Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Animal

Osteogenic Differentiation of Human Umbilical Cord-derived Stromal Cells on Human Amniotic Membrane

Ana Rita Almeida de Oliveira

Mestrado em Biologia Evolutiva e do Desenvolvimento
2011

Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Animal

Osteogenic Differentiation of Human Umbilical Cord-derived Stromal Cells on Human Amniotic Membrane

Ana Rita Almeida de Oliveira

Dissertação orientada por:

Doutora Maria Margarida Gouveia Sancho
Doutora Maria Gabriela Gomes de Figueiredo Rodrigues

Mestrado em Biologia Evolutiva e do Desenvolvimento
2011

Agradecimentos

Muitas pessoas contribuíram de diferentes maneiras para a realização desta tese. Em primeiro lugar agradeço ao Dr Helder Trindade por me receber no CHSul e claro à Dra Margarida Sancho (Margaraides) que me proporcionou esta oportunidade de realizar este projecto, agradeço todo o seu empenho em me acompanhar e ensinar as mais variadas técnicas laboratoriais, sua amizade e aqueles cafés duplos que me ajudaram a sobreviver até ao fim da tese. O meu muito obrigado.

Agradeço também à Professora Gabriela Rodrigues pelos seus conselhos e pela sua sempre pronta disponibilidade em me ajudar.

Agradeço à Cristiana Teixeira (Cristaiane) pela ajuda em tudo o que precisei no laboratório, pela companhia no autocarro das dez para as nove e pela amizade que demonstrou ter para comigo desde que entrei no laboratório. Agradeço à Sofia (Sofs) pelos grandes almoços na sala de refeições e no japonês. Agradeço ainda à Dra Josefina Oliveira e a todos os outros membros do banco de tecido por me ajudarem a obter os meus cordões, à Dra Alice pela ajuda fornecida no laboratório de citometria de fluxo, ao Dr Artur por todas as vezes que me forneceu material fora do dia previsto e ao Dr Dário Ligeiro, por me facilitar o acesso à área pós-PCR.

Agradeço a todos os meus amigos pelo incentivo e apoio nos momentos mais difíceis. Por último e mais importante agradeço à minha família e ao Daniel pelo apoio e carinho que me deram. Em particular, agradeço aos meus pais que me proporcionaram esta oportunidade de efectuar o mestrado e pelo apoio constante em tudo o que precisei. O meu muito obrigado a todos.

Contents

Abbreviations

AM	Amniotic membrane
AP	Alkaline phasphatase
Asc	Ascorbic acid
Atf4	Activating transcription factor 4
ATMP	Advanced therapy medicinal product
β-GP	β-glycerophosphate
BM	Bone marrow
BMP	Bone morphogenic proteins
BSP	Bone sialoprotein
CFU	Colony-forming units
CR	Chemokine receptors
Dex	Dexamethasone
ECM	Extracellular cell matrix
EGF	Epidermal growth factor
FBS	Fetal Bovine Serum
FC	Flow cytometry
FGF	Fibroblast growth factor
GMP	Good Manufacturing Practice
GVHD	Graft-versus-host disease
hABS	Human AB serum
HGF	Hepatocyte growth factor
hP	Human plasma
hPL	Human platelet lysate in medium
hPLP	Human platelet lysate in plasma
hS	Human serum
ICAM	Intracellular adhesion molecule
IGF	Insulin-like growth factor
IL	Interleukin
ISCT	International Society for Cellular Therapy
KGF	Keratinocyte Growth Factor
MFI	Mean fluorescence intensity
MSC	Mesenchymal stromal cells
Osx	Osterix
PDGF	Platelet-derived growth factor
PDEGF	Platelet-derived epidermal growth factor
PF-4	Platelet factor 4

pWJ	Peripheral Wharton's jelly
Runx2/Cbfa1	Core binding factor α1
SFM	Serum free medium
TGF-β	Transforming growth factor-β
tPRP	Thrombin-activated platelet releasate in plasma
tWJ	Total Wharton's jelly
UC	Umbilical cord
UCA	Umbilical cord arteries
UCB	Umbilical cord blood
UCV	Umbilical cord vein
V-UC	Umbilical cord vessels
WJ	Wharton's Jelly
W-UC	Whole umbilical cord

Abstract

Mesenchymal stromal cells (MSC) from the umbilical cord (UC) are a promising tool for regenerative medicine due to their easy acquisition, high expansion potential and the ability to differentiate into multiple lineages. In particular, the capacity for osteogenic differentiation makes them of great interest for clinical application in bone repair.

In this work, 51 MSC lines were isolated from different parts of 26 UC and successfully expanded in fetal bovine serum (FBS)-containing medium. These cells complied with the criteria for MSC classification as they were adherent to plastic, capable of multilineage differentiation, and expressed CD73, CD90, CD44 and CD105 while lacking hematopoietc and endothelial markers. Differentiation of the different MSC lines along the osteogenic lineage was variable depending mostly on the tissue source and isolation method. Moreover, osteogenic differentiation could be modulated by selecting for cells with high expression levels of CD105 (Endoglin) or by the co-culture of MSCs with different osteogenic potential. With the aim of establishing xeno-free clinically compatible culture conditions, StemPro MSCs serum free medium (SP-MSC SFM), human platelet lysate (in medium or in plasma), human plasma and AB serum (hABS) were tested. We have observed that only hABS supplemented medium could efficiently sustain MSC expansion, morphology, immunophenotype and multi-lineage differentiation similarly to FBS-containing medium. Finally, preliminary data indicates that frozen immunologically inert amniotic membrane is a suitable substrate for osteogenic differentiation of MSCs and, therefore, a promising scaffold for producing an osseous matrix for application in regenerative medicine.

Keywords: Amniotic Membrane; Endoglin; human AB serum; Mesenchymal Stromal Cells; Osteogenic Differentiation; Umbilical Cord.

Sumário

A medicina regenerativa é uma área em grande expansão que tenta ultrapassar a escassez e curto tempo de vida dos órgãos e tecidos doados para transplantação e os problemas decorrentes da histocompatibilidade (Chai e Leong 2007). Para tal, células estaminais são expandidas e diferenciadas *in vitro* num sistema de suporte criado artificialmente. As células estaminais têm o potencial de se auto-renovarem por divisão mitótica mantendo a capacidade de se diferenciarem em vários tipos celulares e deste modo de repovoarem um tecido *in vivo*. As células mesenquimais do estroma (MSCs, "mesenchymal stromal cells") representam uma população de células estaminais adultas multipotentes de grande interesse de estudo na área da regeneração de tecidos (Kode *et al*., 2009). Estas células foram inicialmente identificadas como uma população rara de células estaminais não hematopoiéticas presente na medula óssea (Friedenstein *et al*., 1966; Friedenstein *et al*., 1987; Kode *et al*., 2009; Owen e Friedenstein, 1988). Contudo, tem vindo a ser descrito o isolamento destas células de variados tecidos, entre eles o cordão umbilical humano (UC, "umbilical cord") (Lu *et al*., 2006; Nekanti *et al*., 2010; Shetty *et al*., 2010). O UC é constituído por duas artérias, uma veia, e uma matriz circundante de tecido conectivo designada por geleia de Wharton (WJ "Wharton's jelly"), composta por células com morfologia fibroblástica e alguns mastócitos numa substância amorfa rica em proteoglicanos, principalmente ácido hialurónico (Can e Karahuseyinoglu, 2007). MSCs foram já isoladas a partir de todos estes constituintes do UC e constituem uma ferramenta promissora em medicina regenerativa, devido à sua fácil aquisição, elevado potencial de expansão e capacidade de se diferenciar em várias linhagens, nomeadamente osteogénica, adipogénica e condrogénica. Em particular, a sua capacidade de diferenciação osteogénica é de grande interesse para a aplicação clínica destas células em reparação óssea.

Neste trabalho MSCs foram isoladas de diferentes partes de 26 UCs tendo 51 linhas celulares sido obtidas. Em particular, foram isoladas células da geleia de Wharton periférica (pWJ) e total (tWJ), apenas dos vasos (V-UC), e de todo o cordão (W-UC). Para isto aperfeiçoámos um método composto por uma fragmentação mecânica da WJ, V-UC e W-UC em pequenos pedaços seguida de uma digestão enzimática de 3 a 4 horas com colagenase e hialuronidase, deste modo obtendo um bom equilíbrio entre a dissociação do tecido e a viabilidade celular. Para a obtenção de tWJ-UC e V-UC foi necessário efectuar uma digestão enzimática inicial da parte interna do cordão para permitir a remoção dos vasos. As células isoladas foram então expandidas num meio de cultura composto pelo meio basal Dulbecco's Modified Eagle's Medium F12 (DMEM-F12), glutamina e soro fetal bovino (FBS). Estas células preencheram os critérios de classificação para MSCs estabelecidos pela Sociedade Internacional para a Terapia Celular (ISCT), uma vez que: eram aderentes ao plástico; auto-renovaram-se em cultura ao longo de várias passagens; expressam os marcadores de

superfície específicos CD73, CD90, CD44 e CD105, e não marcadores hematopoiéticos nem vasculares como CD45 e CD34; e foram capazes de se diferenciar nas linhagens osteogénica, adipogénica e condrogénica.

Com o objectivo de obter condições de cultura compatíveis com uma aplicação clinica, ou seja sem produtos animais tais como o FBS, foram testados como candidatos o meio de cultura comercial StemPro (SP-MSC SFM), lisado de plaquetas humanas (ressuspendidas em meio- hPL ou em plasma-hPLP), plasma humano (hP) e soro AB humano (hABS). Um meio de cultura apropriado para esta aplicação, deverá ser capaz de sustentar a expansão de MSCs mantendo a sua morfologia, fenótipo e o potencial de diferenciação em diferentes linhagens. Neste estudo, o meio de cultura com hPLP, hPL ou hP apenas permitiu a sobrevivência das MSCs por 3 passagens, ainda que usando placas de cultura revestidas com fibronectina humana e adicionado 250μM de ácido ascórbico (asc), condições que deveriam facilitar a expansão de MSCs (Choi KM *et al*., 2008; Potdar e d'Sousa, 2010), como foi possível verificar no meio com FBS.

No meio de cultura SP-MSC SFM e no meio suplementado com hSAB foi possível expandir as células durante pelo menos 8 passagens. Contudo, em SP-MSC SFM as células sofreram algumas alterações morfológicas e uma diminuição da expressão de CD105 ao longo do tempo em cultura. Por seu lado, MSCs expandidas em hSAB (um *pool* de soro humano AB alogénico) exibiram o comportamento desejado uma vez que nem a sua morfologia nem o imunofenótipo foi alterado quando comparado com células em meio com FBS. De grande importância foi também a observação que, enquanto em MSCs expandidas em SP-MSC SFM houve uma redução na capacidade de diferenciação osteogénica das células, em meio com hSAB esta capacidade foi mantida. Bons resultados no uso de hSAB como suplemento do meio de cultura de MSCs tinham já sido documentados em outros estudos (Yamaguchi *et al*., 2002; Kocaoermer, Kern e Kluter *et al*., 2007).

Para a análise do potencial osteogénico das MSCs foram testados diversos meios basais de cultura (com diferentes concentrações de glucose e aminoácidos) e combinações dos osteoindutores ácido ascórbico, dexametasona e β-glicerofosfato. No entanto, observámos que melhores resultados foram obtidos com o meio de cultura comercial *MesenCult osteogenic stimulatory kit*. A diferenciação osteogénica das células foi avaliada através da análise da expressão génica, imunofenotipagem por citometria de fluxo, marcação para a actividade da fosfatase alcalina (AP), característica da fase de deposição de matriz orgânica (aproximadamente aos 14 dias de diferenciação), e numa fase mais tardia (de mineralização) pela deposição de cálcio detectada com vermelho de Alizarina ou marcação von Kossa. Assim, foi possível observar que durante a osteogénese há uma diminuição inicial da expressão de CD105, CD73 e de CD90 (tanto RNA como proteína), seguida do aparecimento de fosfatase alcalina e osteocalcina, e finalmente um aumento gradual da deposição de cálcio a partir dos 21 dias de diferenciação.

Neste estudo foi observada uma grande variabilidade na capacidade de diferenciação das diferentes linhas de MSCs obtidas. Esta variabilidade parece estar de alguma forma

relacionada com o tecido e método de isolamento utilizado. No entanto, outros factores como a densidade celular no início da cultura, o nível de expressão de CD105 e a interacção entre células parecem também ter uma importante influência na aptidão osteogénica das MSCs

Em diversos aspectos deste estudo o antigénio CD105, pareceu ter um papel relevante, uma vez que a diminuição da sua expressão em SP-MSC-SFM parece inibir a osteogenése, e as células seleccionadas para elevados níveis desta proteína provocam o aumento de diferenciação das MSCs. O CD105, ou Endoglina, é uma glicoproteina membranar de tipo I que faz parte do complexo do receptor TGF-β (Barbara *et al*., 1999; Blanco *et al*., 2005). Assim, a regulação dos níveis de expressão de CD105 pode estar relacionada com a modelação da sinalização em resposta ao TGF-β e esta pode determinar aspectos cruciais da diferenciação osteogénica. Este aspecto deverá ser alvo de estudos futuros.

Outro aspecto importante da diferenciação é a matriz ou substrato onde sobre a qual se encontram as células (Salasznyk *et al*., 2004; Cool e Nurcombe, 2005; Chastain *et al*.,2006). Neste estudo propusemos a utilização de membrana amniótica (AM “Amniotic membrane”) como um substrato adequado para promover a diferenciação osteogénica de MSCs, o que ajudaria a ultrapassar a necessidade de dissociação das células e disrupção de uma matriz óssea para transplantação num paciente. Os nossos dados preliminares indicam que a AM congelada imunologicamente inerte é um substrato adequado para diferenciação osteogénica de MSCs, apesar de dificuldades em prevenir o enrolamento e enrugamento da membrana durante a diferenciação e de esta diferenciação ser menos eficiente que a de células cultivadas em plástico. Um outro aspecto interessante foi a detecção de um nível basal de AP em MSCs sobre AM em meio de expansão. Isto sugere que a AM tem propriedades intrínsecas que lhe permitem induzir osteogénese, como sejam factores de crescimento presentes na AM (Koizumi *et al*., 2000; Valladares *et al*., 2010). Apesar de estudos adicionais serem necessários de modo a melhorar as condições de cultura das MSCs na AM, este parece ser um substrato promissor para a produção de uma matriz óssea para aplicação em medicina regenerativa.

Palavras-chave: Células Mesênquimais do Estroma; Cordão Umbilical; Diferenciação Osteogénica; Endoglina; Membrana Amniotica; Soro AB Humano.

Aims of the thesis

In this study, with the propose improve the knowledge of the biology of UC-MSCs for future application in bone tissue regeneration, we aim to establish culture conditions for the expansion and differentiation of UC-MSCs on amniotic membrane, in order to produce a bone matrix for application in regenerative medicine. With this purpose, the following objectives were defined:

1. To establish an efficient technique for the isolation of MSCs from different parts of umbilical cord (Wharton's jelly, vessels and whole cord). For this, both mechanical and enzymatic methods (combinations of collagenase, hyaluronidase and trypsin) were tested in search for the most reliable and efficient one.

2. To determine appropriate culture conditions for the expansion and differentiation of UC-MSC, compatible with a clinical application. To accomplish this, we tried to replace the animal and chemically undefined media components. A commercially available serum-free medium containing the growth factors PDGF-BB, bFGF and TGF-β1(StemPro MSC serum free medium), human platelet lysate (in medium and plasma), human plasma and human AB serum were tested and the cells grown in this medium were compared to FBS-containing standard medium.

3. To characterise UC-derived MSCs in expansion and differentiation conditions. With this purpose, the cell proliferation, gene expression and MSC surface phenotype were assessed for cells grown in expansion conditions (for various periods of time in culture) and after induction of differentiation. Moreover, some culture conditions and characteristics of these cells that could be related to or help predict the osteogenic potential were analysed.

4. To test the amniotic membrane as substrate for the production of a bone matrix. This step aimed at finding a safe and quickly adapted vehicle for transplantation.

1. Introduction

1.1. Mesenchymal Stromal Cells (MSCs)

Over the years, much progress has been made in the use of biological resources to reconstruct, replace, or repair diseased cells, tissues and organs in order to improve the quality of life of millions of people around the world (Chai and Leong, 2007; Lutolf and Blau, 2009). The emerging field of Regenerative Medicine attempts to overcome the shortage in donor organs and tissues for transplantation (Chai and Leong 2007) by growing and differentiating cells outside the patient in artificially-created support systems.

In the human body an intrinsic stem cell-based regeneration and repair system that uses stem cells is believed to be found in almost in every tissue type (Bajada *et al*., 2008). Stem cells have the potential to self-renew by mitotic division while maintaining the ability to differentiate into many cell types, therefore being able to repopulate a tissue *in vivo*. There are stem cells of several types and origins. Pluripotent stem cells can give rise to all cell types in a body and typically include the following (Xu et al., 2008): embryonic stem cells, derived from the inner cell mass of pre-implantation embryos; epiblast stem cells derived from post-implantation epiblast-stage embryos; germ line stem cells and induced pluripotent stem (iPS) cells obtained by genetic manipulation from somatic cells. Adult stem cells have a more restricted potential and generally give rise only to the differentiated lineages of the tissue in which they reside, being thus referred to as multipotent or unipotent (Watt and Driskell, 2010).

Mesenchymal stromal cells (MSCs) represent a multipotent adult stem cell population (Kode *et al*., 2009) that has been shown to be capable of differentiating *in vitro* and *in vivo* along multiple lineages including those that give rise to bone, cartilage, cardiac and skeletal muscle, neural cells, tendon, adipose, and connective tissue (Nekanti *et al*., 2010), depending on the surrounding environment. These mesenchymal stromal cells, whose existence was first suggested by Cohnheim in 1867, were later identified in the bone marrow (BM) by Friedenstein and colleagues as a rare population of adherent non-hematopoietic stem cells (Friedenstein *et al*., 1966; Friedenstein *et al*., 1987; Kode *et al*., 2009; Owen and Friedenstein, 1988). It was subsequently shown that they can actually be isolated from a variety of other tissues including adipose tissue (Zuk *et al*., 2002), skeletal muscle (Young *et al*., 2001), blood (Zvaifler *et al*., 2000), dental pulp (Gronthos *et al*., 2000), skin (Lorenz *et al*.,2008), amniotic fluid (Tsai *et al*., 2004), umbilical cord blood (Lu *et al*.,2006) and stroma (McElreavey *et al*., 1991; Romanov *et al*., 2003; Seshareddy *et al*. 2008; Troyer and Weiss, 2008; Wang *et al*, 2004), among others (Chamberlain *et al*., 2007; da Silva Meirelles *et al*., 2006; Musina *et al*., 2005; Riekstina *et al*., 2009).

Several studies have focused on the characterization of MSCs, revealing important aspects of their biology and defining molecular markers that allow their identification. As the defining characteristics of MSCs are inconsistent among investigators, The International Society

for Cellular Therapy (ISCT) has proposed three major criteria to define human MSCs (Table 1) : (1) adherence to plastic, (2) a specific surface phenotype, and (3) the ability to differentiate *in vitro* into cells of the osteoblastic, adipogenic and chondrogenic lineages (Dominici *et al*., 2006).

Table1: Summary of criteria to identify MSCs (reproduced from Dominici *et al*., 2006)

1. **Adherence to plastic** in standard culture conditions
2. **Phenotype**

Positive (≥95% positive)	Negative (≤2% positive)
CD105	CD45
CD73	CD34
CD90	CD14 or CD11b
	CD79α or CD19
	HLA-DR

3. ***In vitro* differentiation**: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of in vitro cell culture)

MSCs express a large number of adhesion molecules, extracellular matrix proteins, cytokines and growth factors, which are associated with their function and interactions within the various tissues where they are present (Bobis *et al*., 2006). These cells do not have a unique phenotypic marker but they express a characteristic array of surface proteins including CD73 (5'-nucleotidase), CD90 (Thy-1 cell surface antigen) and the matrix receptors CD44 and CD105 (Endoglin) (Bobis *et al*., 2006; Chamberlain *et al*., 2007). MSCs also express adhesion-related antigens (integrins αvβ3, αvβ5, integrin subunit α4, α5 and β1, intracellular adhesion molecule-1 (ICAM-1) and CD44H), and produce cytokines, chemokines and growth factors (interleukin (IL)-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, leukemia inhibitory factor, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), stem cell factor, macrophage colony-stimulating factor (M-CSF) and fms-like tyrosine kinase-3 ligand (flk-3L)) (Chamberlain *et al*., 2007; Bobis *et al*., 2006; Fox *et al*., 2007). Additionally, they also express cytokine (IL-1R, IL-3R, IL-4R, IL-6R and IL-7R) and chemokine receptors (CCR1, CCR4, CCR7, CXCR5 and CCR10), which may be involved in the directed migration of MSCs for tissue repair (Fox *et al*., 2007; Sordi, 2009). On the other hand, MSCs lack the endothelial and hematopoietic stem cell marker CD34, the pan-leukocyte marker CD45, the endothelial markers von Willebrand factor and CD31 (platelet/endothelial cell adhesion molecule), and the monocyte marker CD14 (monocyte differentiation antigen) (Bobis *et al*., 2006; Chamberlain *et al*., 2007; Dominici *et al*., 2006).

Depending on the tissue source, method of isolation, culture conditions and species differences, MSCs present variable morphology, expression levels of many of the markers mentioned, and proliferative and differentiation potentials (Barry *et al*., 2004; Musina *et al*., 2005; Riekstina *et al*., 2009). Human MSC cultures are morphologically heterogeneous ranging from narrow spindle-shaped to large and flattened polygonal cells and some cuboidal one in confluent cultures, even though there is a predominance of the fibroblastic morphology (Javazon *et al*., 2004; Chamberlain *et al*., 2007).

As previously mentioned, MSCs are able to differentiate *in vitro* and *in vivo* (Figure 1), mostly along, but not restricted to, the osteogenic, chondrogenic and adipogenic lineages (Chamberlain *et al*., 2007; Pittenger *et al*., 1999; Kode *et al*., 2009). Additionally, MSCs have been reported to home to areas of tissue injury, where they set-up a regenerative microenvironment and participate in tissue repair (Chamberlain *et al*., 2007; Fox *et al*., 2007; Sordi, 2009). MSC homing follows a mechanism similar to the one of leukocyte trafficking, even though not involving exactly the same cytokines, chemokines and adhesion molecules (Chamberlain *et al*., 2007; Fox *et al*., 2007). MSCs have also been described to possess anti-inflammatory and immunomodulatory properties that can affect multiple arms of the immune system (Chamberlain *et al*., 2007; Kode *et al*., 2009; Nauta and Fibe, 2007). These features increase the clinical interest in these cells, particularly in regenerative medicine and for tolerance induction in allogeneic transplantation.

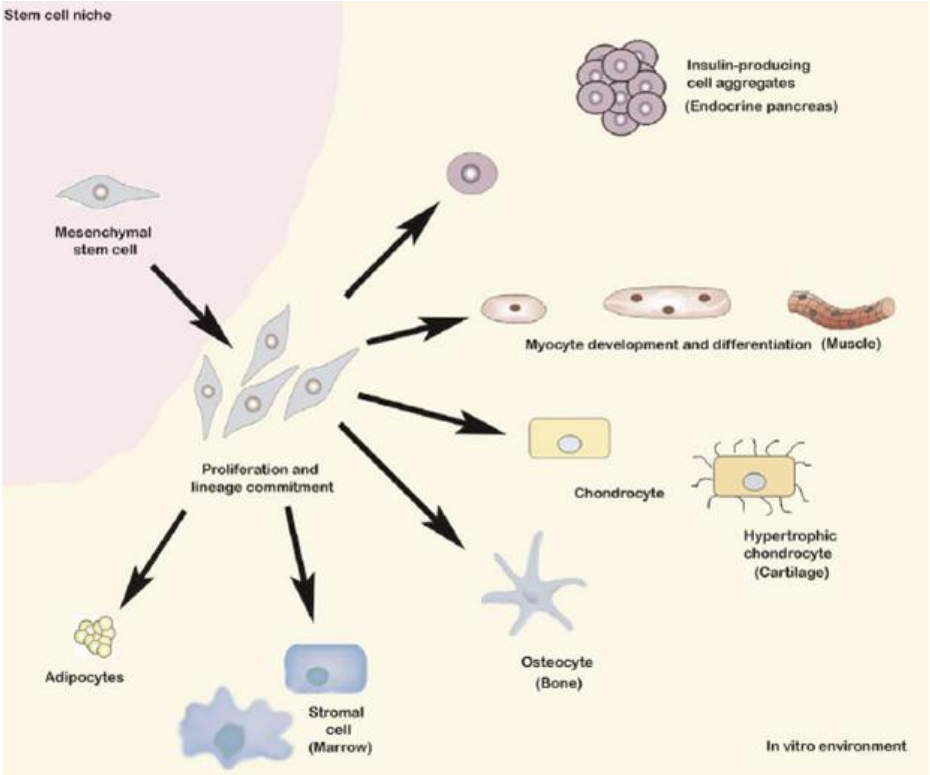


Figure 1. Differentiation potential of MSCs. Pictorial representation of MSCs expanded *in vitro* can be induced to differentiate into several lineages following exposure to specific differentiation factors. Image from Kode *et al*., Cytotherapy 2009, 11:377-91.

1.2. The Umbilical Cord as a stem cell source

Different MSC tissue sources may be especially suited for specific clinical applications as culture-expanded MSCs exhibit heterogeneity in their differentiation potential (Chamberlain *et al*., 2007; Nekanti *et al*., 2010). Although BM-MSCs have been most commonly used in differentiation studies, they are limited by the fact that the collection process is invasive, the number of cells obtained is limited and the quality of the cells in terms of proliferative and

differentiation abilities decreases in aged donors (Kim *et al*., 2010).Therefore, in recent years there has been a lot of interest in the search for novel stem cell sources for specific clinical applications and the umbilical cord is one such source (Nekanti *et al*., 2010).

The Umbilical Cord (UC) constitutes the link between mother and fetus during pregnancy (Can and Karahuseyinoglu, 2007) and is usually discarded after delivery, thus providing an abundant stem cells source in a noninvasive and ethical way (Nekanti *et al*., 2010). The UC inner architecture (Figure 2) is composed of a set of two arteries (UCA) and one vein (UCV) and a surrounding matrix of mucous connective tissue, first described by Thomas Wharton in 1656, and thus named Wharton's Jelly (WJ) (Can and Karahuseyinoglu, 2007). The WJ, which acts as a protective tissue of the UC vessels, comprises specialized fibroblast-like cells and occasional mast cells embedded in an amorphous ground substance rich in proteoglycans, mainly hyaluronic acid (Can and Karahuseyinoglu, 2007).

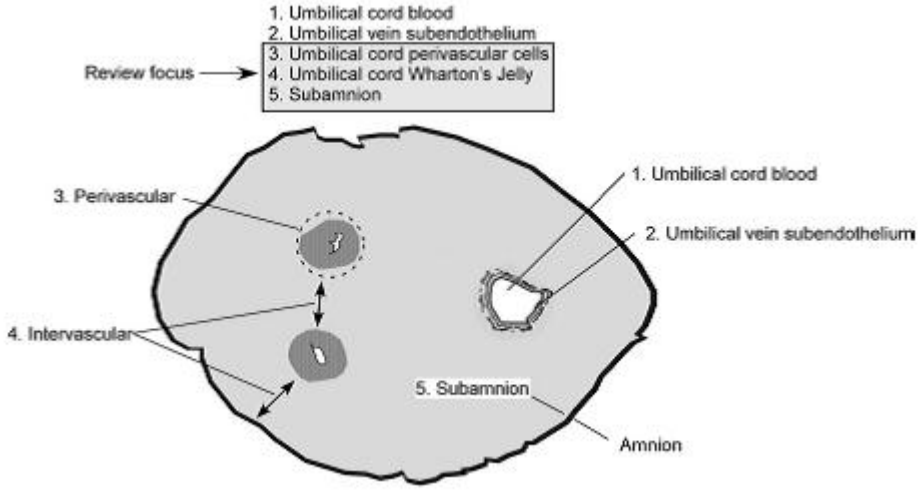


Figure 2. Pictorial representation of different regions of within the umbilical cord. The cord is divided in 5 compartments: 1. umbilical cord blood, 2. umbilical vein subendothelium, 3. perivascular, 4. intervascular and 5. subamnion. Wharton's jelly includes zones 3 through 5. Image from Troyer and Weiss. Stem Cells 2008, 26(3):591-9.

MSCs have been successfully isolated from UC WJ explants (Wang *et al*., 2004; Friedman et al., 2007; Karahuseyinoglu *et al*., 2007; Chen *et al*., 2009; Ishige *et al*., 2009; Fong *et al*., 2011; Hsieh *et al*., 2010;), UC vascular endothelial surface (Romanov *et al*., 2003; Sarugaser, *et al*., 2005;), UC vessels (Panepucci *et al* 2004; Ishige *et al*., 2009) and UC blood (Secco *et al*., 2009; Martins *et al*., 2009; Chang *et al*., 2006; Zeddou *et al*., 2010). They can also be obtained by processing the whole UC tissue (Zeddou *et al*., 2010, Secco *et al*., 2009). Two main methods have been described for isolating UC-MSCs: exclusively mechanical (Mitchell *et al*., 2003; Chen *et al*., 2009; Ishige *et al*., 2009), or the combination of mechanical and one or more enzymatic steps (Wang *et al*., 2004; Seshareddy *et al*. 2008; Karahuseyinoglu *et al*., 2007; Fong *et al*., 2007; Hsieh *et al*., 2010). Regarding UC blood (UCB), some researchers have successfully isolated MSCs from this source although there is great controversy in the literature regarding its efficiency and feasibility (Kim *et al*., 2010; Stronk *et al*., 2007; Martins *et*

al., 2009; Choi *et al*., 2009). Despite the low proportion of MSC obtained from UCB when compared to BM-MSCs, UCB-MSCs have been shown to exhibit a better performance in culture (Bieback *et al*., 2004; Yang *et al*., 2004; Sanchez-Ramos 2006). Nevertheless, more recently, Secco *et al*. (2008) have showed that UC stroma is much richer in MSCs than UCB. UC stroma MSCs have been reported to have several advantages compared to UCB MSCs and BM-MSCs (Troyer *et al*., 2008) such as a higher frequency of colony forming units (CFUs), which can be due to the expression of telomerase by UC stroma cells (Mitchell *et al*., 2003), and a lower immunogenicity (Weiss *et al*., 2008). Comparing UC stroma, UCA and UCV it was not conclusive which one was a better source (Ishige *et al*., 2009). Nevertheless, UCV cells appear to have a higher frequency of CFUs than UC and UCA. All these three tissue sources seem to have the ability to differentiate into chondrocytes, adipocytes and osteoblasts, but UC stroma was described by Ishige and colleagues as the one with the least osteogenic potential and UCA with the greatest (Ishige *et al*., 2009).

When compared with MSCs from BM, UC stroma and UC perivascular MSCs seem to proliferate better than the ones from the BM (Baksh *et al*., 2007; Hsieh *et al*., 2010). Regarding differentiation, UC perivascular MSCs were described to differentiate into osteoblasts more rapidly than BM-MSCs (Baskh *et al*., 2007). When compared to BM-MSCs, UC stroma cells were reported to be more committed to angiogenesis while BM-MSC preferentially undergo osteogenesis (Hsieh *et al*., 2010; Panepucci *et al*., 2004; Chen *et al*., 2009).

1.3. Clinical application of MSCs

As previously mentioned, thanks to their easy attainment, extensive *in vitro* proliferation, wide-range of differentiation potential, homing ability and immunological properties, MSCs are a promising tool in cell therapy, tolerance induction in allogeneic transplantation, and regenerative medicine (Bernardo *et al*., 2011; Kode *et al*., 2009; Krampera *et al*., 2006). For their application in regenerative medicine, stem cells must follow several criteria. These include: production of substantial numbers of cells, harvesting using a minimally invasive procedure, cell multilineage differentiation in a regulated and reproducible manner, safe and effective transplantation to either an autologous or allogeneic host, and manufacture in accordance with current Good Manufacturing Practice guidelines (Gimble *et al*., 2007).

Despite being a great promise for the future, until this date, there are few examples of clinical applicability of stem cells in the field of regenerative medicine. This is due to (Bajada *et al*., 2008): difficulties with extensive *in vitro* cell expansion; cell apoptosis following implantation; problems with vascularisation of the growing tissue; problems in finding viable scaffold biomaterials; high costs associated with prolonged maintenance of the cells in culture; and moral concerns or ethical issues that delay the transition for clinical use.

The increasing interest in the use of MSCs in tissue regeneration and the treatment of immune disorders leads to the need of developing production processes that are in accordance with Good Manufacturing Practices (GMPs). These regulations have the aim of ensuring the

delivery of safe, reproducible, and effective products (Kode *et al*., 2009; Sensebé *et al*., 2011). And therefore cover all aspects of manufacturing (Sensebé *et al*., 2011). In Europe, MSCs are considered advanced therapy medicinal products (ATMPs), as defined by the European Regulation (Sensebé *et al*., 2011).

Regarding the procedures for the production of MCSs for therapeutical purposes, the main parameters to be considered are the sources and collection methods, extent of manipulation (preferentially in closed systems), methods for MSC enrichment, purity, culture medium, cell plating density, passaging, and time in culture, as all these may affect the quality of MSCs (Bourin *et al*., 2008; Sensebé *et al*., 2010; Sensebé *et al*., 2011). For these reasons, finding the optimal culture conditions has been of great interest to researchers.

In particular, the culture medium is crucial for the efficacy and safety of MSC cultures. The media used in culture processes should maintain the phenotype, genotypic stability, proliferation and functions of MSCs during multiple passages. Consensus is lacking on the ideal medium for MSC expansion, but the standard culture condition has been a basal medium supplemented with animal serum, typically at a concentration of 10%, with or without added growth factors. Numerous types of basal media have been tested, and one of the main issues, the glucose content, remains controversial as its effect might be dependent on the cell source or differ between short-term and long-term culture (Sotiropoulou *et al*., 2006; Weil *et al*., 2009; Pal *et al*., 2009; Nekanti *et al*., 2010). Besides glucose, various other factors are important in the culture medium such as additional aminoacids, vitamins, inorganic salts, etc. (Nekanti *et al*., 2010).

Regarding the serum, Fetal Bovine Serum (FBS) is the most commonly and successfully used. However, the animal and poorly defined nature of FBS, the batch to batch variability, the risk of sensitisation due to FBS proteins retained in the cytoplasm of MSCs, and the fact that it is a possible source of bovine prion, viral and zoonose contamination (Schallmoser *et al*., 2007; Chase *et al*., 2010) make it an undesirable component of the culture medium. Despite these hindrances, FBS can be used for GMP production of MSCs as long as it is carefully tested before use and a Transportation Security Administration certificate is obtained to ensure safety in terms of the risk of infectious disease transmission (Sensebé *et al*., 2011). Nevertheless, there still is an increased risk associated with the use of FBS as reported by Selvaggi and collegues that when it was infused into patients lymphocytes expanded in FBS, these patients developed hypersensitivity reactions (Selvaggi *et al*., 1997).

Nevertheless, there still is an increased risk associated with the use of FBS and some examples have previously been reported: alteration of cell behaviour and morphology in the presence of FBS in culture (Hung and Young 2006) and the development of hypersensitivity, malignant ventricular arrhythmias and in some cases sudden death in patients who received cells cultivated in FBS (Selvaggi *et al*., 1997; Chachques *et al*., 2004). Therefore, safety requirements imply replacing FBS with more secure products such as human components or a fully defined serum-free medium.

Replacing FBS with human or humanised components is one of the most challenging aspects of GMP translation in MSC production. The reduced efficiency of animal serum-free culture conditions may be overcome by supplementing the medium with growth factors and cytokines that activate signalling pathways crucial for MSC expansion while preserving their differentiation potential. Some of the growth factors already reported to have an essential role are the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor (TGF)-β, insulin-like growth factor (IGF), and fibroblast growth factor (FGF)-2 (Kilian *et al*., 2004; Choi SC *et al*., 2008; Ng *et al*., 2008; Chase *et al*., 2010; Tamama *et al*., 2010). However, the complete growth factor requirements for MSC expansion are still unknown. In other studies, human serum (hS), or in particular human AB serum (hABS), plasma (hP), platelet lysates (hPL) or thrombin-activated platelet releasate in plasma (tPRP) have also been tested as FBS replacements (Kocaoemer *et al*., 2007; Bieback *et al*., 2009; Doucet *et al*., 2005; Shahdadfar *et al*., 2005; Schallmoser *et al*., 2007; Strunk *et al*., 2007; Avanzini *et al*., 2009; Turnovcova *et al*., 2009). The most promising and most often used is the platelet lysate, human plasma enriched by growth factors released from platelets. Platelet granules contain many growth factor proteins, including PDGF, fibroblast growth factor (FGF), IGF, TGF-β, platelet factor 4 (PF-4), and platelet-derived epidermal growth factor (PDEGF) (van den Dolder *et al*., 2006; Doucet *et al*., 2005; Chevallier *et al*., 2010). These growth factors, which are released from platelet concentrates when subjected to freeze-thaw cycles, have been shown to successfully support MSC expansion *in vitro* (Doucet *et al*., 2005; Schallmoser *et al*., 2007; Müller *et al*., 2006). Regarding human serum, its successful use seems to be restricted to autologous serum (Yamamoto *et al*. 2003; Lin *et al*., 2005; Shahdadfar *et al*., 2005; Mizuno *et al*., 2006), as allogeneic serum has produced contradictory results (Shahdadfar *et al*., 2005; Kocaoemer *et al*., 2007). However, pooled human allogeneic serum would provide a more abundant and immediately available source and would thus be a more advantageous alternative than autologous serum to expand MSCs for clinical application (Kocaoemer *et al*., 2007; Mannello *et al*., 2006). Nevertheless, there still is a need to test the proliferation and functionality of MSCs grown in these media.

1.4. MSC multi-lineage differentiation

As defined by the ISCT, to be classified as MSCs, the cells must differentiate into osteoblasts, chondrocytes and adipocytes under appropriated conditions.

One of the most promising applications of MSCs in regenerative medicine is in bone repair (Krampera *et al*., 2006; Sensebé *et al*., 2010). The bone is a mesenchymal-derived tissue with various functions, including mechanical, metabolic and protective of vital organs (Clarke, 2008; Duplomb *et al*., 2007). It is composed of an organic mineralised connective tissue with two main cell lineages: osteoblasts and osteoclasts (Duplomb *et al*., 2007). Osteoblasts are mesenchymal-derived cells specialised in the production of organic matrix and in the

mineralisation process (Jayakumar and Di Silvio, 2010). Osteoblast cells can progressively differentiate into osteocytes and these two cell types together constitute a cellular network that allows the communication between bone surface and mineral matrix (Clarke, 2008; Jayakumar and Di Silvio, 2010). Bone resorption is controlled by osteoclasts, which differentiate from hematopoietic precursors (Clarke, 2008; Duplomb *et al*., 2007; Jayakumar and Di Silvio, 2010). A balance between osseous tissue formation by osteoblasts and bone resorption by osteoclasts is required for bone homeostasis (Clarke, 2008; Jethva *et al*., 2009). The osseous organic matrix (the osteoid), produced by the osteoblasts, is mainly composed of collagen type I and II, fibronectin and growth factors. This matrix subsequently gets mineralised forming an inorganic matrix that consists of hydroxyapatite crystals (Clarke, 2008).

A good understanding of the osteoblast differentiation process is important for two main reasons: to allow the application of osteoblast committed-cells in regenerative medicine, and to identify new key genes that could be deregulated during pathological processes (bone tumour, osteoporosis, etc). Osteoblast differentiation is controlled by a hierarchy of transcription factors that are expressed in a defined temporal sequence, both *in vivo* and *in vitro* (Franceschi *et al*., 2009; Komori, 2006, Kalajzic *et al*., 2005) (Figure 3). During embryonic development the transcription factor Runx2/Cbfa1 (core binding factor α1) first appears with the formation of mesenchymal condensations in areas destined to become bone, and persists through subsequent stages of bone formation (Franceschi *et al*., 2009). Osterix (Osx) is a zinc finger-containing transcription factor, which is important for the commitment of osteoblast progenitors into the osteoblast lineage (Nakashima *et al*., 2002). In the absence of Osx, no cortical bone and no bone trabeculae were formed through either intramembranous or endochondral ossification (Nakashima *et al*., 2002). Activating transcription factor 4 (Atf4) appears to be important in later phases, and is expressed in mature osteoblasts (Franceschi *et al*., 2009). The activation of these transcription factors is accompanied by the expression of other specific proteins, which can be used as markers to identify the differentiation stage of osteoblasts (Kalajzic *et al*., 2005; Franceschi *et al*., 2009). Type I collagen and osteonectin (a calcium-binding glycoprotein) start being transcribed at the phase of bone matrix formation, following Atf4 activation. When the mineralization phase is initiated a large increase in osteocalcin (bone gamma-carboxyglutamic acid-containing protein) expression is observed (Franceschi *et al*., 2009). Additionally, bone sialoprotein (BSP) and alkaline phosphatase (AP) have also been described to be involved in the mineralisation process (Fauran-Clavel and Oustrin, 1986; Franceschi *et al*., 2009). Osteogenic assessement is usually performed by staining for AP activity, and in a later phase by Alizarin Red or von Kossa techniques that specifically detect for calcium deposition.

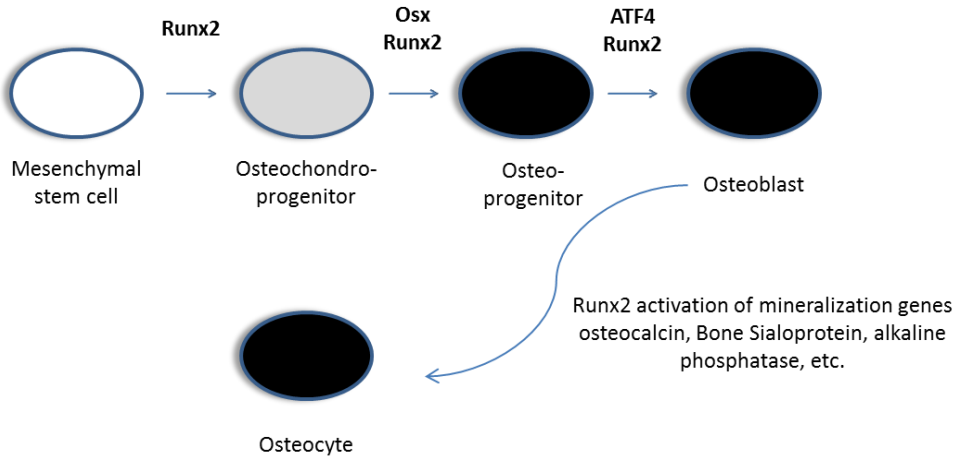


Figure 3. Hierarchy of transcription factors that control osteogenic lineage. Adapted from Franceschi *et al*. Cell Tissues Organs 2009, 189:144-152.

The standard method to induce osteogenic differentiation has been to add combinations of dexamethasone (dex), β-glycerophosphate (β-GP), ascorbic acid (asc), and occasionally 1α,25-dihydroxy vitamin D3, to the culture medium in the presence of FBS (Jaiswal *et al*., 1997; Vater *et al*., 2011).

Dex is a synthetic glucocorticoid essential for osteoprogenitor cell differentiation (Vater *et al*., 2010) but also to enhance cell growth both alone and in combination with β-GP and asc (Coelho and Fernandes 2000). At an initial phase, this compound seems to induce AP activity and later, in the mineralising phase, increases the deposition of calcium (Coelho and Fernandes 2000; Porter *et al*., 2003). However, glucocorticoids have been described to cause bone fragility and bone loss, via the inhibition of bone matrix proteins such as type I collagens and osteocalcin (Iu *et al*., 2005). These contradictory observations suggest that the dex effect on osteogenic differentiation is dependent on both the dosage and the timing of treatment (Porter *et al*., 2003; Iu *et al*., 2005, Park *et al*., 2010) and that dex is not required continuously, but only at particular stages, to generate the osteoblast phenotype (Beloti and Rosa 2005; Park 2010).

β-GP is enzymatically hydrolysed by alkaline phosphatase, serving as a crucial source of inorganic phosphate during matrix mineralisation (Chang *et al*., 2000) and it is almost entirely degraded during the first two weeks of culture (Coelho and Fernandes, 2000). When β-GP is added in combination with asc and dex causes an increase in AP activity (Park, 2010; Vater *et al*., 2010), but on its own leads to a decrease in the activity of this enzyme (Park, 2010). Additionally, β-GP seems to be the only of the three factors that by its own has the ability to form mineral deposits (Coelho and Fernandes, 2000).

Asc, or the more stable form, ascorbic acid 2-phosphate, has a mitogenic effect and is a cofactor for collagen hydroxylation (Vater *et al*., 2011), which is important for its synthesis and maturation (Takamizawa *et al*., 2004). In addition, asc appears to stimulate cell growth at any concentration (Takamizawa *et al*., 2004) with optimal maximum proliferation rate when at 250μM (Choi KM *et al*., 2008).

The osteogenic factors dex, β-GP and asc are generally used in combination with FBS and, as previously discussed, the use of FBS presents several disadvantages when aiming for a clinical application. Therefore, there also is the need for replacing FBS by recombinant growth factors or human components during osteogenic differentiation. Several signalling pathways have been involved in osteoblast differentiation, including the Bone morphogenetic proteins (BMP), FGF, TGF-β, Notch and canonical Wnt pathways (Canalis, 2008; Engin and Lee, 2010; Komori, 2006; Rawadi *et al*., 2003; Valta *et al*., 2006; Yamaguchi *et al*., 1991; Zamurovic *et al*., 2004). In a number of studies, activators and inhibitors of these pathways have been added to the culture medium as a mean to induce or repress differentiation, hence allowing a better understanding of the differentiation process and revealing candidate growth factors to replace FBS.

In a different approach, Chevallier *et al*. (2010), have used platelet lysate, which contains a mixture of growth factors (including PDGF, FGF, IGF, TGF-β, PF-4 and PDEGF), and showed that it enhanced MSC expansion and replaced osteogenic agents during pre-osteoblastic induction. In this study, MSC cultured in platelet lysate showed spontaneous osteoblastic gene expression and enhanced *in vivo* osteogenic capacity during ectopic bone formation (Chevallier *et al*., 2010). Moreover, cells expanded in other animal serum-free supplements, such as with AB hS, autologous hS and hPLP, seem to maintain their osteogenic potential (Kocaoemer *et al*.,2007; Doucet *et al*., 2005; Lange *et al*., 2007; Yamamoto *et al*., 2003; Bernardo *et al*., 2006). Shahdadfar and colleagues observed that osteogenesis was sucessful in cells expanded with autologous hS, even though taking longer for this to be achieved (Shahdadfar *et al*., 2005).

Pre-clinical and clinical studies have been performed for several bone disorders, including the genetic disorders Osteogenesis Imperfecta, infantile hypophosphatasia and osteopetrosis, as well as the acquired disease osteoporosis (Horwitz *et al*., 1999; Arthur *et al*., 2009; Jethva *et al*., 2009). On the other hand, in vivo studies on animal model transplantation showed that MSCs represent a good tool to improve bone regeneration (Jones and Yang, 2011) having, for example, increased bone formation in rat femur (Bruder *et al*., 1998) and osteoporotic rabbit (Wang *et al*., 2006) models. Also, a clinical trial involving the injection of autologous osteoblasts cultured from bone-marrow precursors showed a significant fracture healing improvement (Kim *et al*., 2009a).

MSCs also have the ability to differentiate into chondrocytes. For chondrogenesis to be achieved it requires close cell-to-cell contact and the addition of chemical supplements. The close cell-to-cell contact is generally achieved by promoting MSC concentration forming a three-dimensional a micromass pellet. The cell pellet enhances the upregulation of extracellular cell matrix (ECM) interations, leading to the formation of pre-chondrocytes. After condensation, the cells become highly proliferative and change the ECM composition until reaching a mature chondroblast. Collagen type II is the most abundant fiber in this ECM, which is typical of articular cartilage. During this process, MSC fibroblastic shape changes to a large and round morphology. The three most important factors added to promote chondrogenesis are dex, asc

(which are commonly use in osteogenesis) and TGF- β, but BMPs, FGF and IGF are also commonly added. In animal serum free conditions, chondrogenic differentiation seems to be also maintained in the presence of autologous hS and hPLP, as it was for osteogenesis (Shahdadfar *et al*., 2005; Lange *et al*., 2007; Doucet *et al*., 2005). MSC differentiation into chondrocytes would allow their use in the treatment of several pathologies such as pain in the joints, which is mostly due to cartilage degeneration (Vater *et al*., 2010; Nesic *et al*., 2006) and avoid the need for orthopaedic surgery with all its complications and side effects (Koga *et al*., 2009; Vater *et al*., 2010).

Finally, MSC are also able to differentiate into adipocytes. Adipocyte differentiation begins with the commitment of MSCs into pre-adipocytes that contain small lipid-rich vacuoles (Frith and Genever 2008; Can and Karahuseyionoglu *et al*., 2007). Subsequently, these cells develop into mature adipocytes, when, eventually, the lipid vacuoles fuse and fill the cells (Chamberlain *et al*., 2007). Along this process fibroblastic MSCs are converted to a spherical morphology (Vater *et al*., 2010.) To induce adipogenesis it is normally use dex, 3-isobutyl-1-methyl-xanthine (IBMX), insulin and indomethacin are normally used. Some researchers additionally use triiodothyinine (T3), Asc-2-P and bFGF-2 (Vater *et al*., 2010). Upon induction the cells express specific adipocyte markers such as peroxisome proliferation-activated receptor γ2, lipoprotein lipase, and the fatty acid-binding protein aP2 (Pittenger *et al*., 1999; Chamberlain *et al*., 2007). Adipogenesis evaluation is performed histologically using the Oil Red dye, which marks the lipids in the vacuoles (Can and Karahuseyionoglu *et al*., 2007; Vater *et al*., 2010). The adipogenic differentiation potential of MSCs expanded in animal serum-free conditions is controversial as some authors argue that MSCs growing hPLP supplemented in culture media retain adipogenesis ability (Bernado *et al*., 2006; Doucet *et al*., 2005), whereas others show contradictory results (Lange *et al*., 2007). As for the other mesenchymal lineages, adipocyte differentiation from MSCs would have important clinical applications following procedures or pathologies where large volumes of adipose tissue are lost (Gomillion and Burg 2006; Vater *et al*., 2010).

1.5. Human Amniotic Membrane

The promising use of MSCs in the field of regenerative medicine is undeniable, either through the local implantation of *in vitro* expanded autologous MSCs or of a pre-differentiated tissue matrix loaded onto suitable scaffolds.

The ideal scaffold is expected to be biocompatible and should mimic the structure and biological function of native extracellular matrix. Active research is under way for the development of improved scaffolds, using a variety of natural and synthetic materials and advanced technologies (Sittinger *et al*., 2004; Tsigkou *et al*., 2009; Nöth *et al*., 2010). The human amniotic membrane (AM) is a good candidate for this application. The AM has as primary function the protection of the embryo during its development. Therapeutically, this membrane has been successfully used as a “biological dressing” over more than one hundred

years in cutaneous wounds and burns (Lo and Pope, 2009). However, it was only later that the great potential of the AM in ophthalmological, urological, gynaecological and neurological fields was realised (Tan *et al*., 2009; Burman *et al*., 2004; Sharifiaghdas *et al*., 2009; Ward *et al*., 1989). The AM has recently been used in some studies as a substrate for MSC expansion and differentiation along different lineages with the aim of subsequent transplantation. Among other studies, Tan *et al*. (2011) have reported the successful chondrogenic differentiation of MSCs on AM for use as a cell delivery vehicle, Kim and colleagues showed that grafts of AM loaded with MSCs play an effective role during the healing of skin defects in rabbits (Kim *et al*., 2009b) and, in another report, a layer of somatic epidermal adult stem cells was cultivated and carried on AM into the ocular surface of a goat model of limbal stem cell deficiency (Yang *et al*., 2008).

Importantly, a variant of the AM that is not rejected after allogeneic transplantation is currently prepared at the Tissue Bank of the Centro de Histocompatibilidade do Sul. During the preparation of this AM, apoptosis of the cells in the membrane is induced turning it immunologically inert, while preserving the tissue integrity. Also, the AM contains a variety of cytokines and growth factors (Koizumi *et al*., 2000) which may be responsible for its intrinsic regenerative and antiinflamatory properties (Fernandes *et al*., 2005; Insausti *et al*., 2010; Velez *et al*., 2010; Samandari *et al*., 2011). The high levels of growth factors such as EGF, bFGF, keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF), support epithelialization and regulation of fibrosis and neovascularization process (López-Valladares *et al*., 2010). López-Valladares and colleagues have shown that there are growth factor variations depending on the age of AM donors and the gestational stage, as mothers under 35 years old with 38 to 39 weeks of pregnancy have higher levels of growth factors (López-Valladares *et al*., 2010). Although it has not been proven, it is expected that higher levels of growth factors produce better clinical results (Koisumi *et al*., 2000; López-Valladares *et al*., 2010). The AM is thus a promising substrate for MSC attachment and differentiation and a safe and effective vehicle for transplantation.

2. Materials and Methods

2.1. Isolation of MSCs from different parts of UC

In this study, MSCs were isolated from different parts of 26 human umbilical cords using a variety of methodologies. The UCs were obtained with written informed consent of the parturients from elective caesareans. Human umbilical cords were first washed in Hanks' balanced salt solution (GIBCO; 14175-129) with antibiotics (Penicillin-Streptomycin and Gentamicin) and then processed for MSC isolation. This was accomplished using different combinations of mechanical and/or enzymatic and mechanical methods in search for an optimal one (see supplementary Table 1). MSCs were obtained from WJ (peripheral and total), vessels and whole cord. For the isolation of peripheral Wharton' Jelly (pWJ)), pieces of mesenchymal tissue were cut around the vessels from the outside part of the UC. MSCs from total Wharton's Jelly (tWJ) were obtained by cutting open UC and subjecting the inner part to an initial 1h of enzymatic digestion for removal of the arteries and veins to isolate the WJ. The pWJ and tWJ mesenchymal tissue was then cut into small fragments and directly plated or submitted to a 4 to 16h enzymatic digestion. The arteries and veins obtained by this method were also cut into small pieces and submitted to a 4h enzymatic digestion to isolate MSCs from vessels (V-UC). To obtain the MSCs from whole parts of umbilical cord (W-UC) a cord piece with 2-3cm was cut into small fragments and submitted to a 4h enzymatic digestion. The enzymatic solution used for tissue digestions was composed of 0.1-2 mg/ml collagenase XI (Sigma; C9697) alone or with 100U/ml hyaluronidase (Sigma; H2126). In some cases, a subsequent digestion with Tryple Express® (a trypsin replacement from Invitrogen; 12604-013) was performed. The dissociated cells were then washed in Dulbecco's phosphate buffered saline (DPBS - GIBCO; 14190-169), resuspended in MSC/FBS medium or in StemPro® MSC SFM medium (GIBCO; A10332-01) and cultured on tissue culture plastic plates at 37ºC in a 5% CO_2 humidified incubator.

2.2. MSC *in vitro* culture

For routine *in vitro* MSC expansion, cells were cultured in MSC-FBS medium on tissue culture plastic flasks (Greiner; C6481-200EA) at 37ºC in a 5% CO2 humidified incubator. MSC/FBS medium was composed of DMEM-F12 (1:1) (GIBCO; 21331-020), 2mM L-Glutamine (GIBCO; 25030-024), 15mM HEPES (GIBCO;15630-056), 100U/ml penicillin (GIBCO; 15140-122), 100µg/ml streptomycin (GIBCO; 15140-122), 5 µg/ml Gentamicin (GIBCO; 15710-049) and 10% MSC-qualified Fetal Bovine Serum (FBS – GIBCO; 12662-029). Every 2-3 days, approximately half of the medium was replaced by fresh medium and when reaching approximately 80% confluence the cells were passaged by dissociation using Tryple. The cells were then counted using a Neubauer hemocytometer and replated at 0.5 or $1x10^4$cells/cm^2. For low confluence experiences cells were replated at 200cells/cm^2 in 75-cm^2 flasks (Greiner;

C7106-120EA) MSC-FBS medium. Low passage MSCs were also frozen for later use in DMEM-F12 containing 10% tissue-culture grade dimethyl sulfoxide (DMSO, Merck; K28452252-101) and 10% FBS, kept at -80ºC for a few days and then stored in liquid nitrogen.

2.3. Colony forming units (CFUs) assay

For CFUs analysis, cells were plated at 200cells/cm^2 and cultured in petri dish (Nunc; 150350) in MSC-FBS medium. The medium was replaced twice a week and cultures were stopped at day 14. Colonies were washed with PBS, fixed with 0.05% glutaraldehyde (Sigma; G6257-10X10ML) for 5min and stained with 0.5% aqueous crystal violet (Sigma; C0775-25G). The colonies were then washed 3 times with PBS and the colonies were counted. All experiments were performed in triplicates.

2.4. Immunofluorescence

2.4.1. Flow Cytometry

For the immunophenotypic characterization of UC MSCs, the cells were analysed for the presence of the surface markers CD90, CD73, CD105, CD44, CD34, CD45, CD19, CD14 and HLA-DR using the specific fluorochrome-conjugated monoclonal antibodies listed on table 2. Briefly, the cells were dissociated into a single cell suspension and approximately 1x10^5 cells were incubated with the appropriate monoclonal antibodies (see table 2) in 0.5%BSA/PBS (Sigma;A7284-50ML) for 1 hour in the dark. Following this incubation, the cells were washed in 2mL of PBS and centrifuged. The pellet was then resuspended in PBS and analysed by flow cytometry in a FACSCalibur instrument with CellQuest software (both BD Biosciences).

Table 2: List of monoclonal antibodies used for MSC immunophenotyping.

Antigen	*Antibody Clone*	*Isotype*	*Fluorescence*	*Brand*
CD34	8G12	Mouse IgG1, κ	PerCP-Cy5.5	BD Biosc.
CD34	8G12	Mouse IgG1, κ	PE	BD Biosc.
CD19	HD37	Mouse IgG1, κ	PE	BD Biosc.
CD14	MφP9	Mouse IgG1, κ	PE	BD Biosc.
HLA-DR	L243	Mouse IgG1, κ	PerCP	BD Biosc.
CD44	IM7	Rat IgG2b, κ	FITC	BioLegend
CD44	IM7	Rat IgG2b, κ	PE-Cy7	BioLegend
CD45	HI30	Mouse IgG1, κ	PerCP	BioLegend
CD73	AD2	Mouse IgG1, κ	PE	BioLegend
CD90	5E10	Mouse IgG1, κ	APC	BioLegend
CD105	43A3	Mouse IgG1, κ	FITC	BioLegend
CD105	43A3	Mouse IgG1, κ	APC	BioLegend
bAP	0.G.2	Mouse IgG1, κ	FITC	Abcam
TNAP	B4-78	Mouse IgG1, κ	FITC	Santa Cruz Biotechnology, inc

Osteocalcin	190125	Mouse IgG1, κ	PE	R&D System

2.4.2. Fluorescence Activated Cell Sorting (FACS)

The population enrichment in for $CD105^{low}$ and $CD105^{high}$ or $CD105^{low}$ /$CD90^{low}$ and $CD105^{high}$/$CD90^{high}$ MSCs was performed by FACS sorting. The cells expanded in MSC/FBS medium were dissociated using Tryple and the cell suspension were passed through a 50µm cell strainer (Partec; 04-004-2327) to obtain a single cell suspension and approximately $1x10^6$ cells were incubated with CD105 and CD90 monoclonal antibody (see Table 2) in PBS/0.5%BSA for 1 hour in the dark in a sterile environment. Following this incubation, the cells were washed in 2mL of PBS and centrifuged at 1400rpm. The resuspended pellet in PBS was passed through FACSAria instrument (with FACS Diva software) (both BD Biosciences) for sorting of the populatons of interest. Then, $CD105^{low}$(/$CD90)^{low}$ and $CD105^{high}$(/$CD90)^{high}$ MSC populations were gated and sorted directly to fresh culture medium containing antibiotics. The sorted populations were then plated into tissue culture plates with 1mL of MSC/FBS medium.

2.5. Gene Expression Analysis

The gene expression of markers of MSCs and of different stages of osteoblastic differentiation was analysed by RT-PCR. With this purpose, cells were dissociated, pelleted and immediately stored at -80ºC until RNA extraction. RNA was extracted using the TRIzol reagent (Invitrogen; 15596-026) or the RNeasy mini kit (Qiagen; 74104) in combination with the in column DNase digestion set (Qiagen; 79254), and quantified using a Beckman Coulter DU 530 UV/Vis spectrophotometer or NanoDrop® 2000 spectrophotometer. The extracted RNA was run on a 1.8% agarose gel to verify its quality and integrity. The RNA was then reverse transcribed using the SuperScript III Reverse Transcriptase system (Invitrogen; 18080-129). 1µg of total RNA, 1 µl of 50 µM random primers (Applied Biosystems) and RNase free water (Sigma; 95284-100ML) were mixed to a final volume of 10 µl. After a 5 minute incubation at 65°C the samples were put on ice and a mix containing 0.5mM Deoxynucleotide set (Sigma; DNTP100-1K), 5 mM DTT, 2 U/µl RNaseOUT RNase inhibitor (Invitrogen; 10777-019) and 10 U/µl Superscript III in first strand buffer was added. A reaction mixture without the enzyme was also set up as a negative control (designated “-RT”). The mixture was incubated at 25°C for 5 min, 50°C for 1h and at 70°C for 15 min. The cDNAs were diluted 1:10 in dH2O and kept at -20°C until PCR analysis.

For RT-PCR amplification a reaction mix was set up as follows: 100 ng of cDNA were added to 10 µmol of each primer (Eurofins MWG Operon), 0.2 mM dNTPs, 1 unit of AmpliTaq DNA Polymerase and PCR Buffer (Applied Biosystems; N8080166), in a total volume of 30 µl. Negative controls included the –RT samples and the non-template control (containing dH_2O instead of cDNA). PCR was performed on a GeneAmp PCR System 2720 (Applied Biosystems) and PCR conditions included an initial denaturation step at 94ºC for 5 minutes, followed by 35

amplification cycles of 45 seconds at 94ºC, 30 seconds annealing at 59ºC and 45 seconds extension at 72ºC, followed by a final elongation step of 10 minutes at 72ºC. The resulting PCR products were size-fractionated by electrophoresis in a 1.8 % agarose gel containing ethidium bromide, and visualised under ultraviolet light in a Biorad Gel Doc XR transiluminator. Specific primers for the amplification of genes of interest were designed using the NCBI primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and are listed on table 3.

Table 3: Primers used for the gene expression analysis of markers of MSCs and of osteoblastic differentiation.

Gene symbol	*Forward primer (5'→3')*	*Reverse primer (5'→ 3')*	*Amplicon size (bp)*
ACTB	CCGCCAGCTCACCATGGATGA	TCTTGCTCTGGGCCTCGTCG	197
CD90 (Thy1)	TCGCTCTCCTGCTAACAGTCT	CTCGTACTGGATGGGTGAACT	134
CD73 (NT5E)	AGTGAGGGGTGTGGACGTCGT	TGCCAAAAGCATAGGCCTGGACT	158
CD105 (ENG)	ATCCTCCTCCGACTGGGCCAA	TGCGCAGCTCAGTTCCACCTT	228
RUNX2	TCCTATGACCAGTCTTACCCCT	GGCTCTTCTTACTGAGAGTGGAA	190

2.6. Xeno-free culture of UC-MSCs

Following MSCs isolation, their culture in a chemically defined medium was tested. As a first approach, the commercial serum-free StemPro® MSC SFM medium was used. This medium is supplemented with human platelet-derived growth factor-BB (PDGFBB), basic fibroblast growth factor (bFGF) and TGF-b1, a combination of growth factors that has been shown to be able to promote extensive propagation with retained phenotype, colony-forming unit, and differentiation potential (Ng et al., 2008; Chase et al.,2010).

Subsquentely, human AB serum, platelet-lysate and plasma were test as suitable alternatives for FBS replacement for MSC expansion. Platelet-lysate was obtained by platelet apheresis procedures performed at Instituto Português do Sangue (IPS, Lisbon, Portugal). One pool of platelets was centrifuged twice for 30min at 3850rpm and the platelet pellet was resuspended in DMEM-F12. The plasma supernatant was collected, filtrated in a Stericup (Millipore; SCGPUO1RE), aliquoted and frozen at -80°C (hP). Platelet in medium samples and the remaining platelets in plasmas were frozen at -80°C and subsequently thawed at 37°C to obtain platelet-released growth factors. Platelet bodies were then eliminated by centrifugation of 30min at 3850rpm (twice) and it was obtained supernatant of the platelet-lysate in plasma (hPLP) and the platelet-lysate in medium (hPL). After filtration, hPLP and hPL were aliquoted and frozen at -80°C. For this study a commercial human AB pooled serum (Lonza; 14-498E) was also used in this study.

For the studies of xeno-free media, tissue culture plates were pre-coated with human plasma fibronectin (2µg/cm^2 – Millipore; FC010-1mg) for 30 min. Subsequently, early passage MSCs were dissociated with Tryple, and 1 x 10^4 cells were plated per cm^2 in the different xeno-

free media. To the media supplemented with hPLP and hP was added heparin at a concentration of 1U/mL (Biochron AG, L6510) to avoid media coagulation. To all platelet lysate, plasma and serum conditions were added 250µM asc (Sigma; A8960-5G) to enhance cell proliferation.

2.7. Multilineage differentiation and detection methods

UC-MSCs were induced to adipogenesis, osteogenesis and chondrogenesis and detected by histological staining, RT-PCR and immunophenotypiing.

Osteogenic differentiation of MSCs was induced following protocols relatively well established (Vater et al., 2011). Briefly, a monolayer of MSCs is cultured in the presence of dexamethasone (dex), beta-glycerophosphate (b-GP) and ascorbic acid (asc), leading to the formation of a mineralised matrix in 4 to 6 weeks. In this study, small variations within this osteogenic induction protocol were tested in search for the most efficient one. MSCs were thus plated on tissue-culture plastic, with or without fibronectin precoating. Cell confluences ranged from 0.5 to 2 x 10^4 cells/cm^2, and several differentiation media conditions were tested: the MesenCult osteogenic stimulatory kit (Stem Cell Technologies; 5404), StemPro® Osteogenesis differentiation Kit (GIBCO; A1007201) and DMEM-F12 (1:1), DMEM-KO (GIBCO; 10829-018), DMEM-LG (GIBCO; 11054-001) and MesenCult basal medium supplemented with 10% FBS, 100 nM dexamethasone (Sigma; D4902-25MG), 0.2 mM L-ascorbate (Sigma; A8960), and 10 mM b-glycerophosphate (Sigma;G9422-10G).

After 14 to 21 days, during which the medium was changed every 3 days, osteoblastic differentiation was assessed by flow cytometry immunophenotyping, gene expression analysis, alkaline phosphatase activity assays and by Alizarin Red S (Sigma; A5533-25G) or von Kossa staining for detection of calcium deposition. This characterisation of the differentiating cells in terms of expression of surface markers, gene transcription and specific functions of each of the differentiation phases (osteoblast proliferation, matrix deposition and mineralization phase) allows following the differentiation process.

Alkaline Phosphatase activity of osteoblastic differentiation was assessed using the Alkaline Phosphatase kit from Sigma (Sigma; 86R-1kt). Briefly, cells were fixed in Citrate-acetone-formaldehyde fixative solution for 30 seconds, rinsed in deionised water for 45 seconds and subsequently incubated with alkaline-dye mixture at room temperature for 30 minutes (protected from the light), rinsed for 2 minutes in deionised water and left to dry before analysis.

Mineralised deposits were visualised by von Kossa or Alizarin Red S staining after 21 to 40 days. For von Kossa staining, cells were fixed in 4% PFA for 20 min and exposed to 1% silver nitrate under bright light for 60-90min (this leads to deposition of silver, which can then be visualised as metallic silver, by replacing the calcium reduced by the strong light). Following rinsing in distilled water, unreacted silver was removed with 5% sodium thiosulfate for 5 minutes, rinsed again in distilled water, and finally visualised the brownish mineralized deposits. For Alizarin Red staining, cells were fixed with PBS/10% Formaldehyde (Sigma; 3220-500ML)

for 15 min, washed 3 times with ddH_2O and staining with 0.1% Alizarin Red/ddH_2O working solution (adjusted the pH to 4.1-4.3) for 1 hour with a slow circular agitation. The cells were then rinsed 3 times with PBS for 5-10min with agitation and visualized the reddish mineralized deposits.

Adipogenic differentiation of MSCs was induced with commercial StemPro® adipogenesis differentiation kit (GIBCO; A1007001). MSCs were plated at cells density of $1x10^4$cells/cm^2 and culture medium was replaced every 3 days. After 14 days in culture cells were washed in PBS and fixed with 0.5% glutaraldehyde (Sigma) for 5min. After washing, the cells were stained with 0.5% Oil Red O solution in 60% isopropanol (Sigma; O0626-25G) working solution, washed and visualised.

Chondrogenic differentiation of MSCs was induced with commercial StemPro® chondrogenic differentiation kit (GIBCO; A1007101). MSCs were plated at cells density of $1x10^4$cells/cm^2 in a fibronectin pre-coated tissue culture plates and culture medium was replaced every 3 days. After 21 days in culture cells were washed in PBS and fixed with 0.5% glutaraldehyde for 5min. After washing, the cells were stained with 0.5%Toluidine O blue (Sigma; 198161-5G) working solution, washed and visualised.

2.8. Osteogenic differentiation of MSCs on hAM

MSCs were plated on AM processed by the tissue bank of the Centro de Histocompatibilidade do Sul to test this substrate as a support material for MSC osteogenic differentiation. Two types of processed AM were tested: dehydrated AM on a silicone base, and AM frozen at - 80ºC in 10% DMSO. In both cases the AM was left to hydrate or equilibrate in culture medium for approximately 24h. Due to problems securing the AM straight in the tissue culture plate during these experiments, either the plates were pre-coated with fibronectin or a teflon ring was placed on top of the AM to prevent its deformation. MSCs were then plated on the AM, and induced to differentiate in the appropriated media. Osteoblast differentiation, and in particular the formation of an osseous matrix on the AM was assessed by detection of alkaline phosphatase activity and calcium deposition as previously described. To confirm that AMs were completely decellulerized neutral red staining was performed in AMs with and without MSCs and in MSCs plated in tissue plastic. The neutral red stock solution (4%) was diluted 1:100 in MSC culture medium. The working solution was prepared the day before use and was let at 37ºC overnight. In the next day, the medium was replaced by oil red working solution for 4 hours. After oil red removal, the cells were visualized in PBS to a better reddish staining observation.

3.9. Data Analysis

3.9.1. Statistical Analysis

Statistical analysis was performed using Excel software (Microsoft Office Excel 2007, Microsoft Corporation). Data are presented as the mean ± standard deviation. When appropriate, statistical comparisons were performed using student's *t*-test. Differences were considered statistically significant when $p<0.05$.

3.9.2 Image Acquisition

Images were obtained in Olympus CKX41 microscope with Canon IXUS8015 camera and Olympus IX71 with Olympus DP70 camera.

3. Results

3.1. MSC can be successfully isolated from different parts of UC

In this study we have initially attempted to isolate MSCs from different parts of human UC in order to determine the most suitable stem cell source within this tissue. For the isolation of MSCs from the different parts of the UC, various methods were tested, both exclusively mechanical and combinations of mechanical and enzymatic procedures (see supplemental Table 1). Of the 26 human UC processed, MSCs from WJ (peripheral and total), vessels and whole cord were obtained. MSCs from peripheral WJ tissue (pWJ-UC) were isolated by cutting the mesenchymal tissue from the exterior part of the UC around the vessels into small fragments and directly plating or submitted them to a 4 to 16h enzymatic digestion (with collagenase, and/or hyaluronidase). For the isolation of MSCs from total WJ (tWJ-UC) and vessels (V-UC) the umbilical cord was cut open lengthwise and subjected to an initial 1h enzymatic digestion. The vessels were then separated from the remaining WJ and each of these components was separately digested for 4 to 16h for MSC isolation (see Supplementary Figure 1). To obtain MSCs from whole umbilical cord (W-UC), a segment of the cord (including both vessels and WJ tissue) was cut into small pieces and submitted to a 4 to 16h enzymatic digestion. Some samples were subsequently subjected to a further 20min digestion with the trypsin replacement Tryple.

The exclusively mechanical method was not very efficient as only a minority of single cells was obtained and the bigger pieces did not effectively adhere to the tissue culture flask. The collagenase digestion significantly increased the tissue dissociation, and thus the number of single cells obtained, while it did not meaningfully compromise cell viability. The best cell dissociation/viability ratio was obtained using a collagenase concentration around 0.5mg/ml. Adding hyaluronidase (at a concentration of 100 U/ml) to the digestion increased tissue dissociation and considerably reduced the viscosity of the solution, facilitating the pelleting and washing steps. In addition, the use of hyaluronidase allowed a reduction in the duration of the enzymatic treatment, a crucial factor as it had to be enough to allow tissue dissociation but it also had a decisive effect on cell viability. We observed that 4 hours of enzymatic treatment after cutting the tissue into small fragments was a reasonable time for tissue digestion. Tryple digestion did not significantly improve cell dissociation and therefore this step was eliminated. For some of the samples the digested tissue was finally filtered with a cell strainer, a step that removed undigested cell clumps producing a “cleaner” cell suspension. However, it is not clear whether this improved cell recovery.

Even though different efficiencies were observed between methods and tissue sources, MSC cultures were successfully established from all the 26 UCs (Table 4). From these cords, 13 pWJ-UC, 14 tWJ-UC, 15 W-UC and 12 V-UC were obtained. Of these, the cells of two pWJ-UC and one tWJ-UC got bacterial contamination at the time of the first passage, whereas the remaining MSCs were efficiently propagated in culture for up to over 12 passages in MSC-FBS medium (DMEM:F12 with 2mM L-Glutamine, 15mM Hepes, 10% MSC-qualified FBS and

Table 4. Summary of the characterisation of mother's age, gestational stage, elapse time between harvest and processing the cord (Proces. Time) and MSCs tissue source in each cord. (UC- umbilical cord; pWJ- peripheral Wharton's jelly; tWJ- total Wharton's jelly; W- whole umbilical cord; V- vessels)

UC nº	Mother's Age	Gestational Stage (Weeks)	Proces. Time	Tissue source
1	27	39	1 day	pWJ
2	32	40	same day	pWJ
3	40	39	1 day	pWJ
4	34	37	1 day	pWJ
5	37	39	1 day	tWJ
6	38	38	1 day	pWJ
7	35	38	1 day	pWJ
8	28	39	1 day	tWJ
9	32	39	1 day	pWJ
10	33	39	1 day	pWJ/tWJ
11	34	38	1 day	tWJ
12	37	38	1 day	pWJ /W/V
13	33	38	1 day	pWJ /W/V
14	39	39	1 day	pWJ /W/V
15	35	40	1 day	pWJ /W/V
16	34	39	2 days	tWJ/W/V
17	36	40	1 day	tWJ/W/V
18	39	38	1 day	pWJ/W
19	25	39	1 day	tWJ/W/V
20	31	39	2 days	tWJ/W/V
21	36	38	2 days	tWJ/W/V
22	27	39	Same day	tWJ/W/V
23	31	41	1 day	tWJ/W/V
24	32	38	1 day	tWJ/W/V
25	35	39	1 day	tWJ/W
26	32	41	1 day	tWJ/W

antibiotics (100 U penicillin and 100 µg streptomycin / ml, 5µg/ml Gentamicin and amphotericin B) for the first two passages. The 51 MSC cell lines showed good cell viability at every step of expansion, freezing and thawing.

Some clinical parameters were collected from donors such as mother's age that range from 25 to 40 years old and gestational stage at the time of birth, which range from 37-41 (Table 4). The elapse time between harvest and processing the cord was also analysed and this was not beyond 2 days (Table 4).

The morphology, growth behaviour, immunophenotype, gene expression profile and differentiation abilities of the *in vitro* expanded cells were subsequently investigated.

3.2. Differences in the number of MSCs isolated from WJ-UC and W-UC, but similar MSCs morphology and immunophenotype

Primary cultures obtained from WJ-UC and W-UC initially showed a variable morphology mostly with flattened striated cells but also a significant proportion of fibroblastoid shaped cells (Figure 4A). In some cases, clusters of cells with a typical endothelial morphology were also observed, but these were progressively lost during culture. After few days in culture, MSCs from both tissues grew in colonies and were dissociated and replated 10 to 15 days after plating. MSC from W-UC generally reached confluence and needed passaging after approximately 14 days in culture, a shorter time then cells from other tissue sources. Also, as shown in Figure 4B, at the time of the first passage, more cells per gram of tissue were systematically obtained from W-UC tissue compared to WJ-UC (*n*=4; Figure 4B). After passaging, the cell culture became more homogeneous, containing predominantly spindle-shaped fibroblast-like cells, with no significant differences depending on the tissue source or the isolation method.

In order to characterise the cells isolated from the tWJ-UC and W-UC, the cell surface antigens present on these cells were analysed by flow cytometry (FC) immediately after tissue dissociation. This analysis showed that the majority of the cells from both tissue sources express very low levels of the MSC-characteristic markers CD73 (1,88±1,27 for tWJ and 4,98±0,78 for W-UC), CD105 (2,16±1,39 for tWJ and 6,68±1,84 for W-UC), CD44 (5,23±1,66 for tWJ and 9,56±0,92 for W-UC) and CD90 (13,02±7,41 for tWJ and 30,71±7,64 for W-UC) (Figure 4C). At this stage, a small proportion of cells also expressed hematopoietic markers such as CD34 (1,12±0,77 for tWJ and 3,76±0,38 for W-UC) and CD45 (4,67±2,09 for tWJ and 10,62±2,06 for W-UC) (Figure 4C), although the percentage of CD34 and CD45 expressing cells was 2 to 3 times higher in W-UC than in WJ-UC (*n*=4).

After the first passage, the number of positive cells for the MSC-characteristic markers increased significantly to approximately 80% for CD44 and more than 90% for the other markers, in cells from both tissue sources (Figure 4C). Regarding the negative markers, the expression of CD34 and CD45 remained low for WJ-UC MSCs and decreased to similarly low levels in W-UC (Figure 4C)

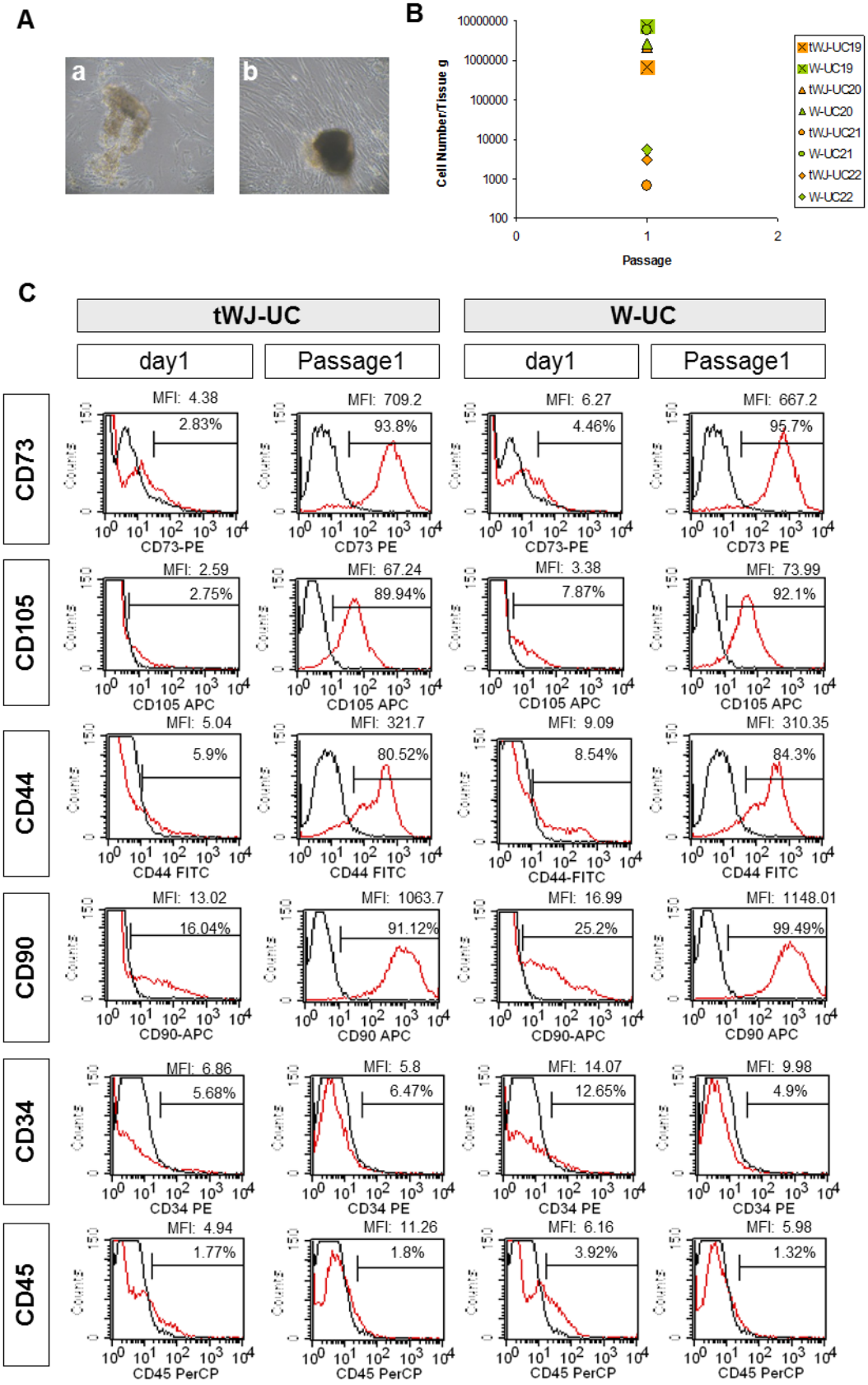


Figure 4. Characterisation of primary cultures of UC-derived MSCs A) Representative phase contrast images of cells isolated from tWJ-UC and W-UC after 15 days of culture in standard MSC-FBS medium. Primary cultures of W-UC (b) were generally more confluent than WJ-UC (a) and both included cells with a variable morphology with flattened as well as fibroblastic-like cells. Image magnification: 400x. **B)** Graphical representation of the number of cells obtained from each different tissue source after 15 days in

culture, normalised to the tissue weight. Identical shaped symbols represent results from the same UC donor. For each donor, more cells were consistently obtained from W-UC (green symbols) than from WJ-UC (orange symbols) **C)** Flow cytometry immunophenotyping of tWJ-UC and W-UC cells on the day of isolation (Day1) and after the first passage (Passage1). Histograms demonstrating the expression of the surface antigens (red line) were plotted against isotype controls (black lines) for unspecific fluorescence. The percentage of positive cells and MFI are given on top of each histogram. During primary culture there is a substantial increase in the expression of CD73, CD105, CD44 and CD90 antigens whereas CD34 and CD45 remained low or further decreased in W-UC. Shown is one representative out of 5 independent experiments.

3.3. MSCs proliferation change from the early to the late passages for different tissue source

During cell passaging, the cell morphology remained relatively homogeneous, predominantly with spindle-shaped fibroblast-like cells, until late passages (passage 12 to 16) (Figure 5A c). After passage 16, MSCs changed to a more stretched and branched morphology (Figure 5A d) for both WJ-UC and W-UC.

In Colony-forming unit (CFU) assay, it was observed great heterogeneity in morphology, size and number of colonies with apparent no correlation with tissue source (Figure 5B a-d).

To assess the growth behaviour of the established cell lines, term growth curves were generated. Cells at an early passage (P4) were thus counted every 24 hours along 4 days. As shown in Figure 5C MSC lines obtained from different tissue sources showed a similar growth profile. The long term growth response of the different cell lines was subsequently studied. For this, the total number of cells at each passage, along 9 passages, was calculated as a ratio of total number of cells harvested to total number of cells seeded, multiplied by the total number of cells from the previous passage (Nekanti *et al*., 2010). Cultured MSCs from the different tissue sources analysed showed similar cell proliferation profiles at low passages (P3 to 8) (Figure 5D). During the initial passages, pWJ-UC exhibited a slightly slower growth when compared to W-UC, even though following a similar growth pattern (Figure 5C and D). . However, from passage 8, the cell proliferation pattern differed depending on the tissue source. While pWJ-UC cells maintained the same tendency of slow proliferation, as for lower passages (Figure 5D), W-UC MSCs further increased their growth rate until passage 10, when a peak was reached (Figure 5D). Finally, tWJ-UC MSCs grew faster in higher than in lower passages and continued to increase beyond passage 12, outpacing the W-UC MSCs growth (Figure 5D).

The immunophenotypic analysis of MSCs isolated from tWJ and W-UC showed that the percentage of positive cells for CD73, CD105, CD44 and CD90 initially increased drastically. Along continuous passaging, MSCs from both tissues remained highly positive for CD73, CD105, CD44 and CD90 (over <90% positive cells) and negative for CD19, CD14, CD45, CD34 and HLA-DR (see Figure 6 and Supplementary Figure 2). In higher passages the mean fluorescence intensity (MFI) for CD73 and CD105 became quite variable independently of tissue source. However, in higher passages, the CD44 MFI increased in almost all the MSCs

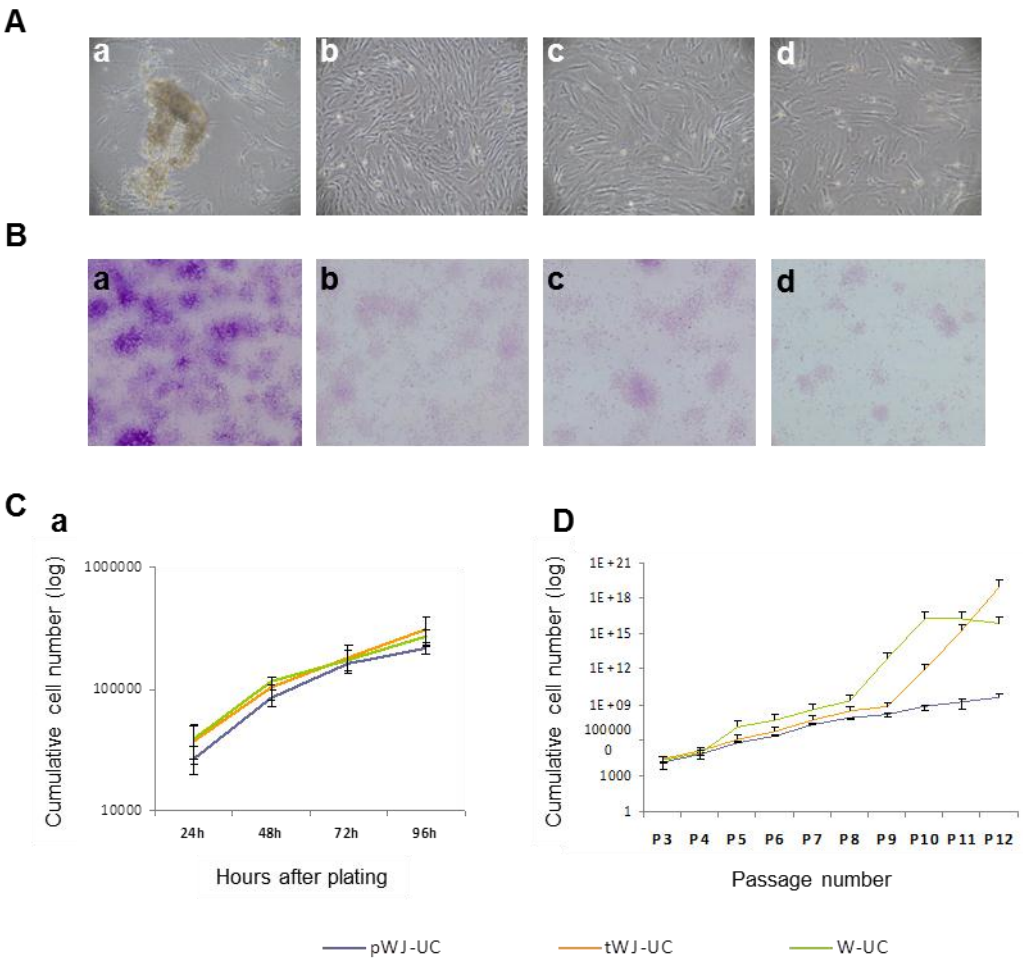


Figure 5. Morphological and proliferative features of UC-MSCs along time in culture. A) Representative phase contrast images of MSC at the time of the first passage (a), and after 3 (b) 12 (c) or 19 (d) passages in standard MSC-FBS medium. Both WJ and W-UC cells initially show a variable morphology with flattened and fibroblastic-like (a) whereas after passaging the cultures become more homogeneous, mainly with spindle shaped or fibroblastoid cells (b-d). Images magnification: 400x. **B)** Representative images of crystal violet stained colony forming units (CFUs) showing the heterogeneous size, morphology and number of colonies of different MSC lines. **C)** Growth curves of MSCs isolated from different UC tissue sources grown in standard MSC-FBS medium for 4 days (96 hours). Data are expressed as mean ± standard deviation. **D)** Long term growth curves representing the cumulative cell expansion rates of pWJ (blue line), tWJ (orange line) and W-UC MSCs (green line) cultured in standard MSC-FBS medium. Total number of cells at each passage was calculated as the ratio of the total number of harvested cells to the total number of cells plated, multiplied by the total number of cells from the previous passage. The results represent the cumulative expansion rates (mean ± standard deviation) of the three different tissue source MSCs plated at a density of 1 x 10^4cells /cm^2 and grown for 12 passages.

analysed (*n*=6/7) and for CD90 this value diminished (*n*=6/7). According to some authors, SSEA-4 and alkaline phosphatase antigens would be expressed in MSCs, which would indicate an evolutionary proximity between these cells and embryonic stem cells (Fong *et al*., 2007). Our results also revealed some positive cells for SSEA-4 (≈1-10%) but alkaline phosphatase antigen was not detected in MSCs (data not shown).

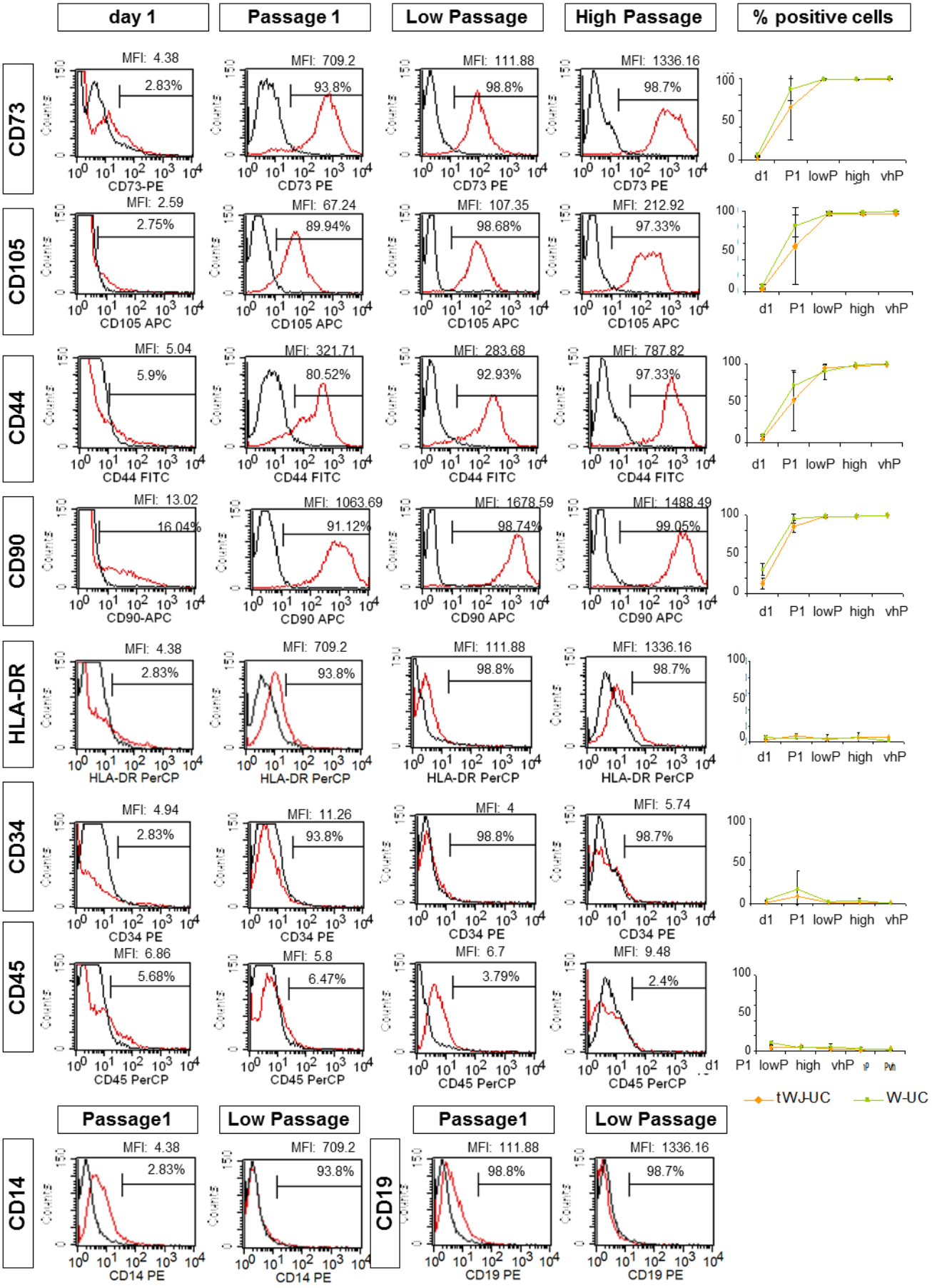


Figure 6. Expression profiles of MSC markers in cells expanded in standard MSC-FBS medium. Flow cytometry analysis of the expression pattern of MSC cell surface markers in primary cultures (P1),

and cells at low passages (P3-P6) and high passages (P12-P16). An increase in CD44, CD73, CD90 and CD105 constant low levels of CD34, CD45, HLA-DR, CD14 and CD19 were observed during the several passages. Representative histograms demonstrating the expression of the surface antigens (red line) were plotted against isotype controls (black lines) for unspecific fluorescence. The percentage of positive cells and MFIs are given on top of each histogram. The last column shows the percentage of positive cells for each marker through several passages. Dots and bars represent the mean ± standard deviation of the results for three different MSCs stem cell lines.

3.4. StemPro MSC SFM induced a decrease in CD105 antigen

An adequate clinically compatible culture medium should support MSC expansion while maintaining the essential properties, phenotype, and multilineage potential of these cells.

Therefore, the expansion potential of the isolated MSCs was tested in the commercial serum-free StemPro® MSC serum free medium (Invitrogen), which is supplemented with human platelet-derived growth factor-BB (PDGF-BB), basic fibroblast growth factor (bFGF) and TGF-β1 (Chase et al., 2010).

Initially, established MSC cell lines were expanded in the StemPro serum free medium (SP-MSC SFM) and the cell growth and phenotype compared to MSC expansion in FBS-containing medium. An immediate limitation observed in the use of the StemPro medium is in terms of cell adhesion since in this condition the cells are not able to efficiently adhere to the tissue culture plastic vessels, requiring pre-coating with an adequate substrate such as human fibronectin.

Therefore, for this experiment the cells were plated on fibronectin-coated plates at the cell density of $1x10^4$cells/cm^2 in the two types of medium. Every 3 days, when confluence was reached, the cells were dissociated and replated. This experiment was extended for eight passages (28 days) and MSC specific markers analysed before experience plating and after one, four and eight passages in each type of medium. MSCs were seeded and reached confluence 4 days after plating.

Morphologically, cells growing in SP-MSC SFM exhibited a narrower and more elongated shape than in FBS medium, although after harvesting MSCs in SP-MSC SFM observed in hemocytometer were smaller than those in FBS-containing medium (not shown).The analysis of the growth curves for the two culture conditions (Figure 7A) showed that MSC growth in SP-MSC SFM followed a similar pattern to that of MSCs in FBS containing medium for up to 8 passages.

The phenotype of cells both in serum-free and serum-containing medium was assessed at the first, fourth and eight passages in terms of cell surface expression of the MSC specific markers (Figure 7B). The percentage of positive cells for the cell surface proteins CD73, CD44 and CD90 was comparable for the two growth conditions whereas CD105 was consistently expressed in a lower percentage MSCs growing in SP-SFM, a difference that increased with time in culture ($p<0.05$) (Figure 7B).

As a further test to the competency of the StemPro MSC SFM, MSC cultures were attempted to be established directly in this medium. This was accomplished with a very low

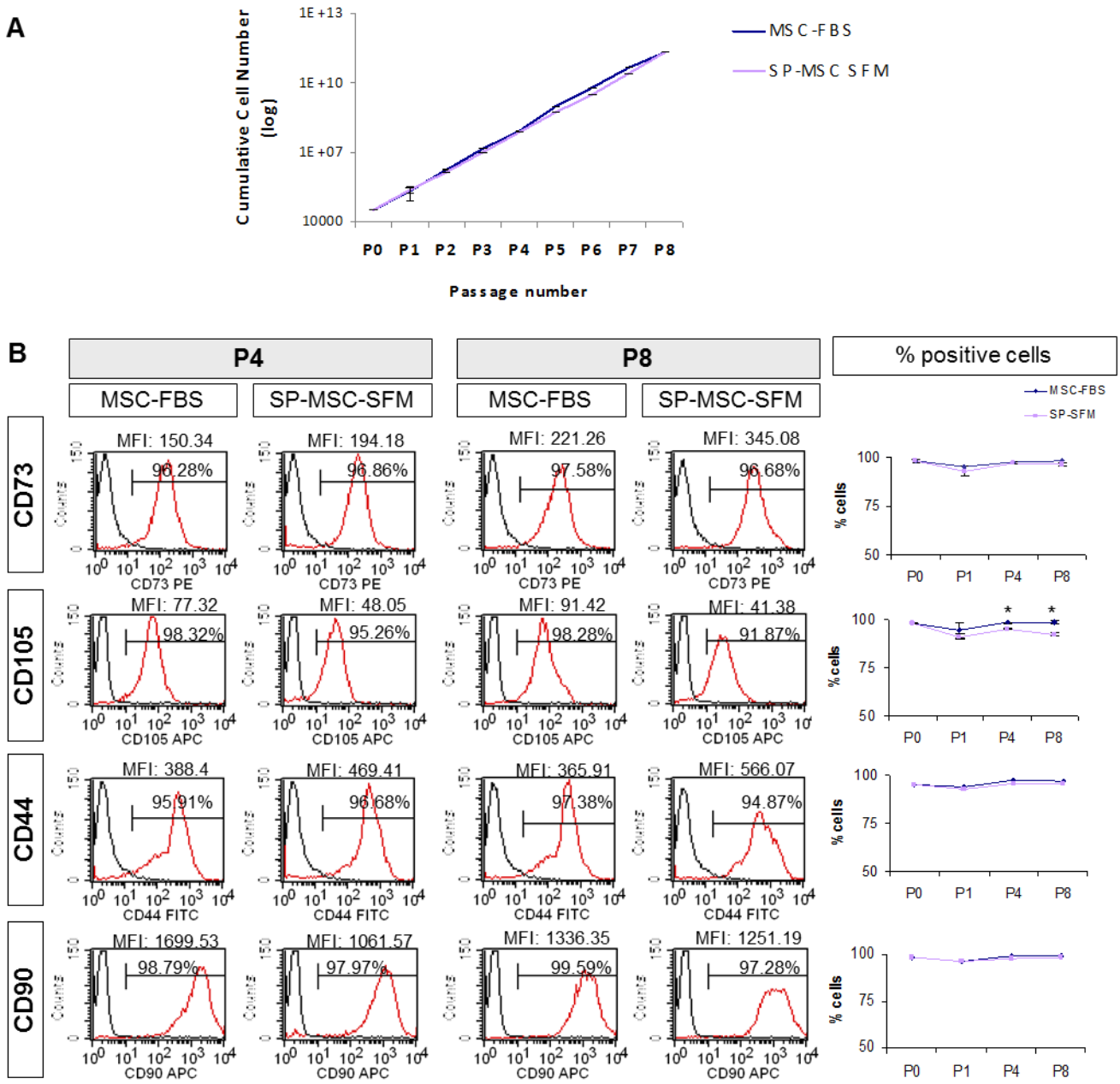


Figure 7. Characterisation of MSCs cultured in StemPro serum-free medium in comparison to MSC/FBS medium. A) Growth curves representing the cumulative cell expansion rates of MSCs cultured in either serum free (SP-MSC SFM) or FBS-containing (MSC-FBS) medium. Total number of cells at each passage was calculated as the ratio of the total number of harvested cells to the total number of cells plated, multiplied by the total number of cells from the previous passage. MSC show a similar growth pattern in both types of medium for up to 8 passages. The results represent the cumulative expansion rates (mean ± standard deviation) of 3 different MSC cell lines plated at a density of 1 x 10^4cells /cm^2. **B)** Flow cytometry immunophenotyping of MSCs expanded on SP-MSC SFM and MSC-FBS medium at passage 4 (P4) and passage 8 (P8). During several passages the high levels of expression of CD73, CD44 and CD90 antigens are maintained in both types of media whereas CD105 marker expression decreases in MSCs expanded in SP-MSC SFM. Histograms demonstrating the expression of the surface antigens (red line) were plotted against isotype controls (black lines) for unspecific fluorescence. The percentage of positive cells and MFI are given on top of each histogram. Shown is one representative out of 3 independent experiments. The last column shows a graphical representation of the average percentage of CD73, CD90, CD44 and CD105-positive MSCs when cultured in MSC-FBS or in SP-MSC SFM medium for 0, 1, 4 and 8 passages. Dots and bars represent the mean ± standard deviation of the results for three different MSC stem cell lines. A high percentage of CD73, CD44 and CD90-positive cells is maintained in SF medium, but the number of CD105 expressing cells decreases in this culture condition compared to the FBS-containing medium (**p<0.01).

efficiency as only 1 in 3 W-UC MSC and 0 in 3 WJ-UC cell lines were successfully established from the primary cultures (data not shown).

The differences observed in morphology, expression of surface antigens and derivation efficiency between MSCs cultured in FBS containing medium and in StemPro-MSC-SFM indicated that this commercial medium is not a very suitable substitute for FBS.

3.5. hABS is a good xeno-free alternative culture supplement for FBS replacement

In this study, human platelet-lysate in plasma (hPLP), human platelet-lysate in DMEM-F12 (hPL), pooled human plasma (hP) and pooled human AB serum (hABS) were tested as FBS substitutes with the aim of obtaining a xeno-free MSC culture condition. Initially, we tested two different concentrations of these supplements and observed that better cell viability was obtained using a concentration of 5% for hPLP, hPL and hP and 10% for hABS. Although MSCs cultured in media containing any of these human component supplements did not require pre-coating with a specific substrate, the use of fibronectin enhanced MSC performance in culture media containing hPLP, hPL and hP (not shown). Also, as previously published results reported (Choi KM *et al*., 2008; Potdar and d'Sousa, 2010), the addition of ascorbic acid (asc) at a concentration of 250 µM improved MSC growth (see Supplementary Figure 3) so this was also included in the culture conditions. Finally, the use of hPLP and hP caused culture media clotting, thus implying the use of an anticoagulant such as heparin which was added to the culture medium at the concentration of 1U/mL. In summary, the experiments aiming at replacing FBS by human components were performed on fibronectin-coated culture vessels and in media containing 250 µM asc, 5% of hPLP, hPL or hP, or 10% FBS or hABS, and, for the hPLP and hP conditions, 1U/mL heparin. MSCs were seeded at $1x10^4$cells/cm^2 in all conditions and passed every 4 days.

Similarly to the analysis done for SP-MSC SFM, the behaviour of MSCs in DMEM-F12 supplemented with 10% FBS (as a control), 5% hPLP, 5% hPL, 5% hP and 10% hABS was analysed for eight continual passages (along 28 days).

During the first passages, MSCs in the hPLP and hP conditions acquired a shorter morphology and looked very bright, whereas in hPL supplemented medium were flattened and translucent (Figure 8A b-d). However, cells in these three conditions did not proliferate or survive, even with fibronectin pre-coating and asc addition. Therefore, hPLP, hPL and hP

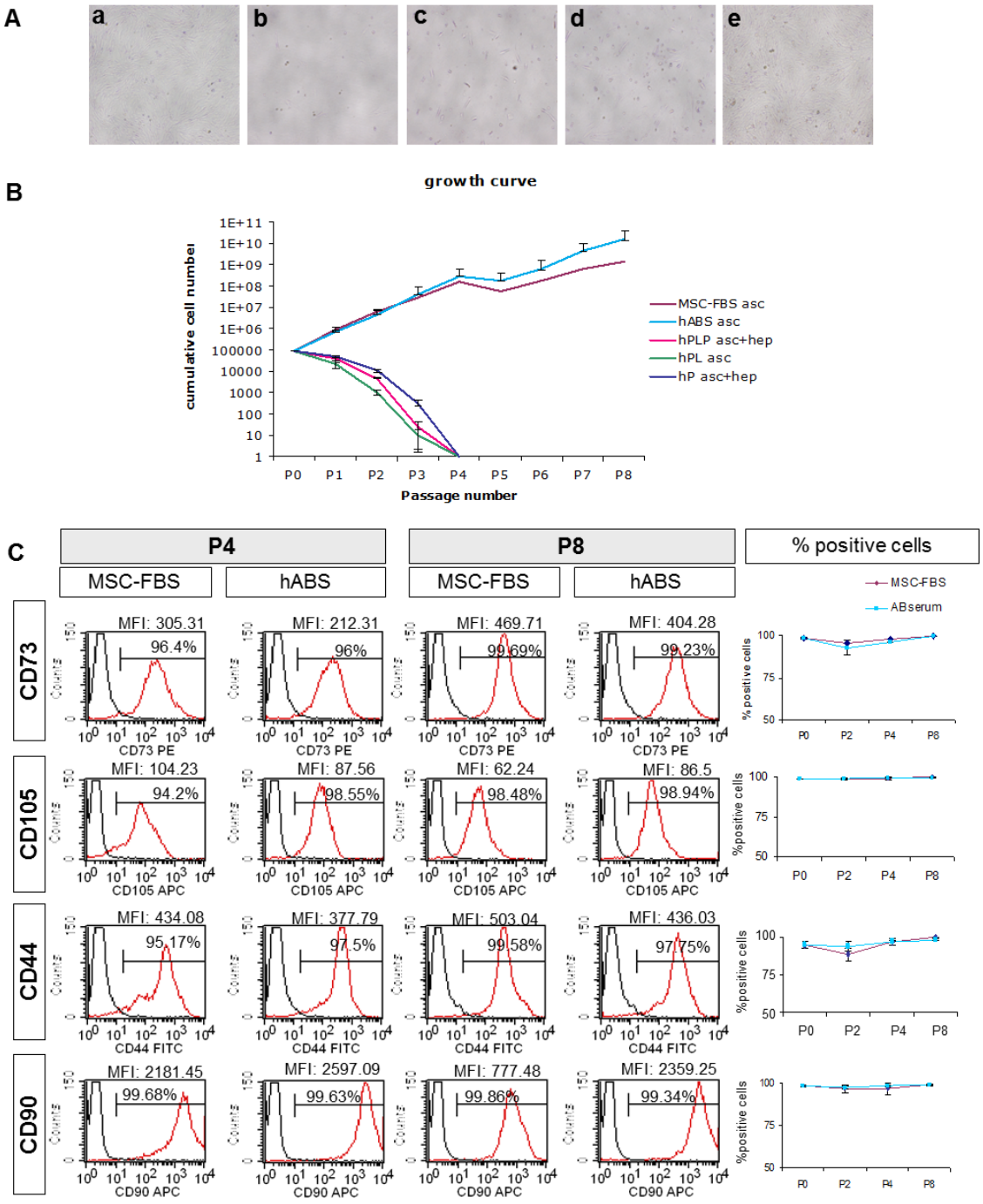


Figure 8. Characterisation of MSCs cultured in hPLP, hPL, hP and hABS supplemented medium. A) Images of MSCs cultivated in 10%FBS (a), 5% hPLP (b), 5% hPL (c), 5% hP (d) and 10% hABS-containing medium (e). MSCs in FBS and hABS-containing medium presented a spindle shaped or fibroblastoid morphology during all passages (a and e). MSCs in hPLP and hP-containing medium were short and bright (b-c) whereas MSCs in hPL-containing medium were flattened and translucent (d). Images magnification: 400x. **B)** Growth curves representing the cumulative cell expansion rates of MSCs cultured in the different types of xeno-free and in FBS-containing (MSC-FBS) medium. MSCs in hPLP, hPL, hP supplemented medium died after 3 passages. MSC growth in hABS and FBS-containing medium presented a similar cumulative growth pattern. The results represent the mean ± standard deviation of cumulative expansion rates (total number of cells at each passage = ratio of the total number of harvested cells to the total number of cells plated, multiplied by the total number of cells from the previous passage) of 2 MSC cell lines plated at a density of 1 x 10^4cells /cm^2 and grown in each medium for 8 passages. **C)** Flow cytometry immunophenotyping of MSCs expanded in hABS and MSC-FBS containing medium at passage 4 (P4) and passage 8 (P8). High levels of expression of the CD73, CD105, CD44 and CD90 antigens were maintained in both types of media for 8 passages. Histograms demonstrating the expression of the surface antigens (red line) were plotted against isotype controls (black lines) for

unspecific fluorescence. The percentage of positive cells and MFI are given on top of each histogram. The last column is a graphical representation of the average percentage of CD73, CD90, CD44 and CD105-positive MSCs when cultured in MSC-FBS or in hABS-containing medium for 1, 4 and 8 passages. Dots and bars represent the mean ± standard deviation of the results for three different MSC stem cell lines. A high percentage of CD73, CD105, CD44 and CD90-positive cells was maintained in hABS supplemented medium for up to 8 passages.

containing media were unable to support MSCs expansion for longer than 3 passages (Figure 8B).

Regarding the MSCs cultured in FBS and hABS containing medium, the fibroblastic-like morphology was maintained with no significant differences between the two culture conditions (Figure8A a and e). Also, the growth curves performed showed that MSCs had a similar growth pattern during the 8 passages in the two types of media (Figure 8B).

The expression of MSC surface antigens was analysed before the beginning of the experiment and after one, four and eight passages in FBS or hABS containing medium, the only conditions that sustained cell expansion for longer periods. As represented in Figure 8C, similarly high percentages of positive cells for the MSC markers CD73, CD105, CD44 and CD90 were present up to passage 8 for both conditions.

3.6. Differentiation potential varies between donors and tissue source

The multilineage potential of MSCs derived from WJ-UC and W-UC was tested by inducing the cells to differentiate into osteoblasts, chondrocytes and adipocytes. For this, commercially available differentiation kits previously described in the literature were used. Chondrocyte and adipocyte differentiation were induced with the StemPro® Chondrogenesis and Adipogenesis differentiation kit, respectively. For osteoblast differentiation the StemPro® osteogenesis differentiation kit, the MesenCult osteogenic stimulatory kit, MesenCult, DMEM-F12, DMEM-KO and DMEM-LG basal media with dex, asc and β-GP addition were used. For adipogenic and chondrogenic differentiation only a few MSC lines were tested, whereas the osteogenic potential was studied more extensively and for almost all the cell lines established.

Cells expanded in MSC-FBS complete medium were thus seeded at $1x10^4$cells/cm^2 and cultured in an appropriate media for the induction of each specific lineage (Figure 9). The presence of chondrocytes after 21 days of MSC culture in chondrogenic induction medium was investigated using Toluidine Blue O, which stains for mucopolysaccharide-rich extracellular matrix. Variable levels of positive staining were observed for 4 out of the 6 cell lines analysed (Figure 9 g-i). The adipogenic differentiation ability was evaluated by Oil Red staining for the detection of lipids in the vacuoles after 14 days in adipogenic induction medium. Of the 6 MSC lines tested, two of them exhibited high and four showed moderate adipogenic differentiation ability (Figure 9 d-f). No correlation was observed between chondrogenic or adipogenic potential and the tissue source from where the cells were derived (pWJ-UC, tWJ-UC and W-UC). However, more cell lines would have to be tested for the results to be representative.

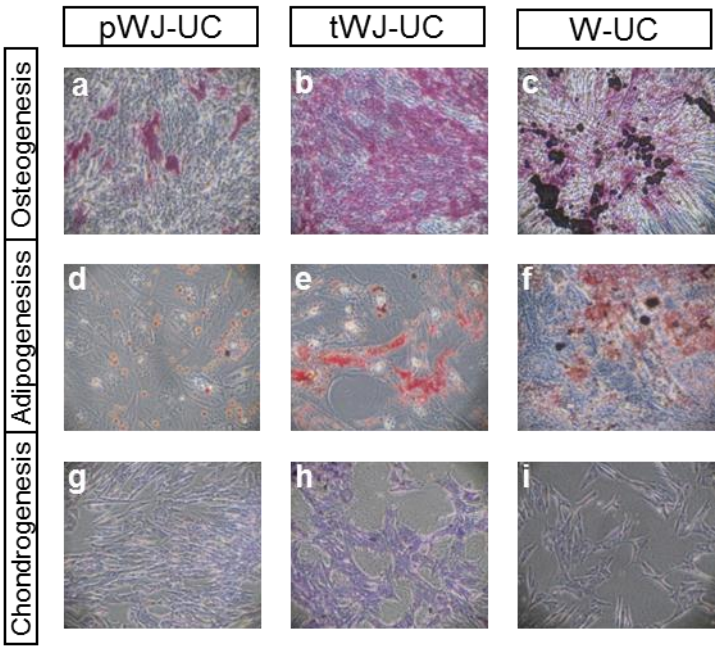


Figure 9. Multilineage capacity of MSCs isolated from peripheral (pWJ), total Wharton's jelly (tWJ) and whole umbilical cord (W-UC). Representative phase contrast images of cells from pWJ, tWJ and W-UC exposed to osteogenic (a-c), adipogenic (d-f) and chondrogenic (g-i) differentiation media. Osteogenic differentiation of MSCs from pWJ, tWJ and W-UC was demonstrated by staining for Alkaline Phosphatase (AP) activity after 14 days of culture in MesenCult Osteogenic medium (a-c). Adipogenesis was detected by the formation of intracytoplasmic lipid droplets stained with oil red O after 14 days in StemPro® Adipogenesis differentiation kit (d-f). Cells were stained with toluidine blue O to confirm chondrogenic differentiation after 21 days in StemPro® Chondrogenesis differentiation kit (g-i). Images magnification: 400x.

Osteogenic differentiation was assessed by staining for alkaline phosphatase activity after 14 days in osteogenic induction medium (Figure 9 a-c). Alkaline Phosphatase (AP) activity is characteristic of the matrix deposition phase of osteoblastic differentiation and, according to the literature, it is higher at around 14 days after osteogenic induction. For the study of later differentiation stages, mineralisation (calcium deposition) was assessed by Von Kossa or Alizarin Red staining. Initially, the effectiveness of the two commercially available kits and the different basal media with osteogenic factors was tested. Comparing the alkaline phosphatase activity in response to the two media we observed that the only MesenCult osteogenic stimulatory kit efficiently induced osteogenesis (Supplementary Figure 4). Therefore, the MesenCult medium was chosen as the standard condition for osteogenic differentiation. From all MSC lines isolated and differentiated using this protocol (*n*=39) only two of them showed a high osteogenic differentiation (Table 5). The majority of the cell lines exhibited a moderate-high differentiation (*n*=25/39) and a minority exhibited a moderate-low (*n*=7/39) or low (*n*=5/39) differentiation ability (Table 5).

Table 5: Summary of the characterisation on the osteogenic ability of the isolated MSCs and relation to the tissue source from which they were obtained. (L- Low; ML- Moderate-Low; MH- Moderate-High; H- high)

	Differentiation ability			
Tissue source	**L**	**ML**	**MH**	**H**
tWJ	2	1	6	1
pWJ	1	4	5	0
W-UC	0	1	8	1
V	2	1	6	0

Interestingly, when correlating the osteogenic ability with the tissue source from which the cells were isolated, we observed that a higher proportion of MSC lines from the tWJ-UC, W-UC and V-UC exhibited a high or moderate-high osteogenic potential than pWJ-UC, which mostly exhibited moderate-low to moderate-high osteogenic differentiation (Table 5).

Gene expression during differentiation was followed RT-PCR and normalised to the housekeeping gene β-actin. Figure 10A shows that a decrease in expression of the MSC-specific genes CD73, CD105 and CD90 was observed during differentiation, even though in an irregular manner. On the other hand, the osteogenic transcription factor Runx2, was abundantly expressed even in undifferentiated MSC, and this expression diminished after 14 days of differentiation.

As previously mentioned, Alkaline Phosphatase (AP) activity, characteristic of the matrix deposition phase of osteoblastic differentiation, has been reported to be higher around 14 days of MSC osteogenic induction. In order to confirm the timing of the peak in AP activity in our specific culture conditions, AP was assayed at 6, 10, 14, and 20 days of differentiation for a high and moderate-high osteogenic potential MSCs (Figure 10B). In fact, for the MSC lines tested, weak AP enzymatic activity was detected at day 6 (Figure 10B a and e), which subsequently increased until around day 14 when the highest activity was detected (Figure 10B c and g), and finally showed fainter and more spread levels at day 20 of differentiation (Figure 10B d and h). As shown in Figure 11B, the differentiating cells presented a compact morphology with the occasional formation of protuberant masses which may be composed of osteoid matrix. AP activity was mainly detected in colonies composed of both AP-expressing and non-expressing cells. The 2 cell lines tested followed a similar staining pattern even though differing on the size of the positive colonies (Figure 10B). At later stages of differentiation, after 21 days in osteogenic medium, the formation of mineralized deposits has been described to take place. To assess the progression of calcium deposits AR staining was performed at 22, 29 and 36 days of differentiation for a MSC line with high osteogenic potential (Figure 10C). Calcium deposits were weakly detected at 22 days in differentiation medium, but subsequently increased until the 36 days of differentiation (Figure 10 a-c). This high concentration of calcium deposits at 36 days of differentiation was also confirmed by Von Kossa staining (Figure 10C d).

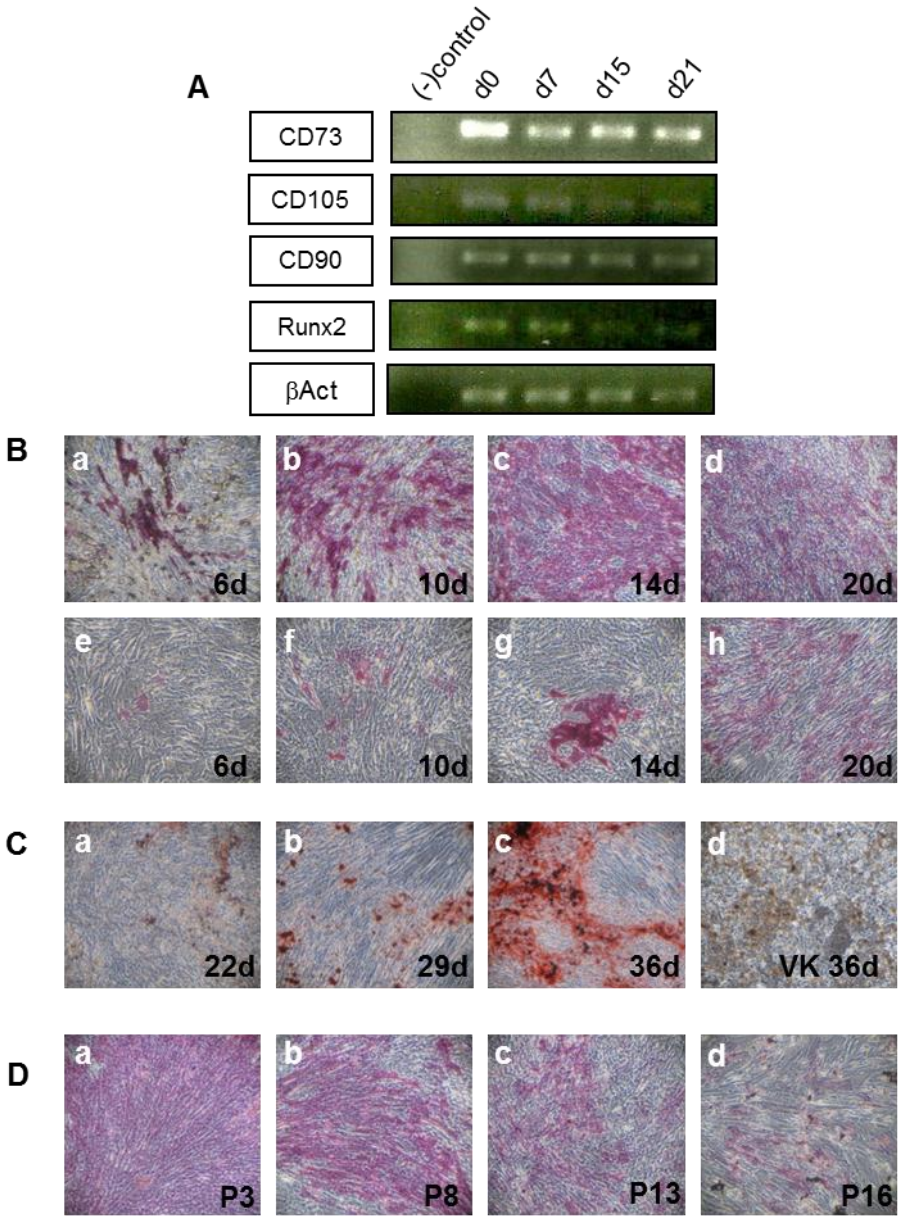


Figure 10. Characterisation of osteogenesis along differentiation time. A) RT-PCR gene expression analysis of CD73, CD105, CD90, Runx2 and the housekeeping gene beta-actin (βAct) for one of the MSCs cell lines in MSC expansion conditions and after 7, 14 and 21 days in osteogenic differentiation culture conditions. **B)** Staining for Alkaline Phosphatase (AP) activity and calcium deposition during osteogenic differentiation in Mesencult Osteogenic medium. An increase in Alkaline Phosphatase activity was observed after 14 days of osteogenic induction, with different intensities depending on the osteogenic potential of the different MSCs lines: (a-d) moderate differentiating and (e-h) highly differentiating cells. AP activity at 6 (a,e), 10 (b,f), 14 (c,g) or 20 (d,h) days of osteogenic differentiation of the MSCs with moderate and high potential. **C)** Detection of calcium deposits by Alizarin Red staining after 22 (i), 29 (j) and 36 (k) and days of osteogenic differentiation or by von Kossa staining after 36 days of osteogenic differentiation (l). **D)** Representative phase contrast images of cells induced to differentiate along the osteogenic lineage when at different passage numbers. Staining for Alkaline Phosphatase (AP) activity after 14 days of differentiation was more intense for cells that were differentiated when at early passages (a,b), and significantly decreased when differentiation was induced in high passage number cells (c,d). Images magnification: 400x.

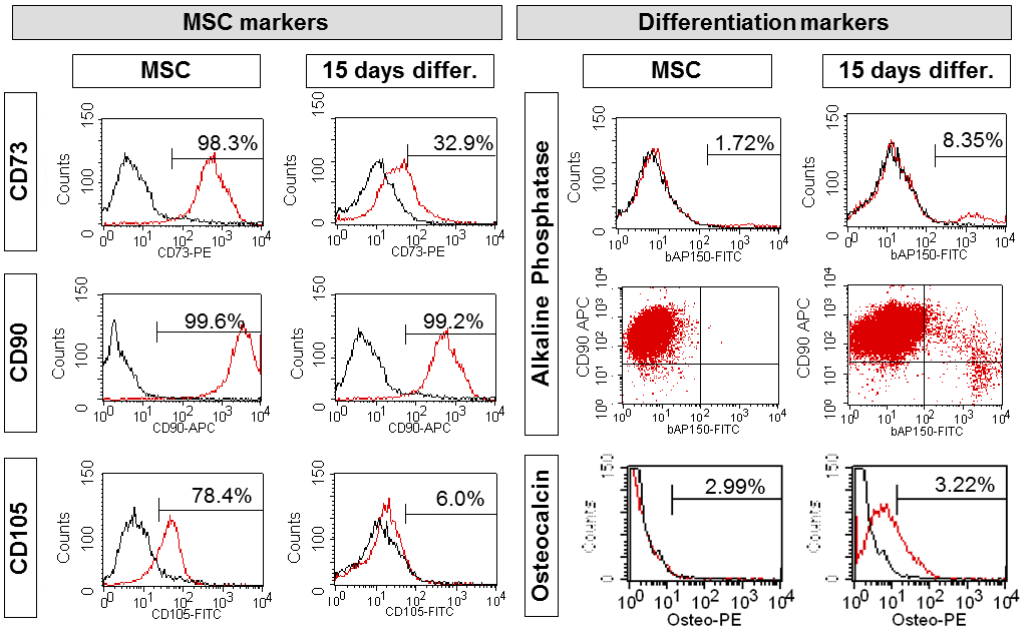


Figure 11. Expression profiles of MSC and osteoblast markers in cells growing in osteogenic medium. Flow cytometric expression analysis for the MSC cell surface antigens CD73, CD90 and CD105, and the osteogenic markers bone Alkaline Phosphatase (bAP) and Osteocalcin, in cells cultured in MSC expansion conditions (MSC-FBS medium) and 15 days after osteogenic induction (in Mesencult Osteogenic medium). During differentiation there is a decrease in expression of MSC markers, and the appearance of a population of osteocalcin and bAP-positive cells. Representative histograms demonstrating the expression of the surface antigens (red line) were plotted against isotype controls (black lines) for unspecific fluorescence. The percentage of positive cells is given on top of each histogram.

The MSC osteogenic potential of cells at different passage numbers was also analysed. As can be seen in Figure 10D, the osteogenic capacity was progressively diminished in cells with increasing passage numbers (longer time in culture), and it was almost lost at passage 16 (Figure 10D).

In order to further characterise the osteogenic differentiation of MSCs, the expression of specific cell surface proteins was analysed by flow cytometry. As expected, after 15 days of differentiation the MSC markers CD73, CD90 and CD105 had significantly decreased (Figure 11), both in terms of the number of positive cells and, in the case of CD90, in number of expressed molecules (which was proportional to the staining MFI). Conversely, Alkaline Phosphatase started being expressed at the surface of a proportion of the differentiating cells. Interestingly, an inverse correlation in the expression of CD90 and AP was observed, as AP-positive cells were generally CD90-negative (Figure 11). Regarding osteocalcin, a later marker of differentiation expressed at the beginning of the mineralisation phase, an increase in its expression was only starting to be detected at this stage.

3.7. hUC-MSC osteogenic ability is related to the cell density, expression levels of CD105 and may be dependent on cell-cell interactions

The differences in osteogenic potential observed in MSCs isolated from the same tissue source and in similar conditions were an intriguing issue that motivated additional studies.

Analysis of the data related to the birth conditions or the newborns did not allow any type of correlation that could help to explain or predict the differential expansion and differentiation potential (Table 4). We therefore attempted to determine which characteristics of these cells could be related to or help predict the osteogenic potential, or which conditions could influence it. For this, the cell density, expression levels of CD105 and cell-cell interactions were analysed.

One of the highly differentiating cell lines was initially expanded from a low cell number due to the poor cell recovery for this particular UC. Therefore, the effect of selecting for highly proliferative MSCs on the differentiation potential was investigated. For that, cells were plated at a low density (200cell/cm^2) in expansion medium and 14 days later, cells from the colonies resulting from the more proliferative cells were harvested, analysed and induced to differentiate (Figure 12). The cell surface expression analysis of MSC specific markers CD73, CD105, CD44 and CD90 showed that the percentage of cells positive for MSCs characteristic markers were maintained (Figure 12A), even though the MFI of CD90 highly increased for both MSC lines tested. These cells were then submitted to osteogenic differentiation conditions which showed that both MSCs with moderate and high osteogenic potential almost lost all the differentiation capacity (Figure 12B).

Another feature that can be related to the differential osteogenic ability of MSCs is the expression level of CD105 and CD90, the MSC markers that showed more variations between cell lines with differing osteogenic abilities and in response to the culture environment. To test this hypothesis, osteogenic competent MSCs were stained with anti-CD105 and anti-CD90 antibodies and the cell populations with high ($CD105^{high}/CD90^{high}$) and low levels ($CD105^{low}/CD90^{low}$) of the two surface proteins were sorted by FACS (Figure 13A). After sorting, the cells were reseeded and were let to recover in MSC-FBS medium for 7 days, at which stage they were harvested and reseeded in osteogenic inducing media. Curiously, the analysis for AP activity and calcium deposition showed that osteogenic differentiation was considerably more efficient in $CD105^{high}/CD90^{high}$ than in $CD105^{low}/CD90^{low}$ MSCs (Figure 13B). However, it was not clear whether this effect was in response to the selection for the CD105 or the CD90 highly expressing population, and if it was specific to this particular cell line. Therefore, cell sorting was repeated but this time based only on the CD105 levels and including another moderately differentiating MSC line. The decision to focus on the CD105 marker, besides the reasons already mentioned, was related to the fact that the expression of CD105 has been reported to decrease over time during the course of multi-lineage differentiation (Choi et al., 2009). After sorting, the cells were cultured in expansion conditions for 7 days and then their immunophenotype was analysed. In terms of percentages of the markers CD73, CD105, CD44 and CD90 no significant differences were observed between $CD105^{high}$ and 105^{low}, cells (Figure 13C). However, analysis of the MFI of MSCs markers in the two sorted populations showed that $CD105^{high}$ cells had not only a higher MFI for CD105 but also for CD90 compared to the $CD105^{low}$ MSCs. When the osteogenic potential of these cells was analysed, an increase in AP activity was once more observed in $CD105^{high}$ MSCs, for both the cell lines (Figure 13D).

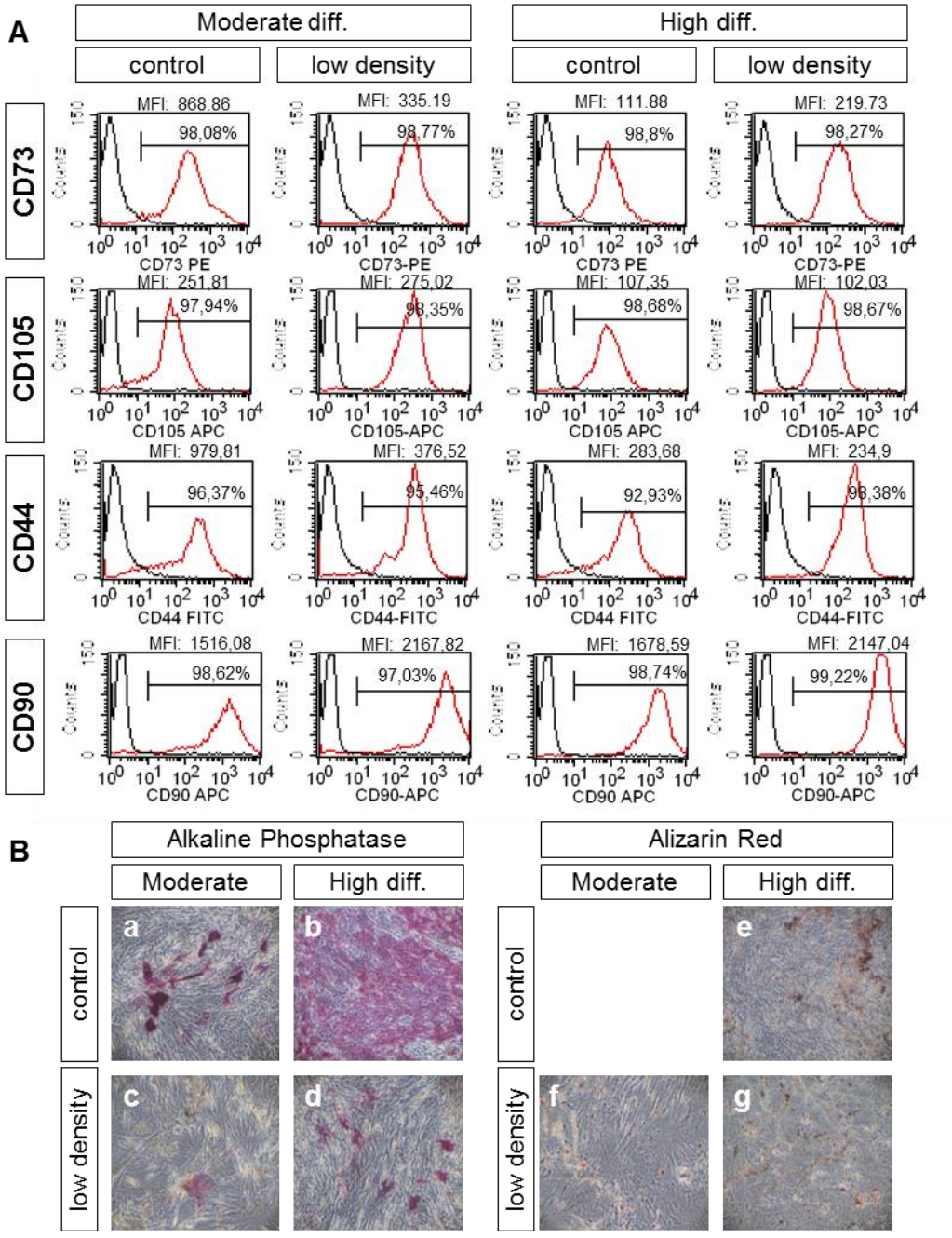


Figure 12. Phenotypical features and differentiation ability of MSCs cultured at low density. A) Flow cytometry immunophenotyping of MSCs expanded for 14 days in standard MSC-FBS medium at normal ($1x10^4$cells/cm^2) or low (200cells/cm^2) plating density. No differences were observed in terms of percentage of the cell surface markers CD73, CD105, CD44 and CD90 but the MFI of CD90 staining highly increased in cells expanded at low density. Histograms demonstrating the expression of the surface antigens (red line) were plotted against isotype controls (black lines) for unspecific fluorescence. The percentage of positive cells and MFI are given on top of each histogram. **B)** Representative phase contrast images of MSCs cultured in osteogenic medium after expansion at normal (a,b,e) or low cell density (c,d,f,g). Staining for Alkaline Phosphatase (AP) activity after 14 days of differentiation and for calcium deposits by Alizarin Red staining after 21 days of differentiation considerably decreased in MSCs previously expanded at low density. Images magnification: 400x.

To determine the effect of cell-cell interaction in MSC differentiation ability, cells with different osteogenic potential were co-cultured in osteogenic-inducing conditions (Mesencult osteogenic stimulatory kit) and the resulting differentiation was assessed by staining for alkaline phosphatase activity. Figure 14A shows that the co-culture of one MSC line with a high differentiation potential with one with moderate potential results in high osteogenic differentiation. Equally, the co-culture of MSCs with high differentiation potential with poorly differentiating ones also showed high osteogenic differentiation (Figure 14B). Additionally, the co-culture of MSCs with a moderate and low differentiation potential produced moderate osteogenesis (Figure 14C). Therefore, cells with a lower propensity for osteogenic differentiation seem to be induced to differentiate when in the presence of more osteogenic prone cells.

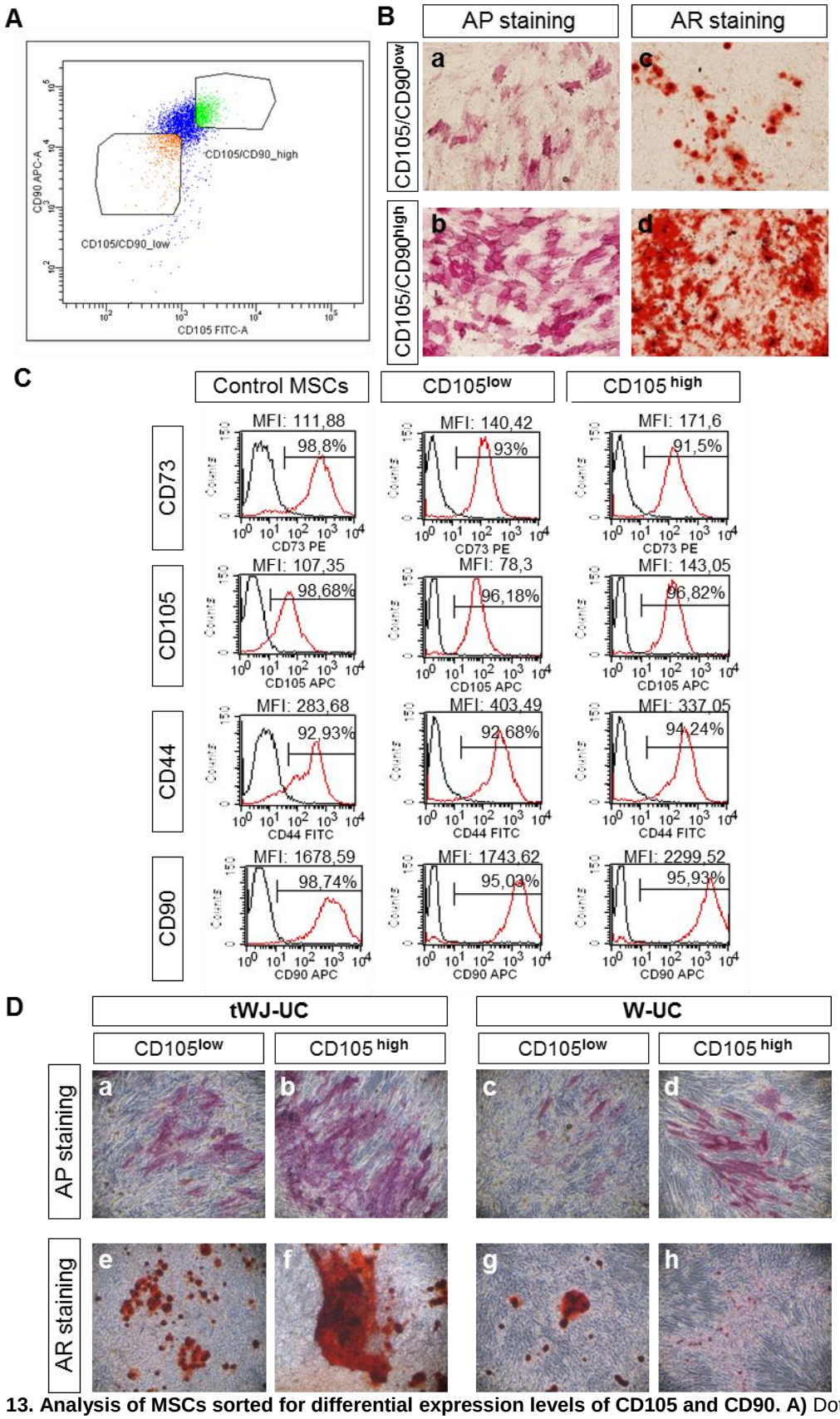


Figure 13. Analysis of MSCs sorted for differential expression levels of CD105 and CD90. A) Dot plot showing the gates used for sorting the CD105 and the CD90 populations. **B)** Representative phase contrast images of MSCs cultured in osteogenic medium after cell sorting for high ($CD90^{high}$/$CD105^{high}$) and low levels of CD105 and CD90 ($CD90^{low}$/$CD105^{low}$) by FACS. Staining for Alkaline Phosphatase (AP) activity after 14 days of differentiation and for calcium deposits by Alizarin Red staining after 21 days of differentiation both showed decreased osteogenic differentiation in $CD90^{low}$/$CD105^{low}$ MSCs (a) and

increased differentiation in CD90^{high}/CD105^{high} cells (b). Images magnification: 400x. **C)** Flow cytometry immunophenotyping of MSCs sorted for low/high CD105 MSCs and expanded in standard MSC-FBS medium for one passage after sorting. No differences in percentages of CD73, CD105, CD44 and CD90 were observed between Low/High CD105MSCs. MSCs CD105^{high} had higher levels of MFIs for CD105 and CD90 than the MSCs CD105^{-} Histograms demonstrating the expression of the surface antigens (red line) were plotted against isotype controls (black lines) for unspecific fluorescence. The percentage of positive cells and MFI are given on top of each histogram. **C)** Representative phase contrast images of MSCs cultured in osteogenic medium after cell sorting for high (CD105^{high}) or low levels of CD105 (CD105^{low}). Staining for Alkaline Phosphatase (AP) activity after 14 days of differentiation and for calcium deposits by Alizarin Red staining after 21 days of differentiation both showed decreased osteogenic differentiation in CD105^{low} MSCs (b,d,f,h) and increased differentiation in CD105^{high} cells (b,c,e,g). Images magnification: 400x.

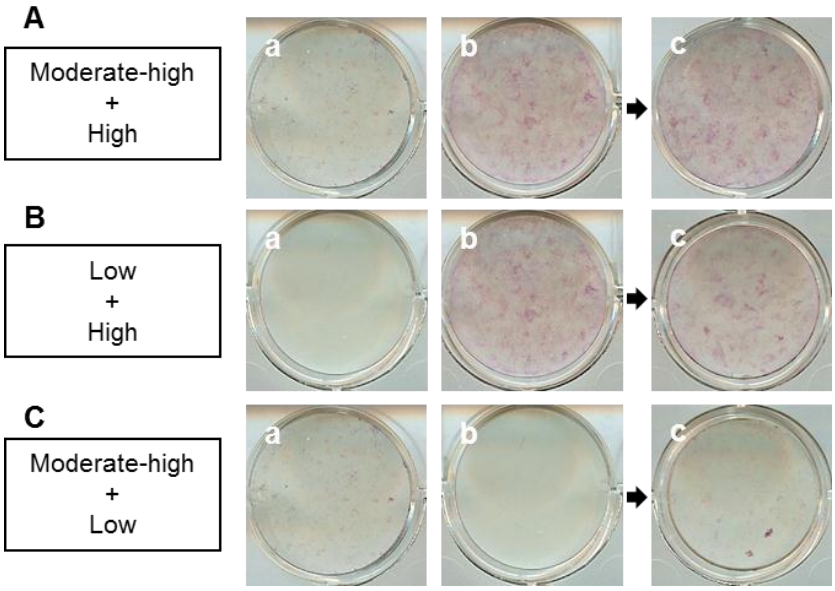


Figure 14. Analysis of differentiation of co-cultures of cells with differential osteogenic potential. Equal cell numbers of two MSC lines with different osteogenic abilities were mixed and co-cultured for 14 days in osteogenic medium, at which point differentiation was assessed by staining for Alkaline Phosphatase (AP) activity. Three different types of co-cultures were experimented: **A**) the mix of moderately differentiating cells (a) and highly differentiating ones (b) resulted in co-culture (c); **B**) cells with low osteogenic potential (a) mixed with high osteogenic potential cells (b) produced co-culture (c); **C**) co-culture (c) was composed of low (a) and moderately (b) differenting MSCs. Staining for AP activity of the MSC co-cultures (A c, B c, C c) consistently looked more similar to the highest differentiating cell line in the original mixture. Images magnification: 400x.

3.8. hABS supplemented expansion medium is capable of maintaining MSC osteogenic potential

As previously mentioned, an adequate clinically compatible culture medium should support MSC expansion while maintaining the essential properties, phenotype, and multilineage potential of these cells. Therefore, we have assessed whether cells expanded in xeno-free media maintained the osteogenic potential (Figure 15). MSCs with moderate to high differentiation potential (assessed after expansion in MSC-FBS medium), when expanded in SP-MSC SFM experienced a significant reduction in their osteogenic potential, as quantified by AP staining (Figure 15 b and e). Conversely, cells expanded in hABS supplemented media succeeded to maintain their osteogenic potential (Figure 15 c and f). Therefore, hABS

supplemented media appears to be a better choice when it is seeking for a more adequate clinically compatible culture medium.

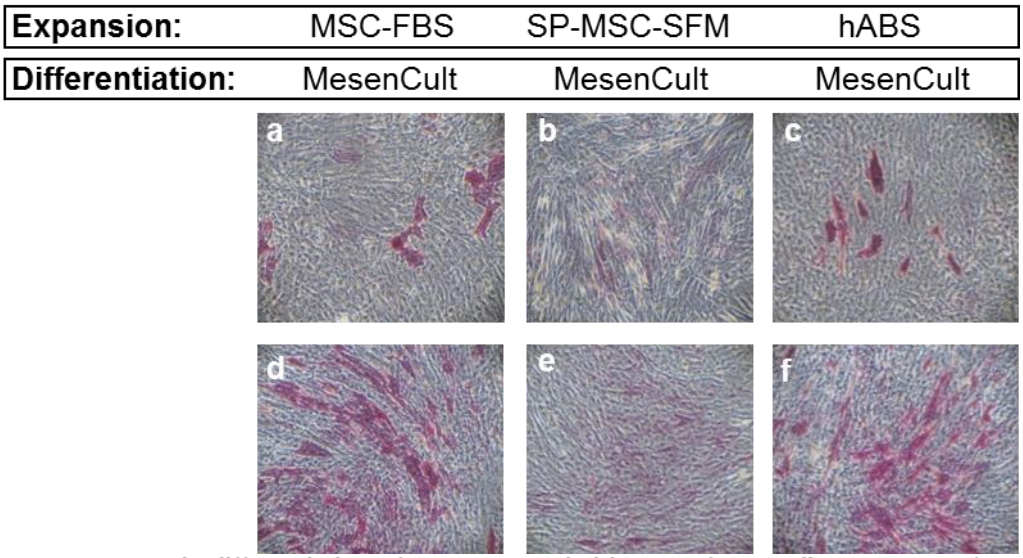


Figure 15. Osteogenic differentiation of MSCs expanded in xeno-free media. Representative phase contrast images of MSCs cultured in MesenCult osteogenic stimulatory kit after expansion for at least three passages in SP-MSC SFM, hABS supplemented medium or the standard MSC-FBS medium. MSCs with moderate to high osteogenic potential experienced a considerable decrease in AP activity after expansion in SP-MSC SFM (c,d) when compared to the MSC-FBS control (a,b). hABS supplemented medium maintained the osteogenic potential of MSCs with moderate (e) and high osteogenic potential (f). Images magnification: 400x.

3.9. The human amniotic membrane is a potentially suitable carrier substrate for a MSC-derived osteoblastic matrix

The last part of this study involved the use of human amniotic membrane as a substrate to support MSC osteogenic differentiation. Two types of AM processed by the CHSul Tissue Bank were tested: dehydrated AM on a silicone base, and AM frozen at -80ºC in 10% DMSO. MSCs were plated on both types of AM and, even though in both cases it was left to hydrate or equilibrate in culture medium for approximately 24h, the dehydrated membrane did not efficiently re-hydrate and so it did not allow cell adhesion. On the other hand, the frozen AM did support MSC adhesion, even though it was difficult to distinguish the MSCs from the AM constituent cells. To resolve this difficulty, neutral red staining was performed (Figure 16A). This has showed that AM is completely decellularised and thus any affect produced in MSCs was exclusively caused by AM extracellularmatrix.

MSCs were then plated and induced to differentiate on the AM, as cells grew on the AM in osteogenic differentiation medium, they started pulling and creasing it. In order to try to hold the AM to the culture plates these were initially pre-coated with fibronectin. However, this did not prevent folding. Subsequently, a teflon ring was placed on top of the AM to try to prevent its deformation, an approach that was reasonably successful.

AP staining of the AM with cells cultured in expansion medium supplemented with 10 or 2%FBS, revealed a modest osteogenic differentiation (Figure 16B b and d) as compared to the negative control of AM without cells (Figure 17B f). Preliminary results of MSCs induced to differentiate on amniotic membrane in osteogenic inducing medium Mesencult or DMEM-F12 with 10 or 2%FBS and the osteogenic supplements dex, asc and β-GP (from Sigma) showed that this substrate allows identical to osteogenic differentiation (Figure 17B a and c), as was assessed by alkaline phosphatase activity. However, differentiation was not as efficient as for cells plated on plastic (Figure 17B e). Analysis of differentiation at later stages was not possible because of the high background obtained when performing von Kossa or Alizarin red staining in the negative control with only amniotic membrane (not shown).

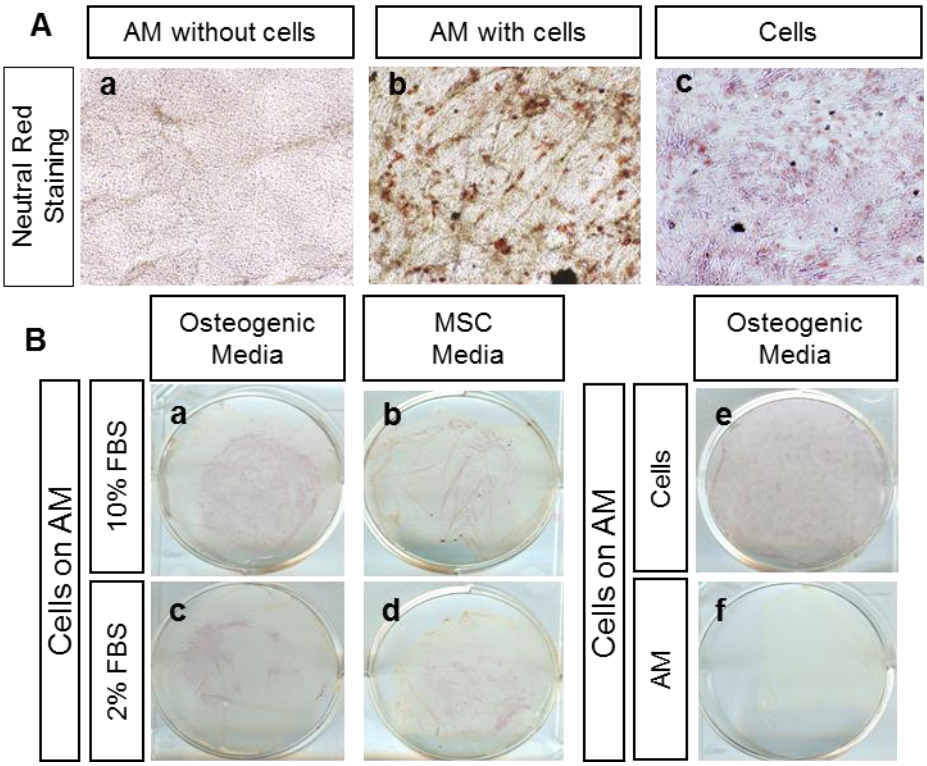


Figure 16. MSC osteogenic differentiation on human amniotic membrane. A) Images representative of neutral red staining of amniotic membrane (AM) only (a), MSCs plated on AM (b) and MSCs seeded in a plastic vessel (c) in osteogenic medium. Only the conditions in which MSCs were present stained positive for neutral red (b, c). **B)** AP staining for MSCs grown for 14 days in different culture conditions. After 14 days in osteogenic media, AM with no added MSCs was negative for AP activity (f) while MSCs plated on AM (a) or in plastic plates (e) exhibited positive AP staining. AP activity of MSCs plated on AM in osteogenic medium with a reduced amount (2%) of FBS (c) was comparable to AP activity of MSCs in complete osteogenic medium (a). In MSC/FBS medium (b) and reduced MSC-FBS (2%FBS) medium (d) cells also showed some AP activity. Images magnification: 400x.

4. Discussion and Future Work

The umbilical cord (UC) is a very promising mesenchymal stromal cell (MSC) source as cells can be easily obtained from this tissue without facing the ethical problems of embryonic stem cells (Can and Karahuseyinoglu, 2007). In addition, they seem to have greater expansion capability (Mitchell *et al*., 2003; Troyer and Weiss, 2008; Karahuseyinoglu *et al.*, 2007) and broader potential (Mitchell *et al*., 2003; Wang *et al*., 2004; Conconi *et al*., 2006; Troyer and Weiss, 2008; Chen *et al*., 2009) than cells from other adult stem cell sources, such as bone marrow (Panepucci et al., 2004; Chen MY et al., 2009).

Isolation of MSCs from distinct parts of the UC and the use of different methods for the isolation of MSCs from UC have been described (Romanov *et al*., 2002; Mitchell *et al*, 2003; Paneppucci *et al*., 2004; Wang *et al*, 2004; Sarugaser *et al*., 2005; Fong *et al*., 2007; Karahuseyinoglu *et al*, 2007; Secco *et al*., 2008; Seshareddy *et al*. 2008; Ishige *et al*, 2009; Zeddou *et al*., 2010). In this work, we successfully established 51distint human MSC lines from 26 UC donors. To determine an efficient isolation procedure from different tissue source we obtained MSCs from 13 peripheral Wharton's Jelly (pWJ), 14 total Wharton's Jelly (tWJ), 15 Whole umbilical cord (W-UC) and 12 vessels (V-UC) tissue. We found that a good balance between tissue dissociation and cell yield was obtained by performing a mechanical disruption of the WJ, vessels and whole cord tissue and performing 3 to 4 hour of enzymatic digestion with collagenase and hyaluronidase. For the isolation of tWJ, an initial 1 hour enzymatic digestion was performed to allow removal of the UC vessels, before following a procedure similar to the remaining tissues.

Cell suspensions immediately after isolation contained a mixture of cell types with variable expression of the markers analysed. Cells from W-UC present higher levels of hematopoietic markers than WJ-UC probably due to blood contamination from the vessels. Expression of the MSCs characteristic markers CD73, CD44, CD90 and CD105 highly increased from the time of the isolation to the first passage. This may be because the culture conditions induced the expression of those proteins, selected for MSCs, or may be related to a lower proliferation of the other cell types. Some embryonic stem cells markers have been reported to be present in MSCs such as SSEA-4 and alkaline phosphatase (Fong *et al*., 2007). In the present study a subpopulation of MSCs lines from different tissue source expressed the primitive stem cell marker SSEA-4 but not alkaline phosphatase. The presence of SSEA-4 suggests that these cells may possess certain embryonic stem cells properties. However, other study demonstrated that this early embryonic glycolipidic antigen expression was restricted to BM-MSCs and not expressed in UC-MSCs (Zeddou *et al.*, 2010). Thus, despite the lack of alkaline phosphatase, this profile indicates an evolutionary proximity between these cells and embryonic stem cells (Fong et al., 2007). Primary cultures obtained from different tissue sources demonstrated a variable morphology with some clusters of cells with typical endothelial morphology. Our culture conditions may thus be inappropriate for endothelial cells since after the first passage the clusters disappeared, as had also been described by other authors (Romanov *et al*., 2002; Secco *et al*., 2007). At the time of the first passage, more cells per gram

of W-UC tissue were systematically obtained compared to tWJ-UC tissue. One possible explanation is that the mixture of cell types obtained from the different parts of UC might potentiate cell adhesion and proliferation at this phase. After passaging, no major differences in the cell cultures were observed for the different tissue sources or isolation methods. In fact, the cultures became more homogeneous with continual passages.

For MSC expansion we used DMEM-F12 based media, a choice that was based in the work of Nekanti *et al.* (2010) who demonstrated that media with higher glucose concentrations sustain a greater growth potential of WJ-MSCs, maintain the typical spindle-shaped fibroblast-like morphology until later passages, and prevent senescence in early and later passages. However, the optimum basal medium, and in particular the ideal glucose content, for MSC expansion is not consensual and some reports argue that a low-glucose content is more appropriate (Sotiropoulou *et al*., 2006). In fact, the most common and, according to many studies, more adequate, basal medium for MSC culture is the low glucose α-MEM (Both *et al*., 2007; Chen *et al*., 2009; Sotiropoulou *et al*., 2006), even though other studies show opposite results (Pal *et al*., 2009; Nekanti *et al*., 2010). This inconsistency may be due to source-related variations, other cell type specific or culture conditions particularities, or differences between short-term and prolonged cultures, as suggested by Pal and colleagues (2009). Therefore, the best culture conditions must be found for each particular system. In this work, the isolated cells could be successfully expanded, while maintaining their essential characteristics, in a culture medium composed of DMEM-F12 with glutamine and 10% FBS. MSCs morphology from different lines in this medium remained relatively homogeneous, predominantly with spindle-shaped fibroblast-like cells, until late passages. At high passages cells changed to a more stretched and branched morphology. MSCs from tWJ and W-UC showed similar growth pattern up to 12 passages, but pWJ showed a less proliferative profile in late passages. In early and late passages, MSCs remained highly positive for CD73, CD105, CD44 and CD90 and negative for CD19, CD14, CD45, CD34 and HLA-DR, despite some irregular values of CD73 and CD105 MFI in higher passages.

Due to the increasing interest in the use on MSCs in regenerative medicine, active research is under way in search for clinically compatible culture media (Klyushnenkova et al., 2005; Kode *et al*., 2009). We have thus attempted to establish xeno-free culture conditions for the expansion and differentiation of the isolated UC-MSCs. Firstly, we used the commercial StemPro MSC serum free medium. Previous studies had described that this medium does not sustain cell adhesion to plastic without the use of a substrate such as fibronectin (Agata *et al*., 2009; Chase *et al*., 2010; Hartmann *et al*., 2010), a fact that is immediately disadvantageous. Therefore, Hartmann and colleagues successfully tried to overcome this issue by adding 2% of human serum (Hartmann *et al*., 2010). In our study only 1 out of 6 MSC lines was successfully isolated directly in this media, a success rate inferior to other studies (Agata *et al*., 2009; Hartmann *et al*., 2010), what can be due to differences in tissue source or isolation method. During expansion, despite some differences in terms of morphology, the growth rate and

immunophenotype of MSCs in SP-MSC SFM were comparable to FBS-containing medium for the same seeding density, except for the expression of the CD105 antigen. The number of CD105 positive MSCs in this serum free medium decreased with time in culture, in opposition to the observations of Agata and colleagues that had reported an increase of CD105 MFI in this media (Agata *et al*., 2009). CD105, also known as Endoglin, is a type I membrane glycoprotein part of the TGF-β receptor complex (Barbara *et al*., 1999; Blanco *et al*., 2005). In addition to MSCs, it is expressed in endothelial cells, activated macrophages, chondrocytes, fibroblasts, and smooth muscle cells (Fonsatti and Maio, 2004). It has been suggested that Endoglin plays an important role in the modulation of the activity of the different branches of the TGF-β signalling pathway, therefore determining the biological response to growth factors belonging to this superfamily (Letamendía *et al*., 1998; Lebrin *et al*., 2004; Blanco *et al*., 2005; Scherner *et al*., 2007; Ishibashi *et al*., 2010). Therefore, the decrease in CD105 levels in SP MSC SFM may be related to the modulation of signalling in response to the TGF-β1 present in this medium.

In addition to this commercial serum-free medium, other culture conditions in which FBS was replaced by human components were tested. Human plasma (hP), platelet lysate (hPLP or hPL), and AB serum (hABS) have been previously reported to support MSC expansion *in vitro* (Doucet *et al*., 2005; Müller *et al*., 2006; Kocaoemer *et al*., 2007; Schallmoser *et al*., 2007; Bieback *et al*., 2009). Preliminary results indicate that in our case hPLP, hPL and hP replacements are not as efficient at supporting MSCs proliferation and viability compared to FBS, even with fibronectin pre-coating and the addition of 250µM asc. Adding ascorbic acid to the culture conditions was shown by others (Choi KM *et al*., 2008; Potdar and d'Sousa, 2010), and confirmed by us, to enhance proliferation while preserving the MSC phenotype and differentiation potency. However, this was not effective in these culture conditions, and the cells progressively died and disappeared from culture at the third passage. The poor performance of MSCs in hPLP medium had also been reported in other studies (Kocaoemer *et al*., 2007; Schallmoser *et al*., 2007), and can be related with the platelet-lysate preparation method where centrifugation at high acceleration clears the platelet membranes, which release small membrane vesicles that have an additional mitogenic effect (Kocaoemer *et al*., 2007). This may have caused the observed decreased proliferative effect of MSCs. However, platelet-lysate containing medium supplemented with bFGF has been shown to improve MSC expansion (Yamaguchi *et al*., 2002; Doucet *et al*., 2005), something that should be experimented in future work.

Most studies concerning the use of human serum as a supplement substitute for FBS focus on the use of autologous serum (Yamamoto *et al*., 2003; Mizuno *et al*., 2006). However, this approach can be limiting in the quantity and quality of the serum for the MSC expansion, especially for some pathological conditions (Kocaoemer *et al*., 2007). Alternatively, a pool of human allogeneic AB serum would provide a more abundant and immediately available source. We show here that MSCs expanded in a hABS supplemented medium had similar morphology, proliferation rate, viability and immunophenotype as in FBS-containing medium. Some groups had already achieved similar results using hABS, even though sometimes also requiring the

addition of bFGF (Yamaguchi *et al*., 2002; Kocaoemer *et al*., 2007), while others have reported death of the cells at the first passage (Shahdadfar *et al*., 2005). In this study, the good performance of MSCs expansion in hABS-supplemented medium indicates that, of the FBS substitutes analysed, this medium is the most promising xeno-free culture condition.

As proposed by The International Society for Cellular Therapy, MSCs should have the ability to differentiate *in vitro* into cells of the osteoblastic, adipogenic and chondrogenic lineages. A heterogenic differentiation potential was observed between MSCs lines, especially between pWJ and tWJ tissue source. The reason for this inconsistency is still unclear. One possibility is that WJ cells made accessible by the two different methods have distinct potentials. In the case of tWJ, the isolation method involved enzymatic digestions for the removal of the vessels and thus enabling the isolation not only of the superficial layer of the WJ but also the inner WJ cells surrounding the vessels. Also, the difficulty in identifying and removing all the vessel pieces when using this method may allow their accidental inclusion in the final dissociated tissue. In fact, some studies have suggested that MSCs in the UC are preferentially located in the perivascular zone, as cells from this region show a higher *in vitro* expansion and differentiation potential (Suragaser *et al*., 2005), or as part of the vessels, with artery-derived cells exhibiting a greater osteogenic potential (Ishige *et al*., 2009).

During osteogenic differentiation, MSCs exhibited a progressive decrease in gene expression of CD73, CD105, CD90 and Runx2. Regarding protein expression of cell surface antigens, the majority of the cells stopped expressing the MSC surface marker CD105, while the CD73 and CD90 antigens only experienced a moderate reduction. Simultaneously, specific osteogenic markers, such as osteocalcin and alkaline phosphatase started to be detected. Importantly, alkaline phosphatase was present mainly at the surface of $CD90^{low}$ or negative cells. However, the proportion of AP-positive cells detected by flow cytometry was significantly lower than the enzymatic alkaline phosphatase activity detected at the same stage of differentiation. This may be due to an inefficiency of the antibodies used, or because the AP is being secreted and therefore only transiently expressed at the cell surface. Similarly, osteocalcin expression by flow cytometry was poorly detected, what may mean that it is not yet strongly expressed at this stage (as it is a later marker of differentiation characteristic of the beginning of mineralisation), or that, since it is generally an intracellular protein, cell surface detection was not adequate. Given that neither AP nor osteocalcin seem to be good cell surface markers of differentiation for flow cytometry detection, it would desirable to find such a phenotypic marker, such as Cadherin 11 (CDH11), or OB-cadherin, that seems to be a good candidate for this role (Cheng *et al*., 1998; Lecanda *et al*., 2000; Kii *et al*, 2004).

The differences observed in osteogenic potential between MSCs from the same tissue source were an intriguing issue for us. Initially, we have analysed whether this tendency was related to specific clinical parameters at the time of birth (gestational stage and mother's age) and isolation method but no correlation was found. We therefore attempted to determine which

characteristics of these cells could be related to or help predict the osteogenic potential, or which conditions could influence it.

Since some MSCs with high osteogenic potential experienced a low cell density during isolation due to low dissociation efficiency, we firstly expanded MSCs from low density cultures. This should select for clonal cells which could be at a more undifferentiated stage. However, MSCs that initially showed a moderate to high osteogenic potential, after low density almost completely lost the differentiation potential suggesting. Some authors affirmed that the fact of MSC culture became homogenous after several passages at high density may cause the loss of their osteogenic differentiation (Colter *et al*. 2001; Smith *et al*., 2004). In contrast, low plating densities seems to maintain heterogeneous cell morphologies and thus favour differentiation (Colter et al., 2001).

Subsequently, the relation between the expression of MSC specific antigens and the differentiation stage or propensity was assessed. As had already been reported by others (Jin *et al*., 2009) we have observed that the expression of MSC markers was reduced during osteogenic differentiation. Most interesting was the decrease in CD105 expression, practically disappearing at 14 days of differentiation, and the appearance of alkaline phosphatase marker at the cell surface of $CD90^{low}$ or negative cells. Also, the expression levels of these markers partially correlated with the osteogenic ability of the cell lines and varied in response to the culture environment. Therefore, when performing the isolation of the $CD105^{low}/CD90^{low}$ cell population by cell sorting and subsequently differentiating it, we expected to obtain increased osteogenic differentiation. However, differentiation of the $CD105^{high}/CD90^{high}$ sorted population produced higher AP activity and calcium deposits compared to both the $CD105^{low}/CD90^{low}$ and to the non-sorted MSCs population. The subsequent analysis, following a similar approach but focusing particularly on the expression of CD105, was based on the fact that higher differentiating MSC lines expressed lower levels of this antigen, and that CD105 expression had been reported to decrease over time during the course of multi-lineage differentiation (Choi et al., 2009). An increase in the osteogenic potential of $CD105^{high}$ MSCs was again obtained. Therefore, MSCs with high expression levels of CD105 seem to be more prone for osteogenic differentiation. This observation is in agreement with the study of Jarocha and who reported that $CD105^{high}$ BM-MSC had an enrichment in molecular markers of early osteogenic progenitors (Jarocha *et al*., 2008). In summary, increased levels of CD90 and CD105, in particular the latter, seem to be predictive of an increased osteogenic propensity, and may be related to a more undifferentiated state. In addition to these, the expression levels of CD146 have also been suggested to correlate with the differentiation potential of MSC-UC and thus should also be investigated (Baksh *et al*., 2007).

Finally, we addressed the question of whether the osteogenic differentiation ability of a MSC was a cell autonomous property or was conditioned by the environment and the neighbouring cells. The co-culture of cells with different osteogenic potential showed that the AP activity of cells with lower differentiation ability was enhanced when co-cultured with higher differentiating ones. This approach needs to be studied in more detail, but it seems that either

growth factors released to the medium by the cells with higher differentiation potential or the cell-to-cell contact are responsible for the increase in osteogenic potential in MSCs.

Therefore, some experiments should be performed in the future, in order to understand the mechanism behind the modulation of osteogenic differentiation in co-cultures. For example, conditioned medium experiments, in which the culture medium from MSCs with high and low osteogenic abilities is exchanged can elucidate whether this osteogenic modulation is mediated by secreted factors whether it requires cell-to-cell contact.

As previously mentioned, an adequate clinically compatible culture medium should, support MSC expansion while maintaining the essential properties, phenotype, and multilineage potential of these cells. Therefore, the osteogenic potential of the cells expanded in different xeno-free media (SP-MSC SFM and hABS) were tested but satisfactory results, were obtained only for the commercial hABS supplemented medium, which succeeded to maintain their osteogenic potential. The low osteogenic potential of MSCs expanded in SP-MSC SFM can be related to the decrease in the CD105 antigen during cell expansion, since the $CD105^{low}$ population has already been shown to have lower osteogenic potential. Published studies are not consensual on the effect of SP-MSC SFM in osteogenic differentiation. Whereas Chase *et al.*, reported the maintenance of the osteogenic potential in BM-MSCs expanded in this medium (Chase *et al*, 2010), Agata and colleagues observed a decrease in AP activity similar to ours (Agata *et al*., 2009). Moreover, Hartmann *et al*., also described the maintenance of osteogenic potential in this medium but requiring the addition of 2% human serum for enhanced results (Hartmann *et al*., 2010). Regarding the hABS supplement, the maintenance of MSC osteogenic capacity was also observed in the studies of Kocaoemer and colleagues (Kocaoemer, kern, Kluter *et al*., 2007).

Therefore, hABS supplemented medium seems to be a good xeno-free supplement for MSC expansion, as it promotes cell proliferation, maintains high levels of expression of the specific MSC markers and retains the potential for osteogenic differentiation. However, further experiments should be performed for the complete validation of the hABS medium, such as the direct isolation of MSCs from the UC in these conditions and the assessment of multilineage differentiation. Besides that, a xeno-free differentiation medium must also be established and thus a hABS supplemented medium with the osteogenic factors dex, asc and β-GP, and possibly with the addition of BMPs or TGF-β growth factors should be tested (Centrella *et al*., 1991; Lecanda *et al*., 1997; Cheng *et al*., 2003; Kanaan and Kanaan, 2006)..

Differentiation is also determined by the substrate on which cells are (Salasznyk *et al*., 2004; Cool and Nurcombe, 2005; Chastain *et al*., 2006) and, in particular, fibronectin has been reported to enhance cell adhesion and differentiation (Hidalgo-Batisda and Cartmell, 2010). Even though in this study fibronectin pre-coating did not have any effect in osteogenic differentiation efficiency, other substrates such as collagen (Salasznyk *et al*., 2004) and, more interestingly, amniotic membrane, could be suitable substrates for differentiation. The use of

human amniotic membrane as a support material for MSC osteogenic differentiation would help overcoming the cell dissociation and disruption of the matrix and of the cell network.

The analysis of the use of AM for this purpose has been hindered by the difficulty in identifying cells on the membrane, to prevent its creasing and to assess cell differentiation. Staining with neutral red helped to distinguish the MSC live cells from the apoptotic AM cells and the use of Teflon rings, a technique previously used in another study (Furie *et al*., 1984), was an efficient option to secure AM to the tissue culture plate. Moreover, frozen AM was preferentially used in our studies since it supports MSC adhesion and proliferation more efficiently than dehydrated AM. For the evaluation of MSC osteogenic differentiation on AM, staining for AP activity was the chosen method since Kossa and Alizarin red staining assay produced high background.

AP activity of MSCs seeded on AM in osteogenic medium showed comparable results with MSCs seeded on plastic. However, a basal osteogenic induction was observed in the MSCs plated on AM in the expansion medium. Therefore, there is the possibility that the acellular AM has some inherent properties capable of inducing osteogenesis, as also previously reported by Samandari *et al.* (2011). This capability could be due to the combination of growth factors described to be present in AM (Koizumi *et al*., 2000; Valladares *et al*., 2010). Consequently, a reduction in the FBS content from 10% to 2% was assessed to evaluate the potential of AM intrinsic factors to sustain osteogenic differentiation. AP activity analysis suggested that the AM could support osteogenic differentiation in a reduced serum environment what further increases the suitability of membrane as a scaffold to support osteogenic differentiation. Once more, it would be important to test the use of hABS-containing medium for MSC osteogenic differentiation on AM.

In conclusion, in this study an efficient method was established for the isolation of cells from the human UC stroma, a promising stem cell source especially due to its easy and non-invasive acquisition. In particular, tWJ-UC seems to be the most adequate tissue source within the UC, given the relatively high efficiency and reproducibility of the isolation, and the fact that it contains mostly free of other contaminant cell types. Also, tWJ-UC MSCs possess all the defining MSC characteristics and exhibit good differentiation ability.

Moreover, although additional studies are needed for the improvement of the MSC culture conditions, the xeno-free hABS-supplemented medium succeeded to maintain UC-MSC proliferation without compromising their morphological and immunophenotypical features, nor the ability for osteogenic differentiation. The applicability of this supplement for osteogenic differentiation should be subject of future analysis.

With this study we were also able to unravel some aspects of MSC osteogenic differentiation, even though many others are still unclear. According to our data, the isolation method and tissue source do influence MSC osteogenic differentiation even though not in a very consistent manner. Additionally, enhanced MSCs osteogenesis was possible by $CD105^{high}$ population enrichment or by MSCs co-culture. The mechanism responsible for these effects

should be investigated. Finally, additional studies should be performed on MSC differentiation on amniotic membrane, since the AM seems to be a promising substrate for the production of an osseous matrix suitable for transplantation.

References

Agata H, Watanabe N, Ishii Y, Kubo N, Ohshima S, Yamazaki M, Tojo A, Kagami H. (2009) Feasibility and efficacy of bone tissue engineering using human bone marrow stromal cells cultivated in serum-free conditions. Biochemical and Biophysical Research Communications. 2009 May 1;382(2):353-8.

Arthur A, Zannettino A, Gronthos S. (2009) The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. Journal of Cellular Physiology; 218(2):237-45

Avanzini MA, Bernardo ME, Cometa AM, Perotti C, Zaffaroni N, Novara F, Visai L, Moretta A, Del Fante C, Villa R, Ball LM, Fibbe WE, Maccario R, Locatelli F. (2009) Generation of mesenchymal stromal cells in the presence of platelet lysate: a phenotypic and functional comparison of umbilical cord blood- and bone marrow-derived progenitors. Haematologica. 94(12):1649-60.

Baksh D, Yao R, Tuan RS. (2007) Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells. 25(6):1384-92.

Bajada S, Mazakova I, Richardson JB, Ashammakhi N. (2008) Updates on stem cells and their applications in regenerative medicine. J Tissue Eng Regen Med. 2(4):169-83.

Barbara NP, Wrana JL, Letarte M (1999) Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-β superfamily. Journal of Biological Chemistry, 274:584-594

Barry FP, Murphy JM. (2004) Mesenchymal stem cells: clinical applications and biological characterization. International Journal of Biochemistry and Cell Biology; 36(4):568-84

Bernardo ME, Avanzini MA, Perotti C, Cometa AM, Moretta A, Lenta E, Del Fante C, Novara F, de Silvestri A, Amendola G, Zuffardi O, Maccario R, Locatelli F. (2006) Optimization of in vitro expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: further insights in the search for a fetal calf serum substitute. J Cell Physiol. 211(1):121-30.

Bernardo ME, Cometa AM, Locatelli F. (2011) Mesenchymal stromal cells: a novel and effective strategy for facilitating engraftment and accelerating hematopoietic recovery after transplantation? Bone Marrow Transplantation [Epub ahead of print]

Bieback K, Hecker A, Kocaömer A, Lannert H, Schallmoser K, Strunk D, Klüter H. (2009) Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells. 27(9):2331-41.

Bieback K, Kern S, Klüter H, Eichler H. (2004) Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells. 22(4):625-34.

Blanco FJ, Santibanez JF, Guerrero-Esteo M, Langa C, Vary CP, Bernabeu C. (2005) Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-beta receptor complex. Journal of Cell Physiology. 204(2):574-84.

Bobis S, Jarocha D, Majka M. (2006) Mesenchymal stem cells: characteristics and clinical applications. Folia Histochemica et Cytobiologica. 44(4):215-30.

Both SK, van der Muijsenberg AJ, van Blitterswijk CA, de Boer J, de Bruijn JD. (2007) A Rapid and Efficient Method for Expansion of Human Mesenchymal Stem Cells. Tissue Engineering.13(1):3-9

Bourin P, Gadelorge M, Peyrafitte JA, Fleury-Cappellesso S, Gomez M, Rage C, Sensebé L. (2008) Mesenchymal Progenitor Cells: Tissue Origin, Isolation and Culture. Transfusion Medicine and Hemotherapy. 35(3):160-167.

Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S (1998) Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. Journal of Orthopaedic Research.

Burman S, Tejwani S, Vemuganti GK, Gopinathan U, Sangwan VS. (2004) Ophthalmic applications of preserved human amniotic membrane: a review of current indications. Cell and Tissue Banking. 5(3):161-75.

Can A, Karahuseyinoglu S (2007) Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. Stem Cells 25:2886-95

Canalis E (2008) Notch signaling in osteoblasts. Science Signaling 1:pag17

Centrella M, McCarthy TL, Canalis E. (1991) Transforming growth factor-beta and remodeling of bone. Journal of Bone and Joint Surgery. 73(9): 1418-28.

Chachques JC, Herreros J, Trainini J, Juffe A, Rendal E, Prosper F, Genovese J. (2004) Autologous human serum for cell culture avoids the implantation of cardioverter-defibrillators in cellular cardiomyoplasty. Int J Cardiol. 95 Suppl 1:S29-33.

Chai C, Leong KW. (2007) Biomaterials approach to expand and direct differentiation of stem cells. Mol Ther. 15(3):467-80.

Chamberlain G, Fox J, Ashton B, Middleton J (2007) Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells 25:2739-49

Chang YL, Stanford CM, Keller JC. (2000) Calcium and phosphate supplementation promotes bone cell mineralization: implications for hydroxyapatite (HA)-enhanced bone formation. Journal of Biomedical Materials Research. 52(2): 270-8

Chang YJ, Shih DT, Tseng CP, Hsieh TB, Lee DC, Hwang SM. (2006) Disparate mesenchyme-lineage tendencies in mesenchymal stem cells from human bone marrow and umbilical cord blood. Stem Cells. 24(3):679-85.

Chase LG, Lakshmipathy U, Solchaga LA, Rao MS, Vemuri MC (2010) A novel serum-free medium for the expansion of human mesenchymal stem cells. Stem Cell Research and Therapy 1:8

Chastain SR, Kundu AK, Dhar S, Calvert JW, Putnam AJ. (2006) Adhesion of mesenchymal stem cells to polymer scaffolds occurs via distinct ECM ligands and controls their osteogenic differentiation. Journal of Biomedical Materials Research, Part A. 78(1):73-85.

Chen MY, Lie PC, Li ZL, Wei X. (2009) Endothelial differentiation of Wharton's jelly-derived mesenchymal stem cells in comparison with bone marrow-derived mesenchymal stem cells. Experimental Hematology. 37(5):629-40.

Chen HH, Decot V, Ouyang JP, Stoltz JF, Bensoussan D, de Isla NG. (2009) In vitro initial expansion of mesenchymal stem cells is influenced by the culture parameters used in the isolation process. Biomedical Materials and Engineering. 19(4-5):301-9.

Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY, He TC. (2003) Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). The Journal of Bone and Joint Surgery. 85-A(8): 1544-52

Cheng SL, Lecanda F, Davidson MK, Warlow PM, Zhang SF, Zhang L, Suzuki S, St John T, Civitelli R. (1998) Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation. Journal of Bone and Mineral Research. 13(4):633-44.

Chevallier N, Anagnostou F, Zilber S, Bodivit G, Maurin S, Barrault A, Bierling P, Hernigou P, Layrolle P, Rouard H (2010) Osteoblastic differentiation of human mesenchymal stem cells with platelet lysate. Biomaterials 31:270-8

Choi KM, Seo YK, Yoon HH, Song KY, Kwon SY, Lee HS, Park JK. (2008) Effect of ascorbic acid on bone marrow-derived mesenchymal stem cell proliferation and differentiation. Journal of Bioscience and Bioengineering. 105(6):586-94.

Choi SC, Kim SJ, Choi JH, Park CY, Shim WJ, Lim DS. (2008) Fibroblast growth factor-2 and -4 promote the proliferation of bone marrow mesenchymal stem cells by the activation of the PI3K-Akt and ERK1/2 signaling pathways. Stem Cells and Development. 17(4):725-36.

Choi SJ, Jin HJ, Park SK, Oh W, Yang YS, Kim SW. (2009) Down-regulation of CD105 is associated with multi-lineage differentiation in human umbilical cord blood-derived mesenchymal stem cells. Biochem Biophys Res Commun. 381(4):676-81.

Clarke B (2008) Normal bone anatomy and physiology. Clinical Journal of the American Society of Nephrology 3:S131-9

Coelho MJ, Fernandes MH. (2000) Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteoblastic differentiation. Biomaterials. 21(11):1095-102.

Colter DC, Sekiya I, Prockop DJ. (2001) Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci U S A. 98(14):7841-5.

cells from Wharton's jelly show in vitro and in vivo myogenic differentiative potential. International Journal of Molecular Medicine. 18(6):1089-96.

Cool SM, Nurcombe V. (2005) Substrate induction of osteogenesis from marrow-derived mesenchymal precursors. Stem Cells and Development. 14(6):632-42.

da Silva Meirelles L, Chagastelles PC, Nardi NB. (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. Journal of Cell Science. 2006 Jun 1;119(Pt 11):2204-13. Epub 2006 May 9.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 8(4):315-7.

Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X, Lataillade JJ. (2005) Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. Journal of Cellular Physiology; 205(2): 228-36

Duplomb L, Dagouassat M, Jourdon P, Heymann D (2007) Concise review: embryonic stem cells: a new tool to study osteoblast and osteoclast differentiation. Stem Cells 25:544-52

Engin F, Lee B (2010) NOTCHing the bone: insights into multi-functionality. Bone 46:274-80

Fauran-Clavel MJ, Oustrin J. (1986) Alkaline phosphatase and bone calcium parameters. Bone. 7(2):95-9.

Fernandes M, Sridhar MS, Sangwan VS, Rao GN. (2005) Amniotic membrane transplantation for ocular surface reconstruction. Cornea. 24(6):643-53.

Fong CY, Richards M, Manasi N, Biswas A, Bongso A. (2007) Comparative growth behaviour and characterization of stem cells from human Wharton's jelly. Reprod Biomed Online. 15(6):708-18.

Fong CY, Chak LL, Biswas A, Tan JH, Gauthaman K, Chan WK, Bongso A. (2011) Human Wharton's jelly stem cells have unique transcriptome profiles compared to human embryonic stem cells and other mesenchymal stem cells. Stem Cell Rev. 7(1):1-16.

Fonsatti E, Maio M. (2004) Highlights on endoglin (CD105): from basic findings towards clinical applications in human cancer. Journal of Translational Medicine. 2(1):18.

Fox JM, Chamberlain G, Ashton BA, Middleton J. (2007) Recent advances into the understanding of mesenchymal stem cell trafficking. British Journal of Haematology; 137(6):491-502.

Franceschi RT, Ge C, Xiao G, Roca H, Jiang D (2009) Transcriptional regulation of osteoblasts. Cells Tissues Organs; 189:144-52

Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. (1966) Osteogenesis in transplants of bone marrow cells. Journal of Embryology & Experimental Morphology. 16(3):381-90

Friedenstein AJ, Chailakhyan RK, Gerasimov UV. (1987) Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell & Tissue Kinetics. 20(3):263-72.

Friedman R, Betancur M, Boissel L, Tuncer H, Cetrulo C, Klingemann H. (2007) Umbilical cord mesenchymal stem cells: adjuvants for human cell transplantation. Biol Blood Marrow Transplant. 13(12):1477-86.

Frith J, Genever P. (2008) Transcriptional Control of Mesenchymal Stem Cell Differentiation. Transfus Med Hemother. 35(3):216-227.

Gimble JM, Katz AJ, Bunnell BA. (2007) Adipose-derived stem cells for regenerative medicine. Circ Res. 100(9):1249-60.

Gomillion CT, Burg KJ. (2006) Stem cells and adipose tissue engineering. Biomaterials. 27(36):6052-63.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences USA. 97(25):13625-30.

Guerrero-Esteo M, Lastres P, Letamendia A, Perez-Alvarez MJ, Langa C, Lopez LA, Fabra A, Garcia-Pardo A, Vera S, Letarte M, Bernabeu C (1999) Endoglin overexpression modulates cellular morphology, migration, and adhesion of mouse fibroblasts. European Journal of Cell Biology. 78:614-623.

Hartmann I, Hollweck T, Haffner S, Krebs M, Meiser B, Reichart B, Eissner G. (2010) Umbilical cord tissue-derived mesenchymal stem cells grow best under GMP-compliant culture conditions and maintain their phenotypic and functional properties. J Immunol Methods. 15;363(1):80-9.

Hidalgo-Bastida LA, Cartmell SH. (2010) Mesenchymal stem cells, osteoblasts and extracellular matrix proteins: enhancing cell adhesion and differentiation for bone tissue engineering.Tissue Engineering. Part B, Reviews. 16(4):405-12.

Hildebrandt C, Büth H, Thielecke H. (2009) Influence of cell culture media conditions on the osteogenic differentiation of cord blood-derived mesenchymal stem cells. Ann Anat. 191(1):23-32.

Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. (1999) Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nature Medicine 5(3):309-13.

Hsieh JY, Fu YS, Chang SJ, Tsuang YH, Wang HW. (2010) Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton's jelly of umbilical cord. Stem Cells and Development. 19(12):1895-910.

Hung CH, Young TH. (2006) Differences in the effect on neural stem cells of fetal bovine serum in substrate-coated and soluble form. Biomaterials. 27(35):5901-8.

Insausti CL, Alcaraz A, García-Vizcaíno EM, Mrowiec A, López-Martínez MC, Blanquer M, Piñero A, Majado MJ, Moraleda JM, Castellanos G, Nicolás FJ. (2010) Amniotic membrane induces epithelialization in massive posttraumatic wounds. Wound Repair and Regeneration. 18(4):368-77.

Ishibashi O, Ikegame M, Takizawa F, Yoshizawa T, Moksed MA, Iizawa F, Mera H, Matsuda A, Kawashima H. (2010) Endoglin is involved in BMP-2-induced osteogenic differentiation of periodontal ligament cells through a pathway independent of Smad-1/5/8 phosphorylation. Journal of Cellular Physiology. 222(2):465-73.

Ishige I, Nagamura-Inoue T, Honda MJ, Harnprasopwat R, Kido M, Sugimoto M, Nakauchi H, Tojo A. (2009) Int J Hematol. 90(2):261-9.

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. Journal of Cellular Biochemistry; 64(2): 295-312.

Jarocha D, Lukasiewicz E, Majka M. (2008) Adventage of mesenchymal stem cells (MSC) expansion directly from purified bone marrow CD105+ and CD271+ cells. Folia Histochem Cytobiol. 46(3):307-14.

Javazon EH, Beggs KJ, Flake AW. (2004) Mesenchymal stem cells: paradoxes of passaging. Experimental Hematology; 32(5): 414-25.

Jayakumar P, Di Silvio L. (2010) Osteoblasts in bone tissue engineering. Proceedings of the Institution of Mechanical Engineers. Part H, Journal of engineering in medicine; 224(12): 1415-40.

Jethva R, Otsuru S, Dominici M, Horwitz EM. (2009) Cell therapy for disorders of bone. Cytotherapy; 11(1):3-17

Kalajzic I, Staal A, Yang WP, Wu Y, Johnson SE, Feyen JH, Krueger W, Maye P, Yu F, Zhao Y, Kuo L, Gupta RR, Achenie LE, Wang HW, Shin DG, Rowe DW. (2005) Expression profile of osteoblast lineage at defined stages of differentiation. J Biol Chem. 280(26):24618-26

Kanaan RA, Kanaan LA. (2006) Transforming growth factor beta1, bone connection. Medical Science Monitor.12(8): RA164-9.

Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, Tukun A, Uckan D, Can A. (2007) Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. Stem Cells. 25(2):319-31.

Kim S, Min WK, Chun S, Lee W, Chung HJ, Choi SJ, Yang SE, Yang YS, Yoo JI (2010) Protein expression profiles during osteogenic differentiation of mesenchymal stem cells derived from human umbilical cord blood. The Tohoku Journal of Experimental Medicine 221:141-50

Kim SJ, Shin YW, Yang KH, Kim SB, Yoo MJ, Han SK, Im SA, Won YD, Sung YB, Jeon TS, Chang CH, Jang JD, Lee SB, Kim HC, Lee SY. (2009a) A multi-center, randomized, clinical study to compare the effect and safety of autologous cultured osteoblast (Ossron) injection to treat fractures. BMC Musculoskeletal Disorders. 12; 10:20.

Kim SS, Song CK, Shon SK, Lee KY, Kim CH, Lee MJ, Wang L. (2009b) Effects of human amniotic membrane grafts combined with marrow mesenchymal stem cells on healing of full-thickness skin defects in rabbits. Cell and Tissue Research. 336(1):59-66.

Kii I, Amizuka N, Shimomura J, Saga Y, Kudo A. (2004) Cell-cell interaction mediated by cadherin-11 directly regulates the differentiation of mesenchymal cells into the cells of the osteo-lineage and the chondro-lineage. Journal of Bone and Mineral Research. 19(11):1840-9.

Kilian O, Flesch I, Wenisch S, Taborski B, Jork A, Schnettler R, Jonuleit T. (2004) Effects of platelet growth factors on human mesenchymal stem cells and human endothelial cells in vitro. Eur J Med Res. 30;9(7):337-44.

Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, Deans RJ, McIntosh KR. (2005) T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci. 12(1):47-57.

Kocaoemer A, Kern S, Klüter H, Bieback K. (2007) Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem Cells. 25(5): 1270-8.

Kode JA, Mukherjee S, Joglekar MV, Hardikar AA (2009) Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. Cytotherapy 11:377-91

Koga H, Engebretsen L, Brinchmann JE, Muneta T, Sekiya I. (2009) Mesenchymal stem cell-based therapy for cartilage repair: a review. Knee Surg Sports Traumatol Arthrosc. 17(11):1289-97.

Koizumi NJ, Inatomi TJ, Sotozono CJ, Fullwood NJ, Quantock AJ, Kinoshita S. (2000) Growth factor mRNA and protein in preserved human amniotic membrane. Current Eye Research. 2000 Mar;20(3):173-7.

Komori T (2006) Regulation of osteoblast differentiation by transcription factors. The Journal of Celular Biochemistry 99:1233-9

Krampera M, Pizzolo G, Aprili G, Franchini M. (2006) Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. Bone. 39(4):678-83.

Lange C, Cakiroglu F, Spiess AN, Cappallo-Obermann H, Dierlamm J, Zander AR. (2007) Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. J Cell Physiol. 213(1):18-26.

Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, ten Dijke P. (2004) Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction.EMBO Journal. 23(20):4018-28.

Lecanda F, Avioli LV, Cheng SL. (1997) Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. Journal of Cellular Biochemistry; 67(3): 386-96.

Lecanda F, Cheng SL, Shin CS, Davidson MK, Warlow P, Avioli LV, Civitelli R. (2000) Differential regulation of cadherins by dexamethasone in human osteoblastic cells. Journal of Cellular Biochemistry. 77(3):499-506.

Letamendía A, Lastres P, Botella LM, Raab U, Langa C, Velasco B, Attisano L, Bernabéu C (1998) Role of endoglin in cellular responses to transforming growth factor-β. Journal of Biological Chemistry. 273:33011–33019

Lin HT, Tarng YW, Chen YC, Kao CL, Hsu CJ, Shyr YM, Ku HH, Chiou SH. (2005) Using human plasma supplemented medium to cultivate human bone marrow-derived mesenchymal stem cell and evaluation of its multiple-lineage potential. Transplant Proc. 37(10):4504-5.

Lo V, Pope E. (2009) Amniotic membrane use in dermatology. International Journal of Dermatology 48(9):935-40.

López-Valladares MJ, Teresa Rodríguez-Ares M, Touriño R, Gude F, Teresa Silva M, Couceiro J. Donor age and gestational age influence on growth factor levels in human amniotic membrane. Acta Ophthalmol. 88(6):e211-6.

Lorenz K, Sicker M, Schmelzer E, Rupf T, Salvetter J, Schulz-Siegmund M, Bader A. (2008) Multilineage differentiation potential of human dermal skin-derived fibroblasts Exp Dermatol. 2008 Nov;17(11):925-32

Lu LL, Liu YJ, Yang SG, Zhao QJ, Wang X, Gong W, Han ZB, Xu ZS, Lu YX, Liu D, Chen ZZ, Han ZC.(2006) Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. Haematologica; 91:1017-26.

Lutolf MP, Blau HM. (2009) Artificial stem cell niches. Adv Mater. 21(32-33):3255-68.

Mannello F, Tonti G, Papa S. (2006) Matrix metalloproteinase inhibitors as anticancer therapeutics. Curr Cancer Drug Targets. 5(4):285-98.

Martins AA, Paiva A, Morgado JM, Gomes A, Pais ML. (2009) Quantification and immunophenotypic characterization of bone marrow and umbilical cord blood mesenchymal stem cells by multicolor flow cytometry. Transplant Proc. 41(3):943-6.

McElreavey KD, Irvine AI, Ennis KT, McLean WH. (1991) Isolation, culture and characterisation of fibroblast-like cells derived from the Wharton's jelly portion of human umbilical cord. Biochemical Society Transactions; 19(1):29S

Mitchell KE, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T, Troyer D, Medicetty S (2003) Matrix cells from Wharton's Jelly form neurons and glia. Stem Cells 21:50–60

Mizuno N, Shiba H, Ozeki Y, Mouri Y, Niitani M, Inui T, Hayashi H, Suzuki K, Tanaka S, Kawaguchi H, Kurihara H. (2006) Human autologous serum obtained using a completely closed bag system as a substitute for foetal calf serum in human mesenchymal stem cell cultures. Cell Biol Int. 30(6):521-4

Müller I, Kordowich S, Holzwarth C, Spano C, Isensee G, Staiber A, Viebahn S, Gieseke F, Langer H, Gawaz MP, Horwitz EM, Conte P, Handgretinger R, Dominici M. (2006) Animal serum-free culture conditions for isolation and expansion of multipotent mesenchymal stromal cells from human BM. Cytotherapy 8(5):437-44.

Musina RA, Bekchanova ES, Sukhikh GT (2005) Comparison of Mesenchymal Stem Cells Obtained from different Human Tissues. Cell Technologies in Biology and Medicine, 1:504-9

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor os terix is required for osteoblast differentiation and bone formation. Cell 108:17-29

Nauta AJ, Fibbe WE. (2007) Immunomodulatory properties of mesenchymal stromal cells. Blood. 110(10): 3499-506

Nekanti U, Rao VB, Bahirvani AG, Jan M, Totey S, Ta M (2010) Long-term expansion and pluripotent marker array analysis of Wharton's jelly-derived mesenchymal stem cells. Stem Cells and Development 19:117-30

Nesic D, Whiteside R, Brittberg M, Wendt D, Martin I, Mainil-Varlet P. (2006) Cartilage tissue engineering for degenerative joint disease. Adv Drug Deliv Rev. 58(2):300-22.

Ng F, Boucher S, Koh S, Sastry KS, Chase L, Lakshmipathy U, Choong C, Yang Z, Vemuri MC, Rao MS, Tanavde V (2008) PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. 112:295-307.

Nöth U, Rackwitz L, Steinert AF, Tuan RS. (2010) Cell delivery therapeutics for musculoskeletal regeneration. Advanced Drug Delivery Reviews. 15;62(7-8):765-83.

Owen M, Friedenstein AJ. (1988) Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symposium; 136:42-60.

Pal R, Hanwate M, Jan M, Totey S. (2009) Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. Journal of Tissue Engineering and Regenerative Medicine. 3(3):163-74.

Panepucci RA, Siufi JL, Silva WA Jr, Proto-Siquiera R, Neder L, Orellana M, Rocha V, Covas DT, Zago MA. (2004) Comparison of gene expression of umbilical cord vein and bone marrow-derived mesenchymal stem cells. Stem Cells. 22(7):1263-78.

Park JB. (2010) The Effects of Dexamethasone, Ascorbic Acid, and β-Glycerophosphate on Osteoblastic Differentiation by Regulating Estrogen Receptor and Osteopontin Expression. J Surg Res. [Epub ahead of print]

Porter RM, Huckle WR, Goldstein AS. (2003) Effect of dexamethasone withdrawal on osteoblastic differentiation of bone marrow stromal cells. J Cell Biochem. 90(1):13-22.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143-7

Potdar PD, D'Souza SB. (2010) Ascorbic acid induces in vitro proliferation of human subcutaneous adipose tissue derived mesenchymal stem cells with upregulation of embryonic stem cell pluripotency markers Oct4 and SOX 2. Human Cell. 23(4):152-5.

Rawadi G, Vayssière B, Dunn F, Baron R, Roman-Roman S (2003) BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. Journal of Bone and Mineral Research 18:1842-53

Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M, Jankovskis G, Muiznieks I, Muceniece R, Ancans J. (2009) Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. Stem Cell Reviews. 5(4):378-86.

Rohde, E., Schallmoser, K., Bartmann, C., Reinisch, A. and Strunk, D. (2007) GMP-Compliant Propagation of Human Multipotent Mesenchymal Stromal Cells, in Pharmaceutical Manufacturing Handbook: Regulations and Quality (ed S. C. Gad), John Wiley & Sons, Inc., Hoboken, NJ, USA.

Romanov YA, Svintsitskaya VA, Smirnov VN. (2003) Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells. 21(1):105-10.

Salasznyk RM, Williams WA, Boskey A, Batorsky A, Plopper GE. (2004) Adhesion to Vitronectin and Collagen I Promotes Osteogenic Differentiation of Human Mesenchymal Stem Cells. Journal od Biomedicine and Biotechnology. 2004(1):24-34.

Samandari MH, Adibi S, Khoshzaban A, Aghazadeh S, Dihimi P, Torbaghan SS, Keshel SH, Shahabi Z (2011) Human amniotic membrane, best healing accelerator, and the choice of bone induction for vestibuloplasty technique (an animal study) Transplant Research and Risk Management. 2011(3):1-8

Sanchez-Ramos J. (2006) Stem cells from umbilical cord blood. Semin Reprod Med. 24(5):358-69.

Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. (2005) Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells. 23(2):220-9.

Schallmoser K, Bartmann C, Rohde E, Reinisch A, Kashofer K, Stadelmeyer E, Drexler C, Lanzer G, Linkesch W, Strunk D. (2007) Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. Transfusion 47(8): 1436-46.

Scherner O, Meurer SK, Tihaa L, Gressner AM, Weiskirchen R. (2007) Endoglin differentially modulates antagonistic transforming growth factor-beta1 and BMP-7 signaling. Journal of Biological Chemistry. 282(19):13934-43.

Secco M, Zucconi E, Vieira NM, Fogaça LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M. (2008) Multipotent stem cells from umbilical cord: cord is richer than blood! Stem Cells. 26(1):146-50.

Secco M, Moreira YB, Zucconi E, Vieira NM, Jazedje T, Muotri AR, Okamoto OK, Verjovski-Almeida S, Zatz M. (2009) Gene expression profile of mesenchymal stem cells from paired umbilical cord units: cord is different from blood. Stem Cell Rev. 5(4):387-401.

Selvaggi TA, Walker RE, Fleisher TA. (1997) Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. Blood. 89(3):776-9.

Sensebé L, Krampera M, Schrezenmeier H, Bourin P, Giordano R. (2010) Mesenchymal stem cells for clinical application. Vox Sanguinis. 98(2):93-107.

Sensebé L, Bourin P, Tarte K. (2011) Good Manufacturing Practices Production of Mesenchymal Stem/Stromal Cells. Human Gene Therapy, 22(1): 19-26.

Sharifiaghdas F, Moghadasali R, Baharvand H, Hosseini-Moghaddam SM, Mahmoudnejad N. (2009) Special characteristics of culturing mature human bladder smooth muscle cells on human amniotic membrane as a suitable matrix. Urol J. 6(4):283-8.

Sittinger M, Hutmacher DW, Risbud MV. (2004) Current strategies for cell delivery in cartilage and bone regeneration. Current Opinion in Biotechnology 15(5):411-8

Smith JR, Pochampally R, Perry A, Hsu SC, Prockop DJ. (2004) Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma. Stem Cells. 22(5):823-31.

Sordi V. (2009) Mesenchymal stem cell homing capacity. Transplantation. 87:S42-5

Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M. (2006) Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells. 24(2):462-71

Takamizawa S, Maehata Y, Imai K, Senoo H, Sato S, Hata R. (2004) Effects of ascorbic acid and ascorbic acid 2-phosphate, a long-acting vitamin C derivative, on the proliferation and differentiation of human osteoblast-like cells. Cell Biol Int. 28(4):255-65.

Tamama K, Kawasaki H, Wells A. (2010) Epidermal growth factor (EGF) treatment on multipotential stromal cells (MSCs). Possible enhancement of therapeutic potential of MSC. Journal of Biomedicine and Biotechnology 2010:795385

Tan SL, Sulaiman S, Pingguan-Murphy B, Selvaratnam L, Tai CC, Kamarul T (2011) Human amnion as a novel cell delivery vehicle for chondrogenic mesenchymal stem cells. Cell and Tissue Banking ;12(1):59-70

Troyer DL, Weiss ML (2008) Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells 26(3):591-9

Tsai MS, Lee JL, Chang YJ, Hwang SM. (2004) Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. Human Reproduction; 19:1450-6.

Tsigkou O, Jones JR, Polak JM, Stevens MM. (2009) Differentiation of fetal osteoblasts and formation of mineralized bone nodules by 45S5 Bioglass conditioned medium in the absence of osteogenic supplements. Biomaterials. 2009 30(21):3542-50.

Turnovcova K, Ruzickova K, Vanecek V, Sykova E, Jendelova P. (2009) Properties and growth of human bone marrow mesenchymal stromal cells cultivated in different media. Cytotherapy; 11(7): 874-85.

Valta MP, Hentunen T, Qu Q, Valve EM, Harjula A, Seppänen JA, Väänänen HK, Härkönen PL (2006) Regulation of osteoblast differentiation: a novel function for fibroblast growth factor 8. Endocrinology 147:2171-82

van den Dolder J, Mooren R, Vloon AP, Stoelinga PJ, Jansen JA. (2006) Platelet-rich plasma: quantification of growth factor levels and the effect on growth and differentiation of rat bone marrow cells. Tissue Engineering 12(11): 3067-73.

Vater C, Kasten P, Stiehler M. (2011) Culture media for the differentiation of mesenchymal stromal cells. Acta Biomaterialia. 7(2): 463-77.

Velez I, Parker WB, Siegel MA, Hernandez M. (2010) Cryopreserved amniotic membrane for modulation of periodontal soft tissue healing: a pilot study. Journal of Periodontology. 81(12):1797-804

Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, Fu YS, Lai MC, Chen CC (2004) Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells 22:1330-7

Wang Z, Goh J, Das De S, Ge Z, Ouyang H, Chong JS, Low SL, Lee EH (2006) Efficacy of bone marrow-derived stem cells in strengthening osteoporotic bone in a rabbit model. Tissue Engineering 12:1753-61

Ward DJ, Bennett JP, Burgos H, Fabre J. (1989) The healing of chronic venous leg ulcers with prepared human amnion. Br J Plast Surg 42:463-467

Watt FM, Driskell RR (2010) The therapeutic potential of stem cells. Philosophical Transactions of the Royal Society of London 365:155-63

Weil BR, Abarbanell AM, Herrmann JL, Wang Y, Meldrum DR. (2009) High glucose concentration in cell culture medium does not acutely affect human mesenchymal stem cell growth factor production or proliferation. Am J Physiol Regul Integr Comp Physiol., 296:R1735-43.

Weiss ML, Anderson C, Medicetty S, Seshareddy KB, Weiss RJ, VanderWerff I, Troyer D, McIntosh KR. (2008) Immune properties of human umbilical cord Wharton's jelly-derived cells. Stem Cells 26:2865-74

Xu Y, Shi Y, Ding S (2008) A chemical approach to stem-cell biology and regenerative medicine. Nature 453:338-44

Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda T, Yoshiki S (1991) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. The Journal of Cell Biology 113:681-7

Yamaguchi M, Hirayama F, Wakamoto S, Fujihara M, Murahashi H, Sato N, Ikebuchi K, Sawada K, Koike T, Kuwabara M, Azuma H, Ikeda H. (2002) Bone marrow stromal cells prepared using AB serum and bFGF for hematopoietic stem cells expansion. Transfusion. 42(7):921-7.

Yamamoto N, Isobe M, Negishi A, Yoshimasu H, Shimokawa H, Ohya K, Amagasa T, Kasugai S. (2003) Effects of autologous serum on osteoblastic differentiation in human bone marrow cells. J Med Dent Sci. 50(1):63-9.

Yang SE, Ha CW, Jung M, Jin HJ, Lee M, Song H, Choi S, Oh W, Yang YS. (2004) Mesenchymal stem/progenitor cells developed in cultures from UC blood. Cytotherapy. 6(5):476-86.

Yang X, Moldovan NI, Zhao Q, Mi S, Zhou Z, Chen D, Gao Z, Tong D, Dou Z (2008) Reconstruction of damaged cornea by autologous transplantation of epidermal adult stem cells. Molecular Vision 14:1064-70

Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J, Duenzl M, Lucas PA, Black AC Jr. (2001) Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec.;264(1):51-62.

Zamurovic N, Cappellen D, Rohner D, Susa M (2004) Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene Hey1 inhibits mineralization and Runx2 transcriptional activity. Journal of Biological Chemistry 279:37704-15

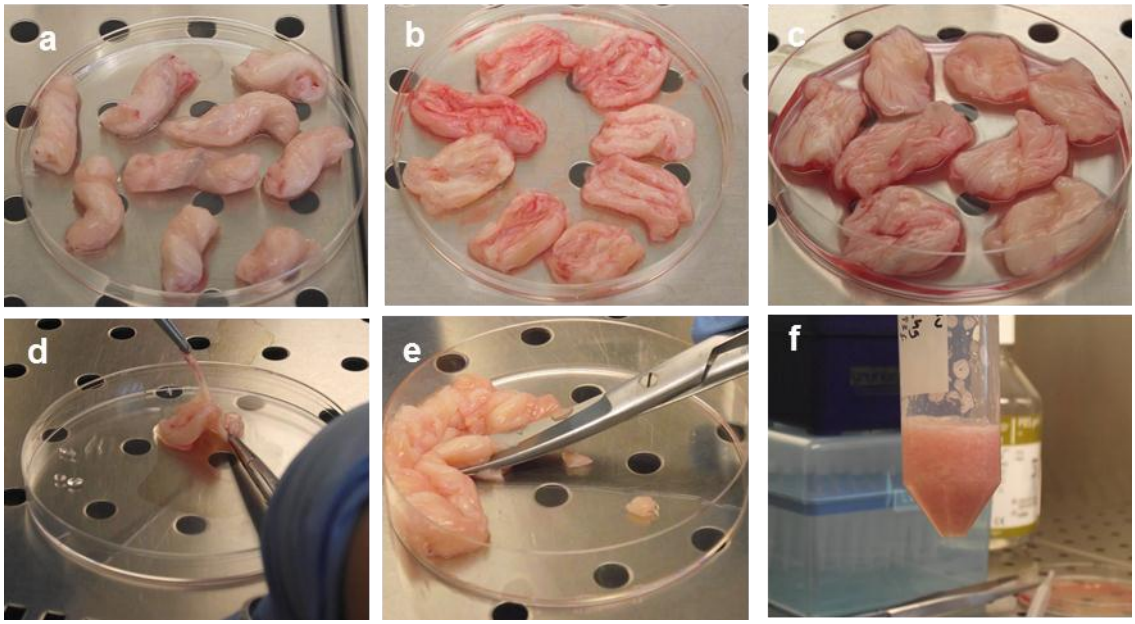
Zeddou M, Briquet A, Relic B, Josse C, Malaise MG, Gothot A, Lechanteur C, Beguin Y. (2010) The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. Cell Biol Int. 34(7):693-701.

Zhang ZY, Teoh SH, Chong MS, Schantz JT, Fisk NM, Choolani MA, Chan J. (2009) Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells. Stem Cells. 27(1):126-37.

Zhao S, Wehner R, Bornhäuser M, Wassmuth R, Bachmann M, Schmitz M. (2010) Immunomodulatory properties of mesenchymal stromal cells and their therapeutic consequences for immune-mediated disorders. Stem Cells and Development; 19(5):607-14

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH.(2002) Human adipose tissue is a source of multipotent stem cells. Molecular Biology of the Cell. 13(12):4279-95

Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, Maini RN. (2000) Mesenchymal precursor cells in the blood of normal individuals. Arthritis Research ;2(6):477-88.



Supplementary Figure 1. Description of total Warthon's Jelly-derived MSCs isolation method. The umbilical cords (UCs) were first washed in Hanks' balanced salt solution with antibiotics and then cut into 2-3cm pieces (a). To obtain MSCs from total Warthon's Jelly (tWJ) each cord piece was cut open lengthwise (b) and the inner part was faced down and subjected to an initial 1h enzymatic solution (c) composed of collagenase and hyaluronidase. After this digestion, the wharton's jelly tissue became softer and the removal of arteries and veins was allowed (d). The tWJ tissue was then cutted into small fragments (e) and submitted to 4 hours of enzymatic digestion (f). After the second digestion, the cell clumps were cultured in expansion medium.

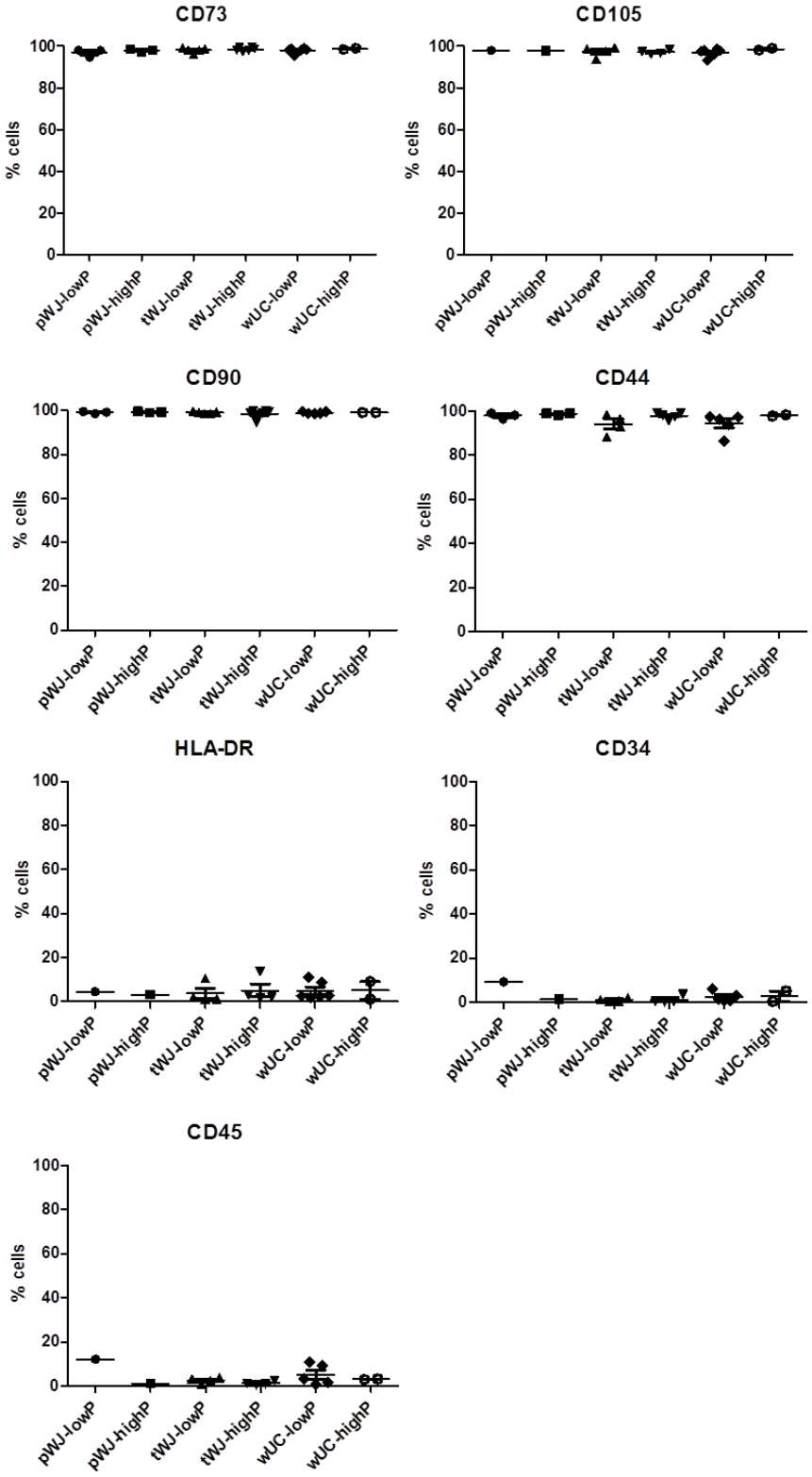
Supplementary Table 1. Description of isolation method for each umbilical cord tissue source and its efficiency.

UC nº	Tissue Source	Isolation Method	Isolation Efficiency
1	pWJ	Mechanical method: cut WJ around the vessels, and then chopped it into small pieces; resuspended the pieces in MSC/FBS medium and plated them straight after.	Isolated cells adhered to the plate but bigger pieces did not; bacterial contamination after 1st passage.
2	pWJ	Cut WJ around the vessels and then digested for 18h in 0.2, 0.8 or 2 mg/ml Collagenase XI (in DMEM:F12 medium without FBS); washed in PBS and plated cells in MSC/FBS medium.	Better efficiency for the 0.2 and 0.8 mg/ml collagenase. Some cells only adhered 3 to 5 days later.
3	pWJ	Washed in PBS with antibiotics; cut WJ around the vessels, then in small pieces and digested ≈20h in 0.5mg/ml Collagenase XI	Some adherent cells but still a significant percentage floating.
4	pWJ	Washed in PBS with antibiotics; cut WJ around the vessels, then in small pieces and digested ≈16h in 0.5mg/ml Collagenase XI; 30min Tryple digestion, washed and plated.	Good efficiency, a lot adherent cells on the day after.
5	tWJ	Cut UC into ~2-3 cm segments, then cut lengthwise and placed with inner part down in enzymatic solution (0.5mg/ml collagenase and 100U/ml hyaluronidase) for 1h; vessels were then removed and the remaining WJ digested for another hour in the same enzymatic solution; the digested tissue was washed in PBS and further digested in Tryple for 20min.	Still very big pieces that did not adhere; "contamination" with red blood cells; low efficiency.
6	pWJ	Cut WJ around the vessels, then into small pieces and digested ≈16h in 0.5 mg/ml collagenase and 100U/ml hyaluronidase; after digestion, the cells were passed through a cell strainer to remove undigested tissue before plating in MSC/FBS medium.	Good efficiency; high number of single cells and % of viable adherent cells.
7	pWJ	Cut WJ around the vessels, then into small pieces, and digest ≈19h in 0.5mg/ml Collagenase XI+50U/ml hyaluronidase; 20min Tryple digestion.	Good efficiency; high number of single, adherent cells.
8	tWJ	Open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h; strip off and discard vessels; cut remaining tissue in small pieces and digest for 2 more hours; undigested pieces were filtered out with a cell strainer before plating the cells in MSC/FBS medium.	Low efficiency; few single cells (not enough digestion) and only a % of them adhered.
9	pWJ	Cut WJ around the vessels, then into small pieces, and digested ≈17h in 0.5mg/ml Collagenase XI+100U/ml hyaluronidase; the day after, washed and plated cells in MSC/FBS medium.	Good efficiency; good tissue dissociation and high cell viability.
10	pWJ/tWJ	pWJ: Cut WJ around the vessels, then into small pieces, and digested ≈4h in 0.5mg/ml Collagenase XI+100U/ml hyaluronidase. tWJ: Open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h; strip off and discard vessels; cut remaining tissue in small pieces and digest for 4 more hours; undigested pieces from both tissues were filtered out with a cell strainer before plating the cells in MSC/FBS medium.	pWJ: low efficiency; few adherent cells and high variable cell morphology; low cell viability. tWJ: very low efficiency; few adherent cells and very low cell viability.

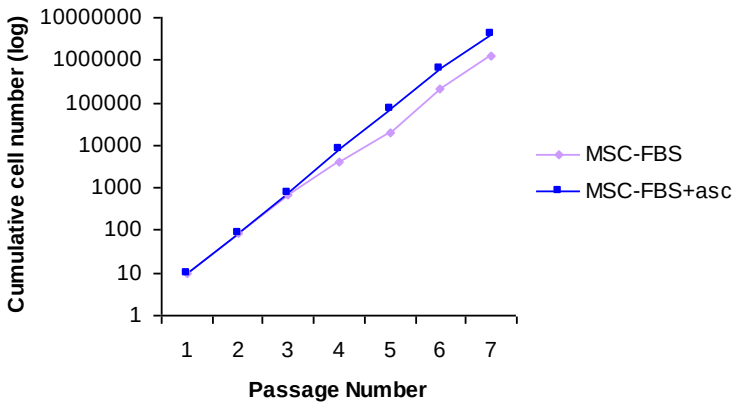
11	tWJ	Open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 45min; strip off and discard vessels; cut remaining tissue in small pieces and digest for 3 more hours; undigested pieces were filtered out with a cell strainer before plating the cells in MSC/FBS medium.	Good efficiency; good tissue dissociation and high cell viability.
12	pWJ /W/V	pWJ: cut WJ around the vessels, then into small pieces, and digested ≈4h in 0.5mg/ml Collagenase XI+100U/ml hyaluronidase. V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h; strip off vessels and the remaining tissue discard; Cut vessels tissue into small pieces and digest for 4 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	pWJ: low efficiency; few adherent cells and high variable cell morphology; low cell viability. W: very high efficiency; high number of single cells and % of viable adherent cells. V: good efficiency; high number of adherent cells clumps.
13	pWJ /W/V	pWJ: cut WJ around the vessels, then into small pieces, and digested ≈4h in 0.5mg/ml Collagenase XI+100U/ml hyaluronidase. V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h; strip off vessels and the remaining tissue discard; Cut vessels tissue into small pieces and digest for 4 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	pWJ: good efficiency; some adherent cells and high variable cell morphology. W: very high efficiency; high number of cells clumps and % of viable adherent cells. V: good efficiency; high number of adherent cells clumps.
14	pWJ /W/V	pWJ: cut WJ around the vessels, then into small pieces, and digested ≈4h in 0.5mg/ml Collagenase XI+100U/ml hyaluronidase. V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 45min; strip off vessels and the remaining tissue discard; Cut vessels tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	pWJ: low efficiency; bacterial contamination after few days in culture. W: good efficiency; Several cells clumps and good % of viable adherent cells. V: good efficiency; some adherent cells clumps.
15	pWJ /W/V	pWJ: cut WJ around the vessels, then into small pieces, and digested ≈4h in 0.5mg/ml Collagenase XI+100U/ml hyaluronidase. V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 45min; strip off vessels and the remaining tissue discard; Cut vessels tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	pWJ: good efficiency; some adherent cells. Low cell viability. W: very high efficiency; high number of single cells and % of viable adherent cells. Some “contamination” with red blood cells; V: good efficiency; high number of adherent cells clumps. Higly “contaminated” culture with red blood cells;
16	tWJ/W/V	tWJ and V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h; cut strip off vessels and the remaining	tWJ: low efficiency; few adherent cell and low cell viability. W: very high efficiency; high number of single

		tissue into small pieces and digest for 4 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	cells and % of viable adherent cells. V: good efficiency; high number of adherent cells clumps.
17	tWJ/W/V	tWJ and V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h15min; cut strip off vessels and the remaining tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 3 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	tWJ: low efficiency; few adherent cell and low cell viability. W: low efficiency; few % of viable adherent cells. V: Low efficiency; few number of adherent cells clumps.
18	pWJ/W	pWJ: cut WJ around the vessels, then into small pieces, and digested ≈3h in 0.5mg/ml Collagenase XI+100U/ml hyaluronidase. W: cut a cord piece with 2-3cm and digest for 3 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	pWJ: good efficiency; some adherent cells. Low cell viability. W: high efficiency; high number of single cells and % of viable adherent cells.
19	tWJ/W/V	tWJ and V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h15min; cut strip off vessels and the remaining tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	tWJ: low efficiency; few adherent cell and low cell viability. W: very high efficiency; high % of viable adherent cells. V: high efficiency; high number of adherent cells clumps. Some "contamination" with red blood cells in all cultures;
20	tWJ/W/V	tWJ and V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h30min; cut strip off vessels and the remaining tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	tWJ/ W/ V: good efficiency; some adherent cell and good cell viability. Some "contamination" with red blood cells in all cultures;
21	tWJ/W/V	tWJ and V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h15min; cut strip off vessels and the remaining tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	tWJ/ W/ V: Very high efficiency; a lot of adherent cell and very high cell viability.
22	tWJ/W/V	tWJ and V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h; cut strip off vessels and the remaining tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	tWJ/W: good efficiency; some number of cell clumps adherent. V: low efficiency; low number of adherent cells clumps.

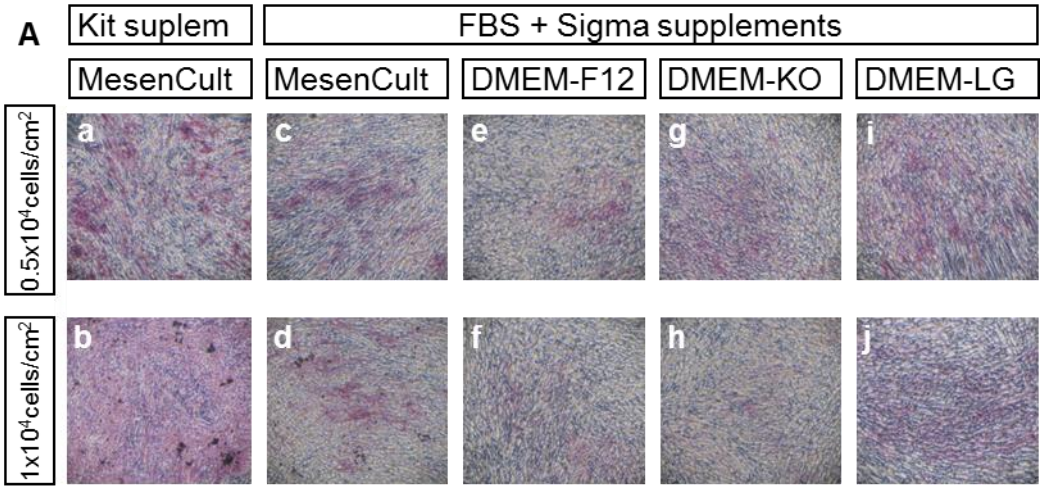
23	tWJ/W/V	tWJ and V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h; cut strip off vessels and the remaining tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS or StemPro serum free medium (only for tWJ and W).	tWJ: very low efficiency; very low adherent single cell in MSC/FBS medium. None adherent cells in SP medium. W: very high efficiency; high % of viable adherent cells. None adherent cells in SP medium. V: very low efficiency; very low number of adherent single cells. Some "contamination" with red blood cells in all cultures;
24	tWJ/W/V	tWJ and V: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h; cut strip off vessels and the remaining tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS or StemPro serum free medium (only for tWJ and W).	tWJ: low efficiency; bacterial contamination after few days in culture in MSC/FBS medium. None adherent cells in SP medium. W: Good efficiency; good % of viable adherent cells. Some adherent cells in SP medium. V: Good efficiency; good number of adherent cells clumps.
25	tWJ/W	tWJ: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h15min; discard strip off vessels and cut the remaining tissue into small pieces and digest for 1 more hours; W: cut a cord piece with 2-3cm and digest for 2 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS or StemPro serum free medium.	tWJ: low efficiency; big few adherent cell clumps in MSC/FBS medium. None adherent cells in SP medium. W: Good efficiency; good % of viable big adherent cells clumps. None adherent cells in SP medium.
26	tWJ/W	tWJ: open cord pieces, place with interior part down on a petri dish with enzymatic solution (0.5mg/ml collagenase+100U/ml hyaluronidase) for 1h15min; discard strip off vessels and cut the remaining tissue into small pieces and digest for 3 more hours; W: cut a cord piece with 2-3cm and digest for 4 hours. Single cells and undigested pieces from all tissues were directly plated in MSC/FBS medium.	tWJ/W: good efficiency; good % number of adherent single cell.



Supplementary Figure 2. MSC markers for different tissue source expanded in standard MSC-FBS medium in high and low passages. Graphical representation of the percentage of positive MSCs characteristic markers from different tissue source in high and low passages. High levels of CD44, CD73, CD90 and CD105 antigens were maintained in high and low passages regardless of MSCs tissue source. Constant low levels of CD34, CD45, HLA-DR were observed along MSCs passages from all tissue sources. Identical shaped symbols represent results from the same UC donor.



Supplementary Figure 3. Characterisation of MSCs cultured in MSC/FBS medium supplemented with ascorbic acid. A) Growth curves representing the cumulative cell expansion rates of MSCs cultured in FBS-containing medium with (MSC-FBS+asc –violet line) or without ascorbic acid (MSC-FBS –blue line). Total number of cells at each passage was calculated as the ratio of the total number of harvested cells to the total number of cells plated, multiplied by the total number of cells from the previous passage. MSC show a similar growth pattern in both types of medium for up to 8 passages. The results represent the cumulative expansion rates (mean ± standard deviation) of 3 different MSC cell lines plated at a density of 1 x 10^4cells /cm^2.



Supplementary Figure 4. Analysis of MSC osteogenic differentiation in response to different osteogenic inductive conditions. Representative phase contrast images of cells cultured in MesenCult osteogenic stimulatory kit (a-b) and MesenCult (c-d), DMEM-F12 (e-f), DMEM-KO (g-h) or DMEM-LG (i-j) basal media supplemented with 100 nM dex, 0.2 asc and 10 mM β-GP (from Sigma). Cultured cells were seeded at 2 different densities: $0.5x10^4$ (a,c,e,g,i) and $1x10^4 cells/cm^2$ (b,d,f,h,j). Osteogenic differentiation of MSCs was assessed by staining for Alkaline Phosphatase (AP) activity after 14 days in differentiation medium. Higher levels of AP activity were obtained from the cell at the density $1x10^4 cells/cm^2$ in MesenCult osteogenic stimulatory kit medium Images magnification: 400x.