Hematopoietic Stem / Progenitor Cells and Placental Vascular Development:

In vitro study on the role of oxygen and stromal-derived factor-1α in the establishment of a stem cell niche

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ABSTRACT

A functional placenta is an absolute requirement for normal embryonic, fetal and post-natal development. During the first trimester of pregnancy, trophoblast expands rapidly, invades the uterine wall and contributes to placental formation. Development of the vascular bed facilitates the absorptive, excretory and respiratory functions of the human placenta. Multiple cell types are present at sites of placental vascular development, including hematopoietic stem cells, endothelial cells, trophoblast and placental stromal fibroblasts. Polarographic measurements made in utero have provided precise information regarding local oxygen tensions (pO2) at the feto-maternal interface during various stages of development. Using these values as a guide, a novel protocol is described that allows simulation of *in utero* oxygen environments with real-time monitoring of oxygen levels. The impact of oxygen on hematopoietic stem/progenitor cell (HSPC) proliferation, cell cycle status, apoptosis and differentiation was investigated. We demonstrate that the low oxygen environment of the developing placenta may help maintain the HSPC stem cell phenotype, while simultaneously inducing the differentiation of monocytes. Locally derived factors also have effects on HSPC in the placental microenvironment. The chemokine SDF-1α / CXCL12 is commonly found in low oxygen environments and is known to effect CXCR4-expressing hematopoietic cells. Results from this investigation indicate that SDF-1α is expressed in chorionic villi throughout gestation. Furthermore, isolated villous stroma-enriched cell fractions secreted SDF-1α *in vitro*. HSPC expression of CXCR4 increased during coculture with placental cells in low oxygen. Interactions of placental-derived SDF-1α with CXCR4 effects HSPC migration, transendothelial migration and adhesion of HSPC. Results indicate the involvement of SDF-1α in attracting CXCR4-expressing HSPC to areas of placental vascular development.

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ABBREVIATIONS

°C	degrees Centigrade
AGM	aorta-gonads-mesonephros
APC	allophycocyanin
bp	base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CLP	common lymphoid progenitors
CMP	common myeloid progenitors
CO_2	carbon dioxide
DMEM	Dulbecco's minimum essential media
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
dpc	days post conception
DTT	1,4-dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunospecific assay
EPC	endothelial progenitor cells
EpCAM	epithelial cell adhesion molecule
ESC	embryonic stem cells
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF4	fibroblast growth factor-4
FITC	fluorescein isothiocyanate
Flt3L	Flt3 ligand
FSC	forward scatter
g	grams
H_2O	water
HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
HIF	hypoxia-inducible factor
HPF	human placental fibroblast; placental stroma-enriched cell fraction
HPF-CM	human placental fibroblast conditioned media
HSC	hematopoietic stem cells
HSPC	hematopoietic stem/progenitor cells
HSPC-GM	hematopoietic stem/progenitor cell conditioned media
HTR	human trophoblast; trophoblast-enriched cell fraction

HTR-CM	human trophoblast conditioned media
HUVEC	human umbilical vein endothelial cells
ICM	inner cell mass
Ig	immunoglobulin
IMDM	Iscove's minimum defined media
IUGR	intrauterine growth deficiency
KCl	potassium chloride
kDa	kilodaltons
l	litres
LT-HSC	long-term hematopoietic stem cells
MACS	magnetic activated cell sorting
$MgCl_2$	magnesium chloride
min	minute(s)
ml	millilitres
mm	millimetres
mmHg	millimetres mercury
MMP	matrix metalloproteinase
MNC	mononuclear cells
N_2	nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
NK	natural killer cells
nM	nanomolar
O_2	oxygen
PBS	phosphate buffered saline
PC	personal computer
PCR	polymerase chain reaction
PE	phycoerythrin
PECAM	platelet-endothelial cell adhesion molecule
PerCP	peridinin chlorophyll protein
pg	picograms
pH	power of hydrogen
PI	propidium iodide
PlGF	placental-derived growth factor
pO_2	partial pressure of oxygen
PS	phosphotidylserine
rh	recombinant human
RNA	ribonucleic acid
ROS	reactive oxygen species
R-PE	R-phycoerythrin

RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor; kit ligand
SDF-1α	stromal-derived growth factor-α
SDR2	Sensor Dish Reader 2
sec	seconds
SRM	serum reduced media
SSC	sideward scatter
TEM	transendothelial migration
TPO	thrombopoietin
TSC	trophoblast stem cells
UCB	umbilical cord blood
UV	ultraviolet
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptor-1; Flt-1
VEGFR-2	vascular endothelial growth factor receptor-2; KDR
VLA-4	very-late antigen-4; α4 integrin
wpc	weeks post conception
YS	yolk sac
µm	micrometres
µM	micromolar

1 INTRODUCTION

Hypoxia is a commonly used term in medicine, especially in reproductive medicine and biology. But, what is hypoxia? Hypoxia can be defined as a pathological or abnormally low level of oxygen at a given site at a defined point in time. This leads logically to the term used to represent physiologically relevant oxygen levels - normoxia. A subtlety quite often overlooked is what is normoxia in the system of interest. In the field of cell biology, this important question has been seemingly ignored. Quite often *in vivo* oxygen levels in the tissue of interest vary markedly from oxygen levels encountered in the incubator. Ambient oxygen during normal tissue culture is convenient and, moreover, mimicking *in vivo* oxygen concentration during *in vitro* culture can be time-consuming, expensive and fraught with multiple problems.

The terms hyperoxia or superoxia are used to describe pathologically high oxygen levels. The mean partial pressure of oxygen (pO_2) in oxygen-rich arterial blood is equivalent to 95 mmHg, thus representing an oxygen concentration of approximately 12% with various local and regional variations (Balin et al., 1984). Healthy blood circulation delivers a mean pO_2 of 32 mmHg to tissues, representing an oxygen concentration of approximately 4%. Ambient atmospheres during traditional cell culture contain around 21% O_2 and, thus, represent clearly hyperoxic conditions. The detrimental effects of oxygen on tissues and cells have been extensively studied.

Physiological oxygen concentrations change throughout pregnancy. The human placenta, for example, encounters different oxygen tensions at specific periods of development. The placenta facilitates these changes and adapts accordingly in order to meet the needs of the developing fetus. At implantation, the fetal derived placenta becomes the primary organ responsible for the transfer of gases from maternal blood to the embryo. Obviously, oxygen levels must be tightly regulated to ensure normal placentogenesis and embryogenesis. Alterations in oxygen tensions during pregnancy have been linked to different pregnancy related disorders (Mayhew et al., 2004).

Development of the placenta is a requirement for development of a healthy, term baby. Cell differentiation, trophoblast invasion and vascular development are part of this tightly regulated process. Aberrant development of the placenta and associated vascular tree has been linked to early pregnancy losses and different pregnancy-related pathologies, such as preeclampsia and intrauterine growth retardation (IUGR). Epidemiological studies point out an increased risk for hypertension, diabetes and cardiovascular diseases in adulthood in association with IUGR (Barker et al., 1990). Oddly enough, the precise mechanisms of early placental vasculogenesis and angiogenesis remain unclear.

1.1 Implantation and the Blastocyst

Early embryological investigations have demonstrated that, after fertilisation, successive cell cleavages result in two-cell, four-cell and eight-cell embryos (Drews, U. Taschenatlas der Embryologie 1993). At the eight-cell stage, compaction of the embryo creates the morula. A blastocyst arises following further cell divisions. The blastocyst is a multicellular stage consisting of an inner cell mass (ICM) encased in a shell of specialised cells called trophectoderm. The ICM will eventually develop into an embryo, while trophectoderm develops into a placenta. In the oxygen-poor environment of the female genital tract, a preimplantation blastocyst spends its first week travelling from the site of conception to the maternal endometrium. The process of implantation commences six to seven days post conception (6-7 dpc).

Successful blastocyst implantation depends on synchronisation of a complex series of molecular and cellular events in the embryo and uterus. Apposition, the first stage of implantation, involves the initial, unstable adhesion of the blastocyst to the uterine wall. Stable adhesion, revealed by an increased contact between the uterine epithelium and trophectodermal layer of the blastocyst, represents the next step during implantation (Figure 1). The blastocyst then invades the uterine endometrium, creating a site where development can proceed. In preparation for implantation, the maternal endometrium undergoes morphological changes, known as decidualization.

Decidualization occurs when endometrial stromal cells proliferate and differentiate into a distinct cell type, called decidual cells. Maternal spiral arteries present in the decidua also prepare for the embryo's arrival. Within the uterine wall, ICM initiates a period of extensive growth and differentiation known as embryogenesis. Simultaneously, trophectoderm grows and differentiates, thereby forming the extra-embryonic organ known as placenta.

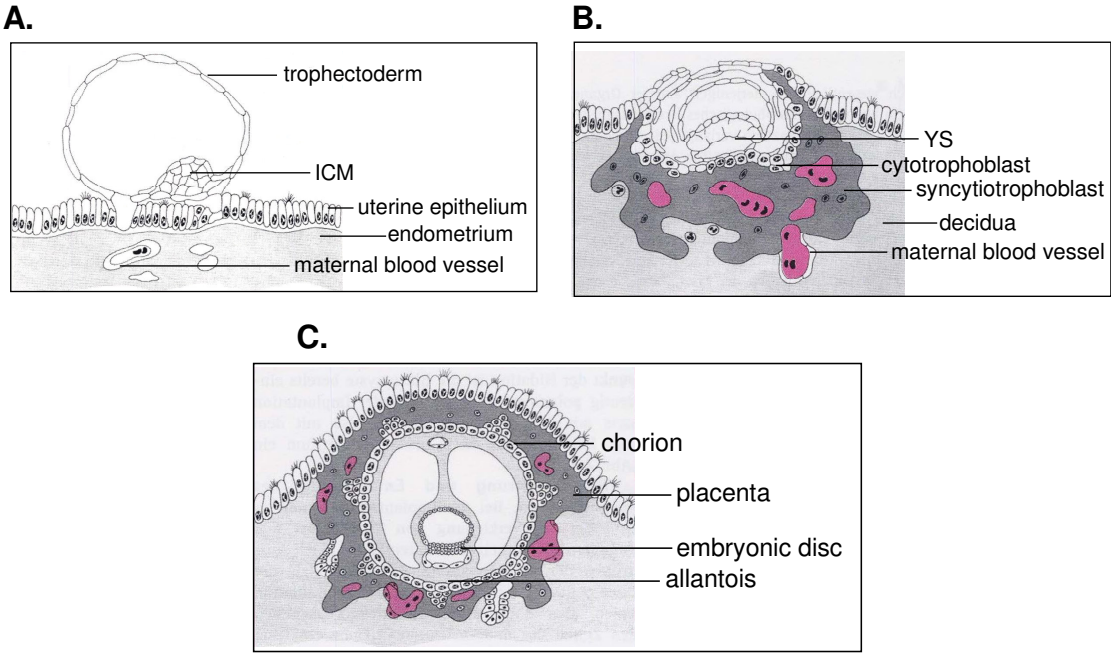


Figure 1. Human blastocyst implantation and early placentation. Implantation involves stable adhesion of blastocyst to uterine epithelium (**A**). As the blastocyst transgresses the epithelial layer, trophectodermal cells proliferate and invade the uterine decidua (**B**). The process of embryogenesis results in formation of the embryonic disc. Extra-embryonic yolk sac (YS) also forms during this time. Further invasion into decidua results in total blastocyst encapsulation (**C**). The chorion arises through differentiation of trophectodermal cells and will eventually consist of cytotrophoblast and syncytiotrophoblast. In humans, fusion of the chorion and allantois results in formation of a chorioallantoic placenta. Figure modified from Moore, 1988.

1.2 The Human Placenta

Placentogenesis initiates as the blastocyst makes contact with the epithelial lining of the uterus. It results from rapid proliferation and differentiation of trophectodermal and adjacent mesenchymal cells of extra-

embryonic origin. The human placenta functions during gestation as a semi-permeable barrier and is responsible for the exchange of gases, nutrients and wastes between the fetus and its mother.

1.2.1 *Placental development and structure*

The placenta is a large, multifunctional organ (Figure 2). As an endocrine organ, the placenta produces a multitude of hormones, growth factors and chemokines. These factors activate autocrine and/or paracrine pathways, thus regulating cellular functions at the feto-maternal interface (Lala and Hamilton, 1996). In addition to its endocrine role, the placenta carries out many of the fetal functions, including feeding, excretion and respiration (Zygmunt, M. in Klinische Pathophysiologe 2006). Furthermore, placenta acts as a protective barrier from potentially harmful substances found in the maternal microenvironment (Lala et al., 1983).

Placentas can be classified according to the extent of their invasiveness into three main types: noninvasive epitheliochorial, moderately invasive endotheliochorial, and highly invasive hemochorial (Benirschke and Kaufmann, 1990). Human placenta is hemochorial. This high degree of invasiveness is necessary for maintenance of the embryonic implantation site and subsequent penetration of maternal blood vessels. Concomitant with trophoblast invasion, placental cells are proliferating thereby forming a large cell population comprised of many cell types, including trophoblasts, fibroblasts, vascular endothelium and immune cells. At term (~40 weeks post conception (wpc)), placenta is the largest endocrine organ weighing approximately 600 grams (Boyd and Hamilton, 1970).

Trophoblast cells of the blastocyst proliferate rapidly throughout the first trimester. During the first week of implantation, the placenta consists of an inner layer of cytotrophoblast surrounded by a multinucleate syncytiotrophoblast layer, derived by fusion of mononucleate cytotrophoblasts (Figure 1). Together, they form a sponge-like network outlining spaces containing tissue fluid called lacunae. Soon, lacunae fill with maternal blood owing to the destruction of maternal capillaries and arterioles within the endometrium by invasive trophoblast cells. As these blood-filled sinusoids expand, maternal blood surrounds finger-like

projections comprised of cytotrophoblast cells and the multinucleate syncytiotrophoblast. These structures are termed chorionic villi and represent the basic structural unit of the placenta (Moore, 1988).

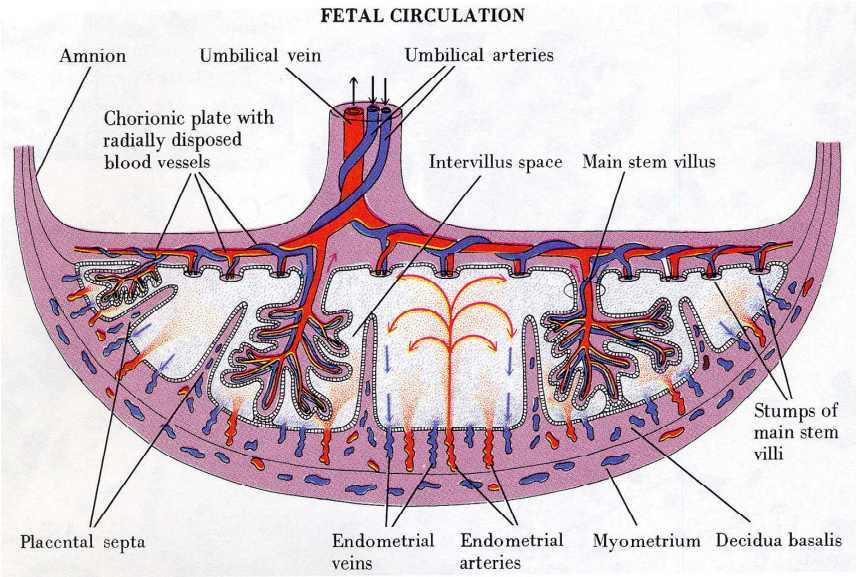


Figure 2. Schematic representation of human placenta at mid-gestation. Invasion of trophoblast cells opens up the endometrial arteries and the placenta becomes bathed in maternal blood. The placenta assumes a respiratory, absorptive and excretory function and formation of the placental vascular tree facilitates the transfer of nutrients, gases and wastes between the developing fetus and mother. Umbilical cord, containing the umbilical vein and umbilical arteries, connects placenta to the fetus. Figure reproduced Ross et al., 1995.

Chorionic villi can be categorised according to structure. Primary chorionic villi consist of two layers - a cytotrophoblast layer surrounded by syncytiotrophoblast. As development proceeds, secondary chorionic villi are formed when extra-embryonic mesoderm cells infiltrate the villous core. The lack of vascular structures within primary and secondary chorionic villi and absence of maternal blood flow suggests that early placentogenesis occurs in an oxygen-poor environment. As the villous core becomes vascularised, the resultant structures are called tertiary villi. Fully functional tertiary chorionic villi can assume two separate structures, each illustrating distinct differentiation pathways of the villous cytotrophoblast. Floating villi do not attach to decidua, but instead project directly into maternal sinusoids where

they are bathed in oxygen-rich maternal blood. Surrounded by a multinucleate syncytiotrophoblast layer, floating villi maintain a primarily absorptive and endocrine role. On the other hand, anchoring villi attach the placenta to the pregnant uterus. These chorionic villi contain highly proliferative villous cytotrophoblast cells at the base that break through the syncytium and form cell columns. A subset of cytotrophoblast cells from the anchoring villi, called extravillous trophoblast (EVT), migrate and invade the uterine decidua (Aplin, 1991; Graham and Lala, 1992). Some EVT form an organised cell layer called the cytotrophoblastic shell, while others remain dispersed in the decidua as interstitial trophoblasts. Yet another subset of EVT invades and modifies maternal spiral arteries. As EVT invade the walls of the uterine spiral arteries, the vessels are adapted into bore conduits capable of delivering an increased supply of oxygen-rich maternal blood to the placenta (Zhou et al., 1997).

1.2.2 *Oxygen levels in the developing placenta*

From implantation and throughout the first trimester, both embryo and placenta develop in an oxygen-poor environment. During the first ten to twelve weeks of gestation (10-12 wpc), the openings of uteroplacental arteries to the intervillous spaces contain plugs of trophoblast cells. After 10 wpc, trophoblastic plugs loosen and blood begins to seep into this space; after 12 wpc, oxygen-rich maternal blood bathes the placenta and oxygen levels begin to rise. This implies that the first trimester human placenta is not truly hemochorial - that is, chorionic cells do not contact maternal blood - until the end of the first trimester (Jauniaux et al., 2001).

Early studies attempted to measure oxygen levels in the feto-placental unit using a variety of techniques. Most techniques involved the use of *in vitro* gas measurements representing partial pressures of oxygen (pO_2). These measurements were often inconsistent. Laboratory gas measurements are fraught with a variety of pitfalls, with the fact that ambient oxygen contains approximately 21% O_2 topping the list. Other difficulties associated with laboratory measurements of *in vitro* pO_2 include: the presence of air bubbles in the sample, diffusion of gases through plastic and changes associated with measuring samples at room temperature. More

recently, Jauniaux and colleagues have used multiparametic sensor probes inserted under ultrasound guidance into the pregnant human uterus (Rodesch et al., 1992; Jauniaux et al., 1999; Jauniaux et al., 2001). This technique has led to more accurate measurements of oxygen concentrations and acid-base gradients present at the feto-maternal interface during the course of gestation. Polarographic probes have likewise been utilised to assess placental and endometrial pO_2 during the first trimester of pregnancy (Rodesch et al., 1992).

In vivo gas measurements have provided further evidence that early human placenta is not in direct contact with maternal blood. At 8-10 wpc, pO_2 in the feto-placental unit was measured at 17.9 ± 6.9 mmHg and a marked pO_2 gradient between the decidua and placental tissue was noted. In fact, the mean pO_2 of the placenta was approximately three times lower than that of the underlying decidua (Jauniaux et al., 2001). After 11 wpc, placental pO_2 increased more than two-fold. By the 12 wpc, placental pO_2 levels were similar to those found in the surrounding decidua, with a mean pO_2 equivalent to 56.2 ± 3.2 mmHg (Rodesch et al., 1992). This increase in oxygen levels corresponds to the establishment of continuous maternal blood flow to the placenta. Maximum oxygen tensions (65.0 ± 3.4 mmHg) were measured in human placentas approximately 16 wpc. After this maximum is reached, oxygen levels in the placenta decline until term, most likely representing the increased oxygen consumption of the feto-placental unit (Jauniaux et al., 1999).

1.2.3 *Placental vasculogenesis and angiogenesis*

The processes regulating vascular development occur, most often, in oxygen-low environments (Coultas et al., 2005). In fact, formation of a new blood vessel *de novo* or through the extension of pre-existing vessels has been shown to be tightly regulated by oxygen. The human placenta is no exception, where formation of the placental vascular tree occurs entirely in a low oxygen environment through the processes of vasculogenesis and angiogenesis.

Vasculogenesis involves *de novo* formation of blood vessels from precursor cells whilst angiogenesis involves creation of new vessels from

pre-existing vessels. Migration and differentiation of endothelial progenitor cells (EPC) to form interconnecting capillaries occurs during the process of vasculogenesis. Communication between endoderm and mesoderm appears to be important during vasculogenesis as well as during subsequent growth and stabilisation of blood vessels. Interestingly, the close spatial proximity of hematopoiesis and vascular development has led to the suggestion of a multipotent cell, the hemangioblast, with the inherent capacity for both vascular and hematopoietic developmental pathways (Park et al., 2005). At the second week of gestation (13-15 dpc), multipotent hemangioblasts emerge at extra-embryonic sites in blood islands of the developing yolk sac. Both primitive hematopoietic and endothelial cells are found in blood islands. Early hematopoietic and vascular cells are also found in temporal and spatial proximity during placental development. The earliest stages of placental vasculogenesis can be seen approximately ten days later (23-26 dpc). Angiogenesis occurs next during placental development as early blood vessels expand and adapt to meet the needs of the embryo.

Improper development of the placental vascular tree has been implicated in a number of devastating pregnancy-related complications. For example, preeclampsia is a potentially lethal complication of the second half of pregnancy, labour or early period after delivery. It affects approximately 3% of women and is characterised by hypertension, abnormal levels of protein in urine and other systematic disturbances (Redman and Sargent, 2005). Oxygen levels in the feto-maternal unit play a key role (Kuenzel and Kirschbaum, 1992). Preeclampsia frequently results from the presence of a hypoxic placenta (Burton and Jauniaux, 2004). Early on in pregnancy, poor placental development leads to the appearance of maternal symptoms such as hypertension and proteinuria. The cellular and molecular mechanisms regulating early placental development remain largely unclear.

1.3 Hematopoietic Stem Cells (HSC)

At the earliest stages of development, the preimplantation blastocyst has absolutely no access to the oxygenated maternal blood supply. In the

multicellular blastocyst, the oxygen concentration deep within the ICM is accordingly low. This environment is home to multipotent cells capable of creating an entire organism and blastocysts have been used in the derivation of a number of human stem cells, including embryonic stem cells (ESC) and trophoblast stem cells (TSC).

Stem cells are defined as cells capable of unlimited self-renewal and the ability to give rise to multiple cell types. These parameters are subject to wide interpretation and depend to some degree on whether the cells are present in their normal environment, residing in the so-called stem cell niche, or in an experimental setting. Pluripotent ESC, derived from the ICM, have unlimited self-renewal properties and give rise to all embryonic tissue types *in vitro* (Thomson et al., 1998; Reubinoff et al., 2000). However, unlimited self-renewal is not a property of cells of the ICM *in situ*, where they differentiate into various tissues thereby losing the pluripotent ESC phenotype. Multipotent adult stem cells, on the other hand, give rise to a wide range of progenitor and mature cells. Many multipotent adult stem cells have unlimited self-renewal - their differentiated progeny have limited self-renewal. Hematopoietic stem cells (HSC) are prototypical multipotent adult stem cells.

Adult stem cells, including HSC, by definition must have both a tremendous capacity for self-renewal and a large differentiation range. In the absence of overt injury, bone marrow-derived HSC maintain the total pool of HSC at a roughly constant level through asynchronous divisions (Cheshier et al., 1999). HSC also give rise to progenitor cells, in this report referred to as hematopoietic stem / progenitor cells (HSPC). Differentiated progeny of HSPC include lineage-restricted progenitor cells. Further differentiation produces all lineages of the hematopoietic system, including monocytes, granulocytes, megakaryocytes and erythrocytes. The cellular signals that influence the choice between self-renewal and differentiation are incompletely defined. Therefore, a long-term goal is precise knowledge about the molecular mechanisms associated with HSC development and lineage decisions. Complicating the matter is that *in vitro* proliferation of HSC inevitably leads to hematopoietic differentiation or death with a subsequent loss of multipotent, self-renewing stem cells (Kondo et al., 2003).

1.3.1 *Hematopoiesis and lineage commitment*

The highly orchestrated process of blood cell production is termed hematopoiesis. During the life of an organism, HSC generate multiple blood cell lineages through a successive series of intermediate progenitors, which involves the progressive loss of developmental potential to other lineages.

Definitive HSC are functionally defined by their capacity for extensive self-renewal. Also called long-term (LT)-HSC, a single cell can give rise to multilineage reconstitution in irradiated patients (Smith et al., 1991). Maturation of LT-HSC leads to a multipotent stem / progenitor cell population (i.e. HSPC) with a more limited self-renewal capacity. The earliest lineage-potential decision that HSPC must make is whether to become a lymphoid or myeloid cell. This first differentiation decision produces lineage restricted progenitor cells called common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). The differentiated progeny downstream of CMP and CLP are even more mature progenitors further restricted in the number and type of lineages they can generate. CMP give rise to myeloid cell lineages, including monocytes, granulocytes, megakaryocytes and erythrocytes, while CLP produce cells of lymphoid lineages, such as B cells, T cells and natural killer (NK) cells. Terminally differentiated cells, such as erythrocytes, are ultimately produced. They have lost all self-renewal capacity and eventually undergo apoptosis. Traditional knowledge has suggested that this step-wise developmental process is linear in the sense that once a cell has made a developmental choice it is permanent (Figure 3). Recent interest in stem cell research has reopened the debate as to whether the process of hematopoiesis is more plastic than traditionally considered.

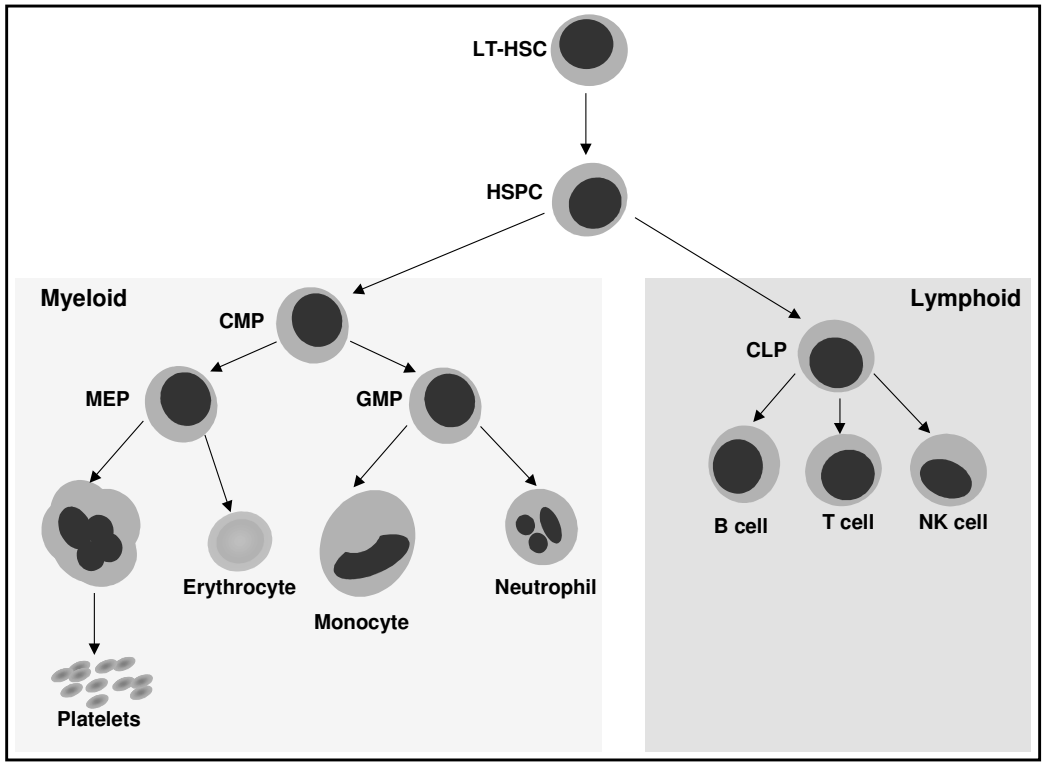


Figure 3. Linear representation of hematopoiesis. Long-term hematopoietic stem cells (LT-HSC) are the most primitive HSC. Proliferation and differentiation produces a population of hematopoietic stem / progenitor cells (HSPC). The first lineage decisions produces common myeloid progenitors (CMP) and common lymphoid progenitors (CLP); subsequent differentiation of HSPC results in more restricted progenitors (MEP, GMP). Further differentiation produces the entire repertoire of hematopoietic cells required during life.

1.3.2 *HSC development*

The primary sites of hematopoiesis change in a temporally and spatially co-ordinated fashion during human development (Figure 4). A close association of endoderm with mesoderm appears to be a typical tissue make-up at sites of hematopoiesis. Until establishment of definitive hematopoiesis in the bone marrow, microenvironmental cues regulate HSC self-renewal and differentiation. In stem cell biology, the local microenvironment is also known as the niche. Locally derived signals are involved in the formation and maintenance of various HSC niches.

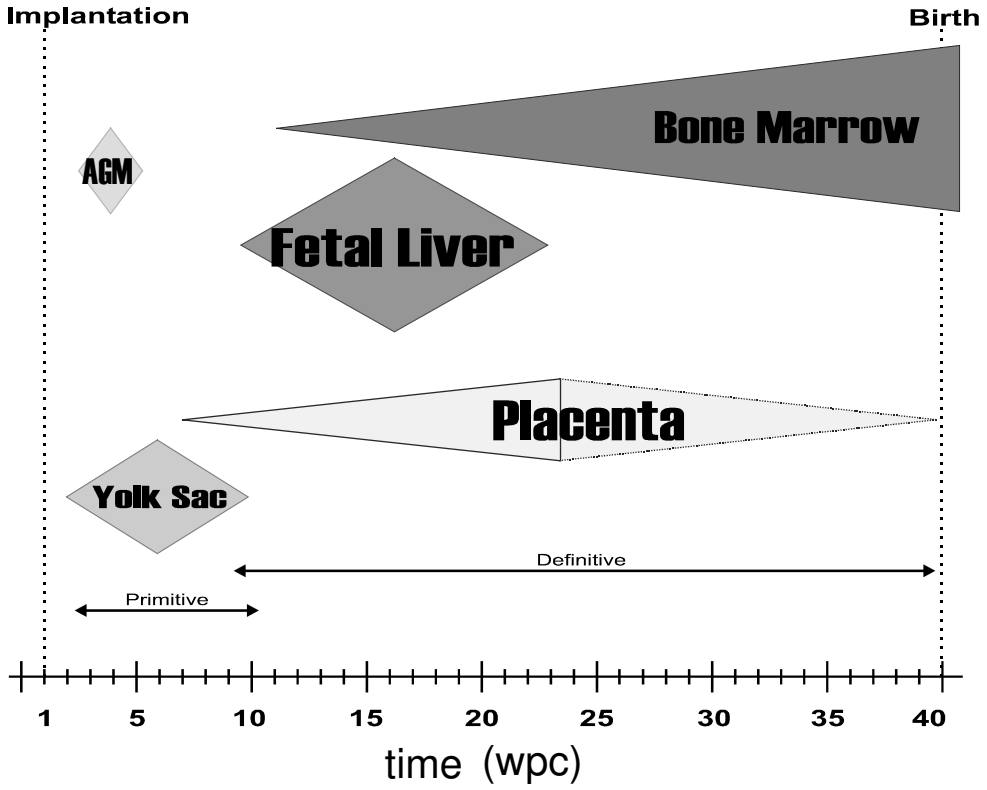


Figure 4. Developmental sites of human hematopoiesis. At implantation, primitive hematopoiesis begins in the yolk sac. Primitive HSC can later be found in the aorta-gonads-mesonephros (AGM) region. Definitive hematopoiesis sites are transient during development. Traditional knowledge states that the fetal liver is responsible for seeding the final HSC niche – bone marrow. It remains unclear whether the placental HSPC niche remains throughout gestation. Figure adapted from Mikkola et al., 2005.

Primitive hematopoiesis in the blood islands of the extra-embryonic yolk sac creates the earliest hematopoietic cells of the developing mammalian embryo (Figure 5). Yolk sac hematopoiesis is biased towards erythropoiesis with primitive erythrocytes indispensable to oxygenation of the developing embryo. Development of a functional yolk sac occurs during implantation of the human blastocyst at approximately 13-15 dpc (Palis and Yoder, 2001). The cellular components of the blood island niche consist originally of undifferentiated mesodermal progenitor cells (i.e. hemangioblasts) and stromal cells apposed to visceral endoderm (Tavian and Peault, 2005). By the time that the secondary yolk sac is formed, mesodermal cells come in contact with cells of the chorion. The first appearance of blood islands is illustrated by hematopoietic and endothelial cell aggregates in the yolk sac stroma. Yolk sac-derived progenitors

contribute to a transient wave of hematopoiesis, while other sites contribute to the hematopoietic needs of the growing embryo.

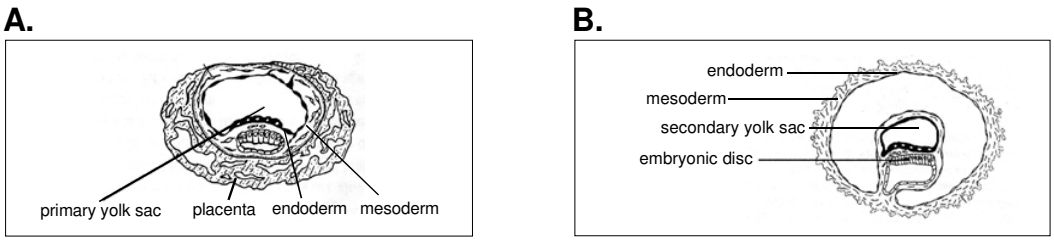


Figure 5. Human yolk sac development. Formation of primary yolk sac occurs during implantation when extra-embryonic mesodermal cells come in direct contact with placental cells (**A**). Extra-embryonic endoderm is directly apposed to mesoderm. Secondary yolk sac appears during embryonic disc formation (**B**). A layer of extra-embryonic mesoderm surrounds the chorion while extra-embryonic endoderm and mesoderm remain in contact. Figure reproduced from Palis and Yoder, 2005.

The next wave of hematopoiesis can be seen during the second month of gestation when clusters of round cells expressing several markers of HSC are observed within major intra-embryonic and extra-embryonic arteries (Labastie et al., 1998). At 27 dpc, cells within the aorta-gonads-mesonephros (AGM) region adhere firmly to the ventral endothelium of the embryonic aorta; these HSC proliferate rapidly and totally disappear by 40 dpc (Tavian et al., 1999). It was observed that expression of the extracellular protein CD34 accompanies early ontogeny of both the hematopoietic and vascular systems. AGM is a unique example of a localised territory where multipotent HSC transiently emerge and proliferate. CD34-positive ($CD34^+$) cells isolated from this region generated both lymphoid and myeloid cells, demonstrating the presence of progenitor (e.g. HSPC, CMP, CLP) cells (Tavian et al., 2001). As HSC in the AGM region are only found during a short developmental window and their number is low at any given time, questions have been raised whether the AGM region alone can supply all HSC in the expanding pool. In humans, a detailed analysis of this region has been hampered with the obvious scarcity of healthy tissue. Nonetheless, Tavian and colleagues (2001) have stated that ventral periaortic mesenchymal cells produce the chemoattractant, stromal-

derived factor (SDF)-1α, although original results remain until now unpublished. The early involvement of cytokines in creation of the AGM niche remains an interesting field of investigation.

Following primitive hematopoiesis in the yolk sac and a transient stage in the AGM, definitive hematopoiesis commences in the liver. Definitive hematopoiesis results in production of all hematopoietic lineages. At approximately 22 dpc, the fetal liver can be identified in the developing human embryo. HSC can be found in the developing liver around 30 dpc. Fetal liver cannot generate its own hematopoietic cells but instead needs to be seeded from another HSC source. Seeding of liver by HSC appears to involve the early fetal circulation. The first vascular connection to fetal liver comes from the yolk sac through the vitelline veins. The second vascular connection is the umbilical vein from the developing placenta. The appearance of progenitor cells (HSPC), as identified by expression of CD34, in embryonic liver and long-term *in vitro* culture of liver cells suggests the multipotentiality of liver hematopoiesis (Labastie et al., 1998). The hematopoietic profile of fetal liver changes during gestation with its repertoire increasing from erythroid differentiation to include megakaryocytic, myeloid, and B lymphoid development. Importantly, fetal liver provides a supportive niche for the growing pool of HSC before they move to their adult home.

Bone marrow is the last blood-forming tissue that develops in ontogeny, when hematopoiesis is extinct in the yolk sac and transiently active in the liver. Bone marrow hematopoiesis begins around 11 wpc after HSC originating in the fetal liver begin to seed developing long bones. Within specialised niches, bone marrow supports self-renewal of the HSC pool and, at the same time, differentiation of progenitor cells to the various hematopoietic lineages. Formation of the bone marrow niche involves the precise interplay between microenvironmental cues, such as oxygen level, cytokine production, and heterotypic cell-cell adhesions. The chemokine SDF-1α seems to play a key role in HSC homing to their bone marrow niche. Using transgenic technology, Nagasawa and colleagues (1996) have demonstrated that mice lacking both copies of the SDF-1α gene had

defective bone marrow hematopoiesis and died perinatally. HSPC in mutant embryos were reduced in bone marrow, demonstrating the importance of chemokines in creating a supportive HSPC niche.

1.3.3 *Hematopoiesis in the placenta*

Recent investigations have demonstrated the presence of a third pre-hepatic HSC niche. One common characteristic of sites of hematopoiesis is that the generation of hematopoietic cells coincides with that of endothelial cells. As previously mentioned, the human placenta is one of the earliest vascularised organs during development and hematopoietic cells have been found within early chorionic villi. In fact, the presence of cells appearing microscopically to be hematopoietic in origin, have been described for quite some time. Transmission electron microscopy demonstrated the presence of hematopoietic cells in first trimester placental tissue (Demir et al., 1989). Furthermore, the name hemangioblastic cord, which has been used to describe the early site of placental vasculogenesis, suggests the multipotentiality of stem cells found in placenta.

Placenta may serve as another supportive niche that facilitates maturation and expansion of HSC. *In vivo* studies in mice have shown that mid-gestation placenta harbours a large pool of multipotent HSC. Placental HSC have the capacity to self-renew and repopulate the entire hematopoietic system (Ottersbach and Dzierzak, 2005; Gekas et al., 2005). Interestingly, the placental HSC pool is transient and diminishes while the fetal liver HSC reservoir expands. As the placenta is located directly upstream of liver in fetal circulation, it is possible that placental cells are an important source of HSC that eventually colonise the fetal liver.

Hemangioblastic cords appear to develop from mesodermal cells in the chorionic villi. However, it is possible that the growing placental HSC pool is supplemented from sites associated with the developing vascular system (e.g. AGM). Both AGM and umbilical artery are immediately upstream of the placenta in fetal circulation. During the third trimester of pregnancy placenta appears to lose its hematopoietic function while bone marrow develops into the main site of hematopoiesis. Notwithstanding, the constantly changing

placental vasculature most likely requires a constant supply of progenitor cells.

1.3.4 *Umbilical cord blood (UCB)*

The umbilical cord contains blood vessels that carry oxygen and nutrients from placenta to the embryo and wastes from the embryo to placenta and the maternal circulation (Figure 2). As umbilical cord is a conduit linking the stem cell-rich placenta and the developing embryo, it contains a large amount of HSPC throughout development. For this reason, stem cells isolated from UCB have been utilised successfully during transplantation for almost twenty years (Kurtzberg et al., 2005).

UCB is typically collected *ex utero* following birth of the baby and expulsion of the placenta. This means that UCB can be considered an ethical source of early stem cells as it can be collected without physical risk to the mother or the baby donor. UCB is an excellent source of HSPC and the ethical collection of UCB has extended the availability of allogenic HSC transplantation. So far, HSPC isolated from UCB has been successfully used in the treatment of patients with leukaemia, lymphoma, hemoglobinopathies, bone marrow failure syndromes, congenital immunodeficiency syndromes and inborn errors of metabolism (Kurtzberg et al., 2005). However, the use of UCB as a source of HSPC is not yet fully optimised and many difficulties are associated with stem cells isolated from UCB.

The use of UCB for transplantation in adults is limited by the small number of HSPC in each graft, resulting in delayed engraftment post transplant (Hofmeister et al., 2007). Initial efforts to expand UCB progenitors *ex vivo* have resulted in expansion of mature HSPC rather than immature stem cells. This is confounded by the inability to measure long-term reconstituting cells aSccurately and reliably. The future of *ex vivo* expansion should include isolation of immature hematopoietic progenitors on the basis of function rather than surface phenotype and will employ physical parameters, secreted factors and stroma to maintain and expand the stem cell populations. Improved understanding of HSPC developmental niches

could promote HSPC *ex vivo* expansion with reduced differentiation and make UCB transplantation available to more patients.

2 OBJECTIVES

The objectives of this study, which examined the impact of oxygen on HSPC development and formation of a placental stem cell niche, included:

I. to establish an *in vitro* model of placental development and vasculogenesis in physiological oxygen environments

II. to examine the influence of different physiological and non-physiological oxygen concentrations on HSPC:
    a) proliferation
    b) cell cycle status
    c) apoptosis
    d) differentiation

III. to characterise the SDF-1α - CXCR4 system during formation of stem / progenitor cell niche in the placenta both *in vivo* and *in vitro*.

3 METHODS AND MATERIALS

3.1 Isolation of Primary Cells from Human Placentas

3.1.1 *HSPC isolation*

Human umbilical cord blood (UCB) was collected from term pregnancies following ceasarian section as approved by the Ethics Committee of the School of Medicine at Justus Liebig University, Giessen, Germany. Immediately following delivery, fetal blood was aspirated from placental blood vessels into syringes containing heparin. This method resulted in an average of 53.2 ml of UCB (range 40-160 ml). Following UCB collection all steps were performed under sterile conditions in a laminar flow hood. UCB was diluted (1:4.5) in 2 µM ethylenediamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS) and 35 ml gently layered on 15 ml of 1.077 g/ml Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). UCB was then subjected to density gradient centrifugation (400 x g, 30 min, 20°C). The mononuclear cell (MNC) fraction was removed and pelleted by centrifugation (400 x g, 10 min, 20°C). The resultant MNC pellet was washed with 50 ml ice-cold MACS Buffer (1X PBS, 5% Bovine Serum Albumin (BSA), 2 µM EDTA), centrifuged (400 x g, 10 min, 4°C) and, depending on pellet size, resuspended in 100-600 µl MACS Buffer.

To obtain pure populations of CD133 expressing MNC, Magnetic Activated Cell Sorting (MACS) with the CD133 Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) was employed. Briefly, following resuspension of the MNC pellet in ice cold MACS Buffer, cells were blocked with 10 µl FcR Blocking Reagent/10^8 MNC (15 min, 4°C). Cells were subsequently labelled with 10 µl CD133 Microbeads/10^8 MNC and incubated 45 min on ice. The cell suspension was then washed with 50 ml cold MACS buffer and centrifuged (400 x g, 10 min, 4°C). The supernatant was removed, cells resuspended in 1 ml MACS Buffer (4°C) and the cell suspension added to pre-rinsed MACS column placed in the magnetic field of a VarioMACS Separator (Miltenyi). Once the cell suspension was allowed to pass through the MACS column, the column was washed 5 times with 4 ml ice cold MACS buffer and the flow-through collected in sterile 50 ml

Falcon tube. The column was removed from the magnetic field and bound cells were eluted and flushed from the column with the supplied plunger into a sterile 5 ml Falcon tube with 5 ml MACS Buffer (4°C). Both collected cell suspensions (i.e. $CD133^-$ and $CD133^+$) were subsequently centrifuged (400 x g, 10 min, 4°C). An aliquot of the cell suspension was removed, dead cells were excluded using trypan blue staining and living cells were enumerated with a hemocytometer. This method resulted in an average retrieval of $1.0x10^6$ $CD133^+$ cells, for future reference labelled hematopoeitic stem / progenitor cells (HSPC).

3.1.2 *Trophoblast- and stroma-enriched cell fractions isolation*

MACS was similarly used to isolate cells from early human placental tissue collected from elective abortions with written consent as approved by the Ethics Committee of the Justus Liebig University School of Medicine. The method used followed previously described protocols (Kliman et al., 1986) with some modifications. Tissue was thoroughly washed in Hank's Balanced Salt Solution (HBSS) to remove blood and chorionic villi were removed from placental tissue and minced with a scalpel. Enzymatic digestion of minced chorionic villi fragments involved 3 times 20 min incubations with trypsin-EDTA. Following enzymatic digestion, collected cells were subjected to magnetic cell sorting using CD326 (epithelial cell adhesion molecule, EpCAM)-coated magnetic beads (Miltenyi Biotech). $CD326^+$ cells were subsequently labelled trophoblast-enriched cell fractions (HTR). Depleted $CD326^-$ cells resulted in a trophoblast-depleted or stroma-enriched fraction. Negatively selected cells are here called human placental fibroblasts (HPF).

3.2 Cell Culture

3.2.1 *HSPC expansion*

Directly following isolation, HSPC were transferred to 15 ml tissue culture flasks (TRP, Biochrom AG, Berlin, Germany) containing HSPC Growth Media (HSPC-GM). HSPC-GM consisted of Iscove's Minimum Defined Media

(IMDM) supplemented with 10% fetal calf serum (FCS), 10 ng/ml stem cell factor (SCF), 20 ng/ml thrombopoietin (TPO) and 50 ng/ml Flt3-Ligand (FLT3L) (all growth factors from PromoCell, Heidelberg, Germany). Culture of hematopoietic stem cells in this media has been shown to result in stem cell proliferation and to maintain cells for extended culture periods (McGuckin et al., 2003; McGuckin et al., 2004; McGuckin et al., 2005). HSPC cell cultures were expanded for 7 days in humidified incubators (37°C) in atmospheres maintained with 5% CO_2. HSPC culture media was replaced every 3-4 days.

3.2.2 *Adherent cell culture*

Trophoblast-enriched (HTR) and placental stroma-enriched (HPF) cell fractions were maintained in culture and used in a variety of experiments. Directly following isolation, HTR in AmnioMax Serum-Free Media (including penicillin/streptomycin) were allowed to attach to 15 mm^2 plastic tissue culture flasks for approximately 1 week in a humidified incubator (37°C, 5% CO_2). On the other hand, HPF were cultured in Dulbecco's Minimum Essential Media (DMEM) containing 10% FCS. HPF cultures were similarly allowed to attach in 75 mm^2 plastic tissue culture flasks for approximately 1 week. Cell cultures were washed with warm media and unattached cells were discarded. HTR and HPF were first passaged when cell cultures reached approximately 80% confluency. Passaging was accomplished using 5 min trypsinisation (Trypsin/EDTA, Gibco) followed by inactivation of the enzyme with DMEM (10% FCS) and centrifugation (400 x g, 5 min). Cell pellets were resuspended in the appropriate media and further incubated. This was considered passage 1 (P1). Both HTR and HPF could be cultured for a maximum of 6-7 passages (P6-7) before senescence. P2-5 cells were used during experimentation.

Conditioned media from HTR and HPF cultures was used in a variety of experiments to test the effects of secreted factors on HSPC. HTR or HPF cells were expanded into 100 mm^2 tissue culture flasks containing the appropriate media (HTR: AmnioMax; HPF: DMEM (10% FCS)) and allowed to reach ~80 confluency. The flasks were washed with 10 ml pre-warmed PBS (3X) and media replaced with 12 ml serum-free IMDM (SFM). Conditioned media was derived during 24 h cell culture. Following cell

culture, media was removed and subjected to high-speed centrifugation (1000 x g, 30 min, 4°C) to pellet cells and cell debris. Media was aspirated, aliquoted and stored -80°C until needed.

3.2.3 *Cell culture in low oxygen environments*

Cell culture in environments containing low oxygen was used to mimic physiologically relevant oxygen concentrations. This was accomplished by transferring cell cultures to a hermetically sealed, humidified Modular Incubator Chamber (Billups-Rothenburg, Del Mar, California USA) (Figure 6A). Modular Incubator Chambers have previously been used in multiple investigations in order to cultivate cells in low oxygen as the air-tight seal and fast gas exchange allows for the rapid creation of a low pO_2 environment (Graven et al., 1994; Lee et al., 1997).

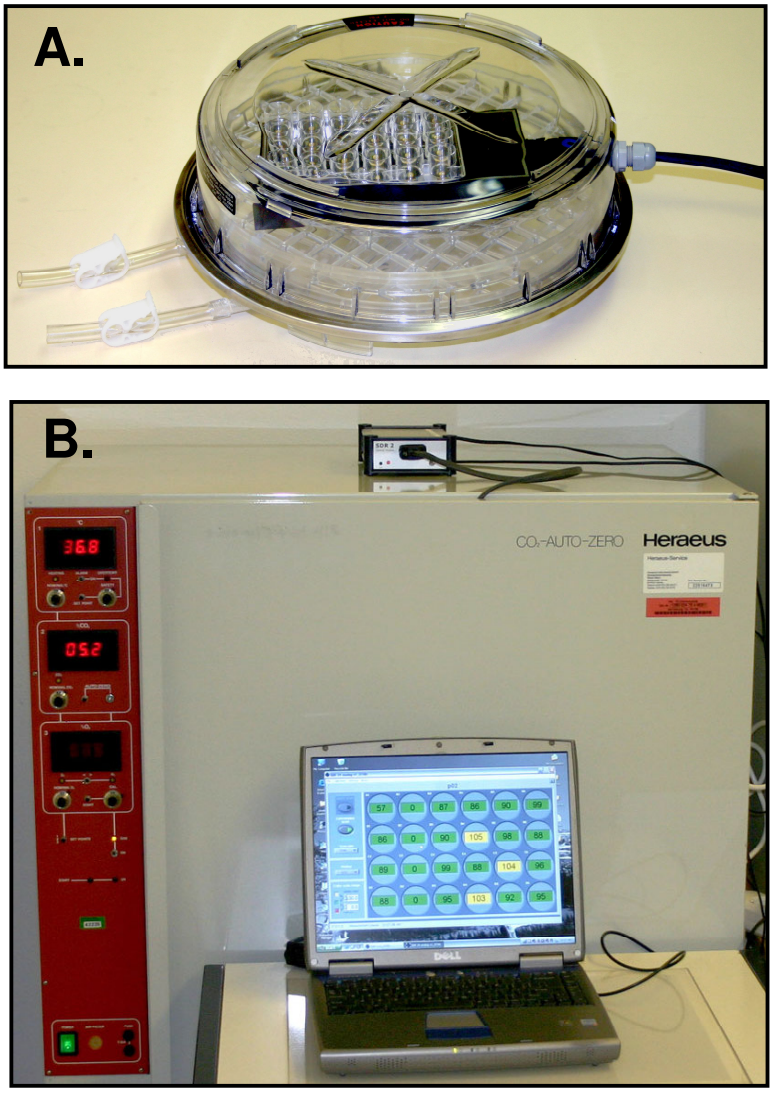


Figure 6. Apparatus for cell culture in physiologically relevant oxygen concentrations with concurrent real-time oxygen monitoring. Cells were cultured in Oxyplate® 24 well plates in a hermetically sealed, humidified cell culture chamber (**A**). Plates were placed on the Sensor Dish Reader (SDR) and the chambers incubated at 37°C. Connection of SDR to computer (**B**) allowed for real-time measurements of oxygen levels present in the cell culture media during experimentation.

In this investigation, Modular Incubator Chambers were used to simulate physiological oxygen tensions during cell culture. Prior to the experiments, all cell culture materials, including plastic cell culture plates and dishes, media and oxygen monitoring equipment were preincubated for 2 h in appropriate oxygen concentrations. The chamber was humidified during the course of the experiment by placing a lidless, plastic petri dish containing 20 ml sterile water on the bottom of the chamber. Following

preincubation, cell cultures were transferred to the Modular Incubator Chamber and the chamber sealed. A Single Flow Meter (Billups-Rothenburg) was attached to inflow tube, connected to the gas flask and was used to control the flow of the gas into the chamber. The outflow tube was opened and the chamber flushed with appropriate gas mixtures for 4 min at a rate of 20-25 l/min. The gas mixtures used in this reports consisted of either 1% or 8% O_2 (5% CO_2 and rest N_2). Inflow and outflow tubes were sealed following gassing, creating a hermetically sealed chamber. The sealed Modular Incubator Chamber was then placed in a 37°C incubator and the atmosphere was replaced with the appropriate gas mixture 3 times daily for the duration of the experiments. As a control, HSPC were cultivated in normal cell culture conditions by incubating cells in atmospheric oxygen (21% O_2, 5% CO_2, rest N_2) in a humidified cell culture incubator (37°C).

3.2.4 *Concurrent real-time oxygen measurements*

During experimentation a prototype oxygen monitoring system was employed in order to concurrently monitor real-time oxygen tension of HSPC culture media. The 24-channel Sensor Dish Reader 2 (SDR2; PreSens, Regensburg, Germany) has been developed to control important parameters, such as pH and oxygen tension, during cell culture. This was accomplished by the integration of opto-chemical sensors in each well of a sterile 24-well multidish (OxyPlates® monitor oxygen; HydroPlates® monitor pH). Sensors consisted of fluorescent dyes embedded in a cell culture compatible polymer placed at the bottom of each well. Cells in cell culture media were then seeded into the wells and the plates placed on the SDR plate reading device. The SDR system was connected to a PC allowing system control and data collection (Figure 6B). Optical signals were collected by the SDR at specific time points (ranging from every 10 sec to every 30 min) and the data transmitted to PC.

The prototype SDR2 system was tested in this investigation and used to measure oxygen tensions in our HSPC cultures. To allow for measurements in low oxygen environments the Modular Incubator Chamber was modified to allow insertion of the SDR2 plate reader into the hermetically sealed chamber. Selection of appropriate gas mixtures allowed

in vivo oxygen tensions to be effectively mimicked during *in vitro* HSPC culture. Similarly, this novel system allowed oxygen tensions in HSPC culture media to be monitored during experimentation.

3.3 HSPC Proliferation Assay

Cell number was determined with a CASY® Cell Counter (Shaerfe Systems, Reutlingen, Germany). The CASY® Cell Counter is an established method for determining cell number utilising the Resistance Measurement Principle. Briefly, 50 µl of cell suspension was removed from each of the groups and mixed with 10 ml CASY®ton electrolyte solution (Schaerfe Systems) and placed in the CASY® Cell Counter. Measurement was performed when the cell suspension was aspirated with a 150 µm capillary and single cells passed through a pore of defined geometry at a constant flow speed. A pulsed low voltage electrical current was applied over the electrolyte-filled measuring pore, representing a defined electrical resistance. As cells passed through the measurement pore a quantity of the electrolyte was displaced. Living intact cells acted as insulators with the resistance representing a dimension for cell volume. Dead cells without an intact cell membrane could no longer act as an electrical barrier and were therefore recorded by the size of the cell nucleus. Thus, living HSPC could be discriminated from dead HSPC and debris based on relative size.

HSPC cultures were expanded for 7 days in HSPC-GM as described above. Attached cells were scraped with a plastic cell scraper (Cellstar, Greiner Bio-One, Frickenhausen, Germany) and both the previously attached and suspended cells were collected. The cell suspension was subsequently centrifuged (400 x g, 10 min), the supernatant removed and the cell pellet resuspended. Initial cell number was determined with trypan blue exclusion. $2x10^5$ HSPC were added to each well of a 24-well plate and the plates incubated in the specified conditions (HSPC-GM, HTR-CM, HPF-CM) in various oxygen concentrations (1%, 8% and 21% O_2). Media was replaced and cell number was determined with a CASY® Cell Counter on days 8, 10, 12 and 14.

3.4 Flow Cytometry

In this investigation flow cytometry was employed to quantify cellular DNA content, and apoptosis, as well as for immunophenotyping.

3.4.1 *Flow cytometric cell cycle analysis*

HSPC cell cycle status and DNA content were quantified using propidium iodide (PI) staining of fixed cells followed by flow cytometric analysis. This method was based on stochiometric intercalation of PI between the bases of double-stranded nucleic acids. Individual cell ploidy could be assessed with a flow cytometer. Resting cells in G0 and cells preparing for DNA synthesis in G1 have a 2n ploidy; cells preparing for cell division in G2 and cells undergoing mitosis (M) have 4n. As PI binding to DNA was stochiometric, the measured fluorescence intensities of PI treated cells was related to the number of DNA molecules. Thus, G0/G1 cells have fluorescence intensities representing one-half of G2/M cells. Cells found between these two peaks represent cells synthesising DNA (S phase) with ploidy between 2n and 4n. The protocol utilised in this investigation was as follows.

HSPC cultures were expanded for 7 days as previously described. HSPC were plated $1.5x10^5$ cells/ml on 12-well plates (Cellstar, Greiner Bio-One, Frickenhausen, Germany) and incubated in environments containing 1%, 8% or 21% O_2. On Days 7, 8, 10 and 14, $1x10^5$ HSPC were removed, centrifuged (400 x g, 10 min, 4°C) and washed two times in ice-cold PBS. HSPC membranes were permeabilised by overnight fixation in 70% ethanol (-20°C). Fixed cells were centrifuged (400 x g, 10 min), the ethanol removed and the cell pellet resuspended in 100 µl 1% BSA in PBS. To avoid propidium iodide (PI) incorporation into double-stranded ribonucleic acid (RNA), cellular RNA was digested with 100 µg/ml DNase-free RNase (Roche Applied Sciences, Mannheim, Germany) for 20 min at 37°C. Cells were subsequently incubated with 10 µg/ml PI (Becton Dickinson (BD), Heidelberg, Germany) for 1 h in the dark. In preparation for flow cytometry, total volume was adjusted to 500 µl and suspensions vortexed to mix the cells.

PI incorporation into HSPC DNA was measured with the FACSCalibur (BD) flow cytometer with CellQuest Pro hardware (BD). Whole cells were first gated based on their Forward Scatter (FSC) and Sideward Scatter (SSC) properties, with particles exhibiting both low FSC and SSC excluded. PI fluorescence intensity was demonstrated by plotting FL2-Area versus FL2-Width. Data was subsequently analysed with ModFit Analysis Software (BD).

3.4.2 *Flow cytometric apoptosis assay*

The level of HSPC apoptosis following various treatments was measured using the Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson). This particular assay utilises Annexin V as a sensitive probe for identifying apoptotic cells. During early apoptosis, loss of the plasma membrane is one of the earliest features. In early apoptotic cells the membrane phospholipid phosphotidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the cellular environment. Annexin V is a phospholipid-binding protein with high affinity for PS. Annexin V conjugation to a fluorochrome such as FITC, therefore, creates a sensitive probe for flow cytometric analysis of cells undergoing apoptosis (Vermes et al., 1995). In the Annexin V-FITC Apoptosis Detection Kit I, PI was utilised as a marker for cells that have lost plasma membrane integrity, such as late apoptotic or dead cells, which further strengthened the assay. A requirement for PI intercalation into cellular nucleic acids is a permeabilised cell membrane that allows passage of PI into the cell. Therefore, PI staining could only be seen in cells that were losing (i.e. late apoptotic cells) or had already lost (i.e. dead cells) cell membrane integrity. Following flow cytometric analysis of the cultures, cells could be categorised. Viable cells were negative for both Annexin V and PI while cells undergoing early apoptosis were positive for Annexin V and negative for PI. Finally, cells that were in late apoptosis or have died as a result of the necrotic pathway were positive for both Annexin V and PI (Figure 7).

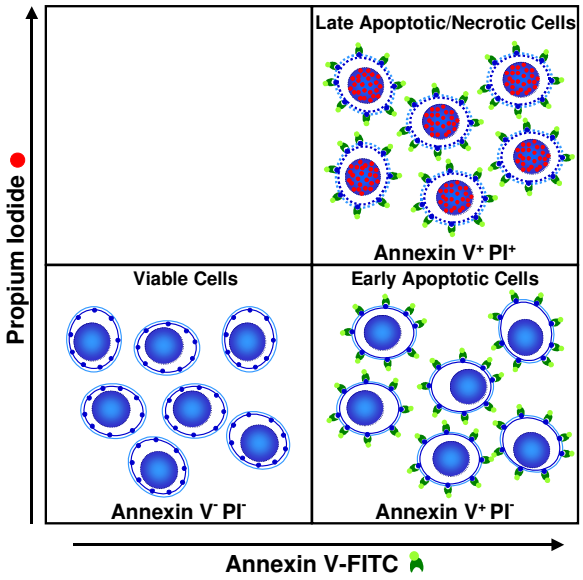


Figure 7. Explanation of data obtained from HSPC apoptosis assay. Results from flow cytometric analysis are displayed as a dot plot diagram. The plot is divided into four quadrants. The bottom left quadrant represents live cells with an intact cell membrane. The bottom left quadrant represents early apoptotic cells where Annexin V-FITC (seen in green) binds to phosphatidylserine in the outer cell membrane. The upper right quadrant represents late apoptotic or dead cells where Annexin V-FITC binds the outer membrane and PI can pass freely into the nucleus.

In this investigation, HSPC were subjected to the Annexin V-FITC Apoptosis Detection Kit (BD). Apoptosis levels were then tracked over a further incubation period in different oxygen concentrations. The supplied protocol was used. Following expansion of HSPC cultures, cell number was quantified with trypan blue exclusion. $4.5x10^5$ HSPC/treatment were removed, divided into three groups ($1.5x10^5$ HSPC/group) and plated in one well of a 12-well cell culture plate (Cellstar, Grener Bio-One, Frickenhausen, Germany). The three groups represented: i. unstained controls for setting the flow cytometer parameters, ii. stained HSPC for the apoptosis assay and iii. staurosporine treated HSPC (Alexis Biochemicals, Gruenberg, Germany). In the third group, HSPC were pre-treated with the apoptosis-inducing antiobiotic (Bertrand et al., 1994) staurosporine (30 nM, 24 h). Plates were then incubated at 37°C in a humidified environment containing 1%, 8% or 21% O_2 as previously described. At each time point, cells were removed from their wells by scraping with a plastic cell scraper (TRP). HSPC were transferred to 12 ml tubes and retrieved from the media by centrifugation.

$2x10^5$ HSPC were washed twice with 5 ml ice cold PBS, resuspended in 1X Annexin V Binding Buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) and transferred to flow cytometry tubes (BD). Excluding the unstained controls, all HSPC were then incubated with 5 µl of both Annexin V-FITC and PI (15 min, RT). Following incubation the final volume was adjusted to 500 µl with 1X Annexin V Binding Buffer. Annexin V-FITC and PI staining of HSPC was subsequently analysed with FACSCalibur (BD).

3.4.3 *Immunophenotypic characterisation*

Following expansion, HSPC cultures were cultured in 6-well plates placed in 1%, 8% and 21% O_2 as described previously. HSPC were subjected to flow cytometric immunophenotyping at days 7, 10 and 14. Cells were removed from 6-well plates by scraping with a plastic cell scraper followed by aspiration of culture media. HSPC were pelleted by centrifugation (400 x g, 10 min, 4°C) and pellets washed twice with ice cold PBS. Following washing, cells were resuspended in 100 µl PBS. Incubating HSPC with human serum for 15 min on ice blocked non-specific binding. Cells were subsequently labelled with specific antibodies (Chart 1). Labelled cells were then quantified with the FACSCalibur flow cytometer and the data analysed with CellQuest Software (BD).

Chart 1. Antibodies for flow cytometry.

Antibody	Fluoro-chrome	Host Species	Ig Subtype	Concentration		Company
				Stock	End	
Fluorochrome-Conjugated Primary Antibodies:						
CD11b	PE	Mouse	IgG_{2a}	50 µg/ml	2.50 µg/ml	BD Biosciences
CD13	APC	Mouse	IgG_1	20µl/10^6 cells	5 µl	BD Biosciences
CD14	PerCP	Mouse	IgG_{2b}	25 µg/ml	1.25 µg/ml	BD Biosciences
CD18	FITC	Mouse	IgG_1	25 µg/ml	1.25 µg/ml	BD Biosciences
CD31	FITC	Mouse	IgG_1	20µl/10^6 cells	5.00 µl	BD Biosciences
CD33	R-PE	Mouse	IgG_1	20µl/10^6 cells	3.00 µl	BD Biosciences
CD34	FITC	Mouse	IgG_{2a}	55 µg/ml	2.75 µg/ml	Miltenyi Biotech
CD45	PerCP	Mouse	IgG_1	25 µg/ml	1.25 µg/ml	BD Biosciences
CD117	APC	Mouse	IgG_1	20µl/10^6 cells	0.80 µl	BD Biosciences

Antibody	Fluoro-chrome	Host Species	Ig Subtype	Concentration		Company
CD133	PE	Mouse	IgG_1	55 µg/ml	2.75 µg/ml	Miltenyi Biotech
CD140b	R-PE	Mouse	IgG_{2a}	20µl/10^6 cells	5 µl	BD Biosciences
Tie-2	PE	Mouse	IgG_1	50 µg/ml	4 µg/ml	R&D Systems
CXCR4	Cy5-PE	Mouse	IgG1	20µl/10^6 cells	5 µl	BD Biosciences
Unconjugated Primary Antibodies:						
CD29/ β1 integrin	--	Mouse	IgG_1	25 µg/ml	1 µg/ml	Serotec
CD49d/ VLA-4/ α4 integrin	--	Mouse	IgG_1	25 µg/ml	5 µg/ml	Serotec
VEGFR-1	--	Mouse	IgG_1	1000 µg/ml	10 µg/ml	Abcam
VEGFR- 2	--	Goat	IgG	1000 µg/ml	10 µg/ml	R&D Systems
Fluorochrome-Conjugated Isotype Control Antibodies:						
	FITC	Mouse	IgG_1	20µl/10^6 cells	5 µl	BD Biosciences
	APC	Mouse	IgG_1	20µl/10^6 cells	5 µl	BD Biosciences
	PE	Mouse	IgG_1	50 µg/ml	4 µg/ml	R&D Systems
	R-PE	Mouse	IgG_1	20µl/10^6 cells	3 µl	BD Biosciences
	FITC	Mouse	IgG_{2a}	20µl/10^6 cells	5 µl	BD Biosciences
	PE	Mouse	IgG_{2a}	20µl/10^6 cells	5 µl	BD Biosciences
	PerCP	Mouse	IgG_1	20µl/10^6 cells	5 µl	BD Biosciences
	Cy5-Pe	Mouse	IgG_1	20µl/10^6 cells	5 µl	BD Biosciences
Unconjugated Isotype Control Antibodies						
	-	Mouse	IgG_1	1000 µg/ml	10 µg/ml	Abcam
	-	Mouse	IgG_1	20µl/10^6 cells	5 µl	BD Pharmingen
	-	Mouse	IgG_1	20µl/10^6 cells	5 µl	Serotec
Fluorochrome-Conjugated Secondary Antibodies						
	APC	Goat (anti-mouse)	IgG	20µl/10^6 cells	1 µl	BD Biosciences
	PE	Rabbit (anti-goat)	IgG	500µg/ml	0.5 ug/ml	Dianova

3.5 Gene Expression Analysis

In this investigation, two-step reverse transcriptase-polymerase chain reaction (RT-PCR) was employed to examine changes in gene expression following incubation of HSPC in different oxygen concentrations.

3.5.1 *RNA isolation*

HSPC were transferred to 6-well plates (CellStar) and incubated in 21%, 8% or 1% O_2 for 7 days. On days 7, 10, 12, and 14 adherent HSPC were first scraped with a plastic cell scraper and the cells aspirated. HSPC were pelleted by centrifugation (400 x g, 10 min, 4°C) and washed two times in ice cold PBS. Cells were lysed with the QIAshredder Kit (QIAGEN, Hilden, Germany) followed by RNA isolation utilising the RNeasy Mini Kit (QIAGEN). RNA isolated using this technique was then quantified with a spectrophotometer (BioRad, SmartSpec 3000), aliquoted and stored at -80°C until required.

3.5.2 *First-strand synthesis*

First strand cDNA synthesis was performed with SuperScript™ II Reverse Transcriptase Kit (Invitrogen Corporation, Karlsruhe, Germany). Reactions were mixed according to provided recipes. 1 µg of RNA isolated from the previous step was mixed with 0.5 µg random primers (Promega, Madison, Wisconsin USA) and deionized H_2O to a total volume of 12 µl. Primers consisted of random hexadeoxynucleotides and allowed for amplification of first strand cDNA from the total cellular messenger RNA (mRNA) pool. The mixture of RNA, random primers and H_2O was subsequently heated to 70°C for 10 min in a PCR cycler (Eppendorf Mastercycler Gradient) followed by cooling 2 min on ice. To this mixture, 4 µl of 5X First-Strand Buffer (250 mM Tris-HCL (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 2 µl 0.1 M DTT and 1 µl of 10 mM dNTP Mix was added. The resultant mixture was then heated for 2 min at 42°C. Finally 1 µl (200 units) of SuperScript™ RT was added and mixed by pipetting. The final 20 µl mixture was then incubated for 50 min at 42°C and the reaction inactivated by heating to 70°C for 15 min. cDNAs were stored at 4°C until required.

3.5.3 *Gene-specific PCR amplification*

Gene-specific primers (Chart 2) were used in combination with AccuPrime™ *Taq* DNA Polymerase (Invitrogen Corporation) to examine changes in HSPC gene expression following incubation in low oxygen. This particular kit includes anti-*Taq* DNA polymerase antibodies that inhibit

polymerase activity, providing a "hot-start" thereby permitting room temperature setup. 50 µl reactions were mixed according to supplied protocol. In 0.2 ml thin-walled PCR Tubes (Biozym Scientific GmBH, Oldendorf, Germany), 200 ng template previously synthesised cDNA was mixed with 10 µM primer, 5 µl of 10X AccuPrime™ PCR Buffer I or II (depending on product length), 1 µl AccuPrime™ *Taq* DNA Polymerase and distilled, deionized H_2O to a 50 µl total volume. PCR amplifications were carried out in the PCR Cycler according to the general program (initial denaturation at 45°C for 2 min, denaturation at 45°C for 30 sec, annealing (see Chart 2 for specific annealing temperatures), elongation at 72°C for 1 min per kbase, 35 cycles, and final elongation at 72°C for 5 min).

Chart 2. Primers employed during RT-PCR.

Name	Primer Sequence (5'→3')	Product Size	T_{Anneal}	Reference
CD117	F: ATTTTCTCTGCGTTCTGCTCCTAC R: CGCCCACGCGGACTATTA	128bp	56°C	(Uccini et al., 2005)
CD133	F: TACCAAGGACAAGGCGTTCAC R: CAGTCGTGGTTTGGCGTTGTA	469bp	55°C	(Baal et al., 2004)
FGF4	F: CTACAACGCCTACGAGTCCTACA R: GTTGCACCAGAAAAGTCAGAGTTG	370bp	55°C	(Henderson et al., 2002)
Flt1	F: GCACCTTGGTTGTGGCTGACT R: CCCTTCTGGTTGGTGGCTTTG	655bp	60°C	(Krussel et al., 1999)
GATA1	F: TCAATTCAGCAGCCTATTCC R: TTCGAGTCTGAATACCATCC	377bp	50°C*	(Chadwick et al., 2003)
GATA2	F: TGTTGTGCAAATTGTCAGACG R: CATAGGTGCCATGTGTCCAGC	275bp	53°C	(Hirasawa et al., 2002)
HIF1α	F: ACAAGTCACCACAGGACA R: AGGGAGAAAATCAAGTCG	168bp	50°C	(Bilton et al., 2005)
KDR	F: AGACTTTGAGCATGGAAG R: CCATTCCACCAAAAGATG	298bp	49°C	(Henderson et al., 2002)
Oct4	F: GGGCTCGAGAAGGATGTGGT R: GGGCTCCCATAGCCTGGG	182bp	55°C	sequence supplied by Chemicon (Temulca, USA)
Rex1	F: CAGCATCCTAAACAGCTCGCAGAAT R: GCGTACGCAAATTAAAGTCCAGA	306bp	55°C	(Henderson et al., 2002)
RUNX1	F: CCGCAGCATGGTGGAGGTA R: AGCGATGGGCAGGGTCTTG	121bp	56°C	(Taniuchi et al., 2002)
SDF-1	F: GTGTCACTGGCGACACGTAG R: TCCCATCCCACAGAGAGAAG	263bp	55°C	(Ceradini et al., 2004)
Sox1	F: CTCACTTTCCTCCGCGTTGCTTCC R: TGCCCTGGTCTTTGTCCTTCATCC	838bp	63°C	(Henderson et al., 2002)
Sox2	F: CCCCCGGCGGCAATAGCA R: TCGGCGCCGGGGAGATACAT	448bp	60°C	(Henderson et al., 2002)
$VEGF_{165}$	F: AGGCCAGCACATAGGAGAGA R: AACAAATGCTTTCTCCGCTC	123bp	54°C	(Hollingsworth et al., 2005)

3.6 Immunofluorescence Microscopy

3.6.1 *Immunohistochemistry*

Placental probes (gestational ages including 6, 12, and 38 wpc) were collected and either directly embedded in Tissue-Tek OTC (Sakura, Torrance, CA) and immersed in liquid nitrogen, or pre-fixed for 1 h in Zamboni´s solution, washed and embedded in OTC before cryoslicing (8 µm).

Tissue slices were blocked with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) and then incubated overnight at 4°C either with monoclonal mouse anti-human CD34 (1:100; DakoCytomation, Hamburg, Germany), or with monoclonal mouse anti-human SDF-1 (1:100; R&D Systems) diluted in PBS supplemented with 1% donkey serum and 0.1% Triton X-100. As secondary antibody, affinity-purified RhodRedX-donkey anti-mouse IgG (Jackson ImmunoResearch), diluted 1:400, was then applied for 1 h at room temperature. After three washing steps, excess mouse IgG-binding activity was blocked by 30 min incubation with 10% mouse serum (Jackson ImmunoResearch) at room temperature. The slices were then incubated for 60 min with FITC-labeled monoclonal mouse anti-human cytokeratin 7 (DakoCytomation), washed, and nuclei were counterstained with Hoechst 33342 (5 µg/ml; Calbiochem, Darmstadt, Germany) before mounting in ProLong Gold antifade reagent (Molecular Probes, Invitrogen, Karlsruhe, Germany). Further slices were first incubated overnight at 4°C with a mixture of polyclonal goat-anti-human vimentin (S-20; 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-human SDF-1 (1:100), and then for 1 h at room temperature with a mixture of affinity purified FITC-donkey anti-goat IgG and RhodRedX-donkey anti-mouse IgG. Controls for nonspecific staining were performed by replacing primary antibodies with mouse isotype controls. Fluorescence was observed with a Leica DM-LB microscope (Wetzlar, Germany) equipped with a Leica DC-200 camera, Leica IM 1000 software and filters for blue light (505 nm), green light (580 nm), and UV-light (400 nm) excitation.

3.6.2 *Immunocytochemistry*

Immunocytochemistry was performed on placental stroma-enriched cell fractions (HPF) cultured in either 1% or 8% O_2 to examine *in vitro* expression of SDF-1α protein. HPF were added to 8-well chamber slides (LabTek, Nunc, Wiesbaden, Germany) and allowed to attach overnight in ambient atmospheres. The following day, HPF were transferred to atmospheres containing the appropriate oxygen concentrations and incubated a further 2 days, as described above. Following incubation, cultures were washed with PBS and fixed for 15 min in Zamboni's solution. Fixed HPF were subsequently washed in PBS (3 X 5 min), blocked for 1 h in 10% donkey serum (PBS; 1% BSA, 0.1% Triton X-100) and incubated for 2 h with a 1:100 dilution of mouse monoclonal anti-human SDF-1α antibody (R&D Systems) or the appropriate isotype-matched control. Slides were washed 3 times in PBS, incubated for 1 h in a 1:100 dilution of Rhodamine Red-conjugated donkey anti-mouse IgG and washed again in PBS (3 X 5 min). Nuclei were stained for 5 min with Hoechst 33342 (5 µg/ml). Cover slides were mounted with ProLong Gold antifade reagent, fluorescent cells visualised with a Nikon fluorescence microscope (Nikon TE2000, Duesseldorf, Germany) and images captured using NIS Elements AR v2.3 software (Nikon).

3.7 Human SDF-1α Immunoassay

SDF-1α protein secretion into media conditioned during culture of mixed placental cells, Jeg3 choriocarcinoma cells, HTR and HPF cells was determined using Quantikine® Human SDF-1α Immunoassay (R&D Systems, Wiesbaden, Germany). This assay employs the quantitative sandwich enzyme immunoassay technique, in which, a specific SDF-1α monoclonal antibody has been pre-coated onto a microplate. Immobilised antibody binds SDF-1α present in samples and standards while unbound substances are washed and removed from the microplate. A second incubation with an enzyme-linked polyclonal antibody specific for SDF-1α facilitates colourimetric detection of bound SDF-1α; following addition of substrate, colour develops in

proportion to the amount of SDF-1α present. SDF-1α ELISA was performed according to the manufacturer's description. Briefly, 200 µl of standard or sample was incubated in the provided 96-well plate for 2 h at room temperature. Wells were washed 3 times and further incubated with SDF-1 conjugate for an additional 2 h with shaking at room temperature. Following this incubation, wells were washed 3 times with wash buffer. 200 µl of substrate solution was added and plates incubated for 30 min (RT). Colourimetric detection determined SDF-1α levels by first measuring absorbance at 450 nm and relating sample absorbance to specific standards. All samples and standards were examined in triplicate. Immunoassays were repeated a minimum of 3 times. In concordance with the manufacturer's description, intra- and interassay variability were within acceptable ranges.

3.8 *In Vitro* Migration Assays

Migratory capacity or the ability of HSPC to migrate through a membrane with 3 µm pores under various treatment conditions was measured using a modified Boyden Chamber assay. Each well of 24-well plate consisted of an upper insert separating the well with a membrane. $2.5x10^5$ cells/200 µl IMDM (± 1 µg/ml AMD3100) was added in the inserts and placed into the lower well containing 800 µl of IMDM, conditioned media (HTR-CM, HPF-CM), or SDF-1α-supplemented IMDM (1% FCS; 0.025% BSA). Plates were incubated in a humidified atmosphere for the specified times at 37ºC. Inserts were removed and migrated HSPC were quantified with a Casy® Cell Counter (Schaerfe Biosystems).

3.8.1 *Transendothelial migration*

Transendothelial migration (TEM) of HSPC was assayed utilising a similar technique. Prior to the addition of HSPC, inserts were coated with a monolayer of human umbilical vein endothelial cells (HUVEC, P3-4) isolated according to established protocols. HSPC were fluorescently stained (PKH26 Red Fluorescent cell stain; Sigma, Munich Germany) before being included. TEM assays were carried out for the specified time under the same conditions as described above. In some cases, the effects of

placental cell types on HSPC TEM were assayed by coculturing specific placental cell types as monolayers coating the bottom well. Where specified, cocultured cells were pretreated for 24 h in different oxygen concentrations (1% or 8% O_2). Migrated HSPC were quantified by measuring the number of PKH^+ event counts in 90 sec with FACSCalibur (BD).

3.9 *In Vitro* Cell-Cell Adhesion Assay

Following expansion, the ability of HSPC to adhere to placental-derived cells was assessed. Briefly, HSPC were fluorescently stained (PKH26) and $2.5x10^5$ HSPC were incubated in 24-well plates containing a human placental cell monolayer. After 6 h incubation in different oxygen concentrations (1%, 8% or 21% O_2), non-adherent HSPC were collected, the wells washed 3 times with cold PBS; all washes were collected. Adherent cells were lifted with trypsin-EDTA and collected. The number of PKH^+ HSPC in both the non-adherent and adherent fractions was determined using FACSCalibur. Cells were quantified by measuring the number of HSPC events in 90 sec and the ratio of adherent HSPC to the total number of HSPC calculated.

4 RESULTS

4.1 Detection of CD34 Positive Cells in Early Human Placental Tissue

Human placental tissue was examined for expression of CD34 during various stages of development (Figure 8). Tissues were probed with specific fluorochrome-coupled antibodies recognising human CD34 and human cytokeratin 7. CD34 is a common marker for both hematopoietic and endothelial progenitor cells. Cytokeratin is commonly used to distinguish endodermal cells from mesenchymal cells and in the human placenta, cytokeratin 7 specifically labels cells of the trophoblast lineage.

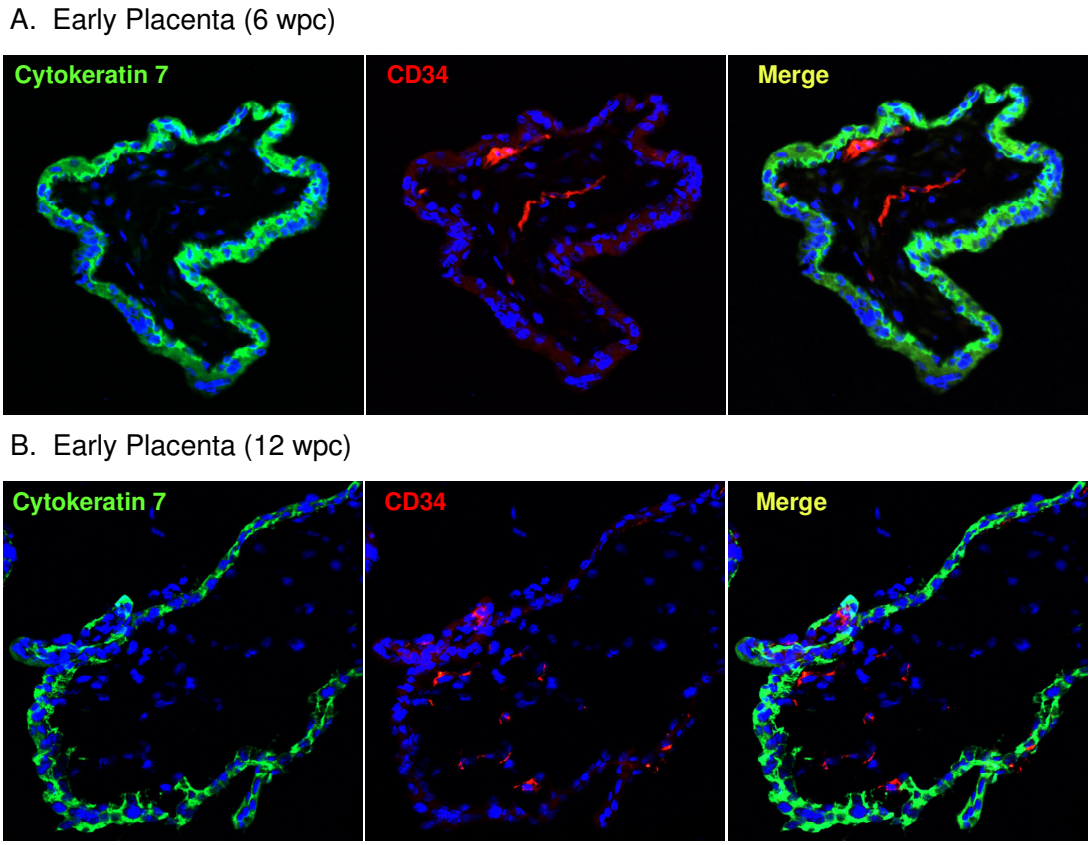


Figure 8. Immunohistochemical analysis of CD34 expression in early human placental tissue. Unfixed human tissue from 6 wpc and 12 wpc were labelled with anti-human CD34 (Red) and anti-human cytokeratin 7 (Green) antibodies. Photomicrographs were made and images merged. CD34 expression was seen on individual cells and cord-like structures in placental villi at 6 wpc (**A**). In 12 week-old placental tissue (**B**), CD34 labelled individual cells and early blood vessels within the chorionic villi. Cell nuclei were stained with Hoechst (Blue). Original magnification 200X.

Early human placental tissue contains $CD34^+$ cells and $CD34^+$ cell clusters at 6 wpc as demonstrated by immunohistochemistry (Figure 8). $CD34^+$ cells did not stain positive for cytokeratin 7. A similar staining pattern was seen in 12 week-old human placental tissue (Figure 8B). At this time point, a greater number of $CD34^+$ cells were situated within the placental stroma, indicating an increase in the number of placental blood vessels. Some $CD34^+$ cells could be in found in proximity to trophoblast.

4.2 Flow Cytometric Analysis of HSPC

HSPC isolated from human UCB from term pregnancies were characterised utilising flow cytometry analysis based on the expression of CD (cluster of differentiation) cellular antigens (Chart 3).

Chart 3. Phenotypic characterization of HSPC (Day 0 and Day 7).

	Positive Cells (%)			
	CD133 / CD34	**CD133 / CD117**	**CD45**	**CD11b / CD14**
Day 0	87.5	84.6	98.6	1.0
Day 7	13.6	15.4	98.3	3.9

In this investigation, HSPC were isolated utilising anti-CD133 antibody coated microbeads. Directly following isolation (Day 0), HSPC were 88% double positive for CD133 and CD34 demonstrating the effectiveness of this technique in the isolation of HSPC. The majority of $CD133^+$ cells were also positive for the stem cell marker CD117 (c-kit / SCF receptor) at the time of isolation. HSPC were also $CD45^+$. Only 1% of freshly isolated HSPC expressed the combination of CD11b and CD14, commonly used as markers for differentiated monocytes.

In our laboratory, seven-day culture expansion was required to provide a sufficient number of HSPC for various functional studies. Furthermore, following seven-day culture expansion in HSPC growth media (HSPC-GM), an appropriate number of HSPC remained in culture. At Day 7, HSPC remained 13.6% CD133 and CD34 double positive (Chart 3). The majority of $CD133^+$

cells also expressed CD117. Almost all cells in the HSPC expressed $CD45^+$ (98.3%). A significant difference was noted in the expression of monocyte markers between Day 0 and Day 7. The number of cells coexpressing the monocyte markers CD11b and CD14 rose from 1% on Day 0 to 4% on Day 7.

These results demonstrate the effective isolation of HSPC expressing CD133, CD34 and CD117. Seven-day expansion of HSPC cultures was also shown to be an effective method for increasing cell number while maintaining a pool of HSPC. In accordance with previously published results, isolated HSPC demonstrated progenitor capabilities (Baal et al, 2004)

4.3 Placental Oxygen Tensions Can Be Effectively Mimicked *In Vitro*

A novel system has been used in our laboratory to measure real-time oxygen concentrations in HSPC media during culture (see Figure 6). Following optimisation of the procedure, three distinct atmospheric compositions were chosen, including mixtures containing either 1% or 8% O_2 (with 5% CO_2) or atmospheric oxygen (21% O_2). Particular low oxygen concentrations were chosen in order to reproduce placental oxygen tensions previously measured *in vivo* and reported (Rodesch et al., 1992; Jauniaux et al., 1999; Jauniaux et al., 2001). Replacement of the gas mixture in the chambers resulted in a rapid reduction in O_2 level followed by a period of equilibration (Figure 9). During standardisation of the protocol it was demonstrated that atmosphere replacement three times daily (1 time/h for 3 h, 4 min gas flow at 25 mm/h) was required to maintain appropriate oxygen tensions over time.

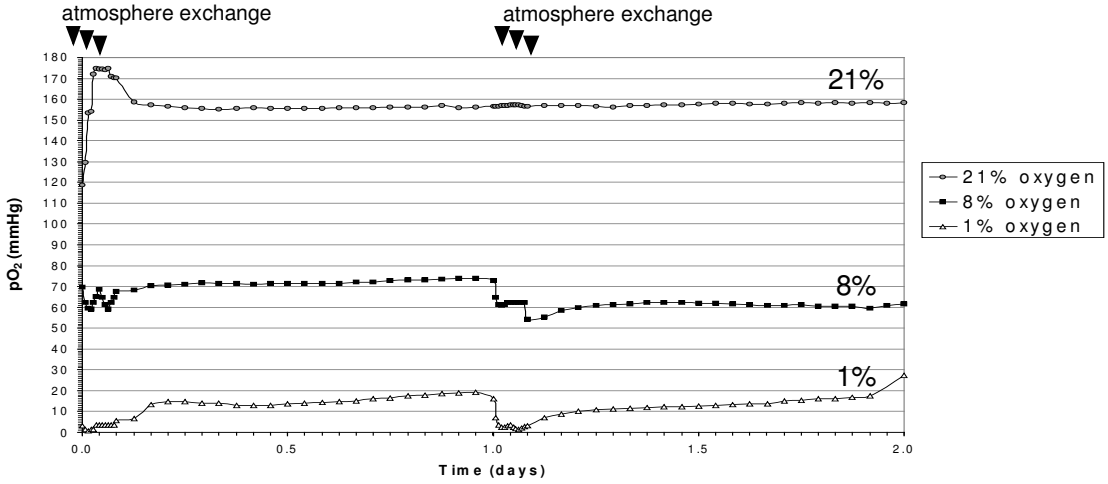


Figure 9. HSPC media oxygen tensions during culture in 1%, 8% or 21% O_2. The SDR system concurrently monitored oxygen concentrations during a two-day experimental period. Representative graph is shown demonstrating *in vitro* oxygen tensions (pO_2) during HSPC culture in low oxygen (1% O_2: open triangles; 8% O_2: black squares). An average oxygen tension of 10.2 ± 5.5 mmHg was encountered when HSPC were cultured in 1% O_2. In 8% O_2, the average oxygen tension was 65 ± 7.5 mmHg. When HSPC were cultured in ambient oxygen (21% O_2: grey circles) the average oxygen tension in HSPC culture media was 157.9 ± 9.2 mmHg. Atmospheres in low oxygen environments were changed three times daily (black arrowheads).

When HSPC were cultured in an atmosphere containing 8% O_2, a mean pO_2 of 65.0 ± 7.5 mmHg was maintained. This environment is similar to measurements made in placental beds from pregnancies at 10-15 wpc (65.0 ± 3.4 mmHg). When HSPC were cultured 1% O_2, the mean pO_2 measured was 10.2 ± 5.5 mmHg. These oxygen tensions were similar to previously described physiological pO_2 in the fetoplacental unit at 8-10 wpc (17.9 ± 6.9 mmHg). This clearly demonstrates the effectiveness of the described, novel system in mimicking actual physiological oxygen tensions at different time points during placental development. As comparison, HPSC cultured in ambient oxygen were similarly assessed. HSPC grown under atmospheric conditions encountered non-physiological, high oxygen environments (Figure 9). A mean *in vitro* pO_2 of 157.9 ± 9.2 mmHg was reached, illustrating that culture in ambient oxygen results in a clearly hyperoxic environment.

4.4 Effect of Physiological Oxygen Levels on HSPC Number

Cell number was measured with a Casy® Counter during HSPC culture in physiological oxygen concentrations (1% and 8% O_2). The effect of low oxygen during HSPC expansion in HSPC-GM was assayed (Figure 10). HSPC cell number in hyperoxic conditions (21% O_2) was likewise quantified.

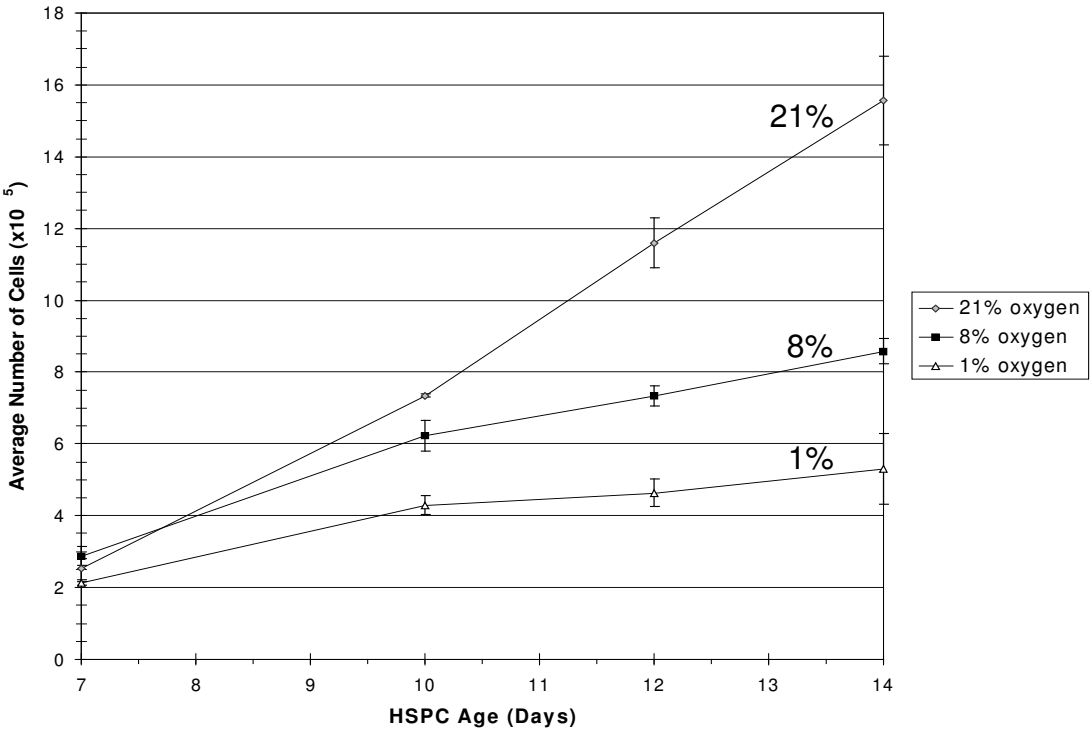


Figure 10. HSPC number during culture in different oxygen concentrations. HSPC (Day 7) were cultured with HSPC-GM in low oxygen environments containing 1% (open triangles) or 8% O_2 (black squares). HSPC number was quantified on days 10, 12, and 14. HSPC expansion in ambient oxygen (21% O_2) was also examined (grey circles). HSPC number positively correlated with oxygen concentration.

HSPC cultivation with HSPC-GM (IMDM, 10% FCS + 50 ng/ml Flt3L, 20 ng/ml SCF, and 10 ng/ml TPO) in ambient oxygen induced expansion of HSPC and was used to amplify HSPC cultures directly following isolation. Results demonstrated that additional culture of HSPC beyond Day 7 with HSPC-GM in 21% O_2, resulted in a >7-fold increase in HSPC number. In fact, previous work in our laboratory has shown that proliferation of cultures continued for ~30 days, after which, HSPC number could be maintained up to 60 days (Baal et al., 2004).

The number of HSPC positively correlated with oxygen concentration (Figure 10). HSPC in high oxygen environments (21% O_2) continued expanding during the 7 day experimental period. HSPC number slightly increased in 1% and 8% O_2 but did not result in substantial expansion when compared to culture in ambient oxygen. Minimal HSPC expansion occurred in atmospheres containing the lowest oxygen concentration (i.e. 1%). Approximately 3-fold and 2-fold increases in cell number were seen after 7 day culture of HSPC in 8% and 1% O_2, respectively. Cell death, as assessed with the Casy® Counter was not affected by culture of HSPC in low oxygen concentrations.

These results demonstrate the effects of oxygen tensions on HSPC cell number. Traditional cell culture conditions have been shown to result in HSPC expansion. However, when HSPC were grown in oxygen tensions more effectively mimicking those found *in utero*, decreased culture expansion was seen.

4.5 HSPC Cell Cycle Analysis

As culture of HSPC in 1% and 8% O_2 was shown to affect cell proliferation, further experiments examined the effects of physiological oxygen tensions on HSPC cell cycle status. This was accomplished using propidium iodide (PI) staining of fixed HSPC followed by quantification of cell ploidy with flow cytometry and ModFit® software.

Cells (Figure 11A, Region 1 (R1)) were first gated based on their size (FSC) and granularity (SSC). Cell aggregates resulting from the fixation process were excluded with further gating (Figure 11B, R2). Using this technique, 2n cells in G2 or M phase of the cell cycle contain twice the amount of PI as 1n cells in the G1 phase of the cell cycle or resting G0 cells. Cells with intermediate levels of PI are considered to be in the S phase. 65% of Day 7 HSPC were 1n and were, thus, considered G0 or G1 phase (Figure 11C). Cells containing twice the amount of DNA (i.e. 2n) were included in HSPC cultures with 2% of Day 7 cells in G2/M.

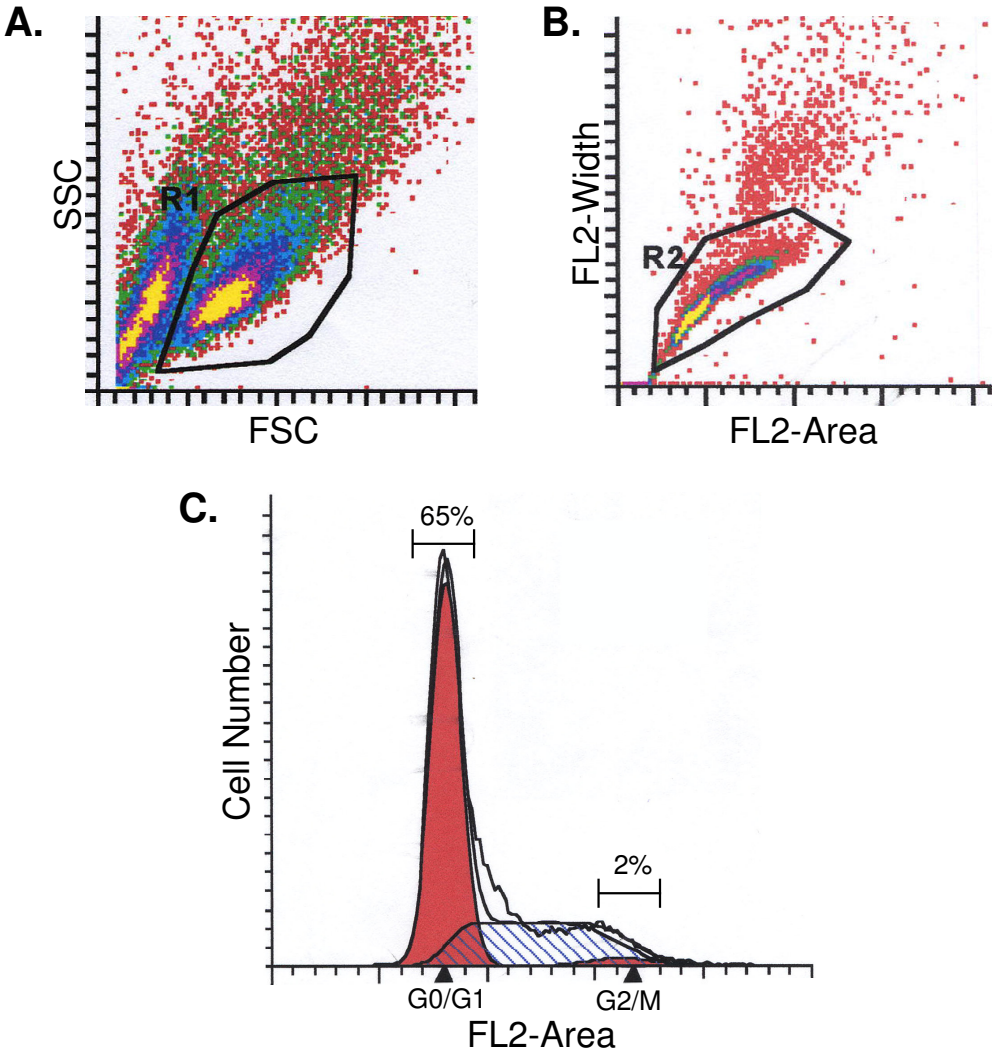


Figure 11. Flow cytometric cell cycle analysis of expanded HSPC (Day 7). Live cells (R1) were gated based on FSC and SSC (**A**). Single cells (R2) were gated excluding cell aggregates resulting from fixation (**B**). Cell ploidy was determined based on the stochastic integration of PI into HSPC DNA (**C**). Results from this experiment demonstrate that following expansion 65% of HSPC were 1n (G0/G1) while 2% of HSPC were 2n (G2/M). The remaining HSPC were in the S phase of the cell cycle (33%). A representative analysis is shown.

Flow cytometric analysis was used to determine cell cycle status of HSPC cultured in atmospheres containing physiological oxygen concentrations. No significant changes were noted between treatments after 1 day (Day 8) in the different oxygen concentrations. When cell cycle status was examined after 3, 5 and 7 days of culture, differences were seen. The greatest difference between treatments was measured on Day 12, following 5 day culture in physiological oxygen concentrations. Culture of cells in both

low oxygen atmospheres increased the number of G0/G1 HSPC, with subsequent reductions in G2/M and S phase cells. This effect was dose-dependent with the greatest increases seen when cells were cultured in 1% O_2. On Day 12, 92% of HSPC in 1% O_2 were G0/G1 compared to 85% and 78% of HSPC in 8% and 21% O_2, respectively.

These results demonstrate the effects of low oxygen on HSPC cell cycle status. Cell culture in atmospheres mimicking early placental development resulted in a rapid increase in resting HSPC. The percentage of resting cells increased with time while the percentage of S/G2/M cells subsequently decreased. HSPC culture in a hyperoxic environment (21% O_2) also increased the percentage of resting HSPC, although not as rapidly as low oxygen environments.

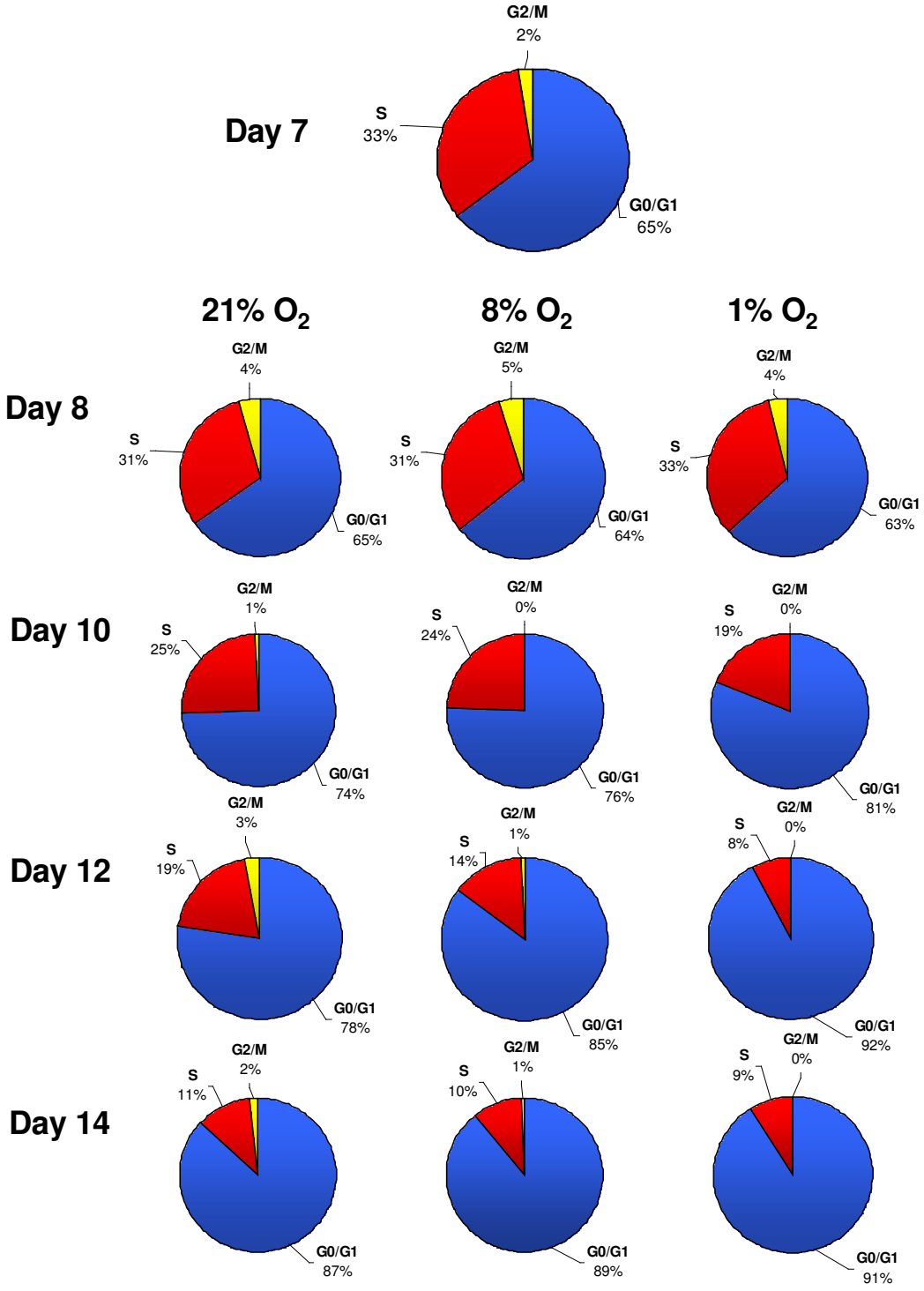


Figure 12. Pie diagrams generated from HSPC cell cycle analysis during culture in different oxygen concentrations. HSPC were grown in 1%, 8% or 21% O_2 and cell cycle status determined on days 7, 8, 10, 12 and 14. Low oxygen dose-dependently increased the number of resting G0/G1 HSPC over the test period. Subsequent reductions in cycling G2/M and S cells were measured. Experiments were repeated 5 times; representative data is shown.

4.6 HSPC Apoptosis Analysis

Cell culture dynamics were further investigated utilising flow cytometric quantification of HSPC apoptosis. Cells were stained with Annexin V-FITC and PI in order to distinguish live cells (Annexin V^-PI^-) from early apoptotic (Annexin V^+PI^-) and late apoptotic/necrotic (Annexin V^+PI^+) cells (see Figure 7). HSPC apoptosis was measured following expansion (Day 7). HSPC were then cultured in low oxygen (1% or 8% O_2) or ambient oxygen (21% O_2) and apoptosis levels quantified on days 8, 10, 12, and 14.

Results from this experiment demonstrated that culture in physiological oxygen concentrations had no significant effects on HSPC apoptosis levels. Upon examination of individual experiments minor differences were noted. For example, when compared to culture in ambient oxygen (21%), higher levels of total apoptosis (early and late apoptosis) were measured after 7 day culture in 1% O_2 (Figure 13). More importantly however, no significant differences were noted when data obtained from multiple experiments (n = 3) was compiled (Chart 4).

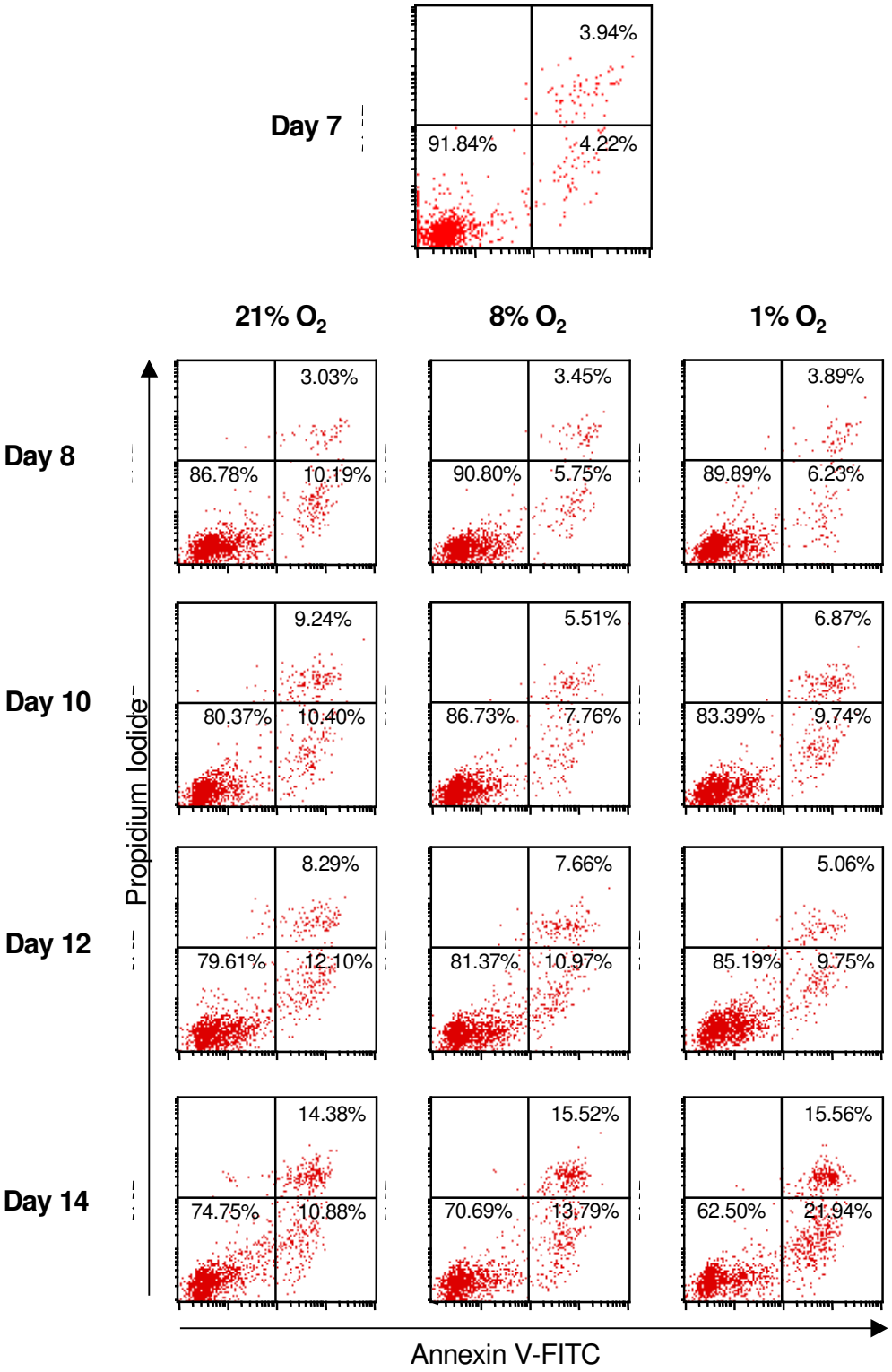


Figure 13. Flow cytometric analysis of HSPC apoptosis. Expanded HSPC (Day 7) apoptosis levels were quantified with flow cytometry following staining with Annexin V-FITC and propidium iodide (PI). Cells were subsequently cultured a further 7 days in 1% or 8% O_2 and analysis performed on days 8, 10, 12 and 14. Apoptosis was similarly quantified during culture in ambient oxygen (21% O_2). Representative results from one of three experiments are shown.

Chart 4. Compiled HSPC apoptosis data following culture in 1%, 8% or 21% O_2.

Treatment	HSPC Age (Days)														
	7			8			10			12			14		
	% Live Cells	% Early Apoptotic Cells	% Late Apoptotic/ Necrotic Cells	% Live Cells	% Early Apoptotic Cells	% Late Apoptotic/ Necrotic Cells	% Live Cells	% Early Apoptotic Cells	% Late Apoptotic/ Necrotic Cells	% Live Cells	% Early Apoptotic Cells	% Late Apoptotic/ Necrotic Cells	% Live Cells	% Early Apoptotic Cells	% Late Apoptotic/ Necrotic Cells
1% O_2 (n=3)	86.7 (±8.6)	5.3 (±3.4)	8.0 (±5.4)	89.6 (±1.6)	4.9 (±2.7)	6.3 (±1.8)	82.8 (±3.3)	9.2 (±2.3)	8.0 (±1.4)	78.0 (±6.3)	17.3 (±2.6)	9.3 (±3.9)	67.5 (±7.4)	18.0 (±4.6)	14.5 (±3.1)
8% O_2 (n=3)				90.7 (±0.5)	5.0 (±1.0)	4.1 (±0.5)	88.3 (±2.3)	6.6 (±1.7)	5.1 (±0.6)	84.3 (±6.1)	8.8 (±3.0)	6.9 (±0.9)	68.2 (±3.6)	15.8 (±2.8)	16.1 (±0.8)
21% O_2 (n=4)				89.5 (±3.6)	5.4 (±3.4)	5.8 (±2.2)	81.5 (±5.7)	7.0 (±3.3)	8.0 (±2.4)	80.3 (±0.8)	9.2 (±2.9)	8.6 (±1.6)	76.8 (±4.1)	10.7 (±1.9)	12.5 (±2.3)

4.7 HSPC Characterisation During Low Oxygen Culture

4.7.1 *Gene expression analysis*

Alterations in HSPC gene expression during incubation in physiological oxygen concentrations were examined with RT-PCR. cDNA was synthesised from extracted total cellular mRNA and probed using primers for various genes known to be expressed during hematopoietic and endothelial cell development.

A representative non-quantitative RT-PCR analysis following seven-day incubation of HSPC in low oxygen (1% or 8% O_2) or atmospheric oxygen (21% O_2) is illustrated (Figure 14). A variety of hematopoietic and endothelial cell mRNA were expressed over the entire culture period. HSPC genes, including CD133, CD117, GATA-1 and -2, Runx-1 and SCL/Tal-1 were expressed in all conditions. The expression of genes associated with vascular development, such as VEGFR-1 and $VEGFA_{165}$, were also expressed in HSPC cultures. Interestingly, expression of VEGFR-2 was not seen in all HSPC cultures tested. HSPC gene expression altered during culture in physiological oxygen concentrations. Alterations noted in gene expression during RT-PCR analysis necessitated further characterisation of HSPC at the protein level.

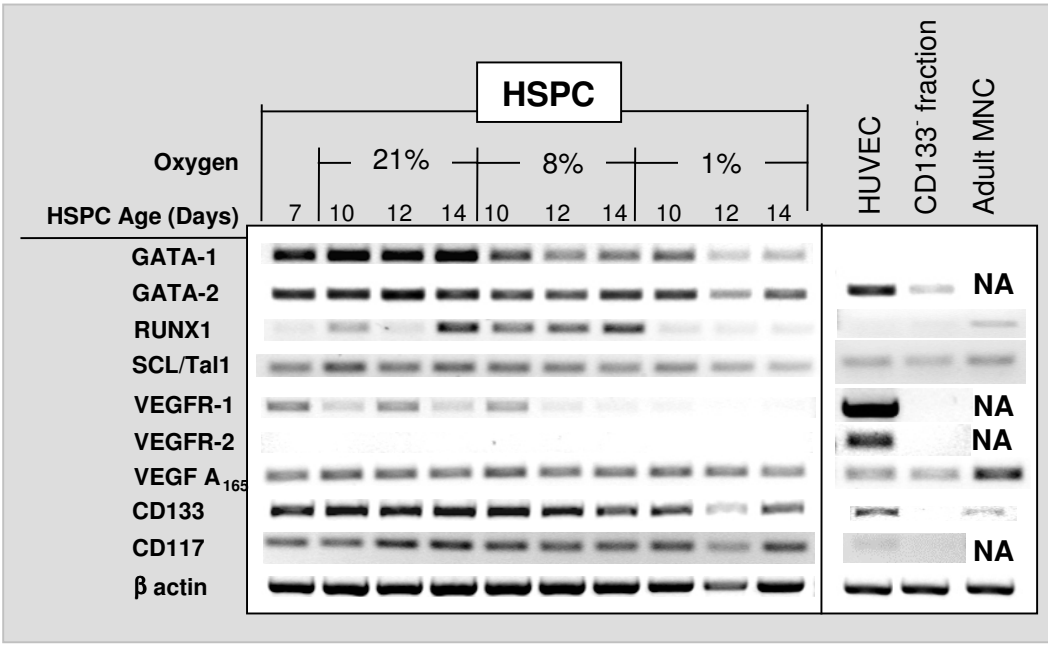


Figure 14. Gene expression analysis of HSPC during culture in low oxygen. Expression of specific hemangioblast genes during 7 day HSPC culture was examined with RT-PCR. The housekeeping gene, β-actin was utilised as a control. Differentiated cells, such as the HSPC negative ($CD133^-$) fraction (D0), adult mononuclear cells (MNC) isolated from peripheral blood and endothelial cells (HUVEC), were concurrently examined. NA: results not available.

4.7.2 *Protein expression analysis*

The effect of simulated placental oxygen tensions on HSPC differentiation was assessed with flow cytometry based on expression of CD markers. Expanded HSPC were cultured for an additional 7 days in HSPC-GM in either atmospheric (21% O_2) or physiological oxygen concentrations (1% or 8% O_2) and analysed with flow cytometry. Flow cytometric analysis was performed on Day 10 and Day 14.

As shown, the majority of expanded HSPC expressed CD45 (88%), an established marker for cells of hematopoietic lineages (Chart 5). Coexpression of CD31 and CD140b was also examined in this investigation. Chart 5 demonstrates the low coexpression of these markers on Day 7 HSPC; no change was seen during culture of HSPC in the various conditions.

Chart 5. Flow cytometric characterisation of HSPC following 7 day culture in atmospheric oxygen (21% O_2) or low oxygen (8% or 1% O_2).

	HSPC CELL AGE (Days after isolation)						
	Day 7	Day 10			Day 14		
		Culture Conditions (% Oxygen)					
MARKER	-	21	8	1	21	8	1
CD45	87.56%	80.44%	87.58%	79.50%	87.83%	89.97%	83.99%
CD34/CD133	10.02%	4.13%	5.07%	4.30%	2.30%	2.42%	2.56%
CD34	27.28%	8.47%	11.80%	7.29%	5.56%	5.88%	3.21%
CD117	61.64%	50.93%	48.03%	28.09%	40.42%	32.31%	19.15%
CD31	71.49%	73.69%	76.14%	86.94%	73.11%	82.82%	83.98%
CD11b/CD14	0.65%	5.01%	10.66%	12.13%	12.78%	18.46%	22.92%
CD31/CD140b	0.53%	0.42%	0.50%	0.69%	1.44%	1.16%	1.44%

Coexpression of CD34, CD133 and CD117 distinguishes a subset of multipotent stem cells. Therefore, expression of these HSPC stem cell markers was examined with flow cytometry during culture (Chart 5 and Figure 15). In this investigation, approximately 12% of expanded HSPC were CD34 and CD133 double positive (Figure 15A); approximately 11% were CD133 and CD117 double positive (Figure 15B). Culture of HSPC in the tested conditions resulted in a decreased percentage of cells expressing stem cell markers. Day 10 HSPC were less than 5% $CD34^+CD133^+$ or $CD133^+CD117^+$ regardless of the oxygen concentration. By Day 14, less than 3% of the cells were either $CD34^+CD133^+$ or $CD133^+CD117^+$ double positive.

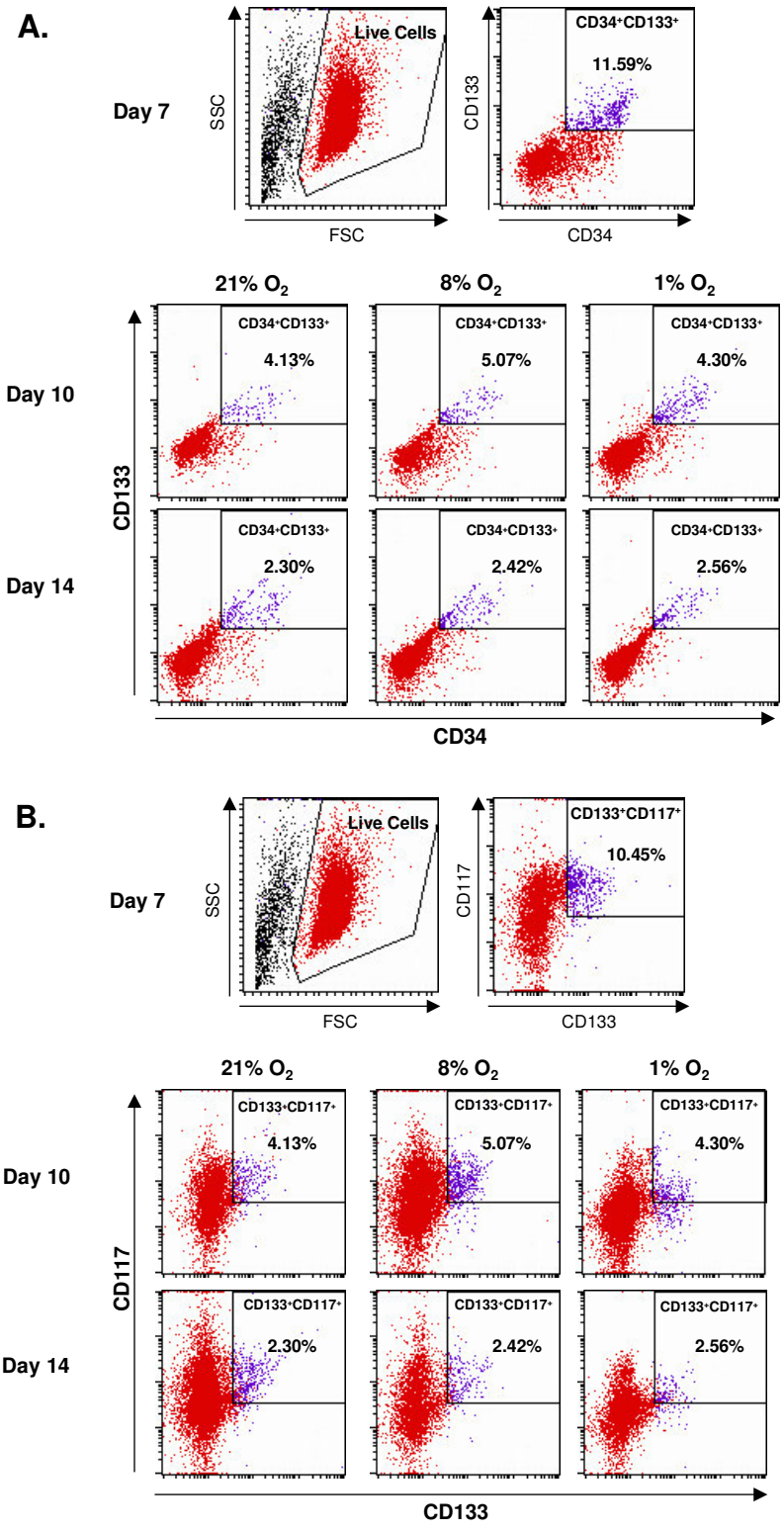


Figure 15. Expression stem cell markers on HSPC during low oxygen culture. Cells were gated and expression of stem cell markers analysed. Approximately 12% of expanded HSPC (Day 7) coexpress CD34 and CD133 (**A**). Approximately 11% of Day 7 HSPC coexpress CD133 and CD117 (**B**). Further culture in atmospheric oxygen (21% O_2) or in physiological oxygen (8% or 1% O_2) for 3 days (Day 10) and 7 days (Day 14) resulted in a decreased percentage of HSPC stem cell marker coexpression.

Another interesting result obtained following flow cytometric analysis of HSPC cultured in physiological oxygen was seen when coexpression of monocyte markers was analysed (Figure 16). Multiparametric flow cytometry allowed the examination of CD11b (α_M integrin) and CD14 coexpression. Double positive cells ($CD11b^+CD14^+$) were gated. $CD11b^+CD14^+$ cells were contained within a population of cells coexpressing CD31 (PECAM) and CD13. Quadruple positive cells ($CD11b^+CD14^+CD31^+CD13^+$) were considered monocytes. HSPC cultures expanded for 7 days consisted of 1% monocytes (Figure 16A). HSPC were then cultured for 1 week in a hyperoxic atmosphere (21% O_2) or in physiological oxygen concentrations (1% or 8% O_2). Media was changed and flow cytometry performed on Day 10 and Day 14. Figure 16B illustrates representative results obtained on Day 14. Day 14 HSPC cultures consisted of 5% monocytes. Low oxygen culture increased the percentage of monocytes in HSPC culture. Day 14 HSPC cultures grown in 8% or 1% consisted of 8% and 11% monocytes, respectively.

These results illustrate the heterogeneity of HSPC cultures and demonstrate the influence of simulating physiological oxygen environments on the cells. The expansion protocol used in this investigation was demonstrated to maintain a population of $CD133^+CD34^+CD117^+$ stem/progenitor cells. At the same time, proliferation and differentiation of cells resulted in an increase in differentiated cells (e.g. monocytes). Cells cultured in physiological oxygen concentrations (1% or 8% O_2) also maintained a population of HSPC and simultaneously increased differentiation. A significant difference was seen between oxygen concentrations when $CD11b^+CD14^+$ monocytes were quantified. Expression of adhesion molecules, such as CD11b (α_M integrin) and CD31 (PECAM) also appear to be affected by low oxygen.

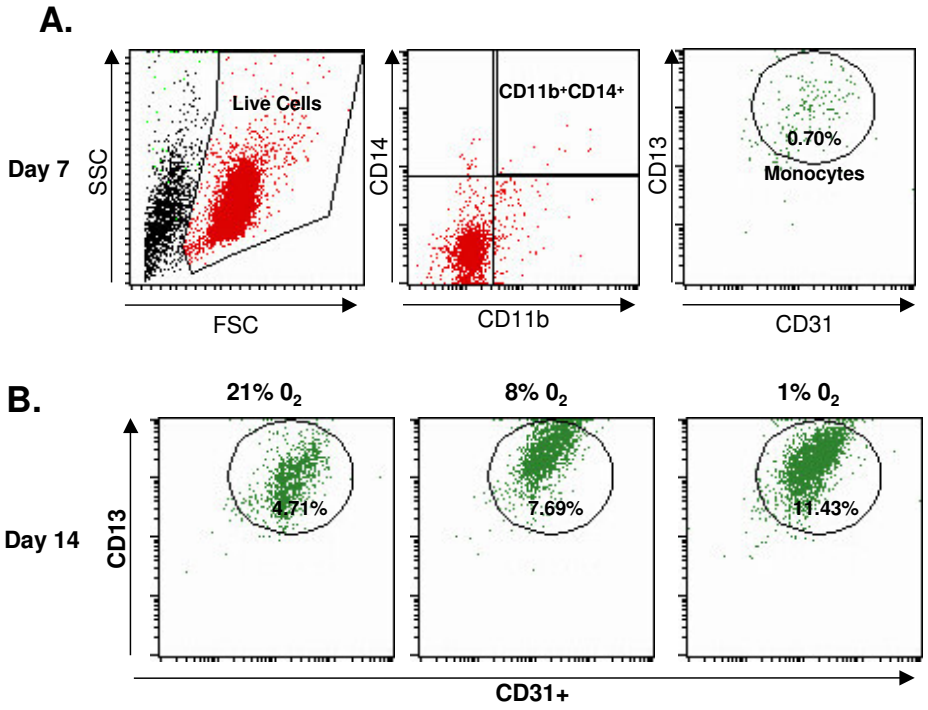


Figure 16. Expression of monocyte markers on HSPC cultivated in low oxygen. Expanded HSPC (Day 7) were incubated with fluorescently labelled antibodies against CD11b, CD14, CD13, and CD31 and subjected to multiparametric flow cytometry. Live cells (**A**) were gated based on size (FSC) and granularity (SSC). Cells coexpressing CD11b and CD14 ($CD11b^+CD14^+$) were further gated. The majority of $CD11b^+CD14^+$ cells were contained within a population of $CD31^+CD13^+$ cells (monocytes). On Day 7, <1% of cells expressed all four markers. Cells expanded a further 7 days (Day 14) were similarly gated and analysed (**B**). In atmospheric oxygen (21% O_2), 5% of cells expressed monocyte markers. The percentage of monocytes increased with decreasing oxygen concentration (8% and 11% of cells in 8% O_2 and 1% O_2, respectively).

4.8 HSPC Matrix Metalloproteinase Activity

The expression of matrix metalloproteinases (MMP) has been shown in hematopoietic stem cells, as well as, differentiated monocytes and macrophages. In this investigation gelatine zymography was employed to test whether culture in low oxygen altered HSPC collagenase (MMP-2 and MMP-9) activity. HSPC cultured for a total of 5 days in 1% or 8% oxygen and the ability of HSPC-derived MMP to digest gelatine was tested. Bands corresponding to active 72 kDa MMP-9 and 49 kDa MMP-2 were apparent in media conditioned by HSPC. Culture of HSPC in decreasing oxygen concentrations (1% and 8% O_2) resulted in an increase in MMP enzymatic

activity (Figure 17). These results demonstrate that HSPC cultures in simulated placental oxygen tensions increased their matrix-degrading ability, suggesting a functional activity of mixed cultures consisting of HSPC and differentiated hematopoietic cells.

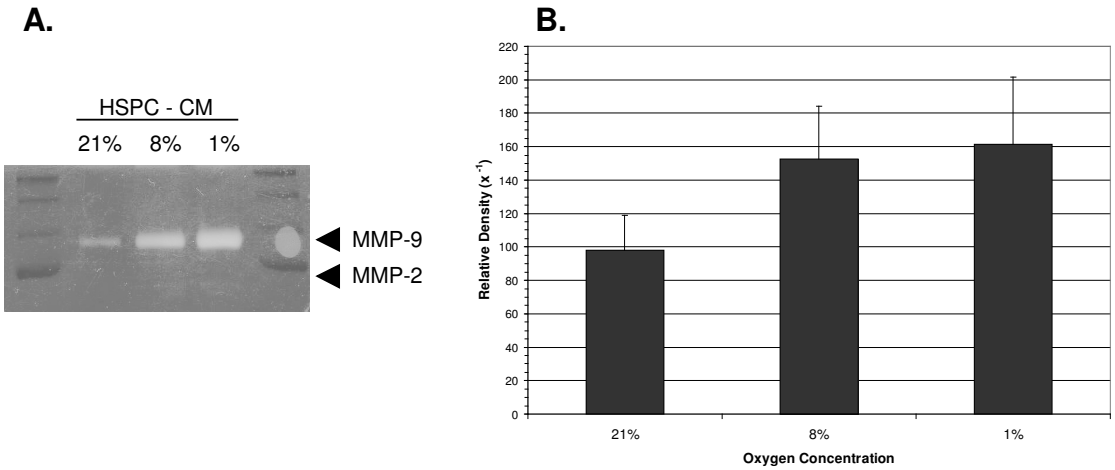


Figure 17. Gelatine zymography following HSPC culture in low oxygen. MMP activity was assessed utilising gel containing 10% gelatine. Secreted proteins present in HSPC-conditioned media were first separated, followed by enzyme reactivation; bands appear where reactivated gelatinases (MMP-2 and MMP-9) digested collagen present in the gel. A representative gelatine zymogram is shown (**A**). HSPC-CM collected during culture in low oxygen concentrations (1% and 8 O_2) contained active MMP-9. Conditioned media from HSPC cultured in a hyperoxic environment (21%) was included for comparison. Densitometry was performed on individual zymograms (n = 5) and the data compiled (**B**). Bars represent standard deviation.

4.9 Placental-Derived Factors Affect HSPC Number

Reducing *in vitro* oxygen concentration dose-dependently decreased HSPC culture expansion in HSPC-GM (see Figure 10). Treatment of HSPC cultures with serum-free conditioned media from trophoblast-enriched (HTR) and stroma-enriched (HPF) cell cultures in physiological oxygen concentrations (1% or 8% O_2) further decreased cell number (Figure 18). Cell number decreased slightly when HSPC were cultured in HPF-conditioned media (HPF-CM). Oxygen concentration had no significant effect on HSPC proliferation in HPF-CM. Incubation of HSPC in HTR-conditioned media (HTR-CM) caused an initial decrease in cell number followed by an increase

in HSPC cell number at Day 14. In contrast to HPF-CM, oxygen concentration had a significant effect on HSPC proliferation in HTR-CM - the lower the oxygen concentration, the greater the HSPC number at Day 14. Seven-day culture of HSPC in serum-reduced media (SRM) resulted in a significant decrease in cell number. Analysis of cell viability demonstrated that culture with SRM greatly reduced HSPC viability.

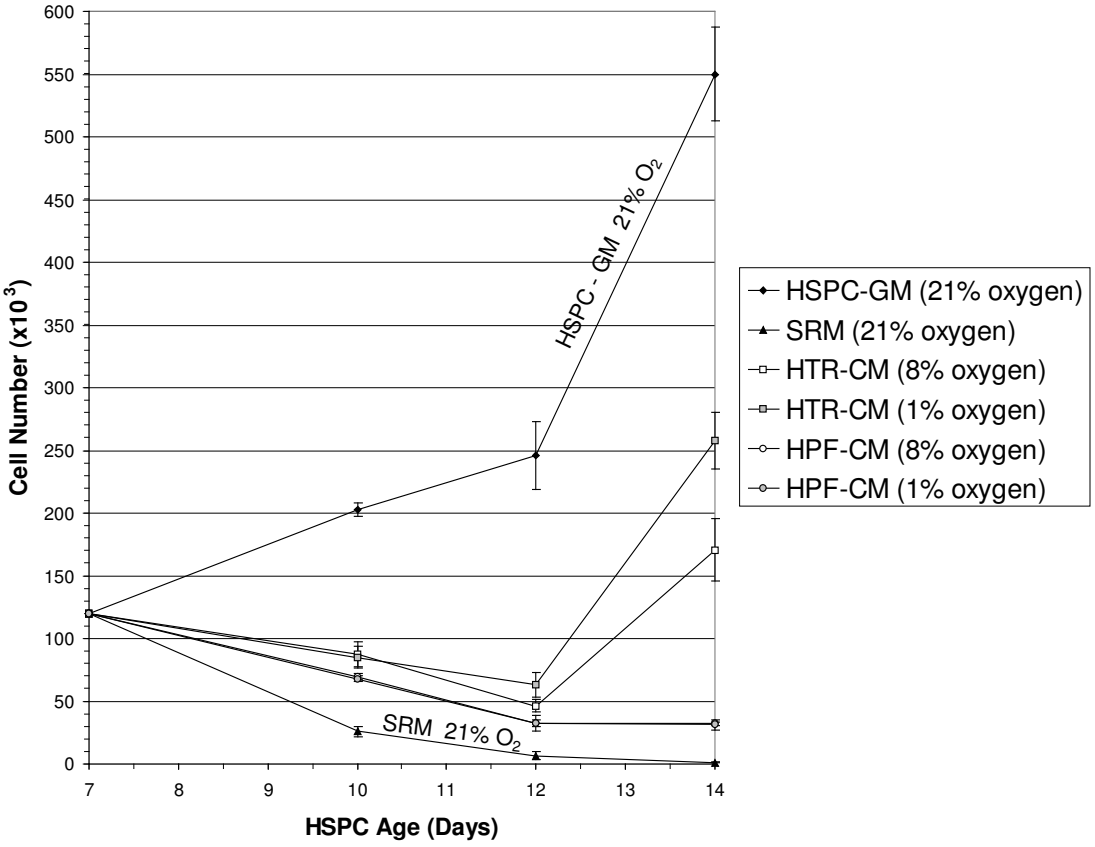


Figure 18. Placental-derived factors and oxygen affect HSPC number. HSPC (Day 7) were cultured with serum-reduced media (SRM) or HSPC Growth Media (HSPC-GM) in ambient oxygen (21% O_2). HSPC were simultaneously cultured in low oxygen (1% or 8% O_2) with trophoblast-conditioned media (HTR-CM) or placental stromal cell-conditioned media (HPF-CM). HSPC number was quantified on Days 10, 12, and 14. HSPC number was affected by culture in HTR-CM and HPF-CM in low oxygen. The decrease in cell expansion was greatest in 1% O_2. On Day 14, cell number increased when HSPC were incubated in HTR-CM (8% and 1% O_2). HSPC culture in SRM resulted in decreased cell number.

These results demonstrate that culture of HSPC in conditioned media from early human placental cells effects HSPC number. HSPC proliferation in trophoblast-enriched (HTR) conditioned media appeared to be affected by oxygen concentration, while HSPC proliferation in trophoblast-deprived (HPF) conditioned media was not affected by oxygen concentration. Neither HTR- nor HPF-CM could expand HSPC cultures as effectively as traditional methods using HSPC-GM in ambient oxygen (21% O_2).

4.10 Placental-Derived Factors Affect CXCR4 Expression on HSPC

Multiparametric flow cytometry was employed to examine CD11b and CXCR4 expression on HSPC cells. Live cells were first gated and isotype-matched control antibodies used to exclude non-specific antibody binding. The percentage of $CD11b^+CXCR4^+$ cells is highlighted (black box in top right quadrant).

Flow cytometric analysis of CXCR4 expression after HSPC expansion (Day 7) demonstrated that only 4% of $CD11b^+$ cells expressed CXCR4 (Figure 19A). Further 7 day expansion in ambient oxygen increased the percentage of $CD11b^+CXCR4^+$ cells to 7% (Figure 19B). In comparison, coculture of HSPC with either placental stroma-enriched (HPF) or trophoblast-enriched (HTR) cell cultures in low oxygen (1% and 8% O_2) markedly altered CD11b and CXCR4 protein expression (Figure 19C). HSPC coculture with HTR or HPF increased the percentage of $CD11b^+CXCR4^+$ cells to approximately 20%. As shown previously in this report, decreasing oxygen tension *in vitro* once again increased the percentage of $CD11b^+$ monocytes in HSPC cultures (see Figure 16). Different expression patterns were noted between the different cocultures. As opposed to coculture with HTR, HSPC coculture with HPF does not significantly alter the number of $CD11b^+$ cells not expressing CXCR4 ($CXCR4^-$). Results from this investigation demonstrate that, along with oxygen, secreted factors present in the placental microenvironment can have significant effects of HSPC phenotype.

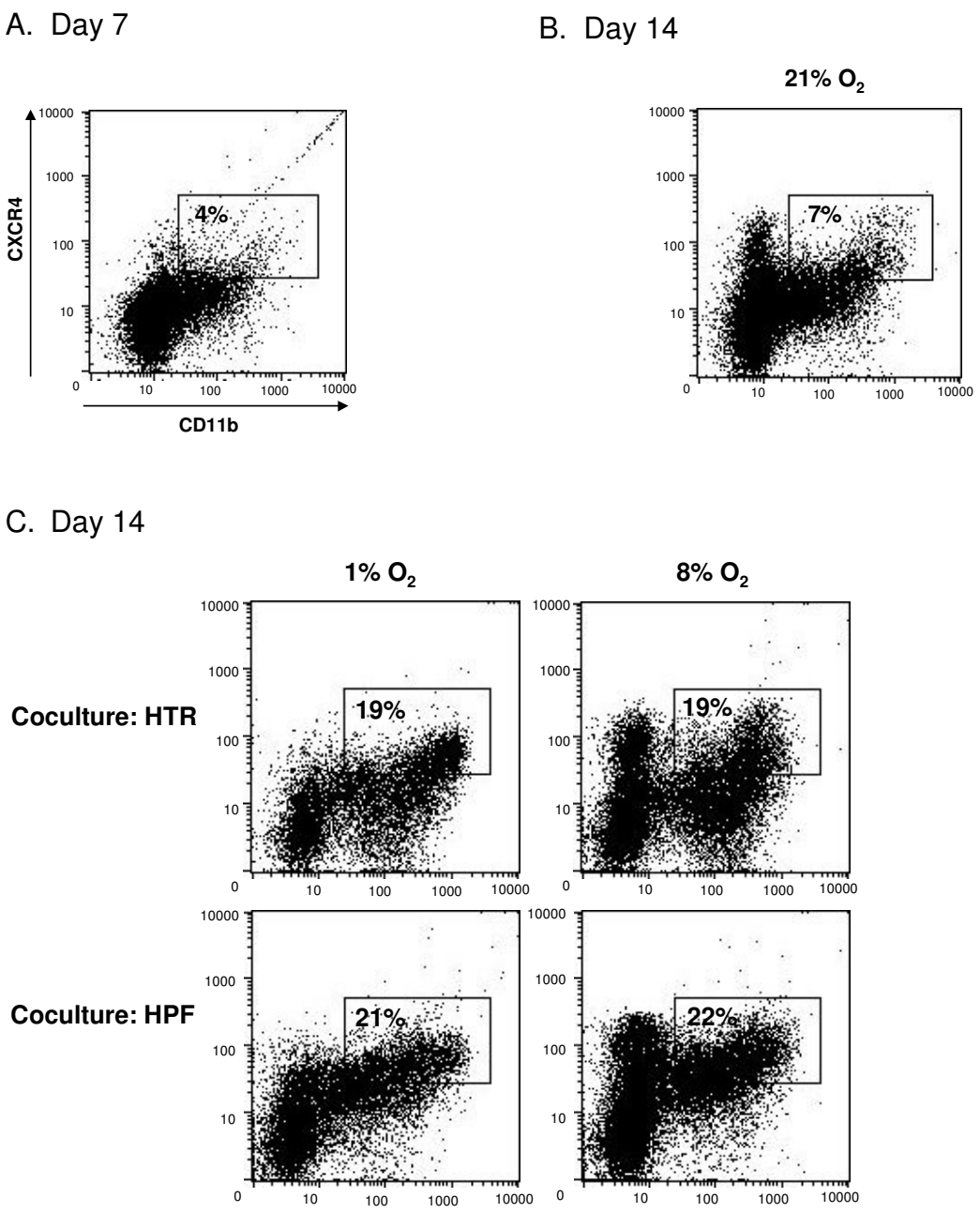


Figure 19. Flow cytometric analysis of CXCR4 expression of expanded HSPC (Day 7) and following coculture with placental cells (HPF and HTR) in low oxygen (1% and 8%). HSPC were subjected to flow cytometric analysis following labelling with anti-CD11b and anti-CXCR4 antibodies. Plots represent gated live cells and demonstrate the percentage $CD11b^{+}CXCR4^{+}$ HSPC (box upper right). Day 7 HSPC were 3% $CD11b^{+}CXCR4^{+}$ (**A**). HSPC were subsequently cultured for a further 7 days with HSPC-GM in atmospheric oxygen (**B**) or cocultured with HTR or HPF in different oxygen concentrations (**C**). When compared to standard HSPC expansion protocol, coculture resulted in significant increases in the percentage of $CD11b^{+}CXCR4^{+}$ cells with no significant differences noted between oxygen concentrations.

4.11 SDF-1α Expression in Human Placenta

Expression of SDF-1α mRNA and protein was investigated in human placental tissue. RT-PCR products from two experiments illustrate SDF-1α mRNA expression in placenta (Figure 20). Results from this experiment clearly demonstrate that SDF-1α is expressed at the mRNA level in early human placental tissue. Expression of SDF-1α mRNA in term placental tissue appears lower than in the first trimester, although direct quantitative analysis was not possible.

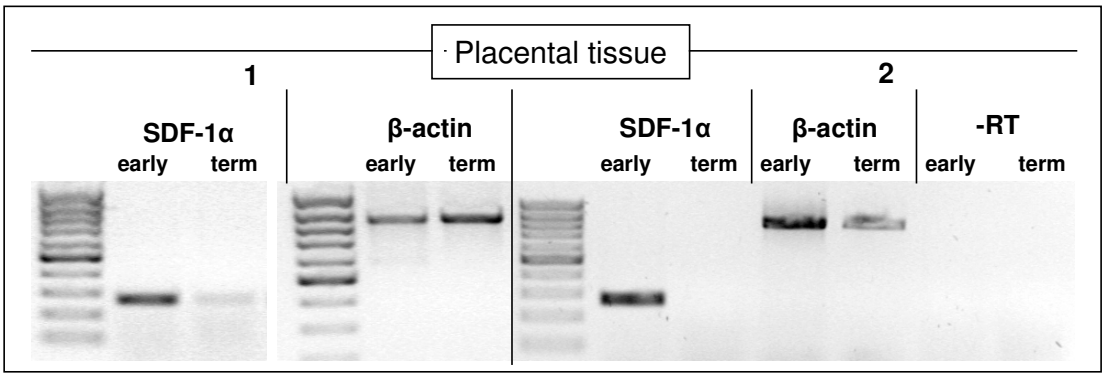


Figure 20. SDF-1α mRNA expression in early and late placental tissue. RNA was extracted from placental tissue of various gestational ages and probed with specific SDF-1α primers. Results from two separate PCR are shown above (1 and 2). SDF-1α mRNA was expressed in early human placental tissue. β-actin and reactions not containing reverse transcriptase (-RT) were used in these investigations as controls.

SDF-1α protein expression was seen throughout human placental tissue in both trophoblast and chorionic villous stromal cells (Figure 21). SDF-1α expression was seen at all time points. Strong SDF-1α expression was noted in a trophoblast layer surrounding the chorionic villi in the earliest tissue sample (6 wpc). Furthermore, SDF-1α was expressed within the chorionic villous stromal core along with the mesodermal cell marker, vimentin. Anti-SDF-1α antibodies stained cell clusters or cords in areas where developing villous blood vessels are located in six-week old placental tissue. At 12 wpc, trophoblast and villous stromal cells continued to express SDF-1α protein. Perivascular regions within the villous core also labelled with SDF-1α

antibodies. This pattern of SDF-1α expression continued throughout gestation. In term placental tissue (38 wpc), vascular structures within the chorionic villi clearly remain SDF-1α positive.

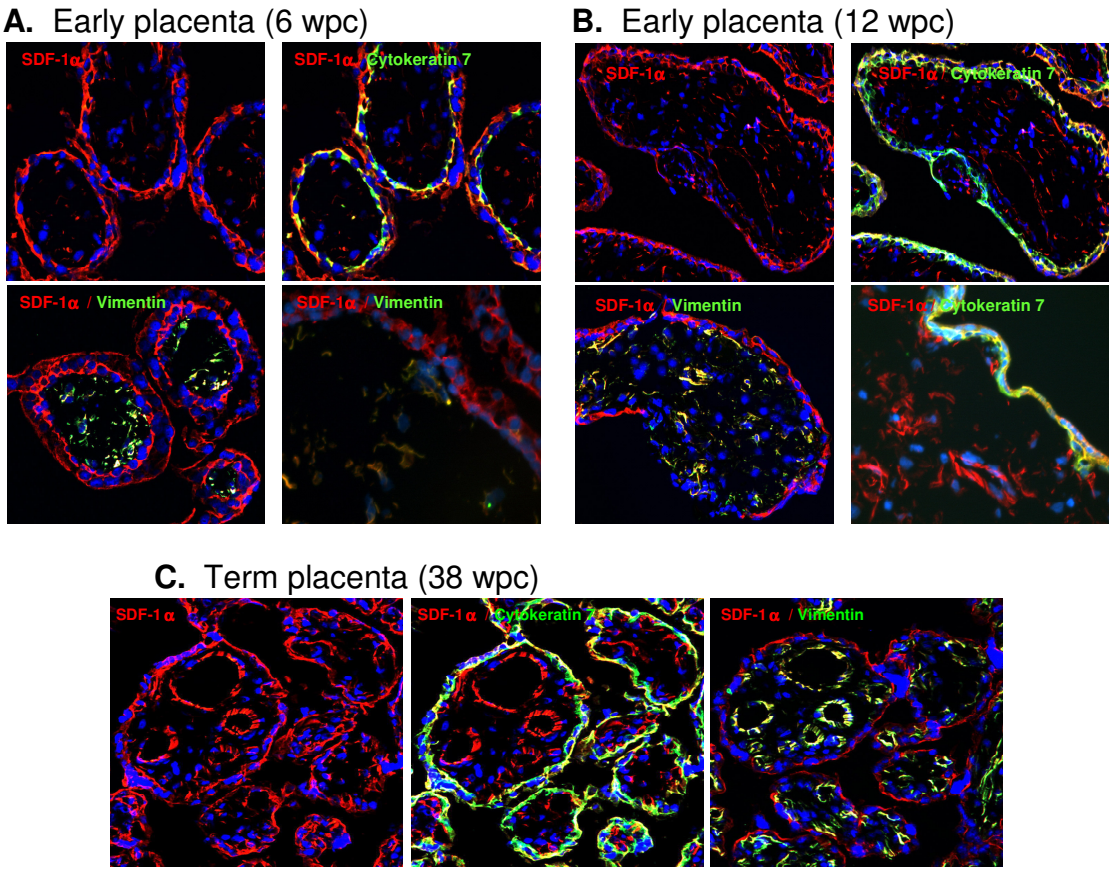


Figure 21. SDF-1α expression in human placental villous tissue. Human placental tissue from 6, 12 and 38 wpc were immunolabelled with anti-human SDF-1α (red). Trophoblast and villous stromal cells were labelled with anti-cytokeratin 7 and vimentin antibodies, respectively (both in green) and double-positive cells can be seen in the merged images (yellow). Multinucleate syncytiotrophoblast cells surrounding the villi expressed SDF-1α at all time points. In the earliest tissue (6 wpc), SDF-1α expression was also noted on vimentin-positive villous stromal cells (**A**). Some double-positive cells appear in cell clumps or cords. In later placental tissue (12 wpc), SDF-1α expression was apparent in perivascular regions (**B**). Blood vessels stained positive for SDF-1α in mature placental tissue (**C**). Original magnification 200X.

4.12 Placental-Derived Cells Express SDF-1α *In Vitro*

RT-PCR and immunochemistry were once again employed to explore expression of SDF-1α mRNA and protein. Purified placental cells, including trophoblast-enriched (HTR) and stroma-enriched (HPF) cell fractions, were examined following 3 day culture in low oxygen (1% or 8% O_2) or atmospheric oxygen (21% O_2). Figure 22A illustrates results from a representative RT-PCR using SDF-1α gene-specific primers. Both HTR and HPF cell cultures expressed SDF-1α mRNA after 3 day culture in a hyperoxic (21% O_2) or low oxygen (1% O_2) environment. Consequently, immunocytochemistry was used to investigate SDF-1α protein expression in placenta stromal cell-enriched (HPF) fractions isolated from human first trimester placental samples. HPF cell cultures were incubated in either 1% or 8% O_2 environments for 2 days before being probed with monoclonal anti-human SDF-1α antibodies. SDF-1α expressing HPF cells (red) and HPF cell nuclei counter-stained with Hoechst (blue) can be seen in Figure 22B. SDF-1α positive cell staining was seen in when HPF cultures were incubated in either 1% or 8% O_2. Protein expression patterns suggest cytoplasmic or extracellular expression. HPF cultures probed with mouse IgG_1 were used to exclude non-specific labelling of secondary antibodies.

These results demonstrate the expression of SDF-1α mRNA and protein in stromal-derived cells isolated from first trimester human chorionic villi. *In vitro* SDF-1α protein expression was shown during culture in low oxygen.

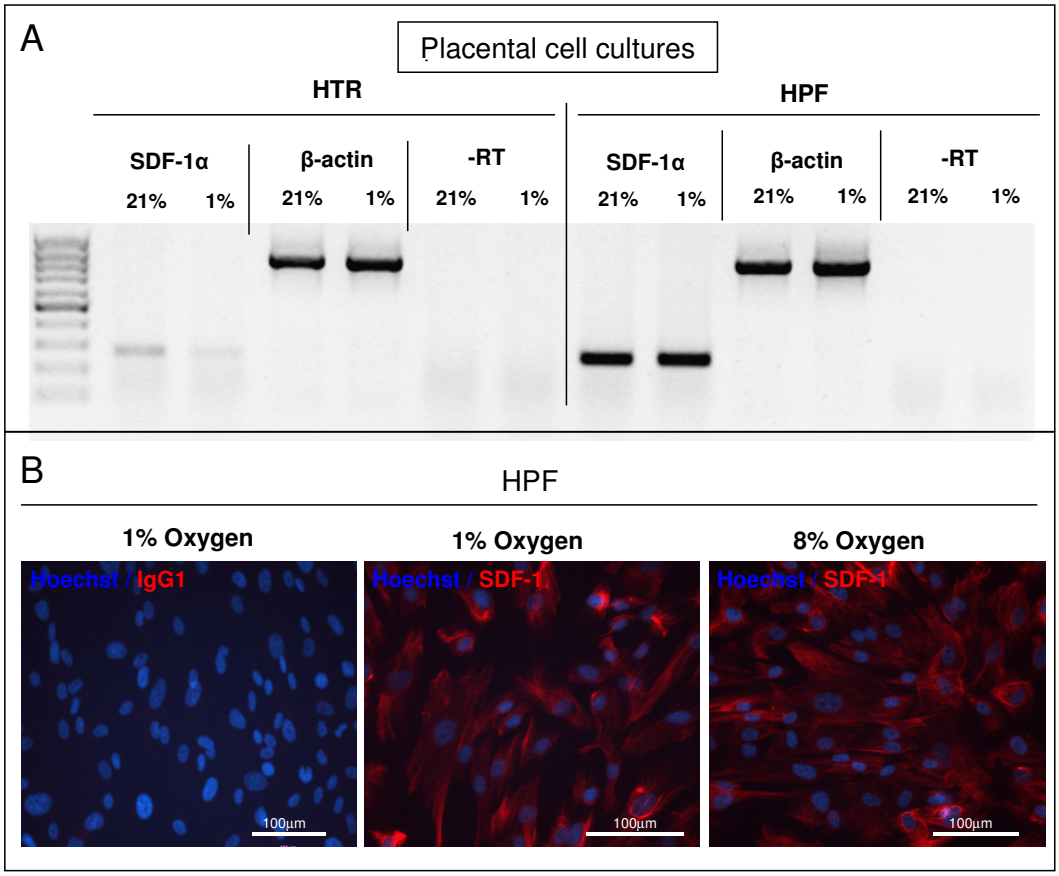


Figure 22. Primary placental cell cultures express SDF-1α mRNA and protein. Trophoblast-enriched (HTR) or stroma-enriched (HPF) were incubated for 3 days in 1% or 8% O_2 or in a hyperoxic environment (21% O_2). Cellular mRNA was isolated and probed for SDF-1α (**A**). Expression of β-actin and reactions where reverse transcriptase was excluded (-RT) were used controls. Both HTR and HPF cultures expressed SDF-1α mRNA. Immunocytochemistry was used to examine SDF-1α expression in HPF cultures cultured in low oxygen (**B**). Fluorescently (PE)-labelled secondary antibodies were used to recognise bound anti-SDF-1α antibodies (red); cell nuclei were stained with Hoechst (blue). Replacement of anti-SDF-1α antibodies with mouse IgG was used as a control (Hoechst/IgG1). Original magnification 200X.

4.13 Placental Cells Secrete SDF-1α Protein

A highly sensitive SDF-1α immunoassay was utilised to determine protein expression levels in media conditioned during culture with different placental-derived cells. Media collected from mixed first trimester placental cell cultures in atmospheric oxygen contained approximately 2-fold more SDF-1α than media conditioned by mixed term placental cells (Figure 23). Likewise, conditioned media from primary and trophoblast tumour cell lines

were analysed. SDF-1α protein was secreted over a 24 h period into media conditioned by both HTR and HPF cells (Figure 23). However, secreted SDF-1α protein was not detected in media conditioned by Jeg3 choriocarcinoma cells. Although SDF-1α protein was detected in conditioned media from trophoblast-enriched (HTR) cell cultures (6.1 ± 0.1 pg/ml), the placental stroma-enriched fraction (HPF) produced considerably greater levels of SDF-1α under these conditions (452.2 ± 0.2 pg/ml).

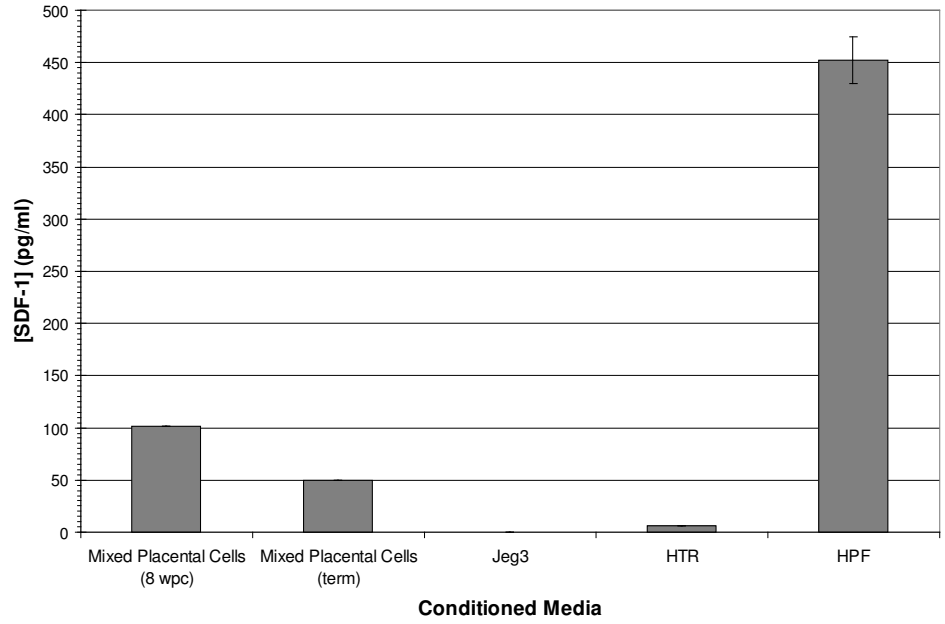


Figure 23. SDF-1α protein secretion by placental-derived cell cultures. Conditioned media was collected following 24-hour incubation and secreted SDF-1α protein assessed utilising ELISA. Media was conditioned during culture with mixed placental cell cultures from early (8 wpc) or term placentas (37 wpc), Jeg3 choriocarcinoma cells, trophoblast-enriched (HTR) or placenta stroma-enriched (HPF) cell fractions from first trimester placentas. SDF-1α was produced and secreted by various placental cells with the greatest SDF-1α production seen in media conditioned by HPF.

To examine whether *in vitro* oxygen level affected SDF-1α protein secretion, placental stroma-enriched (HPF) cultures were incubated for 48 h in low oxygen (1% and 8% O_2) and conditioned media collected. SDF-1α levels were quantified (Figure 24). HPF cultures in hyperoxic tissue culture conditions (21% O_2) were included for comparison. Secretion of SDF-1α protein into conditioned media was greatest when HPF were cultured in

normal atmospheres (21% O_2). However, SDF-1α protein was also found when HPF were incubated in physiological oxygen concentrations. Media conditioned by HPF in 8% O_2 contained 895.3 ± 32.0 pg/ml SDF-1α protein; conditioned media from HPF cultures incubated 48 h in 1% O_2 contained 635.3 ± 42.0 pg/ml SDF-1α protein.

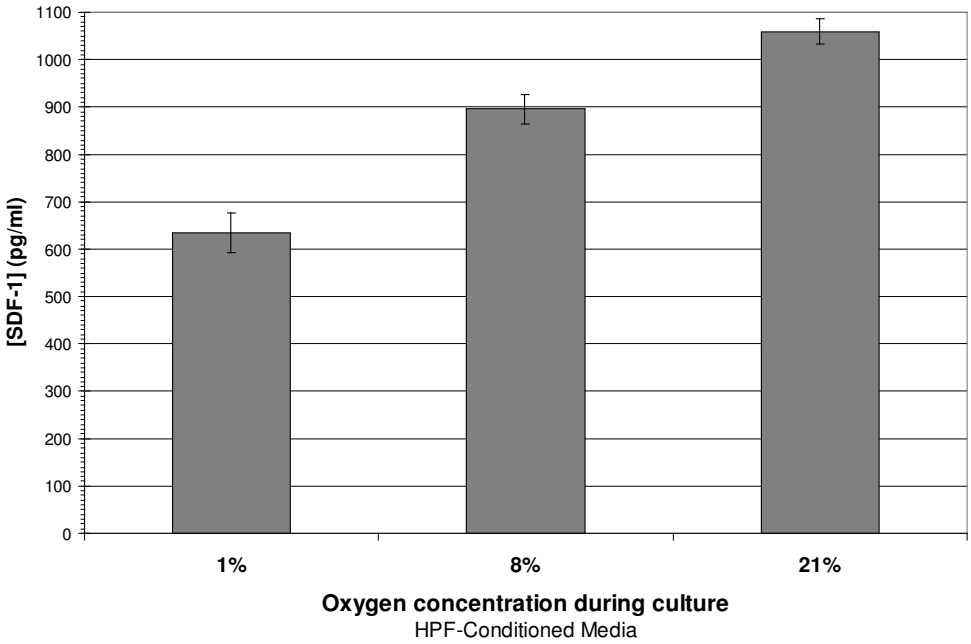


Figure 24. SDF-1α protein secretion by chorionic villous stromal cells (HPF) in low oxygen. ELISA measured secreted SDF-1α protein in media conditioned during 48 h incubation with HPF in low oxygen (1% or 8% O_2) or atmospheric oxygen (21% O_2). The greatest amount of SDF-1α was secreted during HPF culture in hyperoxic conditions (1059 ± 26 pg/ml). Lower levels of SDF-1α were measured in media conditioned during culture in simulated placental oxygen concentrations (635 ± 42 and 895 ± 32 in 1% and 8% O_2, respectively). Standards and samples examined in triplicate; error bars represent standard deviation. A representative experiment is shown.

These results provide further evidence that cells in the developing placenta express and secrete SDF-1α protein, suggesting a role for SDF-1α during early placental development. As demonstrated with RT-PCR, immunochemistry and ELISA, placental stromal (HPF) cells express SDF-1α protein. In this model, HPF cells not only expressed SDF-1α in the hyperoxic environment encountered during tissue culture, but also in simulated placental oxygen concentrations found during early development.

4.14 Placental-Derived Factors Stimulate HSPC Migration

HSPC *in vitro* migration was quantified with a modified Boyden chamber assay. In all experiments, migration of cells in serum-reduced media (SRM) was considered basal HSPC migration. When compared to basal migration, addition of trophoblast-conditioned media (HTR-CM) to the assay stimulated HSPC migration (Figure 25). HTR-CM also stimulated migration above levels induced by 50 ng/ml recombinant SDF-1α. Treatment of HSPC with placenta stromal cell-conditioned media (HPF-CM) also stimulated migration above both basal levels and SDF-1α-induced levels. No significant differences were noted between HSPC migration towards HTR-CM or HPF-CM. This experiment demonstrates paracrine stimulation of HSPC migration by both trophoblast and placenta stromal cells. To assess SDF-1α involvement in the stimulation of migration by HTR-CM or HPF-CM, the specific CXCR4 inhibitor AMD3100 was included in the assay. In this investigation, AMD3100 was shown to inhibit SDF-1α stimulation of migration as the combination of recombinant human SDF-1α and AMD3100 reduced HSPC migration to basal levels. Although no change was seen with the combination of AMD3100 and HTR-CM, the CXCR4 antagonist did, in fact, reduce HPF-CM stimulated migration. This suggests the involvement of placenta stromal-derived SDF-1α in the stimulation of HSPC migration in this investigation. However, SDF-1α does not appear to be the only chemotactic stimulus in the developing placenta, as AMD3100 was unable to reduce HSPC migration to basal levels.

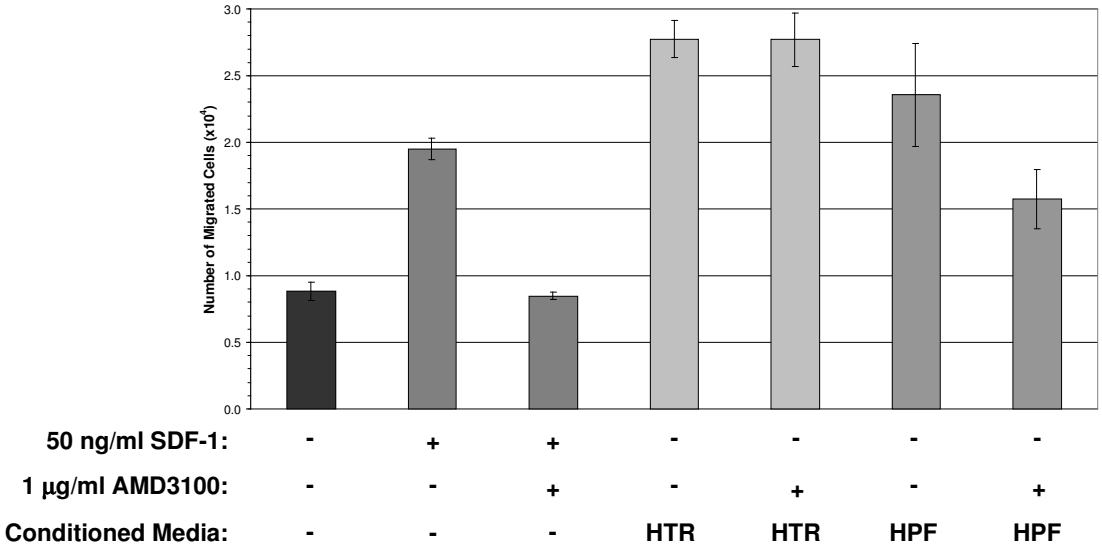


Figure 25. Effect of factors secreted by placental cells on HSPC migration. HSPC were cultured for 8 h and the ability of HTR- or HPF-CM to stimulate migration quantified. Culture of HSPC in the absence of all factors (SRM alone) was considered basal migration. Wells containing 50 ng/ml SDF-1α were used as positive control. The involvement of SDF-1α in the conditioned media-stimulation of migration was examined by including the specific CXCR4 antagonist, AMD3100. Treatments were performed in triplicates; error bars represent standard deviations. A representative experiment is shown.

4.15 Placental-Derived Factors Stimulate HSPC Transendothelial Migration

Further investigations determined whether placental stroma-derived SDF-1α stimulated HSPC transendothelial migration. Following 24 h incubation in either 1% or 8% O_2, HPF stimulated HSPC transendothelial migration above basal levels (Figure 26). Significant differences were not seen between oxygen concentrations. Similar levels of HSPC transendothelial migration were seen when 50 ng/ml SDF-1α was included in the assay. Addition of 1 µg/ml AMD3100, along with HSPC to the upper well of the migration chamber significantly decreased HPF-stimulated HSPC transendothelial migration. This suggests involvement of SDF-1α in the induction of HSPC migration by HPF. As shown for migration, blocking SDF-1α - CXCR4 interactions with AMD3100 did not fully abrogate HPF stimulation

of HSPC transendothelial migration, implying the involvement of other placental-derived factors.

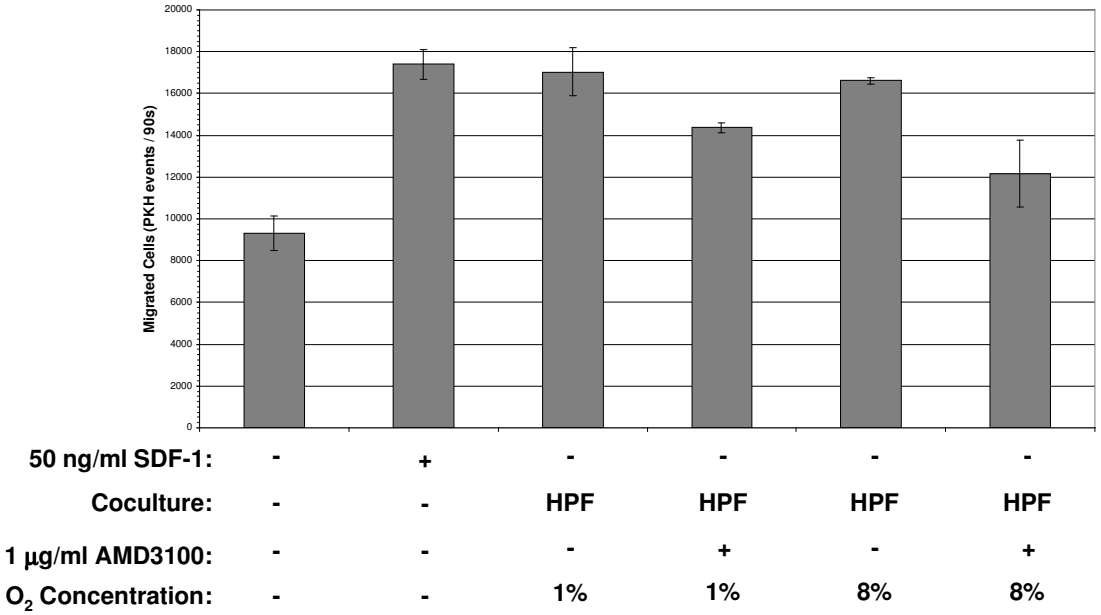


Figure 26. Effect of placental stroma derived factors on HSPC transendothelial migration. Fluorescently labelled HSPC (PKH-HSPC) were cultured for 12 h and their ability to migrate through a HUVEC monolayer quantified. The ability of placental stromal (HPF) cells incubated in 1% or 8% O_2 to act as a migratory stimulus was tested utilising coculture. Basal HSPC transendothelial migration was considered the absence of all factors (SRM). Addition of 50 ng/ml SDF-1α to the assay was used as positive control. Involvement of SDF-1α in the stimulation of migration was examined by including the specific CXCR4 antagonist, AMD3100. Treatments were performed in triplicates; error bars represent standard deviations. A representative experiment is shown.

4.16 HSPC Adhesion to Placental Stromal Cells Involves SDF-1α

Coculture of fluorescently labelled HSPC with various placental-derived cells was utilised to determine whether HSPC adhered to the various cellular components present in developing human placenta. HSPC adhered to mixed placental cells isolated from early human placental tissue (8 wpc), Jeg3 choriocarcinoma cells, trophoblast-enriched (HTR) and stroma-enriched cells when cocultured 48 h in a hyperoxic environment (Figure 27). Relative adhesion varied among the tested cell fractions. Minimal cell-cell adhesion

was measured when HSPC were cocultured with either mixed cells from early placenta (8 wpc) or Jeg3 cells. A significant increase in adhesion was seen when trophoblast-enriched HTR cells were included in the assay. The greatest cell-cell adhesion was measured when HSPC were cocultured with placental stroma cells (HPF). In fact, relative adhesion of HSPC to HPF in normal tissue culture conditions was greater than 2-fold when compared to HSPC adhesion to HTR.

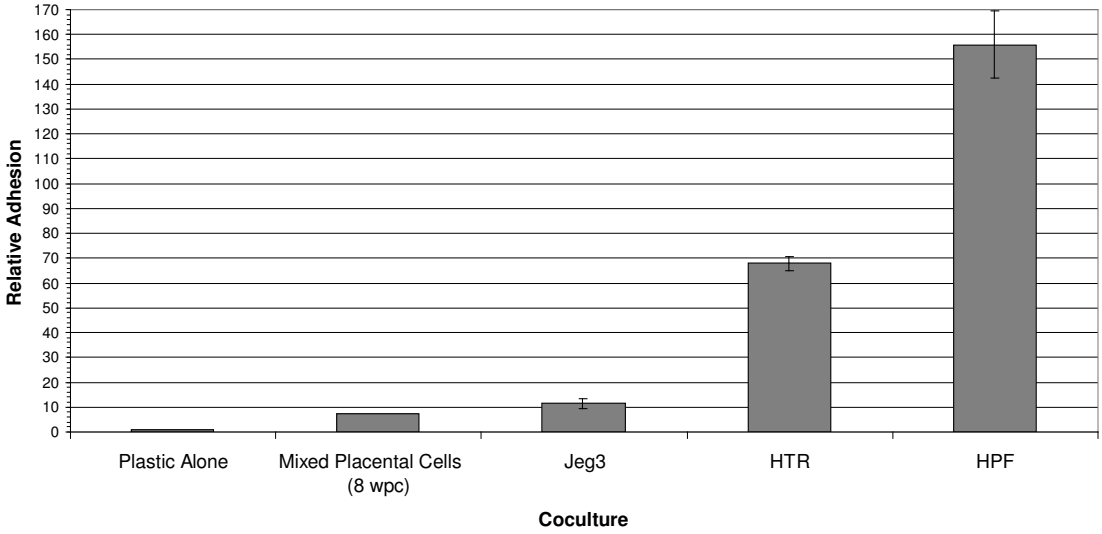


Figure 27. HSPC cell-cell adhesion to placental-derived cells. Fluorescently labelled HSPC were cocultured for 6 h with mixed placental cells, Jeg3 choriocarcinoma cells, HTR or HPF. Following coculture, nonadherent cells were removed and number of adherent HSPC quantified utilising flow cytometry. HSPC adhesion to wells containing no additional cells (plastic alone) was set to 1 and relative adhesion shown. HSPC adhered to all cell cultures tested, although the greatest level of cell-cell adhesion was seen during HPF coculture. Treatments were performed in triplicate; error bars represent standard deviation. A representative experiment is shown.

The effect of low oxygen on HSPC adhesion was also investigated. The greatest number of HSPC adhesion to HTR was seen when cocultures were performed in 1% O_2 (Figure 28), demonstrating that physiological oxygen has a positive effect on HSPC adhesion to trophoblast. In fact, the level of relative adhesion of HSPC to HTR in 1% O_2 was similar to HSPC-HPF adhesion. In 8% O_2, HSPC also adhered to HTR. AMD3100 did not significantly reduce relative HSPC-HTR adhesion. On the contrary, adhesion of HSPC to HPF was not affected by oxygen concentration. Furthermore,

addition of AMD3100 decreased the relative adhesion of HSPC to placental stroma cells, implicating SDF-1α involvement during HSPC adhesion to placental stromal cells.

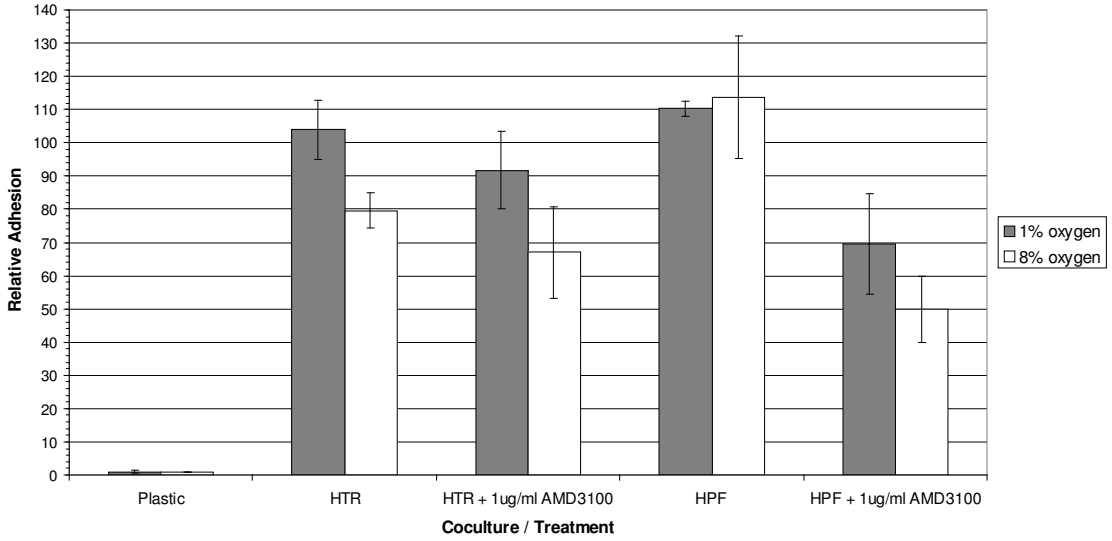


Figure 28. HSPC cell-cell adhesion with placental-derived cells (HTR or HPF) in low oxygen (1% or 8% O_2). Fluorescently labelled HSPC were cocultured for 12 h in the appropriate oxygen concentration (1% or 8% O_2) with HTR or HPF. Following coculture, non-adherent cells were removed and number of adherent HSPC quantified utilising flow cytometry. HSPC adhesion to wells containing no additional cells (plastic alone) was set to 1 and relative adhesions shown. HSPC adhesion to trophoblast cells appeared to be affected by oxygen concentration with the greatest HSPC-HTR adhesion seen in the lowest oxygen concentration (1% O_2). SDF-1α did not appear to play a role in adhesion of HSPC to HTR as no significant change was noted when the CXCR4 inhibitor AMD3100 was included in the assay. HSPC adhesion to HPF cells was not affected by oxygen concentration. AMD3100 significantly reduced HSPC adhesion to HPF. Treatments were performed in triplicate; error bars represent standard deviation. A representative experiment is shown.

4.17 HSPC Integrin Expression is Altered in Low Oxygen

Alterations in HSPC adhesion (see Figure 28), as well as, changes in CD11b (α_M integrin) expression (see Figure 16) suggested that HSPC integrin expression was affected during culture in low oxygen. As previously demonstrated, the expression of α_M integrin subunit (CD11b) increased when HSPC were cultured 7 days in simulated placental oxygen tensions (Figure 29); α_M expression was greatest in environments containing 1% O_2. More than 75% of HSPC expressed the β_2 subunit. No significant differences in β_2 expression were noted between oxygen treatments. Expression of α_4 subunit was also affected during culture in low oxygen environments. Conversely, 7 day culture of HSPC in 8% O_2 resulted in a greater increase in α_4 integrin expression when compared to HSPC cultured 1% O_2. Almost all HSPC expressed the β_1 subunit with no differences noted between oxygen concentrations.

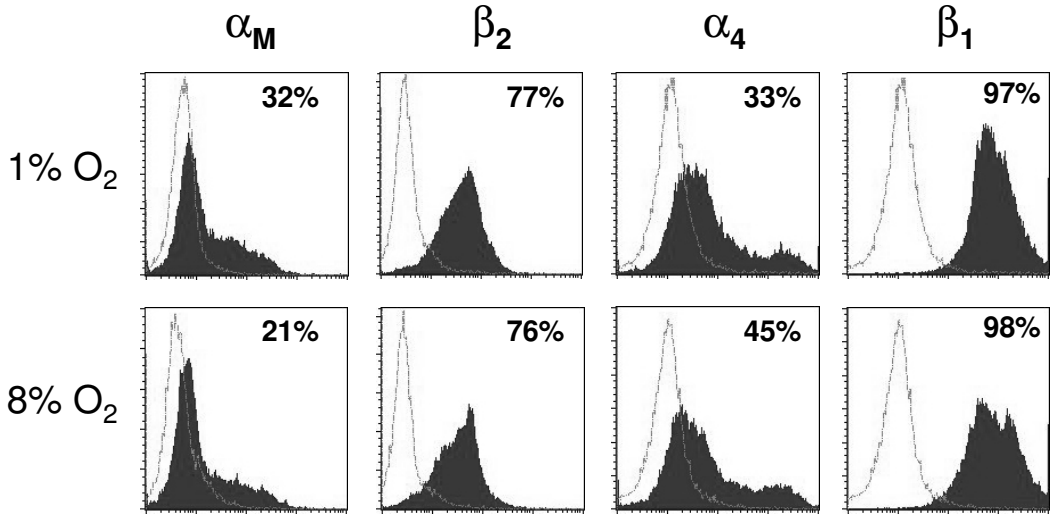


Figure 29. HSPC integrin expression following culture in low oxygen. HSPC were cultured for 7 days in low oxygen (1% or 8% O_2) and the expression of α_M, α_4, β_1 and β_2 integrin subunits on Day 14 examined with flow cytometry. Percentages in top right corner illustrate positive cells. Dotted lines represent the appropriate isotype controls.

5 DISCUSSION

Current understanding of the development of the human feto-placental vascular system is based, almost exclusively, on descriptive structural and immunohistochemical studies. *In vitro* approaches are under-represented due to problems inherent to the placenta, such as ethical limitations and limited availability of early human placental tissue. Likewise, the recent suggestion that the human placenta constitutes a stem cell niche has not attracted much attention, possibly due to the same inherent problems. We describe a novel *in vitro* examination of the human placenta as a HSPC niche. The impact of oxygen and the involvement of SDF-1α during establishment of the placental niche were specifically examined.

5.1 The Human Placenta as a Stem Cell Niche

Hematopoietic cells develop in the embryo in several anatomic locations. Co-ordinated hematopoietic sites ensure both rapid production of differentiated blood cells for immediate needs and establishment of a large pool of undifferentiated stem cells. Hematopoiesis is first observed in the human yolk sac at day 18.5 of pregnancy (Palis and Yoder, 2001). The sites of human hematopoiesis then change throughout embryogenesis and a dogma exists that considers the linear movement of definitive HSC from the aorta-gonads-mesonephros (AGM) region to the fetal liver and finally the bone marrow (BM). However, the ability of the yolk sac and AGM to supply all HSC in the fetal liver has been questioned and the contribution of yet another source of HSC has been suggested (Tavian et al., 2001).

The first evidence of transplantable hematopoietic activity in murine placenta can be found in early transplantation studies by Till and McCulloch (1960) and Dancis and colleagues (1968, 1977). The ability of placental cells to contribute to recipients' hematopoiesis was similar to adult BM, was not dependent on continuous blood supply to placenta and ceased by the end of gestation. These exciting findings in mice were not actively followed up until recently. In 2003, it was demonstrated that the mouse placenta contains multi-potential clonogenic progenitors before colonisation of the liver is initiated (Alvarez-Silva et al., 2003). The most recent evidence has shown

that mid-gestation murine placenta harbours a large pool of HSC with the capacity to self-renew and repopulate the entire hematopoietic system of irradiated mice (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). No signs of hematopoiesis were seen before mesodermal cells originating from the extra-embryonic allantois has fused to the chorionic plate. It is difficult however, to directly relate events during murine placentogenesis to humans as placental development between both species differs significantly.

Some of the first cytological evidence for the appearance of HSC in the human placenta were described using transmission electron microscopy (Demir et al., 1989). The structure of early placental tissue was examined and the appearance of hematopoietic cells in the earliest stages of placental development was demonstrated; expression of HSC markers was not examined. More recently, Challier et al. (2005) provided immunocytological evidence for hematopoiesis in one month-old human placental tissue. Near the end of the first month (25 dpc) typical figures of hematopoiesis, comparable to those described for the yolk sac of the human embryo, were seen. At this time point, placental tissue did not contain cells expressing the hematopoietic marker CD45. Similar results were shown in human placental tissue at 6-7 wpc. By the end of the first trimester (12-14 wpc), $CD45^+$ cells could be found in the human placenta. The expression in early of early markers of primitive and definitive hematopoiesis, such as GATA-2 and CD117 (c-kit), further supported the concept of early placental hematopoiesis. It was also stated that anti-CD34 antibodies did not stain chorionic villi at early time points.

CD34 is a marker for HSPC and is commonly used during the isolation of both hematopoietic stem cells and endothelial progenitor cells (Peichev et al., 2000; Pomyje et al., 2003). In this investigation, approximately 90% of freshly isolated HSPC expressed CD34. Immunohistochemistry also demonstrated that the chorionic villous stroma from six-week old human placental tissue contained $CD34^+$ cells. At this early developmental time point, placental vascular development has been underway for approximately three weeks (Demir et al., 1989). Previous reports have also used CD34 to distinguish early vascular cells in the developing placenta (Challier et al., 2004; te Velde et al., 1997). Thus, immunohistochemical investigations

utilising only CD34 as a marker for HSPC, as reported here, cannot clearly distinguish between HSPC and early vascular cells. Coexpression of other specific HSPC markers is needed. In this investigation, the use of anti-CD133 antibodies to probe for HSPC in placental tissue was excluded due to previous reports that trophoblast also express this marker (Potgens et al., 2001). Attempts to examine the coexpression of other HSPC markers in early human placental tissue were also unsuccessful due to problems, such as non-specific labelling of the available antibodies (e.g. CD117). It would also be of great interest to examine earlier tissue (i.e. >6 wpc) for the appearance of progenitor cells, although the lack of tissue samples prohibited this in our investigation.

5.2 Human Placental Development in Low Oxygen

Placental tissue is plastic and changes constantly throughout gestation to meet and supply the increasing metabolic needs of the growing fetus. The stem cell-rich blastocyst begins as a trophectodermal cell layer surrounding the inner cell mass. After implantation, the human placenta reaches its terminal size short after the end of the first trimester (12 wpc). During this initial period of rapid placental growth, oxygen tensions remain strikingly low. Measurements made with polarographic electrode probes demonstrated that, in the intervillous space prior to 8 wpc, pO_2 is less than 20 mmHg; after 12 wpc placental pO_2 is greater than 50 mmHg (Rodesch et al., 1992; Jauniaux et al., 1999; Jauniaux et al., 2001).

Although the first trimester pO_2 appear quite low, they represent normal values in uncomplicated pregnancies and are relevant for that location and time of development (Charnock-Jones et al., 2004; Kaufmann et al., 2004). Therefore, *in vitro* models should take into account normal physiological pO_2 present during placental development. Concurrent measurements acquired in this investigation during culture of both HSPC and placental cells clearly demonstrate that normal placental oxygen tensions found during placental development can be effectively mimicked *in vitro*. The use of two different gas mixtures, containing either 1% oxygen or 8% oxygen (5% CO_2), simulated *in vivo* oxygen tensions with surprising precision. Measurements made here

also clearly demonstrate that traditional cell culture in atmospheric oxygen (21% O_2) results in a hyperoxic environment with oxygen tensions well above normal pO_2 found in human tissues (157.9 ± 9.2 mmHg).

The use of low oxygen gas mixtures is not novel in placental research as oxygen reduced environments have previously been used to assess trophoblast development and function (Genbacev et al., 1997; Graham et al., 1998; Caniggia et al., 2000). One important difference, however, is that previous investigations did not include real-time oxygen monitoring. As clearly demonstrated in this investigation, oxygen concentrations in low oxygen cell culture systems are in a constant flux and are difficult to maintain at physiological levels. Accordingly, standardisation of experimental parameters (e.g. cell number, media depth and composition, gas replacement intervals, etc.) and extensive testing was required to ensure that actual physiological oxygen concentrations were reached and then maintained. Using standardised protocols, variability between experiments was reduced. Furthermore, and as opposed to previously published results, the SDR oxygen monitoring system provided reliable data concerning *in vitro* oxygen levels during the entire experimental period.

5.3 Low Oxygen Affects HSPC Culture Dynamics

Although tissue culture in 21% oxygen is most frequently used, there is a growing body of evidence suggesting that the use of physiological oxygen levels during cell culture provides a better picture of what is occurring *in vivo*. The effects of low oxygen on cell proliferation and cell cycle kinetics has been previously studied and the results appear to vary depending on oxygen concentration and cell type (reviewed by Csete, 2005).

The exact composition of the media used during *in vitro* HSPC culture varies among different laboratories and a lack of consensus exists as to which factors are essential to maintain stem cell number while inhibiting differentiation. In our laboratory, the HSPC culture media consisted of IMDM (10% FCS) supplemented with TPO, SCF, and Flt3L (HSPC-GM). This particular composition has been previously shown to maintain HSC in culture (McGuckin et al., 2004; McGuckin et al., 2005). Previous publications from

our laboratory have also shown that HSPC culture in ambient oxygen with HSPC-GM results in extensive expansion of HSPC cultures and can maintain cultures for >60 days as assessed by trypan blue exclusion (Baal et al., 2004). In this report, culture with HSPC-GM likewise stimulated HSPC proliferation in 21% oxygen during a seven-day proliferation assay. Results from this assay also demonstrated that when HSPC were cultured in physiological pO_2 cell proliferation was lower. Decreased HSPC culture expansion in low oxygen was dose-dependent with the greatest decrease noted when cells were cultured in oxygen concentrations that mimicked early placental development (1% O_2). The use of a Casy® Counter for this particular proliferation assay allowed cell death to be concurrently assessed; low oxygen did not result in significant alterations in HSPC cell death.

Similar results have been previously described. Using $CD34^+$ cells isolated from adult peripheral blood, Ivanovic and colleagues (2000a, 2000b) have demonstrated that HSPC proliferation was reduced in 1% oxygen. Decreased HSPC culture expansion did not occur at the expense of progenitor cell self renewal. In fact, cd34 mRNA processing and CD34 protein expansion was enhanced in low oxygen environments (Brunet et al., 2006). It was therefore suggested that the enhanced proliferation of HSC in low oxygen may, in part, reflect the normal relatively low oxygen environment of stem cells in their *in vivo* environment – the adult BM. Models of marrow architecture suggest that HSC normally reside in a low oxygen niche (Chow et al., 2001). Delayed proliferative senescence in low oxygen environments with a concurrent reduction in oxidative DNA damage could play a role, although further investigation is needed.

Removal of cells from active cell cycling results in decreased cell proliferation and thus, the transition of cells from G1/M/G2 to the G0 phase can decrease cell number. In the case of HSC, the most primitive stem cells have been shown to be in the resting state (i.e. G0) with a G1/S progression representing a developmental switch between HSC self-renewal and differentiation (Szilvassy et al., 2000; Thornley et al., 2001; Stein et al., 2004). Results in this report demonstrated the effect of placental oxygen tensions on HSPC cell cycle status, where low oxygen rapidly increased the percentage of G0 HSPC. The rapid increase in the number of resting HSPC seen in

placental oxygen concentrations may be partially responsible for the dose-dependent decreases seen in HSPC culture expansion. It is logical to assume that the quicker cells remove themselves from active cycling, decreases in HSPC number would increase over time. In fact, a lower cell number was seen following seven-day culture in 1% oxygen.

Cell death is another factor when determining cell number. The toxic effects of oxygen and more specifically, reactive oxygen species (ROS) on HSC are well described (Ito et al., 2004; Ito et al., 2006). It has even been suggested that ROS may be a major factor driving cellular ageing (Harman, 1956). In our system, HSPC culture in hyperoxic environments did not significantly stimulate apoptosis. HSPC apoptosis was apparently unaffected by low oxygen culture.

The impact of physiologically low oxygen on HSPC dynamics has been demonstrated in this investigation. When different placental oxygen levels are simulated *in vitro*, HSPC transition to resting stem cells occurs. Over time, this results in a reduction of total HSPC number without significantly affecting cell death and/or apoptosis. These results provide further proof that culture of HSPC in physiologically relevant oxygen concentrations can better recreate their *in vivo* environment, perhaps providing a better picture of HSPC dynamics in the stem cell niche.

5.4 HSPC Differentiation in Physiological Oxygen Concentrations

Oxygen has known effects on the regulation of gene expression. In this report the expression of specific genes was examined to determine whether the culture conditions tested had any effects on HSPC differentiation. The genes examined here were chosen due to their involvement in both hematopoiesis and vascular development. In fact, their expression has been noted in the hemangioblast - a common precursor during hematopoietic and endothelial cell development (Choi et al., 1998; Chung et al., 2002; Lacaud et al., 2002). It must be noted, however, that the non-quantitative nature of the RT-PCR results did not allow for solid conclusions to be made about expression levels. Thus, the results obtained from PCR were used simply as a guide for gene expression and to provide clues for changes in HSPC

differentiation. The alterations noted in during HSPC culture in physiological oxygen levels suggested that low oxygen effects HSPC differentiation. These results also suggested that a further examination of HSPC differentiation, through the quantification of protein expression, was necessary.

Recent reports have demonstrated that culture in low oxygen can help maintain pluripotency, a key feature of stem cells. For example, it is well known that ES cells cultured in atmospheric oxygen tend to differentiate spontaneously. However, when ES differentiation was monitored in low oxygen (3% or 5% O_2), cells expressed pluripotency-associated genes (e.g. Oct4) and the expression of differentiation-associated genes and proteins was reduced (Ezashi et al., 2005). Furthermore, the ability of murine HSPC to repopulate bone marrow was enhanced when HSPC were cultured in 1% O_2 (Ivanovic et al., 2000a). In all conditions tested, the percentage of cells expressing stem cells markers decreased over the seven-day period. This is not so unexpected when considering the increases in HSPC cell number due to cell proliferation. Thus, a decrease in the percentage of HSPC expressing the stem cell markers may represent dilution of the stem cells pool. It was shown that the $CD133^+CD34^+$ and $CD133^+CD117^+$ populations were maintained over the entire culture period. Surprisingly, no significant changes in HSPC marker expression between treatments were shown. Further investigations are needed to determine whether HSPC cultured in low oxygen (1% and 8% O_2) or hyperoxia have different functional clonogenic potential or repopulation abilities. Such studies were beyond the scope of this investigation.

Sustaining a balance between self-renewal and differentiation is another inherent property of stem cells. During culture in physiological oxygen tensions the HSPC pool was maintained with a relatively constant number of cells continuing to express known stem cell markers while undergoing low levels of proliferation. Nonetheless, increasing cell number suggested that a population or populations of HSPC were undergoing differentiation.

Consistent with a major role for hypoxia in vasculogenesis and angiogenesis, a large number of genes involved in different steps of angiogenesis are independently responsive to hypoxia in tissue culture. The

effect of hypoxia on the vascular endothelial growth factor (VEGF) axis has received special interest in diverse fields such human development and tumour biology. Most of these studies have focussed on hypoxia-inducible factor (HIF-1)-1α, a transcriptional activator that functions as a master regulator of cellular and systemic oxygen homeostasis. Low oxygen stabilises HIF-1α, which in turn drives expression of VEGF, VEGF receptor-1 (VEGFR-1; a.k.a. Flt-1) and a VEGF isoform, placental-derived growth factor (PlGF). Thus, the influence of hypoxia on endothelial progenitor cells and endothelial cells has been well established. $CD34^+$ cells coexpressing VEGFR-2 and the HSPC marker CD133 have been isolated from human peripheral blood, UCB and fetal liver (Miraglia et al., 1997; Gallacher et al., 2000). Circulating $CD133^+$ cells can even be differentiated to endothelial progenitor cells (EPC) *in vitro* (Peichev et al., 2000; Quirici et al., 2001; Loges et al., 2004). In fact, it has been shown that *in vitro* hypoxic culture of EPC isolated from peripheral blood enhanced therapeutic angiogenesis (Akita et al., 2003). In this investigation, analysis of angioblast and HSC gene expression, such as VEGF, VEGFR-1 and -2, provided no clear pattern. Also, flow cytometric analysis showed no effects on protein expression when HSPC were cultured in low oxygen (data not shown). Several possible explanations exist for these apparent discrepancies. Many of the above mentioned reports involved long-term culture (>1 month) in differentiation media containing a variety of recombinant growth factors. HSPC utilised in this report, on the other hand, were included in assays following short-term culture (≤ 1 week) in exapansion media (i.e. HSPC-GM). Other reports used cells differentiated from the entire mononuclear cell pool present in peripheral blood. It remains possible that different cell populations were used. On the other hand, it is plausible that the complex processes of vasculogenesis and angiogenesis require specific cell populations at different sites and different developmental stages.

While no affects on endothelial markers could be demonstrated, HSPC expression of monocyte-associated proteins during culture in low oxygen was significantly affected. Freshly isolated and expanded HSPC cultures contained less than 1% monocytes, as quantified with multiparametric flow cytometry. Incubation of HSPC in low oxygen dose-dependently increased

the percentage of monocytes. The functional importance of monocyte differentiation in low oxygen remains unclear. Multiple cell types are required in the placental niche. Some may act as accessory cells thereby helping to provide a receptive niche for other cells directly involved in placental vasculogenesis and/or angiogenesis (i.e. hemangioblast).

5.5 Extracellular Matrix Degradation in the Placental HSPC Niche

Extracellular matrix (ECM) degradation is involved in a variety of cellular functions and is primarily mediated by the matrix metalloproteinases (MMP). It has been shown in murine tumour models that the majority of CD11b positive hematopoietic cells located around blood vessels express active MMP-9 (Takakura, 2006). Inhibition of the gelatinases MMP-2 and -9 reduces the homing of $CD34^+$ cells from UCB (Janowska-Wieczorek et al., 2000). Along with their involvement during invasion, MMP have been suggested to play a role in releasing matrix-bound growth factors and chemokines into the microenvironment (Condeelis and Pollard, 2006). Growth factors such as SCF, VEGF and PlGF, as well as the chemokine SDF-1α are known to bind to various components of the ECM. Naturally, degradation of the ECM and release of these factors into the microenvironment could have direct effects on formation of blood vessels. We have demonstrated the secretion of active MMP-9 into media conditioned during HSPC culture with an increase in MMP-9 activity seen in low oxygen concentrations. These data could represent an increase in functional activity subsequent with the induction of monocyte differentiation. However, the cell type responsible for MMP production remains unclear and future experiments must clarify the source of increased gelatinase activity to fully understand the effects of increased MMP-9 in the placental microenvironment.

5.6 HSPC Home to Hypoxia

One obvious question that arises is how a discrete population of cells specifically traffics to hypoxic areas to take part in new blood vessel formation and growth. Are specific factors involved in homing progenitor cells to areas

where they are needed? It is known that endogenous factors home HSC to their niche in adult bone marrow. It remains plausible that similar regulatory mechanisms during developmental HSPC trafficking as well as following injury.

The chemokine SDF-1α has been shown to be critical for homing stem cells expressing the specific receptor CXCR4 to the bone marrow. Studies of mutant mice with targeted gene disruption have revealed that SDF-1α-CXCR4 signalling is essential for bone marrow colonisation by HSC during ontogeny (Nagasawa et al., 1996; Kawabata et al., 1999; Zou et al., 1998). Lethality caused by SDF-1α or CXCR4 gene ablation prevents immediate analysis of their role in adult hematopoiesis and angiogenesis. None of the described abnormalities, however, adequately explains the nearly uniform late gestational lethality in the SDF-1α or CXCR4 null mice. Knock-out placental tissue has not yet been investigated (personal communication with Nagasawa, T.). Among the environmental signals in these tissues that may be involved in recruiting cells is hypoxia.

In murine bone marrow, SDF-1α has been shown to maintain HSC in their low oxygen niche (Sugiyama et al., 2006). Hypoxia may even play a role in the molecular regulation of SDF-1α and/or CXCR4 expression, although results remain relatively unclear (Hitchon et al., 2002). In ischemic tissue, SDF-1α expression is localised to cells in the vascular microenviroment and acts as a signal to facilitate human HSPC adhesion (Peled et al., 2000). Ceradini and colleagues (2004; 2005) have also demonstrated the recruitment of $CXCR4^+$ murine HSPC to regenerating tissue is mediated by hypoxic gradients via HIF-1α-induced expression of SDF-1α.

5.7 SDF-1α in the Human Placenta

As an endocrine organ, cells in the chorionic villi produce a large number of growth factors, hormones and chemokines; these factors can have autocrine and paracrine effects on placental cells (McKinnon et al. 2000; Liang et al., 2004; Zygmunt et al. 2005; Herr et al. 2007). Placental-derived factors are also intricately involved in development and maintenance of the

placental vasculature tree (Zygmunt et al., 2003). Ubiquitous angiogenic factors, such as VEGF-A and Angiopoietin (Ang)-1 and -2 have been shown to be locally produced in the developing placenta and implicated in both placental vasculogenesis and angiogenesis (Geva et al., 2002). The placenta is also a rich source of PlGF, which has been shown to exert effects on endothelial cells at the fetomaternal interface (Zhou et al., 2003). A combination of both VEGF and PlGF at hypoxic sites can recruit progenitor cells to sites of neovasculogenesis (Li et al., 2006).

Chemokines may also play an important role in placental development. *In vitro* expression of SDF-1α and CXCR4 has been previously shown in human placenta, although analysis of expression patterns concentrated primarily on trophoblast cells (Ishii et al., 2000; Wu et al., 2004). In the human placenta, SDF-1α, by signalling through CXCR4 has been demonstrated to stimulate anti-apoptotic pathways in isolated trophoblast cells (Jaleel et al., 2004). Results shown in this report illustrate SDF-1α mRNA and protein expression in early placental tissue isolated from both 6 week-old and 12 week-old pregnancies. At the mRNA level, SDF-1α expression was noted in all early (≤12 wpc) placental tissue samples tested. On the other hand, although most term tissue samples contained SDF-1α mRNA, gene expression was not seen in all tissue examined. Different temporal expression patterns cannot be ruled out. Early placenta exists in a low oxygen environment and SDF-1α has been shown, in some cells, to be regulated by oxygen. Therefore, decreased SDF-1α expression would be expected in term placenta due to the fact that oxygen tensions are higher. However, it is possible that different SDF-1α expression patterns in term placenta also represent patient-specific differences or differences between the actual tissue samples as tissue samples excised for mRNA isolation consisted of different quantities of placental cells. *In situ* SDF-1α protein expression was noted in a variety of locations in developing chorionic villi as well as in term placental tissue. In support of the hypothesis that SDF-1α plays a role in early placental blood vessel development, vimentin-positive villous stroma cells also expressed SDF-1α, with strong expression seen at sites of vascular development. SDF-1α expression patterns continued until the end of

pregnancy. The presence of SDF-1α protein in placental tissue provides the first suggestion that this chemokine may play a role in the developing chorionic villous niche.

5.8 SDF-1α Secretion by Human Placental Cells *In Vitro*

It is known in the field of placentology that human trophoblasts are notoriously difficult to isolate and maintain in culture. The difficulties associated with obtaining early placental material, the variety of trophoblast cell types and a lack of trophoblast specific markers have also hindered research. In our laboratory, a novel method has been used to isolate trophoblast-enriched cell fractions from early placental tissue (Baal, N. et al. manuscript in preparation). Trophoblasts are unique epithelial cells (Bischof and Irminger-Finger, 2005). Thus, microbeads coated with the epithelial-specific cell adhesion molecule (Ep-CAM, also known as CD326) were used to isolate a trophoblast-enriched cell fraction from enzyme-digested human abortus material. Isolated cells were shown to express the pan-trophoblastic marker, cytokeratin 7, and to behave like trophoblasts *in vitro*. $CD326^+$ cells consisting of a trophoblast-enriched cell fraction were called HTR. On the other hand, the CD326-negative fraction, consisting of the placental stroma-enriched cell fraction, was used as a cellular model for the chorionic villi. This particular cell fraction contains cells expressing the mesenchymal marker vimentin and was called HPF. Both HTR and HPF have recently been used in our laboratory in an *in vitro* model of human placenta.

In vitro expression of SDF-1α was subsequently analysed in different placental cells. SDF-1α mRNA expression was demonstrated in both HTR and HPF during culture in 1% oxygen or in hyperoxia (21% O_2) with no differences seen between treatments. Media conditioned by different trophoblast cell models (Jeg3 and HTR) during a 24 h culture period contained only minimal amounts of SDF-1α protein, demonstrating that villous trophoblast cells do not secrete significant amounts of SDF-1α protein. Conversely, placental stroma-enriched cell fractions were shown to secrete large amounts of SDF-1α protein in 1%, 8% and 21% oxygen. Reducing

oxygen concentration decreased SDF-1α secretion from HPF cells. These results were puzzling due to previous reports suggesting that hypoxia HIF-1α regulates expression of SDF-1α in synovial fibroblasts (Hitchon et al., 2002). The reasons for these discrepancies remain unclear, however, they may represent differences between cell types. This report first describes SDF-1α secretion by placental stromal cells. Future molecular and cellular experiments will be required to determine the mechanisms involved during SDF-1α expression in the human placenta. These results provide further proof that this particular chemokine is part of the milieu of factors present in the developing human placenta.

5.9 Secreted Placental Factors Affect HSPC CXCR4 Expression

Intense interest in HSC research has provided a body of evidence supporting a role for paracrine regulatory mechanisms in regulating stem cell development and maintenance. Development of hematopoietic cells is regulated by hematopoietic growth factors, cytokines and chemokines secreted by accessory cells (e.g. fibroblasts, endothelial cells, macrophages and osteoblasts) residing in the various HSC microenvironments (reviewed in Janowska-Wieczorek et al., 2001). Bone marrow, as the adult HSC niche, has been the most extensively studied, however new evidence emphasises the need to determine whether similar paracrine regulatory mechanisms are involved extra-embryonically.

For SDF-1α to induce a response, HSPC must express the appropriate receptor. CXCR4 is a novel chemokine receptor due to its SDF-1α specificity. Cell trafficking in the hematopoietic and vascular systems involves specific progenitor cell populations expressing CXCR4 (Ceradini and Gurtner, 2005). Previously published data has shown that >5% of $CD34^+$ cells isolated from peripheral blood are also positive for CXCR4 (Yamaguchi et al., 2003). Results from our investigation were in accordance with this data, where 4% of expanded HSPC expressed CXCR4.

An accessory role for monocytes / macrophages during angiogenesis has been previously described and $CD11b^+CD14^+$ monocytes have been

shown to express proangiogenic factors, such as VEGF and Ang-1 (Rehman et al., 2003). Furthermore, monocyte-derived macrophages can support tumour angiogenesis through their matrix-degrading capacities, growth factor synthesis and engulfment of apoptotic cells (Condeelis and Pollard, 2006). Therefore, low oxygen-induced HSPC differentiation to monocytes in the placenta could also provide angiogenic support. SDF-1α, as a chemotactic stimulus, could then position monocytes in areas of blood vessel development. In fact, coculture of HSPC with both HTR and HPF cells increased the percentage of $CD11b^+CXCR4^+$ cells over a seven-day period in low oxygen. Further experiments will be needed to determine the involvement of $CXCR4^+$ monocytes during placental vascular development.

5.10 HSPC Migrate in Response to Placental-Derived SDF-1α

A mechanism is needed to stimulate movement of progenitor cells into position around developing blood vessels. In a tumour model, Grunewald and colleagues (2006) demonstrated the involvement of SDF-1α in positioning progenitor cells around developing blood vessels. Likewise, Kaplan and colleagues (2005) illustrated the involvement of SDF-1α in positioning progenitor cells in a pre-metastatic niche. In the placenta, multiple factors have been shown to regulate migration and invasion (Chakraborty et al., 2002). Locally produced SDF-1α may have a similar in the low oxygen environment of the chorionic villous stroma. SDF-1α is a highly potent and efficacious mononuclear cell attractant, inducing migration of many hematopoietic cells, including monocytes (Bleul et al., 1996) and $CD34^+$ stem cells (Mohle et al., 1998; Voermans et al., 1999; Jo et al., 2000).

In our model, conditioned media from both HTR and HPF significantly stimulated HSPC migration. The bicyclam peptidomimetic CXCR4 antagonist AMD3100 is a commonly used inhibitor of SDF-1α - CXCR4 interactions (De Clercq, 2003). Inclusion of AMD3100 in migration assays had no effect on HTR-CM stimulation of migration. On the other hand, AMD3100 inhibited HPF-CM induced HSPC migration. These results demonstrate that SDF-1α locally produced in the chorionic villi stroma can stimulate migration of HSPC.

Inhibition of CXCR4 did not completely abolish HSPC migration illustrating that multiple migratory stimuli are present in the developing placenta. In fact, trophoblast cells appear to secrete a different chemotactic factor or factors. Further investigations will be needed to determine all of the factor(s) involved in stimulating HSPC migration in the human placenta.

In order to enter the tissue of interest, hematopoietic cells must not only migrate but must also transgress the endothelium. To examine whether placental-derived factors had the capacity to stimulate HSPC transendothelial migration an *in vitro* assay was employed. A layer of endothelial cells (i.e. HUVEC) was cultured to confluence on insert membranes, thereby creating a cellular barrier separating HSPC from the chemotactic stimulus. For cells to be quantified, HSPC must first actively move through the endothelial cell monolayer. Stimulation of transendothelial migration was noted when HSPC were cocultured with HPF. Twenty-four hour culture of HPF in either low oxygen environment (1% or 8%) demonstrated no significant differences. The levels of HSPC transendothelial migration were similar to when exogenous SDF-1α was used as a chemotactic stimulus and could be inhibited with AMD3100. As previously discussed for migration, other locally produced factors are likely involved. The results from this particular transendothelial migration assay, while interesting, do not provide a clear picture of the mechanisms involved. For example, inclusion of multiple cell types in an assay increases the number of variables. It remains possible, that increased HSPC transendothelial migration actually represents an effect of HPF cells on the endothelial cell monolayer and not a direct interaction of these factors on HSPC. Nonetheless, inclusion of AMD3100 in the assay provides adequate proof that SDF-1α acting on CXCR4$^+$ HSPC does play a significant role.

5.11 HSPC Adhesion in the Placental Microenvironment

By bringing cells to the places where they are needed, migration is the first step in creating a placental HSPC niche. Once the appropriate cells are in location, a mechanism should exist, which helps retain cells in this niche. True enough, published data indicates that CXCR4 activation is important for the retention of VEGFR-1$^+$ “hemangiocytes” within the neovascular niche (Jin

et al., 2006a). SDF-1α - CXCR4 signalling is implicated as the principal axis regulating migration, retention and mobilisation of HSC during steady-state homeostasis and injury (Lapidot et al., 2005).

One of the monocyte markers examined with flow cytometry, CD11b, is another designation given to the α_M integrin. Thus, the increased expression of CD11b during HSPC culture in placental oxygen tensions suggested an associated alteration in cellular adhesion. Along these lines, the expression of several integrin subunits was examined in our HSPC culture model utilising flow cytometry. The adhesive properties of integrins rely on the association of the various α subunits with different β subunits. Indeed, HSPC were shown to express several important integrin subunits during culture in physiological oxygen concentrations. In this investigation, expression of α_M and the associated β_2 subunit as well as α_4 and β_1 was specifically evaluated. Differences were noted between HSPC cultured in either 1% or 8% oxygen when the expression of α_M and α_4 was examined. Once again, decreasing oxygen concentration resulted in an increase in HSPC α_M expression. Conversely, the expression of the α_4 subunit was highest when cells were cultured in 8% O_2. Whether or not these alterations represent different adhesive properties of HSPC in 1% oxygen versus 8% oxygen remains unclear. Nonetheless, the $\alpha_4\beta_1$ expression has been shown to be important during the homing of progenitor cells to sites of neovascularisation (Jin et al., 2006b). It would be extremely interesting to explore whether the different physiological oxygen tensions have different effects on HSPC function during blood vessel formation.

Besides homing HSC to their BM niche, SDF-1α can also act as a “retention factor”, entrapping angio-competent cells at sites of vascular development in the pre-metastatic niche (Kaplan et al., 2005) and during adult neovascularisation (Grunewald et al., 2006). In ambient oxygen, HSPC adhesion was greatest when the coculture consisted of placental stroma cells. When a similar assay was performed in low oxygen atmospheres, HSPC adhesion to HPF was also greater than control adhesion levels. Again, significant differences were not seen between the two oxygen concentrations (1% and 8% O_2). Another interesting result obtained showed significant

increases in HSPC adhesion to trophoblast (HTR) when the assay was performed in physiologically relevant oxygen concentrations. In this case, HSPC adhesion to HTR was greatest in 1% oxygen reaching levels similar to HSPC-HPF adhesion. In the human placenta, locally derived SDF-1α also appears to act as a retention factor due to the fact that including the CXCR4 antagonist AMD3100 in the experiment inhibited *in vitro* adhesion of HSPC to placental stromal cells. On the contrary, completely different mechanisms appear to be at play in the adhesion of HSPC to trophoblast; these mechanisms remain unclear, but may be dependent on oxygen concentration. This assay provides interesting results regarding cell-cell interactions at a specific point of time. They do not, however, clarify whether this adhesion represents a static condition or part of the dynamic process of HSPC migration. The latter now seems more probable. Recent live-cell imaging experiments have shown that HSPC adhesion to placental stromal cells *in vitro* is a dynamic process with a great number of adhesive cells being constantly in a migratory state (data not shown). At the same time, formation of HSPC colonies also appears to take place. Further experimentation should determine which microenvironmental cues provide the signal for HSPC to stop migrating and colonise the placental HSPC niche.

5.12 Study Limitations

Although this investigation provides interesting data regarding the impact of local oxygen tensions on formation of a placental HSPC niche, an important point should be noted. This qualification concerns the stem / progenitor cells isolated, utilised and reported above. $CD133^+$ HSPC were isolated from term UCB. Isolation of HSPC at this particular time point (i.e. term) results in the culture of, so-called, adult stem cells. These cells are definitive hematopoietic cells by nature. In contrast, the earliest stages of placental development occur during a point of time before definitive hematopoiesis is fully established. Studies into the earliest stages of human development are fraught with a variety of difficulties - the most important being the relative scarcity of healthy material. The collection of early human tissue normally involves samples obtained from elective terminations of pregnancies.

Compounded by the fact that most pregnancies are not discovered until after some time has passed (~1 month), collection of material representing the earliest stages of pregnancy (i.e. ≤1 month) is rare. The material obtained is also small, adding further difficulties to such investigations. Therefore, it was deemed necessary that adult HSPC should be used during experimentation as a general model for hematopoietic stem cells.

5.13 Perspectives

This report attempts to shed light on the cellular mechanisms involved in the establishment of the placental stem cell niche and the involvement of progenitor cells in placental blood vessel formation. Further studies will be needed before a full understanding of these early developmental events is reached. One focus for the future should be to elucidate the precise role that HSPC play during vascular development. A wide variety of angiogenesis assays presently exist (e.g. network forming assay, spheroid formation, sprouting assays). Inclusion of HSPC in such assays in conditions mimicking the *in vitro* placental microenvironment would be an interesting way to examine whether HSPC contain intrinsic hemangioblastic potential. However, the suggestion that HSPC contain a hemangioblast population has not been adequately explored. On the other hand, present experiments suggest that HSPC may have more of an accessory role during *in vitro* vessel formation.

Yet another interesting avenue for research concerns SDF-1α. We have shown that SDF-1α is one of the factors present in the developing placenta and that SDF-1α regulates HSPC functions in this niche. Nonetheless, in our model the intracellular mechanisms induced by SDF-1α binding to CXCR4 remain unclear. Moreover, the molecular events leading to SDF-1α expression in placental stromal cells was also beyond the scope of this study. Future investigations should attempt to clarify the precise molecular mechanisms regulating SDF-1α expression in developing chorionic villi, as well as, explore signalling events stimulated by chemokine binding to $CXCR4^+$ HSPC.

It is interesting to speculate about what other locally derived factors regulate HSPC functions in the placenta. This is a daunting task as the placenta is home to a myriad of growth factors, hormones, and cytokines. Nevertheless, such investigations could be accomplished using the techniques described here. Our novel *in vitro* model could be used in the future to clarify specific molecular and cellular mechanisms involved during formation of a placental stem cell niche, as well as, the involvement of stem / progenitor cells during placental vascular development.

6 CONCLUSIONS

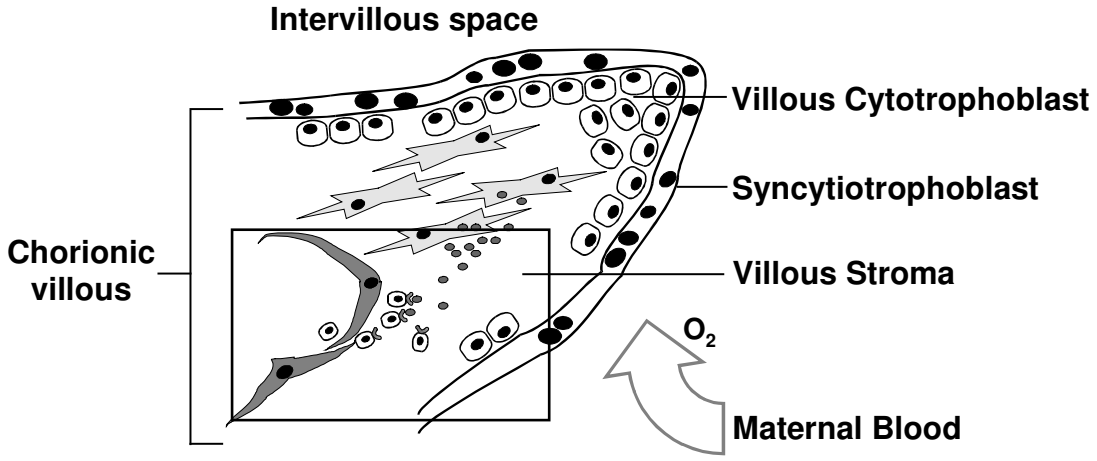
Scientific investigations should make a clear distinction between true hypoxia and physiologically low oxygen concentrations actually representing normoxia. For example, normoxia in the placental stem cell niche is characterised by low oxygen. We provide a novel model allowing for effective simulation and monitoring of physiologically low oxygen levels *in vitro*. Results from this investigation illustrate the involvement of the local oxygen environment during creation of this HSPC niche.

Using HSPC isolated from UCB, we have demonstrated that physiologically low oxygen plays a definite role in cell culture dynamics, maintenance of stem cell characteristics, as well as in the induction of differentiation. We have shown that low oxygen culture in either 1% or 8% O_2 significantly and dose-dependently decreased HSPC culture expansion, rapidly increased the number of G0/G1 cells, and stimulated expression of monocyte markers.

The low oxygen microenvironment of early chorionic villi is also a rich source of placental-derived hormones, growth factors and chemokines. Such factors can be involved during hematopoietic and vascular cell development. In the developing placenta, the chemokine SDF-1α appears to play an important role (Figure 30). Locally derived SDF-1α, produced in the chorionic villous stroma, acts as a “homing” factor inducing migration of HSPC expressing the specific SDF-1α receptor (CXCR4) to areas of vascular development. In this investigation, the direct involvement of SDF-1α during stimulation of HSPC migration, transendothelial migration and cell-cell adhesion was shown. Blocking SDF-1α interactions with CXCR4 using AMD3100 inhibited the placental stroma cell induction of cellular motility and adhesion. Trophoblast, as a major component of the developing placenta also appears to play a role in the promotion of placental vasculogenesis. A direct link between hematopoietic cells and formation of the placental vascular tree has yet to be proven. However, the close developmental and spatial proximity of HSPC and early vasculogenic cells, once again, suggests a connection between their development.

An important goal for future investigations should be to clarify the precise mechanisms involved in placental formation, including growth of the extensive placental vascular system. Only with a better understanding of healthy placental development will it be possible to understand the factors at play during placental malformation and disease.

A.



B.

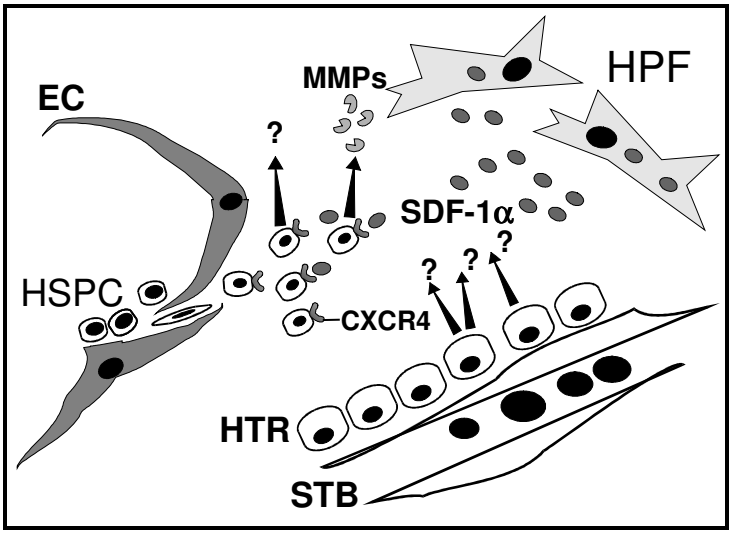


Figure 30. SDF-1α in the chorionic villous microenvironment. Early placental vasculogenesis commences before chorionic villi become bathed in maternal blood (**A**). HSPC are found in the placental blood supply and villous stroma. Syncytiotrophoblast (STB) and villous trophoblast (HTR) surround the villous stroma. Box at lower left magnified in (**B**). SDF-1α produced by chorionic villous stromal cells (HPF) is secreted into the microenvironment. Attracted by local SDF-1α, $CXCR4^+$ HSPC invade the villous stroma. HSPC involvement during placental vasculogenesis and/or angiogenesis is unclear. Secreted factors, such as active MMP may play a role. Likewise, the involvement of trophoblast in this model remains an interesting topic for investigation.

7 ZUSAMMENFASSUNG

Eine physiologische Entwicklung des Gefäßsystems der Plazenta und dessen uneingeschränkte Funktion erlauben einen adäquaten Gasaustausch sowie den Transport von Nährstoffen zwischen der Mutter und dem Fetus und sind somit für eine normale embryonale Entwicklung von entscheidender Bedeutung. In der sich entwickelnden humanen Plazenta ist ein geringer Sauerstoffpartialdruck physiologisch und für eine korrekte Entwicklung dieses Organs von massgeblicher Bedeutung.

Im Rahmen der vorliegenden Studie konnte erstmalig ein Modell entwickelt werden, was die Simulation dieser sauerstoffarmen Umgebung in der Plazenta und die Messung von *in vitro* herrschenden Sauerstoffpartialdrücken ermöglicht. Mit seiner Hilfe wurde der Einfluss der Sauerstoffkonzentration auf die Etablierung der plazentaren Nische für fetale Stammzellen (HSPC) untersucht. Insbesondere wurde die Proliferation, Erhaltung ihres Stammzellphänotyps und Induktion ihrer Differenzierung angesehen. Dabei konnte gezeigt werden, dass die niedrige O_2 Konzentration die Proliferation von HSPC hemmt, zum Anstieg von G0/G1 Zahl führt und die Induktion von monozytären Markern verursacht.

In der reduzierten O_2-Atmosphäre der frühen Plazenta wird zusätzlich eine Vielzahl von Wachstumsfaktoren, Hormonen und Chemokinen produziert, die in hämatopoetischen sowie in vaskulo- und angiogenetischen Vorgängen eine wichtige Rolle spielen. Im hier etablierten Modell wurde die Wirkung des Chemokins SDF-1α, das von plazentaren Stromazellen produziert wird, auf CXCR4 (Rezeptor für SDF1) positive HSPC hinsichtlich Migration, transendotheliale Migration und Adhäsion in der plazentaren Nische untersucht. Es konnte gezeigt werden, dass das durch die plazentare Stromazellen produzierte SDF-1α sowohl die Migration und die transendotheliale Migration als auch die Adhäsion von HPSC stimuliert. Im Gegensatz dazu führte die Zugabe von entsprechenden neutralisierenden Substanzen (AMD3100) zur einer signifikanten Reduktion der zellulären Adhäsion und Mobilität.

Ein wichtiges Ziel für weitere Untersuchungen sollte es sein, die genauen Mechanismen, die der Plazentabildung und Entstehung des weitverzweigten plazentaren Gefässbaumes zugrundeliegen, aufzudecken. Denn nur durch das Verständnis der physiologischen Plazentaentwicklung ist es möglich, die krankhaften Veränderungen dieses Organes zu verstehen.

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Abstracts

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