTCH-regulated ankyrin protein (NRARP) over time, this signaling cascade varies in the angiogenic microenvironment (Phng et al., 2009). Although DLL4 and NOTCH signaling blockade promotes vessel formation, the created vessels are poorly perfused which thus results in hypoxia and growth inhibition in tumors (Thurston et al., 2007).

Signaling by the hedgehog family molecules including sonic hedgehog (shh) and Indian hedgehog (ihh) have a regulatory role in NOTCH expression, thus contributing to vascular structure and arterial formation in embryo (Swift and Weinstein, 2009).

It has been illustrated that endothelial cells express WNT ligands and their frizzled (FZD) receptor that control endothelial cell proliferation. As the vessel branching takes place, WNT signaling is activated by NOTCH in stalk cells (Phng et al., 2009). Suppression of some of WNT and FZD genes (*Wnt2*, *Wnt5a*, *FZD4* and *FZD5*) in mouse resulted in defective vascular structures. Also inactivation of *WNT7a* and *WNT7b* caused an impaired brain and BBB vessel formation (Dejana, 2010).

Enlargement of the vessel lumen is mediated by VE-cadherin, NRARP and FGFs. Hypoxia facilitates sprouting during angiogenesis and cause expression of numerous vasculogenesis regulating genes such as nitric oxide synthase, *VEGF* and *ANG2* (Ferrara, 2002, Nagy et al., 2002). Endothelial cells contain oxygen sensors such as prolyl hydroxylase 2 and hypoxia-inducible factor-2 α (HIF-2 α) necessary for an optimized blood circulation. Endothelial cells are connected through junction proteins such as VE-cadherin and claudin to form the inner layer of micro-vessels. On the outer layer of the newly formed vessels, however, pericytes are localized that inhibit endothelial cell proliferation as they secrete *VEGF* and *ANG-1* to rescue endothelial cells. Upon angiogenic signals like *VEGF-C*, *FGF* or chemokines produced by inflammatory or carcinogenic cells, pericytes are detached from the vessel wall and degraded by matrix metalloproteinases (MMP-2,-3 and -9) (Jain, 2003a). Claudin and VE-cadherin are also degraded in response to *VEGF* resulting in leaky, highly permeable vessels. The plasma proteins leak from these immature vessels and contribute to temporary matrix development that facilitates endothelial cell migration and proliferation in response to *VEGF* (Jain, 2003a, Carmeliet and Jain, 2011).

Ephrins play an important role as environmental guidance molecules to provide information about the vascular microenvironment and collaborating endothelial cells. In the vessel maturation processes, the vessels need to be covered with pericytes. This process is mediated by platelet-derived growth factor B (PDGF-B), sphingosine-1-phosphate (S1P1)/endothelial differentiation sphingolipid G-protein coupled receptor-1 (EDG-1), ANG1/TIE2 as well as transforming growth factor- β (TGF- β) and ephrin-B2 to form stable matured vessels (Carmeliet and Jain, 2011). PDGF-B is released by endothelial cells in response to VEGF and mediates recruitment of mural cells as well as their proliferation and migration in the process of vascular maturation (Hellstrom et al., 2001b). Down regulation of EDG1 receptor might change the endothelial cell matrix production and endothelial cell interaction with stromal mural cells, thus alter the vessel maturation. ANG1 in mural cells and ANG2 in endothelial cells are amongst the main mediators of vessel formation and so are TIE receptors 1 and 2. ANG1 possesses a role in stabilizing the micro-vessels and decrease their permeability presumably by causing mural-endothelial cell interaction. ANG2 serves as an antagonist for Ang1 in the low concentration or absence of VEGF, and therefore destabilizes vessels leading to vessel rupture (Yancopoulos et al., 2000, Loughna and Sato, 2001). In the presence of VEGF, however, ANG2 spreads up the sprouting of vascular structures (Jain, 2003a).

A low amount of FGF is essential for vascular integration while blocking FGFR in endothelial cells result in vessel regression. Aberrant FGF release may cause vessel formation within tumors that are resistant to VEGF and EGFR blocker treatments (Bergers and Hanahan, 2008).

The release of TGF- β 1 is a crucial step for ECM production and mural cell differentiation that result into vessel maturation. Previous findings on knockout mice revealed that TGF- β receptor-1 and 2 and downstream signaling molecules such as ALK1 and Smad5 are involved in initiation of vasculogenesis and vessel maturation (Chambers et al., 2003).

A better understanding of early signaling signature resulting in vessel formation and maturation is highly important for the development of therapeutic strategies in regenerative medicine and cancer therapy. The novel findings that *ex vivo* proteomic profiling can unravel the signaling mediators that are effectively expressed during neo vessel formation *in vivo* and the impact of caspase signaling to promote neo-vasculogenesis through ECFC is part of the results of this dissertation.

4.4. Signaling Pathways

Cellular activities are governed by a series of molecular events resulting in cellular crosstalk. This regulatory cell signaling implicates protein-protein interactions as the major cell process regulators as well as cell-microenvironment interactions which are necessary for differentiation, tissue homeostasis and repair (Kolch and Pitt, 2010, Gadbois et al., 1992). Aberrant cell signaling could result in cell malfunction or diseases such as cancer and diabetes (Kolch and Pitt, 2010). Understanding cell signaling through system biology approaches provides information about cellular behavior and thus can have an important role in effective therapeutic strategies as well as regenerative interventions.

Although cell signaling has been studied intensively within the single cell types, it may also happen between two different cell types as occurs when the surface embryonic cells are being attached and the embryo is implanted to the endometrial tissue mediated by β -catenin signaling molecule in the process of WNT signaling (Mohamed et al., 2005).

Many signaling pathways are regulated via protein kinases, a group of enzymes involved in protein phosphorylation resulting in functional alteration of these proteins (Mustonen and Alitalo, 1995).

4.4.1. Protein Kinase Signaling Pathways

Kinases mediate cleavage of phosphate group(s) from a nucleotide tri-phosphate molecule and covalent attachments to free hydroxyl group-containing amino acids. A major group of kinases are active on both serine and threonine (termed serine/threonine kinases), while a group of kinases act on tyrosine specifically (tyrosine kinases) (Dhanasekaran, 1998, Dhanasekaran and Premkumar Reddy, 1998). There exist also protein kinases involved in other amino acids phosphorylation, such as histidine kinases (Besant et al., 2003).

Serine/threonine protein kinases are involved in specific cellular processes such as DNA cleavage and damage as well as chemical signal transduction process like Ca^{2+} /calmodulin signaling. A well-known sub-group is mitogen activated protein (MAP) kinases participating in MAPK signaling pathway (Orton et al., 2005).

4.4.1.1. MAPK signaling pathway

MAP kinases are categorized amongst the serine/threonine protein kinase group and possess similarities to cyclin-dependent kinases (CDKs) in structure and function (Manning et al., 2002). The major role of MAP kinases consists of participating in signal transduction through receptor tyrosine kinases (RTKs) in response to extracellular stimuli such as stress (osmotic, temperature), mitogens as well as inflammatory cytokines that activate the receptor and, consequently, start downstream signaling mechanisms.

RTKs are cell-surface allosteric enzymes consisting of a single trans-membrane domain separating an intracellular kinase domain from an extracellular ligand-binding domain. Ligand binding induces receptor homo- or hetero-dimerization that is essential for activation of the tyrosine kinase and subsequent recruitment of target proteins which initiate a complex signaling cascade 1 and 2 (Franklin et al., 2004) .

MAP kinase signaling as an evolutionary conserved signaling pathway regulates crucial cellular processes including proliferation, differentiation, gene expression and survival in various cell types (Pearson et al., 2001) Aberrant regulation of mediators in this signaling pathway can give rise to different pathological manifestations such as cancer (Lemmon and Schlessinger, 2010).

The cell cycle regulatory information is mostly produced as a result of protein serine/threonine phosphorylation. S6 protein of the 40S ribosomal subunit is involved in cell cycle processing. This protein, later referred to as RSK, was shown to be regulated by serine/threonine phosphorylation (Maller et al., 1991, Erikson, 1991). Soon after, extracellular signal regulated kinase (ERK) was identified as a mitogen activated protein kinase that has a role in reactivation of RSK after being

dephosphorylated (Sturgill et al., 1988). Knowing that MAP kinases need tyrosine phosphorylation for becoming active lead the researchers to speculate a switching role for MAP kinase for tyrosine-to-serine/threonine phosphorylation (Anderson et al., 1990). Nevertheless, like any other signaling pathway regulating cell cycle and proliferation, MAP kinase signaling was expected to be a complex pathway with numerous checkpoints and inherent regulatory processes (Blenis, 1993). The key regulators in this signaling pathway consist of the ERK family, the p38 kinase family and the c-Jun *N*-terminal kinase (JNK) family. The MAPK cascade is formed by three main components known as MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK. Moreover, some regulatory mediators and scaffold proteins can activate and influence this pathway. For instance, the transmembrane RTKs, integrins and G protein-coupled receptors (GPCR) at upstream of MAPKs can start a downstream signaling upon activation through phosphorylation, that leads to recruitment of MAPK mediators to the signaling zone. It was also shown that epidermal growth factor receptor (EGFR) as one ligand binds to transmembrane monomeric RTKs and dimerizes the receptor which promotes transphosphorylation of the receptor proteins (Zhou et al., 1993, Yarden and Schlessinger, 1987). Long ago it was revealed that two signaling components, growth factor receptor-bound protein 2 (Grb2) and son of sevenless homologue (Sos) are responsible for linking phosphorylated EGFR and Ras localized upstream of MAPK pathway start point (Buday and Downward, 1993, Egan et al., 1993). Grb2 binds through its Src homology (SH) domain to EGFR (phosphorylated tyrosine 1068 of EGFR (SH2 domain)) and proline-enriched region in Sos (SH3 domain). Sos requires GTP as a crucial component for Ras-mediated activation of MAPK pathway. This has been confirmed through the

inability of Ras-negative mutants to promote the downstream signaling cascade (Nakielnny et al., 1992). Ras's active form (attached to GTP) binds to Ras-binding domain (RBD) of Raf that is a serine/threonine kinase facilitating Raf entrance to the plasma membrane that enrolls ERK signaling (Rossomando et al., 1992, Seger et al., 1992, Robbins et al., 1992).

Having been introduced as p21^{src} in early publications, rat sarcoma (RAS) genes were identified in acute transforming retroviruses serving as transduced oncogenes (Kirsten and Mayer, 1969, Harvey, 1964). Ras superfamily oncoproteins consist of GTPases and associated effector/regulator molecules that participate in signal transduction pathways (Colicelli, 2004). RAS proteins operate through a common mechanism that is associated with conformational changes regulated by nucleotides (Vetter and Wittinghofer, 2001). When bound to GTP, they possess a surface that promotes binding of downstream effector proteins with high affinity, e.g. HRAS-GTP displays a K_d (as a dissociation constant) for the protein kinase RAF1 (Herrmann et al., 1996).

However, the conformation of the high-affinity effector-binding RAS is unstable as GTP is hydrolyzed and γ -phosphate is released, resulting in re-orientation of effector binding site molecules and reduction of affinity that leads to the release of effector proteins and attenuation of downstream signaling (Colicelli, 2004).

On the other hand, RAS subfamily members are mostly small proteins that consist of 183 to 340 amino acids and display a high conservation status within the G1, G3, G4, and G5 boxes. These small proteins do not have any functional motifs other than those defining their relatedness to RAS. RAS subfamily proteins are most likely located in the plasma membrane. Post-translational modifications including C-terminal prenylation and cysteine farnesylation occur in the

endoplasmic reticulum (ER) before these proteins are transported into the plasma membrane (Choy et al., 1999).

Some RAS subfamily members possess fatty acid acylation signals. In particular, HRAS, NRAS, ERAS, RRAS1 and RAP2A, RAP2B, and RAP2C contain palmitoylated cysteine residues and, therefore, they need to be transited via the Golgi apparatus (Apolloni et al., 2000). Some studies suggest that RAS proteins act as signal transducers in ER-Golgi as well as cellular endomembrane (Roy et al., 2002, Jiang and Sorkin, 2002, Chiu et al., 2002). Although it has been revealed that RAS subfamily protein HRAS undergoes serine phosphorylation (Ballester et al., 1987) and nitrosylation (Mallis et al., 2001, Baker et al., 2000, Williams et al., 2003) and RRAS1 may undergo tyrosine phosphorylation (Zou et al., 1999, Zou et al., 2002); the functions related to these post translational modifications are yet to be studied.

Mutated forms of RAS proteins such as HRAS (H-RAS), KRAS (K-RAS) and NRAS (N-RAS) were isolated from human tumor cells by means of transfection assays (Perucho et al., 1981, Krontiris and Cooper, 1981, Shih et al., 1981). High levels of K-RAS mutation have been identified in lung cancer (Mitsuuchi and Testa, 2002), colon adenocarcinoma (Grady and Markowitz, 2002) as well as pancreatic carcinoma (Jaffee et al., 2002) making it a common human onco-protein (Jaffee et al., 2002, Grady and Markowitz, 2002, Mitsuuchi and Testa, 2002). HRAS- or NRAS-activating mutations have also been detected in some tumors. Many malignant poorly differentiated or undifferentiated thyroid tumors show a mutation in K-RAS, H-RAS, or N-RAS (Garcia-Rostan et al., 2003). Additional to mutational activation, overexpression or amplification of RAS genes

have been detected in some tumors (Vageli et al., 1996). For instance, RAS-activating mutations do not happen frequently in case of breast cancer, however, RAS activity increases as a response to elevated signaling from the receptor tyrosine kinase Her2 (ERBB2) (von Lintig et al., 2000). Other mechanisms resulting in RAS overexpression in tumors include the impairment of genes encoding negative regulators such as NF1, a GAP for RAS, in neuronal cancers (Xu et al., 1990b, Xu et al., 1990a, Martin et al., 1990, Ballester et al., 1990) and over activation of positive regulators such as SOS1, a GEF for RAS, in renal tumors (Shinohara et al., 1997). These all suggest a major role for RAS in cellular transformation.

4.4.1.2. ERK Signaling Pathway

ERK signaling is one of the most well-known MAPK pathways amongst mammals that controls cell proliferation and is deregulated in many types of carcinogenic cells. ERK1 and ERK2 as initiator components of this module are activated upon MEK phosphorylation promoted by Raf. In carcinogenic cells, ERK receptor tyrosin kinase, Ras and Raf mutations have been detected. It was also shown that aberrant overexpression or inhibition of Ras/Raf nuclear transcription factors such as *myc* can lead to cancer cell development (Giancotti and Ruoslahti, 1999). As mentioned previously, the abundance of activating mutations centered around the Ras-Raf axis suggests that this is a regulatory hotspot of this pathway (Orton et al., 2005). Moreover, mathematical and computational modeling predicts that Ras and Raf activation are crucial points of regulation that affect the overall activation profile (Orton et al., 2005). In addition, a high frequency of B-Raf and Ras

mutations happening within various cancer cells suggests a more effective signaling role for Raf in human cancer development (Petti et al., 2006).

Ras-GTPases are effector molecules in MAPK/ERK signaling pathways that regulate numerous downstream events. The mutations in *K-Ras* and *N-Ras* genes specifically in codons 12, 13 and 61, have been detected in various cancer cell types (Downward, 2003, Sebolt-Leopold and Herrera, 2004). The mutations lead to non-efficient GTP hydrolysis and consequently, conformation alterations that promote Ras oncogen activation. Activation and trafficking of high number of Ras oncogenes has been shown to play an important role in signaling events that lead to tumor formation; however, further investigations are required to better understand the mechanisms through which Ras-oncogene overexpression affects cellular proliferation and function (Dhillon et al., 2007).

Other effectors of Ras consist of Raf kinases having three isoforms in mammalian cells (Raf-1, B-Raf and A-Raf). These isoforms differ by their regulation, tissue localization and potential to activate MEK (Wellbrock et al., 2004). Raf kinases bind to Ras and become activated upon phosphorylation and other molecular interactions. This process activates the kinase domain by hampering the kinase auto-inhibition process. Raf-1 activation requires a complex module of phosphorylation changes as well as dephosphorylation of S259 (inhibitory site) and phosphorylation of S338 (an important activating site), which are all required for maximal activation of Raf. These inhibition and activation sites are highly conserved in the A-Raf isoform and have been shown to follow similar patterns to that of Raf-1. However, B-Raf can be activated by Ras alone, whereas Raf-1 needs other signaling components for activation. Which Raf isoform is responsible

for activating ERK pathway, and whether it differs in different cell types has remained to be studied (Galabova-Kovacs et al., 2006).

Raf activates MEK1 and MEK2 upon serine phosphorylation. While B-Raf and Raf-1 are potent MEK kinases, A-Raf possesses weak MEK kinase activity and only activates MEK1. There are two distinct MEK binding regions on the Raf-1 protein and phosphorylation of these regions significantly increases the binding of MEK. Interestingly, these two MEK binding sites possess a higher negative charge in B-Raf that may explain the better and more efficient binding of MEK by B-Raf (Marais et al., 1998, Emuss et al., 2005). In addition, scaffolds such as kinase suppressor of Ras (KSR) are shown to affect the ability of Raf to activate MEK efficiently (Morrison and Davis, 2003).

Activated ERKs phosphorylate many nuclear and cytoplasmic components such as kinases, transcription factors as well as cytoskeleton proteins. Depending on which cell type it is getting activated in, ERK signaling pathway can regulate processes such as proliferation, differentiation, migration, angiogenesis and chromatin remodeling (Dunn et al., 2005, Yoon and Seger, 2006). This diverse potential might be due to differences in the expression level of ERK as well as its localization within the cellular components in various cell types (Murphy and Blenis, 2006). It has been shown in a previous study that alterations in expression and phosphorylation pattern of some gene products such as Fos, Jun and Myc may serve as a sensor for ERK signaling pathway dynamics and the frequency of expression of Erk (Murphy et al., 2002). A higher level of ERK signaling leads to an overexpression of CDK inhibitor protein (e.g. P21 and P27) resulting in cell cycle arrest. On the other hand, the affinity to proliferate leads particular cancer

cells to increase Rho signaling to counteract ERK-mediated induction of CDK inhibitor proteins resulting in higher cell proliferation (Mirza et al., 2004).

ERK has been shown to play an important role in cancer, thus it has been targeted for drug discovery purposes with Ras, Raf and MEK as its main effectors. Raf kinase inhibitor sorafenib (BAY43-9006) is an example of an anti-tumor drug that is used in melanomas where B-Raf mutations are the major tumor inducers (Downward, 2003, Eisen et al., 2006). The clinical trials testing this and other Raf kinases and/or druggable Ras and MEK kinases as cancer treatment drugs are under progress (Gollob, 2005, Eisen et al., 2006, Dhillon et al., 2007).

4.4.1.3. ERBB Signaling Pathway

As described previously cellular information flow in form of signaling cascades from the extracellular environment (ECE) through cellular cytoplasm and compartments is the hallmark of functional biological machinery.

These signaling cascades are mediated mostly by receptor tyrosine kinases located on the cell surface contributing to determination of cellular processes like proliferation, migration, differentiation or death. A well-known family of receptor tyrosine kinases is referred to as ErbB family (named due to their homology to the erythroblastoma viral gene product, *v-erbB*) and consists of four receptors: ERBB-1, (also known as epidermal growth factor receptor (EGFR)), ERBB-2 (HER2 or Neu), ERBB-3 and ERBB-4 reside on the surface of the cell. Because of an extended receptor-receptor interaction, this signaling cascade possesses the capacity of conveying enormous variety of biological messages (Alroy and Yarden, 1997). There exist 13 polypeptides as extra cellular ligands including neuregulins

families (also known as Neu differentiation factors, NDFs, or heregulins) for ErbB-1 receptor. Each ligand has been shown to stabilize the receptor dimers with certain affinity and different preferences. In addition, it has been shown that the receptor dimers have different tyrosine autophosphorylation regions that are binding sites for specific SH2-protein units, thus each receptor dimer emits signals through a specific signaling module upon induction by the ligand. In the ERBB network, ERBB-2 promotes stable dimerization; however, no ligand has been distinguished for it and it fails to interact with a growth-factor ligand (Karunakaran et al., 1996, Klapper et al., 1999). ERBB-3 possesses the ability to bind to SH2-containing proteins; however, no kinase activity has been assigned to this ERBB receptor (Pinkas-Kramarski et al., 1996, Guy et al., 1994). These are important defining features of the ERBB family making ERBB network distinct from other receptor tyrosine kinase-mediated signaling pathways. It should be indicated, though, that ERBB-2 and ERBB-3 are still able to generate effective cellular signals and participate in the signaling cascade (Citri and Yarden, 2006).

ERBB1 has the potential to dock to diverse ligands and form homodimers, as well as three functional heterodimers. ERBB1 contains numerous tyrosine-based motifs that, upon phosphorylation, have the potential to bind to signal inducers such as the adaptor proteins growth-factor-receptor bound-2 (GRB2) and Src-homology-2 (Shc) (Schulze et al., 2005). These signal transducers recruit Ras and activate MAPK pathway. Signal transducer/activator of transcription-5 (STAT5) is another example of a direct substrate for ERBB1. No direct recognition site for phosphatidylinositol 3-kinase (PI3K) on ERBB1 has been recognized; therefore, ERBB1 lacks the ability to activate PI3K-AKT/protein kinase B (PKB) cascade.

Nevertheless it can couple to Ras–MAPK and/or Ras–PI3K–AKT/PKB pathways to emit signals (Franklin et al., 2004).

A previous study on *ErbB1* knockout mice unraveled a major role for ERBB1 receptor in epithelial cell formation and development. These knockout mice died either at mid-gestation or postnatal day 20 depending on their genetic background (Miettinen et al., 1995) suffering from severe brain deficiencies as well as impaired proliferation, migration and differentiation of epithelial cells in organs such as lung, and intestine (Threadgill et al., 1995, Miettinen et al., 1995).

Unlike ERBB-1, ERBB2 has been considered as a non-autonomous amplifier for the ERBB network. It does not dock to the EGF ligands, rather works as a heterodimeric unit of the other three ERBB receptors (Tzahar et al., 1996). A handful number of phosphotyrosine-binding proteins bind to heterodimer ERBB2 that due to its low speed endocytosis property, frequently gets back to the surface of the cell (Baulida et al., 1996).

As a non-autonomous, kinase-defective ERBB receptor, ERBB3 undergoes tyrosine phosphorylation through its cytoplasmic domain and recruits PI3K and Shc to separated regions to protect them from ligand induced degradation (Waterman et al., 1999). ERBB3 causes a robust activation of PI3K, particularly when it is docked to ERBB2 as its preferred heterodimeric unit (Wallasch et al., 1995).

ERBB4 is an autonomous receptor possessing similar recognition and signaling patterns to those of ERBB1 such as the linkage ability to wide range of ligands (e.g. betacellulin and heparin-binding ligand (HB-EGF)). Use of mRNA splicing methods has revealed numerous isoforms for ERBB4 such as juxtamembrane

variant (JM-a) with a 23 amino acid insertion site allowing membrane proteinases to cleave it and promote ectodomain shedding (Rio et al., 2000).

ERBB4 and ERBB1 both recruit GRB2, Shc and STAT5, however, it has been reported that CYT-1, a ERBB4 isoform, can recruit and activate PI3K as well (Elenius et al., 1999).

ERBB signaling pathway is associated with various pathological circumstances. The involvement of ERBB receptor in oncogenic transformation has been studied and the effect has been investigated both *in vitro* and *in vivo* (Olayioye et al., 2000). It has been reported in a previous study that co-expression of ERBB1 and ERBB2 in fibroblasts (NIH 3T3) has increased the influence of EGF on the phenotype of oncogenic transformation (Kokai et al., 1989). In other studies, it was revealed that ERBB3 up regulation augments ERBB2-mediated transformation giving rise to cancer development in NIH 3T3 cells. Furthermore, it has been previously shown that fibroblasts can be transformed through NRG-1 inducer component by ERBB4 and the whole procedure requires co-expression of either ERBB1 or ERBB2 (Alimandi et al., 1995, Cohen et al., 1996b, Cohen et al., 1996a, Zhang et al., 1996). The cells having high potential of ERBB expression are shown to be more susceptible to oncogenic transformation presumably due to diversity of signaling modules that can be affected by ERBB receptor combination. Such contributions have potential to deregulate cell proliferation machinery associated with tumor development and progression. Up regulation of ERBB2 has been detected in ovarian and breast cancer cells and has become an important characteristic for poor prognosis (Slamon et al., 1989).

The co-expression of ERBB2 and ERBB4 has been observed in $\geq 50\%$ medulloblastoma in children and overexpression of NRG-1 (ERBB4 ligand) has

been detected in these tumors. Moreover, ERBB3 expression has been found in several cancer cells having high expression levels of ERBB2 such as breast, bladder and melanoma tumors (Rajkumar et al., 1996, Bodey et al., 1997, Siegel et al., 1999).

In the recent years, technological approaches have been made to identify and characterize ERBB network mediators due to evidence indicating the involvement of aberrant ERBB signaling not only in several types of cancer (e.g. breast and lung cancer) but also in psoriasis and atherosclerosis (Citri and Yarden, 2006). cDNA and proteomic array technologies have decoded various ERBB signaling components with druggability properties leading to ongoing drug discovery (Kitano, 2003). Trastuzumab (targets ERBB2) has shown promising effects in breast cancer treatment and cetuximab (targets ERBB1) is currently used for treatment of colorectal cancer. Other therapeutic strategies are designated to target ERBB2 heterodimerization or use combination of antibodies to cause receptor degradation (Spiridon et al., 2002, Friedman et al., 2005). Gefitinib and erlotinib, two small molecules that block the nucleotide-binding site of ERBB proteins, have been shown to effectively treat tumors expressing hyperactive ERBB1 mutants (Lynch et al., 2004). A dual specificity inhibitor usage in case of severe, complex cancers would be profitable such as lapatinib and EKB-569 that target both ERBB1 and -2 (Xia et al., 2002).

Heat shock proteins (e.g.HSP90) are amongst the main components of ERBB network contributing to ERBB2 stability and maintenance (Citri et al., 2004), and are thus attractive targets for specific ERBB2 blockade (Citri et al., 2002).

Recently there has been a lot of attention towards therapeutic strategies that target multiple mediators in the ERBB signaling cascade since genetic

heterogeneity and cellular dynamic alterations often lead to drug resistant tumors (Goldie and Coldman, 1979, Ye et al., 1999). Coupling ERBB blocker drugs with appropriate chemo- and/or radiotherapy procedures has been shown to improve the treatment outcome (Slamon et al., 2001, Citri and Yarden, 2006). A cartoon illustrating ERB signaling events and associated cellular manifestations is shown in **Figure 1**.

4.4.1.4. Focal Adhesion Kinase Pathway

Focal adhesion kinase (FAK) is a non-receptor signaling (Parsons, 2003, Schaller, 2001) tyrosine kinase localized in cytoplasm mostly involved in cellular migration as a crucial step in biological processes such as embryonic growth as well as diseases like cancer and cardiovascular deficiencies (Erickson, 1990, Stoker and Gherardi, 1991). During cell migration, multiple steps including edge protrusion and assembly of focal adhesion molecules to generate efficient and appropriate signal take place (Ridley et al., 2003). Integrin receptors as the main cellular receptors reside in ECM involved in cell adhesion play an important role in several steps of cell migration (Hynes, 2002a). Integrins possess the ability to dock to several cytoskeletal and signaling mediators via their short cytoplasmic domain thus interacting with focal adhesions in adherent cell types (Parsons, 2003, Schlaepfer and Mitra, 2004).

In early 90's FAK was first identified as a ~120 kDa integrin-dependent tyrosine phosphorylated protein that resided in focal adhesions (Guan et al., 1991). It was further characterized when various substrates of v-Src oncogene were identified and shown to possess high level of tyrosine phosphorylation in cells undergoing v-

Src-mediated transformation (Kanner et al., 1990). One substrate was later identified to be a tyrosine kinase and referred to as FAK because it is localized within focal adhesions (Schaller et al., 1992, Guan and Shalloway, 1992). Integrin-mediated regulation of cell migration through focal adhesions has been studied in many cell types as well as involvement of FAK in migration processes in cancer development (Guan and Shalloway, 1992, McLean et al., 2005). FAK has been shown to be the major integrin signaling mediator as well as a crucial component for other cell surface receptor signaling modules and is thus an attractive therapeutic target for cancer and cardiovascular disease treatment. Some small molecule FAK blockers have been already used in clinical trials (Lim et al., 2008, Parsons et al., 2008).

The FAK-encoding gene is conserved in different living systems such as mammals, rodent and single cell organisms (Schaller et al., 1992, Hanks et al., 1992). FAK protein consist of a kinase domain that binds to an N-terminal FERM (Band 4.1, four-point-one, ezrin, radixin, moesin) and a C-terminal domain containing the focal adhesion targeting (FAT) part. The FERM site has the same structure and signaling mediators as that of cytoskeletal talin and ezrin–radixin–moesin (ERM) proteins as well as signaling molecules such as JAK family of tyrosine kinases (Chishti et al., 1998, Girault et al., 1999).

FAK signaling network is thus mediated through interactions with integrins, growth factor receptors (Sieg et al., 2000) as well as intra-molecular interaction with the FAK kinase site (Cooper et al., 2003, Cohen and Guan, 2005). The C-terminus of FAK possesses two sequences rich in amino acid proline promoting linkage of SH3-domain containing proteins like p130cas (Polte and Hanks, 1995), Graf (Hildebrand et al., 1996) and endophilin A2 (Wu et al., 2005). In addition, some of

the SH3 domain-containing proteins like p130cas have the ability to recruit additional signaling mediators (Hildebrand et al., 1996). Various tyrosine phosphorylation sites have been detected on FAK protein involved in regulation of this protein's activity such as the auto-phosphorylation site Y397 involved in a number of FAK functions.

It was observed that the expression of FAK increases in keratinocytes upon wound healing which requires migration of these cells to the epidermal level (Romer et al., 1994). In another study, the activity of FAK was shown to increase upon endothelial cell migration to the wound region in an *ex vivo* model (Gates et al., 1994). Augmentation in the FAK expression level was detected in metastatic tumor cells in humans (Weiner et al., 1993, Owens et al., 1995). In a study using FAK knockout mice, FAK^{-/-} phenotype led to development of embryonic fibroblasts with impaired migration potential as an evidence for the involvement of FAK in cell migration (Illic et al., 1995). Another study reported that microinjection of FRNK (FAK C-terminal recombinant protein) blocked FAK activity and impaired endothelial and fibroblast cell migration (Gilmore and Romer, 1996).

All these findings suggest a role for FAK and its downstream signaling components in cell migration such as the pathway associated with phosphorylation of p130cas by the FAK/Src unit (Cary et al., 1996, Cary et al., 1998). Inability of FAK to dock to Src or p130cas molecules block p130cas phosphorylation regions resulting in hampered cell migration (Cary et al., 1996, Owen et al., 1999, Cary et al., 1998). Paxillin as a signaling mediator in cytoskeleton is an important substrate for FAK/Src kinase unit, the phosphorylation of which leads to Crk and Cas recruitment to the signalosome (Zhao and Guan, 2011a). A second signaling pathway in which PI3K and adaptor molecule Grb7 interact was shown to mediate

FAK involvement in cell migration (Han and Guan, 1999, Han et al., 2000). This finding was first observed upon detecting a FAK mutant with PI3K and Grb7 impaired binding sites but active Src docking region and Cas phosphorylation properties that lack the ability to promote cell migration (Reiske et al., 1999). This finding led the scientists to speculate that active form of PI3K can stimulate migration of the cells via Rac, an effective regulator component in actin filaments (Mitra et al., 2005). Furthermore, PI3K linkage to Grb7 results in Grb7 conformation changes, and consequently, augmentation of Grb7 phosphorylation mediated by FAK (Shen et al., 2002). Mutant-based studies have unraveled the importance of FAK-Grb7 binding as well as Grb7 phosphorylation in promoting cell migration (Han et al., 2000). Considering that FAK can bind independently to PI3K and Grb7, both FAK/Grb7 and FAK/PI3K units are shown to be involved in FAK mediated cell migration.

FAK stimulates cell migration via Rho molecule of GTPases resulting in alterations in actin filament assembly patterns within cytoskeleton. It was previously reported that disturbance of FAK in fibroblasts results in RhoA activity that stimulates cellular spreading and hampers their migration. This can be covered up by FAK expression in the cells (Ren et al., 2000, Chen et al., 2002). FAK was shown in turn to possess the ability to stimulate Rho by mediating phosphorylation of p190RhoGEF, a RhoA-specific guanine nucleotide component that interacts with microtubules (Zhai et al., 2003). FAK was also shown to facilitate normal and transformed cell invasion. FAK has been detected in cells that undergo v-Src transformation, mediating phosphorylation of Src and reducing interaction of this molecule with dynamin. In turn, dynamin plays a key role in endocytosis via regulating metalloprotease MT1-MMP on the cell surface and decrease its

entrance into the cytosol. This leads to accumulation of MT1-MMP on the cell surface and promotes v-Src transformed cell invasion (Wu et al., 2005).

Moreover, it has been shown that FAK interacts with v-Src-Cas-Crk-Dock180 signaling module through which it indirectly stimulates MMP2 and MMP9 expression in tumor cells (Hsia et al., 2003, Shibata et al., 1998) leading to an increased tumor invasion.

Due to the regulatory role of FAK in endothelial cell migration, the scientists speculated that it might affect angiogenesis (Rizzo, 2004, Zhao and Guan, 2011b, Lechertier and Hodivala-Dilke, 2012). The concept was taken into more serious consideration upon detecting levels of FAK expression in vessel development during embryonic growth (Polte et al., 1994). Furthermore, a study showed that cardiovascular deficiencies are associated with early miscarriage in FAK knockout mice (Illc et al., 1995).

FAK-PIK3 association was shown to increase when VEGF receptor-2 was stimulated via VEGF-A, facilitating migration of endothelial cells (Qi and Claesson-Welsh, 2001).

ANG1 as an angiogenesis growth factor for endothelial cells was shown to promote FAK phosphorylation (Kim et al., 2000a). Moreover, integrins as major components upstream of FAK in the signaling pathway were shown to be involved in tumor angiogenesis. Inhibition of integrin $\alpha\beta 3$ using small molecule chemical blockers diminished tumor vessel formation in numerous *in vivo* studies (Brooks et al., 1994a, Brooks et al., 1995, Eliceiri and Cheresh, 1999).

In conclusion, FAK can be regulated by some angiogenic growth factor receptors and, therefore, it is crucial for normal vessel formation and might also be an attractive target for cancer therapy (Zhao and Guan, 2011a).

4.4.2. Death-Associated Signaling Pathways

Different types of cell death are grouped into apoptotic, necrotic and autophagic categories. Cell death is defined according to the involvement of various types of proteases such as caspases and nucleases, on functional, physiological and/or pathological aspects of the cell (Galluzzi et al., 2007). Although caspase-dependent apoptosis is the dominant pathway, a different cell death pathway has been recognized and referred to as “autophagic cell death” taking place along with autophagic vacuolization.

Major types of programmed cell death are defined as necrotic cell death, autophagic cell death, pyroptosis and apoptotic cell death. Previously necrosis was considered as an uncontrolled mode of cellular death with no involvement of signaling events. However, there is evidence showing the involvement of caspase-independent cell death pathways in necrosis (Chautan et al., 1999). Necrosis is characterized through swelling of cytoplasm and organelles, loss of cell membrane integrity and release of the cellular content into the extracellular environment. Although the default cell death pathway is apoptosis, if the caspases are hampered, necrotic cell death takes place. Although FAS associated death domain (FADD) has been shown to be an important protein in FAS- and TRAIL-R-induced necrosis, its importance in TNF-induced necrosis is a matter of debate (Lin et al., 2004, Holler et al., 2000). A study on TNF receptor type 1-associated DEATH domain (TRADD) knockout mice revealed that TRADD is necessary for TNF-induced necrosis within MEF cells (Pobezinskaya et al., 2008). RIP1 is another essential initiator of death receptor-mediated necrosis (Festjens et al., 2007). RIP1 has kinase activity that was shown not to be essential for NF- κ B and MAPKs

activation, however, it is required for necroptosis (Holler et al., 2000, Degterev et al., 2005, Chan et al., 2003).

Necrotic cell death contributes to activation-induced cell death (AICD) in T lymphocytes as a crucial mechanism for limiting the number of T cells upon an immune response (Holler et al., 2000). However, it should be underlined that in most cases, necrotic cell death comes along either with apoptosis or in the presence of caspase blockers, suggesting a back-up role for necrosis in addition to apoptosis.

Autophagy pathway has been conserved during evolution and allows for degradation and recycling of cellular components in eukaryotes. Proteins and organelles are trapped in special bi-layer membrane vesicles to form autophagosomes as typical units for autophagic cells. Autophagy at its normal level maintains cellular homeostasis; however, several studies have suggested that it has important roles in cellular processes, such as cellular stress, differentiation and development (Duprez et al., 2009). It has been controversial whether or not autophagy possesses a crucial role in cellular death. The autophagic vacuoles have been detected in dying cells leading to its discovery; however, like necrosis, this pathway has also been considered to be a cell death back-up mechanism (Maiuri et al., 2007). To-date, several autophagy types have been characterized such as chaperone-mediated autophagy, microautophagy and macroautophagy. The latter is also known as “autophagy” which occurs as a typical event in case of severe stress and works through *de novo* autophagosomes formation. In this specialized vacuoles, proteins are trapped in advance of being introduced to the lysosomes (Xie and Klionsky, 2007).

The classical autophagic signaling pathway functions through mTOR (mammalian target of rapamycin), a crucial protein kinase in translation and cell-cycle regulation as well as negative regulation of autophagy. Upon mTOR blockade as a result of starvation or rapamycin treatment, the ULK-Atg13-FIP200 complex is activated, leading to stimulation of autophagosome formation (Jung et al., 2009). The formation of specific autophagic vacuoles needs vesicle nucleation that, upon formation of another unit, develops the PI3KC3 (Vps34)-complex. Within this complex, Beclin-1 (Atg6) acts as a platform for linking PI3KC3 (Vps34), UVRAG, Bif-1 and Ambra-1, all of which are responsible for PI3KC3 positive regulation (Pattingre et al., 2008).

Surprisingly, Bcl-2 has been shown to hamper autophagy by binding to Beclin-1, thus eliminating autophagic signal (Pattingre et al., 2005). Bcl-2 becomes phosphorylated upon starvation. This mediates release of Beclin-1 and induces autophagy (Wei et al., 2008). PI3KC3 (Vps34)-complex activation eventually leads to PI3P generation. As a consequence, other autophagy-related (Atg) proteins mediating vesicle membrane formation are recruited and ubiquitin-like systems are activated resulting in an Atg12eAtg5eAtg16(L) multimer complex formation that is involved presumably in vesicle curvature (Mizushima et al., 2003). Furthermore, LC3 (Atg8) binds to phosphatidylethanolamine (PE) and thereafter docks specifically to autophagic membranes. In the final step, the autophagosome attaches to a lysosome to release its content into the lysosome lumen for hydrolysis.

Autophagic signaling mostly exerts pro-survival function by protecting cells via elimination of harmful and damaged organelles and aberrant protein components during stress. However, several studies have shown the involvement of autophagy

in cell death (Scarlatti et al., 2009). For instance, in cells with perturbed apoptotic signaling another cell death program similar to necrosis takes place as a back-up, which can be characterized as autophagy because of typical LC3-II accumulation. It was shown in a study that apoptosis-deficient Bax/Bak double knockout MEF cells treated with DNA-damaging or ER-stress-inducing chemical agents resulted in non-apoptotic cell death, depending on the presence of Beclin-1 and Atg5 (Lum et al., 2005). Another study unraveled the involvement of FADD and caspase-8 in regulating autophagic signals in proliferative T cells (Bell et al., 2008). Lack of FADD or caspase-8 in those T cells caused them to undergo autophagy, whereas autophagy blockade rescued the cells from death.

Autophagic cell death has been shown to play a role in developmental processes that need destruction and elimination of unwanted cells. For instance, disruption of salivary gland cells during metamorphosis in *Drosophila* is associated with Atg expression and autophagy stimulation along with caspase activation and DNA fragmentation (Lee and Baehrecke, 2001), indicating an important role of autophagy for proper degradation pattern of salivary glands (Berry and Baehrecke, 2007).

Pyroptosis has been recognized more recently and has been described as regulated cell death with morphological and biochemical characteristics different from necrosis and apoptosis (Labbe and Saleh, 2008). Pyroptosis has been studied in monocytes, macrophages and dendritic cells exposed to microbial pathogens such as *Salmonella* and *Legionella*. Pyroptosis is dependent on caspase-1 activation (Bergsbaken et al., 2009). Caspase-1 as a member of the inflammatory caspases, is not involved in apoptotic cell death (Li et al., 1995); vice versa, the apoptotic caspases usually are not involved in pyroptosis (Lamkanfi et

al., 2008). Inactive caspase-1 is localized in the cytosol. Upon caspase-9 activation in the apoptosome, caspase-1 is also activated in a complex referred to as inflammasome. This molecular complex consists of nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family members responsible for recruiting caspase-1 via adaptor molecules such as ASC/Pycard. To-date, four inflammasomes have been identified and referred to after their NLR (Nalp-1, Nalp-3 and Ipaf) or HIN-200 protein (AIM2) (Bergsbaken et al., 2009, Schroder et al., 2009).

Inflammasomes develop upon NLRs recognition by intracellular bacterium, virus or host danger signals. For instance, Nalp-1 identifies cytosolic delivery of *Bacillus anthracis* lethal toxin, Ipaf distinguishes cytosolic flagellin, and Nalp-3 is responsible to respond to various damage- and pathogen-associated molecular pattern (DAMP and PAMP) molecules (Bergsbaken et al., 2009).

Most NLRs contain three specific domains: an N-terminal caspase activation and recruitment domain (CARD) or alternatively a pyrin effector domain (PYD), a central nucleotide binding/oligomerization domain (NACHT) as well as various C-terminal leucine-rich repeats (LRRs). Furthermore, Nalp-1 has a C-terminal part that docks to a CARD domain.

In contrast to human Nalp-1, the mouse Nalp-1b does not contain a N-terminal PYD domain. Upon induction, NLRs are oligomerized through the NACHT domain. The NLRs attaches to the adaptor protein ASC through PYD. Additionally, Nalp-3 binds to the cardinal adaptor in its inflammasome. These adaptor molecules are working to recruit caspase-1 via CARD-CARD interactions, leading to its oligomerization and activation (Duprez et al., 2009). Cells degrading through pyroptosis show biochemical and morphological characteristics of both apoptotic

as well as necrotic cells (Bergsbaken et al., 2009). Pyroptotic cells fail to sustain their mitochondrial membrane potential and plasma membrane integrity leading to release of cytoplasmic contents into the extracellular environment. Similar to apoptosis, pyroptotic cells are subjected to DNA fragmentation as well as nuclear condensation. However, this caspase-1-dependent nuclease-mediated cleavage of DNA does not possess the same fragmentation pattern as it is observed in apoptosis (Brennan and Cookson, 2000). In addition, activation of poly ADP ribose polymerase (PARP)-1 associated with pyroptotic cell death is not essential for the initiation of cell lysis (Fink and Cookson, 2006).

Pyroptosis has been considered as a part of the host defense system against pathogens, because infectious host cell death ruins the pathogen's niche leading into eliminated bacterial or viral replication. Paradoxically, host cell death is beneficial for some pathogens letting them have access to other host cells. Pathogens have thus developed strategies to block or stimulate host cell pyroptosis. For instance, poxvirus has been shown to encode several PYD-containing proteins that inhibit caspase-1 activation through inflammasomal ASC interactions, thereby facilitating the infection (Johnston et al., 2005). Since pyroptosis depends on caspase-1 activity, it is associated with the inflammatory response, which increases upon release of cytosolic content after cell lysis.

A previous study on caspase-1-deficient mice revealed that these animals are more sensitive to *Salmonella* infection than wild-type and IL-1 β and IL-18 double-knockout mice (Lara-Tejero et al., 2006), whereas caspase-1 impairment provides protection against sepsis induced by *E. coli* which contrasted with wild-type and IL-1 β and IL-18 double-knockout mouse models (Sarkar et al., 2006). These findings lead the scientists to speculate that the phenotypic characteristic associated with

caspase-1 impairment is not only because of the lack of these proinflammatory cytokines, but rather pyroptosis or further caspase-1-dependent events control and regulate the infection. Aberrant activation of caspase-1 causes several disorders including myocardial infarction as well as cerebral ischemia and endotoxic shock (Bergsbaken et al., 2009).

4.5. Apoptosis

The term “apoptosis” was first used in 1972 to explain a form of cell death that could be characterized due to specific morphological alterations such as cell shrinkage, blebbing of the cell membrane, and condensation of chromatin in the nucleus. Apoptosis has been defined as an intrinsic programmed mechanism resulting in regulated cellular breakdown into apoptotic bodies recognizable for phagocytes.

Bcl-2 family of proteins and cysteinyl aspartate-specific proteases, i.e. caspases, as two major protein families have been shown to be involved in apoptosis by controlling mitochondrial integrity (Youle and Strasser, 2008) and regulating the execution step, of apoptosis (Fuentes-Prior and Salvesen, 2004), respectively.

Twelve human caspases classified into apoptotic and inflammatory subfamilies have been characterized. Apoptotic caspases have been further categorized into initiator (caspases-2, -8, -9, -10) and executioner (effector) caspases (caspase-3, -6, -7). Caspases -1, -4 and -12 are also considered as inflammatory caspases. They are all expressed as proenzymes possessing an N-terminus with variable lengths, a large subunit (p20) containing protein-protein interaction domain (also known as death effector domains (DED) as well as caspase activation and

recruitment domains (CARD) and a tiny small C-terminal subunit (p10). Protein-protein interaction motifs recruit inactive initiator caspases and subject them to oligomerization and proximity-induced autoproteolysis for activation (Salvesen and Riedl, 2008). Prodomain caspases are also produced in the cell and their activation is dependent on upstream caspases (Salvesen and Riedl, 2008, Duprez et al., 2009). Intrinsic or the extrinsic apoptotic pathways are responsible for caspase activation in mammalian cells. Numerous stimuli like DNA damage and cytotoxic agents activate the intrinsic pathway that works through mitochondria and is regulated via Bcl-2 family of proteins (Youle and Strasser, 2008). Upon homeostatic conditions, the anti-apoptotic Bcl-2 family proteins inhibit pro-apoptotic Bcl-2 family member Bax and Bak thus protecting mitochondria from being damaged. Upon stress, Bcl-2-homology 3 (BH3)-only proteins become active serving as antagonists for anti-apoptotic Bcl-2 proteins. As a result, the BAX and/or Bak molecules are no longer inhibited and thus become oligomerized forming a channel through which cytochrome C enters the intracellular environment. Upon cyt c association with Apaf-1 and ATP, a platform (apoptosome) is developed that recruits and activates procaspase-9 (Riedl and Salvesen, 2007). Activated caspase-9 is thus responsible for cleavage and activation of downstream effector caspases-3, -6 and -7 necessary for apoptotic cell death execution. Moreover, pro-apoptotic components released from the mitochondria participate in the cell suicidal process. For instance, Smac/Diablo molecule serves as antagonist for inhibitory action of apoptotic proteins like XIAP, cIAP1, and cIAP2 (LaCasse et al., 2008). Upon docking to Smac/Diablo, XIAP inhibits caspase-9, -3 and -7 (LaCasse et al., 2008, Shaw et al., 2008). In contrast to XIAP, cIAP1 and cIAP2 are not involved in direct inhibition of caspases, rather

they interact with tumor necrosis factor receptor (TNFR)-associated factor2 (TRAF2) resulting in TNFR1 recruitment and NF- κ B activation through ubiquitination of RIP1 (LaCasse et al., 2008). Knowing that NF- κ B mediates anti-apoptotic gene expression within the cell, cIAP1 and cIAP2 indirectly serve as apoptosis blockers.

The extrinsic pathway of apoptosis is promoted upon TNFR family receptor induction (e.g. TNFR, Fas and TRAIL-R). Signaling through these receptors might stimulate numerous cellular processes such as cell death as well as proliferation and cellular differentiation. Cell death (apoptosis) is stimulated via death-inducing signaling complex (DISC) formation. Within this complex, Fas-associated death domain (FADD) serves as recruiter and initiator for caspases-8 and -10 through death motif interactions (Peter and Krammer, 2003). In contrast to Fas and TRAIL-R, accumulation of TNFR1 causes development of two other complexes (Wilson et al., 2009): Complex I including TNFR1, TRADD, TRAF2, RIP1, cIAP1 and cIAP2 (crucial mediators for TNF-induced activation of NF- κ B and MAPKs) is developed at the plasma membrane.

Endocytosis of TNFR1 initiates the development of complex II, an analogue to the receptor-proximal DISC, which consists of TRADD, FADD, and caspase-8 and/or -10 that can be activated by FasL and TRAIL. Caspase-8 and -10 in turn activate downstream executioner caspases resulting in apoptosis. Additionally, caspase-8 mediates BH3-only protein Bid cleavage that increases the death receptor-induced cell death program through activation of the mitochondrial pathway (Duprez et al., 2009).

A previous study has shown that TNF stimulates one of the two caspase-8 activation pathways according to the cellular condition (Wang et al., 2008). One

pathway has been shown to be independent from RIP1 and can be blocked by cFLIP, a caspase-8 homologue that competes with caspase-8 for binding to FADD. The second pathway was deciphered upon TNF and Smac mimetics treatment and has been shown to depend on active form of RIP1 (kinase). Smac mimetics have been shown to stimulate auto-degradation of cIAP1 and cIAP2 that facilitates RIP1 release from the complex to create caspase-8 activating platform formed by RIP1, FADD, and caspase-8 (Wang et al., 2008).

Apoptotic cell death possesses a crucial role in organ and tissue remodeling during development. Its role has been investigated in development and shaping of the inner ear, muscle development as well as omitting the interdigital webs (Penaloza et al., 2006). Moreover, it has been shown that during immune and nervous system development, not only cellular overproduction takes place, but also elimination of excess cells through apoptosis occurs (Elmore, 2007). Apoptosis has been shown to play an important role in organ homeostasis (Segura et al., 2002, Elmore, 2007, Krysko and Vandenabeele, 2008).

Apoptosis of host cells during infections serves as a defense mechanism by eliminating the places in which the pathogen replicates. Pathogens therefore use several strategies to prevent cellular apoptosis e.g. via preventing cyt c release from mitochondria, inducing cellular survival pathways or blocking caspase activation and/or functionality (Galluzzi et al., 2008, Faherty and Maurelli, 2008).

Disruption or aberrant apoptosis might lead to several disorders such as autoimmune diseases and cancer and accounts for tissue damage in diseases like sepsis, ischemia and diabetes (Duprez et al., 2009).

In summary, caspase proteins play crucial roles in different death-associated pathways, such as initiator (caspases-2, -8, -9, -10) and effector caspases

(caspase-3, -6 and-7) in apoptosis, caspases-1 in pyroptosis and the indirectly caspase-8 in autophagy (Figure 2).

4.5.1. Non-Apoptotic Role of Caspases in Developmental Biology

Although inflammatory caspases have been shown to participate in some forms of cell death (e.g. caspases-1 involvement in pyroptosis), their most well-known function is processing inflammatory cytokines to their active and functional forms. A previous study showed that caspase-1/caspase-11 knockout mice failed to activate IL-1, and also caspase-11 knockout animals could not activate caspase-1 in response to lipopolysaccharide (Wang et al., 1998, Mosley et al., 1987). However, it should be noted that *in vitro* studies have not shown direct crosstalk between caspase-11 and caspase-1. Caspase-1 knockout animals lack the capability of IL-1 activation by proteolytic cleavage as well as IL-1 release. This suggests that caspase-1 can cleave and mediate release of IL-1 from the living organisms. In survival processes of cardiomyocytes as well as in neuronal apoptosis, activation of apoptotic caspases has been detected. Activity of initiator caspases such as caspase-8 and -9 has been observed in neurons within Alzheimer's lesions. These caspases, however, failed to convey apoptotic signals to stimulate effector downstream caspases such as caspase 3, 6, and 7 (Raina et al., 2001).

Aberrant apoptosis has been shown in reverse modeling of cardiocytes (Narula et al., 1999, Haider et al., 2002). Furthermore, activated caspase components have been detected in myocardial biopsy samples from patients that have undergone implantation of ventricular assisting device; however, the cellular mitochondria and

nuclei were not damaged. These findings indicate that activation of the caspases does not necessarily result in DNA fragmentation and cell death, rather each step can be stopped resulting in incomplete apoptosis.

It has been reported that caspase-2 knockout mice showed an overgrowth of the germ cells in ovaries; however, during development in the absence of neuronal growth factors, death of neurons was accelerated (Bergeron et al., 1998) suggesting that the role of mammalian caspases might not be restricted to the cell death (Nhan et al., 2006).

Activation-induced proliferation of T cells was shown to be hampered in FADD knockout mice or mice with a dominant-negative FADD protein (Zhang et al., 1998, Newton et al., 1998, Zornig et al., 1998). Studies on transgenic mice for dominant-negative FADD and impairment in RAG-1 function additionally support a role for FADD in proliferation (Zhang et al., 1998, Newton et al., 1998, Zornig et al., 1998). Furthermore, T-cells with non-functional FADD arrest at the G0/G1 phase of cell cycle. T cell precursors of these animals lack the capability of proliferation when induced by CD3. Signaling events via pre-T-cell receptor (pre-TCR) and death receptors appeared to trigger cell proliferation, survival as well as differentiation, whereas signaling through death receptors in thymocytes did not include pre-TCR induced apoptosis (Newton and Strasser, 2000). Serine protein kinases have been shown to phosphorylate FADD during G2/M phase in cell cycle, but not in cells that are arrested in G1/S phase (Scaffidi et al., 2000).

These events could relate FADD and presumably caspase-8 and FLIP to the cell cycle. Another indirect support for the involvement of FADD/FLIP/caspase-8 in cell growth is derived from an study showing that proliferation of primary T cells is

blocked by small molecule, cell-permeable caspase blockers such as zVAD-fmk (Kennedy et al., 1999, Alam et al., 1999, Chau et al., 2005).

A previous report showed that expression of the caspase-8 inhibitor CrmA in T cells totally inhibited apoptosis through CD95 whereas it did not have an impact on T-cell proliferation (Newton et al., 1998). Furthermore, initiator caspases-6 and -8 are cleaved and activated in induced B cells for entering G1 phase of the cell cycle (Olson et al., 2003).

Caspases might become activated during terminal differentiation. During the differentiation of the lens cells, high level of caspase-3 is expressed in the equatorial epithelium cells (Weber and Menko, 2005). Additionally, staurosporine (a broad spectrum kinase inhibitor that induces caspase-dependent apoptosis) has been shown to induce *in vitro* differentiation of lens cells, which was accompanied by BCL-2 expression and cyt c release from mitochondria, and low level caspase-9 and -3 activation (Weber and Menko, 2005). These data support the involvement of caspases, in addition to being major pro-apoptotic components, in non-apoptotic cellular processes such as cell survival, proliferation and differentiation.

4.6. Goal of this thesis

The precise mechanisms regulating the cellular crosstalk during neo-vessel formation *in vivo* are not clear. The goal of this thesis was to better understand the cellular communication during neo-vasculogenesis. We wanted to know whether an *ex vivo* proteomic profiling approach can unravel effective signaling events that take place early during vessel formation *in vivo*. We studied the early *in vivo* signaling events in an established model of experimental neo-vasculogenesis using antibody array analysis. Our result revealed significant alteration of components of inflammatory, effector and executioner caspases, human ErbB2

receptor tyrosine kinase as well as calcium/calmodulin-dependent and DNA protein kinases. We also found that blocking caspase-4 or caspases in general considerably diminished capillary-like structure formation *in vitro* and significantly impaired vessel formation *in vivo*. Our findings indicate that caspases can modulate mesenchymal-endothelial stem/progenitor cell interaction toward vessel formation, and also suggest a novel role for caspase-4 during early signaling events in the initiation of vasculogenesis. These data can expand our insights into cellular crosstalk during vascular regeneration and bring about new ideas for developing more effective vascular regenerative strategies.

5. MATERIALS AND METHODS

(The following chapter follows the publication Rohban et al. 2013 (Rohban et al., 2013))

5.1. Materials

All cell culture media were purchased from Sigma-Aldrich; St. Louis; MO, USA; www.sigmaaldrich.com) thereafter were supplemented with 100 µg/mL Streptomycin, 100 U/mL penicillin, 2 mM L-Glutamine (all Sigma-Aldrich) as well as manufacturer-provided aliquots containing vascular endothelial cell growth factor (VEGF), human epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), human basic fibroblast growth factor (bFGF).

The culture media were also supplemented with 10% pooled human platelet lysate (pHPL) (Reinisch et al., 2007, Schallmoser et al., 2007b, Reinisch et al., 2009, Hofmann et al., 2012b) instead of fetal bovine serum (FBS). Prior to pHPL

supplementation, an amount of 10 U/mL of Heparin (Biochrom AG, Berlin, Germany) was added to the medium.

Trypsin (0.25% trypsin/1mM ethylenediaminetetraacetic acid [EDTA]), was purchased from Sigma-Aldrich. An amount of 30 $\mu\text{L}/\text{cm}^2$ of trypsin was used to detach cells from a 80-90 % confluent cell culture flasks of different areas.

Cell surface markers that were assessed in FACS analysis were purchased from Becton, Dickinson and Company (BD Biosciences), NJ, USA. For the list of the employed cell markers and their original and applied concentration see the Table below).

In vitro angiogenesis assay kit (Cat. No. ECM 625) was purchased from Millipore, Billerica, MA, USA). An amount of 300 $\mu\text{L}/\text{plug}$ and 250 $\mu\text{L}/9.5 \text{ cm}^2$ of this extracellular matrix was used for *in vivo* and *in vitro* assays, respectively.

Caspase-4 inhibitor Z-LEVD-FMK (original concentration 2 mM) and pan-caspase inhibitor EZ-SolutionTM Q-VD-OPh (original concentration 10 mM) were both purchased from BioVision Research Products, CA, USA. These chemical inhibitors were used at the diluted concentrations (Z-LEVD-FMK from 0.2 μM to 5 μM , and Q-VD-OPh from 0.08 μM to 50 μM with 5-fold dilution steps) for *in vitro* as well as *in vivo* studies. An amount of 2 μM of caspase-4 inhibitor (Bian et al., 2009) and 10 μM of pan-caspase inhibitor (Caserta et al., 2003) as irreversible chemical inhibitors were used for *in vivo* studies For the dilution of chemical inhibitors, dimethyl sulfoxide (DMSO) was purchased from WakChemieMedical GmbH, Steinbach, Germany, to make the required dilutions.

Triton X-100 lysis buffer, phosphatase- and proteinase-inhibitors were purchased from Roche, IN, USA.

Caspase-4-specific primary antibody (N-15), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, FL-335) and peroxidase-conjugated secondary antibodies (donkey anti-goat, and goat anti-rabbit) were all purchased from Santa Cruz, CA, USA. The applied concentrations for caspase-4, GAPDH and both secondary antibodies were 0.1 µg/mL, 0.025 µg/mL and 0.025 µg/mL, respectively. The antibodies were diluted in PBS (Gibco life technologies, Grand Island, NY, USA). ECL reagent was purchased from Amersham, UK. Signal intensity of the visualized bands of western blot was quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Annexin V apoptosis detection kit (Cat. No. 88-8103, ebioscience, SD, USA) and Staurosporin (Cat. No. 1285/100U, TocrisetTM, Tocris bioscience, Bristol, UK) were used according to the manufacturer's protocol and at the concentration of 2 µM, respectively.

For cell enumeration purposes, trypan blue solution (0.4%) was purchased from Gibco life technologies, Grand Island, NY, USA.

Mayer's hematoxylin and eosin phloxin were both purchased from Sigma-Aldrich. Unconjugated monoclonal mouse anti-human antibodies against CD31 (clone: JC70A, 5.15 µg/mL, Dako), CD90 (clone: EPR3132, Abcam, Cambridge, MA, USA) Vimentin (clone: V9, 0.78 µg/mL, Dako), von Willbrand factor (vWF, clone F8/86, Dako) and isotype control IgG1 (BD) were used for immune histochemical experiments.

For development of immune histochemical signal Ultravision LP large volume detection system horseradish peroxidase (HRP) polymer was purchased from Thermo Scientific, PA, USA. Diaminobenzidine (DAB) or alkaline phosphatase

detection system using either fast blue or fast red according to the manufacturer's protocols were also purchased from Vector, CA, USA.

Avidin-biotin inhibiting from Vector was used before staining with biotinylated polyclonal rabbit anti-human CD90 (EPR3132, Abcam) and biotinylated monoclonal goat anti-rabbit IgG1 (BD). Streptavidin-horse radish peroxidase conjugate and diaminobenzidine (DAB) were purchased from Dako for detection of immunohistochemical signal. All the reagents used for Movat's pentachrom histological staining were purchased from Dako, CA, USA.

Table 1 below shows the antibodies used for flowcytometrical characterization of the employed cells.

Antibody	Label	Clone	Isotype	Concentrations	Species & Clonality	Used conc.	Producer	Cat. No
CD3	APC	SK7	IgG1	50 µg/ml	Mouse monoclonal	:25	BD	345767
CD3	PE	SK7	IgG1	12.5 µg/ml	Mouse monoclonal	:100	BD	347347
CD3	FITC	SK7	IgG1kappa	100 µg/ml	Mouse monoclonal	:100	DAKO	F0818
CD13	APC	WM15	IgG1kappa	100 tests	Mouse monoclonal	:20	BD	557454
CD15	FITC	MMA	IgM	100 µg/ml	Mouse Monoclonal	:50	BD	347423
CD15	PE	HI98	IgG1	100 µg/ml	Mouse monoclonal	:20	Pharminogen	555402

CD19	PE	SJ25C1	IgG1	12.5 µg/ml	Mouse monoclonal	:20	BD	345789
CD19	FITC	4G7	IgG1	25 µg/ml	Mouse monoclonal	:25	BD	345776
CD29	PE	HUTS- 21	IgG2kappa	100 tests	Mouse monoclonal	:20	BD	556049
CD31	FITC	WM59	IgG1kappa	100 tests	Mouse monoclonal	:100	BD	555445
CD44	FITC	G44-26	IgG2kappa	25 tests	Mouse monoclonal	:20	BD	560977
CD49-a	PE	SR84	IgG1kappa	100 tests	Mouse monoclonal	:20	BD	559596
CD63	PE	H5C6	IgG1kappa	100 tests	Mouse monoclonal	:20	BD	556020
CD73	PE	AD2	IgG1kappa	100 tests	Mouse monoclonal	:100	BD	550257
CD90	FITC	F15.42	IgG1	100 tests	Mouse monoclonal	:90	Immunotech	1839
CD105	PE	266	IgG1kappa	50 tests	Mouse Monoclonal	:20	BD	562380
CD166	PerCP	3A6	IgG1kappa	50 tests	Mouse Monoclonal	:10	BD	559263
HLAABC	FITC	W6/32	IgG2a	100 Tests	Mouse monoclonal	:50	Lab	VTF- 114-10

HLA-DR	APC	L243	IgG2a	50 µg/ml	Mouse monoclonal	:50	BD	340549
STRO-1	FITC	Clone STRO-1	IgM	100 Tests	Mouse monoclonal	:20	Biolegend	340106

5.2. Experimental Devices

Table 2 below summarizes the devices that were used in the experiments of this project.

Bürker Türk hemocytometer	Bio-Rad, CA, USA
Cell factory (CF-4, 2,528 cm ²)	Thermo Fisher Scientific, Freemont, CA, USA
Centrifuge Heraeus microfuge	Thermo Fisher Scientific, CA, USA
CO ₂ incubator	BINDER GmbH, Germany
Color View III camera microscope with the analySIS B acquisition software	Olympus IX51, all Olympus, Hamburg, Germany
Confocal microscope LSM 510	Carl Zeiss, Germany
Culture flasks 75, 225 cm ²	Corning Inc., Acton, MA, USA- BDBiosciences
Density gradient centrifuge	Ficoll-Paque TM -PLUS, StemCell Technologies Inc., Vancouver, Canada
FACS Calibur instrument	BD, Bioscience, CA, USA
Fluorescence microscope BX51	Olympus, Hamburg, Germany
Freezer -20	Thermo Fisher Scientific, CA, USA
Freezer -80	Thermo Fisher Scientific, CA, USA
ImageJ software	http://rsbweb.nih.gov/ij/
ImaGene 8.0 BioDiscovery	El Segundo, CA, USA
Inline filtration for leukocyte depletion	PALL Autostop, Pall, Dreieich, Germany
Laminar flow Hood	Life technologies, NY,USA
MagNAlyser Centrifuge	Imlab, Boutersem, Belgium
Microtome HM300	Artisan technology, IL, USA
Navios cytometer	Beckman Coulter, www.bc-cytometry.com
Perkin-Elmer ScanArray Reader laser scanner	Waltham, MA, USA
Sonicator	Imlab, Boutersem, Belgium
Spectramax instrument	Molecular Devices, Sunnyvale, CA, USA
Stereomicroscope SZX12	Olympus, Hamburg, Germany
Ultra-centrifuge	Beckman Coulter GmbH, Vienna, Austria

5.3. Experimental Procedures

5.3.1. Preparation of ECFC and MSPC Culture Medium

ECFC isolation and expansion was carried out using endothelial basal medium (EBM-2) supplemented with 100 µg/mL Streptomycin, 100 U/mL penicillin, 2 mM L-Glutamine (all Sigma) as well as manufacturer-provided aliquots containing vascular endothelial cell growth factor (VEGF), human epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), human basic fibroblast growth factor (bFGF). The medium was also supplemented with 10% pooled human platelet lysate (pHPL) replacing fetal bovine serum (FBS) as described previously (Reinisch et al., 2007, Schallmoser et al., 2007b, Reinisch et al., 2009, Hofmann et al., 2012b). Prior to pHPL supplementation, an amount of 10 U/mL of Heparin (Biochrom AG) was added to avoid pHPL solidification within the liquid medium.

MSPC isolation and expansion were performed using α -modified minimum essential medium (α -MEM, M4526; Sigma-Aldrich; St. Louis; MO, USA; www.sigmaaldrich.com) supplemented with 100 µg/mL Streptomycin as well as 100 U/mL Penicillin (to minimize the bacterial contamination), 2 mM L-Glutamine (all Sigma;) and 10% pHPL (Schallmoser et al., 2007a, Bartmann et al., 2007, Schallmoser and Strunk, 2009). To avoid platelet lysate solidification in the medium, heparin (2 U/mL, Biochrom AG) was added to the α -MEM medium prior to pHPL supplementation.

5.3.1.1. Pooled Human Platelet Lysate (pHPL)

The preparation of pHPL was performed by Katharina Schallmoser and Claudia Url, Stem Cell Research Unit, Department of Blood Group Serology and Transfusion Medicine, Medical University of Graz.

An amount of 450 ± 45 mL of the blood was collected according to the informed consent from healthy individuals following the Austrian protocol for blood donation. In order to produce platelet enriched plasma, the buffy coat was subjected to red blood cell and plasma separation procedures automatically and thereafter transferred into satellite containers (Compomat, G3, NPBI, Amsterdam, Netherlands). Platelet-rich plasma (PRP) was further exposed to leukocyte depletion infiltration procedures (PALL Autostop, Pall, Dreieich, Germany) and thereafter stored at -30°C to allow for platelet cleavage. To avoid the incidence of individual donor variations, a minimum number of 10 platelet lysate units were pooled and stored at -30°C until further use (Schallmoser et al., 2007a, Schallmoser and Strunk, 2009).

5.1.2. ECFC and MSPC Isolation and Expansion

The cell isolation, purification and expansion procedures were carried out together with Birgit Feilhauer and Daniela Thaler at the Division of Hematology, Medical University of Graz.

MSPC were isolated and expanded from umbilical cord, umbilical cord blood, white adipose tissue, amnion and bone marrow (Shahdadfar et al., 2005, Szoke et al., 2012, Kita et al., 2010, Miao et al., 2006). Umbilical cord blood-derived mononuclear cells (UCB-MNC) were obtained from the interface using density gradient centrifugation (Ficoll-PaqueTM-PLUS, StemCell Technologies Inc., Vancouver, Canada, www.stemcell.com). The mononuclear cells were washed with phosphate buffered saline (PBS) prior to be seeded in the culture flasks (Corning Inc., Acton, MA, USA) with a density of 1×10^6 mononuclear cells /cm² directly in supplemented α -MEM (Sigma). A complete medium replacement was

carried out 2-3 days after seeding to promote the removal of non-adherent cells. The fresh medium was provided to the cultures twice per week. Mononuclear cell colonies consisting of approximately 50-80 cells in the primary cultures were subjected to trypsin (0.25% trypsin/1mM ethylenediaminetetraacetic acid [EDTA], 1-5 min, 37°C; Sigma) after 2-3 weeks and were seeded into new flasks until reaching 80-90% confluence; thereafter, they were trypsinized and quantified prior to storage in liquid nitrogen. The cells were thawed for experimental assessment of expansion (Reinisch et al., 2007, Schallmoser et al., 2007b, Reinisch et al., 2009, Hofmann et al., 2012b).

Umbilical cord-derived MSC were isolated from Wharton's jelly dissected from umbilical cord and cut into pieces (4-6 mm²). The tissue pieces were placed on the culture plate to allow for plastic attachment prior to addition of the culture medium (http://www.jove.com/author/Andreas_Reinisch). The cells were allowed to grow for 2-3 weeks.

ECFC were isolated and expanded from human umbilical cord, umbilical cord blood, adult peripheral blood and white adipose tissue (Prasain et al., 2012, Hofmann et al., 2009, Reinisch and Strunk, 2009, Mead et al., 2008, Szoke et al., 2012). For ECFC isolation from blood, cell culture was initiated no more than two hours after blood donation. To minimize cell loss, any unnecessary manipulation of freshly collected blood including red blood cell lysis or density gradient centrifugation was avoided. The heparin-containing blood was diluted in EGM-2 in a ratio of 1:4. The cultures were incubated at 37°C, 5% CO₂ overnight and washed twice with pre-warmed PBS to remove non-adherent cells prior to addition of the fresh pre-warmed EGM-2 medium. The medium was replaced 2-3 times per week

until the cobblestone-shaped ECFC colonies appeared (Reinisch et al., 2007, Schallmoser et al., 2007b, Reinisch et al., 2009, Hofmann et al., 2012b).

In order to expand ECFC and MSPC, the frozen cells of the first passage were thawed and seeded in 2,528 cm² cell factories (CF-4, Thermo Fisher Scientific, Freemont, CA) using 500 mL EGM-2 and α -MEM medium, respectively. The cell-containing CF4s were maintained at 37°C, 5% CO₂ and 95% humidity in an incubator. The cultures were subjected to medium replacement twice weekly to allow for the required nutrition accessibility and removal of the waste. Upon reaching 70-80% confluence, the cells were harvested using pre-warmed trypsin (50 mL per CF-4, 4-5 min., 37°C; Sigma Aldrich). After two washes with pre-warmed PBS and centrifugation (5 min, 300xg, 4°C), the harvested cells were quantified and checked for viability by means of Bürker Türk hemocytometer and trypan blue exclusion, respectively.

5.3.2.1. Flow Cytometric Characterization of ECFC and ECFC

ECFC and MSPC characterization was performed together with Eva Rohde, Paracelsus Medical University of Salzburg.

Umbilical cord-derived ECFC and MSPC were stained with cell surface markers (for the list of the employed cell markers see [Figure 5 A and B](#)) and the reactivity was evaluated using flow cytometry (Facs Calibur Flow cytometer, BD) according to the manufacturer's instructions ([see 5.1.](#)).

5.3.3. Animal Experiments

Animal experiments were performed together with Nathalie Etchart and Andreas Reinisch, Department of Hematology and Stem Cell Research Unit, Medical University of Graz).

Animal experiments in this project were performed following the 2010/63/EU guidance of the European Parliament on the welfare of laboratory animals. Protocols were approved by the Animal Care and Use Committee of the Veterinary University of Vienna on behalf of the Austrian Ministry of Science and Research. Immune-deficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were purchased from the Jackson laboratory (Bar Harbor, ME, USA), housed in specific pathogen-free (SPF) facility of the Medical University of Graz and were used for the *in vivo* experiments between seven to 18 weeks of age. The immune-compromised NSG mice were used in order to avoid the immune response leading to the rejection of injected human cells (transplants). ECFC/MSPC were re-suspended with a ratio of 80:20 % in ice-cold extracellular matrix (300 mL per plug, Cat. No. ECM 625, Millipore, Billerica, MA, USA), thereafter injected in form of subcutaneous plugs into the flank of the NSG mice (Melero-Martin et al., 2007, Au et al., 2008). Prior to injection, mice were anesthetized following the approval for animal handling (BMWF:-66.010/0082-II/10b/2009).

Mice were observed for the indicated time periods of two to 24 weeks after injection. At the defined time point, the mice were sacrificed by cervical dislocation and the plugs were surgically dissected from the subcutaneous tissue, photographed using stereomicroscope (SZX12, Olympus) and fixed in neutral buffered 4% paraformaldehyde overnight to be used for histological assessments.

5.3.4. Vessel Formation *In Vivo*

Cell culture procedures required for this experiment were performed together with Daniela Thaler and Birgit Feilhauer, Division of Hematology, Medical University of Graz

ECFC and MSPC were subjected to an optimized isolation and purification procedure as previously indicated (Reinisch and Strunk, 2009) (see also Figure 3 A). ECFC were seeded in EGM-2 (Lonza) at a density of 1,000 cells/cm² and MSPC in α -MEM (Sigma-Aldrich, St. Louis, MO) at a density of 500 cells/cm² in 2,528 cm² cell factories (CF-4, Thermo Fisher Scientific, Fremont, CA).

In a different set of *in vivo* vascularization experiments, a total number of 2x10⁶, 8x10⁶ or 32x10⁶ cells were used to investigate vessel formation. Two, eight or 32 million MSPC (MSPC only), two, eight or 32 million ECFC (ECFC only) or the combination of 1.6 x 10⁶, 6.4x10⁶ or 24x10⁶ ECFC with 0.4 x 10⁶, 1.6x10⁶ or 8x10⁶ MSPC (ECFC+MSPC) were re-suspended in 300 μ L ice-cold extracellular matrix (*In vitro* angiogenesis assay kit, Millipore, Billerica, MA, USA) and injected subcutaneously into immune-deficient NSG mice (four plugs per mouse were injected, for every cell condition three mice were used). For every set of *in vivo* experiments, cell-free 'Matrix only' plugs (Millipore) were injected and used as controls (Figure 3 B).

Mice were sacrificed at day one, 14, 56 or 168 after implantation, and plugs were harvested from the subcutaneous sites (three mice and three plugs per cell combination per time point; Figure 3 C and D). Three plugs explanted after one day were subjected to protein lysis procedure to investigate the expression of early signaling molecules (Figure 3 E), whereas parallel plugs explanted at two, eight and 24 weeks (14, 56 and 168 days, respectively) were used for histological experiments for confirmation of functional and stable vessel formation in a time-course analysis (Figure 3 F and G).

To investigate the impact of caspase inhibition on *in vivo* vascularization, either ECFC or MSPC or both cell types were cultured in the 225 cm² flasks and

incubated in 37°C, 95% humidity. Upon reaching 70-80% confluence, the cells were pre-treated with the small-molecule caspase-4 inhibitor Z-LEVD-FMK (2 µM), pan-caspase inhibitor EZ-Solution™ Q-VD-OPh (10 µM; both BioVision Research Products, CA, USA) or vehicle solvent (dimethyl sulfoxide, DMSO, WakChemieMedical GmbH, Steinbach, Germany) for 8 hours followed by a washing step with pre-warmed PBS (5 min, 300 x g, 4°C). Viability of the cells was checked using trypan blue staining and cells were re-counted prior to implantation (Figure 3).

5.3.5. Proteomic Profiling: Detection of Signaling Molecules

5.3.5.1. Sample Preparation

Sample preparation for proteomic experiment was performed together with Eva Rohde, Paracelsus Medical University of Salzburg

In order to detect early signaling molecules, ECFC of three different donors were seeded separately in EGM-2 (Lonza) at a density of 1,000 cells/cm² and MSPC from three donors were cultured separately in α-MEM (Sigma-Aldrich, St. Louis, MO) at a density of 500 cells/cm² in 2,528 cm² cell factories (CF-4, Thermo Fisher Scientific, Fremont, CA) and were kept in an incubator (37°C, 5% CO₂; Binder) for approximately 8-10 days until the cells reached 70-80% confluence. Upon harvesting, an amount of 32x10⁶ MSPC, 32x10⁶ ECFCs or the combination of 6.4x10⁶ MSPC + 25.6x10⁶ ECFC (1:5) were re-suspended in 300 µL ice-cold extracellular matrix (*In Vitro* Angiogenesis Assay Kit, Millipore, Billerica, MA, USA) and were injected subcutaneously into immune-deficient NSG mice. Three

transplants per condition containing 32×10^6 cells (the appropriate amount of protein lysate required for protein microarray analysis) were harvested after one day (three mice and three plugs per condition were used). Explants were snap-frozen in liquid nitrogen for one day and subjected to the protein lysis protocol (Figure 3 E). Plugs were homogenized using Triton X-100 lysis buffer (400 μ l) supplemented with phosphatase- and proteinase-inhibitors (Roche, IN, USA). The plugs were further homogenized using MagNAlyser Centrifugation (700 x g, 20 s) and sonification (5 times for 10 s with 10 s ice-incubation intervals; Imlab, Boutersem, Belgium). The proteolysis procedure was finalized using ultracentrifugation (100,000 x g, 30 min; Beckman Coulter GmbH, Vienna, Austria) to allow for maximum release of the expressed proteins. Protein concentrations were identified by a Bradford assay (Bio-Rad, CA, USA) and optical density (OD) was measured with a Spectramax instrument (Molecular Devices, Sunnyvale, CA, USA). The extracted protein materials were stored in form of 100 and 500 μ g aliquots at -80°C until further use. Upon detecting vessel formation in the parallel transplants harvested at day 14 and 56, protein lysates derived from various cellular combinations were subjected to the KinexTM antibody microarray as a customized service (Kinexus Bioinformatics Corp., Vancouver, Canada, www.kinexus.ca) to identify and compare protein expression alterations.

5.3.5.2. Antibody-mediated Detection of Signaling Molecules

In order to detect phosphorylated proteins, protein lysates derived from the 24 h plugs were subjected to KinexTM antibody microarray (Kinexus Bioinformatics Corp., Vancouver, Canada, www.kinexus.ca). The microarrays were designed to

compare various cellular combinations and allow for detection of changes in protein expression and phosphorylation (Figure 3 E). Briefly, an amount of 50 μ g of protein lysates were labeled with appropriate fluorescent dye, and thereafter subjected to blocking steps to minimize non-specific binding. For protein detection, dual-chamber incubation slides were used with the possibility to load two samples side by side on the same chip (Figure 3 E). After sample-antibody incubation and washing steps for removing unbound proteins, a pair of 16-bit images from each array was captured with a Perkin-Elmer ScanArray Reader laser scanner (Waltham, MA, USA). Signal quantification was performed using ImaGene 8.0 from BioDiscovery (El Segundo, CA, USA) using predetermined settings for spot partitioning as well as background minimization. Use of a single dye, non-competitive binding method in this technology helped to reduce the risk of false negative/positive binding, however as this issue is inevitable in protein microarray technique, the validation of microarray results for the target of interest through an alternative method (e.g. immunoblotting) is crucial. A cartoon indicating the principle of antibody-mediated detection of signaling molecules using microarray technology is depicted in Figure 4.

To identify the significantly expressed targets, statistical analysis was used according to the manufacturer's protocol. Z normalization of the results was used based on its greater stability when testing signal localization in the broad distribution of values within the sample, rather than adjusting all of the signals in a sample based on a single common value. Z scores were calculated by subtracting the average intensity of all spots within a sample from the raw intensity for each spot, and dividing it by the standard deviations (SD) of all of the measured intensities within each sample (Cheadle et al., 2003). Z ratios were further

calculated using the difference between the averages of the detected protein Z scores and dividing by the SD of all of the differences for that particular comparison.

5.3.5.3. Data Analysis

Proteomic microarray data analysis was performed with the help of Kinexus customer service (www.kinexus.ca) and Dr. Mario Gimona, Paracelsus Medical University of Salzburg

Z normalization for the data was used to identify relevant regulated targets (Cheadle et al., 2003). Calculated Z ratios can be used in various comparisons without further reference to the unique standard deviations by which they were derived. Z ratio of equal to or more than ± 1.2 was considered as a significant change based on the manufacturer's instructions. The protein microarray data representing detected signaling proteins as well as significantly expressed targets were analyzed using Uniprot (www.uniprot.org) and Swissprot database (www.swissprot.org). Putative protein-protein interactions were further investigated using String database 8.3 (www.string-db.org). Differentially expressed signaling proteins were selected based on the frequency of detection in the samples as well as their biological relevance. A summary of the project strategy is shown in **Figure 3**. Array results were submitted to Gene Expression Omnibus (GEO accession N° GSE45896).

5.3.5.4. Target confirmation: Western Blot Analysis

Western blot experiment has been performed together with Ehsan Bonyadi Rad, Medical University of Graz.

In order to confirm the expression of the target of interest in proteomic assessment, protein lysates were further subjected to Western blot analysis for human caspase-4. Briefly, the stored protein lysates were thawed and their concentrations were re-measured using Bradford assay (BioRad). An amount of 15 µg protein lysate per lane was run on a 10% SDS-polyacrylamide gel. The bands within the gel were transferred to polyvinylidenedifluoride (PVDF) membrane. Membranes were thereafter incubated with caspase-4-specific primary antibody (N-15, 0.1 µg/mL, Santa Cruz, CA) overnight or with glyceraldehyde 3-phosphate dehydrogenase (GAPDH, FL-335, 0.025µg/mL, Santa Cruz) serving as house-keeping protein control, followed by incubation with peroxidase-conjugated secondary antibodies (donkey anti-goat, 0.025 µg/mL and goat anti-rabbit, 0.025 µg/mL, both Santa Cruz, for caspase-4 and GAPDH antibodies, respectively). In order to develop the signals, the blot was incubated with ECL reagent (Amersham) and exposed to x-ray film. Signal intensity of the visualized bands was quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>).

5.3.6. *In vivo* signaling molecule inhibition in MSPC and ECFC

Cell preparation was performed together with Daniela Thaler and Birgit Feilhauer. Plug preparation was carried out together with Andreas Reinisch, Department of Hematology, Medical University of Graz.

Caspases were selected from the proteomic data to investigate their impact on vasculogenesis. In order to identify the role of pre-selected signaling molecules in vessel formation, caspase-4 and pan-caspase chemical inhibitors (Caspase-4 inhibitor Z-LEVD-FMK and pan-caspase inhibitor EZ-Solution™ Q-VD-OPh (both BioVision) were used to investigate the influence of significantly regulated

caspases displayed in the co-transplant array. For titration of the highest non-toxic inhibitory concentration of the selected kinase inhibitors, ECFC and MSPC were seeded in triplicate at a density of 500 cells/cm² and treated with serially diluted inhibitor concentrations (Z-LEVD-FMK from 0.2 µM to 5 µM, and Q-VD-OPh from 0.08 µM to 50 µM with 5-fold dilution steps) for up to 48 hours. The cells were treated with the appropriate amount of DMSO as a control (see Figure 17). To determine whether caspase inhibition affected the viability of ECFC and MSPC, we performed annexin V staining to evaluate the respective proportion of apoptotic cells by flow cytometry according to the manufacturer's protocol (Navios cytometer, Beckman Coulter, www.bc-cytometry.com) as well as trypan blue exclusion to directly count and quantify viable and dead cells. Cell suspensions were washed once with phosphate-buffered saline (PBS) and centrifuged (5 min, 300 x g, 4°C). Cell pellets were re-suspended in 100 µL ice-cold binding buffer and stained with 10 µL of annexin V. After a 15 min incubation in the dark at room temperature, cells were analyzed on a FACS Calibur instrument (BD, Bioscience, CA, USA) within 30 min. Staurosporin-treated (2 µM; TocrisetTM, Tocris bioscience, Bristol, UK) and DMSO-treated cells were used as positive and negative apoptosis controls, respectively. For trypan blue staining, trypsinized cells were incubated with 0.4% trypan blue solution (Gibco life technologies, Grand Island, NY, USA) and enumerated using a Bürker-Türk counting chamber (Bio-Rad, CA, USA).

5.3.7. *In vitro* capillary-like structure formation

Umbilical cord blood, umbilical cord, white adipose tissue, bone marrow, amnion and peripheral blood-derived ECFC were seeded in 225 cm² tissue culture flasks

(BD Biosciences) and incubated in 37°C until confluence. Upon trypsinization followed by a washing step with pre-warmed PBS, 7.5×10^4 ECFCs were re-suspended in 2 mL EGM-2 supplemented with 10% pHPL and seeded on 9.2 cm² polymerized Matrix (Angiogenesis assay kit; Millipore, Billerica, MA, USA) as described previously (Rohde et al., 2007). To assess the impact of small molecule caspase inhibitors on capillary-like network formation, ECFCs were incubated with 2 µM of caspase-4 inhibitor (Bian et al., 2009) and 10 µM of pan-caspase inhibitor (Caserta et al., 2003) in 225 cm² tissue culture flasks for eight hours, washed with pre-warmed PBS (1X) and seeded on the solidified Matrix. After 24 hours, endothelial networks were captured using a Color View III camera on an Olympus IX51 microscope with the analySIS B acquisition software (all Olympus, Hamburg, Germany). Numbers of branching points were evaluated using ImageJ software (<http://rsbweb.nih.gov>) as described (Hofmann et al., 2012a).

5.3.8. Histological Experiments and Analysis

Plugs harvested after 2, 8, and 24 weeks were fixed using 3.7% neutral-buffered formalin in 4°C overnight thereafter dehydrated in a graded series of ethanol prior to being embedded in paraffin. Paraffin-embedded tissue sections (4µm) were deparaffinized using xylene and descending alcohol series prior to Hematoxylin/Eosin (H&E), Movat's pentachrome as well as immune histochemistry staining.

5.3.8.1. Hematoxylin-Eosin Staining (H&E)

Sections (4 μm) of formalin-fixed (3.7% neutral buffered, overnight) paraffin-embedded plugs were deparaffinized using heat (68°C hot chamber) for an hour followed by treatment with xylene and descending alcohol series (5 min each) and stained by routine hematoxylin-eosin (H&E) protocol (Lillie, 1965). The sections were stained using Mayer's hematoxylin (Sigma Aldrich) for 3 min, thereafter rinsed in running tap water and consequently stained with eosin phloxin (Sigma Aldrich) for 30 sec. Sections were then washed with running water, rehydrated in ascending alcohol series (2 min each) and mounted using TissueTek[®] Glas[™] mounting media (Sakura). The nuclei, cytoplasm and red blood cells were differentially stained blue, purple and red, respectively (Lillie, 1965). The protocol has been optimized based on the specimen properties.

To quantify vessel formation, luminal structures containing red blood cells were considered as perfused micro-vessels and counted using ImageJ (<http://rsbweb.nih.gov>). For each condition, 5 high power fields (200x original magnification) of H&E-stained slides were examined by two independent observers using counting function in ImageJ software. For *in vivo* vasculogenesis experiments, micro-vessel density was quantified by analyzing the number of red blood cell-containing luminal structures additionally at three different cutting depths (150 μm intervals).

5.3.8.2. Immune Histochemistry Staining

Sections (1.5 μm) of formalin-fixed (3.7% neutral buffered, overnight) paraffin-embedded plugs were de-waxed prior to antigen retrieval using heat (68°C/160 W, 40 min) at either high or low pH (pH 9 or 6, respectively) depending on the protein

target and antibody properties followed by a descending alcohol series (2x xylol, 5 min each; 1x ethanol 100%, 5 min; 1x ethanol 90%, 5 min; 1x ethanol 70%, 5 min; 1x ethanol 50%, 5 min; 1x PBS, 5 min, distilled water , 5 min) as previously described (Hofmann et al., 2012a). Endogenous peroxidases were inhibited using H₂O₂ (15 min) and non-specific antibody binding was minimized using Ultra V Block (Thermo Scientific; 7-10 min), mouse-on-mouse blocking (MOM, Vector; 90 min) and serum-free protein inhibiting reagent (Dako; 30 min). Sections were incubated (45 min, RT) with unconjugated monoclonal mouse anti-human antibodies against CD31 (clone: JC70A, 5.15 µg/mL, Dako), CD90 (clone: EPR3132, Abcam, Cambridge, MA, USA) Vimentin (clone: V9, 0.78 µg/mL, Dako), von Willbrand factor (vWF, clone F8/86, Dako) or appropriate amount of IgG1 (BD) as the control. The signal was developed using Ultravision LP large volume detection system horseradish peroxidase (HRP) polymer (Thermo Scientific) followed by diaminobenzidine (DAB) or alkaline phosphatase detection system using either fast blue or fast red (Vector) according to the manufacturer's protocols. Avidin-biotin inhibiting (Vector) was used before staining with biotinylated polyclonal rabbit anti-human CD90 (EPR3132, Abcam) and biotinylated monoclonal goat anti-rabbit IgG1 (BD). Streptavidin-horse radish peroxidase conjugate (Dako) and diaminobenzidine (DAB) were used to detect and visualize the positive signal. Sections were counter-stained using hematoxylin for 1-2 min. The protocol has been optimized based on the specimen and antibodies properties.

In order to quantify vasculogenesis in ECFC+MSPC plugs, luminal structures filled with RBCs representing hu-CD31 and vimentin positive signal were considered as perfused functional vessels and quantified by two independent observers using

five high power microscopic fields (200x original magnification) of H&E-stained sections from the related plugs using ImageJ software. Additionally, sections from three different cutting depths (150 μ m intervals) were assessed for histology analysis using five high-power microscopic fields with 200x original magnification using bright field microscope (BX51, Olympus). The number of perfused vessels was quantified using ImageJ software (<http://rsbweb.nih.gov>).

5.3.8.3. Movat's Pentachrome Staining

Paraffinized sections (4 μ m) were hydrated using distilled water and stained in alcian blue (stain for ground substance and mucin, Dako, 20 min). The slides were washed under running water for 10 min followed by alkaline alcohol treatment (to convert the alcian blue into insoluble monastral fast blue) for 1-2h and washing steps (10 min) to remove alkaline alcohol. The sections were incubated with Verheoff's hematoxylin solution (15 min, Sigma Aldrich) to promote nuclei and elastic fiber staining. Upon rinsing with dH₂O (4 times, each time for 2 min), the slides were exposed to 2% aqueous ferric chloride several times to promote differential staining of the different tissues. The differentiation process was checked microscopically for black elastic fiber staining versus gray background. Upon washing steps using sodium thiosulfate (1 min) and distilled water (5 min), the sections were stained with scarlet-acid fuchsine (8:2, Dako) for 90 sec (Stain for fibrinoid, fibrin-intense red, muscle-red). After rinsing steps in distilled water and 0.5% acetic acid water, the slides were incubated with 5% aqueous phosphotungstic acid (5-10 min) and checked microscopically for further differentiation to collagen indicator (pale pink) versus bluish ground substance.

After differentiation was completed, the slides were rinsed in 0.5% acetic acid water (5 min, 2X) followed by 100% alcohol (5 min, 3X).

5.3.9. Tunnel Assay

In order to test apoptosis *in vivo* and *in vitro*, ECFC+MSPC plugs harvested one, 14 and 56 days post transplantation and ECFC+MSPC admixed with matrix incubated at 37°C for three and five days *in vitro* were analyzed for apoptosis using TdT-mediated dUTP nick end labeling (TUNEL Assay; Dead End™ Fluorometric System, Promega) in accordance with manufacturer's instructions. Nuclei were counter-stained using propidium iodide. To develop a positive control, slides were incubated with 1U DNase 1 (10 min, RT, Fermentas). For a negative control, incubation buffer was prepared without rTdT. Signals were documented using confocal microscopy (LSM510 Meta, Zeiss).

5.3.10. Statistics

All data are shown as mean $\pm$ standard deviation (SD). Statistical differences were determined using unpaired student's t-test or ANOVA using post-hoc Bonferroni test for multiple comparisons. Differences were considered significant (*) when the P-value was less than 0.05, very significant (**) when P-value was less than 0.001 and extremely significant (***) when P-value was less than 0.0001.

6. RESULTS

(The following chapter follows the publication Rohban et al. 2013 (Rohban et al., 2013))

6.1. Contribution of ECFC and MSPC in Neo-Vasculogenesis

ECFC and MSPC, and the co-culture of both cell types were phenotypically characterized using various surface markers (Figure 5 A) including CD90, CD73, CD105, CD19, CD15 and CD31 markers (Figure 5 B). In addition other surface markers such as CD3, STRO-1, CD166, HLA-ABC, CD13, CD29, CD49a, CD63 and CD44 were assessed for their presence on MSPC and ECFC surface (Figure 5 A). CD90 (also known as thymocyte antigen (Thy-1)) is a glycosylphosphatidylinositol-anchored protein on the surface of human neurons, fibroblasts, hematopoietic stem cells, early T and B cells as well as mesenchymal stem cells and pericytes (Blair et al., 1997, Dominici et al., 2006, Bergers and Song, 2005). CD90 is involved in some non-immunological cell processes such as cell adhesion and migration, cancer cell growth as well as cell death (Rege and Hagood, 2006).

CD73 antigen is a 70 kDa glycoprotein expressed on subsets of T and B lymphocytes, follicular dendritic cells, endothelial and mesenchymal stem cells (MSCs) (Pittenger et al., 1999).

CD105 antigen, also known as endoglin, acts as a receptor for TGF- β 1 and TGF- β 3. An epitope of CD105 antigen is identified by the SH-2 antibody (Barry et al., 1999) which is produced against human mesenchymal stem cells (MSC) representing mesodermal commitment capacity (Majumdar et al., 2000). Therefore, this marker can be used for investigations on mesengensis. Mature

endothelial cells and some leukemic cells with B lymphoid origin may also express CD105 (Majumdar et al., 2000).

Human CD19 antigen is a 95 kDa transmembrane glycoprotein of the immunoglobulin superfamily. It is present on B cells during most stages of cell differentiation. CD19 is also expressed on follicular dendritic cells. On B cells, CD19 together with CD81, CD21 and CD225 (Leu-13) forms a complex that serves as signal transducer (Pesando et al., 1989).

CD15 antigen is located on human myelomonocytes. It is also found on neutrophils and eosinophils but basophils or lymphocytes lack this surface protein (Kerr and Stocks, 1992) .

The CD15 antibody identifies the 3-fucosyl-N-acetyl-lactosamine structure which is also known as stage-specific embryonic antigen 1 (SSEA-1) and serves as a common surface marker for pluripotency on mouse embryonic stem cells and iPS cells. On human cells however, SSEA-1 is present exclusively on early differentiating cells (Kerr and Stocks, 1992).

CD31 is a single-chain transmembrane glycoprotein of 120–140 kDa. Nearly all monocytes, platelets, lymphocytes and granulocytes express this marker (Kimmig et al., 2002) . CD31 is expressed with high frequency on endothelial cells, notably at their juncture (Kimmig et al., 2002).

CD13 is a transmembrane glycoprotein of 150–170 kDa. It is present on myeloid progenitors, endothelial cells, granulocytes, and epithelial as well as granular lymphoid cells. It is highly expressed in tissues such as kidney, intestine, and placenta. CD13 deficiency may result in different types of lymphoma and/or leukemia (Look et al., 1989, Yeager et al., 1992, Favaloro et al., 1993).

CD29, a 130 kDa single-pass membrane protein is also known as integrin beta (Brakebusch et al., 1997). It makes heterodimers with integrin α (s) to form functional integrin receptor that binds to various extracellular molecules such as collagen, fibronectin and laminin. The cytoplasmic domain of CD29 can cross-talk with cytoskeletal and signaling molecules. In humans, CD29 can be found in four different isoforms (β 1A, β 1B, β 1C, β 1D) (Brakebusch et al., 1997, Altruda et al., 1990, Shih et al., 1993).

Also known as integrin α 1, CD49a is a heterodimeric integrin receptor of 200 kDa. It interacts with integrin β 1 (CD29) to make the VLA-1 complex which serves as a receptor for collagen and laminin (Boiret et al., 2005). Integrin α 1 participates in cell-cell adhesion and is expressed by NK cells, fibroblasts, activated T cells, neuronal cells as well as mesenchymal cells (Boiret et al., 2005, Miettinen et al., 1993, Hemler et al., 1985).

CD63 is a member of tetraspanin glycoprotein family, as it is recognized by four membrane spanning domains. CD63 is expressed in the plasma membrane and membrane of several cellular compartments including endosomes and lysosomes. MHC class II molecules in dendritic cells and B lymphocytes interact with CD63 glycoprotein (Mahmudi-Azer et al., 2002, Vischer and Wagner, 1993, Pols and Klumperman, 2009).

CD44 is a marker for several types of cancer stem cells (CSC) such as breast CSC that have higher potential to develop metastatic tumors (Al-Hajj et al., 2003), colorectal (Dalerba et al., 2007), pancreatic (Li et al., 2007), and prostate CSC (Patrawala et al., 2006, Collins et al., 2005). CD44 also affects tumor cell migration and adhesion; therefore it elevates tumor cell aggregation and outgrowth (Aruffo et

al., 1990). CD44 is also present on cells with mesodermal origin including fibroblasts, hematopoietic and glial cells (Aruffo et al., 1990).

STRO-1 is a 75 kD cell surface marker that is produced by stromal cells in human bone marrow (Simmons and Torok-Storb, 1991, Ning et al., 2011). In the bone marrow-derived cell population, the frequency of fibroblast colony-forming cells (CFU-F) is approximately 100-fold higher in the Glycophorin A-/STRO-1+ population rather than in the Glycophorin A+/STRO-1+ colonies (Simmons and Torok-Storb, 1991, Ning et al., 2011). It has been revealed that the BM-derived cell populations that are enriched in STRO-1+ fraction can differentiate into mesenchymal cell lineages such as hematopoiesis-supportive stromal cells that are phenotypically similar to vascular smooth muscle-like cells as well as osteoblasts, chondrocytes and adipocytes (Gronthos et al., 1994, Encina et al., 1999, Oyajobi et al., 1999, Dennis et al., 2002).

CD3 biomarker is present in the cytoplasm of pro-thymocytes from which T-cells are differentiated in the thymus (Singer, 1974). The pro-thymocytes form common thymocytes, which ultimately differentiate into medullary thymocytes; in the latter stage CD3 antigen migrates to the cell membrane. CD3 is expressed on all mature T-cells as well as a vast majority of T-cell lymphomas and thus can be used to distinguish those malignancies from B-cell and myeloid neoplasms (Williams et al., 1990, Singer, 1974, 1991).

CD166 is a 100-105 kD transmembrane glycoprotein. In humans the ALCAM gene is responsible for encoding this protein (Bowen et al., 1995, Davies and Jiang, 2010). CD166 is expressed on activated T cells, monocytes, epithelial cells, fibroblasts, neural and melanoma cells. CD166 has been shown to mediate adhesion of thymic epithelial cells and CD6+ cells during intrathymic T cell

development. CD166 has also been introduced as a potential marker for cancer stem cell recognition (Bowen et al., 1995, Davies and Jiang, 2010).

HLA-ABC refers to a number of human genes located on chromosome 6 encoding proteins that transport antigens from inner cell to the cell surface. The related proteins are also termed MHC, or major histocompatibility complex, consist of specific loci referred to as HLA-A, HLA-B, HLA-C, and HLA-D (Parham and Ohta, 1996) .

The ECFC and MSPC cellular populations were checked for purity using marker profiling prior to use for *in vitro* and *in vivo* experiments (Figure 5). Accordingly, trypsinized cell populations were considered pure when they were $\geq 95\%$ positive for their positive markers and $\leq 2\%$ positive for their negative markers (Dominici et al., 2006). MSPC and ECFC populations were thereafter stored long term to be used for *in vitro* and *in vivo* assays.

Transplantation of sole ECFC or MSPC or co-transplantation of both cell types in a defined 5:1 (80:20) ratio subcutaneously into the NSG mouse (Reinisch et al., 2009) was carried out to investigate the early molecular interactions that allow these two mesodermal-derived stem cell types to contribute to vessel formation *in vivo* (Figure 3 B). Upon plug harvesting after two, eight and 24 weeks (6 months), vessel formation was evaluated macroscopically and histologically in the explants (three mice and three implants per condition and per time point were used; Figure 3 D). Macroscopic features of the explanted plugs indicated the development of perfused red blood cell-containing vessels in the ECFC+MSPC plugs *in vivo* that was further confirmed by immune histochemistry analysis. The red color of the plugs explanted after two and eight weeks macroscopically indicated patent red

blood cell-containing mature micro-vessel formation after ECFC+MSPC co-transplantation (Figure 6 A and B, upper panels).

The human origin of these newly formed vessels was confirmed using human vimentin immune histochemistry (Figure 6 A, B, lower panels). The contribution of ECFC in the neo-vessel establishment was evaluated using anti-human von Willebrand factor (vWF) and CD31 immune histochemistry as surface markers for human ECFC (Figure 6 A, B). The intimate interaction of ECFC forming the inner layer of the vascular lumen and MSPC serving as pericytes providing stability of the vasculatures was also confirmed by human CD31 and CD90 analysis in an extended time course (3 mice and 3 plugs per condition, per time course were used; Figure 7). In contrast, the vessels in the MSPC only plugs did not stain for human vimentin and CD31; therefore, these vessels were presumably due to murine cell invasion into the plug leading to murine vessel in-growth (Figure 6 A, B) as indicated previously in other studies (Allen et al., 2011, Hofmann et al., 2012a). A longer time course (8 weeks) of MSPC-only implantation *in vivo* led to an early chondrogenesis state (Figure 6 B, middle panel), whereas a higher cell number and extended implantation time resulted in vascularization in ECFC-only plugs *in vivo* (Figure 8, 9, 10). The micro-vessels were formed in the plugs containing a combination of MSPC+ECFC *in vivo* 2 weeks after implantation, suggesting a supportive role for MSPC serving as pericytes stabilizing the developed vasculatures as indicated previously (Reinisch et al., 2009). The stability and functionality of the established micro-vessels *in vivo* was further confirmed in implants harvested after 6 months (24 weeks) using anti-human vimentin immune histochemistry as well as Movat's staining (Figure 11). The use of human anti-vimentin immune histochemistry in this study was performed with

regard to the mesodermal origin of ECFC and MSPC which are known to express vimentin as a surface marker (three mice and three plugs per condition per time course were used). These micro-vessels derived from the combination of ECFC+MSPC remained stable in the plugs regardless of the original implanted cell density (Figure 8, 9, 10, see also figure 11) whereas vasculogenesis in ECFC only plugs *in vivo* depended on the number of injected cells (Figure 8, 9, 10, see also figure 12), tissue of origin (Figure 12) as well as time point (Figure 8, 9, 10). Plugs containing solely ECFCs with a higher transplantation cell number and later in the time course were able to establish functional micro-vessels (the number of implanted and harvested plugs was three per condition and time point). ECFC and MSPC from different tissue sources such as bone marrow, white adipose tissue, peripheral blood, umbilical cord and umbilical cord blood were tested to investigate their potential of vessel formation *in vivo* (Figure 13 A, B and C, see also figure 14). The result showed that both cell types derived from white adipose tissue and umbilical cord blood, and MSPC derived from bone marrow are the most potent cells in course of neo-vasculogenesis, but also the umbilical cord blood-derived MSPC co-transplanted white adipose tissue-derived ECFC led to a higher number of established micro-vessels within the matrix *in vivo* (Figure 13 C).

6.2. Expression of Signaling Molecules upon Co-transplantation

In order to get insights into the early signaling events in the cross-talk between MSPC and ECFC that could be of potential relevance for neo-vasculogenesis, we analyzed the expression of signaling molecules using an established vasculogenesis model (Figure 3).

ECFC and MSPC in this experiment were isolated and used from the same donors (autologous cell pairs) to avoid likely inter-individual variations as well as histocompatibility antigen-dependent protein expression that could potentially confound the results of protein microarray analysis.

To investigate the early signaling molecule expression during neo-vessel formation, protein lysates derived from plugs of ECFC or MSPC or co-transplantation of both cell types in a defined 5:1 ratio (20:80) were harvested one day (24 hours) after implantation prior to vessel assembly and were subjected to protein microarray analysis (www.kinexus.ca). The antibody-based protein microarray was used to detect up regulated signaling molecules in explants harvested one day after subcutaneous implantation (Figure 3 C; three mice and three plugs per condition per time point were used). The protein array results were submitted to Gene Expression Omnibus (accession number GSE45896).

The communication between these cell types has led to successful vasculogenesis as demonstrated in parallel transplants (Figure 3 D, F, G and Figure 6 A, B). This strategy allowed us to identify signaling events that were activated in either cell population as compared to the co-transplant model (Figure 15). Protein expression analysis in extracellular matrix without admixed cells was meant to subtract background signaling serving as a control.

Protein microarray analysis via target protein clustering revealed that most instances of significantly (Z-ratio equal to or more than ± 1.2) different expression of signaling proteins in neo-vasculogenesis-competent co-transplants belonged either to the kinase or caspase families (Table 3).

In particular, caspase-4 and 7 were up regulated in all four co-transplant microarrays, caspase-2 and 12 were up regulated in three out of four while

caspase-5 and -6 were expressed only in one out of four co-transplant microarrays compared to ECFC (group I) or MSPC transplants (group II), cell-free matrix (group III) or ECFC/MSPC co-cultured *in vitro* (group IV) (Table 3 and Figure 15).

DNA protein kinase (DNAPK), calcium/calmodulin-dependent protein kinase-2 (CaMK-2), never-in-mitosis-related protein-serine kinase 2 (Nek2), and the human ErbB2 receptor-tyrosine kinase as the kinase family proteins were expressed with higher frequency in 4/4 and 3/4 co-transplant microarrays, respectively.

Extracellular signal-regulated serine protein kinase (Erk 1/2) was expressed in 2/4 co-transplant arrays. The FLJ35932 serine kinase (STK33), serine phosphatase 1 catalytic subunit (PP1/Cb) and nerve growth factor receptor tyrosine kinase (NTRK1) were down regulated in four, three and two comparative microarrays, respectively (Table 3).

Up regulation of caspase-4 protein in ECFC/MSPC co-transplants 24h after transplantation was further confirmed by Western blot analysis of the protein lysates derived from the same explants that were subjected to protein array analysis (Figure 16).

6.3. Blocking Caspase Signaling Leads to Impaired ECFC Network Formation *in vitro*

Vascular-like network formation is a frequently used assay for studying ECFC vasculogenic capacity *in vitro* (Grant et al., 1989, Davis and Camarillo, 1995, Rohde et al., 2007). To test its relevance for *in vivo* neo-vasculogenesis, the impact of caspase-4 and general caspase signaling on *in vitro* network formation was analyzed. To determine the potential toxicity of the caspase inhibitors

applicable to *in vitro* as well as *in vivo* assessments, concentrations of caspase-4 and broad-spectrum caspase inhibitors were titrated based on previous studies (Caserta et al., 2003, Bian et al., 2009, Kuzelova et al., 2011) (Figure 17).

Untreated umbilical cord-derived ECFC, used as positive control, were capable of developing vascular-like networks covering the matrix surface after one day (24 hours) (Figure 18 A). ECFC pre-treatment with caspase-4 and general-caspase inhibitor led to significantly impaired vascular like-network formation. ECFC network quantification confirmed a considerable reduction in the number of branching points in caspase-4 and general-caspase inhibitor-treated ECFC, whereas capillary like-network formation and number of branching points remained unaffected when only the inhibitor vehicle (DMSO) was used for pre-treatment of the ECFC (Figure 18, B-E). The experiment was carried out using three different ECFC donors of each origin.

6.4. Blocking Caspase Signaling Results in Diminished Neo-vasculogenesis *in vivo*

Since the co-transplantation of MSPC together with ECFC is proven to be a stable and reproducible model for neo-vasculogenesis, this model was used to verify the impact of caspase signaling on vessel formation *in vivo*.

A decrease in the number of developed micro-vessels was detected in plugs containing umbilical cord-derived ECFC + umbilical cord-derived MSPC pretreated either with caspase-4 or pan-caspase inhibitor and harvested two weeks post implantation (two mice and two plugs per condition were used; Figure 19 B).

To address a possible debate on whether the role of caspases in vessel formation was restricted to umbilical cord-derived cells or caspase signaling has impact on vessel formation regardless of the tissue of origin, other cell sources including MSPC from bone marrow ,ECFC from white adipose tissue and MSCP and ECFC from human umbilical cord blood were additionally assessed. Based on the vasculogenesis potential of various cell sources (Figure 13 A, B), the combination of white adipose tissue-derived ECFC and umbilical cord blood-derived MSPC (Figure 20), and umbilical cord blood-derived ECFC and bone marrow-derived MSPC (Figure 21) were additionally tested for caspases-mediated vessel formation. Despite variation in the total numbers of vessels developed, caspase inhibition diminished vasculogenesis regardless of the ECFC/MSPC sources (Figures 19 B, 20 B and 21 B); two mice and two plugs per condition were used).

A significant reduction in vasculogenesis was observed when ECFC were treated by a caspase-4 inhibitor prior to co-transplantation regardless of the ECFC source (six mice and six plugs per condition were used for analysis, pooled from the experiments shown in Figures 19, 20 and 21). Although caspase-4 inhibition of MSPC did not have a significant impact on vasculogenesis, blocking caspases-4 both cell types resulted in diminished vessel formation comparable to that of ECFC only. These findings were again regardless of the ECFC or MSPC source (Figure 22 A). In contrast, neo-vessel formation was significantly reduced upon general-caspase blocking of either cell type solely or the combination (six plugs per pre-treatment per cell source; Figure 22 B).

Although co-transplants containing white adipose tissue-ECFC and umbilical cord blood-MSPC revealed even higher potential of neo-vasculogenesis (Fig 13 B),

caspase blockade significantly impaired vessel formation (Figure 20), irrespective of the sources combined (Figures 19, 20 and 21 A, B). To exclude a possible bias because of un-even distribution of developed vessels within the three-dimensional transplant model, the number of created micro-vessels was further quantified in three different depths of sections within the implants harvested after two weeks. Despite an existing difference in the number of micro-vessels in various depths, vascular structure density did not alter significantly in untreated samples or samples pre-treated with caspase-4 or pan caspase inhibitors. (Figure 23 A, B).

7. DISCUSSION

(The following chapter follows the publication Rohban et al. 2013 (Rohban et al., 2013))

De novo vessel formation (neo-vasculogenesis) is an essential step which takes place in course of organ regeneration, wound healing, inflammation as well as tumor growth (Segura et al., 2002, Elmore, 2007, Krysko and Vandenabeele, 2008). Vascular structure formation consists of migration and replication of endothelial colony forming cells (ECFC) as the backbone of newly formed vessel (Segura et al., 2002, Elmore, 2007, Krysko and Vandenabeele, 2008) and mesenchymal stem and progenitor cells (MSPC) as pericytes which serve as vessel supporters and maintain micro-vessel stability (Reinisch et al., 2007, Schallmoser et al., 2007b, Reinisch et al., 2009, Hofmann et al., 2012b). In the process of neo-vasculogenesis, the cross talk between the two cellular

components through mediators and signaling molecules is inevitable, yet poorly understood.

A number of studies emphasized the importance of several members of the TGF signaling cascade in the process of vessel formation, stability and function. Genetic inactivation of *tgfb1* (Dickson et al., 1995) and impairment of genes encoding its receptors such as activin receptor-like kinase 1 (*alk1*) (Oh et al., 2000, Urness et al., 2000, Courtoy and Boyles, 1983, Oshima et al., 1996), TGF receptor II (*t_rll*) (Oshima et al., 1996) as well as *endoglin* (type III TGF receptor) (Li et al., 1999, Bourdeau et al., 1999, Arthur et al., 2000) and its effector Smad (Courtoy and Boyles, 1983, Yang et al., 1999, Chang et al., 1999) in mice have been shown to cause significant cardiovascular deficiency and embryonic lethality. In humans, mutations in endothelial cells expressed *ENDOGLIN* and *ALK1* may lead to bleeding due to vascular malformations (McAllister et al., 1994, Johnson et al., 1996). TGF-signaling has been shown to affect endothelial cells; various endothelial responses such as proliferation and differentiation are regulated through ALK1/Smad1/5 and ALK5/Smad2/3, respectively (Goumans et al., 2002). Endoglin has also been shown to promote ALK1 signaling, shifting the TGF response toward proliferation (Lebrin et al., 2004). Carvalho and colleagues reported that the aberrant TGF signaling in endothelial cells led to impaired TGF/ALK5 signaling in mesenchymal cells, inhibiting their differentiation into vascular smooth muscle cells (VSMC) and their participation in endothelial tube formation (Carvalho et al., 2004). TGF signaling in endothelial cells has been shown to elevate TGF expression and release which, also induces differentiation of vascular smooth muscle cells from surrounding cells (Carvalho et al., 2004).

Previous findings have also unraveled the involvement of angiopoietin–Tie2 signaling cascade in the endothelial-pericyte interaction (Maisonpierre et al., 1997, Witzanbichler et al., 1998, Uemura et al., 2002). Mutations in *TIE2* have been shown to result in malformed and malfunctioned human vessels and deficient vascular smooth muscle cells (Vikkula et al., 1996). Tie2 receptor has also been reported to be an endothelial-specific molecule (Sato et al., 1995, Davis et al., 1996) whereas Ang1 (its agonistic ligand) has been shown to be expressed mainly by mural cells (Suri et al., 1996, Sundberg et al., 2002). The findings delineate that Ang1 is a paracrine signal derived from pericytes and affects endothelial cells; therefore the Ang1–Tie2 signaling loop is crucial for vessel maturation and stabilization. Earlier reports support this idea showing that *Ang1*- or *tie2*-null mice die as a result of cardiovascular failure. The embryos of these animals show impaired angiogenesis, their blood vessels are poorly organized and not adequately covered by pericytes (Sato et al., 1995, Suri et al., 1996, Dumont et al., 1994) suggesting that Ang1 as a pericyte-derived signal regulates vascular maturation and quiescence (Armulik et al., 2005).

Another report by Iivanainen's group delineated that attachment of Ang-1 to Tie-2 results in up regulation of endothelial Heparin-binding epidermal growth factor -like growth factor (HB-EGF), which leads to vascular smooth muscle cell migration via binding to ErbB1 and ErbB2 (Iivanainen et al., 2003). Mice with HB-EGF impairment has been shown to suffer from severe heart failure similar to *erbB1* and *erbB2* knockouts (Iwamoto et al., 2003). Tie2 might affect mural cells directly since *in vitro* studies suggest that cultured vascular smooth muscle-like cells express Tie2 and respond to Ang1 (Iurlaro et al., 2003, Metheny-Barlow and Li, 2003). In the aorta ring sprouting assay, it has been observed that Ang1 elevates

endothelial sprout formation as well as mural cell recruitment; however, only the latter could be blocked by p38 mitogen-activated protein kinase inhibitors suggesting that Ang1 may affect both endothelial cells and pericytes in this system (Zhu et al., 2003).

PDGFR pathway has been shown to play an important role in pericyte recruitment to newly formed vessels (Hoch and Soriano, 2003, Betsholtz, 2004). During angiogenesis, sprouting endothelial cells release PDGF-B, which signals through PDGFR expressed by pericytes leading to migration and propagation of mural cells in the process of vessel maturation. The knockouts of *pdgfb* and *pdgfrb* display vascular dysfunction (Hellstrom et al., 1999, Lindahl et al., 1997, Crosby et al., 1998) due to lack of functional pericytes (Leveen et al., 1994, Soriano, 1994).

Vascular dysfunction might cause a compensatory up regulation of vascular endothelial growth factor A (VEGF-A) which can be detected in *pdgfb* and *pdgfrb* knockouts. Elevated levels of VEGF-A could also cause further malfunction such as vascular hemorrhage and leakage (Hellstrom et al., 2001a). PDGF-B signaling is not crucial for pericyte induction but pericyte populations lacking PDGF-B cannot expand along the vasculature (Hellstrom et al., 1999). The PDGF-B/PDGFR pathway is therefore critical for the pericyte population to migrate and spread along the vessel (Hellstrom et al., 1999).

Sphingosine-1-phosphate (S1P) plays a role in cellular crosstalk through certain G-protein coupled receptors (S1P1 to 5). S1P has been shown to trigger cytoskeletal alterations which affect cell proliferation, migration, and survival (Allende and Proia, 2002). Disruption of the *s1p1* (*edg1*) gene in mice is associated with vascular abnormalities including impaired pericyte coverage of micro-vessels (Liu et al., 2000). S1P1 expression was first recognized in

endothelial cells, however these receptors have been shown to be present in majority of cultured cells including endothelial and mesenchymal cells (Liu et al., 2000) . It has been shown, however, that endothelial-specific knockout of *s1p1* caused the mural cell impairments that also occurred in the full *s1p1* knockouts, suggesting that pericyte vessel coverage is mediated by the activity of S1P1 in the endothelial cells (Allende and Proia, 2002).

Notch signaling mediators play an important role in the development of arterio-venous (A-V) and venous endothelial structure in the establishing vessel (Lawson et al., 2001, Shawber and Kitajewski, 2004). In endothelial cells, activation of Notch signaling leads to induction of many arterial markers including ephrin B2 and CD44, and suppression of venous markers such as EphB4 (Lawson et al., 2001, Fischer et al., 2004).

Beside the signaling pathways indicated above, other mediators such as junctional molecules including connexin (Pries et al., 2010), claudin and occludin (Dejana et al., 2009), VE-cadherin (Strilic et al., 2009), chemokines and chemokine receptors including SDF-1 α and CXC chemokine receptor type 4 (CXCR4), as well as G-protein coupled receptors including GPR124 (Duda et al., 2011, Visentin et al., 2006, Kuhnert et al., 2010) and oxygen sensors such as the prolyl hydroxylase domain proteins PHD1-3 and hypoxia-inducible factor proteins (Majmundar et al., 2010, Hofmann et al., 2012a) have been shown to affect and govern vessel formation. N-cadherins in endothelial cells have been shown to be responsible for endothelium–pericyte crosstalk; knockout of N-cadherin in endothelium has been shown to significantly diminish VE-cadherin levels resulting in severe defective vasculature with impaired junctions (Luo and Radice, 2005).

Extracellular matrix (ECM) has also been shown to modulate the process of vessel formation either by promoting or inhibiting vessel assembly. Several ECM molecules, such as laminin, collagen and fibronectin facilitate endothelial cell migration, survival, growth and their ability to form capillary like structures, thus they are being considered to have proangiogenic properties. Fibronectin has an impact on endothelial cell adhesion, migration, proliferation and survival, and is therefore crucial for angiogenesis *in vivo* (Kim et al., 2000b, George et al., 1997). Fibronectin as an important component of extracellular matrices is present throughout the body tissues (Hynes and Yamada, 1982, Hynes, 2009). In a vessel, fibronectin is localized within the ECM surrounding endothelial cells, and is also present in the outer layers of the vessel wall (Labat-Robert et al., 1985, Takasaki et al., 1991) . Endothelial cells and vascular smooth muscle cells are also able to produce fibronectin locally, so that it can be found in form of a circulating soluble substance in the plasma (Hynes and Yamada, 1982, Hynes, 2009). Soluble fibronectin has been shown to return into tissue ECM by a cell-dependent regulated process (Sechler and Schwarzbauer, 1997, Akiyama et al., 1989). Disruption of the existing fibronectin or inhibition of its deposition in the matrix blocks the growth of endothelial cells (Mercurius and Morla, 1998). These and other data suggest a crucial role for matrix fibronectin in modulating cell growth (Kim et al., 2000b, George et al., 1997, Hynes and Yamada, 1982, Hynes, 2009, Labat-Robert et al., 1985, Takasaki et al., 1991, Sechler and Schwarzbauer, 1997, Akiyama et al., 1989, Mercurius and Morla, 1998). Endothelial cell survival and migration have been shown to be promoted by fibronectin (Re et al., 1994, Kim et al., 2002). Moreover, fibronectin attaches to VEGF and promotes migration of endothelial cells and activation of MAP kinase induced by VEGF (Wijelath et al.,

2002). Additionally, both fibronectin and its receptor $\alpha 5\beta 1$ integrin are overexpressed in the vascular tissue upon treatment with angiogenesis-promoting factors *in vivo* (Kim et al., 2000b). These findings indicate that a number of endothelial cell functions crucial for vessel formation are regulated by soluble and ECM fibronectin. Chemical inhibitors that block fibronectin matrix deposition have been shown to block the deposition of other proteins (e.g. thrombospondin-1) in the ECM (Sottile and Hocking, 2002), therefore fibronectin may modulate vessel formation by regulating ECM modeling in addition to regulating endothelial cell function. Fibronectin-knockout mice die during embryogenesis due to impaired vessel development and/or function (George et al., 1993, George et al., 1997). A number of other matrix molecules such as laminin, vitronectin and different types of collagen have also been shown to regulate endothelial cell adhesion and survival. Laminin 8 is the predominant laminin found in vascular basement membranes, and according to the literature the only laminin that could be found in capillary basement membranes at the time of embryonic development (Thyboll et al., 2002). Mice with defective laminin $\alpha 4$ chain (crucial for expression of laminin 8), fail to develop matured and functional micro-vessels (Thyboll et al., 2002) which could be due to defective capillary basement membrane assembly, as collagen IV, another vessel formation regulator in ECM (Xu et al., 2001) has been also shown to be interrupted in the absence of laminin 8 (Thyboll et al., 2002).

It has been unraveled that collagen I, III, and fibrillin play a role in stabilizing vessel wall as mutations in these proteins can result in vessel rupture (Lohler et al., 1984, Liu et al., 1997). In terminal stages of vessel formation, pericytes and smooth muscle cells home to the outer side of developing vessels to contribute to vessel wall stabilization partly through production of ECM components (Carmeliet and

Collen, 2000, Folkman and D'Amore, 1996). Although proangiogenic role of ECM components has been well studied, ECM molecules may also possess anti-angiogenic effects. Extracellular matrix molecules, including thrombospondin-1 and -2, have been recognized as anti-angiogenic factors (Good et al., 1990, Streit et al., 1999). Thrombospondin-1, initially described as a component of platelet alpha granules and secreted upon platelet activation is produced by endothelial as well as smooth muscle cells and fibroblasts (Mosher et al., 1982, Jaffe et al., 1983, Raugi et al., 1982). Thrombospondin-1 has been shown to block vessel formation stimulated by FGF, in case of wound healing and tumorigenesis (Good et al., 1990, Tolsma et al., 1993, Volpert et al., 1998). Thrombospondin-1 has been considered to induce endothelial cell apoptosis and thus blocks angiogenesis (Jimenez et al., 2000, Guo et al., 1997). This idea has been supported using thrombospondin-1 knockout mice, in which vessel formation is elevated in pancreatic islets and dermis (Crawford et al., 1998). The role of tumor suppressor p53 to serve as an anti-angiogenic factor is dependent on p53-mediated suppression of thrombospondin-1 (Dameron et al., 1994). Thrombospondin-2, a member of the thrombospondin gene family (Bornstein and Sage, 1994), can be found in tissue extracellular matrices (Bornstein and Sage, 1994). Thrombospondin-2 has been shown to inhibit endothelial cell outgrowth and block angiogenesis and tumor growth (Streit et al., 1999, Bornstein et al., 2000). Notably, thrombospondin-2 knockout mice illustrate an elevated vascular density in various tissues (Bornstein et al., 2000, Kyriakides et al., 2001).

Another extracellular matrix molecule that has been shown to serve as an antiangiogenic component is decorin, a proteoglycan that is crucial for collagen network arrangement (Danielson et al., 1997). Despite a report indicating a

proangiogenic role for decorin in collagen matrices (Schonherr et al., 1999), other findings show that decorin blocks the migration of endothelial cells (Kinsella et al., 2000, Davies Cde et al., 2001), and thereby inhibits tube-like formation (Davies Cde et al., 2001). Tumor cells engineered to express a higher level of decorin show less growth and impaired vascularization *in vivo* (Grant et al., 2002) which might be due to decorin's ability to down regulate VEGF production (Grant et al., 2002). Although not significantly up regulated, PDGFR, VEGFR, Smad and junction protein connexin were expressed in our co-transplant microarrays presumably due to the involvement of these signaling molecules in vasculogenesis processes but not necessarily during the initial phase of neo-vessel formation (for the extended array results see Gene Array Omnibus (GEO accession N°GSE45896)). The previous findings give an insight into the signaling events and mediators that play a role throughout vessel formation and maturation processes, however, in the current study we aimed to unravel the signaling pathways that are mediating the initial phase of neo-vessel formation upon the first 24 hours of ECFC and MSPC co-transplantation *in vivo*.

The aim was accomplished by (1) identifying 57 significantly expressed signaling molecules using antibody-based proteomic profiling of early (24 h)-harvested ECFC/MSPC co-transplants and (2) demonstrating the involvement of caspase pathways, in particular caspase-4, in neo-vessel formation by *in vivo* blocking experiments.

In this study we used an antibody-based proteomic strategy to detect signaling molecules operative during neo-vessel formation in early co-transplants. The strategy of comparative analysis allowed us to monitor signals that are up regulated upon co-transplantation as compared to transplants of either sole cell

population. Protein expression analysis in cell free extracellular matrix was meant to subtract the signals expressed from the background. In a fourth group the *in vivo* early vasculogenesis module (ECFC+MSPC co-transplantation, harvested after one day) was compared with ECFC plus MSPC mixed and cultured *in vitro* (to identify and exclude signals expressed upon mixture of both progenitor cell populations in the absence of vasculogenesis (The experimental procedure for protein microarray in this study has been described in the materials and methods, section 5.5.1. Sample preparation). Our proteomic profiling of the early *in vivo* signaling events revealed considerable manifestation of tyrosine and serine protein kinases as well as the death-associated components including caspases, the death-associated protein 6 (DAXX) and p53 involved in programmed cell death-associated pathways.

Apoptosis has been shown to play a crucial role in course of tissue repair and remodeling amongst vertebrates throughout life and allows them to deal with alterations produced by tissue homeostasis, infection and injury (Meier et al., 2000, Segura et al., 2002); thus it has been considered as a pro-survival mechanism (Meier et al., 2000). It has been reported previously that aberrant apoptosis pathways can lead to impaired angiogenesis (Segura et al., 2002). The role of the activation of death-associated pathways through the caspase cascade during the early phase of vasculogenesis or enhanced sensitization to irradiation of cancer cells using caspase inhibitor pretreatment has been studied in various models of vessel formation and tumorigenesis (Segura et al., 2002, Kim et al., 2008). It has been also shown that general-caspase inhibitor Z-VAD and chemical blockers of caspase-3 and -8 enhance radiation sensitivity of the cancer cells preventing tumor growth and expansion *in vivo* (Kim et al., 2008, Moretti et al.,

2009). The ability of a caspase-8 blocker to induce deficient adhesion and migration of endothelial progenitor cells (EPC) leading to impaired neo-vasculogenesis has also been reported (Scharner et al., 2009). Contradictory earlier studies supported the notion that activation of apoptosis signaling components can inhibit vessel formation (Brooks et al., 1994b, Schechner et al., 2000). Despite that controversy on the impact of apoptosis in angiogenesis, the precise role of caspases and in particular caspase-4 during regenerative vasculogenesis has not been addressed yet.

As a member of the caspase-1 subfamily, human caspase-4 is an inflammatory caspase located in the endoplasmic reticulum (ER) membrane that has not been well characterized to present (Hitomi et al., 2004, Martinon and Tschopp, 2004, Nadiri et al., 2006). However, it has been reported that caspase-4 activates the inflammosome through caspase-1 regulation (Sollberger et al., 2012) and has an impact in ER stress-induced apoptosis (Hitomi et al., 2004, Bian et al., 2009). In the current study, we found that caspase-4 was significantly up regulated in all four co-transplant protein microarrays, which suggests an activatory role for this inflammatory caspase during early phase of neo-vasculogenesis, along with numerous initiator and executioner caspases like caspase-2, -5, -6 and -7. The broad-spectrum caspase blocker used in the present study (Caserta et al., 2003) chemically inhibits executioner caspase-3 (Walsh et al., 2008), initiator caspases - 8, -9, and -10 (Chen and Wang, 2002) and inflammatory caspase-12 (Kersse et al., 2007). Previous studies indicate the impact of initiator caspase-8 and executioner caspase-3 and caspase-7 on endothelial cell survival during *in vivo* angiogenesis (Segura et al., 2002, Kim et al., 2008, Scharner et al., 2009, Moretti et al., 2009), suggesting the importance of these caspases during the initial stage

of vasculogenesis. Our data indicate a yet unknown function of the inflammatory caspase-4 in modulating vascular-like network formation *in vitro* and neo-vessel formation *in vivo*. In this study a caspase-4 inhibitor (Z-FMK-LEVD) and a non-selective-caspase inhibitor (Q-VD-OPh) were used which can inhibit caspase activity irreversibly without inducing cytotoxicity (Caserta et al., 2003, Bian et al., 2009, Kuzelova et al., 2011), thus indicating the druggability of caspases when targeted in therapeutic application. Whether caspase-4 activity during vasculogenesis is restricted to progenitor cells remains to be addressed in future studies (Ryoo and Bergmann, 2012).

A number of protein kinases expressed significantly in our study are also known to play a role in vasculogenesis. Extracellular signal-regulated protein kinases 1 and 2 (Erk1/2) and a class of mitogen-activated protein kinases (p38d MAPK) are members of the mitogen-activated protein kinase superfamily involved in MAPK/ERK signaling pathway responsive to stress stimuli and mediate cell proliferation, differentiation and apoptosis (Mebratu and Tesfaigzi, 2009). In a previous study the role of ERK-MAPK signaling in survival and migration of endothelial cells was investigated (Mavria et al., 2006) showing that expression of dominant-negative MEK1 can result in ERK-MAPK signaling blockade and tumor angiogenesis suppression (Mavria et al., 2006) as a consequence of up regulation of Rho-kinase signaling. Inhibition of Rho-kinase showed to undo the effect of ERK-MAPK blockade both *in vivo* and *in vitro* by promoting vessel elongation in tumors, suggesting a mediatory role for Rho-kinase through ERK-MAPK during vessel formation. The data suggests a mechanism through which ERK-MAPK signaling facilitates the survival and migration of endothelial cells through

suppression of Rho-kinase signaling (Mavria et al., 2006) leading to an effective vessel formation.

Ephrin B (EphB) receptor tyrosine kinases and the associated transmembrane ephrin-B2 ligands have been shown to play an essential role in vasculogenesis in the embryo (Gale and Yancopoulos, 1999). The EphB/ephrin-B2 expression pattern *in vivo* was interrupted using a transgenic mouse model (CAGp-ephrin-B2Tg) capable of expressing ephrin-B2 under control of a ubiquitous promoter, CMV enhancer-actin promoter-globin splicing acceptor (CAG) (Oike et al., 2002) to investigate the vasculogenesis alterations. A disrupted arrangement of microvessels has been detected in these animals whereas such deficiency was not observed in Tie-2p-ephrin-B2 Tg animal models with elevated levels of ephrin-B2 in vascular endothelial cells (Oike et al., 2002). These data show that non-endothelial cells expressing ephrin-B2 might cause alterations in the migration pattern of EphB receptor producing-endothelial cells into the inter-somitic microenvironment where there is a lack of ephrin-B2 expression. Consequently, the CAGpephrin- B2 Tg mice undergo neonatal death due to aortic problems as a result of impaired recruitment of vascular smooth muscle cells to the aorta (Oike et al., 2002), suggesting an important role for EphB/ephrin-B2 signaling between endothelial and mesenchymal cells during vessel formation and maturation. Another study suggests that Ephrin A1 and Slit2, a protein produced and secreted by floor plate or midline glia cells keeping the longitudinal axons from crossing the midline of the central nervous system (CNS) (Brose et al., 1999, Farmer et al., 2008), may cooperate to orchestrate a balance between angiostasis and angiogenesis (Dunaway et al., 2011). The findings of Dunaway and colleagues delineate that Slit2 promotes vessel formation via mTOR-dependent stimulation of

Akt and Rac GTPase when applied as a single agent *in vitro* and *in vivo*, whereas Slit2 has been shown to block vasculature remodeling in the presence of ephrin-A1 (Dunaway et al., 2011). Additionally, Akt and Rac GTPase have been shown to be inactive in the presence of Ephrin A1, while co-stimulation of Rac or Akt rescues the motility of endothelial cells and vasculature assembly (Dunaway et al., 2011), suggesting that Slit2 differentially mediates angiogenesis in concert with ephrin A1. In accordance with the previous findings, EphrinA1 might possess an early pro-angiogenic regulatory role during neo-vasculogenesis.

Protein tyrosine kinase ErbB2 (HER2) is known to be involved in cancer progress, tumor cell proliferation, cell migration and invasion as well as cell survival (Holbro et al., 2003). During the incidence of tumor-induced neo-vasculogenesis, endothelial cells undergo proliferation and differentiation; the ErbB signaling network has an impact on these processes. TGF- α -expressing cancer cells have been shown to induce ErbB1 expression in endothelial cells in orthotopic mouse tumor model (Baker et al., 2002). In addition, ErbB1 in endothelial cells may function indirectly during vessel formation process (Vinals and Pouyssegur, 2001) in a way that TGF- β stimulates autocrine production of TGF- α which has a crucial impact on promoting endothelial cell survival and differentiation into capillary-like network *in vitro*. Blocking ErbB1 activity in endothelial cells has been shown to keep TGF- β from stimulating differentiation in these cells (Vinals and Pouyssegur, 2001). A study on glioblastoma cell line has also revealed that ErbB1 mediates VEGF promoter activity through MAPK and PI-3K independent from the hypoxia-induced factor (HIF-1) pathway (Maity et al., 2000). In other reports cell lines of breast and lung cancer with ErbB2 activity displayed an increased VEGF mRNA level (Yen et al., 2000, Xiong et al., 2001, Yen et al., 2002).

Using small molecule kinase blockers to inhibit ErbB kinase activity resulted in a decreased level of VEGF expression with dramatic effects on the migration potential of the endothelial cells toward tumor cells *in vitro* (Hirata et al., 2002) as well as tumor-associated endothelial cell mortality (Bruns et al., 2000).

Calcium/calmodulin-dependent protein-serine kinases (CaMKs) are main components of calcium signaling during cellular responses such as motility, proliferation, and eukaryotic cell growth (Means and Dedman, 1980). Calcium ion (Ca^{2+}) is the most common signal transduction component in prokaryotic as well as eukaryotic cells. Different levels of Ca^{2+} and ion localization may lead to different messages through various calcium-binding proteins like calmodulin (CaM) with four Ca^{2+} -docking motifs. Upon Ca^{2+} attachment, the Ca^{2+} /CaM unit docks to target proteins, e.g. Ser/Thr protein kinases known as calcium calmodulin kinases (CaMKs), and regulate their function (Wayman et al., 2011). Calmodulin activates CaMKs, a group of effector enzymes mediating Ca^{2+} -signal transduction (Anderson, 2005, Wayman et al., 2011). Treating cells with agonists promotes selective CaMKs activation of the ones localized close to the cellular Ca^{2+} entrance point. Potential and key sarcoplasmic reticulum and handling proteins (RyR2, PLN) are modulated by CaMKII presumably with proarrhythmic outcomes. This has been also confirmed by computational model studies (Grandi et al., 2007) which unravels the therapeutic capacity of CaMKII inhibition in cardiac diseases such as heart failure. However the feasibility of CaMKII blockade as a therapeutic intervention has yet to be investigated (e.g. through clinical trials) and validated before taking in to account for treatment purposes (Fischer et al., 2013).

CaMKs are also known to play a role in tumor progression via modulating several signaling pathways (Rodriguez-Mora et al., 2005, Das and Sharma, 2005). They

can activate the hypoxia-inducible transcription factor (HIF-1) through which vascular endothelial growth factor (VEGF) expression and vessel growth occur (Mukhopadhyay and Akbarali, 1996). It has been recently reported that ECFC can be rescued by MSPC from hypoxia-induced apoptosis during vessel formation via a HIF-1-dependent mechanism (Hofmann et al., 2012a). In this study we also detected the expression of inflammatory caspase-12 in the majority (three of four) of co-transplant arrays, which may serve as additional evidence showing the involvement of inflammatory caspases in neo-vessel formation. The exact mechanism through which caspase activity and hypoxia sensing contribute to a functional interaction between ECFC and MSPC during vessel formation needs to be addressed in future studies.

Focal adhesion tyrosine kinase (FAK), detected significantly in our protein array data, has been shown to play a role in early integrin signaling reducing vasculature establishment when down regulated in glioma cells. Further, suppression of VEGF, protein kinase B (Akt) and FAK inhibited vasculogenesis in nude mice treated with human umbilical cord blood cells (Dasari et al., 2010). Significant expression of CaMK, EphA1, Erk, ERB and FAK during efficient neo-vasculogenesis in this study demonstrates the relevance of the current strategy.

Fibroblast growth factor receptor 1 (FGFR1) was also significantly expressed in early vasculogenesis array in our study. Fibroblast growth factors (FGF) are a family of heparin-binding growth factors displaying pro-angiogenic potential by interacting with several surface receptors on endothelial cells such as tyrosine kinase receptors, heparan-sulfate proteoglycans, and integrins (Presta et al., 2005). Stimulation of FGFR1 or FGFR2 by angiogenic FGF such as FGF1, FGF2, FGF4 and FGF8b isoform (Mattila et al., 2001) have been shown to promote

endothelial cell proliferation (Cross and Claesson-Welsh, 2001). The interaction between several FGF members, vascular endothelial growth factors (VEGF), and inflammatory cytokines may have an impact on the regulation of vasculature expansion in various pathological conditions like cancer (Presta et al., 2005). FGF has been shown to play a role in extracellular matrix degradation as an important step during early angiogenesis. This degradation takes place in cooperation with matrix metalloproteinases (MMP) and/or plasmin–plasminogen activator system (Liotta et al., 1991). Expression of FGFR1 in the early vasculogenesis array in our study might support the idea of involvement of growth factor-mediated signaling pathways, notably that of FGF during the first step of vessel formation *in vivo*.

A group of proteins that are stimulated by high temperature are referred to as heat-shock proteins (HSP). The most well-known members of this group are a class of proteins involved in regulation of folding and unfolding of other proteins. Heat shock proteins are up regulated when cells are exposed to increased temperature and other stress conditions (De Maio, 1999) including infection, inflammation, exposure to toxic reagents, starvation and hypoxia. Consequently, the heat shock proteins are also referred to as stress proteins that are expressed as a part of stress response (Santoro, 2000).

Small molecule heat-shock protein 27 (HSP27) acts in the process of cellular protection in response to stress including heat shock, toxins as well as oxidative stress. It is also involved in regulation of cell activities through a crosstalk with the actin cytoskeleton. A study carried out by Lee et al. showed that up regulation of HSP27 in HSP27-overexpressing NIH3T3 cells governs focal adhesion kinase (FAK) formation and phosphorylation in concert with polymerization of integrin-mediated actin in the cytoskeleton (Lee et al., 2008) . Furthermore, cell migration

and invasion in terms of wound healing process has been shown to be disrupted in this model according to the wound healing assays (Lee et al., 2008). Such disrupted wound healing mediated by HSP27 was correlated with diminished matrix metalloproteinase-2 (MMP-2) expression as well as less phosphorylated transcription factor for MMP-2 expression and signal transducer and activator of transcription 3 (STAT3). Transfection of the cells with a phosphomimetic form of HSP27 decreased the migration and invasion of the cells through the FAK/STAT3/MMP-2 signaling pathway, whereas a non-phosphorylated form of HSP27 inhibited HSP27-mediated phenotypes, suggesting a role for HSP27 in activation of cell adhesion and regulation of cell migration and invasion via the expression of FAK-dependent actin and STAT3-dependent MMP-2 (Lee et al., 2008). The phosphorylation of HSP27 is necessary for the regulation of this signaling pathway (Lee et al., 2008).

Furthermore, a recent study has elucidated that extracellular HSP27 regulates angiogenesis via Toll-like receptor 3 (TLR3) (Thuringer et al., 2013). It has been reported in this study that the interaction between HSP27 and TLR3 stimulates NF- κ B activation which results in VEGF-mediated cell migration and vessel formation (Thuringer et al., 2013). HSP27 was abundantly expressed in early vasculogenesis array in our study suggesting the impact of stress in signaling pathways that govern the initial steps of neo-vessel formation *in vivo*. As it has been shown in the previous studies that overexpression of HSP27 interrupts cell migration, one can speculate that HSP27 has a dual impact both on pro- and anti-angiogenesis processes depending on its level of expression within the cell.

The involvement of hemeoxygenase-1 (HO-1) in angiogenesis was investigated by Abraham and colleagues (Deramaudt et al., 1998) who delineated that up

regulation of HO-1 in endothelial cells elevated their proliferation potential. Further studies confirmed that HO-1 facilitates the progression of cell cycle in endothelial cells (Li Volti et al., 2005). Blocking HO-1 by means of antisense strategies abolished the proliferation potential of endothelial cells and *in vitro* network formation, an effect that was associated with augmentation of p21 and p27, blockers of the cell cycle (Li Volti et al., 2005). The reports delineating a role for HO-1 in vessel formation have suggested that proangiogenic factors like VEGF (Bussolati et al., 2004, Dulak et al., 2004) and SDF-134 promote expression of HO-1 in endothelial cells. Moreover, local HO blockade with zinc protoporphyrin inhibits angiogenesis and tumor expansion and growth *in vivo* (Fang et al., 2003). In parallel, *in vivo* findings have shown that up regulation of HO-1 in tumor-infiltrating macrophages promotes angiogenesis in human glioma cells (Nishie et al., 1999). Knockout animals with defective HO-1 gene do not display any particular phenotype suggestive for impaired angiogenesis, however HO-1^{-/-} homozygote animals may have impaired pregnancy and the rate of mortality is significantly higher in HO-1^{-/-} animal embryos (Poss and Tonegawa, 1997) indicating the impact of HO-1 on prenatal vessel formation.

As reported by Bussolati et al. (Bussolati et al., 2004, Bussolati and Mason, 2006) HO-1 activity is required in VEGF-induced vessel formation process, whereas inflammation-induced angiogenesis was abolished by overexpression of HO-1 (Bussolati et al., 2004, Bussolati and Mason, 2006). In accordance with these findings, HO-1 was expressed significantly in our early *in vivo* arrays suggesting a role for this enzyme in early phase of vessel establishment.

While several members of protein serine and tyrosine kinase subfamily such as CaMK, Erk, MAPK, Never-in-mitosis-related protein serine kinase (NeK2) and

protein kinase C (PACSLN1) as well as nerve growth factor (TrkA) were significantly expressed in our early vasculogenesis module of protein microarray, a number of protein serine kinases including FLJ932 protein serine kinase (STK33), protein serine kinase C epsilon (PKCe) and integrin-linked protein serine kinase 1 (ILK1) were down regulated in this module, suggesting a dual involvement of protein serine kinases in early regulation of neo-vessel formation.

P53 (cellular tumor antigen p53), encoded by the TP53 gene in human has been shown to play an important role in regulation of the cell cycle and has been described to be crucial in maintaining genome stability and keeping the genome from mutations (Strachan and Read, 1999). In our study, P53 has been significantly expressed in the MSPC+ECFC array compared to that of cell free matrix, suggesting a potential role for this tumor suppressor protein in the initial phase of vasculogenesis. It has also been shown in a study that P53 plays a crucial role in differentiation, propagation and spontaneous transformation of MSC reporting that MSC derived from p53KO mice showed a higher level of proliferation while the doubling time of the cell populations was decreased, and they were showing morphologic and phenotypic alterations compared to MSC from wild-type animal. Additionally, P53 has been shown to affect and control MSC differentiation process since the lack of this protein alters the speed of differentiation (Armesilla-Diaz et al., 2009). Interestingly, P53 has not been expressed in the MSPC-only array in our experiment, which might be due to incompetency of UC-derived MSC to differentiate into osteoblasts, whereas P53 has been expressed in early vasculogenesis array (MSPC+ECFC *in vivo*) which requires the early differentiation of MSC into pericytes that maintain the stability of the micro-vessels.

Death associated protein 6 (DAXX) was significantly expressed in two out of four early vasculogenesis arrays. This protein possesses pro-apoptotic receptors like FAS and TGF β receptor II (Yang et al., 1997, Perlman et al., 2001) which allows for recruitment of the FAS-associated death domain (FADD) protein and consequently stimulation of caspase-8 and downstream caspases. Daxx has been also suggested to act as a pro-apoptotic mediator downstream of FAS through activation of the c-Jun-N-terminal kinase (JNK) pathway independently from FADD (Yang et al., 1997, Chang et al., 1998). The JNK pathway itself governs stress-induced cell death and is crucial for programmed cell death in the neural cells (Weston and Davis, 2007). Controversial reports, however, have advocated a potential anti-apoptotic role for Daxx showing that Daxx^{-/-} embryonic stem cells have an increased level of spontaneous apoptosis (Ishov et al., 2004, Michaelson et al., 1999, Nefkens et al., 2003). In our study DAXX was significantly expressed in the co-transplant arrays presumably due to its involvement in cell survival and rearrangement during neo-vessel formation.

This study suggests a non-apoptotic role for caspases expressed during vessel formation that may lead us to a better understanding towards the contribution of progenitor/stem cell types in tissue regeneration and novel therapeutic strategies. Additionally, our data can be used to create a platform for evaluating small molecules that inhibit essential signaling pathways during vascular regeneration and/or pruning. Using a subtractive strategy by pre-treating one or the other cell type to block caspase-4 in ECFC or MSPC was meant to specify whether both or just one of the two cell types utilize caspase-4 during de novo vessel formation. Our results (Figures 15C, 16C, 17C and Figure 18) demonstrate a particular role of caspases in the ECFC compartment.

We show that blockade of an inflammatory caspase (caspase-4) activity through the action of Z-LEVD-FMK, produces significant alterations in the regular network formation *in vitro* (ECFC involved), similar to the impact of Q-VD-OPh, a broad spectrum caspase inhibitor which blocks mostly effector and initiator caspases. The same result has been observed for caspase blockade with respect to neo-vasculogenesis *in vivo*. In addition, caspase-4 inhibition in MSPC alone with unaffected ECFC in co-transplant model did not lead to significantly impaired vessel formation. This suggests that inflammatory caspase-4 has a major impact in regulating neo-vasculogenesis through endothelial cells (ECFC) whereas initiator and effector caspases regulate both MSPC and ECFC in the process of vasculogenesis. Moreover, up regulation of ASK1 (apoptosis signal-regulating protein serine kinase) in “ECFC only” plugs in microarray analysis provides additional evidence that caspase mediated neo-vasculogenesis is regulated mostly by ECFC in process of vessel formation initiation.

In summary, these findings may provide a new understanding of the involvement of cell death associated components as early signaling molecules which drive cells to enter a pro-survival and differentiation-based process such as neo-vasculogenesis.

The significantly expressed signaling molecules within the displayed signaling signature of early vessel formation provide a source of attractive targets for therapeutic applications in this model. The significantly expressed targets and the related signaling pathways provide a platform through which the pro- and anti angiogenic properties of the signaling molecules could be assessed to be used for novel therapeutic strategies. Moreover, this model provides a platform for the signaling molecules that were significantly expressed in MSPC only array. MSPC

as multi-potent progenitor cells are able to differentiate to at least three lineages (chondrogenic, osteogenic and adipogenic) upon being cultured under defined conditions (Bruder et al., 1994, Mackay et al., 1998, Pittenger et al., 1999). Mitogen activated protein kinases (MAP kinase) have been shown to regulate MSC differentiation to osteo- or adipogenic lineage (Jaiswal et al., 2000) with a significant expression of p38, Erk2 and JNK2 in a time-dependent manner through phosphorylation during the process of differentiation (Jaiswal et al., 2000) suggesting a crucial role for protein kinase signaling molecules and their phosphorylation status during differentiation. The signaling molecules that were significantly expressed in our MSC-only array therefore could be used to investigate the early signaling events during chondrogenesis to be used for further regenerative interventions.

One drawback of this study is that the relatively ischemic implants in this model do not identically display a real organ injury situation; therefore, cells under more physiological conditions may show different expression patterns of the signals. Additional analysis in other vasculogenesis models are warranted to evaluate which of the selected signaling molecules can be targeted to regulate vasculogenesis most efficiently in diseases with aberrant vascularization.

8. CONCLUSION

In this study, we focused on the interactions of ECFC and MSC and the underlying signaling signature during neo-vasculogenesis, which modulates patent and functional vessel establishment. We determined significantly up regulated signaling mediators including caspase cascade proteins through which the early

interaction occurs. Our hypothesis that effective functional signaling signature during early vessel formation can be identified by *ex vivo* proteomic profiling was confirmed as we could demonstrate that defective endothelial network formation *in vitro* and vessel formation *in vivo* resulted from inhibiting caspase molecules. These findings led us to conclude that inflammatory caspase-4 has an impact in the initiation of vasculogenesis by ECFC+MSPC co-transplantation. A better understanding of the mechanism underscoring de novo vessel formation will be helpful to develop robust therapeutic strategies.

9. FIGURES

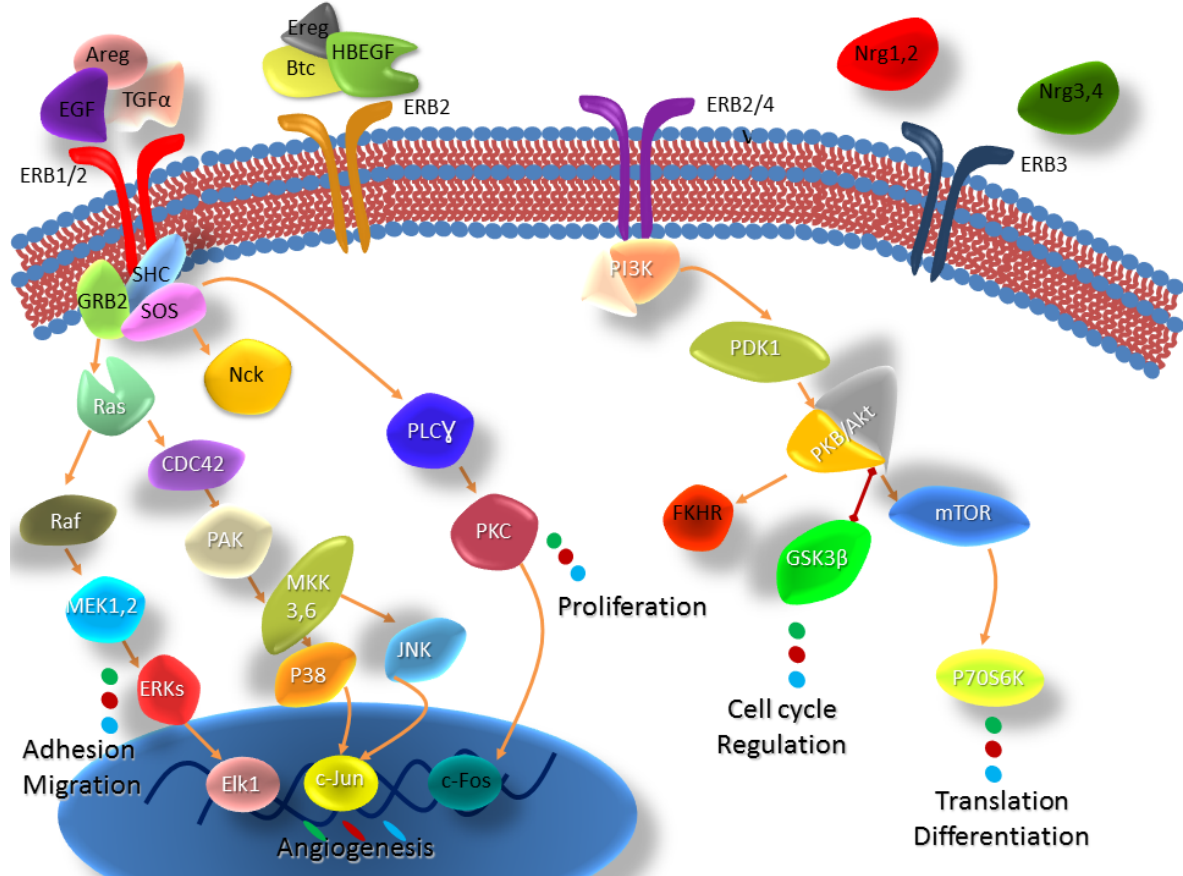


Figure 1. ERB signaling pathway and associated cellular manifestations

ERB cascade consists of signaling protein kinases such as mammalian target of rapamycin (mTOR) and protein kinase PKB/Akt that regulate cellular manifestations such as differentiation, cell cycle regulation, proliferation as well as cellular migration and adhesion.

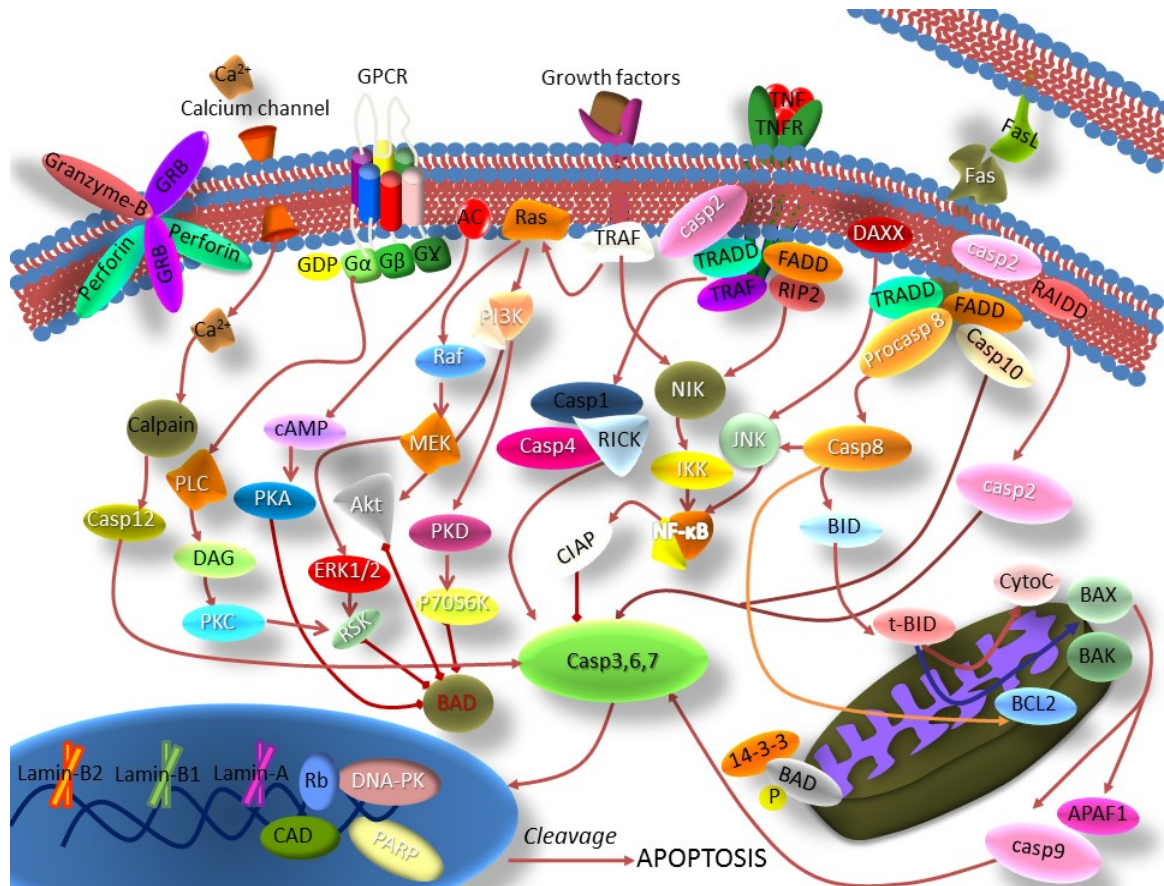


Figure 2. Death-associated signaling pathway

A number of initiator, effector and executioner caspases as well as protein kinases such as PKA, PKC and PKD are involved in this pathway leading to cellular apoptosis.

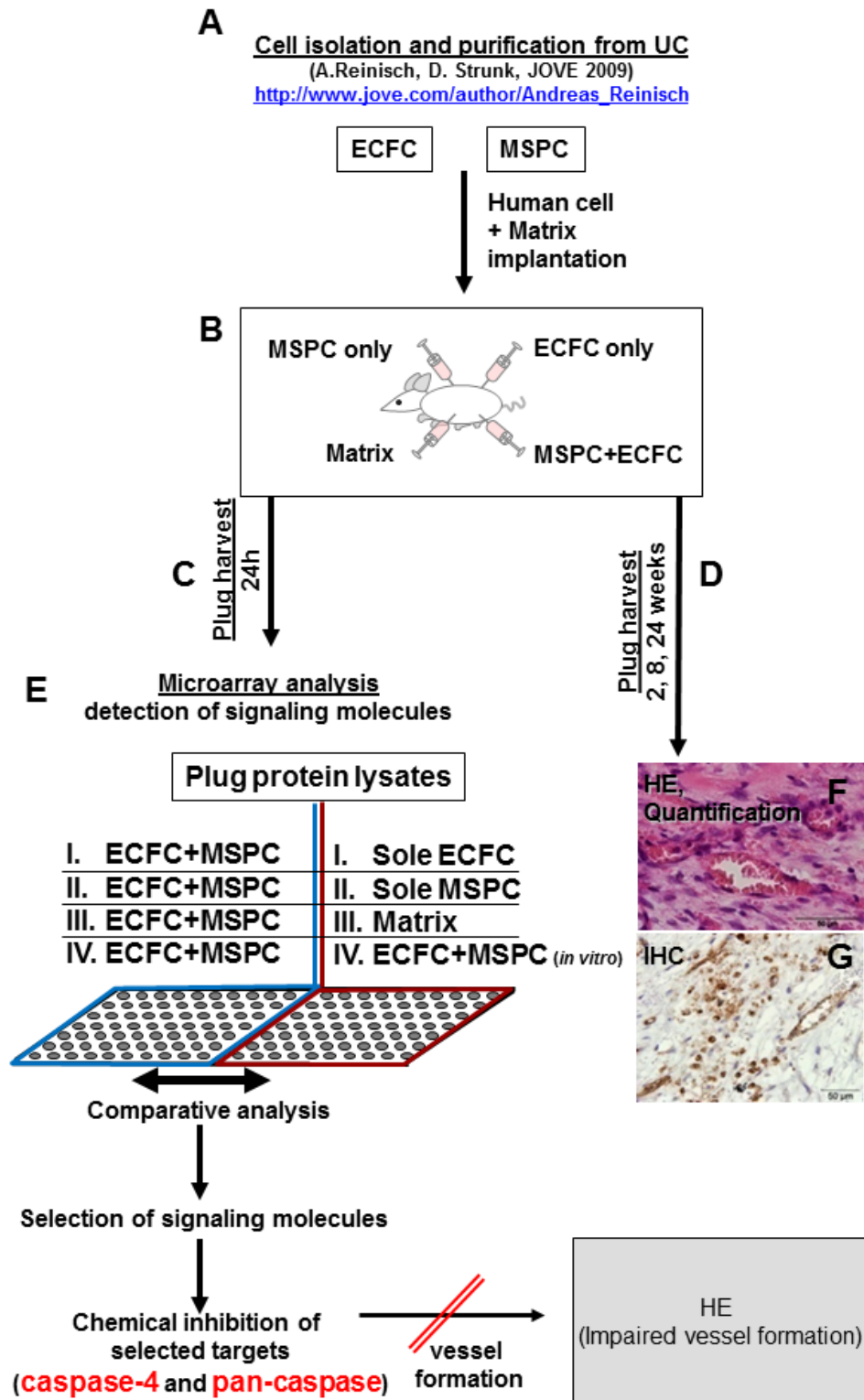


Figure 3. Overview of the project strategy

Autologous pairs of mesenchymal stem/progenitor cells (MSPC) and endothelial colony-forming progenitor cells (ECFC) were isolated, purified and expanded from the same human umbilical cord as described (http://www.jove.com/author/Andreas_Reinisch) (**A**). Cells were combined with matrix and injected subcutaneously in four 300 μ L implants per animal into the flank of immune-compromised NSG mice as specified (**B**). Plugs were explanted 24 hours after implantation (**C**) to detect the signaling signature operative during the early phase of therapeutic vasculogenesis using proteomic profiling on customized antibody microarrays comparing either co-transplants of ECFC+MSPC with transplants containing sole ECFC, MSPC or matrix *in vivo* (groups I – III), or co-transplants *in vivo* with a mixture of ECFC+MSPC created *in vitro* (group IV) (**E**). Macroscopic view of plugs explanted after 2, 8 and 24 weeks (**C and D**). Histology and morphometry were performed to visualize vessel formation and stability in plugs in a time course (for up to 24 weeks) (**F and G**). Small molecule inhibition of selected targets (from the array analysis) was used as a proof-of-principle confirming druggability of significantly regulated proteins within the early vasculogenesis signaling signature.

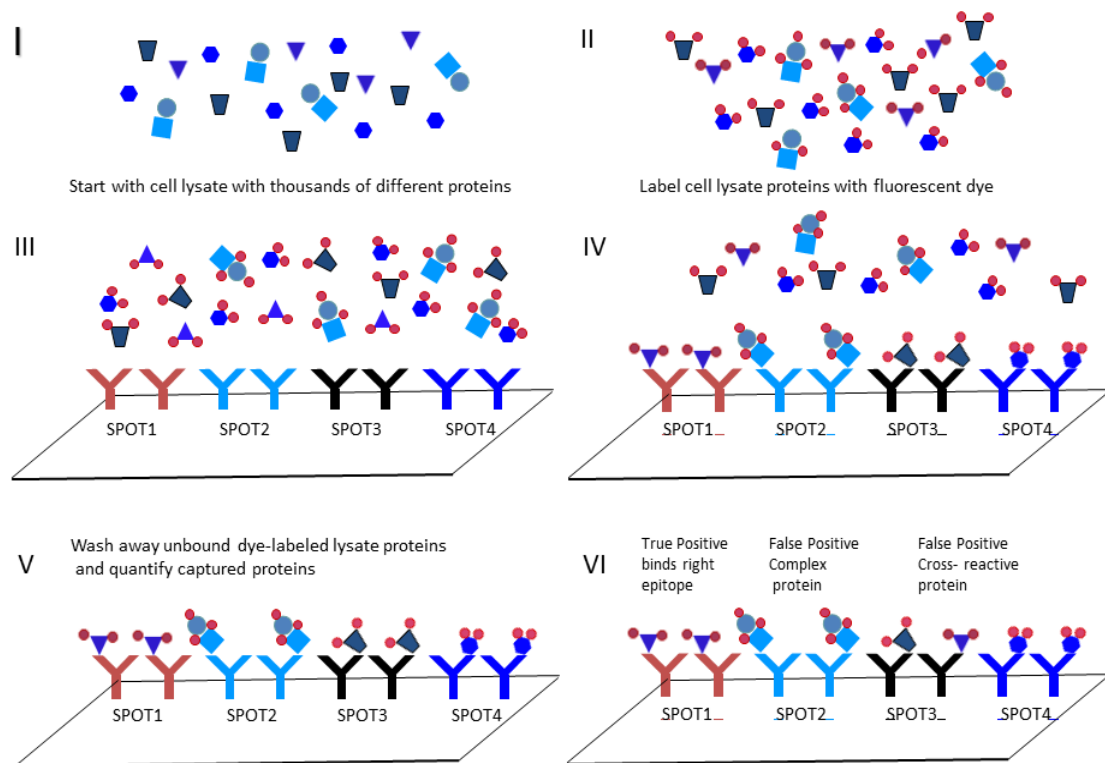
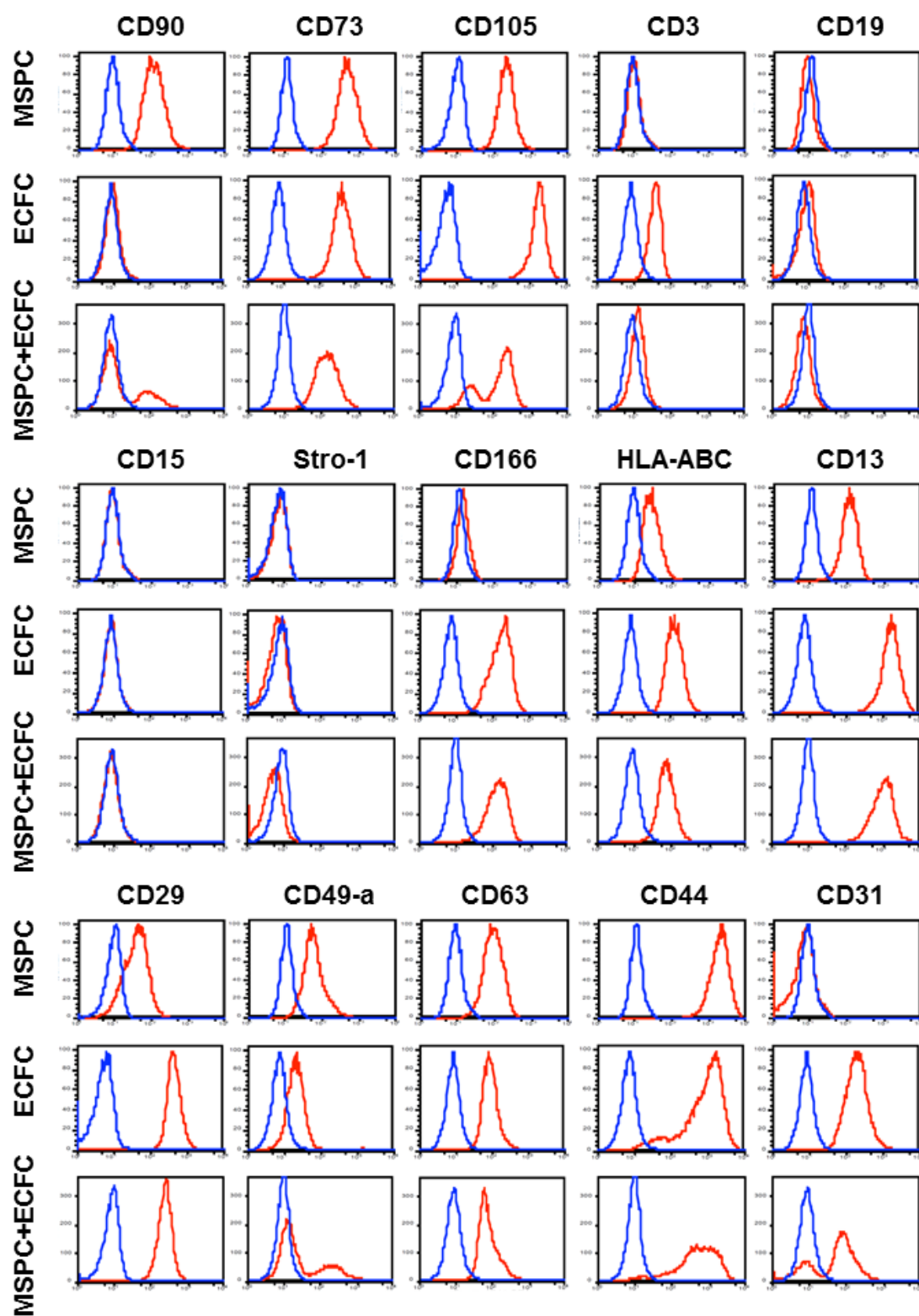


Figure 4. The principal of antibody mediated detection of signaling molecules

Cells were isolated from the explanted plugs and were lysed using an optimized lysis protocol as described in Material and Methods section (I). The lysate was labeled with fluorescent dyes (II) and was loaded on a dual chamber, antibody-coated array chip with various spots (III) to allow the labeled proteins to bind specifically (IV). After the incubation time, the unbound labeled lysate proteins were removed by washing steps (V) and true positive bounds to the right epitope were distinguished from those which represent complex protein or cross-reactive protein bound (false positive) (VI). The use of a single dye, non-competitive binding method in this technology helps to reduce the risk of false negative/positive binding, however as this issue is inevitable in protein microarray technique, the validation of microarray results for the target of interest through an alternative method (e.g. immunoblotting) is crucial.

A



B

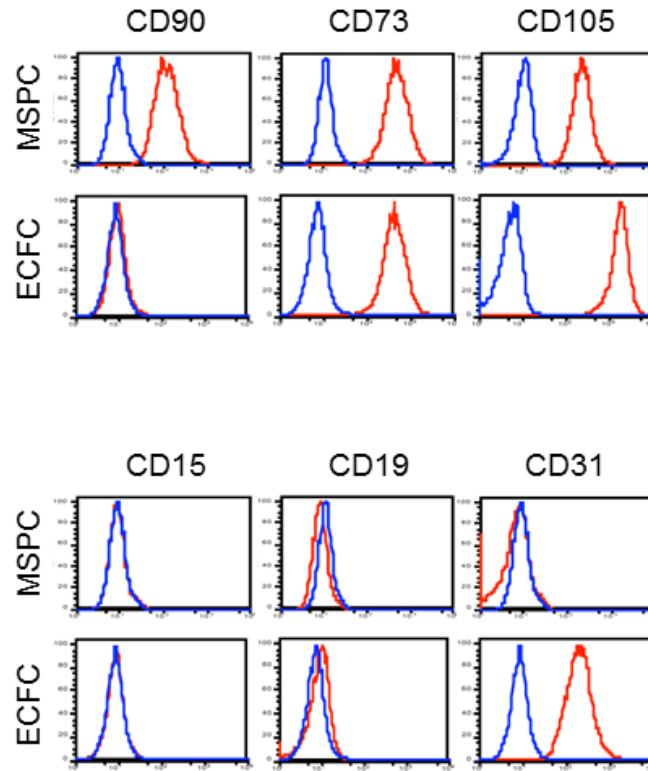
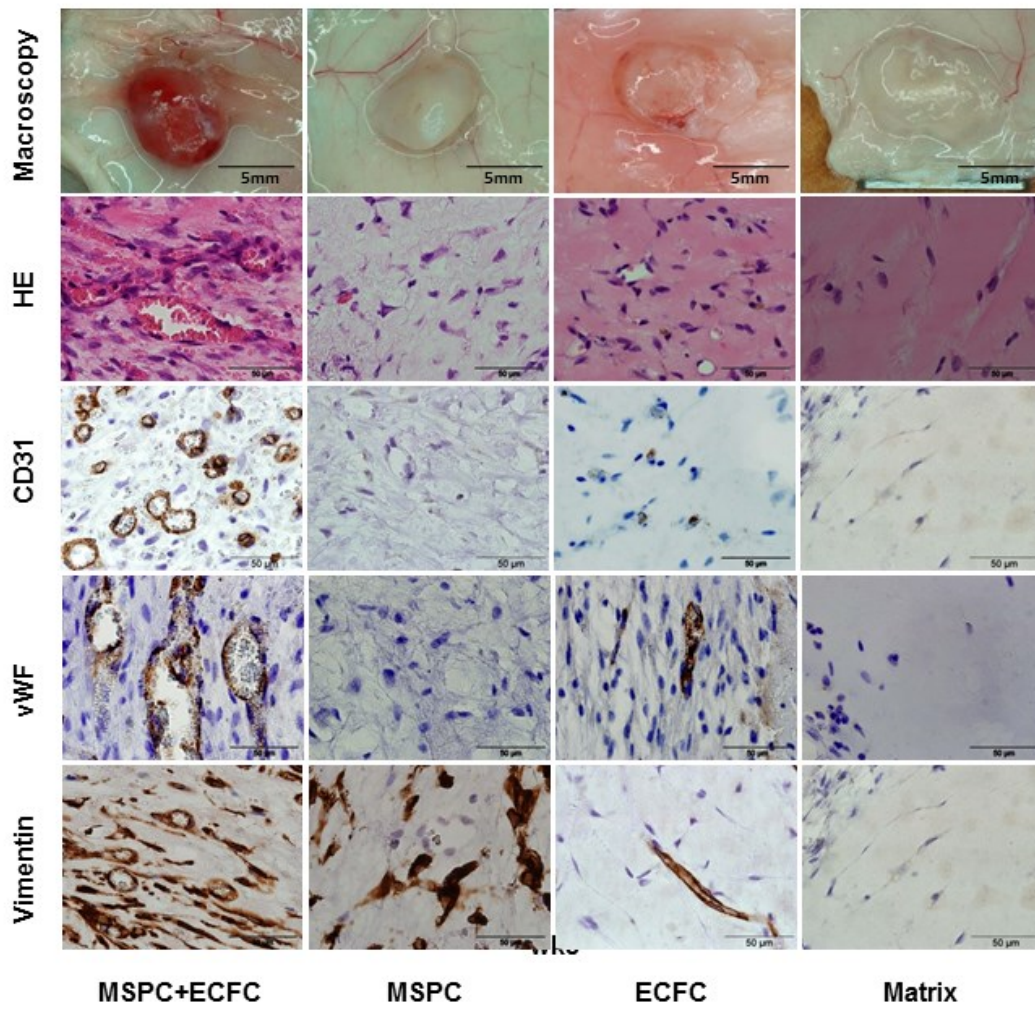


Figure 5. Immune phenotype of culture-expanded cells

Culture-expanded mesenchymal stem/progenitor cells (MSC; upper rows) and endothelial colony-forming progenitor cells (ECFC; lower rows) as used for co-transplantation were analyzed by flow cytometry for their immune phenotype. The co-culture of MSC and ECFC was also assessed for phenotypic differential display (3rd row, **A**). Red lines indicate target antibody and blue lines isotype control reactivity. All MSC used were >95% positive for CD90, CD73 and CD105 fulfilling common ISSCR (International Society for Stem Cell Research) criteria and lacked reactivity with hematopoietic markers (<2%) as exemplified by CD15 and CD19 (**B**). ECFC were completely CD90-negative excluding MSC contamination and their purity was confirmed by CD31 reactivity (**B**). One representative example out of at least three experiments is shown.

A



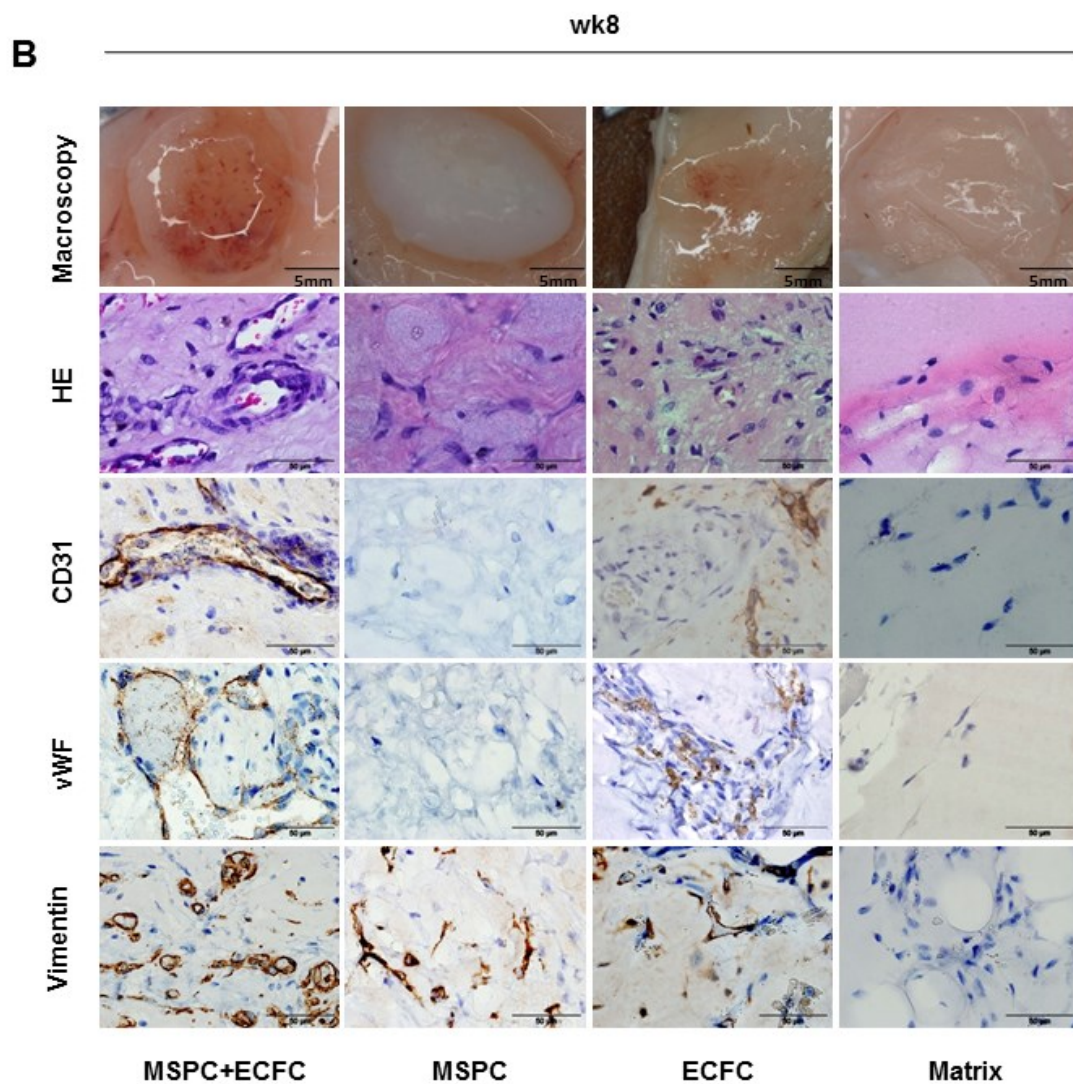


Figure 6. Co-transplantation of ECFC and MSPC induce neo-vasculogenesis

Plugs were harvested two (**A**) and eight weeks (**B**) after subcutaneous co-transplantation of ECFC+MSPC (ratio 80 : 20, left column), MSPC only (2nd column), ECFC only (3rd column), and cell-free matrix (right column). Representative macroscopic pictures of plugs in subcutaneous location (upper rows), micrographs of hematoxylin and eosin staining (H&E, middle rows) and immune histochemistry combining human CD31 (third rows from above), von Willebrand factor (vWF, forth rows from above) and Vimentin (lower rows) colored brown with hematoxylin counter-stain are depicted. Total cell number within the plugs was 2×10^6 per 300 μ L matrix (representative of $n = 3$ per group and time point).

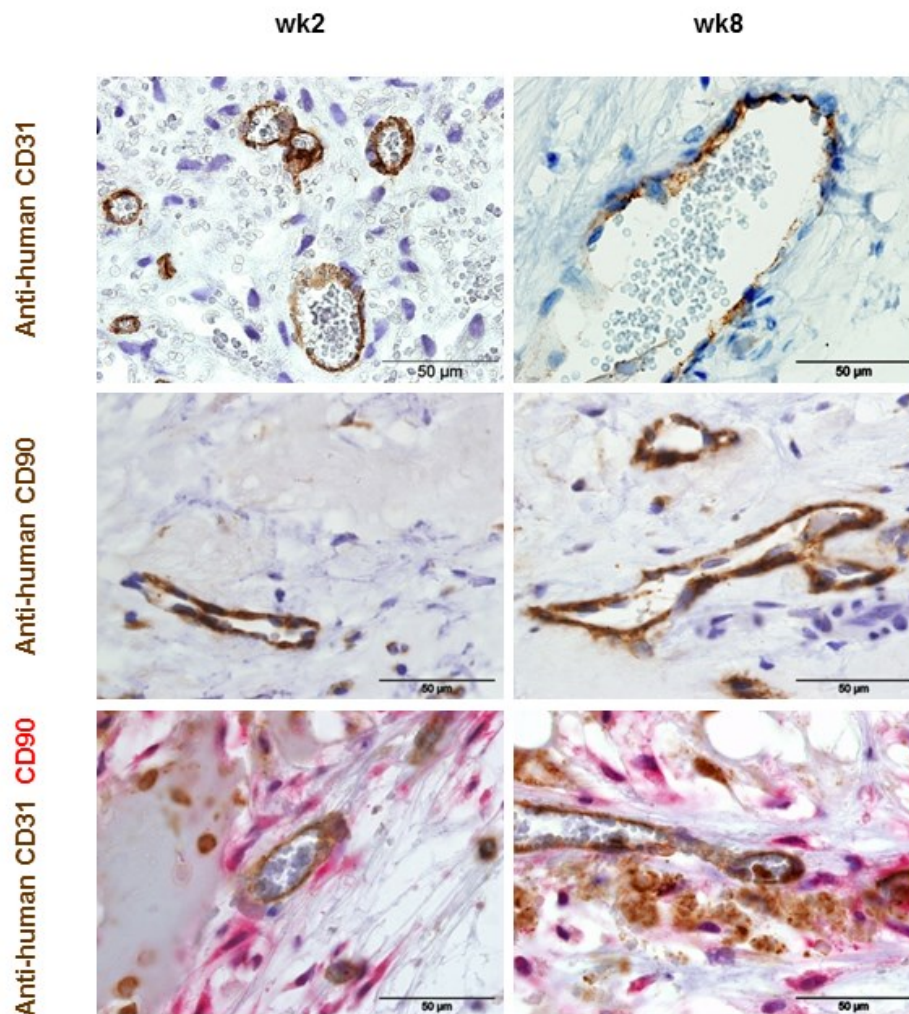
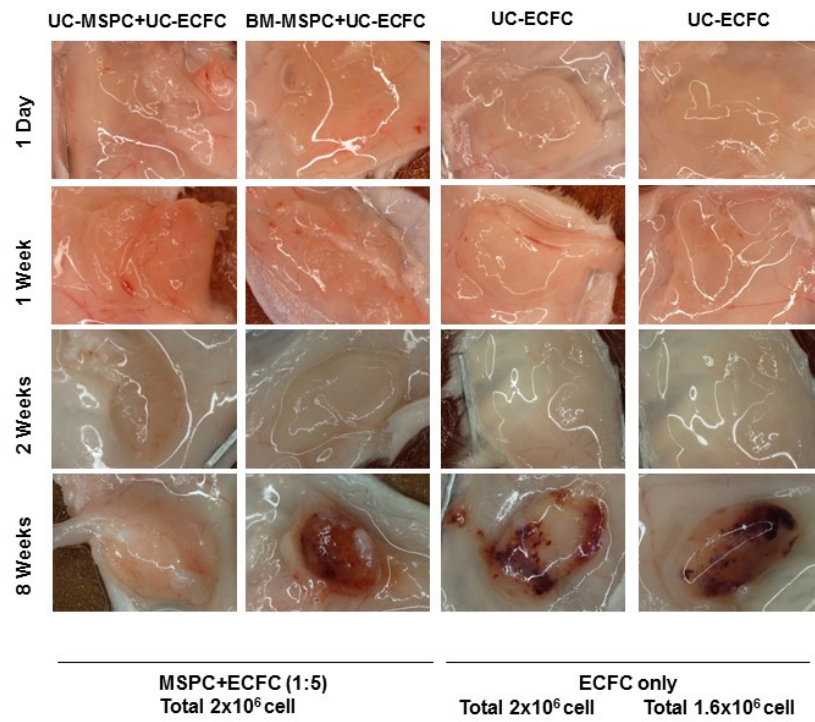


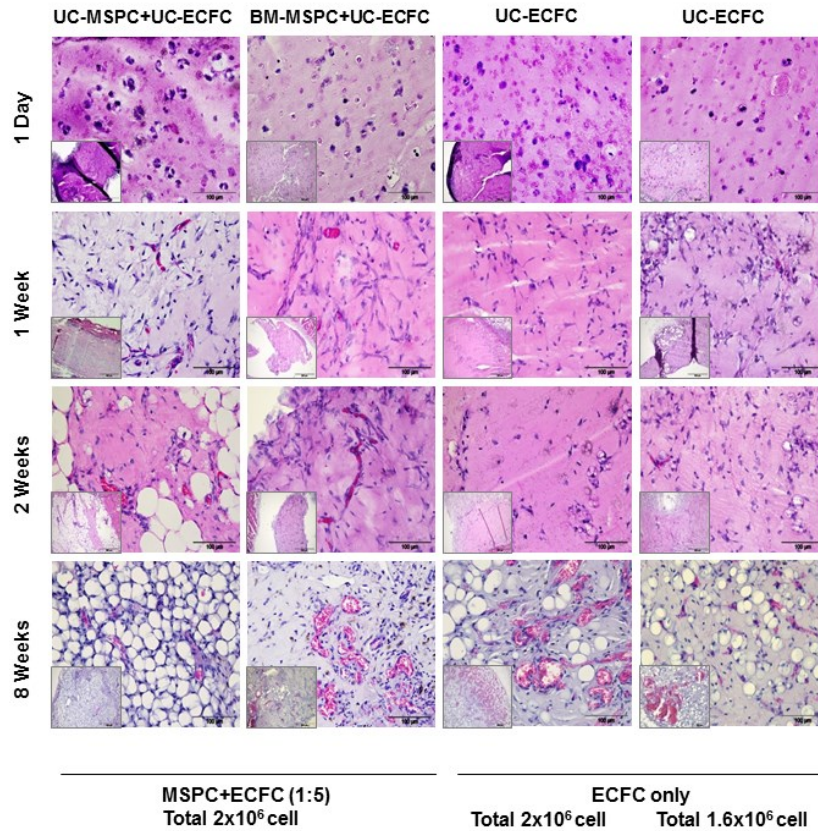
Figure 7. Anti-human CD31 and CD90 immune histochemistry of ECFC plus MSPC co-transplants

Perfused vessels within ECFC+MSPC co-transplants harvested after two weeks (wk2, left column) and eight weeks (wk8, right column) were visualized by anti-human CD31 (upper row, CD31-positive signal in brown) and anti-human CD90 immune histochemistry (middle row, positive signal in brown), verifying contribution of ECFC to vascular lumen and presence of MSPC around capillaries. Human CD31/CD90 double staining (lower row, CD31+ in brown, CD90+ in red) depicts intimate localization of MSPC to ECFC in vascular structures. Nuclei were counterstained with hematoxylin (blue). A total number of 32×10^6 cells have been used with a ratio of 1:5 (MSPC: ECFC). Micrographs are representative of $n = 3$ per group and time point.

A



B



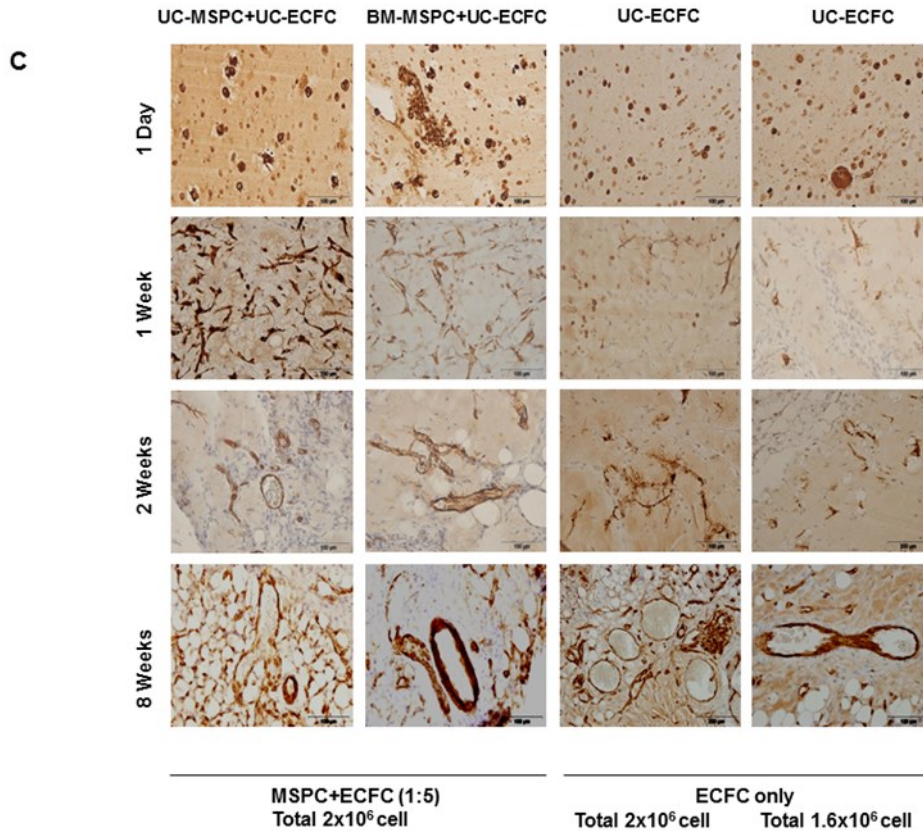


Figure 8. Neo-vasculogenesis potential of MSPC+ECFC co-transplants compared to that of ECFC only

Macroscopic features of co-transplants containing umbilical cord-derived MSPC and ECFC (UCMSPC+UC ECFC, **A**, left column), bone marrow derived MSPC and umbilical cord derived ECFC (BM-MSPC+UC-ECFC, **A**, second column from left) are shown. A total number of 2×10^6 cells were injected within the co-transplantation plugs at a ratio of 1:5 (MSPC: ECFC).

The right columns depict the macroscopic features of transplants containing umbilical cord derived ECFC (UC-ECFC) solely with a number of 1.6×10^6 (**A**, right column), and 2×10^6 cells (**A**, the second column from right). The plugs were harvested after one day, one week, two weeks and eight weeks, respectively. Images are representative of $n = 2$ per group.

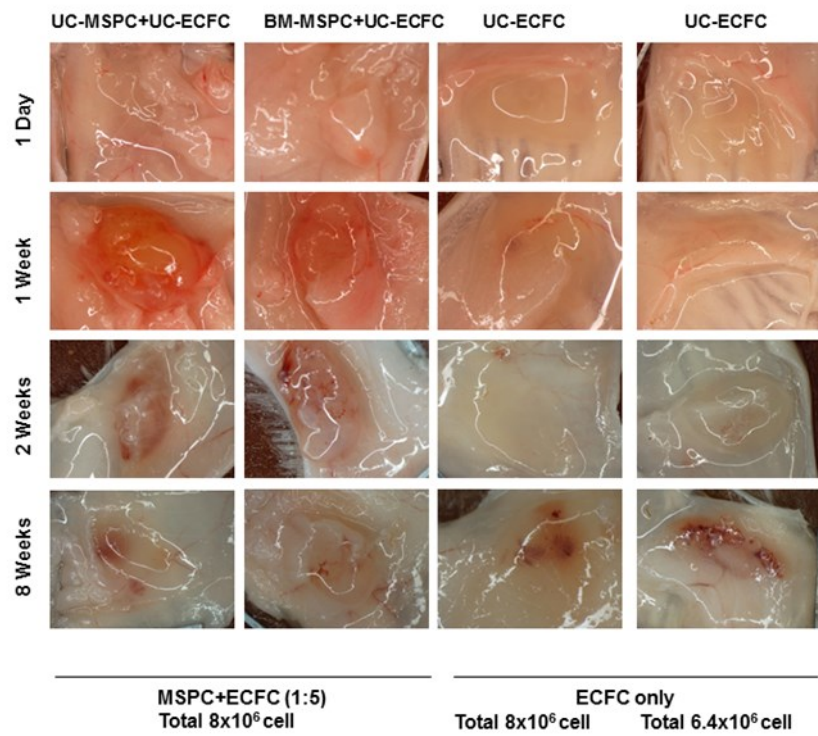
H&E staining of co-transplants containing umbilical cord-derived MSPC and ECFC (UCMSPC+UC ECFC, **B**, left column), bone marrow derived MSPC and umbilical cord derived ECFC (BM-MSPC+UC-ECFC, **B**, second column from left) are shown. A total number of 2×10^6 cells were injected within the co-transplantation plugs at a ratio of 1:5 (MSPC: ECFC).

The right columns depict the H&E staining of the transplants containing umbilical cord derived ECFC (UC-ECFC) solely with a number of 1.6×10^6 (**B**, right column), and 2×10^6 cells (**B**, the second column from right). The plugs were harvested after one day, one week, two weeks and eight weeks. Images are representative of $n = 2$ per group.

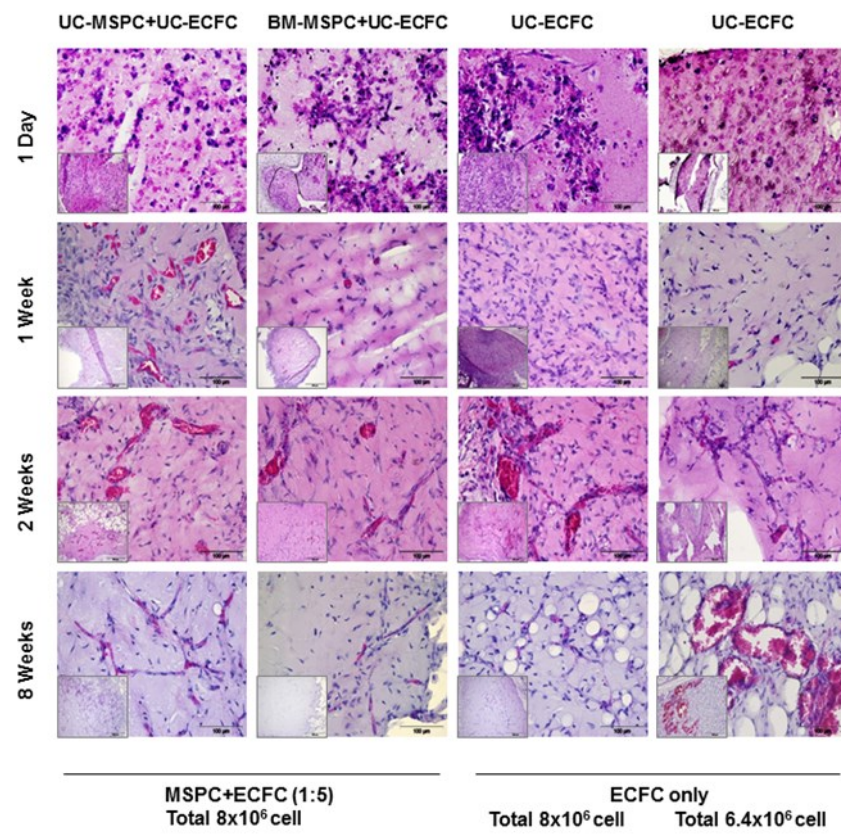
Anti-human vimentin immune histochemistry of co-transplants containing umbilical cord-derived MSPC and ECFC (UCMSPC+UC ECFC, **C**, left column), bone marrow derived MSPC and umbilical cord derived ECFC (BM-MSPC+UC-ECFC, **C**, second column from left) are depicted. A total number of 2×10^6 cells were injected within the co-transplantation plugs at a ratio of 1:5 (MSPC: ECFC).

The right columns depict the anti-human vimentin immunohistochemistry of the transplants containing umbilical cord derived ECFC (UC-ECFC) solely with a number of 1.6×10^6 (**C**, right column), and 2×10^6 cells (**C**, the second column from right), respectively. The plugs were harvested after one day, one week, two weeks and eight weeks, respectively. Images are representative of $n = 2$ per group.

A



B



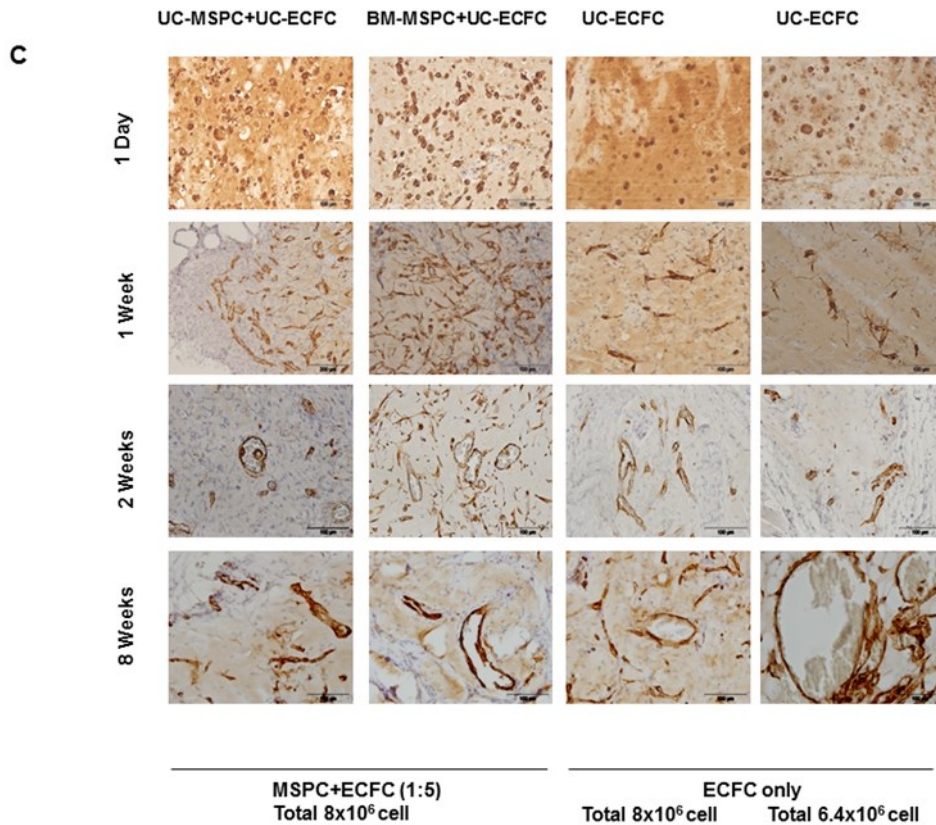


Figure 9. *In vivo* vessel formation capacity of MSPC+ECFC co-transplants compared to that of sole ECFC

Macroscopic features of co-transplants containing umbilical cord-derived MSPC and ECFC (UCMSPC+UC ECFC, **A**, left column), bone marrow derived MSPC and umbilical cord derived ECFC (BM-MSPC+UC-ECFC, **A**, second column from left) are shown. A total number of 8×10^6 cells were injected within the co-transplantation plugs at a ratio of 1:5 (MSPC: ECFC).

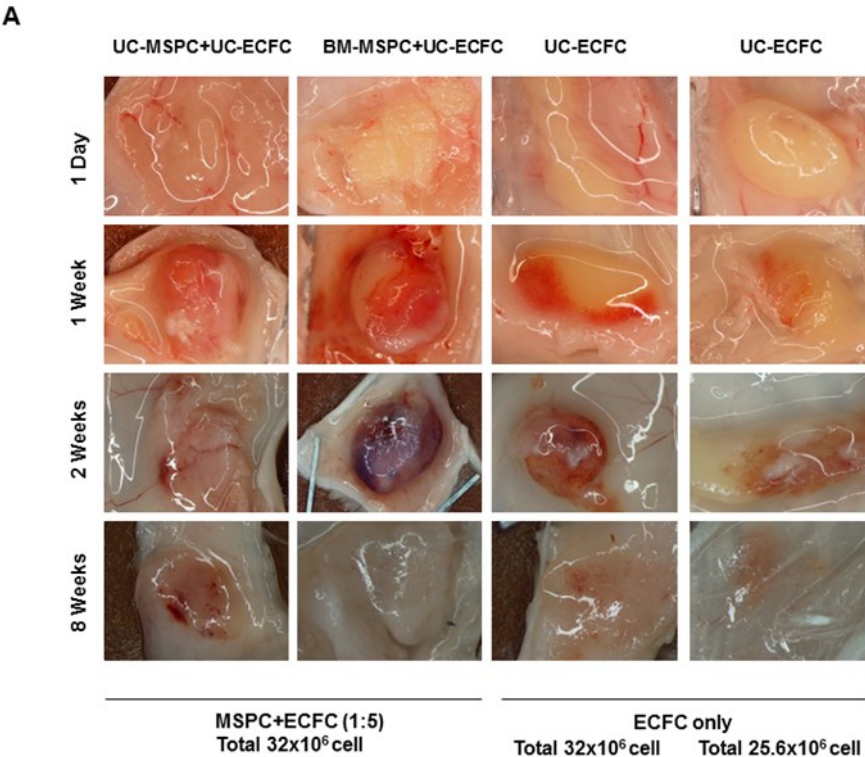
The right columns depict the macroscopic features of transplants containing umbilical cord derived ECFC (UC-ECFC) solely with a number of 6.4×10^6 (**A**, right column), and 8×10^6 cells (**A**, the second column from right). The plugs were harvested after one day, one week, two weeks and eight weeks. Images are representative of $n = 2$ per group.

H&E staining of co-transplants containing umbilical cord-derived MSPC and ECFC (UCMSPC+UC ECFC, **B**, left column), bone marrow derived MSPC and umbilical cord derived ECFC (BM-MSPC+UC-ECFC, **B**, second column from left) are shown. A total number of 8×10^6 cells were injected within the co-transplantation plugs at a ratio of 1:5 (MSPC: ECFC).

The right columns depict the H&E staining of the transplants containing umbilical cord derived ECFC (UC-ECFC) solely with a number of 6.4×10^6 (**B**, right column), and 8×10^6 cells (**B**, the second column from right). The plugs were harvested after one day, one week, two weeks and eight weeks, respectively. Images are representative of $n = 2$ per group.

Anti-human vimentin immune histochemistry of co-transplants containing umbilical cord-derived MSPC and ECFC (UCMSPC+UC ECFC, **C**, left column), bone marrow derived MSPC and umbilical cord derived ECFC (BM-MSPC+UC-ECFC, **C**, second column form left) are depicted. A total number of 8×10^6 cells were injected within the co-transplantation plugs at a ratio of 1:5 (MSPC: ECFC).

The right columns depict the anti-human vimentin immunohistochemistry of the transplants containing umbilical cord derived ECFC (UC-ECFC) solely with a number of 6.4×10^6 (**C**, right column), and 8×10^6 cells (**C**, the second column from right), respectively. The plugs were harvested after one day, one week, two weeks and eight weeks, respectively. Images are representative of $n = 2$ per group.



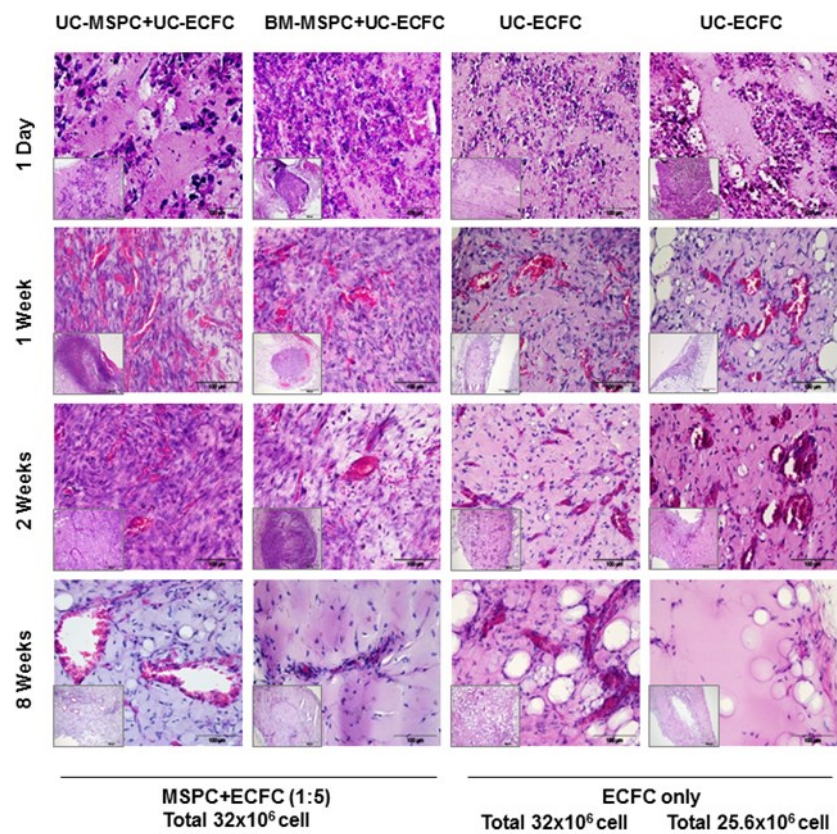
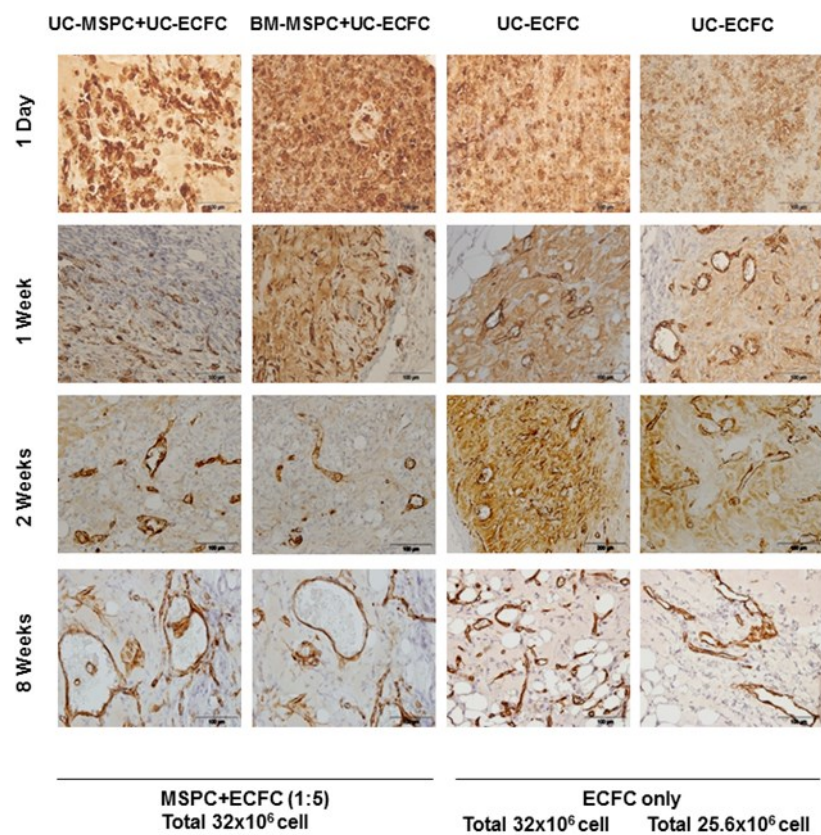
B**C**

Figure 10. MSPC and ECFC co-transplantation as well as sole ECFC transplantation lead to long term stable perfused vessels *in vivo*

Macroscopic features of co-transplants containing umbilical cord-derived MSPC and ECFC (UCMSPC+UC ECFC, **A**, left column), bone marrow derived MSPC and umbilical cord derived ECFC (BM-MSPC+UC-ECFC, **A**, second column from left) are shown. A total number of 8×10^6 cells were injected within the co-transplantation plugs at a ratio of 1:5 (MSPC: ECFC).

The right columns depict the macroscopic features of transplants containing umbilical cord derived ECFC (UC-ECFC) solely with a number of 25.6×10^6 (**A**, right column), and 32×10^6 cells (**A**, the second column from right). The plugs were harvested after one day, one week, two weeks and eight weeks, respectively. Images are representative of $n = 2$ per group.

H&E staining of co-transplants containing umbilical cord-derived MSPC and ECFC (UCMSPC+UC ECFC, **B**, left column), bone marrow derived MSPC and umbilical cord derived ECFC (BM-MSPC+UC-ECFC, **B**, second column from left) are shown. A total number of 8×10^6 cells were injected within the co-transplantation plugs at a ratio of 1:5 (MSPC: ECFC).

The right columns depict the H&E staining of the transplants containing umbilical cord derived ECFC (UC-ECFC) solely with a number of 25.6×10^6 (**B**, right column), and 32×10^6 cells (**B**, the second column from right). The plugs were harvested after one day, one week, two weeks and eight weeks, respectively. Micrographs are representative of $n = 2$ per group.

Anti-human vimentin immune histochemistry of co-transplants containing umbilical cord-derived MSPC and ECFC (UCMSPC+UC ECFC, **C**, left column), bone marrow derived MSPC and umbilical cord derived ECFC (BM-MSPC+UC-ECFC, **C**, second column from left) are depicted. A total number of 8×10^6 cells were injected within the co-transplantation plugs at a ratio of 1:5 (MSPC: ECFC).

The right columns depict the anti-human vimentin immunohistochemistry of the transplants containing umbilical cord derived ECFC (UC-ECFC) solely with a number of 25.6×10^6 (**C**, right column), and 32×10^6 cells (**C**, the second column from right), respectively. The plugs were harvested after one day, one week, two weeks and eight weeks, respectively. Images are representative of $n = 2$ per group.

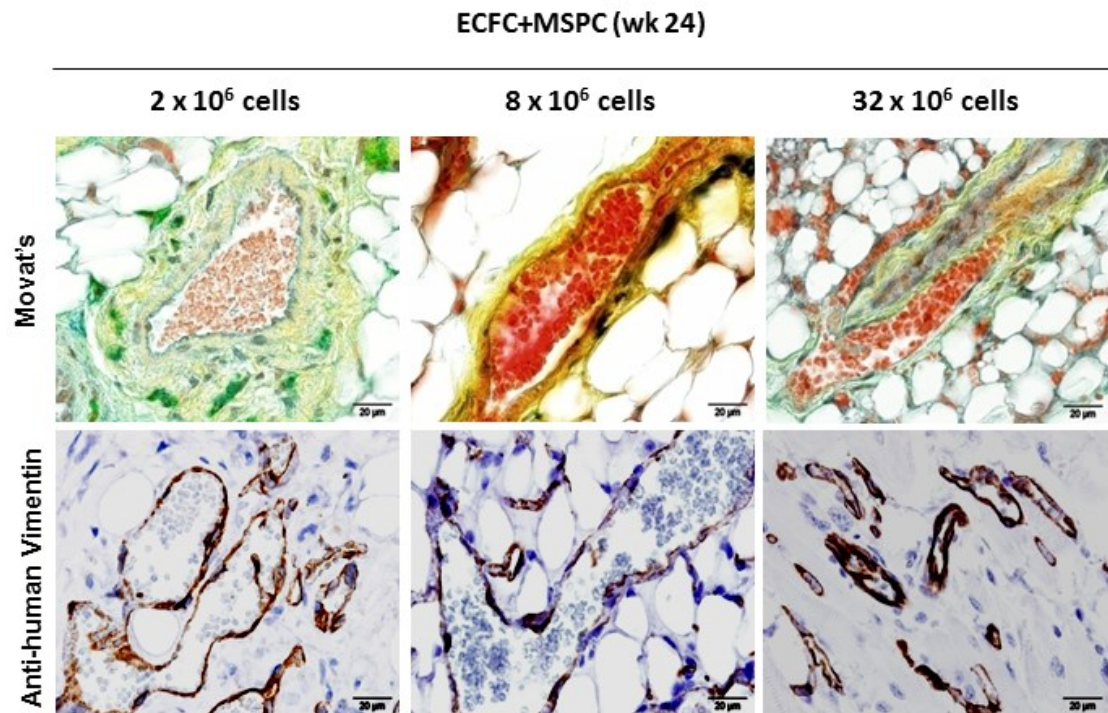


Figure 11. Stability of the established vessels during a 24 week-time period

Plugs containing MSPC/ECFC with a total cell number of 2×10^6 (left column), 8×10^6 (middle) and 32×10^6 (right column) were harvested 24 weeks after implantation and stained with Movat's pentachrom (upper row). The color indicator displays red for red blood cells and muscle, yellow for collagen fibers, green for mucin and brown to black for elastic fibers for Movat's pentachrom staining. Brown and blue (lower row) display anti-human vimentin and nuclei as counter stained by hematoxilin, respectively. Micrographs are representative of $n = 3$ per group and time point.

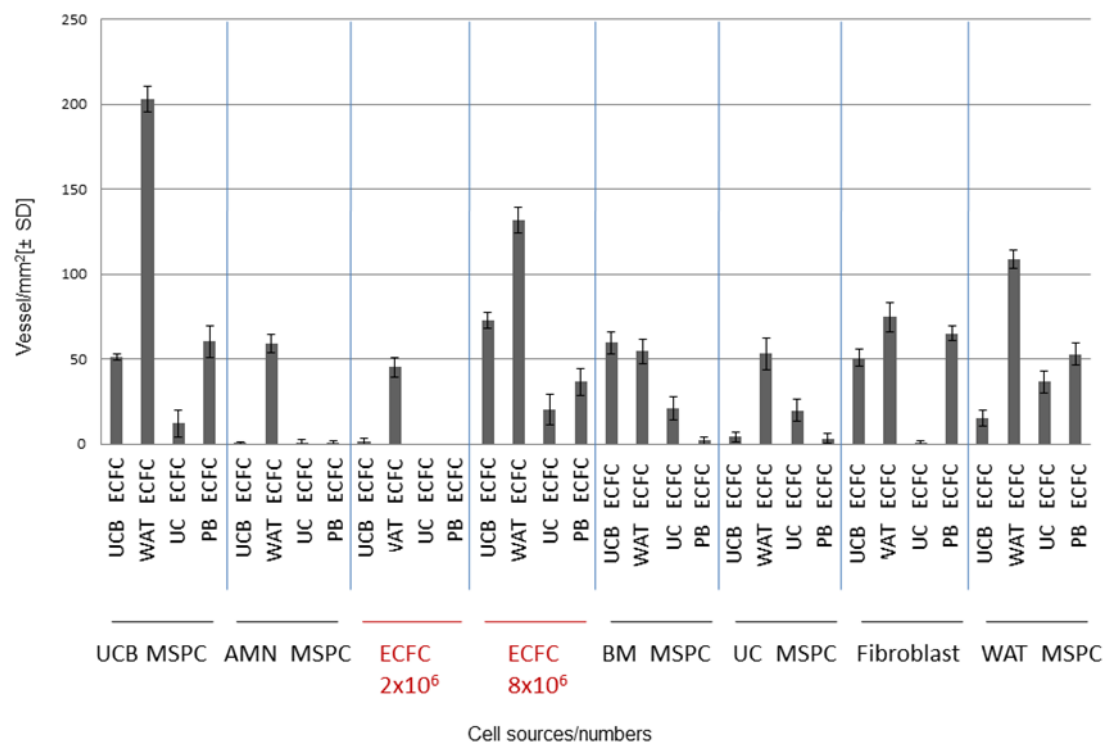
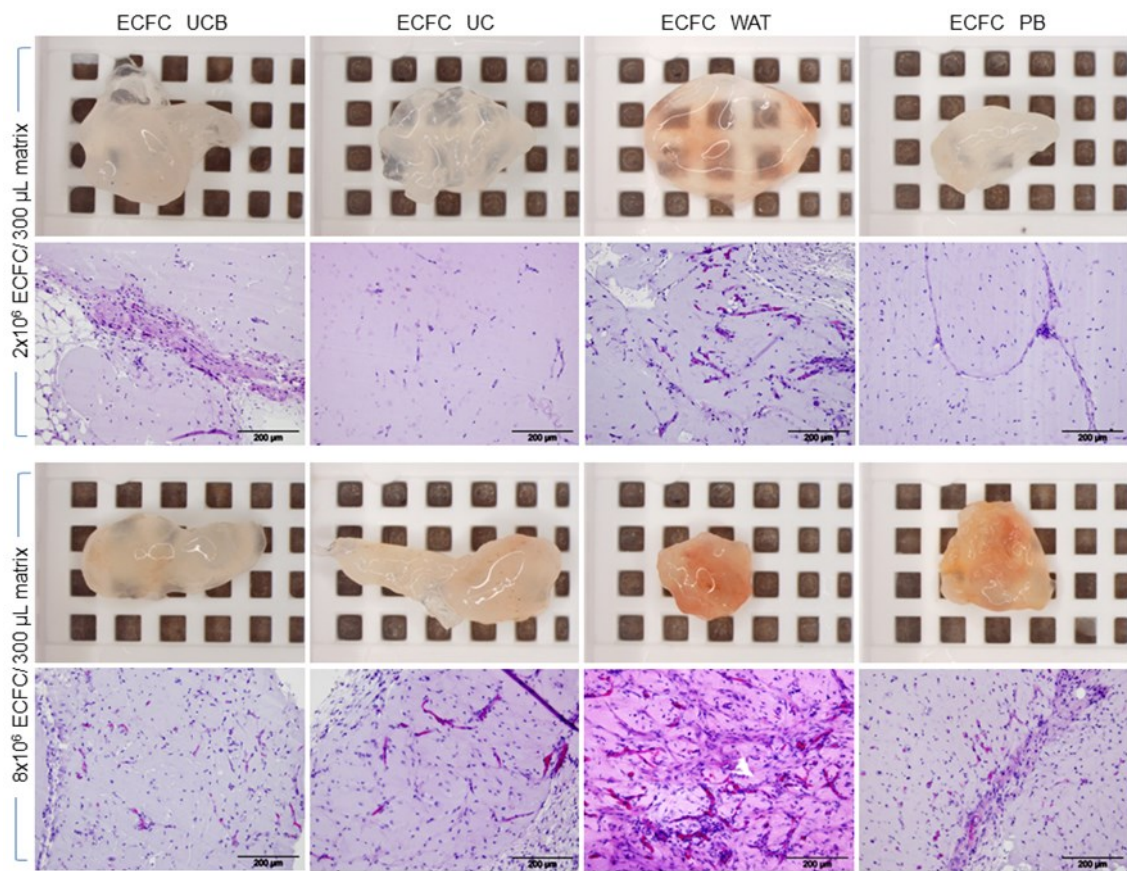


Figure 12. Vasculogenesis potential of sole ECFC *in vivo*

Macroscopic features and H&E stained micrographs of transplants containing 2×10^6 (upper row) and 8×10^6 (lower row) ECFC derived from umbilical cord blood (UCB), umbilical cord (UC), white adipose tissue (WAT) and peripheral blood (PB) harvested after two weeks are depicted (upper panel). Images are representative of $n = 2$ per group.

The potential of vasculogenesis in ECFC+MSPC co-transplants from various sources (umbilical cord blood (UCB), umbilical cord (UC), white adipose tissue (WAT), amniotic membrane (AMN) and peripheral blood (PB)) comparable to sole ECFC transplants containing 2×10^6 and 8×10^6 cells is depicted (lower panel). The plugs were harvested after 2 weeks. A number of two mice were used per condition ($n=2$). Quantifications of micro-vessels in different co-transplants are shown as mean $\pm$ standard deviation.

C

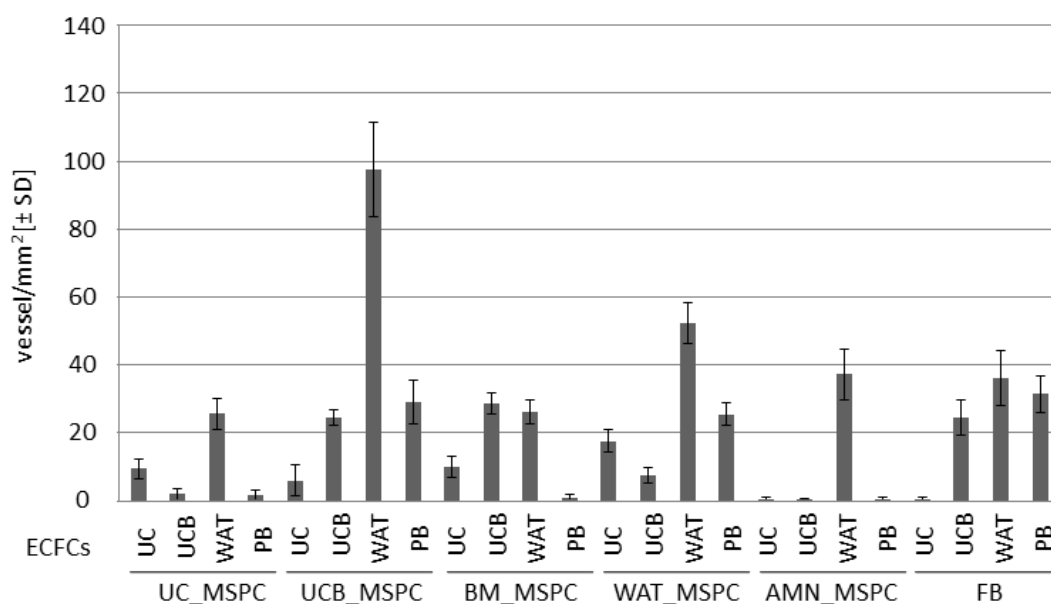


Figure 13. Vasculogenic capacity of MSC and ECFC combinations from different sources

Macroscopic (**A**) and microscopic hematoxylin/eosin (HE)-stained (**B**) appearance of MSC from umbilical cord (UC), umbilical cord blood (UCB), white adipose tissue (WAT), bone marrow (BM), amnion (AMN) and fibroblasts (FB) combined with ECFCs from UC, UCB, WAT and peripheral blood (PB) as indicated. Images are representative of $n = 3$ per group. Vessel numbers in different co-transplants are shown as mean $\pm$ standard deviation in **C**.

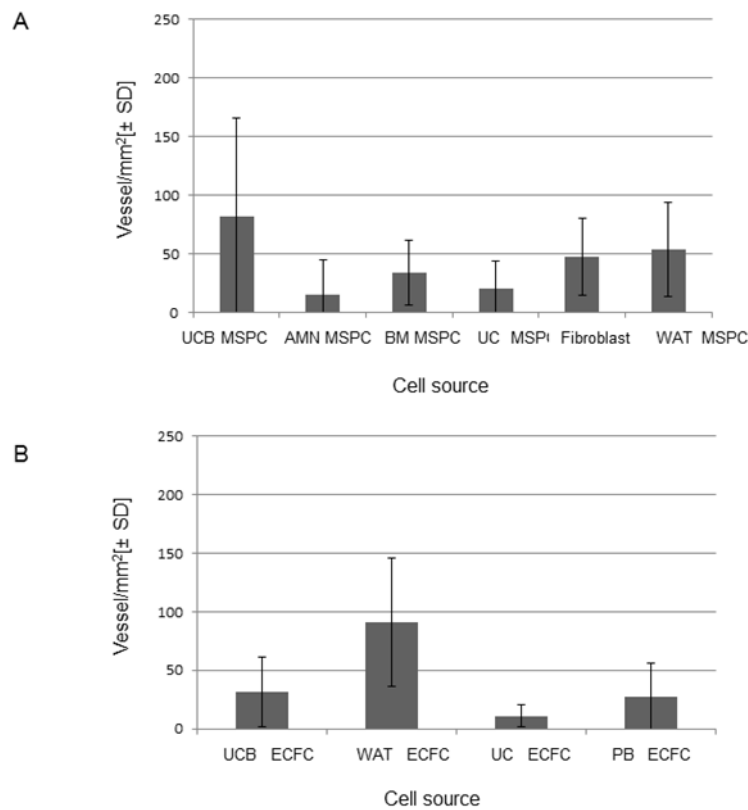


Figure 14. Capability of MSC and ECFC of various sources in vessel formation *in vivo*

MSC derived from umbilical cord blood (UCB), amniotic membrane (AMN), bone marrow (BM) and white adipose tissue (WAT) as well as fibroblast cells were assessed and quantified for the potential to participate in vasculogenesis regardless of ECFC source combined (**A**).

ECFC derived from umbilical cord blood (UCB), white adipose tissue (WAT), umbilical cord (UC) as well as Peripheral blood (PB) were examined for the vessel formation potential *in vivo*, thereafter the number of perfused vessels were quantified regardless of MSC source combined (**B**). Vessel numbers are shown as mean ± standard deviation in **A** and **B**.

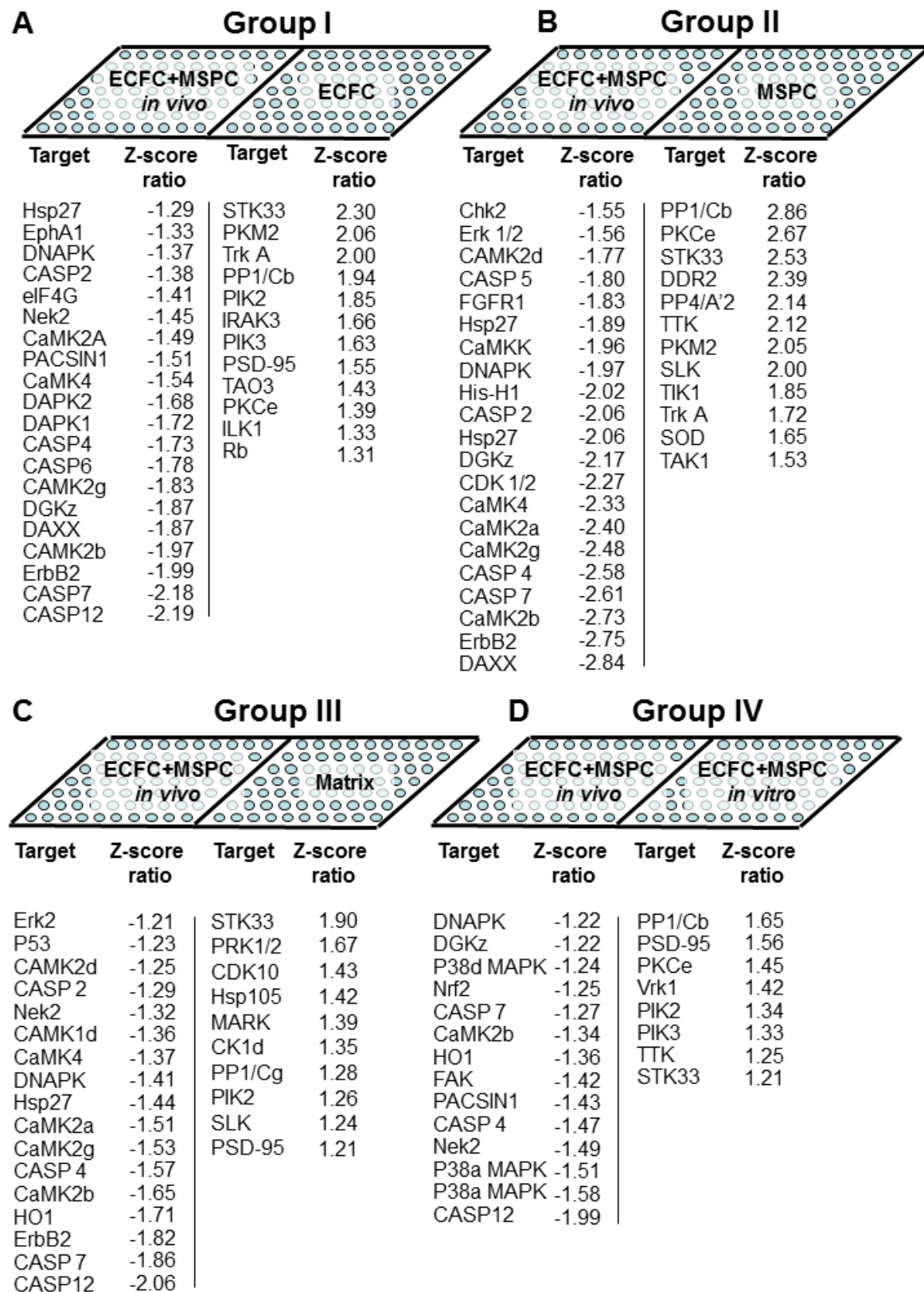


Figure 15. Proteomic profiling of significantly expressed signaling molecules allocated to the respective comparative arrays

Signaling molecules expressed in differential lysates on microarrays as indicated and corresponding Z-ratio values are delineated. Z-ratio greater than ± 1.2 was considered significant. Signaling molecule profiles of in vivo ECFC+MSPC co-transplant lysates compared to ECFC only (A) MSPC only (B) human cell free matrix (C) and ECFC+MSPC mixture in vitro lysate (D) are delineated. Three plugs were subjected to protein extraction per condition (n=3). Full names for abbreviated targets are illustrated below.

CaMK4: Calcium/calmodulin-dependent protein-serine kinase 4; **CaMKK** (CaMKK2): Calcium/calmodulin-dependent protein-serine kinase kinase; **CAMK2a**: Calcium/calmodulin-dependent protein-serine kinase 2 alpha; **CAMK2b**: Calcium/calmodulin-dependent protein-serine kinase 2 beta; **CAMK2d**: Calcium/calmodulin-dependent protein-serine kinase 2 delta; **CAMK2g**: Calcium/calmodulin-dependent protein-serine kinase 2 gamma; **Casp2**: Caspase 2 (ICH1 protease); **Casp12**: Caspase 12 (mouse); **Casp4**: Caspase 4 (ICH2 protease, ICE(rel)-II); **Casp5**: Caspase 5 (ICH3 protease, ICE(rel)-III); **Casp6**: Caspase 6 (apoptotic protease Mch2); **Casp7**: Caspase 7 (ICE-like apoptotic protease 3 (ICE-LAP3, Mch3); **CDK1/2**: Cyclin-dependent protein-serine kinase 1/2; **CDK10**: Cyclin-dependent protein-serine kinase 10; **Chk2**: Checkpoint protein-serine kinase 2; **CK1d**: Casein protein-serine kinase 1 delta; **DAXX**: Death-associated protein 6 (BING2); **DGKz**: Diacylglycerol kinase zeta **DNAPK**: DNA-dependent protein kinase catalytic subunit; **eIF4G**: eukaryotic translation initiation factor 4 gamma 1; **EphA1**: Ephrin type-A receptor 1 protein-tyrosine kinase; **ErbB2**: ErbB2 (Neu) receptor-tyrosine kinase; **Erk1/2**: Extracellular regulated protein-serine kinase 1 (p44 MAP kinase); **FAK**: Focal adhesion protein-tyrosine kinase; **FGFR1**: Fibroblast growth factor receptor-tyrosine kinase 1; **HO-1**: Hemeoxygenase 1; **Hsp27**: Heat shock 27 kDa protein beta 1 (HspB1); **Hsp105**: Heat shock 105 kDa protein; **ILK1**: Integrin-linked protein-serine kinase 1; **IRAK3**: Interleukin 1 receptor-associated kinase 3; **JIK**: STE20-like protein-serine kinase (TAO3); **MARK**: MAP/microtubule affinity-regulating protein-serine kinase 1; **Nek2**: NIMA (never-in-mitosis)-related protein-serine kinase 2; 1 (HspB1); **Nrf2**: Nuclear factor erythroid 2-related factor 2; **PACIN1**: Protein kinase C + casein kinase substrate in neurons protein 1; 2 (p42MAP kinase); **P53**: Tumor suppressor protein p53 (antigenNY-CO-13); **PIK2**: Polo-

like protein kinase 2 (serum -inducible kinase (SNK)); **PIK3**: Polo-like protein kinase 3 (cytokine-inducible kinase (CNK)); **PKCe**: Protein-serine kinase C epsilon; **PKM2**: Pyruvate kinase, isozymes M1/M2; **PP4/A'2**: Protein-serine phosphatase 4 - regulatory subunit (PPX/A'2); **PP1/Cb**: Protein-serine phosphatase 1 - catalytic subunit - beta isoform; **PP1/Cg**: Protein-serine phosphatase 1-catalytic subunit-gamma isoform; **PRK1/2**: Protein kinase C-related protein-serine kinase 1/2; **PSD-95**: Disks large homolog 4; **p38MAPK**: Mitogen-activated protein-serine kinase p38; **Rb**: Retinoblastoma-associated protein 1; **SLK**: STE20-like protein-serine kinase; **SOD**: Superoxide dismutase 1; **STK33**: FLJ35932 protein-serine kinase; **TAK1**: TGF-beta-activated protein-serine kinase 1; **Tik1**: Tousled-like protein-serine kinase 1; **TrkA**: Nerve growth factor (NGF) receptor-tyrosine kinase; **TTK**: Dual specificity protein kinase; **Tyro10**: Neurotrophic receptor-tyrosine kinase of discoidin domain receptor family, member 2 precursor; **Vrk1**: Vaccinia related protein-serine kinase 1

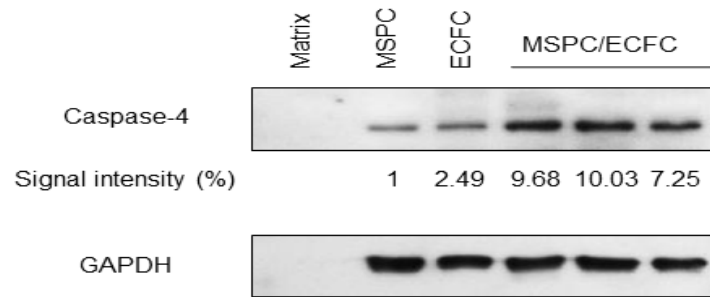


Figure 16. Western blot analysis of protein lysates from early transplants

Expression of human caspase-4 in MSC, ECFC and co-transplants of the two cell populations, as indicated, was analyzed in protein lysates obtained from plugs harvested 24h after transplantation. The protein lysate content of three plugs (n=3) with the same cell type/number but different cell donors in three different mice (n=3) were pooled for the Western blot as a confirmation for the proteomic data. A higher relative caspase-4 expression in MSC/ECFC co-transplants as compared to the sole populations is illustrated (top graph) and verified using densitometry with ImageJ software relative to the GAPDH signal. The quantification values (caspase-4/GAPDH area) were measured and the MSC-only signal set as one. The Western blot was optimized and performed as a method for confirmation of the proteomic analysis in which a higher expression of caspase-4 in MSC+ECFC co-transplant plugs compared to other conditions (including Matrix only, MSC only and ECFC only) was detected.

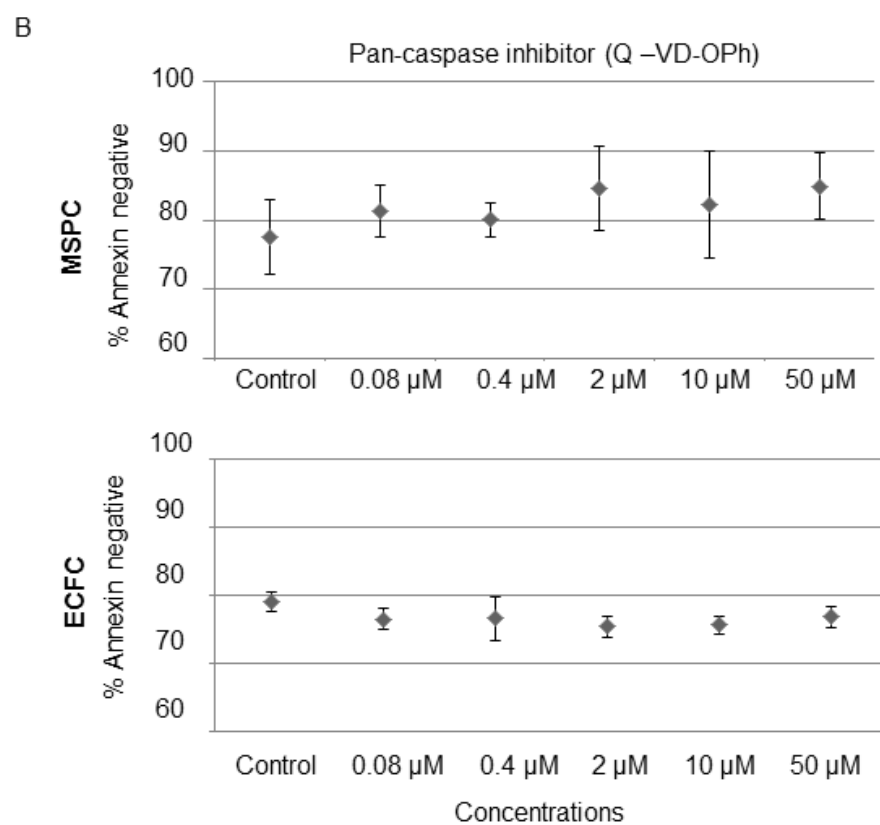
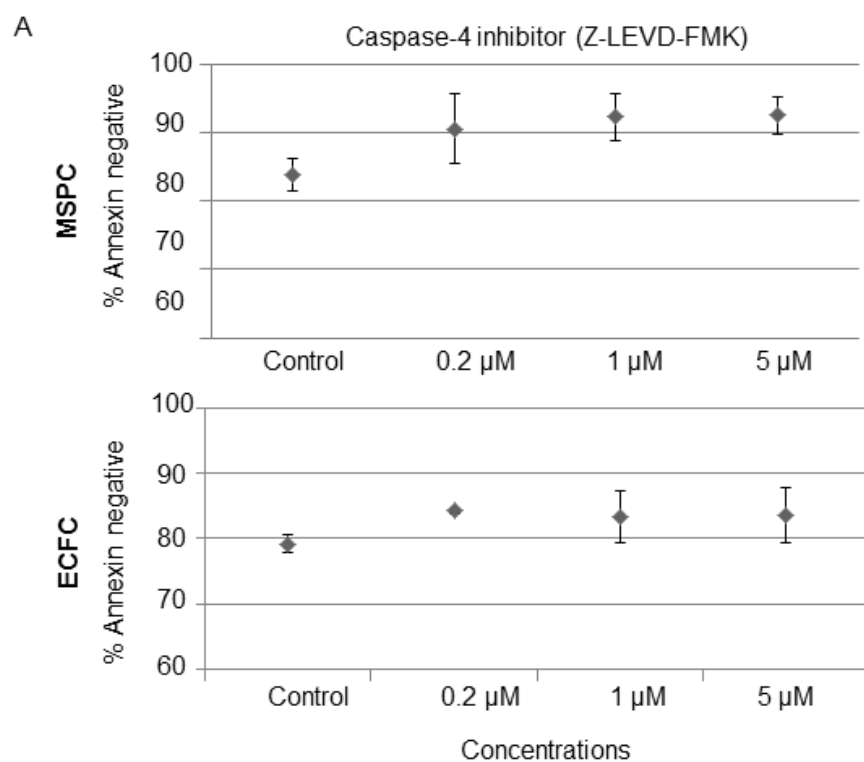


Figure 17. Limited cytotoxicity of caspase inhibitors

MSPC and ECFC were treated with vehicle (DMSO) or increasing concentrations of DMSO-dissolved **(A)** caspase-4 or **(B)** pan-caspase inhibitor for 8h. Cells were washed 2x and Annexin-V-reactivity was determined by flow cytometry to measure apoptotic and dead cells. Percentages of viable annexin negative cells are depicted (mean $\pm$ SD of three experiments).

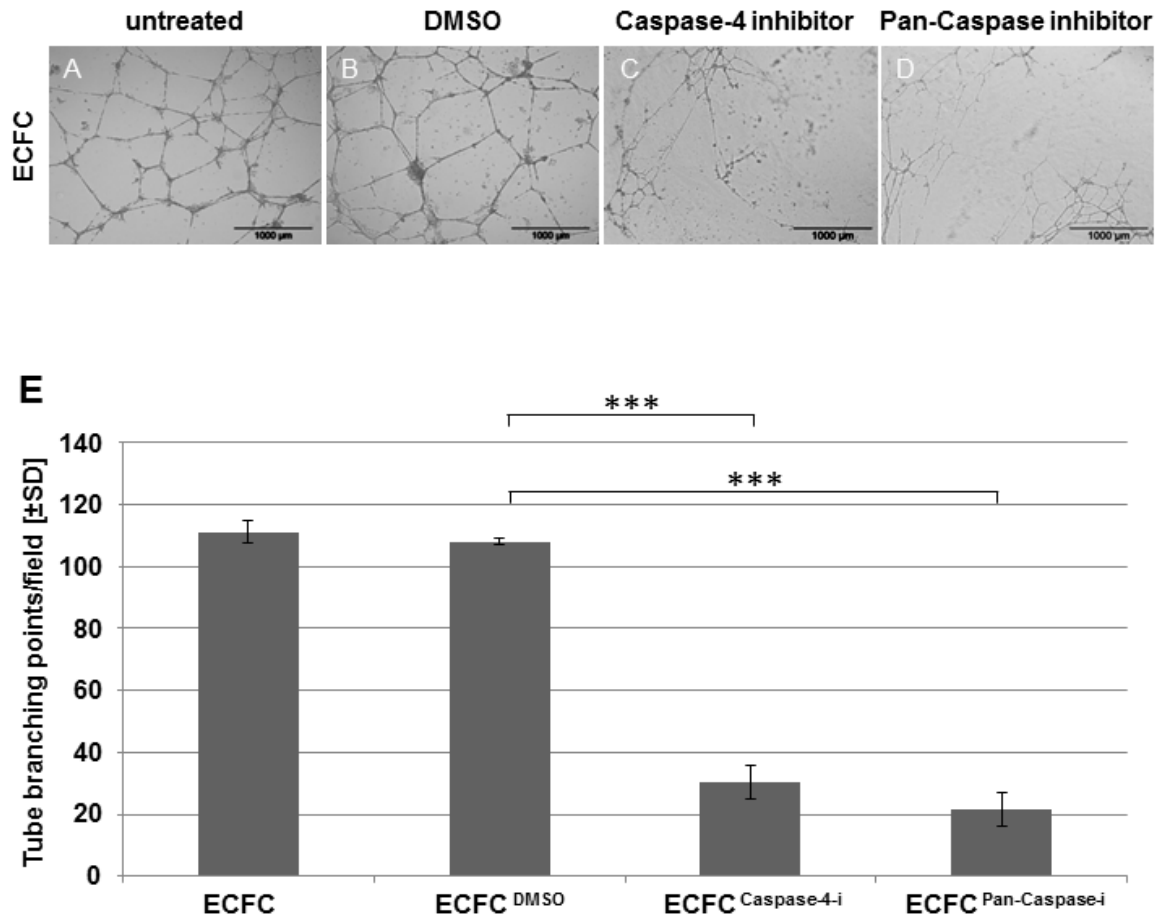


Figure 18. Effect of caspase inhibitors on ECFC network formation *in vitro*

ECFCs were tested un-treated (A) and upon eight hour pretreatment with either DMSO (B), caspase-4 inhibitor Z-LEVD-FMK (C) or pan-caspase inhibitor Q-VD-OPh (D) in a 24 hour network formation assay *in vitro*. Quantification of cell network branching points and corresponding un-paired t-test results (***) $p \leq 0.0001$ are depicted (E). Each field represents an area of 9.45 mm². Scale bar: 1000 µm. Mean ± standard deviation are shown of three experiments per condition.

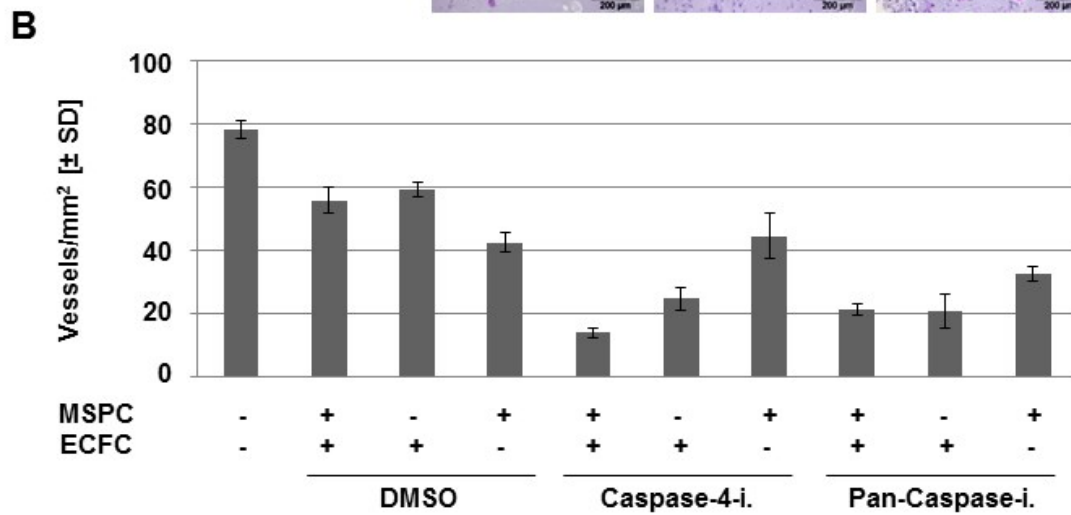
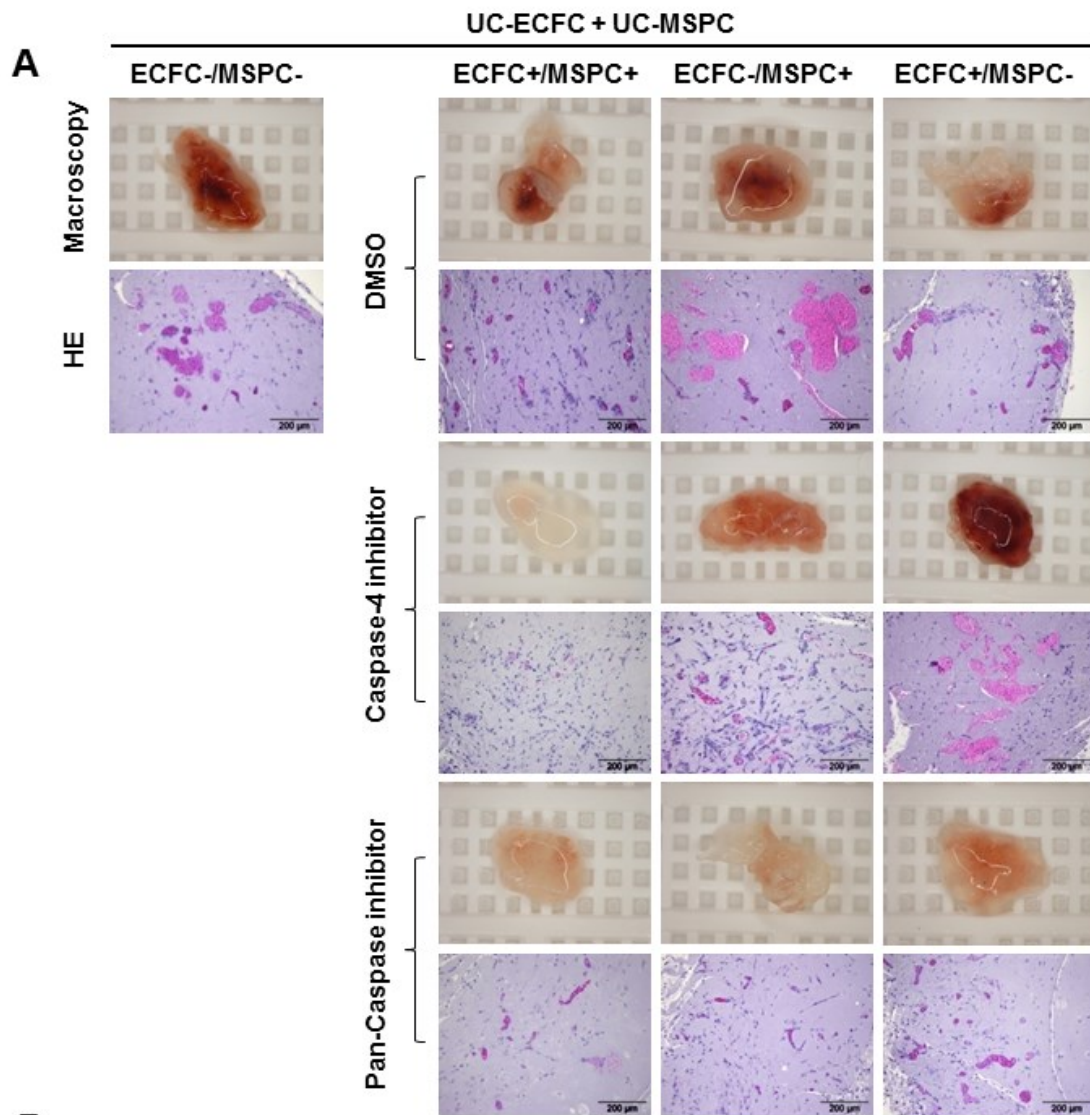


Figure 19. Caspase inhibition hampers umbilical cord progenitor cell-derived vasculogenesis *in vivo*

Macroscopic view and micrographs of hematoxylin/eosin (HE) staining of plugs after 2 weeks containing either untreated (-) or treated (+) umbilical cord (UC)-derived autologous ECFC+MSPC pairs, each from the same cord (**A**). Quantification of vascular structures upon treatment with DMSO (left panel), caspase-4 inhibitor (middle panel) and pan-caspase inhibition (right panel) compared to untreated conditions are shown (**B**). Mean $\pm$ standard deviation are shown of two plugs from two mice per condition.

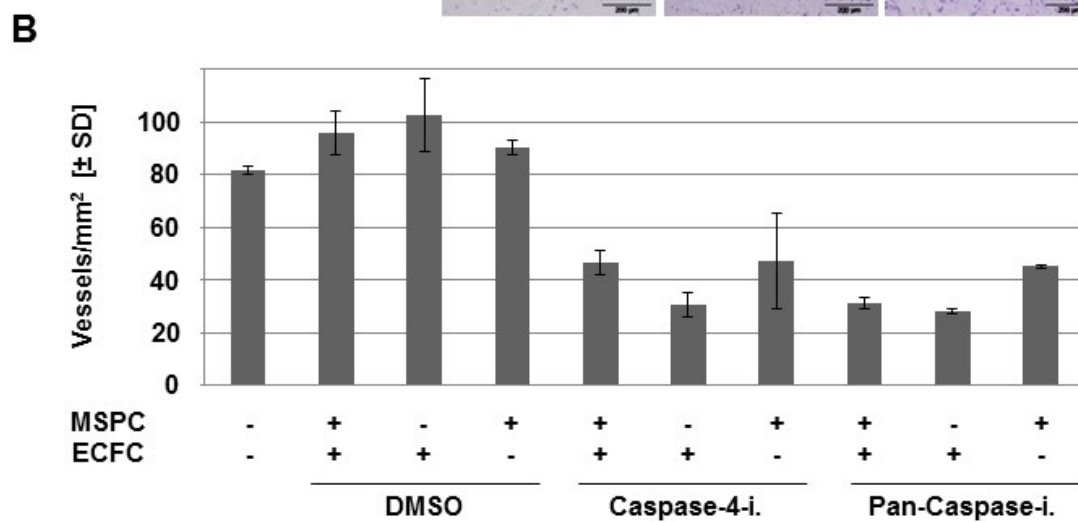
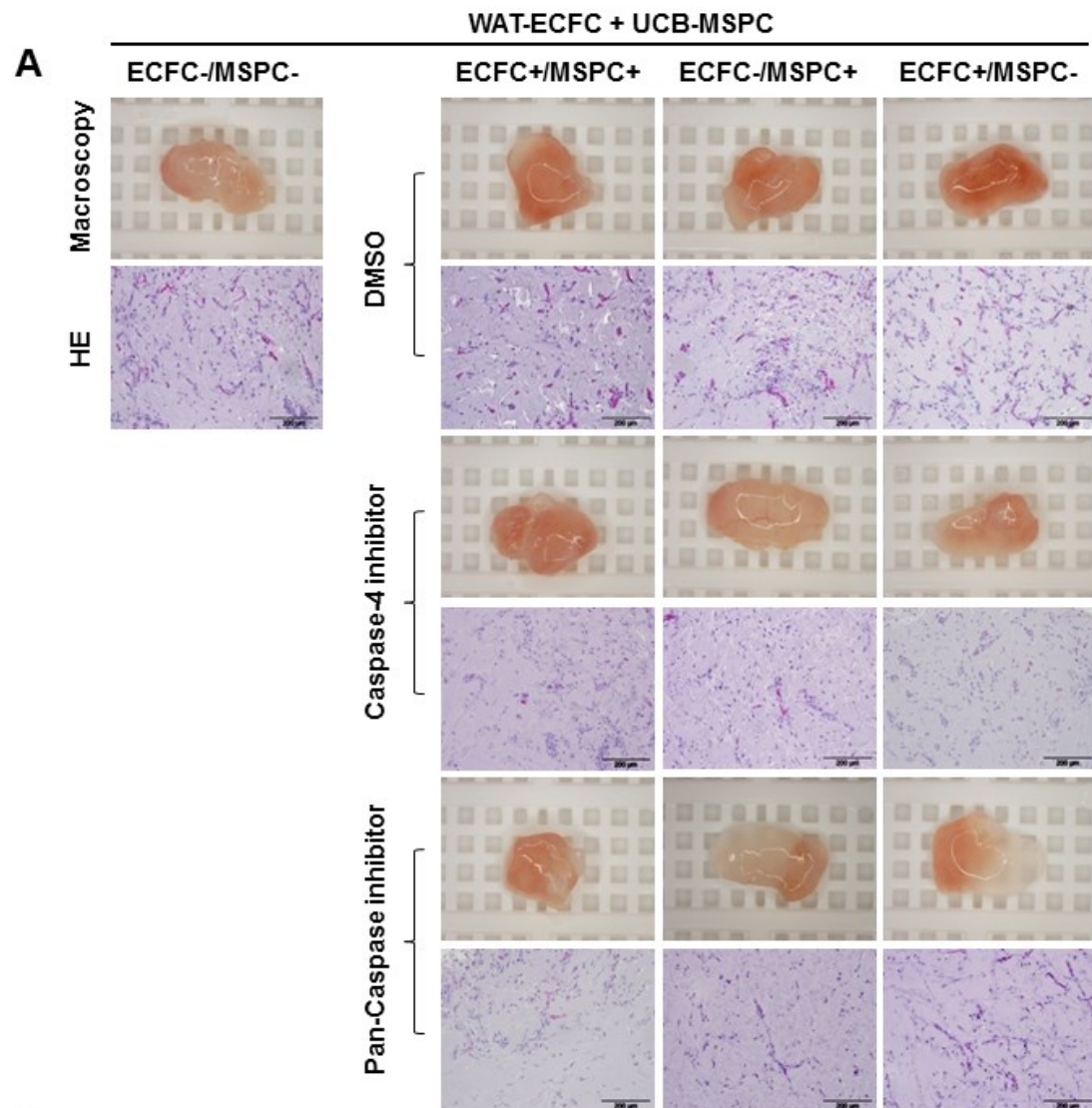


Figure 20. Vasculogenesis is decreased after caspase inhibition in white adipose tissue-derived ECFC and umbilical cord blood-derived MSPC *in vivo*

Macroscopic view and micrographs of hematoxylin/eosin (H&E) staining of plugs after 2 weeks containing either untreated (-) or treated (+) white adipose tissue (WAT)-derived autologous ECFC and umbilical cord blood (UCB)-derived MSPC (**A**). Quantification of vascular structure upon DMSO (left panel), caspase-4 (middle panel) and pan-caspase inhibition compared to untreated conditions are shown (**B**). Mean $\pm$ standard deviation are shown of two plugs from two mice per condition.

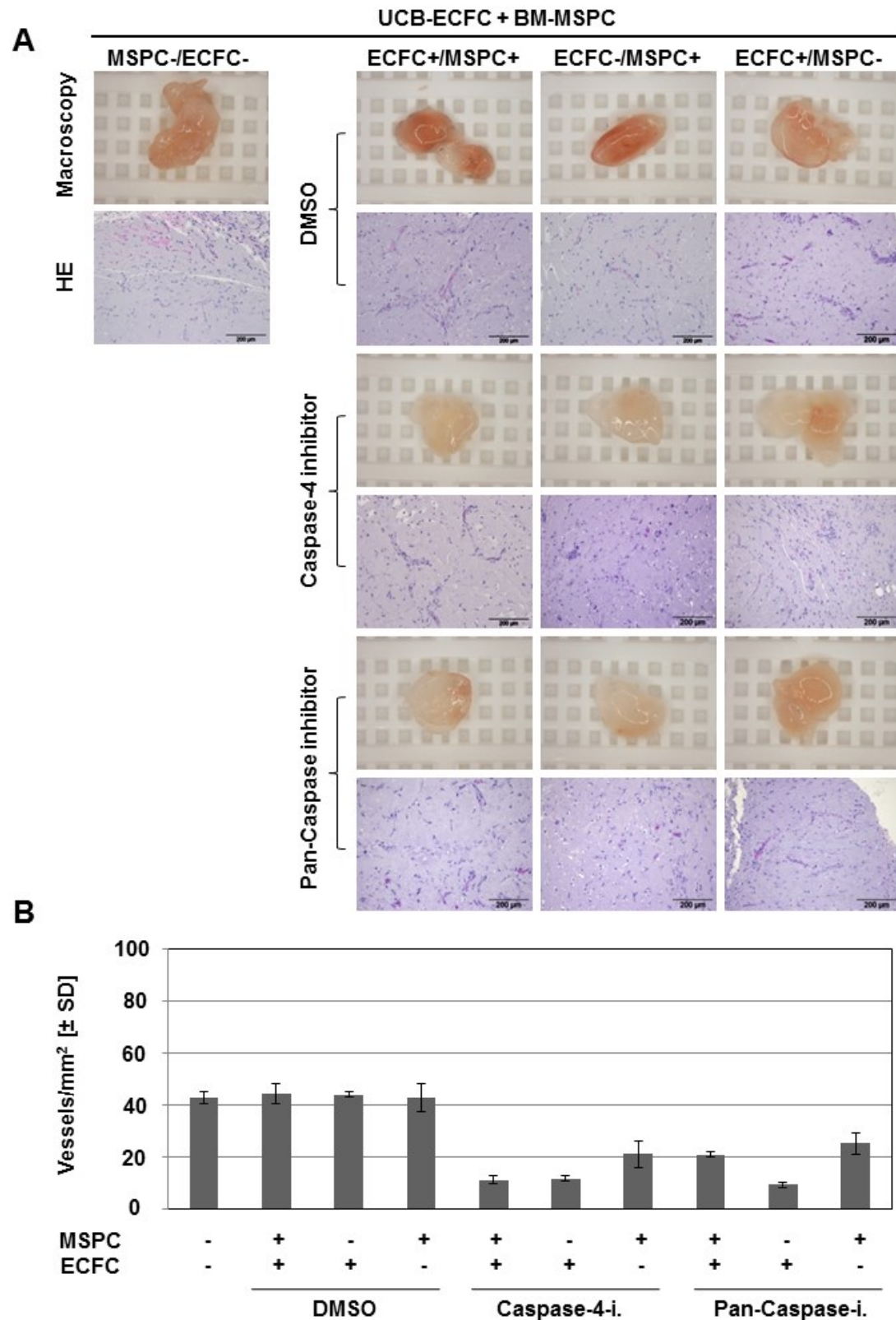


Figure 21. Vasculogenesis is decreased after caspase inhibition in umbilical cord blood-derived ECFC and bone marrow-derived MSPC *in vivo*

Macroscopic view and micrographs of hematoxylin/eosin (H&E) staining of plugs after 2 weeks containing either untreated (-) or treated (+) umbilical cord blood (UCB)-derived ECFC and bone marrow (BM)-derived MSC (A). Quantification of vascular structure upon DMSO (left panel), caspase-4 (middle panel) and pan-caspase inhibition compared to untreated conditions are shown (B). Mean $\pm$ standard deviation are shown of two plugs from two mice per condition.

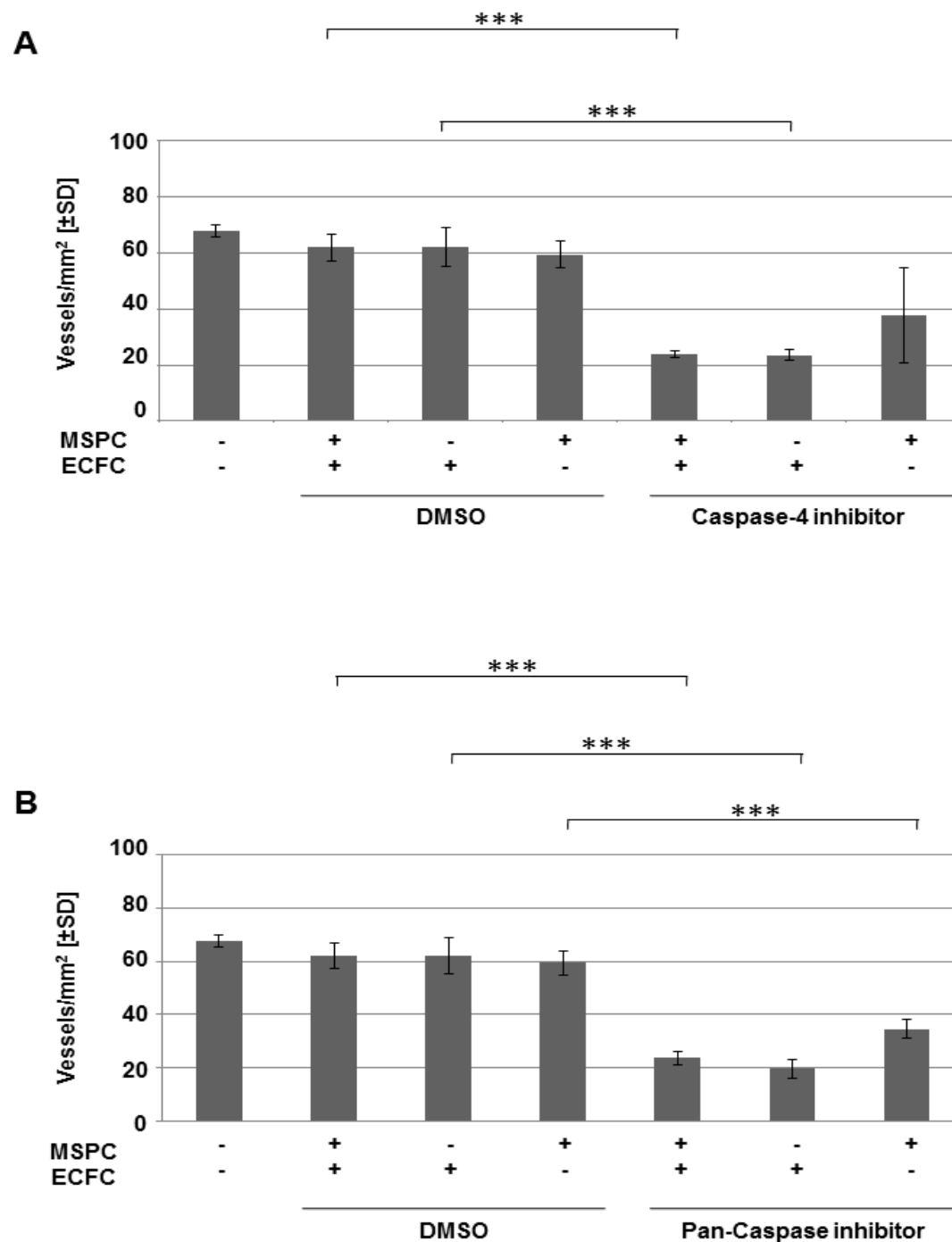
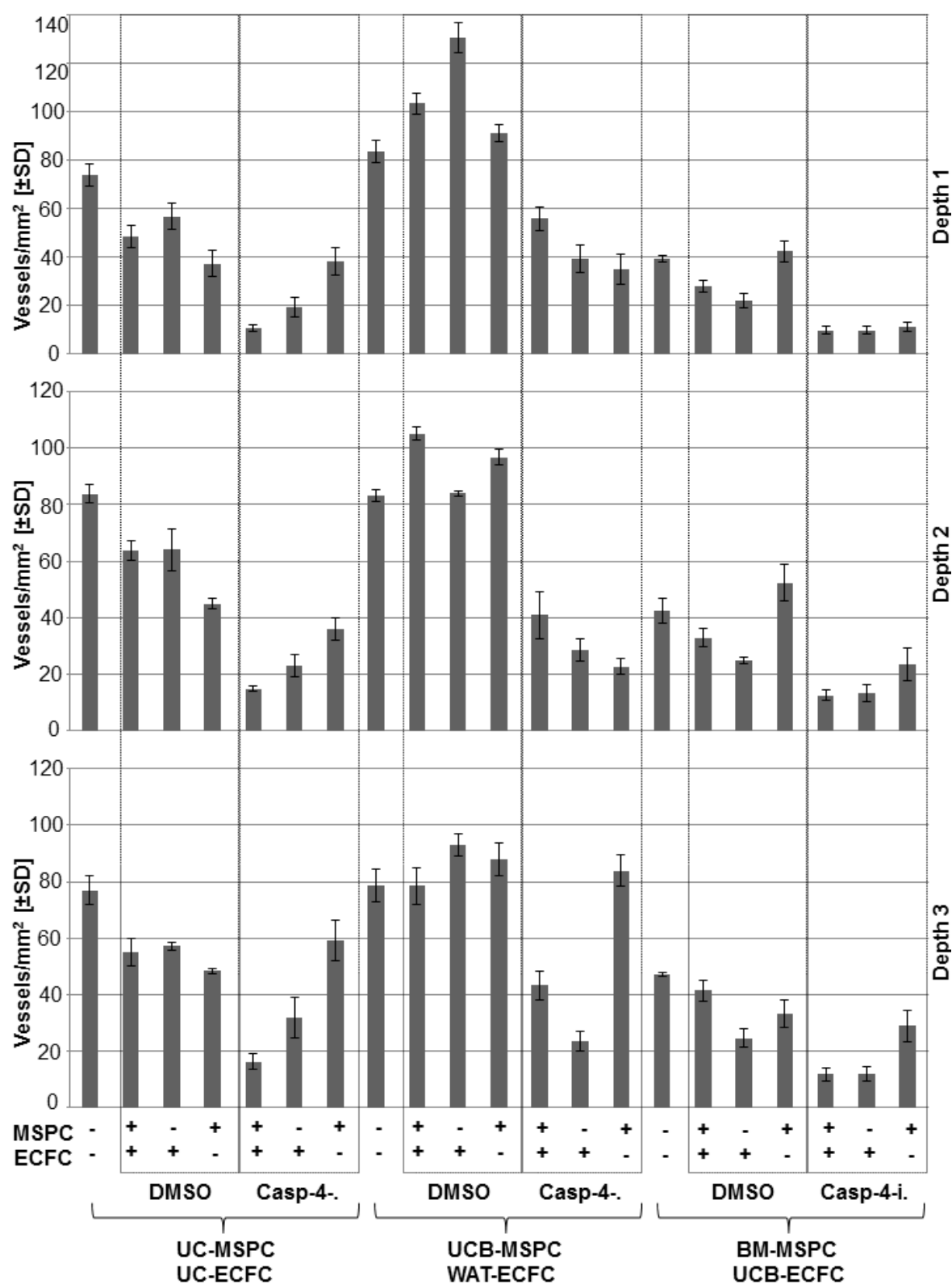


Figure 22. Vessel formation is impaired upon caspase-4 and pan-caspase inhibition

Mean number of vessels created by co-transplantation of MSPC+ECFC pairs derived from three different human organs (combined analysis of the data seen Figures 18, 19 and 20: umbilical cord-derived ECFC and umbilical cord-derived MSPC; white adipose tissue-derived ECFC and umbilical

cord blood-derived MSPC; umbilical cord blood-derived ECFC and bone marrow-derived MSPC) comparing untreated progenitor cells with those pretreated either with DMSO (**A** and **B**, left panels), caspase-4 inhibitor (**A**, right panels) or pan-caspase inhibitor (**B**, right panel) (** $p < 0.001$, *** $p \leq 0.0001$). Mean $\pm$ standard deviation are shown of six plugs from six mice per condition.

A



B

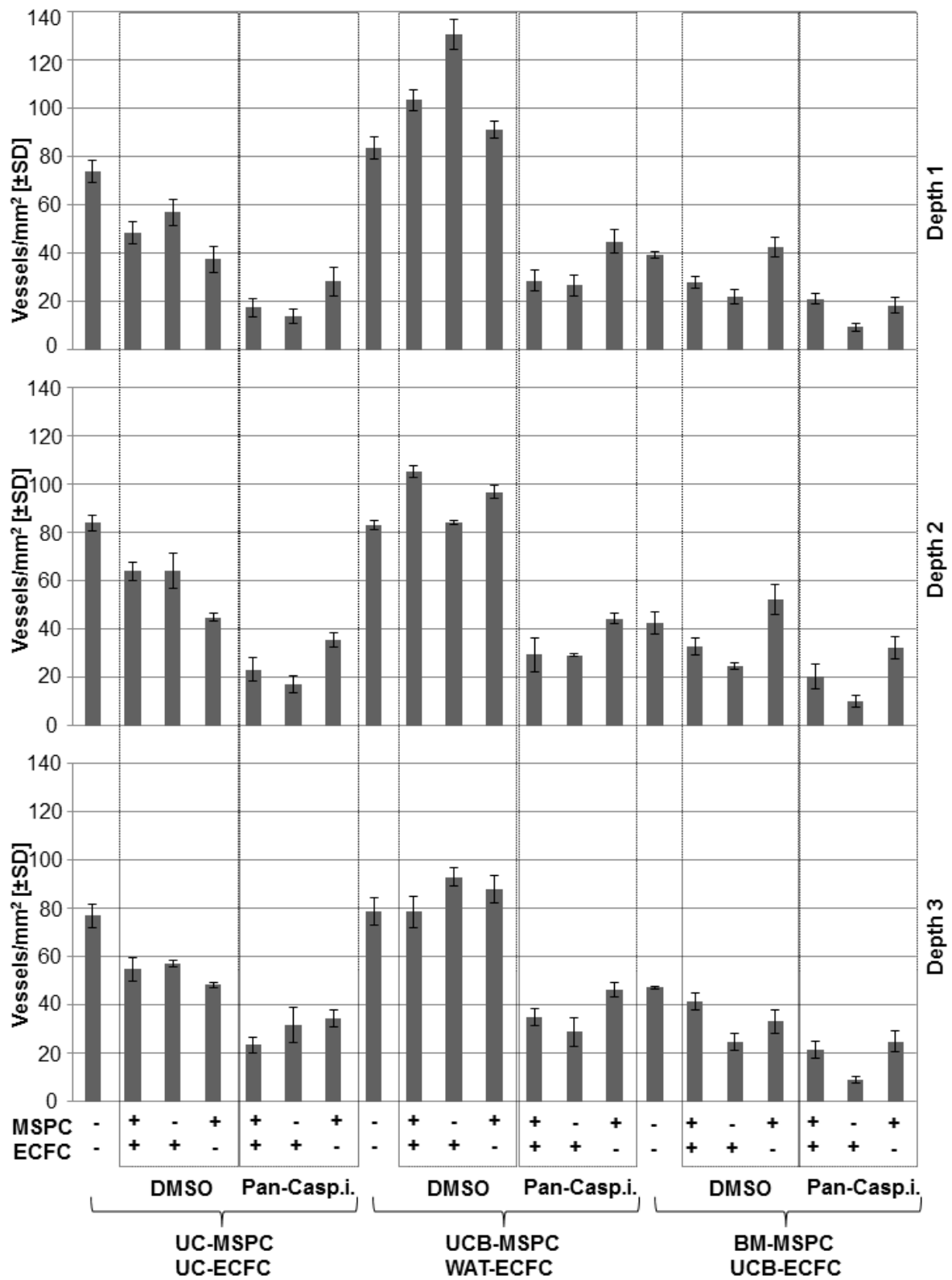


Figure 23. Micro-vessel density upon caspase-4 inhibition as quantified in various depths of the implants

Plugs consisted of umbilical cord (UC)-ECFC and UC-MSPC (left panel), white adipose tissue (WAT)-ECFC and umbilical cord blood (UCB)-MSPC (middle panel), and UCB-ECFC and (BM)-MSPC (right panel), and contained either un-treated cells or cells treated with caspase 4 (**A**) or pan-caspase blocker (**B**). Explanted plugs were cut in three depths with approximately 150 μm intervals and the micro-vessels were counted in different sections. This strategy was used to address a hypothetical uneven vessel distribution within plugs in micro-vessel quantification. DMSO (-) or caspase inhibitor (+) pretreatment as indicated. Mean $\pm$ standard deviation are shown of $n=2$ plugs per condition per time point. For each condition a number of two mice were used.

Table 3. Signaling signature specific for effective ECFC+MSPC co-transplantation in antibody microarrays

<i>ECFC+MSPC</i>	<i>I. ECFC</i>	<i>II. MSPC</i>	<i>III. Matrix</i>	<i>IV. in vitro</i>
CASP 2	↑ 1.38	↑ 2.06	↑ 1.29	
CASP 4	↑ 1.73	↑ 2.58	↑ 1.57	↑ 1.47
CASP 5		↑ 1.80		
CASP 6	↑ 1.78			
CASP 7	↑ 2.18	↑ 2.61	↑ 1.86	↑ 1.27
CASP 12	↑ 2.19		↑ 2.06	↑ 1.99
DAXX	↑ 1.87	↑ 2.84		
CAMK 2 α	↑ 1.49	↑ 2.40	↑ 1.51	
CAMK 2 β	↑ 1.97	↑ 2.73	↑ 1.65	↑ 1.34
CAMK 2 γ	↑ 1.83	↑ 2.48	↑ 1.53	
CAMK 2 δ			↑ 1.77	↑ 1.25
DNAPK	↑ 1.37	↑ 1.97	↑ 1.41	↑ 1.22
PACSIN 1	↑ 1.51			↑ 1.43
DGKz	↑ 1.87	↑ 2.17		↑ 1.22
TTK		↓ 2.12		↓ 1.25
Erk 1/2		↑ 1.34	↑ 1.21	
Nek 2	↑ 1.45		↑ 1.32	↑ 1.49
PP1/Cb	↓ 1.94	↓ 2.86		↓ 1.65
STK 33	↓ 2.30	↓ 2.53	↓ 1.90	↓ 1.21
ErbB 2	↑ 1.99	↑ 2.75	↑ 1.82	
NTRK 1(TrkA)	↓ 2.00	↓ 1.72		

ECFC+MSPC	ECFC	MSPC	Matrix	<i>in vitro</i>
PKM2	↓ 2.06	↓ 2.05		
HSP27	↑ 1.29	↑ 1.89	↑ 1.44	
TTK		↓ 2.12		↓ 1.25
HO-1			↑ 1.71	↑ 1.36
P53			↑ 1.23	
DAPK1	↑ 1.72			
DAPK2	↑ 1.68			
JIK	↓ 1.43			
PKCe	↓ 1.39	↓ 2.67		↓ 1.45
ILK1	↓ 1.33			
PP4/A'2		↓ 2.14		
FAK				↑ 1.42
FGFR1		↑ 1.83		
DDR2		↓ 2.39		
Plk2	↓ 1.85		↓ 1.26	↓ 1.34
Plk3	↓ 1.63			↓ 1.33
IRAK3	↓ 1.66			
P38MAPK				↑ 1.51

ECFC+MSPC	ECFC	MSPC	Matrix	<i>in vitro</i>
PSD-95	↓ 1.55		↓ 1.21	↓ 1.56
Chk2		↑ 1.55		
SLK		↓ 2.00	↓ 1.26	
TIK1		↓ 1.85		
SOD(Cu/Zn)		↓ 1.65		
TAK1		↓ 1.53		
Rb	↓ 1.31			
PRK 1/2			↓ 1.67	
CDK10			↓ 1.43	
HSP105			↓ 1.42	
MARK			↓ 1.39	
CK1d			↓ 1.35	
Histone H1		↑ 2.02		
eIF4G	↑ 1.41			
CDK1/2		↑ 2.27		
PP1/Cg			↓ 1.28	
Vrk1				↓ 1.42
Nrf2				↑ 1.25

Footnote Table 3

Signaling molecules expressed significantly in co-transplants of ECFC+MSPC *in vivo* compared to those detected in equal amounts of ECFC only, MSPC only, matrix only without cells (all harvested 24h after transplantation) and ECFC+MSPC mixture *in vitro*.

The total number of microarrays is four with each target antibody determined in duplicate (1,760 spots per condition). ↑arrows indicate up-regulation while ↓arrows show down-regulation of the target in ECFC+MSPC *in vivo* compared to the four different groups. Z-ratio of more than ±1.2 was considered to be significant. The results have been submitted to Gene Expression Omnibus (accession number GSE45896).

Table abbreviations

CaMK4: Calcium/calmodulin-dependent protein-serine kinase 4; **CaMKK** (CaMKK2): Calcium/calmodulin-dependent protein-serine kinase kinase; **CAMK2a**: Calcium/calmodulin-dependent protein-serine kinase 2 alpha; **CAMK2b**: Calcium/calmodulin-dependent protein-serine kinase 2 beta; **CAMK2d**: Calcium/calmodulin-dependent protein-serine kinase 2 delta; **CAMK2g**: Calcium/calmodulin-dependent protein-serine kinase 2 gamma; **Casp2**: Caspase 2 (ICH1 protease); **Casp12**: Caspase 12 (mouse); **Casp4**: Caspase 4 (ICH2 protease, ICE(rel)-II); **Casp5**: Caspase 5 (ICH3 protease, ICE(rel)-III); **Casp6**: Caspase 6 (apoptotic protease Mch2); **Casp7**: Caspase 7 (ICE-like apoptotic protease 3 (ICE-LAP3, Mch3); **CDK1/2**: Cyclin-dependent protein-serine kinase 1/2; **CDK10**: Cyclin-dependent protein-serine kinase 10; **Chk2**: Checkpoint protein-serine kinase 2; **CK1d**: Casein protein-serine kinase 1 delta; ; **DAXX**: Death-associated protein 6 (BING2); **DGKz**: Diacylglycerol kinase zeta **DNAPK**: DNA-dependent protein kinase catalytic subunit; **elF4G**: eukaryotic translation initiation factor 4 gamma 1; **EphA1**: Ephrin type-A receptor 1 protein-tyrosine kinase; **ErbB2**: ErbB2 (Neu) receptor-tyrosine kinase; **Erk1/2**: Extracellular regulated protein-serine kinase 1 (p44 MAP kinase); **FAK**: Focal adhesion protein-tyrosine kinase; **FGFR1**: Fibroblast growth factor receptor-tyrosine kinase 1; **HO-1**: Hemeoxygenase 1; **Hsp27**: Heat shock 27 kDa protein beta 1 (HspB1); **Hsp105**: Heat shock 105 kDa protein; **ILK1**: Integrin-linked protein-serine kinase 1; **IRAK3**: Interleukin 1 receptor-associated kinase 3; **JIK**: STE20-like protein-serine kinase (TAO3); **MARK**: MAP/microtubule affinity-regulating protein-serine kinase 1; **Nek2**: NIMA (never-in-mitosis)-related protein-serine kinase 2; 1 (HspB1); **Nrf2**: Nuclear factor erythroid 2-related factor 2; **PACIN1**: Protein kinase C + casein kinase substrate in neurons protein 1; 2 (p42MAP kinase); **P53**: Tumor suppressor protein p53 (antigenNY-CO-13); **PIK2**: Polo-like protein kinase 2 (serum -inducible kinase (SNK)); **PIK3**: Polo-like protein kinase 3 (cytokine-inducible kinase (CNK)); **PKCe**: Protein-serine kinase C epsilon; **PKM2**: Pyruvate kinase, isozymes M1/M2; **PP4/A'2**: Protein-serine phosphatase 4 - regulatory subunit (PPX/A'2); **PP1/Cb**: Protein-serine phosphatase 1 - catalytic subunit - beta isoform; **PP1/Cg**: Protein-serine phosphatase 1-catalytic subunit-gamma isoform; **PRK1/2**: Protein kinase C-related protein-serine kinase 1/2; **PSD-95**: Disks large homolog 4; **p38MAPK**: Mitogen-activated protein-serine kinase p38; **Rb**: Retinoblastoma-associated protein 1; **SLK**: STE20-like protein-serine kinase; **SOD**: Superoxide

dismutase 1; **STK33**: FLJ35932 protein-serine kinase; **TAK1**: TGF-beta-activated protein-serine kinase 1; **Tlk1**: Tousled-like protein-serine kinase 1; **TrkA**: Nerve growth factor (NGF) receptor-tyrosine kinase; **TTK**: Dual specificity protein kinase; **Tyro10**: Neurotrophic receptor-tyrosine kinase of discoidin domain receptor family, member 2 precursor; **Vrk1**: Vaccinia related protein-serine kinase 1

10. REFERENCES

1991. Cellular and molecular networks in clinical immunology and allergy. 18th symposium, Collegium Internationale Allergologicum. Funchal, Madeira, September 22-26, 1990. Proceedings. *Int Arch Allergy Appl Immunol*, 94, 1-376.
- ADAM, G. C., SORENSEN, E. J. & CRAVATT, B. F. 2002. Proteomic profiling of mechanistically distinct enzyme classes using a common chemotype. *Nat Biotechnol*, 20, 805-9.
- AKIYAMA, S. K., YAMADA, S. S., CHEN, W. T. & YAMADA, K. M. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J Cell Biol*, 109, 863-75.
- AL-HAJJ, M., WICHA, M. S., BENITO-HERNANDEZ, A., MORRISON, S. J. & CLARKE, M. F. 2003. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*, 100, 3983-8.
- ALAM, A., COHEN, L. Y., AOUAD, S. & SEKALY, R. P. 1999. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *J Exp Med*, 190, 1879-90.
- ALHAMDANI, M. S., SCHRODER, C. & HOHEISEL, J. D. 2009. Oncoproteomic profiling with antibody microarrays. *Genome Med*, 1, 68.
- ALIMANDI, M., ROMANO, A., CURIA, M. C., MURARO, R., FEDI, P., AARONSON, S. A., DI FIORE, P. P. & KRAUS, M. H. 1995. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene*, 10, 1813-21.
- ALLEN, P., MELERO-MARTIN, J. & BISCHOFF, J. 2011. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. *J Tissue Eng Regen Med*, 5, e74-86.
- ALLENDE, M. L. & PROIA, R. L. 2002. Sphingosine-1-phosphate receptors and the development of the vascular system. *Biochim Biophys Acta*, 1582, 222-7.
- ALROY, I. & YARDEN, Y. 1997. The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett*, 410, 83-6.
- ALTRUDA, F., CERVELLA, P., TARONE, G., BOTTA, C., BALZAC, F., STEFANUTO, G. & SILENGO, L. 1990. A human integrin beta 1 subunit with a unique cytoplasmic domain generated by alternative mRNA processing. *Gene*, 95, 261-6.
- ANDERSON, M. E. 2005. Calmodulin kinase signaling in heart: an intriguing candidate target for therapy of myocardial dysfunction and arrhythmias. *Pharmacol Ther*, 106, 39-55.
- ANDERSON, N. G., MALLER, J. L., TONKS, N. K. & STURGILL, T. W. 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature*, 343, 651-3.
- ANDERSON, N. L. & ANDERSON, N. G. 1998. Proteome and proteomics: new technologies, new concepts, and new words. *Electrophoresis*, 19, 1853-61.

- APOLLONI, A., PRIOR, I. A., LINDSAY, M., PARTON, R. G. & HANCOCK, J. F. 2000. H-ras but not K-ras traffics to the plasma membrane through the exocytic pathway. *Mol Cell Biol*, 20, 2475-87.
- ARAI, F. & SUDA, T. 2007. Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche. *Ann N Y Acad Sci*, 1106, 41-53.
- ARMESILLA-DIAZ, A., ELVIRA, G. & SILVA, A. 2009. p53 regulates the proliferation, differentiation and spontaneous transformation of mesenchymal stem cells. *Exp Cell Res*, 315, 3598-610.
- ARMULIK, A., ABRAMSSON, A. & BETSHOLTZ, C. 2005. Endothelial/pericyte interactions. *Circ Res*, 97, 512-23.
- ARTHUR, H. M., URE, J., SMITH, A. J., RENFORTH, G., WILSON, D. I., TORSNEY, E., CHARLTON, R., PARUMS, D. V., JOWETT, T., MARCHUK, D. A., BURN, J. & DIAMOND, A. G. 2000. Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol*, 217, 42-53.
- ARUFFO, A., STAMENKOVIC, I., MELNICK, M., UNDERHILL, C. B. & SEED, B. 1990. CD44 is the principal cell surface receptor for hyaluronate. *Cell*, 61, 1303-13.
- ASAHARA, T. & KAWAMOTO, A. 2004. Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol*, 287, C572-9.
- AU, P., DAHERON, L. M., DUDA, D. G., COHEN, K. S., TYRRELL, J. A., LANNING, R. M., FUKUMURA, D., SCADDEN, D. T. & JAIN, R. K. 2008. Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. *Blood*, 111, 1302-5.
- AUGUSTIN, H. G., KOH, G. Y., THURSTON, G. & ALITALO, K. 2009. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat Rev Mol Cell Biol*, 10, 165-77.
- BAKER, C. H., KEDAR, D., MCCARTY, M. F., TSAN, R., WEBER, K. L., BUCANA, C. D. & FIDLER, I. J. 2002. Blockade of epidermal growth factor receptor signaling on tumor cells and tumor-associated endothelial cells for therapy of human carcinomas. *Am J Pathol*, 161, 929-38.
- BAKER, T. L., BOODEN, M. A. & BUSS, J. E. 2000. S-Nitrosocysteine increases palmitate turnover on Ha-Ras in NIH 3T3 cells. *J Biol Chem*, 275, 22037-47.
- BALLESTER, R., FURTH, M. E. & ROSEN, O. M. 1987. Phorbol ester- and protein kinase C-mediated phosphorylation of the cellular Kirsten ras gene product. *J Biol Chem*, 262, 2688-95.
- BALLESTER, R., MARCHUK, D., BOGUSKI, M., SAULINO, A., LETCHER, R., WIGLER, M. & COLLINS, F. 1990. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell*, 63, 851-9.
- BARFIELD, R. C., HALE, G. A., BURNETTE, K., BEHM, F. G., KNAPP, K., ELDRIDGE, P. & HANDGRETINGER, R. 2007. Autologous transplantation of CD133 selected hematopoietic progenitor cells for treatment of relapsed acute lymphoblastic leukemia. *Pediatr Blood Cancer*, 48, 349-53.
- BARRY, F. P., BOYNTON, R. E., HAYNESWORTH, S., MURPHY, J. M. & ZAIA, J. 1999. The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). *Biochem Biophys Res Commun*, 265, 134-9.

- BARTMANN, C., ROHDE, E. & SCHALLMOSER, K., ET AL. 2007. Two steps to functional MSC for clinical application. *Transfusion*.
- BAULIDA, J., KRAUS, M. H., ALIMANDI, M., DI FIORE, P. P. & CARPENTER, G. 1996. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J Biol Chem*, 271, 5251-7.
- BEENKEN, A. & MOHAMMADI, M. 2009. The FGF family: biology, pathophysiology and therapy. *Nat Rev Drug Discov*, 8, 235-53.
- BELL, B. D., LEVERRIER, S., WEIST, B. M., NEWTON, R. H., ARECHIGA, A. F., LUHRS, K. A., MORRISSETTE, N. S. & WALSH, C. M. 2008. FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. *Proc Natl Acad Sci U S A*, 105, 16677-82.
- BELLE, A., TANAY, A., BITINCKA, L., SHAMIR, R. & O'SHEA, E. K. 2006. Quantification of protein half-lives in the budding yeast proteome. *Proc Natl Acad Sci U S A*, 103, 13004-9.
- BENEDITO, R., ROCA, C., SORENSEN, I., ADAMS, S., GOSSLER, A., FRUTTIGER, M. & ADAMS, R. H. 2009. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell*, 137, 1124-35.
- BERGERON, L., PEREZ, G. I., MACDONALD, G., SHI, L., SUN, Y., JURISICOVA, A., VARMUZA, S., LATHAM, K. E., FLAWS, J. A., SALTER, J. C., HARA, H., MOSKOWITZ, M. A., LI, E., GREENBERG, A., TILLY, J. L. & YUAN, J. 1998. Defects in regulation of apoptosis in caspase-2-deficient mice. *Genes Dev*, 12, 1304-14.
- BERGERS, G. & HANAHAN, D. 2008. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer*, 8, 592-603.
- BERGERS, G. & SONG, S. 2005. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol*, 7, 452-64.
- BERGERS, G., SONG, S., MEYER-MORSE, N., BERGSLAND, E. & HANAHAN, D. 2003. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest*, 111, 1287-95.
- BERGSBAKEN, T., FINK, S. L. & COOKSON, B. T. 2009. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol*, 7, 99-109.
- BERNARDO, M. E., EMONS, J. A., KAPERIEN, M., NAUTA, A. J., WILLEMZE, R., ROELOFS, H., ROMEO, S., MARCHINI, A., RAPPOLD, G. A., VUKICEVIC, S., LOCATELLI, F. & FIBBE, W. E. 2007. Human mesenchymal stem cells derived from bone marrow display a better chondrogenic differentiation compared with other sources. *Connect Tissue Res*, 48, 132-40.
- BERRY, D. L. & BAEHRECKE, E. H. 2007. Growth arrest and autophagy are required for salivary gland cell degradation in *Drosophila*. *Cell*, 131, 1137-48.
- BESANT, P. G., TAN, E. & ATTWOOD, P. V. 2003. Mammalian protein histidine kinases. *Int J Biochem Cell Biol*, 35, 297-309.
- BETSHOLTZ, C. 2004. Insight into the physiological functions of PDGF through genetic studies in mice. *Cytokine Growth Factor Rev*, 15, 215-28.
- BHATTACHARYA, D., ROSSI, D. J., BRYDER, D. & WEISSMAN, I. L. 2006. Purified hematopoietic stem cell engraftment of rare niches corrects severe lymphoid deficiencies without host conditioning. *J Exp Med*, 203, 73-85.
- BIAN, Z. M., ELNER, S. G. & ELNER, V. M. 2009. Dual involvement of caspase-4 in inflammatory and ER stress-induced apoptotic responses in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*, 50, 6006-14.

- BLACKSTOCK, W. P. & WEIR, M. P. 1999. Proteomics: quantitative and physical mapping of cellular proteins. *Trends Biotechnol*, 17, 121-7.
- BLAGOEV, B., KRATCHMAROVA, I., ONG, S. E., NIELSEN, M., FOSTER, L. J. & MANN, M. 2003. A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nat Biotechnol*, 21, 315-8.
- BLAIR, A., HOGGE, D. E., AILLES, L. E., LANSDORP, P. M. & SUTHERLAND, H. J. 1997. Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. *Blood*, 89, 3104-12.
- BLÉNIS, J. 1993. Signal transduction via the MAP kinases: proceed at your own RSK. *Proc Natl Acad Sci U S A*, 90, 5889-92.
- BOBIS, S., JAROCHA, D. & MAJKA, M. 2006. Mesenchymal stem cells: characteristics and clinical applications. *Folia Histochem Cytobiol*, 44, 215-30.
- BODEY, B., BODEY, B., JR., GROGER, A. M., LUCK, J. V., SIEGEL, S. E., TAYLOR, C. R. & KAISER, H. E. 1997. Clinical and prognostic significance of the expression of the c-erbB-2 and c-erbB-3 oncoproteins in primary and metastatic malignant melanomas and breast carcinomas. *Anticancer Res*, 17, 1319-30.
- BOIRET, N., RAPATEL, C., VEYRAT-MASSON, R., GUILLOUARD, L., GUERIN, J. J., PIGEON, P., DESCAMPS, S., BOISGARD, S. & BERGER, M. G. 2005. Characterization of nonexpanded mesenchymal progenitor cells from normal adult human bone marrow. *Exp Hematol*, 33, 219-25.
- BORNSTEIN, P., KYRIAKIDES, T. R., YANG, Z., ARMSTRONG, L. C. & BIRK, D. E. 2000. Thrombospondin 2 modulates collagen fibrillogenesis and angiogenesis. *J Invest Dermatol Symp Proc*, 5, 61-6.
- BORNSTEIN, P. & SAGE, E. H. 1994. Thrombospondins. *Methods Enzymol*, 245, 62-85.
- BOURDEAU, A., DUMONT, D. J. & LETARTE, M. 1999. A murine model of hereditary hemorrhagic telangiectasia. *J Clin Invest*, 104, 1343-51.
- BOWEN, M. A., PATEL, D. D., LI, X., MODRELL, B., MALACKO, A. R., WANG, W. C., MARQUARDT, H., NEUBAUER, M., PESANDO, J. M., FRANCKE, U. & ET AL. 1995. Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand. *J Exp Med*, 181, 2213-20.
- BRAKEBUSCH, C., HIRSCH, E., POTOCHNIK, A. & FASSLER, R. 1997. Genetic analysis of beta1 integrin function: confirmed, new and revised roles for a crucial family of cell adhesion molecules. *J Cell Sci*, 110 (Pt 23), 2895-904.
- BRENNAN, M. A. & COOKSON, B. T. 2000. Salmonella induces macrophage death by caspase-1-dependent necrosis. *Molecular Microbiology*, 38, 31-40.
- BRITTAN, M. & WRIGHT, N. A. 2002. Gastrointestinal stem cells. *J Pathol*, 197, 492-509.
- BROOKS, P. C., CLARK, R. A. F. & CHERESH, D. A. 1994a. Requirement of Vascular Integrin Alpha(V)Beta(3) for Angiogenesis. *Science*, 264, 569-571.
- BROOKS, P. C., MONTGOMERY, A. M., ROSENFELD, M., REISFELD, R. A., HU, T., KLIER, G. & CHERESH, D. A. 1994b. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell*, 79, 1157-64.

- BROOKS, P. C., STROMBLAD, S., KLEMKE, R., VISSCHER, D., SARKAR, F. H. & CHERESH, D. A. 1995. Antiintegrin Alpha-V-Beta-3 Blocks Human Breast-Cancer Growth and Angiogenesis in Human Skin. *Journal of Clinical Investigation*, 96, 1815-1822.
- BROSE, K., BLAND, K. S., WANG, K. H., ARNOTT, D., HENZEL, W., GOODMAN, C. S., TESSIER-LAVIGNE, M. & KIDD, T. 1999. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell*, 96, 795-806.
- BROXMEYER, H. E., DOUGLAS, G. W., HANGOC, G., COOPER, S., BARD, J., ENGLISH, D., ARNY, M., THOMAS, L. & BOYSE, E. A. 1989. Human Umbilical-Cord Blood as a Potential Source of Transplantable Hematopoietic Stem Progenitor Cells. *Proceedings of the National Academy of Sciences of the United States of America*, 86, 3828-3832.
- BRUDER, S. P., FINK, D. J. & CAPLAN, A. I. 1994. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J Cell Biochem*, 56, 283-94.
- BRUNS, C. J., SOLORZANO, C. C., HARBISON, M. T., OZAWA, S., TSAN, R., FAN, D., ABBRUZZESE, J., TRAXLER, P., BUCHDUNGER, E., RADINSKY, R. & FIDLER, I. J. 2000. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res*, 60, 2926-35.
- BRY, M., KIVELA, R., HOLOPAINEN, T., ANISIMOV, A., TAMMELA, T., SORONEN, J., SILVOLA, J., SARASTE, A., JELTSCH, M., KORPISALO, P., CARMELIET, P., LEMSTROM, K. B., SHIBUYA, M., YLA-HERTTUALA, S., ALHONEN, L., MERVAALA, E., ANDERSSON, L. C., KNUUTI, J. & ALITALO, K. 2010. Vascular endothelial growth factor-B acts as a coronary growth factor in transgenic rats without inducing angiogenesis, vascular leak, or inflammation. *Circulation*, 122, 1725-33.
- BRYDER, D., ROSSI, D. J. & WEISSMAN, I. L. 2006. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am J Pathol*, 169, 338-46.
- BUDAY, L. & DOWNWARD, J. 1993. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell*, 73, 611-20.
- BURGER, J. A., SPOO, A., DWENGER, A., BURGER, M. & BEHRINGER, D. 2003. CXCR4 chemokine receptors (CD184) and alpha4beta1 integrins mediate spontaneous migration of human CD34+ progenitors and acute myeloid leukaemia cells beneath marrow stromal cells (pseudoemperipolesis). *Br J Haematol*, 122, 579-89.
- BUSSOLATI, B., AHMED, A., PEMBERTON, H., LANDIS, R. C., DI CARLO, F., HASKARD, D. O. & MASON, J. C. 2004. Bifunctional role for VEGF-induced heme oxygenase-1 in vivo: induction of angiogenesis and inhibition of leukocytic infiltration. *Blood*, 103, 761-6.
- BUSSOLATI, B. & MASON, J. C. 2006. Dual role of VEGF-induced heme-oxygenase-1 in angiogenesis. *Antioxid Redox Signal*, 8, 1153-63.
- CAIRO, M. S. & WAGNER, J. E. 1997. Placental and/or umbilical cord blood: an alternative source of hematopoietic stem cells for transplantation. *Blood*, 90, 4665-78.
- CANN, H. M., DE TOMA, C., CAZES, L., LEGRAND, M. F., MOREL, V., PIOUSFRE, L., BODMER, J., BODMER, W. F., BONNE-TAMIR, B.,

- CAMBON-THOMSEN, A., CHEN, Z., CHU, J. Y., CARCASSI, C., CONTU, L., DU, R. F., EXCOFFIER, L., FERRARA, G. B., FRIEDLAENDER, J. S., GROOT, H., GURWITZ, D., JENKINS, T., HERRERA, R. J., HUANG, X. Y., KIDD, J., KIDD, K. K., LANGANEY, A., LIN, A. A., MEHDI, S. Q., PARHAM, P., PIAZZA, A., PISTILLO, M. P., QIAN, Y. P., SHU, Q. F., XU, J. J., ZHU, S., WEBER, J. L., GREELY, H. T., FELDMAN, M. W., THOMAS, G., DAUSSET, J. & CAVALLI-SFORZA, L. L. 2002. A human genome diversity cell line panel. *Science*, 296, 261-262.
- CANQUE, B., CAMUS, S., DALLOUL, A., KAHN, E., YAGELLO, M., DEZUTTER-DAMBUYANT, C., SCHMITT, D., SCHMITT, C. & GLUCKMAN, J. C. 2000. Characterization of dendritic cell differentiation pathways from cord blood CD34(+)CD7(+)CD45RA(+) hematopoietic progenitor cells. *Blood*, 96, 3748-56.
- CARMELIET, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat Med*, 6, 389-95.
- CARMELIET, P. 2003. Angiogenesis in health and disease. *Nat Med*, 9, 653-60.
- CARMELIET, P. & COLLEN, D. 2000. Transgenic mouse models in angiogenesis and cardiovascular disease. *J Pathol*, 190, 387-405.
- CARMELIET, P. & JAIN, R. K. 2011. Molecular mechanisms and clinical applications of angiogenesis. *Nature*, 473, 298-307.
- CARVALHO, R. L., JONKER, L., GOUMANS, M. J., LARSSON, J., BOUWMAN, P., KARLSSON, S., DIJKE, P. T., ARTHUR, H. M. & MUMMERY, C. L. 2004. Defective paracrine signalling by TGFbeta in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. *Development*, 131, 6237-47.
- CARY, L. A., CHANG, J. F. & GUAN, J. L. 1996. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *Journal of Cell Science*, 109, 1787-1794.
- CARY, L. A., HAN, D. C., POLTE, T. R., HANKS, S. K. & GUAN, J. L. 1998. Identification of p130(Cas) as a mediator of focal adhesion kinase-promoted cell migration. *Journal of Cell Biology*, 140, 211-221.
- CASERTA, T. M., SMITH, A. N., GULTICE, A. D., REEDY, M. A. & BROWN, T. L. 2003. Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. *Apoptosis*, 8, 345-52.
- CHAMBERS, R. C., LEONI, P., KAMINSKI, N., LAURENT, G. J. & HELLER, R. A. 2003. Global expression profiling of fibroblast responses to transforming growth factor-beta(1) reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. *American Journal of Pathology*, 162, 533-546.
- CHAN, F. K., SHISLER, J., BIXBY, J. G., FELICES, M., ZHENG, L., APPEL, M., ORENSTEIN, J., MOSS, B. & LENARDO, M. J. 2003. A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. *J Biol Chem*, 278, 51613-21.
- CHAN, J., WADDINGTON, S. N., O'DONOGHUE, K., KURATA, H., GUILLOT, P. V., GOTHERSTROM, C., THEMIS, M., MORGAN, J. E. & FISK, N. M. 2007. Widespread distribution and muscle differentiation of human fetal mesenchymal stem cells after intrauterine transplantation in dystrophic mdx mouse. *Stem Cells*, 25, 875-84.
- CHANG, H., HUYLEBROECK, D., VERSCHUEREN, K., GUO, Q., MATZUK, M. M. & ZWIJSEN, A. 1999. Smad5 knockout mice die at mid-gestation due to

- multiple embryonic and extraembryonic defects. *Development*, 126, 1631-42.
- CHANG, H. Y., NISHITOH, H., YANG, X., ICHIJO, H. & BALTIMORE, D. 1998. Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science*, 281, 1860-3.
- CHANG, T. W. 1983. Binding of cells to matrixes of distinct antibodies coated on solid surface. *J Immunol Methods*, 65, 217-23.
- CHANG, Y. J., SHIH, D. T., TSENG, C. P., HSIEH, T. B., LEE, D. C. & HWANG, S. M. 2006. Disparate mesenchyme-lineage tendencies in mesenchymal stem cells from human bone marrow and umbilical cord blood. *Stem Cells*, 24, 679-85.
- CHARRON, D. J. 2009. Jean Dausset (1916-2009) OBITUARY. *Nature*, 460, 338-338.
- CHAU, H., WONG, V., CHEN, N. J., HUANG, H. L., LIN, W. J., MIRTSOS, C., ELFORD, A. R., BONNARD, M., WAKEHAM, A., YOU-TEN, A. I., LEMMERS, B., SALMENA, L., PELLEGRINI, M., HAKEM, R., MAK, T. W., OHASHI, P. & YEH, W. C. 2005. Cellular FLICE-inhibitory protein is required for T cell survival and cycling. *J Exp Med*, 202, 405-13.
- CHAUTAN, M., CHAZAL, G., CECCONI, F., GRUSS, P. & GOLSTEIN, P. 1999. Interdigital cell death can occur through a necrotic and caspase-independent pathway. *Curr Biol*, 9, 967-70.
- CHEADLE, C., VAWTER, M. P., FREED, W. J. & BECKER, K. G. 2003. Analysis of microarray data using Z score transformation. *J Mol Diagn*, 5, 73-81.
- CHEN, B. H., TZEN, J. T. C., BRESNICK, A. R. & CHEN, H. C. 2002. Roles of Rho-associated kinase and myosin light chain kinase in morphological and migratory defects of focal adhesion kinase-null cells. *Journal of Biological Chemistry*, 277, 33857-33863.
- CHEN, M. & WANG, J. 2002. Initiator caspases in apoptosis signaling pathways. *Apoptosis*, 7, 313-9.
- CHIMENTI, I., SMITH, R. R., LI, T. S., GERSTENBLITH, G., MESSINA, E., GIACOMELLO, A. & MARBAN, E. 2010. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. *Circ Res*, 106, 971-80.
- CHISHTI, A. H., KIM, A. C., MARFATIA, S. M., LUTCHMAN, M., HANSPAL, M., JINDAL, H., LIU, S. C., LOW, P. S., ROULEAU, G. A., MOHANDAS, N., CHASIS, J. A., CONBOY, J. G., GASCARD, P., TAKAKUWA, Y., HUANG, S. C., BENZ, E. J., BRETSCHER, A., FEHON, R. G., GUSELLA, A. F., RAMESH, V., SOLOMON, F., MARCHESI, V. T., TSUKITA, S., TSUKITA, S., ARPIN, M., LOUVARD, D., TONKS, N. K., ANDERSON, J. M., FANNING, A. S., BRYANT, P. J., WOODS, D. F. & HOOVER, K. B. 1998. The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends in Biochemical Sciences*, 23, 281-282.
- CHIU, V. K., BIVONA, T., HACH, A., SAJOUS, J. B., SILLETTI, J., WIENER, H., JOHNSON, R. L., 2ND, COX, A. D. & PHILIPS, M. R. 2002. Ras signalling on the endoplasmic reticulum and the Golgi. *Nat Cell Biol*, 4, 343-50.
- CHOY, E., CHIU, V. K., SILLETTI, J., FEOKTISTOV, M., MORIMOTO, T., MICHAELSON, D., IVANOV, I. E. & PHILIPS, M. R. 1999. Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell*, 98, 69-80.

- CITRI, A., ALROY, I., LAVI, S., RUBIN, C., XU, W., GRAMMATIKAKIS, N., PATTERSON, C., NECKERS, L., FRY, D. W. & YARDEN, Y. 2002. Drug-induced ubiquitylation and degradation of ErbB receptor tyrosine kinases: implications for cancer therapy. *EMBO J*, 21, 2407-17.
- CITRI, A., KOCHUPURAKKAL, B. S. & YARDEN, Y. 2004. The achilles heel of ErbB-2/HER2: regulation by the Hsp90 chaperone machine and potential for pharmacological intervention. *Cell Cycle*, 3, 51-60.
- CITRI, A. & YARDEN, Y. 2006. EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol*, 7, 505-16.
- CIVIN, C. I., STRAUSS, L. C., BROVALL, C., FACKLER, M. J., SCHWARTZ, J. F. & SHAPER, J. H. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol*, 133, 157-65.
- COHEN, B. D., GREEN, J. M., FOY, L. & FELL, H. P. 1996a. HER4-mediated biological and biochemical properties in NIH 3T3 cells. Evidence for HER1-HER4 heterodimers. *J Biol Chem*, 271, 4813-8.
- COHEN, B. D., KIENER, P. A., GREEN, J. M., FOY, L., FELL, H. P. & ZHANG, K. 1996b. The relationship between human epidermal growth-like factor receptor expression and cellular transformation in NIH3T3 cells. *J Biol Chem*, 271, 30897-903.
- COHEN, L. A. & GUAN, J. L. 2005. Residues within the first subdomain of the FERM-like domain in focal adhesion kinase are important in its regulation. *Journal of Biological Chemistry*, 280, 8197-8207.
- COLICELLI, J. 2004. Human RAS superfamily proteins and related GTPases. *Sci STKE*, 2004, RE13.
- COLLINS, A. T., BERRY, P. A., HYDE, C., STOWER, M. J. & MAITLAND, N. J. 2005. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res*, 65, 10946-51.
- COOPER, L. A., SHEN, T. L. & GUAN, J. L. 2003. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. *Molecular and Cellular Biology*, 23, 8030-8041.
- COURTOY, P. J. & BOYLES, J. 1983. Fibronectin in the microvasculature: localization in the pericyte-endothelial interstitium. *J Ultrastruct Res*, 83, 258-73.
- CRAWFORD, S. E., STELLMACH, V., MURPHY-ULLRICH, J. E., RIBEIRO, S. M., LAWLER, J., HYNES, R. O., BOIVIN, G. P. & BOUCK, N. 1998. Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell*, 93, 1159-70.
- CROSBY, J. R., SEIFERT, R. A., SORIANO, P. & BOWEN-POPE, D. F. 1998. Chimaeric analysis reveals role of Pdgf receptors in all muscle lineages. *Nat Genet*, 18, 385-8.
- CROSS, M. J. & CLAEISSON-WELSH, L. 2001. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol Sci*, 22, 201-7.
- DA SILVA MEIRELLES, L., CHAGASTELLES, P. C. & NARDI, N. B. 2006. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci*, 119, 2204-13.
- DALERBA, P., DYLLA, S. J., PARK, I. K., LIU, R., WANG, X., CHO, R. W., HOEY, T., GURNEY, A., HUANG, E. H., SIMEONE, D. M., SHELTON, A. A., PARMIANI, G., CASTELLI, C. & CLARKE, M. F. 2007. Phenotypic

- characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci U S A*, 104, 10158-63.
- DAMERON, K. M., VOLPERT, O. V., TAINSKY, M. A. & BOUCK, N. 1994. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science*, 265, 1582-4.
- DANIELSON, K. G., BARIBAULT, H., HOLMES, D. F., GRAHAM, H., KADLER, K. E. & IOZZO, R. V. 1997. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J Cell Biol*, 136, 729-43.
- DAS, S. B. & SHARMA, R. K. 2005. Potential role of calmodulin-dependent phosphodiesterase in human brain tumor (review). *Oncol Rep*, 14, 1059-63.
- DASARI, V. R., KAUR, K., VELDULA, K. K., DINH, D. H., TSUNG, A. J., MOHANAM, S. & RAO, J. S. 2010. Downregulation of Focal Adhesion Kinase (FAK) by cord blood stem cells inhibits angiogenesis in glioblastoma. *Aging (Albany NY)*, 2, 791-803.
- DAVIES CDE, L., MELDER, R. J., MUNN, L. L., MOUTA-CARREIRA, C., JAIN, R. K. & BOUCHER, Y. 2001. Decorin inhibits endothelial migration and tube-like structure formation: role of thrombospondin-1. *Microvasc Res*, 62, 26-42.
- DAVIES, S. & JIANG, W. G. 2010. ALCAM, activated leukocyte cell adhesion molecule, influences the aggressive nature of breast cancer cells, a potential connection to bone metastasis. *Anticancer Res*, 30, 1163-8.
- DAVIS, G. E. & CAMARILLO, C. W. 1995. Regulation of endothelial cell morphogenesis by integrins, mechanical forces, and matrix guidance pathways. *Exp Cell Res*, 216, 113-23.
- DAVIS, S., ALDRICH, T. H., JONES, P. F., ACHESON, A., COMPTON, D. L., JAIN, V., RYAN, T. E., BRUNO, J., RADZIEJEWSKI, C., MAISONPIERRE, P. C. & YANCOPOULOS, G. D. 1996. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell*, 87, 1161-9.
- DE CLERCQ, E. 2005. Potential clinical applications of the CXCR4 antagonist bicyclam AMD3100. *Mini Rev Med Chem*, 5, 805-24.
- DE MAIO, A. 1999. Heat shock proteins: facts, thoughts, and dreams. *Shock*, 11, 1-12.
- DE PALMA, M., VENNARI, M. A., GALLI, R., SERGI SERGI, L., POLITI, L. S., SAMPAOLESI, M. & NALDINI, L. 2005. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell*, 8, 211-26.
- DEGTEREV, A., HUANG, Z., BOYCE, M., LI, Y., JAGTAP, P., MIZUSHIMA, N., CUNY, G. D., MITCHISON, T. J., MOSKOWITZ, M. A. & YUAN, J. 2005. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol*, 1, 112-9.
- DEJANA, E. 2010. The role of wnt signaling in physiological and pathological angiogenesis. *Circ Res*, 107, 943-52.
- DEJANA, E., TOURNIER-LASSERVE, E. & WEINSTEIN, B. M. 2009. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell*, 16, 209-21.
- DELORME B, CHATEAUVIEUX S & CHARBORD P 2006. The concept of mesenchymal stem cells. *Regen Med*, 1(4), 497-509.

- DENNIS, J. E., CARBILLET, J. P., CAPLAN, A. I. & CHARBORD, P. 2002. The STRO-1+ marrow cell population is multipotential. *Cells Tissues Organs*, 170, 73-82.
- DERAMAUDT, B. M., BRAUNSTEIN, S., REMY, P. & ABRAHAM, N. G. 1998. Gene transfer of human heme oxygenase into coronary endothelial cells potentially promotes angiogenesis. *J Cell Biochem*, 68, 121-7.
- DHANASEKARAN, N. 1998. Cell signaling: an overview. *Oncogene*, 17, 1329-30.
- DHANASEKARAN, N. & PREMKUMAR REDDY, E. 1998. Signaling by dual specificity kinases. *Oncogene*, 17, 1447-55.
- DHILLON, A. S., HAGAN, S., RATH, O. & KOLCH, W. 2007. MAP kinase signalling pathways in cancer. *Oncogene*, 26, 3279-90.
- DICKSON, M. C., MARTIN, J. S., COUSINS, F. M., KULKARNI, A. B., KARLSSON, S. & AKHURST, R. J. 1995. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development*, 121, 1845-54.
- DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. & HORWITZ, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, 8, 315-7.
- DOWNWARD, J. 2003. Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer*, 3, 11-22.
- DUDA, D. G., KOZIN, S. V., KIRKPATRICK, N. D., XU, L., FUKUMURA, D. & JAIN, R. K. 2011. CXCL12 (SDF1alpha)-CXCR4/CXCR7 pathway inhibition: an emerging sensitizer for anticancer therapies? *Clin Cancer Res*, 17, 2074-80.
- DULAK, J., LOBODA, A., ZAGORSKA, A. & JOZKOWICZ, A. 2004. Complex role of heme oxygenase-1 in angiogenesis. *Antioxid Redox Signal*, 6, 858-66.
- DUMONT, D. J., GRADWOHL, G., FONG, G. H., PURI, M. C., GERTSENSTEIN, M., AUERBACH, A. & BREITMAN, M. L. 1994. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev*, 8, 1897-909.
- DUNAWAY, C. M., HWANG, Y., LINDSLEY, C. W., COOK, R. S., WU, J. Y., BOOTHBY, M., CHEN, J. & BRANTLEY-SIEDERS, D. M. 2011. Cooperative signaling between Slit2 and Ephrin-A1 regulates a balance between angiogenesis and angiostasis. *Mol Cell Biol*, 31, 404-16.
- DUNN, K. L., ESPINO, P. S., DROBIC, B., HE, S. & DAVIE, J. R. 2005. The Ras-MAPK signal transduction pathway, cancer and chromatin remodeling. *Biochem Cell Biol*, 83, 1-14.
- DUPREZ, L., WIRAWAN, E., VANDEN BERGHE, T. & VANDENABEELE, P. 2009. Major cell death pathways at a glance. *Microbes Infect*, 11, 1050-62.
- EGAN, S. E., GIDDINGS, B. W., BROOKS, M. W., BUDAY, L., SIZELAND, A. M. & WEINBERG, R. A. 1993. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature*, 363, 45-51.
- EISEN, T., AHMAD, T., FLAHERTY, K. T., GORE, M., KAYE, S., MARAIS, R., GIBBENS, I., HACKETT, S., JAMES, M., SCHUCHTER, L. M., NATHANSON, K. L., XIA, C., SIMANTOV, R., SCHWARTZ, B., POULIN-COSTELLO, M., O'DWYER, P. J. & RATAIN, M. J. 2006. Sorafenib in

- advanced melanoma: a Phase II randomised discontinuation trial analysis. *Br J Cancer*, 95, 581-6.
- ELENIUS, K., CHOI, C. J., PAUL, S., SANTIESTEVEAN, E., NISHI, E. & KLAGSBRUN, M. 1999. Characterization of a naturally occurring ErbB4 isoform that does not bind or activate phosphatidylinositol 3-kinase. *Oncogene*, 18, 2607-15.
- ELICEIRI, B. P. & CHERESH, D. A. 1999. The role of α_v integrins during angiogenesis: insights into potential mechanisms of action and clinical development. *Journal of Clinical Investigation*, 103, 1227-1230.
- ELMORE, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol*, 35, 495-516.
- EMUSS, V., GARNETT, M., MASON, C. & MARAIS, R. 2005. Mutations of C-RAF are rare in human cancer because C-RAF has a low basal kinase activity compared with B-RAF. *Cancer Res*, 65, 9719-26.
- ENCINA, N. R., BILLOTTE, W. G. & HOFMANN, M. C. 1999. Immunomagnetic isolation of osteoprogenitors from human bone marrow stroma. *Lab Invest*, 79, 449-57.
- ERICKSON, C. A. 1990. Cell migration in the embryo and adult organism. *Curr Opin Cell Biol*, 2, 67-74.
- ERIKSON, R. L. 1991. Structure, expression, and regulation of protein kinases involved in the phosphorylation of ribosomal protein S6. *J Biol Chem*, 266, 6007-10.
- FAHERTY, C. S. & MAURELLI, A. T. 2008. Staying alive: bacterial inhibition of apoptosis during infection. *Trends Microbiol*, 16, 173-80.
- FANG, J., SAWA, T., AKAIKE, T., AKUTA, T., SAHOO, S. K., KHALED, G., HAMADA, A. & MAEDA, H. 2003. In vivo antitumor activity of pegylated zinc protoporphyrin: targeted inhibition of heme oxygenase in solid tumor. *Cancer Res*, 63, 3567-74.
- FARMER, W. T., ALTICK, A. L., NURAL, H. F., DUGAN, J. P., KIDD, T., CHARRON, F. & MASTICK, G. S. 2008. Pioneer longitudinal axons navigate using floor plate and Slit/Robo signals. *Development*, 135, 3643-53.
- FAVALORO, E. J., BROWNING, T. & FACEY, D. 1993. CD13 (GP150; aminopeptidase-N): predominant functional activity in blood is localized to plasma and is not cell-surface associated. *Exp Hematol*, 21, 1695-701.
- FERRARA, N. 2002. VEGF and the quest for tumour angiogenesis factors. *Nature Reviews Cancer*, 2, 795-803.
- FERRARA, N. 2009. VEGF-A: a critical regulator of blood vessel growth. *Eur Cytokine Netw*, 20, 158-63.
- FESTJENS, N., VANDEN BERGHE, T., CORNELIS, S. & VANDENABEELE, P. 2007. RIP1, a kinase on the crossroads of a cell's decision to live or die. *Cell Death Differ*, 14, 400-10.
- FINK, S. L. & COOKSON, B. T. 2006. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cellular Microbiology*, 8, 1812-1825.
- FISCHER, A., SCHUMACHER, N., MAIER, M., SENDTNER, M. & GESSLER, M. 2004. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev*, 18, 901-11.

- FISCHER, C., MAZZONE, M., JONCKX, B. & CARMELIET, P. 2008. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nat Rev Cancer*, 8, 942-56.
- FISCHER, T. H., NEEF, S. & MAIER, L. S. 2013. The Ca-calmodulin dependent kinase II: a promising target for future antiarrhythmic therapies? *J Mol Cell Cardiol*, 58, 182-7.
- FLAMME, I., FROLICH, T. & RISAU, W. 1997. Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *J Cell Physiol*, 173, 206-10.
- FOLKMAN, J. & D'AMORE, P. A. 1996. Blood vessel formation: what is its molecular basis? *Cell*, 87, 1153-5.
- FRANKLIN, M. C., CAREY, K. D., VAJDOS, F. F., LEAHY, D. J., DE VOS, A. M. & SLIWKOWSKI, M. X. 2004. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell*, 5, 317-28.
- FRIEDENSTEIN, A. J., CHAILAKHYAN, R. K., LATSINIK, N. V., PANASYUK, A. F. & KEILISS-BOROK, I. V. 1974. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation*, 17, 331-40.
- FRIEDENSTEIN, A. J., PIATETZKY, S., II & PETRAKOVA, K. V. 1966. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol*, 16, 381-90.
- FRIEDMAN, L. M., RINON, A., SCHECHTER, B., LYASS, L., LAVI, S., BACUS, S. S., SELA, M. & YARDEN, Y. 2005. Synergistic down-regulation of receptor tyrosine kinases by combinations of mAbs: implications for cancer immunotherapy. *Proc Natl Acad Sci U S A*, 102, 1915-20.
- FRIEDRICH, E. B., WALENTA, K., SCHARLAU, J., NICKENIG, G. & WERNER, N. 2006. CD34-/CD133+/VEGFR-2+ endothelial progenitor cell subpopulation with potent vasoregenerative capacities. *Circ Res*, 98, e20-5.
- FUENTES-PRIOR, P. & SALVESEN, G. S. 2004. The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J*, 384, 201-32.
- FUNG, E. T., THULASIRAMAN, V., WEINBERGER, S. R. & DALMASSO, E. A. 2001. Protein biochips for differential profiling. *Curr Opin Biotechnol*, 12, 65-9.
- GADBOIS, D. M., CRISSMAN, H. A., TOBEY, R. A. & BRADBURY, E. M. 1992. Multiple kinase arrest points in the G1 phase of nontransformed mammalian cells are absent in transformed cells. *Proc Natl Acad Sci U S A*, 89, 8626-30.
- GALABOVA-KOVACS, G., KOLBUS, A., MATZEN, D., MEISSL, K., PIAZZOLLA, D., RUBIOLO, C., STEINITZ, K. & BACCARINI, M. 2006. ERK and beyond: insights from B-Raf and Raf-1 conditional knockouts. *Cell Cycle*, 5, 1514-8.
- GALE, N. W. & YANCOPOULOS, G. D. 1999. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev*, 13, 1055-66.
- GALLUZZI, L., BRENNER, C., MORSELLI, E., TOUAT, Z. & KROEMER, G. 2008. Viral control of mitochondrial apoptosis. *PLoS Pathog*, 4, e1000018.
- GALLUZZI, L., MAIURI, M. C., VITALE, I., ZISCHKA, H., CASTEDO, M., ZITVOGEL, L. & KROEMER, G. 2007. Cell death modalities: classification and pathophysiological implications. *Cell Death Differ*, 14, 1237-43.
- GARCIA-ROSTAN, G., ZHAO, H., CAMP, R. L., POLLAN, M., HERRERO, A., PARDO, J., WU, R., CARCANGIU, M. L., COSTA, J. & TALLINI, G. 2003.

- ras mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. *J Clin Oncol*, 21, 3226-35.
- GATES, R. E., KING, L. E., HANKS, S. K. & NANNEY, L. B. 1994. Potential Role for Focal Adhesion Kinase in Migrating and Proliferating Keratinocytes near Epidermal Wounds and in Culture. *Cell Growth & Differentiation*, 5, 891-899.
- GEORGE, E. L., BALDWIN, H. S. & HYNES, R. O. 1997. Fibronectins are essential for heart and blood vessel morphogenesis but are dispensable for initial specification of precursor cells. *Blood*, 90, 3073-81.
- GEORGE, E. L., GEORGES-LABOUESSE, E. N., PATEL-KING, R. S., RAYBURN, H. & HYNES, R. O. 1993. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development*, 119, 1079-91.
- GEPSTEIN, L. 2002. Derivation and potential applications of human embryonic stem cells. *Circ Res*, 91, 866-76.
- GIANCOTTI, F. G. & RUOSLAHTI, E. 1999. Integrin signaling. *Science*, 285, 1028-32.
- GILMORE, A. P. & ROMER, L. H. 1996. Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Molecular Biology of the Cell*, 7, 1209-1224.
- GIRAULT, J. A., LABESSE, G., MORNON, J. P. & CALLEBAUT, I. 1999. The N-termini of FAK and JAKs contain divergent band 4.1 domains. *Trends in Biochemical Sciences*, 24, 54-57.
- GOLDIE, J. H. & COLDMAN, A. J. 1979. A mathematic model for relating the drug sensitivity of tumors to their spontaneous mutation rate. *Cancer Treat Rep*, 63, 1727-33.
- GOLLOB, J. A. 2005. Sorafenib: scientific rationales for single-agent and combination therapy in clear-cell renal cell carcinoma. *Clin Genitourin Cancer*, 4, 167-74.
- GOOD, D. J., POLVERINI, P. J., RASTINEJAD, F., LE BEAU, M. M., LEMONS, R. S., FRAZIER, W. A. & BOUCK, N. P. 1990. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci U S A*, 87, 6624-8.
- GOUMANS, M. J., VALDIMARSDOTTIR, G., ITOH, S., ROSENDAHL, A., SIDERAS, P. & TEN DIJKE, P. 2002. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J*, 21, 1743-53.
- GRADY, W. M. & MARKOWITZ, S. D. 2002. Genetic and epigenetic alterations in colon cancer. *Annu Rev Genomics Hum Genet*, 3, 101-28.
- GRANDI, E., PUGLISI, J. L., WAGNER, S., MAIER, L. S., SEVERI, S. & BERS, D. M. 2007. Simulation of Ca-calmodulin-dependent protein kinase II on rabbit ventricular myocyte ion currents and action potentials. *Biophys J*, 93, 3835-47.
- GRANT, D. S., TASHIRO, K., SEGUI-REAL, B., YAMADA, Y., MARTIN, G. R. & KLEINMAN, H. K. 1989. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. *Cell*, 58, 933-43.

- GRANT, D. S., YENISEY, C., ROSE, R. W., TOOTELL, M., SANTRA, M. & IOZZO, R. V. 2002. Decorin suppresses tumor cell-mediated angiogenesis. *Oncogene*, 21, 4765-77.
- GRIFFIOEN, A. W. & MOLEMA, G. 2000. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev*, 52, 237-68.
- GRIFFITHS, M. J., BONNET, D. & JANES, S. M. 2005. Stem cells of the alveolar epithelium. *Lancet*, 366, 249-60.
- GRONTHOS, S., GRAVES, S. E., OHTA, S. & SIMMONS, P. J. 1994. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood*, 84, 4164-73.
- GUAN, J. L. & SHALLOWAY, D. 1992. Regulation of Focal Adhesion-Associated Protein Tyrosine Kinase by Both Cellular Adhesion and Oncogenic Transformation. *Nature*, 358, 690-692.
- GUAN, J. L., TREVITHICK, J. E. & HYNES, R. O. 1991. Fibronectin Integrin Interaction Induces Tyrosine Phosphorylation of a 120-Kda Protein. *Cell Regulation*, 2, 951-964.
- GUO, N., KRUTZSCH, H. C., INMAN, J. K. & ROBERTS, D. D. 1997. Thrombospondin 1 and type I repeat peptides of thrombospondin 1 specifically induce apoptosis of endothelial cells. *Cancer Res*, 57, 1735-42.
- GUY, P. M., PLATKO, J. V., CANTLEY, L. C., CERIONE, R. A. & CARRAWAY, K. L., 3RD 1994. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci U S A*, 91, 8132-6.
- GYGI, S. P., ROCHON, Y., FRANZA, B. R. & AEBERSOLD, R. 1999. Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol*, 19, 1720-30.
- HAIDER, N., NARULA, N. & NARULA, J. 2002. Apoptosis in heart failure represents programmed cell survival, not death, of cardiomyocytes and likelihood of reverse remodeling. *J Card Fail*, 8, S512-7.
- HAN, D. C. & GUAN, J. L. 1999. Association of focal adhesion kinase with Grb7 and its role in cell migration. *Journal of Biological Chemistry*, 274, 24425-24430.
- HAN, D. C., SHEN, T. L. & GUAN, J. L. 2000. Role of Grb7 targeting to focal contacts and its phosphorylation by focal adhesion kinase in regulation of cell migration. *Journal of Biological Chemistry*, 275, 28911-28917.
- HANKS, S. K., CALALB, M. B., HARPER, M. C. & PATEL, S. K. 1992. Focal Adhesion Protein-Tyrosine Kinase Phosphorylated in Response to Cell Attachment to Fibronectin. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 8487-8491.
- HARVEY, J. J. 1964. An Unidentified Virus Which Causes the Rapid Production of Tumours in Mice. *Nature*, 204, 1104-5.
- HELLBERG, C., OSTMAN, A. & HELDIN, C. H. 2010. PDGF and vessel maturation. *Recent Results Cancer Res*, 180, 103-14.
- HELLSTROM, M., GERHARDT, H., KALEN, M., LI, X., ERIKSSON, U., WOLBURG, H. & BETSHOLTZ, C. 2001a. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *J Cell Biol*, 153, 543-53.
- HELLSTROM, M., GERHARDT, H., KALEN, M., LI, X. R., ERIKSSON, U., WOLBURG, H. & BETSHOLTZ, C. 2001b. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *Journal of Cell Biology*, 153, 543-553.

- HELLSTROM, M., KALEN, M., LINDAHL, P., ABRAMSSON, A. & BETSHOLTZ, C. 1999. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development*, 126, 3047-55.
- HEMLER, M. E., JACOBSON, J. G. & STROMINGER, J. L. 1985. Biochemical characterization of VLA-1 and VLA-2. Cell surface heterodimers on activated T cells. *J Biol Chem*, 260, 15246-52.
- HERBERT, S. P., HUISKEN, J., KIM, T. N., FELDMAN, M. E., HOUSEMAN, B. T., WANG, R. A., SHOKAT, K. M. & STAINIER, D. Y. 2009. Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. *Science*, 326, 294-8.
- HERRMANN, C., HORN, G., SPAARGAREN, M. & WITTINGHOFFER, A. 1996. Differential interaction of the ras family GTP-binding proteins H-Ras, Rap1A, and R-Ras with the putative effector molecules Raf kinase and Ral-guanine nucleotide exchange factor. *J Biol Chem*, 271, 6794-800.
- HILDEBRAND, J. D., TAYLOR, J. M. & PARSONS, J. T. 1996. An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase. *Molecular and Cellular Biology*, 16, 3169-3178.
- HIRATA, A., OGAWA, S., KOMETANI, T., KUWANO, T., NAITO, S., KUWANO, M. & ONO, M. 2002. ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res*, 62, 2554-60.
- HITOMI, J., KATAYAMA, T., EGUCHI, Y., KUDO, T., TANIGUCHI, M., KOYAMA, Y., MANABE, T., YAMAGISHI, S., BANDO, Y., IMAIZUMI, K., TSUJIMOTO, Y. & TOHYAMA, M. 2004. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. *J Cell Biol*, 165, 347-56.
- HOCH, R. V. & SORIANO, P. 2003. Roles of PDGF in animal development. *Development*, 130, 4769-84.
- HOFMANN, N. A., ORTNER, A., JACAMO, R. O., REINISCH, A., SCHALLMOSER, K., ROHBAN, R., ETCHART, N., FRUEHWIRTH, M., BEHAM-SCHMID, C., ANDREEFF, M. & STRUNK, D. 2012a. Oxygen sensing mesenchymal progenitors promote neo-vasculogenesis in a humanized mouse model in vivo. *PLoS One*, 7, e44468.
- HOFMANN, N. A., REINISCH, A. & STRUNK, D. 2009. Isolation and large scale expansion of adult human endothelial colony forming progenitor cells. *J Vis Exp*.
- HOFMANN, N. A., REINISCH, A. & STRUNK, D. 2012b. Endothelial colony-forming progenitor cell isolation and expansion. *Methods Mol Biol*, 879, 381-7.
- HOLBRO, T., CIVENNI, G. & HYNES, N. E. 2003. The ErbB receptors and their role in cancer progression. *Exp Cell Res*, 284, 99-110.
- HOLLER, N., ZARU, R., MICHEAU, O., THOME, M., ATTINGER, A., VALITUTTI, S., BODMER, J. L., SCHNEIDER, P., SEED, B. & TSCHOPP, J. 2000. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol*, 1, 489-95.
- HORWITZ, E. M., LE BLANC, K., DOMINICI, M., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F. C., DEANS, R. J., KRAUSE, D. S. & KEATING, A. 2005. Clarification of the nomenclature for MSC: The

- international society for cellular therapy position statement. *Cytotherapy*, 7, 393-395.
- HSIA, D. A., MITRA, S. K., HAUCK, C. R., STREBLOW, D., NELSON, J. A., ILIC, D., HUANG, S., LI, E. G., NEMEROW, G. R., LENG, J., SPENCER, K. S. R., CHERESH, D. A. & SCHLAEPFER, D. D. 2003. Differential regulation of cell motility and invasion by FAK. *Journal of Cell Biology*, 160, 753-767.
- HYNES, R. O. 2002a. Integrins: bidirectional, allosteric signaling machines. *Cell*, 110, 673-87.
- HYNES, R. O. 2002b. A reevaluation of integrins as regulators of angiogenesis. *Nat Med*, 8, 918-21.
- HYNES, R. O. 2009. The extracellular matrix: not just pretty fibrils. *Science*, 326, 1216-9.
- HYNES, R. O. & YAMADA, K. M. 1982. Fibronectins: multifunctional modular glycoproteins. *J Cell Biol*, 95, 369-77.
- IIVANAINEN, E., NELIMARKKA, L., ELENIOUS, V., HEIKKINEN, S. M., JUNTILA, T. T., SIHOMBING, L., SUNDVALL, M., MAATTA, J. A., LAINE, V. J., YLA-HERTTUALA, S., HIGASHIYAMA, S., ALITALO, K. & ELENIOUS, K. 2003. Angiopoietin-regulated recruitment of vascular smooth muscle cells by endothelial-derived heparin binding EGF-like growth factor. *FASEB J*, 17, 1609-21.
- ILLC, D., FURUTA, Y., KANAZAWA, S., TAKEDA, N., SOBUE, K., NAKATSUJI, N., NOMURA, S., FUJIMOTO, J., OKADA, M., YAMAMOTO, T. & AIZAWA, S. 1995. Reduced Cell Motility and Enhanced Focal Adhesion Contact Formation in Cells from Fak-Deficient Mice. *Nature*, 377, 539-544.
- IRISH, J. M., KOTTECHA, N. & NOLAN, G. P. 2006. Mapping normal and cancer cell signalling networks: towards single-cell proteomics. *Nat Rev Cancer*, 6, 146-55.
- ISHOV, A. M., VLADIMIROVA, O. V. & MAUL, G. G. 2004. Heterochromatin and ND10 are cell-cycle regulated and phosphorylation-dependent alternate nuclear sites of the transcription repressor Daxx and SWI/SNF protein ATRX. *J Cell Sci*, 117, 3807-20.
- IURLARO, M., SCATENA, M., ZHU, W. H., FOGEL, E., WIETING, S. L. & NICOSIA, R. F. 2003. Rat aorta-derived mural precursor cells express the Tie2 receptor and respond directly to stimulation by angiopoietins. *J Cell Sci*, 116, 3635-43.
- IWAMOTO, R., YAMAZAKI, S., ASAKURA, M., TAKASHIMA, S., HASUWA, H., MIYADO, K., ADACHI, S., KITAKAZE, M., HASHIMOTO, K., RAAB, G., NANBA, D., HIGASHIYAMA, S., HORI, M., KLAGSBRUN, M. & MEKADA, E. 2003. Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc Natl Acad Sci U S A*, 100, 3221-6.
- JAFFE, E. A., RUGGIERO, J. T., LEUNG, L. K., DOYLE, M. J., MCKEOWN-LONGO, P. J. & MOSHER, D. F. 1983. Cultured human fibroblasts synthesize and secrete thrombospondin and incorporate it into extracellular matrix. *Proc Natl Acad Sci U S A*, 80, 998-1002.
- JAFFEE, E. M., HRUBAN, R. H., CANTO, M. & KERN, S. E. 2002. Focus on pancreas cancer. *Cancer Cell*, 2, 25-8.
- JAIN, R. K. 2003a. Molecular regulation of vessel maturation. *Nature Medicine*, 9, 685-693.
- JAIN, R. K. 2003b. Molecular regulation of vessel maturation. *Nat Med*, 9, 685-93.

- JAIN, R. K., DUDA, D. G., WILLETT, C. G., SAHANI, D. V., ZHU, A. X., LOEFFLER, J. S., BATCHELOR, T. T. & SORENSEN, A. G. 2009. Biomarkers of response and resistance to antiangiogenic therapy. *Nat Rev Clin Oncol*, 6, 327-38.
- JAISWAL, R. K., JAISWAL, N., BRUDER, S. P., MBALAVIELE, G., MARSHAK, D. R. & PITTENGER, M. F. 2000. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. *J Biol Chem*, 275, 9645-52.
- JIANG, X. & SORKIN, A. 2002. Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells. *Mol Biol Cell*, 13, 1522-35.
- JIMENEZ, B., VOLPERT, O. V., CRAWFORD, S. E., FEBBRAIO, M., SILVERSTEIN, R. L. & BOUCK, N. 2000. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med*, 6, 41-8.
- JOHNSON, D. W., BERG, J. N., BALDWIN, M. A., GALLIONE, C. J., MARONDEL, I., YOON, S. J., STENZEL, T. T., SPEER, M., PERICAK-VANCE, M. A., DIAMOND, A., GUTTMACHER, A. E., JACKSON, C. E., ATTISANO, L., KUCHERLAPATI, R., PORTEOUS, M. E. & MARCHUK, D. A. 1996. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat Genet*, 13, 189-95.
- JOHNSTON, J. B., BARRETT, J. W., NAZARIAN, S. H., GOODWIN, M., RICUTTIO, D., WANG, G. & MCFADDEN, G. 2005. A poxvirus-encoded pyrin domain protein interacts with ASC-1 to inhibit host inflammatory and apoptotic responses to infection. *Immunity*, 23, 587-598.
- JUNG, C. H., JUN, C. B., RO, S. H., KIM, Y. M., OTTO, N. M., CAO, J., KUNDU, M. & KIM, D. H. 2009. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell*, 20, 1992-2003.
- KANNER, S. B., REYNOLDS, A. B., VINES, R. R. & PARSONS, J. T. 1990. Monoclonal-Antibodies to Individual Tyrosine-Phosphorylated Protein Substrates of Oncogene-Encoded Tyrosine Kinases. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 3328-3332.
- KARUNAGARAN, D., TZAHAR, E., BEERLI, R. R., CHEN, X., GRAUS-PORTA, D., RATZKIN, B. J., SEGER, R., HYNES, N. E. & YARDEN, Y. 1996. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J*, 15, 254-64.
- KEMENY, D. M. & MACARY, P. A. 2007. Immunology: making magic bullets. *BMJ*, 334 Suppl 1, s13.
- KENNEDY, N. J., KATAOKA, T., TSCHOPP, J. & BUDD, R. C. 1999. Caspase activation is required for T cell proliferation. *J Exp Med*, 190, 1891-6.
- KERR, M. A. & STOCKS, S. C. 1992. The role of CD15-(Le(X))-related carbohydrates in neutrophil adhesion. *Histochem J*, 24, 811-26.
- KERSSE, K., VANDEN BERGHE, T., LAMKANFI, M. & VANDENABEELE, P. 2007. A phylogenetic and functional overview of inflammatory caspases and caspase-1-related CARD-only proteins. *Biochem Soc Trans*, 35, 1508-11.
- KESSLER, D. A., SIEGEL, J. P., NOGUCHI, P. D., ZOON, K. C., FEIDEN, K. L. & WOODCOCK, J. 1993. Regulation of somatic-cell therapy and gene therapy by the food and drug administration. *N Engl J Med*, 329, 1169-73.

- KIEL, M. J., YILMAZ, O. H., IWASHITA, T., YILMAZ, O. H., TERHORST, C. & MORRISON, S. J. 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*, 121, 1109-21.
- KIM, I., KIM, H. G., MOON, S. O., CHAE, S. W., SO, J. N., KOH, K. N., AHN, B. C. & KOH, G. Y. 2000a. Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. *Circulation Research*, 86, 952-959.
- KIM, K. W., MORETTI, L. & LU, B. 2008. M867, a novel selective inhibitor of caspase-3 enhances cell death and extends tumor growth delay in irradiated lung cancer models. *PLoS One*, 3, e2275.
- KIM, S., BAKRE, M., YIN, H. & VARNER, J. A. 2002. Inhibition of endothelial cell survival and angiogenesis by protein kinase A. *J Clin Invest*, 110, 933-41.
- KIM, S., BELL, K., MOUSA, S. A. & VARNER, J. A. 2000b. Regulation of angiogenesis in vivo by ligation of integrin $\alpha 5 \beta 1$ with the central cell-binding domain of fibronectin. *Am J Pathol*, 156, 1345-62.
- KIMMIG, S., PRZYBYLSKI, G. K., SCHMIDT, C. A., LAURISCH, K., MOWES, B., RADBRUCH, A. & THIEL, A. 2002. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J Exp Med*, 195, 789-94.
- KINSELLA, M. G., FISCHER, J. W., MASON, D. P. & WIGHT, T. N. 2000. Retrovirally mediated expression of decorin by macrovascular endothelial cells. Effects on cellular migration and fibronectin fibrillogenesis in vitro. *J Biol Chem*, 275, 13924-32.
- KIRSTEN, W. H. & MAYER, L. A. 1969. Malignant lymphomas of extrathymic origin induced in rats by murine erythroblastosis virus. *J Natl Cancer Inst*, 43, 735-46.
- KITA, K., GAUGLITZ, G. G., PHAN, T. T., HERNDON, D. N. & JESCHKE, M. G. 2010. Isolation and characterization of mesenchymal stem cells from the sub-amniotic human umbilical cord lining membrane. *Stem Cells Dev*, 19, 491-502.
- KITANO, H. 2003. Cancer robustness: tumour tactics. *Nature*, 426, 125.
- KLAPPER, L. N., GLATHE, S., VAISMAN, N., HYNES, N. E., ANDREWS, G. C., SELA, M. & YARDEN, Y. 1999. The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc Natl Acad Sci U S A*, 96, 4995-5000.
- KOBE, B. & KEMP, B. E. 1999. Active site-directed protein regulation. *Nature*, 402, 373-6.
- KOKAI, Y., MYERS, J. N., WADA, T., BROWN, V. I., LEVEA, C. M., DAVIS, J. G., DOBASHI, K. & GREENE, M. I. 1989. Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts. *Cell*, 58, 287-92.
- KOLCH, W. & PITT, A. 2010. Functional proteomics to dissect tyrosine kinase signalling pathways in cancer. *Nat Rev Cancer*, 10, 618-29.
- KRONTIRIS, T. G. & COOPER, G. M. 1981. Transforming activity of human tumor DNAs. *Proc Natl Acad Sci U S A*, 78, 1181-4.
- KRYSKO, D. V. & VANDENABEELE, P. 2008. From regulation of dying cell engulfment to development of anti-cancer therapy. *Cell Death Differ*, 15, 29-38.

- KUCIA, M., WOJAKOWSKI, W., RECA, R., MACHALINSKI, B., GOZDZIK, J., MAJKA, M., BARAN, J., RATAJCZAK, J. & RATAJCZAK, M. Z. 2006. The migration of bone marrow-derived non-hematopoietic tissue-committed stem cells is regulated in an SDF-1-, HGF-, and LIF-dependent manner. *Arch Immunol Ther Exp (Warsz)*, 54, 121-35.
- KUHNERT, F., MANCUSO, M. R., SHAMLOO, A., WANG, H. T., CHOKSI, V., FLOREK, M., SU, H., FRUTTIGER, M., YOUNG, W. L., HEILSHORN, S. C. & KUO, C. J. 2010. Essential regulation of CNS angiogenesis by the orphan G protein-coupled receptor GPR124. *Science*, 330, 985-9.
- KUMAR, D., KAMP, T. J. & LEWINTER, M. M. 2005. Embryonic stem cells: differentiation into cardiomyocytes and potential for heart repair and regeneration. *Coron Artery Dis*, 16, 111-6.
- KUZELOVA, K., GREBENOVA, D. & BRODSKA, B. 2011. Dose-dependent effects of the caspase inhibitor Q-VD-OPh on different apoptosis-related processes. *J Cell Biochem*, 112, 3334-42.
- KYRIAKIDES, T. R., ZHU, Y. H., YANG, Z., HUYNH, G. & BORNSTEIN, P. 2001. Altered extracellular matrix remodeling and angiogenesis in sponge granulomas of thrombospondin 2-null mice. *Am J Pathol*, 159, 1255-62.
- LABAT-ROBERT, J., SZENDROI, M., GODEAU, G. & ROBERT, L. 1985. Comparative distribution patterns of type I and III collagens and fibronectin in human arteriosclerotic aorta. *Pathol Biol (Paris)*, 33, 261-5.
- LABBE, K. & SALEH, M. 2008. Cell death in the host response to infection. *Cell Death Differ*, 15, 1339-49.
- LACASSE, E. C., MAHONEY, D. J., CHEUNG, H. H., PLENCHETTE, S., BAIRD, S. & KORNELUK, R. G. 2008. IAP-targeted therapies for cancer. *Oncogene*, 27, 6252-75.
- LAMKANFI, M., KANNEGANTI, T. D., VAN DAMME, P., VANDEN BERGHE, T., VANOVERBERGHE, I., VANDEKERCKHOVE, J., VANDENABEELE, P., GEVAERT, K. & NUNEZ, G. 2008. Targeted peptidecentric proteomics reveals caspase-7 as a substrate of the caspase-1 inflammasomes. *Mol Cell Proteomics*, 7, 2350-63.
- LARA-TEJERO, M., SUTTERWALA, F. S., OGURA, Y., GRANT, E. P., BERTIN, J., COYLE, A. J., FLAVELL, R. A. & GALAN, J. E. 2006. Role of the caspase-1 inflammasome in Salmonella typhimurium pathogenesis. *Journal of Experimental Medicine*, 203, 1407-1412.
- LAWLER, J. 2000. The functions of thrombospondin-1 and-2. *Curr Opin Cell Biol*, 12, 634-40.
- LAWSON, N. D., SCHEER, N., PHAM, V. N., KIM, C. H., CHITNIS, A. B., CAMPOS-ORTEGA, J. A. & WEINSTEIN, B. M. 2001. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development*, 128, 3675-83.
- LEBRIN, F., GOUMANS, M. J., JONKER, L., CARVALHO, R. L., VALDIMARSDOTTIR, G., THORIKAY, M., MUMMERY, C., ARTHUR, H. M. & TEN DIJKE, P. 2004. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J*, 23, 4018-28.
- LECHERTIER, T. & HODIVALA-DILKE, K. 2012. Focal adhesion kinase and tumour angiogenesis. *J Pathol*, 226, 404-12.
- LEE, C. Y. & BAEHRECKE, E. H. 2001. Steroid regulation of autophagic programmed cell death during development. *Development*, 128, 1443-55.

- LEE, J. W., KWAK, H. J., LEE, J. J., KIM, Y. N., LEE, J. W., PARK, M. J., JUNG, S. E., HONG, S. I., LEE, J. H. & LEE, J. S. 2008. HSP27 regulates cell adhesion and invasion via modulation of focal adhesion kinase and MMP-2 expression. *Eur J Cell Biol*, 87, 377-87.
- LEE, S., CHEN, T. T., BARBER, C. L., JORDAN, M. C., MURDOCK, J., DESAI, S., FERRARA, N., NAGY, A., ROOS, K. P. & IRUELA-ARISPE, M. L. 2007. Autocrine VEGF signaling is required for vascular homeostasis. *Cell*, 130, 691-703.
- LEMMON, M. A. & SCHLESSINGER, J. 2010. Cell signaling by receptor tyrosine kinases. *Cell*, 141, 1117-34.
- LEVEEN, P., PEKNY, M., GEBRE-MEDHIN, S., SWOLIN, B., LARSSON, E. & BETSHOLTZ, C. 1994. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev*, 8, 1875-87.
- LI, C., HEIDT, D. G., DALERBA, P., BURANT, C. F., ZHANG, L., ADSAY, V., WICHA, M., CLARKE, M. F. & SIMEONE, D. M. 2007. Identification of pancreatic cancer stem cells. *Cancer Res*, 67, 1030-7.
- LI, D. Y., SORENSEN, L. K., BROOKE, B. S., URNESS, L. D., DAVIS, E. C., TAYLOR, D. G., BOAK, B. B. & WENDEL, D. P. 1999. Defective angiogenesis in mice lacking endoglin. *Science*, 284, 1534-7.
- LI, P., ALLEN, H., BANERJEE, S., FRANKLIN, S., HERZOG, L., JOHNSTON, C., MCDOWELL, J., PASKIND, M., RODMAN, L., SALFELD, J. & ET AL. 1995. Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell*, 80, 401-11.
- LI VOLTI, G., SACERDOTI, D., SANGRAS, B., VANELLA, A., MEZENTSEV, A., SCAPAGNINI, G., FALCK, J. R. & ABRAHAM, N. G. 2005. Carbon monoxide signaling in promoting angiogenesis in human microvessel endothelial cells. *Antioxid Redox Signal*, 7, 704-10.
- LILLIE, R. D. 1965. Histochemistry - Theory Practice and Bibliography. *Journal of Histochemistry & Cytochemistry*, 13, 305-&.
- LIM, S. T., MIKOLON, D., STUPACK, D. G. & SCHLAEPFER, D. D. 2008. FERM control of FAK function - Implications for cancer therapy. *Cell Cycle*, 7, 2306-2314.
- LIN, Y., CHOKSI, S., SHEN, H. M., YANG, Q. F., HUR, G. M., KIM, Y. S., TRAN, J. H., NEDOSPASOV, S. A. & LIU, Z. G. 2004. Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *J Biol Chem*, 279, 10822-8.
- LINDAHL, P., JOHANSSON, B. R., LEVEEN, P. & BETSHOLTZ, C. 1997. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science*, 277, 242-5.
- LIOTTA, L. A., ESPINA, V., MEHTA, A. I., CALVERT, V., ROSENBLATT, K., GEHO, D., MUNSON, P. J., YOUNG, L., WULFKUHLE, J. & PETRICIOIN, E. F., 3RD 2003. Protein microarrays: meeting analytical challenges for clinical applications. *Cancer Cell*, 3, 317-25.
- LIOTTA, L. A., STEEG, P. S. & STETLER-STEVENSON, W. G. 1991. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell*, 64, 327-36.
- LIU, X., WU, H., BYRNE, M., KRANE, S. & JAENISCH, R. 1997. Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. *Proc Natl Acad Sci U S A*, 94, 1852-6.

- LIU, Y., WADA, R., YAMASHITA, T., MI, Y., DENG, C. X., HOBSON, J. P., ROSENFELDT, H. M., NAVA, V. E., CHAE, S. S., LEE, M. J., LIU, C. H., HLA, T., SPIEGEL, S. & PROIA, R. L. 2000. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest*, 106, 951-61.
- LO, B. & PARHAM, L. 2009. Ethical issues in stem cell research. *Endocr Rev*, 30, 204-13.
- LOHLER, J., TIMPL, R. & JAENISCH, R. 1984. Embryonic lethal mutation in mouse collagen I gene causes rupture of blood vessels and is associated with erythropoietic and mesenchymal cell death. *Cell*, 38, 597-607.
- LOOK, A. T., ASHMUN, R. A., SHAPIRO, L. H. & PEIPER, S. C. 1989. Human myeloid plasma membrane glycoprotein CD13 (gp150) is identical to aminopeptidase N. *J Clin Invest*, 83, 1299-307.
- LOUGHNA, S. & SATO, T. N. 2001. Angiopoietin and Tie signaling pathways in vascular development. *Matrix Biology*, 20, 319-325.
- LUM, J. J., BAUER, D. E., KONG, M., HARRIS, M. H., LI, C., LINDSTEN, T. & THOMPSON, C. B. 2005. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell*, 120, 237-48.
- LUO, Y. & RADICE, G. L. 2005. N-cadherin acts upstream of VE-cadherin in controlling vascular morphogenesis. *J Cell Biol*, 169, 29-34.
- LYNCH, T. J., BELL, D. W., SORDELLA, R., GURUBHAGAVATULA, S., OKIMOTO, R. A., BRANNIGAN, B. W., HARRIS, P. L., HASERLAT, S. M., SUPKO, J. G., HALUSKA, F. G., LOUIS, D. N., CHRISTIANI, D. C., SETTLEMAN, J. & HABER, D. A. 2004. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*, 350, 2129-39.
- MA, Y., XU, Y., XIAO, Z., YANG, W., ZHANG, C., SONG, E., DU, Y. & LI, L. 2006. Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. *Stem Cells*, 24, 315-21.
- MACKAY, A. M., BECK, S. C., MURPHY, J. M., BARRY, F. P., CHICHESTER, C. O. & PITTENGER, M. F. 1998. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng*, 4, 415-28.
- MAEHR, R., CHEN, S., SNITOW, M., LUDWIG, T., YAGASAKI, L., GOLAND, R., LEIBEL, R. L. & MELTON, D. A. 2009. Generation of pluripotent stem cells from patients with type 1 diabetes. *Proc Natl Acad Sci U S A*, 106, 15768-73.
- MAHMUDI-AZER, S., DOWNEY, G. P. & MOQBEL, R. 2002. Translocation of the tetraspanin CD63 in association with human eosinophil mediator release. *Blood*, 99, 4039-47.
- MAISONPIERRE, P. C., SURI, C., JONES, P. F., BARTUNKOVA, S., WIEGAND, S. J., RADZIEJEWSKI, C., COMPTON, D., MCCLAIN, J., ALDRICH, T. H., PAPADOPOULOS, N., DALY, T. J., DAVIS, S., SATO, T. N. & YANCOPOULOS, G. D. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science*, 277, 55-60.
- MAITY, A., PORE, N., LEE, J., SOLOMON, D. & O'ROURKE, D. M. 2000. Epidermal growth factor receptor transcriptionally up-regulates vascular endothelial growth factor expression in human glioblastoma cells via a pathway involving phosphatidylinositol 3'-kinase and distinct from that induced by hypoxia. *Cancer Res*, 60, 5879-86.

- MAIURI, M. C., ZALCKVAR, E., KIMCHI, A. & KROEMER, G. 2007. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol*, 8, 741-52.
- MAJDIC, O., STOCKL, J., PICKL, W. F., BOHUSLAV, J., STROBL, H., SCHEINECKER, C., STOCKINGER, H. & KNAPP, W. 1994. Signaling and induction of enhanced cytoadhesiveness via the hematopoietic progenitor cell surface molecule CD34. *Blood*, 83, 1226-34.
- MAJETI, R., PARK, C. Y. & WEISSMAN, I. L. 2007. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell*, 1, 635-45.
- MAJMUNDAR, A. J., WONG, W. J. & SIMON, M. C. 2010. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell*, 40, 294-309.
- MAJUMDAR, M. K., BANKS, V., PELUSO, D. P. & MORRIS, E. A. 2000. Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. *J Cell Physiol*, 185, 98-106.
- MALLER, J. L., ROY, L. M. & IZUMI, T. 1991. Cell cycle and mitotic control in *Xenopus* eggs. *Cold Spring Harb Symp Quant Biol*, 56, 533-8.
- MALLIARAS, K., LI, T. S., LUTHRINGER, D., TERROVITIS, J., CHENG, K., CHAKRAVARTY, T., GALANG, G., ZHANG, Y., SCHOENHOFF, F., VAN EYK, J., MARBAN, L. & MARBAN, E. 2012. Safety and efficacy of allogeneic cell therapy in infarcted rats transplanted with mismatched cardiosphere-derived cells. *Circulation*, 125, 100-12.
- MALLIS, R. J., BUSS, J. E. & THOMAS, J. A. 2001. Oxidative modification of H-ras: S-thiolation and S-nitrosylation of reactive cysteines. *Biochem J*, 355, 145-53.
- MANNING, G., WHYTE, D. B., MARTINEZ, R., HUNTER, T. & SUDARSANAM, S. 2002. The protein kinase complement of the human genome. *Science*, 298, 1912-34.
- MARAS, R., LIGHT, Y., MASON, C., PATERSON, H., OLSON, M. F. & MARSHALL, C. J. 1998. Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. *Science*, 280, 109-12.
- MARTIN, G. A., VISKOCHIL, D., BOLLAG, G., MCCABE, P. C., CROSIER, W. J., HAUBRUCK, H., CONROY, L., CLARK, R., O'CONNELL, P., CAWTHON, R. M. & ET AL. 1990. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell*, 63, 843-9.
- MARTINON, F. & TSCHOPP, J. 2004. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell*, 117, 561-74.
- MATTILA, M. M., RUOHOLA, J. K., VALVE, E. M., TASANEN, M. J., SEPPANEN, J. A. & HARKONEN, P. L. 2001. FGF-8b increases angiogenic capacity and tumor growth of androgen-regulated S115 breast cancer cells. *Oncogene*, 20, 2791-804.
- MAVRIA, G., VERCOULEN, Y., YEO, M., PATERSON, H., KARASARIDES, M., MARAS, R., BIRD, D. & MARSHALL, C. J. 2006. ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. *Cancer Cell*, 9, 33-44.
- MAYS, R. W., VAN'T HOF, W., TING, A. E., PERRY, R. & DEANS, R. 2007. Development of adult pluripotent stem cell therapies for ischemic injury and disease. *Expert Opin Biol Ther*, 7, 173-84.

- MCALLISTER, K. A., GROGG, K. M., JOHNSON, D. W., GALLIONE, C. J., BALDWIN, M. A., JACKSON, C. E., HELMBOLD, E. A., MARKEL, D. S., MCKINNON, W. C., MURRELL, J. & ET AL. 1994. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet*, 8, 345-51.
- MCCARTY, M. F., SOMCIO, R. J., STOELTZING, O., WEY, J., FAN, F., LIU, W., BUCANA, C. & ELLIS, L. M. 2007. Overexpression of PDGF-BB decreases colorectal and pancreatic cancer growth by increasing tumor pericyte content. *J Clin Invest*, 117, 2114-22.
- MCLEAN, G. W., CARRAGHER, N. O., AVIZIENYTE, E., EVANS, J., BRUNTON, V. G. & FRAME, M. C. 2005. The role of focal-adhesion kinase in cancer. A new therapeutic opportunity. *Nature Reviews Cancer*, 5, 505-515.
- MEAD, L. E., PRATER, D., YODER, M. C. & INGRAM, D. A. 2008. Isolation and characterization of endothelial progenitor cells from human blood. *Curr Protoc Stem Cell Biol*, Chapter 2, Unit 2C 1.
- MEANS, A. R. & DEDMAN, J. R. 1980. Calmodulin--an intracellular calcium receptor. *Nature*, 285, 73-7.
- MEBRATU, Y. & TESFAIGZI, Y. 2009. How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle*, 8, 1168-75.
- MEIER, P., FINCH, A. & EVAN, G. 2000. Apoptosis in development. *Nature*, 407, 796-801.
- MELERO-MARTIN, J. M., KHAN, Z. A., PICARD, A., WU, X., PARUCHURI, S. & BISCHOFF, J. 2007. In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood*, 109, 4761-8.
- MELTON, L. 2004. Protein arrays: proteomics in multiplex. *Nature*, 429, 101-7.
- MERCURIUS, K. O. & MORLA, A. O. 1998. Inhibition of vascular smooth muscle cell growth by inhibition of fibronectin matrix assembly. *Circ Res*, 82, 548-56.
- METHENY-BARLOW, L. J. & LI, L. Y. 2003. The enigmatic role of angiopoietin-1 in tumor angiogenesis. *Cell Res*, 13, 309-17.
- MIAO, Z., JIN, J., CHEN, L., ZHU, J., HUANG, W., ZHAO, J., QIAN, H. & ZHANG, X. 2006. Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. *Cell Biol Int*, 30, 681-7.
- MICHAELSON, J. S., BADER, D., KUO, F., KOZAK, C. & LEDER, P. 1999. Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. *Genes Dev*, 13, 1918-23.
- MIETTINEN, M., CASTELLO, R., WAYNER, E. & SCHWARTING, R. 1993. Distribution of VLA integrins in solid tumors. Emergence of tumor-type-related expression. Patterns in carcinomas and sarcomas. *Am J Pathol*, 142, 1009-18.
- MIETTINEN, P. J., BERGER, J. E., MENESES, J., PHUNG, Y., PEDERSEN, R. A., WERB, Z. & DERYNCK, R. 1995. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature*, 376, 337-41.
- MIMEAULT, M. & BATRA, S. K. 2006. Recent advances on multiple tumorigenic cascades involved in prostatic cancer progression and targeting therapies. *Carcinogenesis*, 27, 1-22.

- MIMEAULT, M., HAUKE, R. & BATRA, S. K. 2007. Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. *Clin Pharmacol Ther*, 82, 252-64.
- MIRZA, A. M., GYSIN, S., MALEK, N., NAKAYAMA, K., ROBERTS, J. M. & MCMAHON, M. 2004. Cooperative regulation of the cell division cycle by the protein kinases RAF and AKT. *Mol Cell Biol*, 24, 10868-81.
- MITCHELL, P. 2002. A perspective on protein microarrays. *Nat Biotechnol*, 20, 225-9.
- MITRA, S. K., HANSON, D. A. & SCHLAEPFER, D. D. 2005. Focal adhesion kinase: In command and control of cell motility. *Nature Reviews Molecular Cell Biology*, 6, 56-68.
- MITSUUCHI, Y. & TESTA, J. R. 2002. Cytogenetics and molecular genetics of lung cancer. *Am J Med Genet*, 115, 183-8.
- MIZUSHIMA, N., KUMA, A., KOBAYASHI, Y., YAMAMOTO, A., MATSUBAE, M., TAKAO, T., NATSUME, T., OHSUMI, Y. & YOSHIMORI, T. 2003. Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J Cell Sci*, 116, 1679-88.
- MOHAMED, O. A., JONNAERT, M., LABELLE-DUMAIS, C., KURODA, K., CLARKE, H. J. & DUFORT, D. 2005. Uterine Wnt/beta-catenin signaling is required for implantation. *Proc Natl Acad Sci U S A*, 102, 8579-84.
- MOORE, K. A. & LEMISCHKA, I. R. 2006. Stem cells and their niches. *Science*, 311, 1880-5.
- MORETTI, L., KIM, K. W., JUNG, D. K., WILLEY, C. D. & LU, B. 2009. Radiosensitization of solid tumors by Z-VAD, a pan-caspase inhibitor. *Mol Cancer Ther*, 8, 1270-9.
- MORRISON, D. K. & DAVIS, R. J. 2003. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. *Annu Rev Cell Dev Biol*, 19, 91-118.
- MOSHER, D. F., DOYLE, M. J. & JAFFE, E. A. 1982. Synthesis and secretion of thrombospondin by cultured human endothelial cells. *J Cell Biol*, 93, 343-8.
- MOSLEY, B., URDAL, D. L., PRICKETT, K. S., LARSEN, A., COSMAN, D., CONLON, P. J., GILLIS, S. & DOWER, S. K. 1987. The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. *J Biol Chem*, 262, 2941-4.
- MUKHOPADHYAY, D. & AKBARALI, H. I. 1996. Depletion of [Ca²⁺]_i inhibits hypoxia-induced vascular permeability factor (vascular endothelial growth factor) gene expression. *Biochem Biophys Res Commun*, 229, 733-8.
- MURPHY, L. O. & BLENIS, J. 2006. MAPK signal specificity: the right place at the right time. *Trends Biochem Sci*, 31, 268-75.
- MURPHY, L. O., SMITH, S., CHEN, R. H., FINGAR, D. C. & BLENIS, J. 2002. Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol*, 4, 556-64.
- MURRAY, L., CHEN, B., GALY, A., CHEN, S., TUSHINSKI, R., UCHIDA, N., NEGRIN, R., TRICOT, G., JAGANNATH, S., VESOLE, D. & ET AL. 1995. Enrichment of human hematopoietic stem cell activity in the CD34⁺Thy-1⁺Lin⁻ subpopulation from mobilized peripheral blood. *Blood*, 85, 368-78.
- MUSTONEN, T. & ALITALO, K. 1995. Endothelial receptor tyrosine kinases involved in angiogenesis. *J Cell Biol*, 129, 895-8.

- NADIRI, A., WOLINSKI, M. K. & SALEH, M. 2006. The inflammatory caspases: key players in the host response to pathogenic invasion and sepsis. *J Immunol*, 177, 4239-45.
- NAGY, J. A., DVORAK, A. M. & DVORAK, H. F. 2007. VEGF-A and the induction of pathological angiogenesis. *Annu Rev Pathol*, 2, 251-75.
- NAGY, J. A., VASILE, E., FENG, D., SUNDBERG, C., BROWN, L. F., MANSEAU, E. J., DVORAK, A. M. & DVORAK, H. 2002. VEGF-A induces angiogenesis, arteriogenesis, lymphangiogenesis, and vascular malformations. *Cold Spring Harbor Symposia on Quantitative Biology*, 67, 227-237.
- NAKIELNY, S., CAMPBELL, D. G. & COHEN, P. 1992. MAP kinase kinase from rabbit skeletal muscle. A novel dual specificity enzyme showing homology to yeast protein kinases involved in pheromone-dependent signal transduction. *FEBS Lett*, 308, 183-9.
- NARULA, J., PANDEY, P., ARBUSTINI, E., HAIDER, N., NARULA, N., KOLODIE, F. D., DAL BELLO, B., SEMIGRAN, M. J., BIELSA-MASDEU, A., DEC, G. W., ISRAELS, S., BALLESTER, M., VIRMANI, R., SAXENA, S. & KHARBANDA, S. 1999. Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. *Proc Natl Acad Sci U S A*, 96, 8144-9.
- NEFKENS, I., NEGOREV, D. G., ISHOV, A. M., MICHAELSON, J. S., YEH, E. T., TANGUAY, R. M., MULLER, W. E. & MAUL, G. G. 2003. Heat shock and Cd2+ exposure regulate PML and Daxx release from ND10 by independent mechanisms that modify the induction of heat-shock proteins 70 and 25 differently. *J Cell Sci*, 116, 513-24.
- NEWMAN, R. E., YOO, D., LEROUX, M. A. & DANILKOVITCH-MIAGKOVA, A. 2009. Treatment of inflammatory diseases with mesenchymal stem cells. *Inflamm Allergy Drug Targets*, 8, 110-23.
- NEWTON, K., HARRIS, A. W., BATH, M. L., SMITH, K. G. & STRASSER, A. 1998. A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *EMBO J*, 17, 706-18.
- NEWTON, K. & STRASSER, A. 2000. Cell death control in lymphocytes. *Adv Immunol*, 76, 179-226.
- NHAN, T. Q., LILES, W. C. & SCHWARTZ, S. M. 2006. Physiological functions of caspases beyond cell death. *Am J Pathol*, 169, 729-37.
- NING, H., LIN, G., LUE, T. F. & LIN, C. S. 2011. Mesenchymal stem cell marker Stro-1 is a 75 kd endothelial antigen. *Biochem Biophys Res Commun*, 413, 353-7.
- NISHIE, A., ONO, M., SHONO, T., FUKUSHI, J., OTSUBO, M., ONOUE, H., ITO, Y., INAMURA, T., IKEZAKI, K., FUKUI, M., IWAKI, T. & KUWANO, M. 1999. Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas. *Clin Cancer Res*, 5, 1107-13.
- OH, S. P., SEKI, T., GOSS, K. A., IMAMURA, T., YI, Y., DONAHOE, P. K., LI, L., MIYAZONO, K., TEN DIJKE, P., KIM, S. & LI, E. 2000. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A*, 97, 2626-31.
- OIKE, Y., ITO, Y., HAMADA, K., ZHANG, X. Q., MIYATA, K., ARAI, F., INADA, T., ARAKI, K., NAKAGATA, N., TAKEYA, M., KISANUKI, Y. Y., YANAGISAWA, M., GALE, N. W. & SUDA, T. 2002. Regulation of

- vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells. *Blood*, 100, 1326-33.
- OLAYIOYE, M. A., NEVE, R. M., LANE, H. A. & HYNES, N. E. 2000. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J*, 19, 3159-67.
- OLSEN, J. V., BLAGOEV, B., GNAD, F., MACEK, B., KUMAR, C., MORTENSEN, P. & MANN, M. 2006. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell*, 127, 635-48.
- OLSON, N. E., GRAVES, J. D., SHU, G. L., RYAN, E. J. & CLARK, E. A. 2003. Caspase activity is required for stimulated B lymphocytes to enter the cell cycle. *J Immunol*, 170, 6065-72.
- ORTON, R. J., STURM, O. E., VYSHEMIRSKY, V., CALDER, M., GILBERT, D. R. & KOLCH, W. 2005. Computational modelling of the receptor-tyrosine-kinase-activated MAPK pathway. *Biochem J*, 392, 249-61.
- OSHIMA, M., OSHIMA, H. & TAKETO, M. M. 1996. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev Biol*, 179, 297-302.
- OWEN, J. D., RUEST, P. J., FRY, D. W. & HANKS, S. K. 1999. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. *Molecular and Cellular Biology*, 19, 4806-4818.
- OWENS, L. V., XU, L. H., CRAVEN, R. J., DENT, G. A., WEINER, T. M., KORNBERG, L., LIU, E. T. & CANCE, W. G. 1995. Overexpression of the Focal Adhesion Kinase (P125(Fak)) in Invasive Human Tumors. *Cancer Research*, 55, 2752-2755.
- OYAJOB, B. O., LOMRI, A., HOTT, M. & MARIE, P. J. 1999. Isolation and characterization of human clonogenic osteoblast progenitors immunoselected from fetal bone marrow stroma using STRO-1 monoclonal antibody. *J Bone Miner Res*, 14, 351-61.
- PARDALI, E., GOUMANS, M. J. & TEN DIJKE, P. 2010. Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends Cell Biol*, 20, 556-67.
- PARHAM, P. & OHTA, T. 1996. Population biology of antigen presentation by MHC class I molecules. *Science*, 272, 67-74.
- PARK, I. H., ZHAO, R., WEST, J. A., YABUUCHI, A., HUO, H., INCE, T. A., LEROU, P. H., LENSCH, M. W. & DALEY, G. Q. 2008. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*, 451, 141-6.
- PARSONS, J. T. 2003. Focal adhesion kinase: the first ten years. *Journal of Cell Science*, 116, 1409-1416.
- PARSONS, J. T., SLACK-DAVIS, J., TILGHMAN, R. & ROBERTS, W. G. 2008. Focal adhesion kinase: Targeting adhesion signaling pathways for therapeutic intervention. *Clinical Cancer Research*, 14, 627-632.
- PATAN, S. 2000. Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. *J Neurooncol*, 50, 1-15.
- PATRAWALA, L., CALHOUN, T., SCHNEIDER-BROUSSARD, R., LI, H., BHATIA, B., TANG, S., REILLY, J. G., CHANDRA, D., ZHOU, J., CLAYPOOL, K., COGHLAN, L. & TANG, D. G. 2006. Highly purified CD44+ prostate cancer

- cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene*, 25, 1696-708.
- PATTERSON, S. D. 2003. Proteomics: evolution of the technology. *Biotechniques*, 35, 440-4.
- PATTINGRE, S., ESPERT, L., BIARD-PIECHACZYK, M. & CODOGNO, P. 2008. Regulation of macroautophagy by mTOR and Beclin 1 complexes. *Biochimie*, 90, 313-23.
- PATTINGRE, S., TASSA, A., QU, X., GARUTI, R., LIANG, X. H., MIZUSHIMA, N., PACKER, M., SCHNEIDER, M. D. & LEVINE, B. 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell*, 122, 927-39.
- PEARSON, G., ROBINSON, F., BEERS GIBSON, T., XU, B. E., KARANDIKAR, M., BERMAN, K. & COBB, M. H. 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev*, 22, 153-83.
- PELECH, S. 2004. Tracking cell signaling protein expression and phosphorylation by innovative proteomic solutions. *Curr Pharm Biotechnol*, 5, 69-77.
- PENALOZA, C., LIN, L., LOCKSHIN, R. A. & ZAKERI, Z. 2006. Cell death in development: shaping the embryo. *Histochem Cell Biol*, 126, 149-58.
- PERLMAN, R., SCHIEMANN, W. P., BROOKS, M. W., LODISH, H. F. & WEINBERG, R. A. 2001. TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol*, 3, 708-14.
- PERUCHO, M., GOLDFARB, M., SHIMIZU, K., LAMA, C., FOGH, J. & WIGLER, M. 1981. Human-tumor-derived cell lines contain common and different transforming genes. *Cell*, 27, 467-76.
- PESANDO, J. M., BOUCHARD, L. S. & MCMASTER, B. E. 1989. CD19 is functionally and physically associated with surface immunoglobulin. *J Exp Med*, 170, 2159-64.
- PETER, M. E. & KRAMMER, P. H. 2003. The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ*, 10, 26-35.
- PETTI, C., MOLLA, A., VEGETTI, C., FERRONE, S., ANICHINI, A. & SENSI, M. 2006. Coexpression of NRASQ61R and BRAFV600E in human melanoma cells activates senescence and increases susceptibility to cell-mediated cytotoxicity. *Cancer Res*, 66, 6503-11.
- PHNG, L. K. & GERHARDT, H. 2009. Angiogenesis: a team effort coordinated by notch. *Dev Cell*, 16, 196-208.
- PHNG, L. K., POTENTE, M., LESLIE, J. D., BABBAGE, J., NYQVIST, D., LOBOV, I., ONDR, J. K., RAO, S., LANG, R. A., THURSTON, G. & GERHARDT, H. 2009. Nrarp coordinates endothelial Notch and Wnt signaling to control vessel density in angiogenesis. *Dev Cell*, 16, 70-82.
- PINKAS-KRAMARSKI, R., SHELLY, M., GLATHE, S., RATZKIN, B. J. & YARDEN, Y. 1996. Neu differentiation factor/neuregulin isoforms activate distinct receptor combinations. *J Biol Chem*, 271, 19029-32.
- PITTENGER, M. F., MACKAY, A. M., BECK, S. C., JAISWAL, R. K., DOUGLAS, R., MOSCA, J. D., MOORMAN, M. A., SIMONETTI, D. W., CRAIG, S. & MARSHAK, D. R. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science*, 284, 143-7.
- POBEZINSKAYA, Y. L., KIM, Y. S., CHOKSI, S., MORGAN, M. J., LI, T., LIU, C. & LIU, Z. 2008. The function of TRADD in signaling through tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors. *Nat Immunol*, 9, 1047-54.

- POLS, M. S. & KLUMPERMAN, J. 2009. Trafficking and function of the tetraspanin CD63. *Exp Cell Res*, 315, 1584-92.
- POLTE, T. R. & HANKS, S. K. 1995. Interaction between Focal Adhesion Kinase and Crk-Associated Tyrosine Kinase Substrate P130(Cas). *Proceedings of the National Academy of Sciences of the United States of America*, 92, 10678-10682.
- POLTE, T. R., NAFTILAN, A. J. & HANKS, S. K. 1994. Focal Adhesion Kinase Is Abundant in Developing Blood-Vessels and Elevation of Its Phosphotyrosine Content in Vascular Smooth-Muscle Cells Is a Rapid Response to Angiotensin-II. *Journal of Cellular Biochemistry*, 55, 106-119.
- POSS, K. D. & TONEGAWA, S. 1997. Heme oxygenase 1 is required for mammalian iron reutilization. *Proc Natl Acad Sci U S A*, 94, 10919-24.
- PRASAIN, N., MEADOR, J. L. & YODER, M. C. 2012. Phenotypic and functional characterization of endothelial colony forming cells derived from human umbilical cord blood. *J Vis Exp*.
- PRESTA, M., DELL'ERA, P., MITOLA, S., MORONI, E., RONCA, R. & RUSNATI, M. 2005. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev*, 16, 159-78.
- PRIES, A. R., HOPFNER, M., LE NOBLE, F., DEWHIRST, M. W. & SECOMB, T. W. 2010. The shunt problem: control of functional shunting in normal and tumour vasculature. *Nat Rev Cancer*, 10, 587-93.
- QI, J. H. & CLAEISSON-WELSH, L. 2001. VEGF-induced activation of phosphoinositide 3-kinase is dependent on focal adhesion kinase. *Experimental Cell Research*, 263, 173-182.
- QUAEGEBEUR, A., SEGURA, I. & CARMELIET, P. 2010. Pericytes: blood-brain barrier safeguards against neurodegeneration? *Neuron*, 68, 321-3.
- RAFII, S. & LYDEN, D. 2003. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med*, 9, 702-12.
- RAINA, A. K., HOCHMAN, A., ZHU, X., ROTTKAMP, C. A., NUNOMURA, A., SIEDLAK, S. L., BOUX, H., CASTELLANI, R. J., PERRY, G. & SMITH, M. A. 2001. Abortive apoptosis in Alzheimer's disease. *Acta Neuropathol*, 101, 305-10.
- RAJKUMAR, T., STAMP, G. W., PANDHA, H. S., WAXMAN, J. & GULLICK, W. J. 1996. Expression of the type 1 tyrosine kinase growth factor receptors EGF receptor, c-erbB2 and c-erbB3 in bladder cancer. *J Pathol*, 179, 381-5.
- RAUGI, G. J., MUMBY, S. M., ABBOTT-BROWN, D. & BORNSTEIN, P. 1982. Thrombospondin: synthesis and secretion by cells in culture. *J Cell Biol*, 95, 351-4.
- RE, F., ZANETTI, A., SIRONI, M., POLENTARUTTI, N., LANFRANCONE, L., DEJANA, E. & COLOTTA, F. 1994. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. *J Cell Biol*, 127, 537-46.
- REGE, T. A. & HAGOOD, J. S. 2006. Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *FASEB J*, 20, 1045-54.
- REINISCH, A., BARTMANN, C., ROHDE, E., SCHALLMOSER, K., BJELIC-RADISIC, V., LANZER, G., LINKESCH, W. & STRUNK, D. 2007. Humanized system to propagate cord blood-derived multipotent mesenchymal stromal cells for clinical application. *Regen Med*, 2, 371-82.

- REINISCH, A., HOFMANN, N. A., OBENAUF, A. C., KASHOFER, K., ROHDE, E., SCHALLMOSER, K., FLICKER, K., LANZER, G., LINKESCH, W., SPEICHER, M. R. & STRUNK, D. 2009. Humanized large-scale expanded endothelial colony-forming cells function in vitro and in vivo. *Blood*, 113, 6716-25.
- REINISCH, A. & STRUNK, D. 2009. Isolation and animal serum free expansion of human umbilical cord derived mesenchymal stromal cells (MSCs) and endothelial colony forming progenitor cells (ECFCs). *J Vis Exp*.
- REISKE, H. R., KAO, S. C., CARY, L. A., GUAN, J. L., LAI, J. F. & CHEN, H. C. 1999. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. *Journal of Biological Chemistry*, 274, 12361-12366.
- REN, X. D., KIOSSES, W. B., SIEG, D. J., OTEY, C. A., SCHLAEPFER, D. D. & SCHWARTZ, M. A. 2000. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *Journal of Cell Science*, 113, 3673-3678.
- RENGACHARY, S. S., COLEN, C. & GUTHIKONDA, M. 2008. Charles-Edouard Brown-Sequard: An eccentric genius. *Neurosurgery*, 62, 954-964.
- RIDLEY, A. J., SCHWARTZ, M. A., BURRIDGE, K., FIRTEL, R. A., GINSBERG, M. H., BORISY, G., PARSONS, J. T. & HORWITZ, A. R. 2003. Cell migration: integrating signals from front to back. *Science*, 302, 1704-9.
- RIEDL, S. J. & SALVESEN, G. S. 2007. The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol*, 8, 405-13.
- RINGDEN, O. 2007. Immunotherapy by allogeneic stem cell transplantation. *Adv Cancer Res*, 97, 25-60.
- RIO, C., BUXBAUM, J. D., PESCHON, J. J. & CORFAS, G. 2000. Tumor necrosis factor-alpha-converting enzyme is required for cleavage of erbB4/HER4. *J Biol Chem*, 275, 10379-87.
- RISAU, W. & FLAMME, I. 1995. Vasculogenesis. *Annu Rev Cell Dev Biol*, 11, 73-91.
- RIZZO, M. T. 2004. Focal adhesion kinase and angiogenesis. Where do we go from here? *Cardiovasc Res*, 64, 377-8.
- ROBBINS, D. J., CHENG, M., ZHEN, E., VANDERBILT, C. A., FEIG, L. A. & COBB, M. H. 1992. Evidence for a Ras-dependent extracellular signal-regulated protein kinase (ERK) cascade. *Proc Natl Acad Sci U S A*, 89, 6924-8.
- RODRIGUEZ-MORA, O. G., LAHAIR, M. M., MCCUBREY, J. A. & FRANKLIN, R. A. 2005. Calcium/calmodulin-dependent kinase I and calcium/calmodulin-dependent kinase kinase participate in the control of cell cycle progression in MCF-7 human breast cancer cells. *Cancer Res*, 65, 5408-16.
- ROHBAN, R., REINISCH, A., ETCHART, N., SCHALLMOSER, K., HOFMANN, N. A., SZOKE, K., BRINCHMANN, J. E., BONYADI RAD, E., ROHDE, E. & STRUNK, D. 2013. Identification of an effective early signaling signature during neo-vasculogenesis in vivo by ex vivo proteomic profiling. *PLoS One*, 8, e66909.
- ROHDE, E., BARTMANN, C., SCHALLMOSER, K., REINISCH, A., LANZER, G., LINKESCH, W., GUELLY, C. & STRUNK, D. 2007. Immune cells mimic the morphology of endothelial progenitor colonies in vitro. *Stem Cells*, 25, 1746-52.

- ROMER, L. H., MCLEAN, N., TURNER, C. E. & BURRIDGE, K. 1994. Tyrosine Kinase-Activity, Cytoskeletal Organization and Motility in Human Vascular Endothelial-Cells. *Molecular Biology of the Cell*, 5, 349-361.
- ROSSOMANDO, A. J., WU, J., MICHEL, H., SHABANOWITZ, J., HUNT, D. F., WEBER, M. J. & STURGILL, T. W. 1992. Identification of Tyr-185 as the site of tyrosine autophosphorylation of recombinant mitogen-activated protein kinase p42mapk. *Proc Natl Acad Sci U S A*, 89, 5779-83.
- ROY, S., WYSE, B. & HANCOCK, J. F. 2002. H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis. *Mol Cell Biol*, 22, 5128-40.
- RYOO, H. D. & BERGMANN, A. 2012. The role of apoptosis-induced proliferation for regeneration and cancer. *Cold Spring Harb Perspect Biol*, 4, a008797.
- SALVESEN, G. S. & RIEDL, S. J. 2008. Caspase mechanisms. *Adv Exp Med Biol*, 615, 13-23.
- SANTORO, M. G. 2000. Heat shock factors and the control of the stress response. *Biochem Pharmacol*, 59, 55-63.
- SARKAR, A., HALL, M. W., EXLINE, M., HART, J., KNATZ, N., GATSON, N. T. & WEWERS, M. D. 2006. Caspase-1 regulates Escherichia coli sepsis and splenic B cell apoptosis independently of interleukin-1 beta and interleukin-18. *American Journal of Respiratory and Critical Care Medicine*, 174, 1003-1010.
- SATO, T. N., TOZAWA, Y., DEUTSCH, U., WOLBURG-BUCHHOLZ, K., FUJIWARA, Y., GENDRON-MAGUIRE, M., GRIDLEY, T., WOLBURG, H., RISAU, W. & QIN, Y. 1995. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature*, 376, 70-4.
- SCAFFIDI, C., VOLKLAND, J., BLOMBERG, I., HOFFMANN, I., KRAMMER, P. H. & PETER, M. E. 2000. Phosphorylation of FADD/ MORT1 at serine 194 and association with a 70-kDa cell cycle-regulated protein kinase. *J Immunol*, 164, 1236-42.
- SCARLATTI, F., GRANATA, R., MEIJER, A. J. & CODOGNO, P. 2009. Does autophagy have a license to kill mammalian cells? *Cell Death Differ*, 16, 12-20.
- SCHALLER, M. D. 2001. Biochemical signals and biological responses elicited by the focal adhesion kinase. *Biochimica Et Biophysica Acta-Molecular Cell Research*, 1540, 1-21.
- SCHALLER, M. D., BORGMAN, C. A., COBB, B. S., VINES, R. R., REYNOLDS, A. B. & PARSONS, J. T. 1992. Pp125fak, a Structurally Distinctive Protein-Tyrosine Kinase Associated with Focal Adhesions. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 5192-5196.
- SCHALLMOSER, K., BARTMANN, C. & ROHDE, E., ET AL. 2007a. Human platelet lysate can replace fetal bovine serum for clinical scale expansion of functional MSC. *Transfusion*.
- SCHALLMOSER, K., BARTMANN, C., ROHDE, E., REINISCH, A., KASHOFER, K., STADELMEYER, E., DREXLER, C., LANZER, G., LINKESCH, W. & STRUNK, D. 2007b. Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. *Transfusion*, 47, 1436-46.

- SCHALLMOSER, K. & STRUNK, D. 2009. Preparation of pooled human platelet lysate (pHPL) as an efficient supplement for animal serum-free human stem cell cultures. *J Vis Exp*.
- SCHARNER, D., ROSSIG, L., CARMONA, G., CHAVAKIS, E., URBICH, C., FISCHER, A., KANG, T. B., WALLACH, D., CHIANG, Y. J., DERIBE, Y. L., DIKIC, I., ZEIHNER, A. M. & DIMMELER, S. 2009. Caspase-8 is involved in neovascularization-promoting progenitor cell functions. *Arterioscler Thromb Vasc Biol*, 29, 571-8.
- SCHATTEMAN, G. C., DUNNWALD, M. & JIAO, C. 2007. Biology of bone marrow-derived endothelial cell precursors. *Am J Physiol Heart Circ Physiol*, 292, H1-18.
- SCHECHNER, J. S., NATH, A. K., ZHENG, L., KLUGER, M. S., HUGHES, C. C., SIERRA-HONIGMANN, M. R., LORBER, M. I., TELLIDES, G., KASHGARIAN, M., BOTHWELL, A. L. & POBER, J. S. 2000. In vivo formation of complex microvessels lined by human endothelial cells in an immunodeficient mouse. *Proc Natl Acad Sci U S A*, 97, 9191-6.
- SCHLAEPFER, D. D. & MITRA, S. K. 2004. Multiple connections link FAK to cell motility and invasion. *Current Opinion in Genetics & Development*, 14, 92-101.
- SCHONHERR, E., O'CONNELL, B. C., SCHITTNY, J., ROBENEK, H., FASTERMANN, D., FISHER, L. W., PLENZ, G., VISCHER, P., YOUNG, M. F. & KRESSE, H. 1999. Paracrine or virus-mediated induction of decorin expression by endothelial cells contributes to tube formation and prevention of apoptosis in collagen lattices. *Eur J Cell Biol*, 78, 44-55.
- SCHRODER, K., MURUVE, D. A. & TSCHOPP, J. 2009. Innate immunity: cytoplasmic DNA sensing by the AIM2 inflammasome. *Curr Biol*, 19, R262-5.
- SCHULZE, W. X., DENG, L. & MANN, M. 2005. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol Syst Biol*, 1, 2005 0008.
- SCHWARTZ, J. D., ROWINSKY, E. K., YOUSSEF, H., PYTOWSKI, B. & WU, Y. 2010. Vascular endothelial growth factor receptor-1 in human cancer: concise review and rationale for development of IMC-18F1 (Human antibody targeting vascular endothelial growth factor receptor-1). *Cancer*, 116, 1027-32.
- SCHWEITZER, B., PREDKI, P. & SNYDER, M. 2003. Microarrays to characterize protein interactions on a whole-proteome scale. *Proteomics*, 3, 2190-9.
- SEBOLT-LEOPOLD, J. S. & HERRERA, R. 2004. Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer*, 4, 937-47.
- SECHLER, J. L. & SCHWARZBAUER, J. E. 1997. Coordinated regulation of fibronectin fibril assembly and actin stress fiber formation. *Cell Adhes Commun*, 4, 413-24.
- SEGER, R., AHN, N. G., POSADA, J., MUNAR, E. S., JENSEN, A. M., COOPER, J. A., COBB, M. H. & KREBS, E. G. 1992. Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells. *J Biol Chem*, 267, 14373-81.
- SEGURA, I., SERRANO, A., DE BUITRAGO, G. G., GONZALEZ, M. A., ABAD, J. L., CLAVERIA, C., GOMEZ, L., BERNAD, A., MARTINEZ, A. C. & RIESE, H. H. 2002. Inhibition of programmed cell death impairs in vitro vascular-like structure formation and reduces in vivo angiogenesis. *FASEB J*, 16, 833-41.

- SHAHDAFAR, A., FRONSDAL, K., HAUG, T., REINHOLT, F. P. & BRINCHMANN, J. E. 2005. In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. *Stem Cells*, 23, 1357-66.
- SHAW, T. J., LACASSE, E. C., DURKIN, J. P. & VANDERHYDEN, B. C. 2008. Downregulation of XIAP expression in ovarian cancer cells induces cell death in vitro and in vivo. *Int J Cancer*, 122, 1430-4.
- SHAWBER, C. J. & KITAJEWSKI, J. 2004. Notch function in the vasculature: insights from zebrafish, mouse and man. *Bioessays*, 26, 225-34.
- SHEN, T. L., HAN, D. C. & GUAN, J. L. 2002. Association of Grb7 with phosphoinositides and its role in the regulation of cell migration. *Journal of Biological Chemistry*, 277, 29069-29077.
- SHIBATA, K., KIKKAWA, F., NAWA, A., THANT, A. A., NARUSE, K., MIZUTANI, S. & HAMAGUCHI, M. 1998. Both focal adhesion kinase and c-ras are required for the enhanced matrix metalloproteinase 9 secretion by fibronectin in ovarian cancer cells. *Cancer Research*, 58, 900-903.
- SHIH, C., PADHY, L. C., MURRAY, M. & WEINBERG, R. A. 1981. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature*, 290, 261-4.
- SHIH, D. T., EDELMAN, J. M., HORWITZ, A. F., GRUNWALD, G. B. & BUCK, C. A. 1993. Structure/function analysis of the integrin beta 1 subunit by epitope mapping. *J Cell Biol*, 122, 1361-71.
- SHINOHARA, N., OGISO, Y., TANAKA, M., SAZAWA, A., HARABAYASHI, T. & KOYANAGI, T. 1997. The significance of Ras guanine nucleotide exchange factor, son of sevenless protein, in renal cell carcinoma cell lines. *J Urol*, 158, 908-11.
- SIEG, D. J., HAUCK, C. R., ILIC, D., KLINGBEIL, C. K., SCHAEFER, E., DAMSKY, C. H. & SCHLAEPFER, D. D. 2000. FAK integrates growth-factor and integrin signals to promote cell migration. *Nature Cell Biology*, 2, 249-256.
- SIEGEL, P. M., RYAN, E. D., CARDIFF, R. D. & MULLER, W. J. 1999. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. *EMBO J*, 18, 2149-64.
- SIMMONS, P. J. & TOROK-STORB, B. 1991. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood*, 78, 55-62.
- SINGER, S. J. 1974. Molecular biology of cellular membranes with applications to immunology. *Adv Immunol*, 19, 1-66.
- SLAMON, D. J., GODOLPHIN, W., JONES, L. A., HOLT, J. A., WONG, S. G., KEITH, D. E., LEVIN, W. J., STUART, S. G., UDOVE, J., ULLRICH, A. & ET AL. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*, 244, 707-12.
- SLAMON, D. J., LEYLAND-JONES, B., SHAK, S., FUCHS, H., PATON, V., BAJAMONDE, A., FLEMING, T., EIERMANN, W., WOLTER, J., PEGRAM, M., BASELGA, J. & NORTON, L. 2001. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*, 344, 783-92.
- SMALL, T. N., YOUNG, J. W., CASTRO-MALASPINA, H., PROCKOP, S., WILTON, A., HELLER, G., BOULAD, F., CHIU, M., HSU, K.,

- JAKUBOWSKI, A., KERNAN, N. A., PERALES, M. A., O'REILLY, R. J. & PAPADOPOULOS, E. B. 2007. Intravenous busulfan and melphalan, tacrolimus, and short-course methotrexate followed by unmodified HLA-matched related or unrelated hematopoietic stem cell transplantation for the treatment of advanced hematologic malignancies. *Biol Blood Marrow Transplant*, 13, 235-44.
- SOLLBERGER, G., STRITTMATTER, G. E., KISTOWSKA, M., FRENCH, L. E. & BEER, H. D. 2012. Caspase-4 is required for activation of inflammasomes. *J Immunol*, 188, 1992-2000.
- SORA, F., PICCIRILLO, N., CHIUSOLO, P., LAURENTI, L., MARRA, R., BARTOLOZZI, F., LEONE, G. & SICA, S. 2006. Mitoxantrone, carboplatin, cytosine arabinoside, and methylprednisolone followed by autologous peripheral blood stem cell transplantation: a salvage regimen for patients with refractory or recurrent non-Hodgkin lymphoma. *Cancer*, 106, 859-66.
- SORIANO, P. 1994. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev*, 8, 1888-96.
- SOTTILE, J. & HOCKING, D. C. 2002. Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. *Mol Biol Cell*, 13, 3546-59.
- SPANGRUDE, G. J., HEIMFELD, S. & WEISSMAN, I. L. 1988. Purification and characterization of mouse hematopoietic stem cells. *Science*, 241, 58-62.
- SPIRIDON, C. I., GHETIE, M. A., UHR, J., MARCHES, R., LI, J. L., SHEN, G. L. & VITETTA, E. S. 2002. Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antigrowth activity of a human breast cancer cell line in vitro and in vivo. *Clin Cancer Res*, 8, 1720-30.
- STARZL, T. E. 2000. History of clinical transplantation. *World J Surg*, 24, 759-82.
- STARZL, T. E., MURASE, N., DEMETRIS, A., TRUCCO, M. & FUNG, J. 2000. The mystique of hepatic tolerogenicity. *Seminars in Liver Disease*, 20, 497-510.
- STELLOS, K. & GAWAZ, M. 2007. Platelets and stromal cell-derived factor-1 in progenitor cell recruitment. *Semin Thromb Hemost*, 33, 159-64.
- STOCKMANN, C., DOEDENS, A., WEIDEMANN, A., ZHANG, N., TAKEDA, N., GREENBERG, J. I., CHERESH, D. A. & JOHNSON, R. S. 2008. Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis. *Nature*, 456, 814-8.
- STOKER, M. & GHERARDI, E. 1991. Regulation of cell movement: the motogenic cytokines. *Biochim Biophys Acta*, 1072, 81-102.
- STORB, R. & THOMAS, E. D. 1983. Allogeneic Bone-Marrow Transplantation. *Immunological Reviews*, 71, 77-102.
- STRACHAN, T. & READ, A. P. 1999. *Human Molecular Genetics*. 2nd ed. New York.
- STREIT, M., RICCARDI, L., VELASCO, P., BROWN, L. F., HAWIGHORST, T., BORNSTEIN, P. & DETMAR, M. 1999. Thrombospondin-2: a potent endogenous inhibitor of tumor growth and angiogenesis. *Proc Natl Acad Sci U S A*, 96, 14888-93.
- STRILIC, B., KUCERA, T., EGLINGER, J., HUGHES, M. R., MCNAGNY, K. M., TSUKITA, S., DEJANA, E., FERRARA, N. & LAMMERT, E. 2009. The molecular basis of vascular lumen formation in the developing mouse aorta. *Dev Cell*, 17, 505-15.

- STUPACK, D. G. & CHERESH, D. A. 2002. Get a ligand, get a life: integrins, signaling and cell survival. *J Cell Sci*, 115, 3729-38.
- STURGILL, T. W., RAY, L. B., ERIKSON, E. & MALLER, J. L. 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature*, 334, 715-8.
- SUNDBERG, C., KOWANETZ, M., BROWN, L. F., DETMAR, M. & DVORAK, H. F. 2002. Stable expression of angiopoietin-1 and other markers by cultured pericytes: phenotypic similarities to a subpopulation of cells in maturing vessels during later stages of angiogenesis in vivo. *Lab Invest*, 82, 387-401.
- SURI, C., JONES, P. F., PATAN, S., BARTUNKOVA, S., MAISONPIERRE, P. C., DAVIS, S., SATO, T. N. & YANCOPOULOS, G. D. 1996. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell*, 87, 1171-80.
- SWIFT, M. R. & WEINSTEIN, B. M. 2009. Arterial-venous specification during development. *Circ Res*, 104, 576-88.
- SZOKE, K., BECKSTROM, K. J. & BRINCHMANN, J. E. 2012. Human adipose tissue as a source of cells with angiogenic potential. *Cell Transplant*, 21, 235-50.
- TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. & YAMANAKA, S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, 131, 861-72.
- TAKAHASHI, K. & YAMANAKA, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126, 663-76.
- TAKASAKI, I., CHOBANIAN, A. V. & BRECHER, P. 1991. Biosynthesis of fibronectin by rabbit aorta. *J Biol Chem*, 266, 17686-94.
- TEN DIJKE, P. & ARTHUR, H. M. 2007. Extracellular control of TGFbeta signalling in vascular development and disease. *Nat Rev Mol Cell Biol*, 8, 857-69.
- THOMAS, E. D. 1999. Bone marrow transplantation: A review. *Seminars in Hematology*, 36, 95-103.
- THOMSON, M., LIU, S. J., ZOU, L. N., SMITH, Z., MEISSNER, A. & RAMANATHAN, S. 2011. Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. *Cell*, 145, 875-89.
- THREADGILL, D. W., DLUGOSZ, A. A., HANSEN, L. A., TENNENBAUM, T., LICHTI, U., YEE, D., LAMANTIA, C., MOURTON, T., HERRUP, K., HARRIS, R. C. & ET AL. 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science*, 269, 230-4.
- THURINGER, D., JEGO, G., WETTSTEIN, G., TERRIER, O., CRONIER, L., YOUSFI, N., HEBRARD, S., BOUCHOT, A., HAZOUME, A., JOLY, A. L., GLEAVE, M., ROSA-CALATRAVA, M., SOLARY, E. & GARRIDO, C. 2013. Extracellular HSP27 mediates angiogenesis through Toll-like receptor 3. *FASEB J*, 27, 4169-83.
- THURSTON, G., NOGUERA-TROISE, I. & YANCOPOULOS, G. D. 2007. The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nat Rev Cancer*, 7, 327-31.
- THYBOLL, J., KORTESMAA, J., CAO, R., SOININEN, R., WANG, L., IIVANAINEN, A., SOROKIN, L., RISLING, M., CAO, Y. & TRYGGVASON, K. 2002. Deletion of the laminin alpha4 chain leads to impaired microvessel maturation. *Mol Cell Biol*, 22, 1194-202.

- TILL, J. E. & MC, C. E. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res*, 14, 213-22.
- TOLSMA, S. S., VOLPERT, O. V., GOOD, D. J., FRAZIER, W. A., POLVERINI, P. J. & BOUCK, N. 1993. Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. *J Cell Biol*, 122, 497-511.
- TVOROGOV, D., ANISIMOV, A., ZHENG, W., LEPPANEN, V. M., TAMMELA, T., LAURINAVICIUS, S., HOLNTHONER, W., HELOTERA, H., HOLOPAINEN, T., JELTSCH, M., KALKKINEN, N., LANKINEN, H., OJALA, P. M. & ALITALO, K. 2010. Effective suppression of vascular network formation by combination of antibodies blocking VEGFR ligand binding and receptor dimerization. *Cancer Cell*, 18, 630-40.
- TZAHAR, E., WATERMAN, H., CHEN, X., LEVKOWITZ, G., KARUNAGARAN, D., LAVI, S., RATZKIN, B. J. & YARDEN, Y. 1996. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol*, 16, 5276-87.
- UEMURA, A., OGAWA, M., HIRASHIMA, M., FUJIWARA, T., KOYAMA, S., TAKAGI, H., HONDA, Y., WIEGAND, S. J., YANCOPOULOS, G. D. & NISHIKAWA, S. 2002. Recombinant angiopoietin-1 restores higher-order architecture of growing blood vessels in mice in the absence of mural cells. *J Clin Invest*, 110, 1619-28.
- URNESS, L. D., SORENSEN, L. K. & LI, D. Y. 2000. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet*, 26, 328-31.
- VAGELI, D., KIARIS, H., DELAKAS, D., ANEZINIS, P., CRANIDIS, A. & SPANDIDOS, D. A. 1996. Transcriptional activation of H-ras, K-ras and N-ras proto-oncogenes in human bladder tumors. *Cancer Lett*, 107, 241-7.
- VAIDYANATHAN, G. 2012. Redefining clinical trials: the age of personalized medicine. *Cell*, 148, 1079-80.
- VETTER, I. R. & WITTINGHOFFER, A. 2001. The guanine nucleotide-binding switch in three dimensions. *Science*, 294, 1299-304.
- VIKKULA, M., BOON, L. M., CARRAWAY, K. L., 3RD, CALVERT, J. T., DIAMONTI, A. J., GOUMNEROV, B., PASYK, K. A., MARCHUK, D. A., WARMAN, M. L., CANTLEY, L. C., MULLIKEN, J. B. & OLSEN, B. R. 1996. Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. *Cell*, 87, 1181-90.
- VINALS, F. & POUYSSEGUR, J. 2001. Transforming growth factor beta1 (TGF-beta1) promotes endothelial cell survival during in vitro angiogenesis via an autocrine mechanism implicating TGF-alpha signaling. *Mol Cell Biol*, 21, 7218-30.
- VISCHER, U. M. & WAGNER, D. D. 1993. CD63 is a component of Weibel-Palade bodies of human endothelial cells. *Blood*, 82, 1184-91.
- VISENTIN, B., VEKICH, J. A., SIBBALD, B. J., CAVALLI, A. L., MORENO, K. M., MATTEO, R. G., GARLAND, W. A., LU, Y., YU, S., HALL, H. S., KUNDRA, V., MILLS, G. B. & SABBADINI, R. A. 2006. Validation of an anti-sphingosine-1-phosphate antibody as a potential therapeutic in reducing growth, invasion, and angiogenesis in multiple tumor lineages. *Cancer Cell*, 9, 225-38.

- VOLPERT, O. V., LAWLER, J. & BOUCK, N. P. 1998. A human fibrosarcoma inhibits systemic angiogenesis and the growth of experimental metastases via thrombospondin-1. *Proc Natl Acad Sci U S A*, 95, 6343-8.
- VON LINTIG, F. C., DREILINGER, A. D., VARKI, N. M., WALLACE, A. M., CASTEEL, D. E. & BOSS, G. R. 2000. Ras activation in human breast cancer. *Breast Cancer Res Treat*, 62, 51-62.
- WAGNER, J. E. & GLUCKMAN, E. 2010. Umbilical cord blood transplantation: the first 20 years. *Semin Hematol*, 47, 3-12.
- WALLASCH, C., WEISS, F. U., NIEDERFELLNER, G., JALLAL, B., ISSING, W. & ULLRICH, A. 1995. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *EMBO J*, 14, 4267-75.
- WALSH, J. G., CULLEN, S. P., SHERIDAN, C., LUTHI, A. U., GERNER, C. & MARTIN, S. J. 2008. Executioner caspase-3 and caspase-7 are functionally distinct proteases. *Proc Natl Acad Sci U S A*, 105, 12815-9.
- WANG, H. & KEISER, J. A. 1998. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. *Circ Res*, 83, 832-40.
- WANG, L., DU, F. & WANG, X. 2008. TNF-alpha induces two distinct caspase-8 activation pathways. *Cell*, 133, 693-703.
- WANG, S., MIURA, M., JUNG, Y. K., ZHU, H., LI, E. & YUAN, J. 1998. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell*, 92, 501-9.
- WATERMAN, H., ALROY, I., STRANO, S., SEGER, R. & YARDEN, Y. 1999. The C-terminus of the kinase-defective neuregulin receptor ErbB-3 confers mitogenic superiority and dictates endocytic routing. *EMBO J*, 18, 3348-58.
- WAYMAN, G. A., TOKUMITSU, H., DAVARE, M. A. & SODERLING, T. R. 2011. Analysis of CaM-kinase signaling in cells. *Cell Calcium*, 50, 1-8.
- WEBER, G. F. & MENKO, A. S. 2005. The canonical intrinsic mitochondrial death pathway has a non-apoptotic role in signaling lens cell differentiation. *J Biol Chem*, 280, 22135-45.
- WEI, Y., PATTINGRE, S., SINHA, S., BASSIK, M. & LEVINE, B. 2008. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Mol Cell*, 30, 678-88.
- WEINER, T. M., LIU, E. T., CRAVEN, R. J. & CANCE, W. G. 1993. Expression of Focal Adhesion Kinase Gene and Invasive Cancer. *Lancet*, 342, 1024-1025.
- WEISS, M. L., MEDICETTY, S., BLEDSOE, A. R., RACHAKATLA, R. S., CHOI, M., MERCHAV, S., LUO, Y., RAO, M. S., VELAGALETI, G. & TROYER, D. 2006. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells*, 24, 781-92.
- WELLBROCK, C., KARASARIDES, M. & MARAIS, R. 2004. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol*, 5, 875-85.
- WESTON, C. R. & DAVIS, R. J. 2007. The JNK signal transduction pathway. *Curr Opin Cell Biol*, 19, 142-9.
- WIJELATH, E. S., MURRAY, J., RAHMAN, S., PATEL, Y., ISHIDA, A., STRAND, K., AZIZ, S., CARDONA, C., HAMMOND, W. P., SAVIDGE, G. F., RAFII, S. & SOBEL, M. 2002. Novel vascular endothelial growth factor binding

- domains of fibronectin enhance vascular endothelial growth factor biological activity. *Circ Res*, 91, 25-31.
- WILLIAMS, J. G., PAPPU, K. & CAMPBELL, S. L. 2003. Structural and biochemical studies of p21Ras S-nitrosylation and nitric oxide-mediated guanine nucleotide exchange. *Proc Natl Acad Sci U S A*, 100, 6376-81.
- WILLIAMS, M. E., LICHTMAN, A. H. & ABBAS, A. K. 1990. Anti-CD3 antibody induces unresponsiveness to IL-2 in Th1 clones but not in Th2 clones. *J Immunol*, 144, 1208-14.
- WILSON, A. & TRUMPP, A. 2006. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol*, 6, 93-106.
- WILSON, N. S., DIXIT, V. & ASHKENAZI, A. 2009. Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nat Immunol*, 10, 348-55.
- WITZENBICHLER, B., MAISONPIERRE, P. C., JONES, P., YANCOPOULOS, G. D. & ISNER, J. M. 1998. Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. *J Biol Chem*, 273, 18514-21.
- WOLBANK, S., PETERBAUER, A., FAHRNER, M., HENNERBICHLER, S., VAN GRIENSVEN, M., STADLER, G., REDL, H. & GABRIEL, C. 2007. Dose-dependent immunomodulatory effect of human stem cells from amniotic membrane: a comparison with human mesenchymal stem cells from adipose tissue. *Tissue Eng*, 13, 1173-83.
- WRIGHT, D. E., WAGERS, A. J., GULATI, A. P., JOHNSON, F. L. & WEISSMAN, I. L. 2001. Physiological migration of hematopoietic stem and progenitor cells. *Science*, 294, 1933-6.
- WU, X. Y., GAN, B. Y., YOO, Y. & GUAN, J. L. 2005. FAK-mediated Src phosphorylation of endophilin A2 inhibits endocytosis of MT1-MMP and promotes ECM degradation. *Developmental Cell*, 9, 185-196.
- XIA, W., MULLIN, R. J., KEITH, B. R., LIU, L. H., MA, H., RUSNAK, D. W., OWENS, G., ALLIGOOD, K. J. & SPECTOR, N. L. 2002. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene*, 21, 6255-63.
- XIE, Z. & KLIONSKY, D. J. 2007. Autophagosome formation: core machinery and adaptations. *Nat Cell Biol*, 9, 1102-9.
- XIONG, S., GRIJALVA, R., ZHANG, L., NGUYEN, N. T., PISTERS, P. W., POLLOCK, R. E. & YU, D. 2001. Up-regulation of vascular endothelial growth factor in breast cancer cells by the heregulin-beta1-activated p38 signaling pathway enhances endothelial cell migration. *Cancer Res*, 61, 1727-32.
- XU, G. F., LIN, B., TANAKA, K., DUNN, D., WOOD, D., GESTELAND, R., WHITE, R., WEISS, R. & TAMANOI, F. 1990a. The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of *S. cerevisiae*. *Cell*, 63, 835-41.
- XU, G. F., O'CONNELL, P., VISKOCHIL, D., CAWTHON, R., ROBERTSON, M., CULVER, M., DUNN, D., STEVENS, J., GESTELAND, R., WHITE, R. & ET AL. 1990b. The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell*, 62, 599-608.
- XU, J., RODRIGUEZ, D., PETITCLERC, E., KIM, J. J., HANGAI, M., MOON, Y. S., DAVIS, G. E. & BROOKS, P. C. 2001. Proteolytic exposure of a cryptic site

- within collagen type IV is required for angiogenesis and tumor growth in vivo. *J Cell Biol*, 154, 1069-79.
- YAMADA, Y., YOKOYAMA, S., WANG, X. D., FUKUDA, N. & TAKAKURA, N. 2007. Cardiac stem cells in brown adipose tissue express CD133 and induce bone marrow nonhematopoietic cells to differentiate into cardiomyocytes. *Stem Cells*, 25, 1326-33.
- YAMASHITA, A., NISHIKAWA, S. & RANCOURT, D. E. 2010. Microenvironment modulates osteogenic cell lineage commitment in differentiated embryonic stem cells. *PLoS One*, 5, e9663.
- YANCOPOULOS, G. D., DAVIS, S., GALE, N. W., RUDGE, J. S., WIEGAND, S. J. & HOLASH, J. 2000. Vascular-specific growth factors and blood vessel formation. *Nature*, 407, 242-248.
- YANG, X., CASTILLA, L. H., XU, X., LI, C., GOTAY, J., WEINSTEIN, M., LIU, P. P. & DENG, C. X. 1999. Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development*, 126, 1571-80.
- YANG, X., KHOSRAVI-FAR, R., CHANG, H. Y. & BALTIMORE, D. 1997. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell*, 89, 1067-76.
- YARDEN, Y. & SCHLESSINGER, J. 1987. Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. *Biochemistry*, 26, 1434-42.
- YE, D., MENDELSON, J. & FAN, Z. 1999. Augmentation of a humanized anti-HER2 mAb 4D5 induced growth inhibition by a human-mouse chimeric anti-EGF receptor mAb C225. *Oncogene*, 18, 731-8.
- YEAGER, C. L., ASHMUN, R. A., WILLIAMS, R. K., CARDELLICHIO, C. B., SHAPIRO, L. H., LOOK, A. T. & HOLMES, K. V. 1992. Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature*, 357, 420-2.
- YEN, L., BENLIMAME, N., NIE, Z. R., XIAO, D., WANG, T., AL MOUSTAFA, A. E., ESUMI, H., MILANINI, J., HYNES, N. E., PAGES, G. & ALAOUI-JAMALI, M. A. 2002. Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers. *Mol Biol Cell*, 13, 4029-44.
- YEN, L., YOU, X. L., AL MOUSTAFA, A. E., BATIST, G., HYNES, N. E., MADER, S., MELOCHE, S. & ALAOUI-JAMALI, M. A. 2000. Heregulin selectively upregulates vascular endothelial growth factor secretion in cancer cells and stimulates angiogenesis. *Oncogene*, 19, 3460-9.
- YOON, S. & SEGER, R. 2006. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors*, 24, 21-44.
- YOULE, R. J. & STRASSER, A. 2008. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*, 9, 47-59.
- ZENGIN, E., CHALAJOUR, F., GEHLING, U. M., ITO, W. D., TREEDE, H., LAUKE, H., WEIL, J., REICHENSPURNER, H., KILIC, N. & ERGUN, S. 2006. Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development*, 133, 1543-51.
- ZHAI, J. B., LIN, H., NIE, Z. Y., WU, J. H., CANETE-SOLER, R., SCHLAEPFER, W. W. & SCHLAEPFER, D. D. 2003. Direct interaction of focal adhesion kinase with p190RhoGEF. *Journal of Biological Chemistry*, 278, 24865-24873.
- ZHANG, D. Y., YE, F., GAO, L., LIU, X., ZHAO, X., CHE, Y., WANG, H., WANG, L., WU, J., SONG, D., LIU, W., XU, H., JIANG, B., ZHANG, W., WANG, J.

- & LEE, P. 2009. Proteomics, pathway array and signaling network-based medicine in cancer. *Cell Div*, 4, 20.
- ZHANG, J., CADO, D., CHEN, A., KABRA, N. H. & WINOTO, A. 1998. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature*, 392, 296-300.
- ZHANG, K., SUN, J., LIU, N., WEN, D., CHANG, D., THOMASON, A. & YOSHINAGA, S. K. 1996. Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. *J Biol Chem*, 271, 3884-90.
- ZHAO, X. & GUAN, J. L. 2011a. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Advanced Drug Delivery Reviews*, 63, 610-615.
- ZHAO, X. & GUAN, J. L. 2011b. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Adv Drug Deliv Rev*, 63, 610-5.
- ZHOU, M., FELDER, S., RUBINSTEIN, M., HURWITZ, D. R., ULLRICH, A., LAX, I. & SCHLESSINGER, J. 1993. Real-time measurements of kinetics of EGF binding to soluble EGF receptor monomers and dimers support the dimerization model for receptor activation. *Biochemistry*, 32, 8193-8.
- ZHU, H. & SNYDER, M. 2003. Protein chip technology. *Curr Opin Chem Biol*, 7, 55-63.
- ZHU, W. H., HAN, J. & NICOSIA, R. F. 2003. Requisite role of p38 MAPK in mural cell recruitment during angiogenesis in the rat aorta model. *J Vasc Res*, 40, 140-8.
- ZORNIG, M., HUEBER, A. O. & EVAN, G. 1998. p53-dependent impairment of T-cell proliferation in FADD dominant-negative transgenic mice. *Curr Biol*, 8, 467-70.
- ZOU, J. X., LIU, Y., PASQUALE, E. B. & RUOSLAHTI, E. 2002. Activated SRC oncogene phosphorylates R-ras and suppresses integrin activity. *J Biol Chem*, 277, 1824-7.
- ZOU, J. X., WANG, B., KALO, M. S., ZISCH, A. H., PASQUALE, E. B. & RUOSLAHTI, E. 1999. An Eph receptor regulates integrin activity through R-Ras. *Proc Natl Acad Sci U S A*, 96, 13813-8.