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“Amniotic fluid stem cells from second and third trimester, comparison and potency for regenerative medicine”

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RIASSUNTO

Le cellule staminali del liquido amniotico (AFSCs) possono essere isolate dal liquido amniotico in seguito ad amniocentesi cui le donne incinte si sottopongono durante il secondo trimestre della gravidanza, questa popolazione è già stata caratterizzata e condivide proprietà comuni fra le cellule staminali embrionali e le cellule staminali adulte mesenchimali. Le AFSCs possono essere identificate tramite specifici marcatori; in questo studio – com'è già stato pubblicato – si è puntata l'attenzione sulla frazione positiva per CD117 (c-Kit). Queste cellule sono di grande interesse ai fini della medicina rigenerativa, considerando il loro potenziale di differenziamento e la relativa costante disponibilità, inoltre il recupero di cellule prenatali di origine autologa offre la possibilità di nuove strategie per curare i neonati con malformazioni congenite o altre malattie.

In questo studio si è voluto esplorare la possibilità di isolare le AFSCs al termine della gravidanza (quindi al terzo trimestre, recuperando il liquido amniotico durante il parto). Sono stati investigati i fenotipi e le potenzialità differenziative di entrambi i trimestri. Queste cellule hanno anche un grande potenziale poiché possono essere mantenute in cultura e il loro numero significativamente aumentato per successive applicazioni.

L'ipossia è un ben conosciuto fattore che influenza la coltura delle cellule staminali; coltivare le cellule in basse tensioni di ossigeno è già stato dimostrato avere un effetto benefico su altre cellule staminali, è stato quindi deciso di provare quest'approccio per migliorare l'espansione delle AFSCs.

Sono stati effettuati esperimenti *in vivo* per verificare il potenziale angiogenico delle AFSCs.

ABSTRACT

Amniotic Fluid Stem Cells (AFSCs) can be isolated from the amniotic fluid after amniocentesis that pregnant women undergo during the second trimester of pregnancy, this population has already been characterised and share common features between embryonic stem cells and adult mesenchymal stem cells. AFSCs are identified by specific markers; this study – as already published – focuses on the CD117 (c-Kit) positive fraction of AFSCs. Those cells are of great interest for regenerative medicine purposes considering their potential of differentiation and the relative constant availability, moreover retrieving prenatal autologous cells can offer new strategies for new-borns with congenital malformations or diseases.

This study was intended to explore the possibility of isolating AFSCs at term of pregnancy (so at the third trimester, retrieving the amniotic fluid during delivery). Comparison on phenotype and test of potential for different lineages differentiation has been investigated on cells from both trimesters. These cells have a great potential also because they can be kept in culture and their number significantly increased for successive applications.

Hypoxia is also a well-known factor that influence culture of stem cells, cultivating cells at lower oxygen tension has already demonstrated various beneficial effects on other stem cells so it has been decided to try this approach to ameliorate the expansion of AFSCs.

In vivo experiments were performed to verify *in vitro* results on angiogenic potential of AFSCs.

INTRODUCTION

The main goal of regenerative medicine is to restore a normal functionality and structure in a tissue after an injury, a pathologic debilitation/condition or a congenital malformation.

In the human body tissues are continuously maintained in a homeostatic state by self-reparative processes that contribute to the growth and subsequently to the lifetime of an adult person. This regenerative capacity decline with the lifespan and eventually is poorly able to sustain its role in elder people, pathological conditions can also diminish or inhibit this property. Obviously in presence of a genetic disease the cells that are going to substitute the “malfunctioning” ones will have the same impairment and so the defect is not replaced but maintained (and at some point this futile regeneration will stop for lack of cells). Also in case of congenital malformations usually the body is not able to repair the compromised state occurred in the development.

The “R³” paradigm

Therapeutically speaking these concepts can be unified under the “R³” paradigm that state a balance of three repair strategies: rejuvenation, replacement and regeneration (Nelson *et al.*, 2008) (Figure 1).

- **Rejuvenation:** it's the most basic process in which self-renew occur; resident tissue specific precursors cells take the place of naturally compromised or senescent cells in the tissue, this allows to maintain a fully functional organism able to be make front to the stress and tolerate a normal survival. This process allows us to recover after an injury but with limitations due to tissue-specific progenitors capacity. Acting on this mechanism will boost the response of an organism to repair itself with its own precursor cells: like using pharmacological treatments to induce resident dormant cells to reactivate and contribute to healing processes (Surani and McLaren, 2006). This strategy is somehow limited, for example it is unlikely to envisage this kind of strategy to restore heart muscle after a critical event like a myocardial infarction.
- **Replacement:** maybe this is the most familiar strategy know, the most common example is transplantation of an organ to replace one that failed its role; obviously this most familiar strategy has also a series of problematic like the low availability of “replacement parts” and the difficulty to match a donor-host transplant without risk of rejection. In this field cell-based replacement is a promising strategy, the aim is to recreate a transplantable

tissue(s) using precursors from a donor or the patient itself (avoiding rejection risks) and manufacture *in vitro* a new transplantable organ or a part of it.

- **Regeneration:** a step forward in respect of replacement strategies, this refers to the engraftment of progenitor cells able to grow and differentiate directly *in vivo* and restore the damaged tissue. An extraordinary example of this approach are haematopoietic stem cell transplantations, in this procedure some precursors derived from a donor can be injected inside a host and there they will repopulate the bone marrow niche and restore the ability to generate all cells in the haematopoietic system. The main problem of this strategy is to find a specific cell population able not only to restore damaged tissue but also to restore a functional reserve of precursors that will remain in the regenerated site.

All these point have greatly benefitted from the discoveries made in the field of stem cell research. It is clear how the two are strictly linked, providing information on the identity and the mechanism of differentiation about cells compatible for clinical approaches.

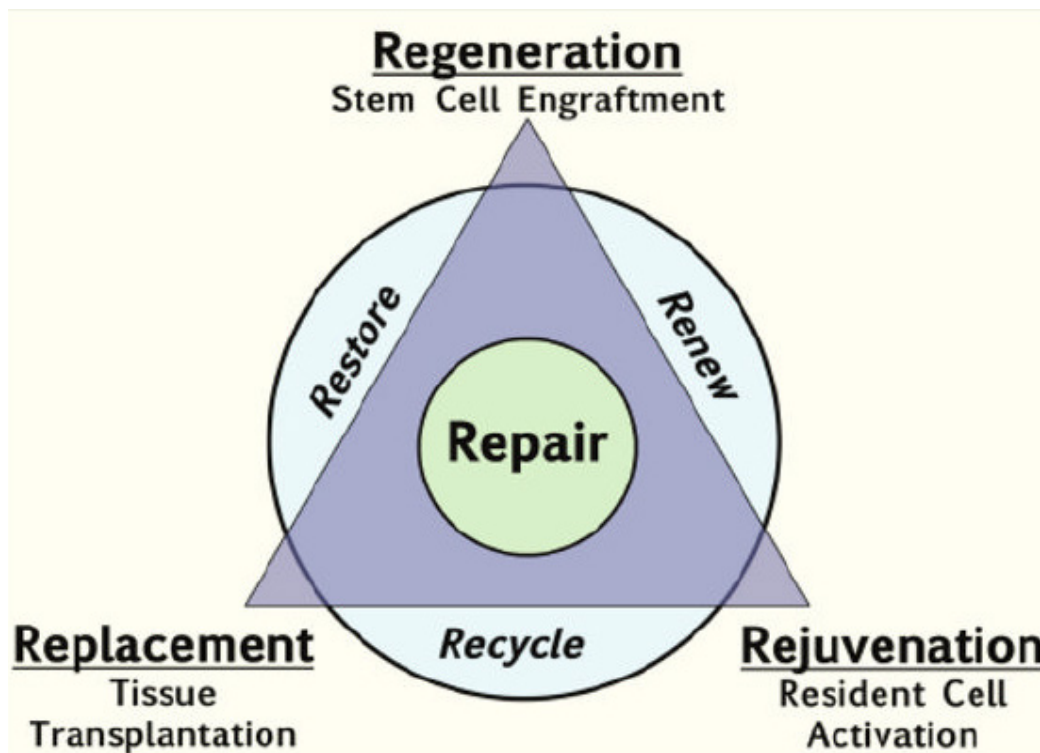


Figure 1 an exemplificative scheme of the R³ paradigm (Nelson *et al.*, 2008)

Stem cell types and sources

A stem cell is characterised by simply two criteria: ability to divide maintaining this property of duplication throughout generations and capacity to differentiate into one or more specialised cell types.

The first property is called “self-renewal” and could be theorised and observed already two centuries ago when the concept of “cell” as the basic unit of life was determined. Notably stem cells maintain the pool of undifferentiated cells after each division, two types of cell division are observed: asymmetric and symmetric (Figure 2 B). Asymmetric division produce two different daughter cells from a father stem cell, one will be equal to the original and the other will become a differentiated cell losing the stem cell criteria. Symmetric division (or stochastic differentiation) implies that one father stem cell produce two identical daughter cell that are differentiated, at the same time, to maintain the original pool of undifferentiated cells, another stem cell in the tissue will undergo a symmetric division producing two daughter stem cells (Magnus *et al.*, 2008).

The second property is called “potency” and was classified in different manners during the years; nowadays a common classification is based on how broadly a single cell is able to differentiate (Figure 2 A):

- Totipotent: cells able to form all embryonic and extraembryonic types of cell; when a sperm fertilises an egg a zygote is formed, this is the first totipotent cell and maintain its characteristics for the first few divisions (until the 16-cell stage of the morula).
- Pluripotent: these cells can differentiate into all the three germ layers (endoderm, mesoderm and ectoderm) and give rise to all types of foetal and adult cells; however they cannot form a developing embryo because they lose the ability to form extraembryonic tissue such as the placenta.
- Multipotent: stem cells able to differentiate into a limited (yet various) family of cells, meaning they cannot originate cells from all the three germ layers (*e.g.* human foetal liver multipotent progenitor cell able to differentiate as liver and as fat, cartilage, bone, and endothelial cells (Dan *et al.*, 2006)).
- Oligopotent: is the characteristic of cells sometimes called “progenitor cells”, they have the ability to differentiate into few cell types, an example of progenitor are Vascular Stem Cells that are able to become endothelial but also smooth muscle cells (Klein *et al.*, 2011). The main difference of progenitor cells compared to stem cells is that the first can divide only a limited number of times, for that reason there is still controversy about calling them real stem cells.
- Unipotent: those cells are also called “precursor cells”, they have lost almost all their potency, they retain the self-renew ability but they can differentiate in only one cellular type.

As already told, the main problem is to find the best cellular source for regenerative medicine aims, based on: grade of potency in regard of the purposes, availability from donors and easiness of isolation methods.

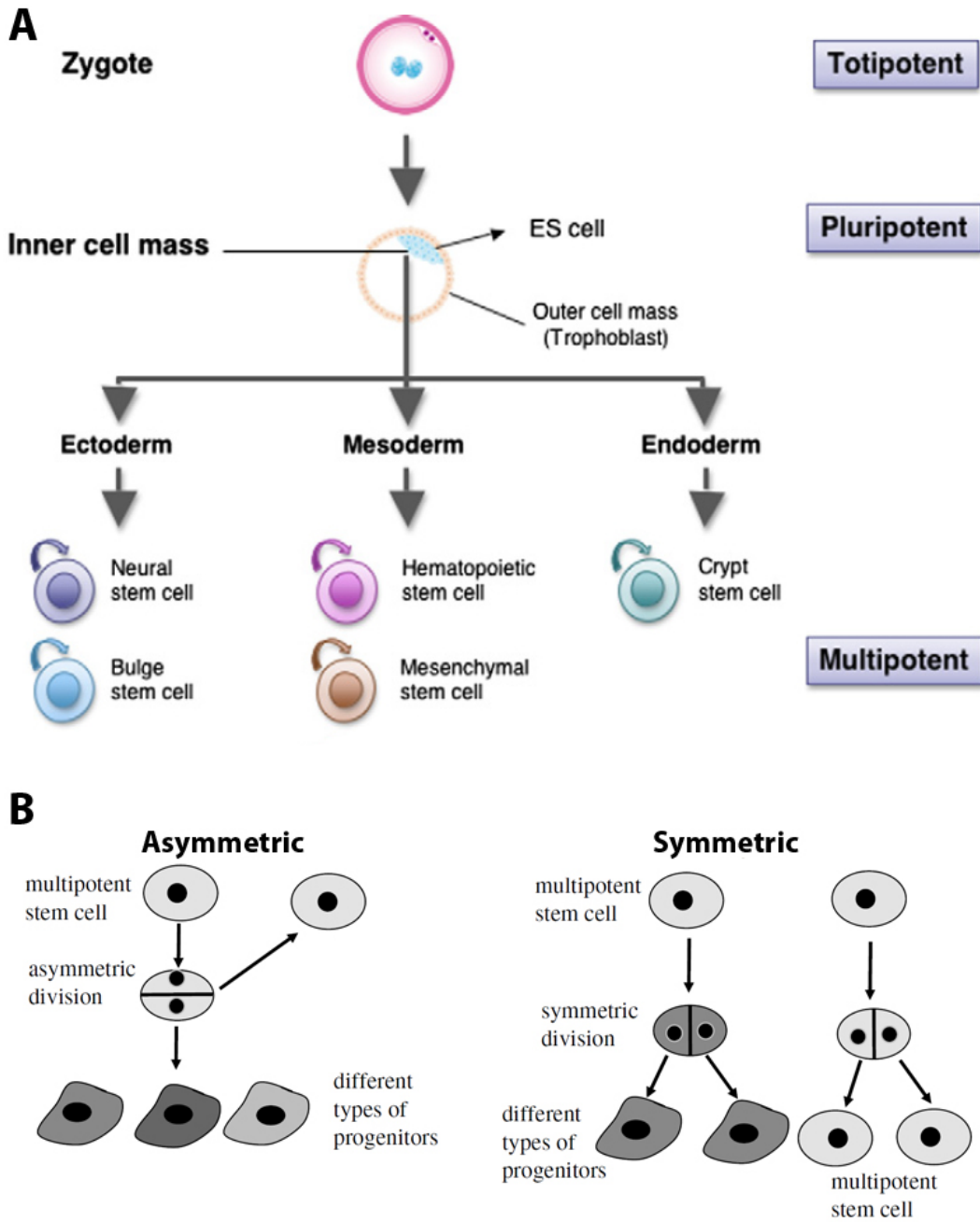


Figure 2 A: potency degree of stem cells. B: the two main type of division to guarantee the stem cell pool survival adapted from (Magnus *et al.*, 2008; Kyo *et al.*, 2011).

Embryonic stem cells

Stem cells can be obtained from a host at different times in his life, starting from the very moment of conception and ending at the end of life itself. Potency is greatly influenced by development and so more potent stem cells are found only in early stages of embryonic divisions.

Embryonic Stem (ES) cells comprise totipotent stem cells if they derive from the whole fertilised egg before the formation of the blastocyst or pluripotent stem cells if they came from Cells of the Inner Mass (IMC). In both cases they are normally excluded to be considered a good source of cells: using those cells means destroy a zygote, which raises a series of ethical questions.

In 1981 the first embryonic stem cell line were derived from inner cell masses of late blastocysts (Evans and Kaufman, 1981; Martin, 1981) and after that for almost twenty years scientist tried to obtain a human ES cell line.

In 1998 Thomson and colleague were able to do that, obtaining five ES cell lines starting from a high number of fresh or frozen human embryo produced but not used during *in vitro* fertilisation procedures (Thomson, 1998).

In 2006 there was then the discovery of a technique that permits to derive ES cells from single blastomers without harming the embryo development (Klimanskaya *et al.*, 2006).

Even with those great steps the process for obtaining ES cells is still laborious, time demanding, with a low yielding and mostly it is limited by the low oocytes availability.

Somatic Cell Nuclear Transfer

The nucleus of an unfertilised oocyte can be taken out and replaced with another from a different cell, this is called Somatic Cell Nuclear Transfer (SCNT). These experiments initiated originally with nuclei from *Xenopus* extracted from embryonic cells and then implanted into a new egg, leading to a new developing embryo (Briggs and King, 1952; Gurdon *et al.*, 1958). Many years later it was also demonstrated that not only embryonic cells but also adult and so terminally differentiated cells permits this process to obtain a new frog larvae (Gurdon, 2006; Pasque *et al.*, 2011). The proof of concept about these experiments is that somehow the oocyte cytoplasmic environment is able to reprogram the somatic nucleus to a pluripotent state and erase all the epigenetic imprinting acquired during development. These embryos if implanted will eventually leads to a clone of an already existing creature – the donor of the nucleus – as widely known from the experiments on sheep resulting in “Dolly”, the first cloned being produced from an adult mammal cell (Campbell *et al.*, 1996).

Shortly after the discovery of SCNT from Gurdon and colleagues, in 2000 for the first time mouse ES cells were isolated from a cloned blastocyst after nuclear transfer (Munsie *et al.*, 2000). This was aimed to establish a procedure to obtain ES cells and then transfer the technique to possible human applications in order to obtain pluripotent patient-specific replacement cells. Unfortunately experiments on primates revealed that SCNT is really hard to achieve, early experiments showed a blockage to early-cleavage stage on monkey embryos (Pan *et al.*, 2012). After improvements in the technique, two ES cell lines were isolated from over 300 starting oocytes, displaying ES morphology, pluripotency markers and teratoma formation capability when injected *in vivo* (Byrne *et al.*, 2007), this was the first result confirming that cloning in human might be possible.

As already discussed for primates, also human SCNT presented a lot of technical challenges, probably the manipulation of the oocyte during the nuclear transfer impair the factors inside the cytoplasm and leads to an early arrest of blastocyst divisions (Chung *et al.*, 2009b). Up to now the only successful result in obtaining ES cells from SCNT in human was achieved by Noggle and colleagues: they introduced the nucleus of a somatic cell inside an egg without taking first out the oocyte's nucleus (Noggle *et al.*, 2011). This leads to a triploid set of chromosome not suitable for perspective applications in human medicine; also, without detailed analysis of gene profiling and epigenetic signature remodelling, these results are to take with proper caution.

Even with those discoveries SCNT as a source for ES cells for regenerative medicine is not a very good choice if someone consider the efficiency of the process (the huge numbers of starting oocytes required to obtain few pluripotent cells) moreover this technique has all a series of ethical concerns.

Adult cells

Induced Pluripotent Stem cells

Avoiding all kinds of ethical issues and starting from an easily accessible source of cells, in 2006 Takahashi and Yamanaka first developed a technique for obtaining ES-like cells from mouse somatic cells without the needs of an oocyte. Inspired by the work on SCNT they hypothesised that some reprogramming factors specific of ES cell could substitute the unique environment of the oocyte and thus reprogram a somatic cell without the need of SCNT. A series of transcription factors were transduced, via retroviral infection, into mouse fibroblasts and then culturing them under ES cell conditions (over Mouse Embryonic Fibroblasts (MEF) feeder-layer and supplementing the culture with Leukaemia Inhibitory

Factor (LIF, a molecule responsible for cell growth and inhibition of differentiation)) resulted in the formation of colonies with pluripotency characteristics. Subsequently, after an exclusion process, four transcription factors (*i.e.* Oct3/4, Sox2, c-Myc, and Klf4) were found to be sufficient in inducing this transformation. The obtained cells (called iPS (induced Pluripotent Stem) cells) show a morphology, growth and markers expression typical of ES cells, they are also able to form teratoma when injected in vivo (formation of tissue from all the three germ layers) (Klimanskaya *et al.*, 2006) and if injected into a dividing blastocyst they generate a chimera with the blastomeres and contribute to the formation of the embryo (Wernig *et al.*, 2007).

These results were subsequently obtained also with human fibroblasts by transfecting them with the homologous transcription factors of mice (Takahashi *et al.*, 2007).

The first striking thing about this approach is the origin of material, potentially almost each cell of the organism can be reprogrammed into an iPS cell, this means obtaining samples with non-invasive procedures from donors (e.g. a simple skin biopsy but someone would also go further and say that starting cells could be obtained even from a cheek swab (Cyranoski, 2007)). The second thing is that with this method one can generate replacing parts for each cell type of the body, regardless of the original material.

Even with those promising perspectives there are some issues related to iPS cells generation. First of all there is the problem of the reprogramming, the four reprogramming factors constructs are usually delivered with retrovirus or lentivirus (Park *et al.*, 2008), this “integrational” strategies cause the insertion of the constructs inside the genome of the original cell so without the possibility of take out the factors once they are integrated. Some strategies using inducible promoters (*e.g.* doxycycline controlled expression (Carey *et al.*, 2009)) or excisable transcription factors (using constructs flanked with LoxP regions that allows the excision by CRE recombinase enzyme (Sommer *et al.*, 2010)) have been developed but even with controllable expression of the exogenous factors there is one problem that remain bounded to this integrational strategy and this is “insertional mutagenesis”: this phenomenon is the result of random events where the constructs integrate inside the genome disrupting the function of important genes implied in cell maintenance, also there is the possibility that insertion occurs in sites of some endogenous oncogenes.

Numerous strategies are been investigated to find a method with no DNA integration but so far there has been a lot of results with “low probability” of genomic integration and the only few “integration free” methods have very low efficiency or require too much complicated culture techniques (see Table 1).

INTRODUCTION

DELIVERY METHOD	CHARACTERISTICS	GENOMIC INTEGRATION	HIGH EFFICIENCY	TECHNICAL ISSUES
Retrovirus	Limited to dividing cells, possibility of viral transgene reactivation.	+	+/-	+
Lentivirus	Inducible transgene expression, excision possible.	+	+	-
ΦC31 phage Integrase	Site-specific DNA integration	+	+	+
Transposon	Temporary integration.	(+)	+	+/-
Sendai Virus	No integration but complex method.	-	+/-	+
Minicircle vector	Low efficiency.	+/-	-	+
Plasmid	Very low possibility of genomic integration but also very low efficiency.	(-)	--	-
Synthetic mRNA	Require continuous transfection for up to 2 weeks.	-	+	++
Protein	Recombinant proteins of the four factors, very low efficiency.	-	--	+

Table 1. Adapted from (Hussein and Nagy, 2012; Mochiduki and Okita, 2012).

Whether a full conversion to a pluripotent state or not is achieved with one of these methods, there are some issues related to the generation of iPS cells. One should consider that, starting from a somatic cell, there is the possibility of “bring back” mutations developed by the adult cell during its development. Another aspect to be considered in the iPS cells generation process is that of epigenetic: even if an adult cell does not present mutations that could alter its fate during reprogramming, there are certain boundaries by which the pluripotency is not fully achieved. After differentiation into a specific cell lineage, some genome loci are not more accessible and became silenced, so if a cell is brought back to the pluripotent state, and then induced to differentiate towards another lineage, it is not clear to which extent this process is effective and what are the limits of generating other cell types with different fate pathway of than the original cell history. A lot of controversies are present nowadays among scientists about genetic imprinting and capability of iPS cells (Figure 3).



Figure 3 resuming studies on comparison between iPS and ES cells (Sindhu *et al.*, 2012).

Interestingly SCNT is able to produce cells that from the transcriptional point of view are indistinguishable from ES cells (Brambrink *et al.*, 2006), also the DNA analysis of methylation sites reveals a high similarity profile, iPS cells instead show an epigenetic memory of the originating cell (Kim *et al.*, 2010) and different gene expression signatures (Chin *et al.*, 2009), rising questions on their application for regenerative medicine purposes.

Direct Reprogramming

Another important point in cell reprogramming is about “direct reprogramming”. Even before the discovery of the four genes to revert cells to a pluripotent state, it was observed that it is possible to convert one cell type into another without passing through a pluripotent state. Taylor and Jones first demonstrated that fibroblasts exposed in culture to a hypomethylating agent (called 5-azacytidine) spontaneously differentiate into adipocytes and myocytes (Taylor and Jones, 1979). Then another important step was the finding that expressing the basic helix-loop-helix transcription factor MyoD inside fibroblasts will induce them to transform into contracting myocytes (Davis *et al.*, 1987). Notably a full conversion could not be achieved if MyoD was expressed inside cells originating from a different germ layer (Weintraub *et al.*, 1989). It was just until a couple of years ago that nobody was able to convert adult cells to other cell types if not to those closely related to the originals; in 2010 it was demonstrated that fibroblast (mesoderm origin) expressing three transcription factors (Brn2, Ascl1, and Myt1l) could be converted into functional neural cells

(ectoderm origin) (Vierbuchen and Wernig, 2012); from that moment on direct reprogramming was achieved by expressing different transcriptional factors and generating new cell types from not related somatic cells of origin (Figure 4).

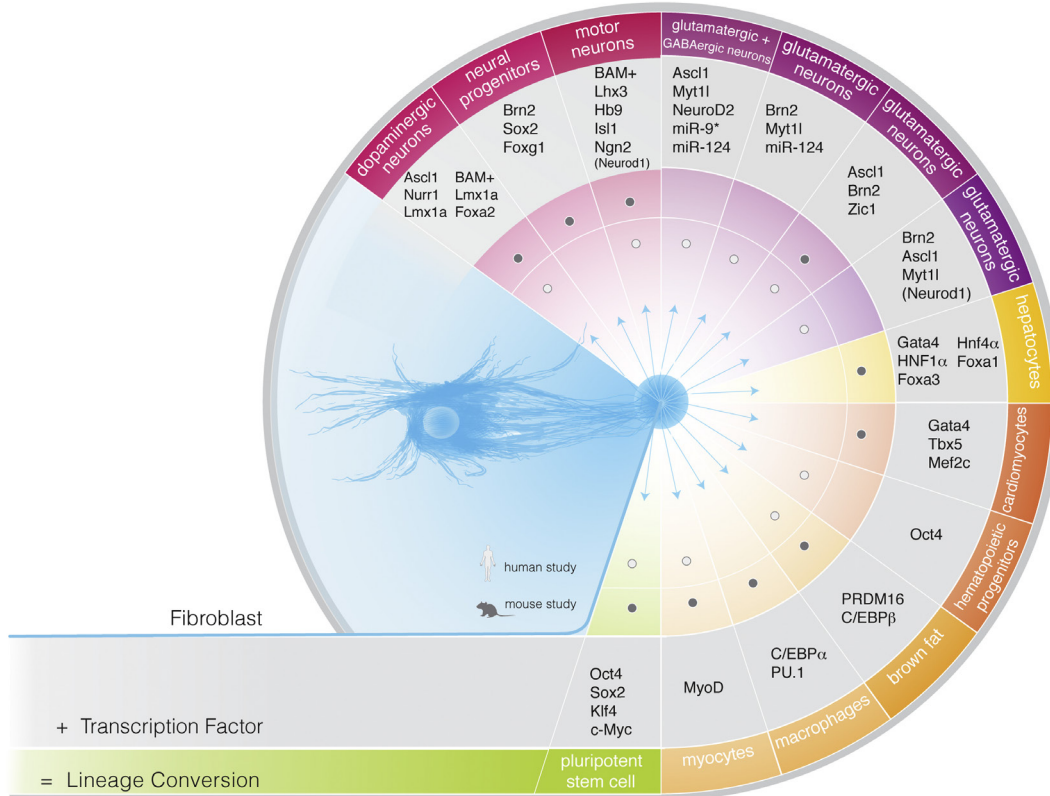


Figure 4 Direct reprogramming successful factors and specific lineage conversion (Vierbuchen and Wernig, 2012).

Mesenchymal stem cells

Cells present in the bone marrow (BM) are mainly divided in two categories: Haematopoietic Stem Cells (HSC) and stromal supportive cells; where the first type has a clear identification (stem cells capable of originating all the cells in the haematopoietic system) the second group are quite heterogeneously described. Pioneer experiments in the 19th century showed that BM transplantation in different anatomical regions generated *de novo* ectopic bone and marrow (Goujon, 1869). Almost a century later Tavassoli and Crosby demonstrated the presence of osteogenic progenitors inside BM (Tavassoli and Crosby, 1968) but these progenitors were fully identified as BM and not bone derived only in 1974 by Friedenstein and co-workers; they described those cells as a “non-haematopoietic” source of cells inside the stromal portion of the marrow capable of clonogenic activity in an *in vitro* assay called Colony-Forming Unit Fibroblast (CFU-F) (Friedenstein *et al.*, 1974). They called these “BM stromal cells” in light of later demonstration that in the bone they function as a supportive tissue for HSC, later they also demonstrated that non only bone but

also cartilage, adipose and fibrous tissue were generated by those cells when injected *in vivo* (Owen and Friedenstein, 1988).

A new search begun to find multipotent stem cells resident in adult tissues containing stroma with function of mechanical and biological support. Soon enough BM stromal cells were found and isolated into a variety of other tissues like: adipose tissue, periosteum, synovial membrane, muscle, dermis, pericytes, blood, and trabecular bone (Tuan *et al.*, 2003).

These was demonstrated to be true not only in adult tissues but also in birth-associated tissues like: placenta, amnion, umbilical cord, cord blood, and Wharton's jelly (Hass *et al.*, 2011).

It was clear that calling those cells "BM stromal cells" was no more adequate, so in 1991 Caplan used for the first time the term "Mesenchymal Stem Cell" (MSC).

Both definitions are quite misunderstandable in spite of different aspects (Porcellini, 2009):

- Mesenchyme is an embryonic tissue of mesodermal origin that gives rise to haematopoietic cells and connective tissues: as already told, Mesenchymal Stem Cells (MSCs) "do not" differentiate into haematopoietic lineages.
- Stromal cells are present in, and form, the connective tissue in which the functional cells carry out their role: it is quite evident that MSC also contribute to tissue functions, have an active role in tissue repair and maintenance.
- MSC, even if derived not only from BM and demonstrated to have multipotent abilities, lack the capacity to reconstitute an entire organ: in light of that, someone suggested to change the meaning of the acronym to "Multipotent Stromal Cell".

After the fine characterisation of Friedenstein and colleagues about the ability of MSCs to differentiate into bone, cartilage and adipose tissue, other works demonstrated an even more broad potential of those cells.

MSCs derived from different tissues displayed myogenic properties: they were able to differentiate into skeletal muscle cells (Ferrari *et al.*, 1998), smooth muscle cells (Lee *et al.*, 2006; Rodriguez *et al.*, 2006) and also cardiac muscle cells (Planat-Benard *et al.*, 2004).

Differentiation into endothelial cells (Wosnitza *et al.*, 2007; Heydarkhan-Hagvall *et al.*, 2008) and hepatocyte-like cells (Seo *et al.*, 2005) was also obtained with MSCs.

More interestingly it was later demonstrated that MSCs have the capacity to differentiate *in vitro* towards neuron-like cells, specifically in dopaminergic neuron-like cells (Pacary *et al.*, 2006; Scuteri *et al.*, 2011); more importantly they

are also able to differentiate into mature astrocytes and neurons when implanted in the brain of neonatal mouse (Kopen *et al.*, 1999).

This major finding demonstrated that MSCs have the capability to go beyond the limit of proper multipotent stem cells: they are able to differentiate into tissue of germ layers other than mesoderm.

After demonstrating the neuroectoderm potency of MSCs, soon came the demonstration that these cells can be induced to differentiate into insulin-producing cells, making them a notable candidate for diabetes therapies (Limbirt and Seufert, 2009).

This intrinsic plasticity of MSCs is somehow heterogeneous, these multipotent cells isolated from different anatomical sources show disparity on the potential to differentiate in some lineage, a well-known example are MSCs isolated from the Umbilical Cord Blood (UCB-MSCs) that seem unable to give rise to advanced adipogenesis when cultured in specific media (see Figure 5) (Lee *et al.*, 2012).

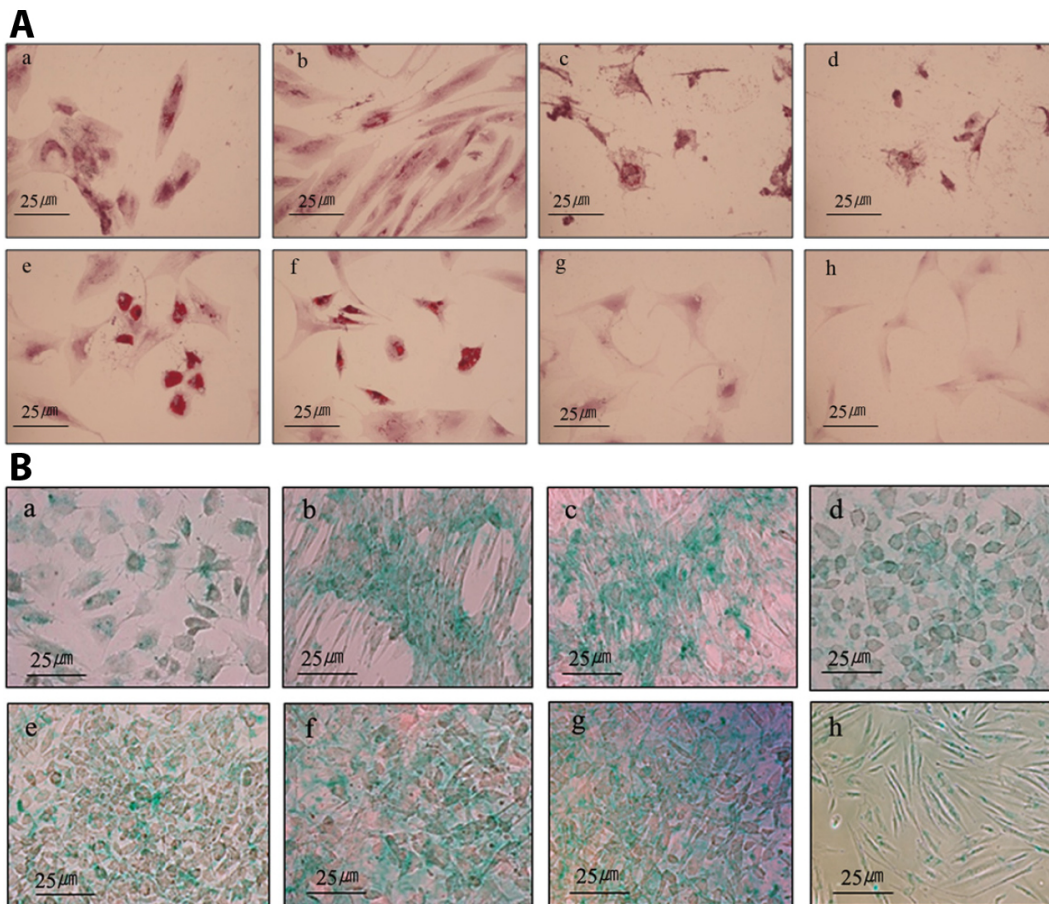
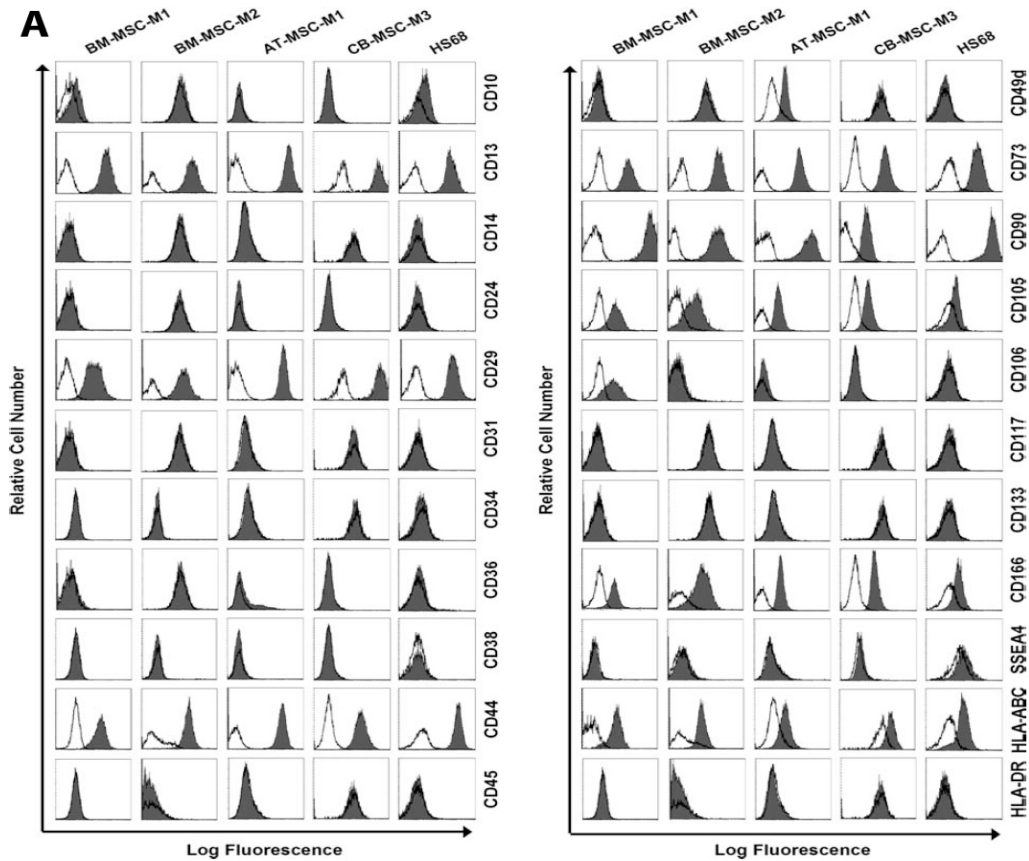


Figure 5 MSC comparison of different potential. A: adipogenic differentiation as shown by Oil Red O pigment (red) uptake. B: hepatic differentiation as shown by indocyanine green (light green) uptake. a to h: in order there are amnion MSCs, chorionic villi MSCs, chorionic villi MSCs, Wharton's jelly MSCs, adipose derived MSCs, bone marrow MSCs, umbilical cord MSCs and control fibroblasts (Lee *et al.*, 2012).

Surface marker identification by flow cytometry reveals a consistent phenotype among MSCs from different tissues, even if some slight variation in terms of percentage of positivity is observed and some specific markers are expressed only in one cell type. For example CD49d is found expressed in Adipose-Tissue-derived MSC (AT-MSCs) compared to BM-MSCs (where instead CD106 is expressed, see Figure 6).



Antibody	BM (%) (n = 9)	UCB (%) (n = 10)	AT (%) (n = 9)
CD44	97.5 ± 5.1	99.7 ± 0.5	99.8 ± 0.2
CD73	90.0 ± 20.0	99.3 ± 1.3	99.6 ± 0.5
CD90	99.1 ± 2.5	97.8 ± 7.1	99.9 ± 0.2
CD14	1.2 ± 1.1	0.8 ± 0.9	2.4 ± 5.0
CD34	1.6 ± 1.4	1.2 ± 1.5	5.0 ± 5.1
CD45	5.2 ± 3.7	3.8 ± 3.6	3.8 ± 5.1
CD105 ^a	88.1 ± 7.4	72.4 ± 20.0	90.4 ± 5.9
CD133	1.3 ± 1.1	2.9 ± 5.5	2.9 ± 3.5
CD29	99.0 ± 1.5	99.8 ± 0.4	99.5 ± 1.3
HLA I	95.2 ± 6.0	94.3 ± 6.8	98.8 ± 2.8
CD106 ^b	66.3 ± 22.7	70.0 ± 23.6	30.3 ± 18.6
HLA II	4.2 ± 6.1	0.8 ± 1.2	4.4 ± 6.2
CD144	4.5 ± 9.3	2.4 ± 4.0	2.4 ± 2.5

The table shows mean values of the percentage of positive cells ± standard deviation to the total number of cells analyzed.
^aSignificant differences were observed between UCB compared with BM and AT ($p < .05$).
^bSignificant differences were observed between AT compared with BM and UCB ($p < .05$).
 Abbreviations: AT, adipose tissue; BM, bone marrow; UCB, umbilical cord blood.

Figure 6 A: flow cytometry graphs showing expression of markers on various MSCs and HS68 fibroblasts (Wagner *et al.*, 2005). B: table showing percentages of flow cytometric analysis (Kern *et al.*, 2006).

Another point is related to the ability to isolate MSCs starting from different tissues, for example the ability to form CFU-F described by Friedenstein and co-workers can be used to evaluate the score of MSCs obtainable; using this type of comparison BM-MSCs and AT-MSCs show similar results whereas UCB-MSCs seems more difficult to obtain.

Following the continuous search for new sources of MSCs in new tissues – more amenable to obtain, and maybe more powerful in term of potency – the International Society for Cellular Therapy (ISCT) defined some rules that should be followed to identify new types of MSCs (Dominici *et al.*, 2006).

- First of all the cells should be able to adhere on the plastic surface when cultured in standard conditions.
- They should possess a specific phenotype characterised by flow cytometry.
 - expression of CD105, CD73 and CD90 (with at least 95% of positive cells)
 - no staining for CD45, CD34, CD14, CD11b, CD79a, CD19 or Human Leukocyte Antigen (HLA) class II (tolerated values < 2%).
- Cells have to be able to generate *in vitro* osteoblasts, adipocytes and chondroblasts (this should be demonstrated by specific stainings).

Another interesting and really appealing aspect of MSCs for regenerative medicine purposes is about their immunoprivileged as well as immunosuppressive properties.

MSCs display a peculiar phenotype in terms of molecules on the cell membrane. First of all they have low levels of MHC class I antigens (almost no HLA type A, B or C detectable) and a low presence/activity of the internal proteasome-related antigen processing machinery (Le Blanc *et al.*, 2003). As already told they also lack the presence of HLA class II molecules, components of the Major histocompatibility complex (MHC) class II, a typical molecule found on Antigen Presenting Cells (APCs). They also lack the FAS Ligand (CD95L), CD40 or CD40L (implied in recognition on APCs by CD4⁺ helper T cells), B7-1 and B7-2 (also called CD80 and CD86) that are known as co-stimulators for T cell activation (so if MSCs lack these molecules the only way to be exposed to CD8⁺ cytotoxic T cells is via signalling by helper T cells or other cytokines) (Deans and Moseley, 2000).

On the other hand MSCs express a variety of adhesion molecules and are able to migrate into injured tissues after adhering to endothelial cells (Chamberlain *et al.*, 2007), this migration can be caused also if MSCs are stimulated by ligands of Toll Like Receptor (TLRs) that these cells express on their membrane and it has been demonstrated that these TLRs are implicated in proliferation, differentiation, migration, survival, and immunosuppressive abilities of MSCs.

The immunomodulation effect exerted by MSCs is a relatively new field in which it is now becoming clear how these cells interact with the immune system.

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As already told, TLRs expressed on the surface acts in MSCs behaviour, it has been suggested that this is the start of a downstream signalling that ultimately results in release of soluble factors like indoleamine 2,3 dioxygenase (IDO), prostaglandin E2 (PGE-2) or nitric oxide (NO), all demonstrated to have an effect in the immune system (see Figure 7) (DelaRosa and Lombardo, 2010).

Soluble factors	Function
IDO	Inhibition of proliferation through reduction of tryptophan
NO	Inhibition of cell activation
HGF	Inhibition of proliferation, cytotoxicity
sHLA-G TGF-β	Inhibition of proliferation, cytotoxicity, promotion of Treg generation
PGE-2	Inhibition of proliferation: cytotoxicity, stimulation of cell activation and inhibition of DC and Treg stimulation
IFN-γTNF-αIL-1β	Promotes chemokine production and immunosuppressive factors such as NO or IDO
IL-6	Regulates migration, stimulates mitosis and angiogenesis
IL-10	Inhibition of apoptosis
VEGF	Inhibition of apoptosis, stimulates angiogenesis
LIF	Inhibition of apoptosis
SCF	Supports growth and differentiation
Jagged1	Enhances differentiation
CCLs CXCLs	Promotes migration of leukocytes

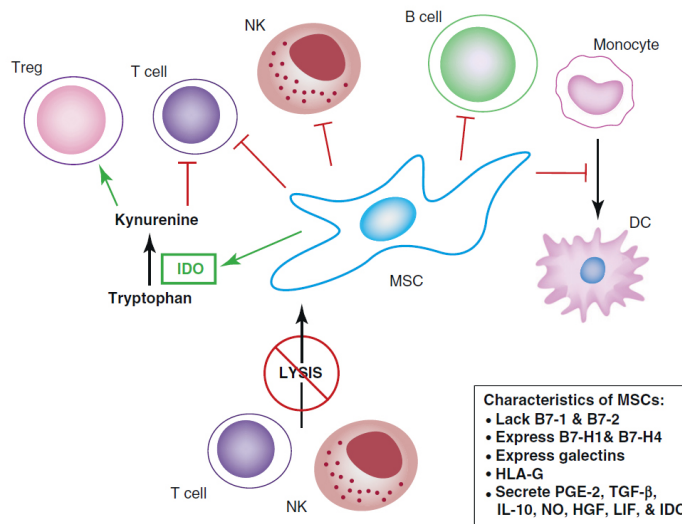


Figure 7 Immunomodulatory properties and soluble factors related to MSCs (Gebler *et al.*, 2012).

It has already been discussed about what surface molecules MSCs do not present and so why they are immune-tolerated, but also they present molecules that directly influence the immune system. Among these we have the presence of HLA-G (Human Leukocyte Antigen class G), a common antigen found in organs that have an immune-privileged environment (*e.g.* testes in males and ovaries in females, also some cells during embryogenesis) and acts mainly via inhibition of APCs.

A variety of other factors released by MSCs are related to specific cell subpopulations inhibition, both from innate and adaptive immunity (Shi *et al.*, 2011).

Given all these considerations one should clearly understand the interest of regenerative medicine towards MSCs. Not only they are an appealing source for regenerating damaged or non-functional parts, with a good chance of not being rejected and to integrate with the patient, but they can also be used as a regulatory tool for creating a permissive environment for treated areas.

They are nowadays many clinical trials about MSCs (see Figure 8), one of the few in phase 3 (final testing phase where the treatment is given to large groups of people) involves treatment with MSCs for creating an immunosuppressant environment to treat GVHD (Graft-Versus-Host Disease) in steroid refractory acute cases and in newly diagnosed acute GVHD (Kebriaei and Robinson, 2011).

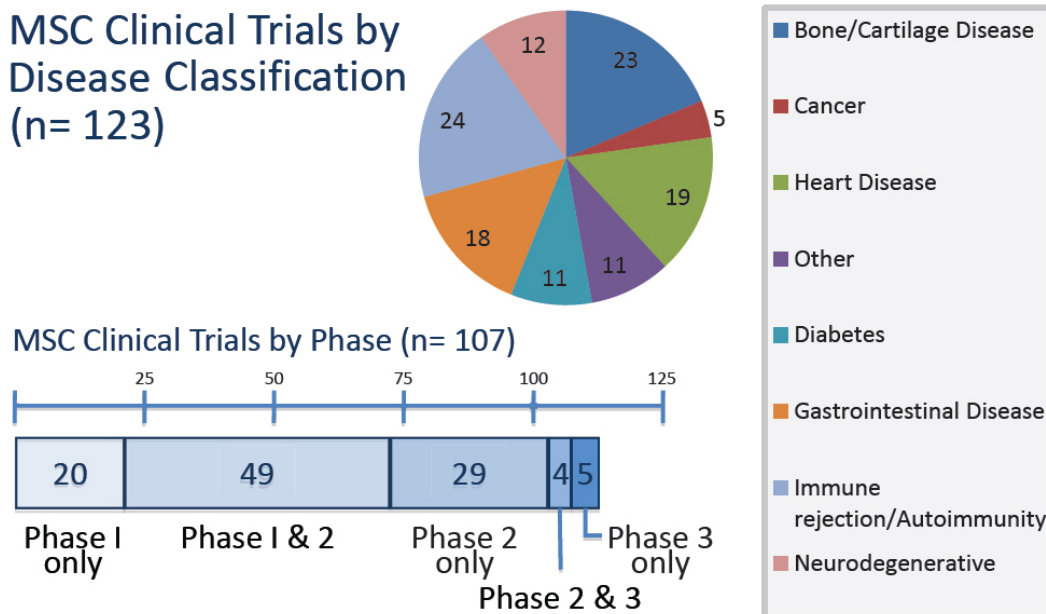


Figure 8 Actual clinical trials regarding MSCs (Trounson *et al.*, 2011).

Foetal stem cells: amniocytes

The amniotic fluid (AF) is an aqueous environment contained by the membrane “amnion” that surrounds the foetus during development; it has a function of sustain for the embryo and protect it from outside injuries like movements or blows but it also allow to maintain a constant temperature (Underwood *et al.*, 2005). Among other functions it allows the passage of nutrients from the placenta to the foetus that can absorb them before skin keratinisation (Jauniaux *et al.*, 1999), but also after that it is a source of nutrients and also hormones that can be assimilated via foetal swallowing (Mulvihill *et al.*, 1986). It was also known that AF contains some cells, even if not fully identified their source it is taught a

shared origin from both embryonic and extra-embryonic tissues, those cells derive from all the three germ layers (Cremer *et al.*, 1981). AF cells – or amniocytes – were not of great interest considering their identities of fully differentiated and non-replicating cells but then, in the 1990s, some subpopulation with proliferating behaviour were isolated for the first time, then there were demonstrated to be some kind of haematopoietic progenitor (Torricelli *et al.*, 1993) and some year later also cells with myogenic potential were identified (Streubel *et al.*, 1996).

In the early 2000s an MSC-like population was first described based on the proliferation rate and some marker expression (Kaviani *et al.*, 2001), these cells were phenotypically more characterised and their multilineage potential was verified, giving them a full title of MSCs (In 't Anker *et al.*, 2003).

The great interest on cells deriving from AF is related mainly for the source *per se* considering that the AF is easily obtainable from procedures that pregnant women undergo around the second trimester of gestation, namely amniocentesis and amnioreduction. During the first procedure, few millilitres (2-3 mL) are available for research; differently during amnioreduction, which is usually performed during the third trimester in case of polydramnios, more than 500 mL can be retrieved for cells extraction.

This source of cells overcomes the ethical issues related to the use of human embryonic cells because of their foetal but non-embryonic origin; amniocytes also make front to the donor associated morbidities of postnatal tissue biopsies.

As reviewed by Prusa and colleagues, the cellular component of AF consist of a heterogeneous pool of subpopulations different for morphological and also characteristics in culture (Prusa and Hengstschlager, 2002). When an amniocentesis sample is cultured in proper conditions, viable cells could be identified both attached to the surface of the culture material and some other floating in the medium. The adherent fraction normally takes up to four days to show up, meaning the cells need some time to adapt in the new environment, inside these cells we have some cell that do not proliferate and other as already told that are able to replicate and form colonies like those observed for MSCs.

These colony-forming cells are also a heterogeneous population that include:

- epitheloid (E-type) cells: have the classical morphology and form colonies typical of epithelial cells, soon after the beginning of the culture they stop replicating and disappear through passages.
- amniotic fluid specific (AF-type) cells: they persist along different passages in culture, they present a spindle-shaped morphology, they are believed to originate from trophoblastic tissue as they produce estrogen, human chorionic gonadotropin and progesterone.

- fibroblastic (F-type) cells: they show up in culture at later passages and not every sample is able to generate those types of cell.

Both AF and F-type cells share the same MHC phenotype (no HLA class II and low levels of HLA class I molecules) with MSCs.

Amniocytes have been categorised “between” ES cells and adult stem cells; they share a more potent state compared to MSCs because they have been demonstrated able to differentiate into a more broader range of cell types (including all three germ layers): osteogenic, chondrogenic, adipogenic, hepatic, myogenic, renal, and more importantly into hematopoietic lineages (In 't Anker *et al.*, 2003; Tsai *et al.*, 2004; Bossolasco *et al.*, 2006; Kim *et al.*, 2007; Kolambkar *et al.*, 2007; De Coppi *et al.*, 2007; Perin *et al.*, 2007; Perin *et al.*, 2010; Siegel *et al.*, 2009; Siegel *et al.*, 2010; Ditadi *et al.*, 2009; Hauser *et al.*, 2010).

Considering their characteristics, amniocytes are now considered to contain a pluripotent fraction and so they are also called AF Stem Cells (AFSCs). Given the importance of standardise experiments, and be sure to work on the same cellular type, identification based solely on morphology is not desirable; there are four main techniques performed to isolate amniocytes nowadays (see Figure 9):

- Single-step: the cellular fraction of AF is put in culture and left undisturbed for one up-to three weeks without medium change (In 't Anker *et al.*, 2004; Antonucci *et al.*, 2009).
- Two-step: five days after starting the primary culture unattached cells are retrieved and cultivated further (Tsai *et al.*, 2004).
- Selection: a specific antigen is used for positively identify a specific population within the amniocytes pool and isolate it (De Coppi *et al.*, 2007).
- Starter-cell: fibroblastoid colonies can be selectively cloned after their appearance and characterised later on (Phermthai *et al.*, 2010).

Given these four methods it is important to notice that cell potential referred to *in vitro* differentiation protocols, if tested from a heterogeneous pool, may derive from already committed progenitors inside the sample rather than obtain differentiation of AFSCs. It is then clear that the only two specific methods, to identify with a certain confidence “real” AFSCs, are those based on positive antigen selection or clonal expansion. The selection of specific antigen on cells require a deep study to identify possible markers of pluripotency associated to specific cell types; on the other hand the starter-cell protocol have the advantage of produce more rapidly large quantities of cells but one should also consider that the method is time-consuming and also it later needs to characterise each clone (see Figure 10).

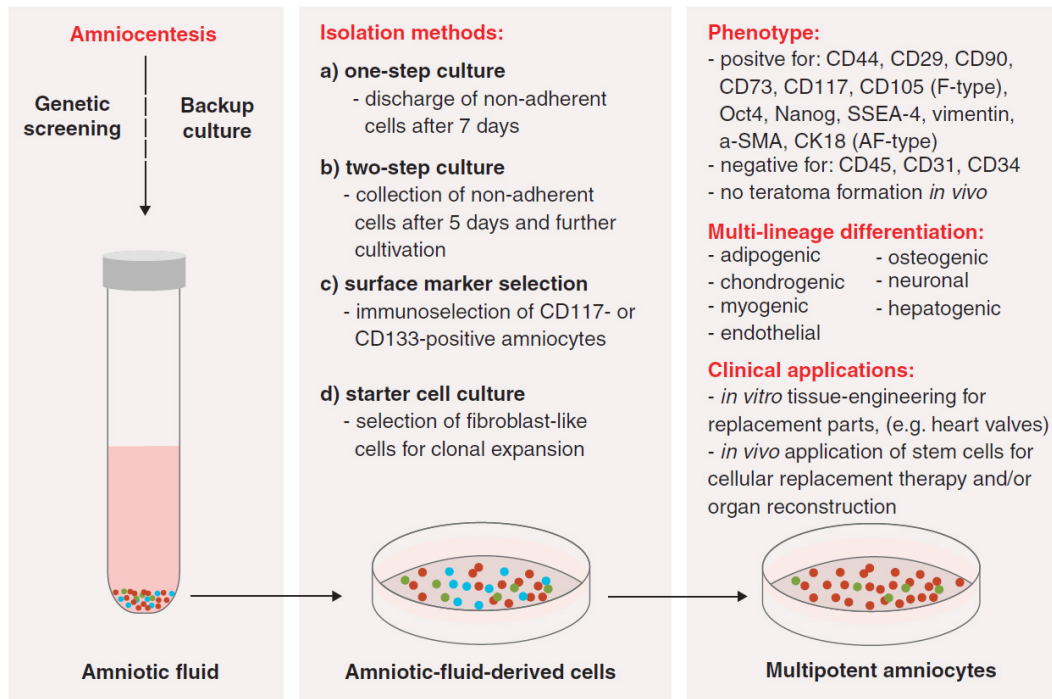


Figure 9 The four principal methods of culturing and selecting AFSCs (Klemmt *et al.*, 2011).

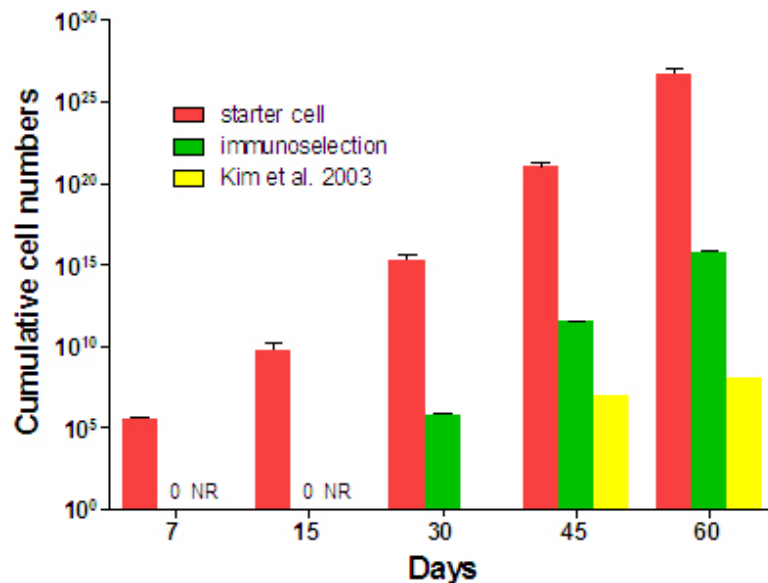


Figure 10 Growing curves of AFSCs deriving from different methods of isolation (Phermthai *et al.*, 2010).

One important thing about AFSCs' origin: giving the fact that they are obtained from procedures before the end of the pregnancy, one could expand those cells and also store them for a future purpose of treat the same foetus/new-born where they came from. This is particularly intriguing because it would be a transplantation without the risk of rejection (giving the autologous source of the cells) and moreover if one consider pathologies like neonatal malformation it should be considered that in some cases it's practically impossible to find a suitable replacement from a donor that fits the specific needs of an infant.

AFSCs could also be suitable for therapies aimed to cure genetic diseases incompatible with life; the autologous cells – the amniocytes – could be retrieved during gestation, genetically remodelled and then re-injected *in-utero* before delivery to correct the defect.

Perinatal stem cells

Amniotic fluid can also be retrieved from pregnant women undergoing caesarean section; after cutting through the uterine wall and exposing the amniotic sac, a syringe can aspirate the AF.

In 2008 and later also in 2009 a group from Heilongjiang published two papers reporting the discovery of MSCs from third-trimester AF. You and co-workers reported that these cells were positive for the pluripotency marker OCT4 (one of the factors used by Yamanaka to reprogram fibroblasts to iPS cells) at gene expression and protein level, showed consistent surface markers with MSCs and demonstrated able to differentiate towards the osteogenic lineage. There are a lot of questions opened on their work: first of all the fail to give proof of OCT4 presence at protein level because the immunofluorescence staining are not convincing; osteogenic differentiation is demonstrated only by RT-PCR and not by specific stainings for calcium deposition or osteoblasts markers; as a final point they use a “single-step” method to isolate the cells.

Hypoxia

Routine cell culture protocols are based on the fact that pioneer work of cellular growth seemed to confirm that ambient air was suitable. These studies concentrated more on the components of the culture milieu to substitute the *in vivo* environment (nutrients, pH, minerals, grow factors), giving poor importance to the gas status inside physiological tissues (Shooter and Gey, 1952).

Later studies demonstrated that oxygen levels inside the human body are very dissimilar from that of the initial partial pressure of oxygen (or pO_2 , referred also as “oxygen tension” when talking of gas in a solution like the blood) inhaled inside the lungs. Levels ranging from 2% to 9% (14-16 mm Hg) have been reported *in vivo* (see Figure 11) (Brahimi-Horn and Pouyssegur, 2007).

Recently some experiments demonstrated that, during *in vitro* culture, these pO_2 values are able to sustain the cells and so the oxygen tension of ambient air usually considered “normoxic” may not represent the physiological normoxia condition (Simon and Keith, 2008).

However, when translated to the culture protocols of MSCs, contrasting results have been reported. This is probably due to a poor standardisation of culture conditions (media, oxygen tension, cell type and method of isolation). Although it

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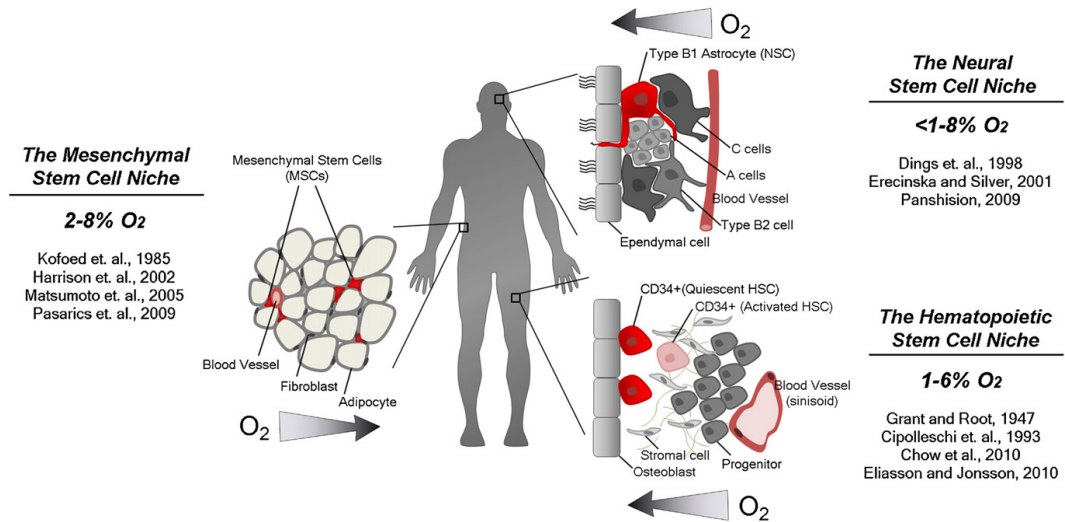


Figure 11 Hypoxic region of the human body related to specific stem cell niches (Mohyeldin *et al.*, 2010).

is generally accepted that hypoxic condition improve cell proliferation and maintain a more undifferentiated state (see Figure 12) (Tsai *et al.*, 2012). About the differentiation potential of hypoxic-cultured MSCs there is a bit of confusion in literature. The majority of authors describe an inhibition of osteogenic differentiation in hypoxia but not all articles are concordant (Yang *et al.*, 2011; Wagegg *et al.*, 2012; Xu *et al.*, 2012). About adipogenesis some authors report diminished achievement of differentiated adipocytes, other refer pre-adipocyte stage blocking and some other report increased adipogenesis (Chung *et al.*, 2009a). Chondrogenesis is a well oxygen-dependended process (Schipani, 2005) and MSCs in hypoxia seems to be more prone to form chondrocytes (Wang *et al.*, 2005; Xu *et al.*, 2007). Also angiogenesis seems enhanced when culturing cells in low oxygen tension (Hung *et al.*, 2007; Zhou *et al.*, 2013). Finally MSCs cultured in an hypoxic ambient show a more migratory phenotype than normoxia-cultivated cells (Rosova *et al.*, 2008).

MSC source	Effect on proliferation	O2 (%) or hypxia mimicking; seeding density (cells/cm ²); period tested
BM, murine	promotion	8%; 10,000; 7-8 days
BM, murine	inhibition	CoCl ₂ or DFX; 10,000; 7-8 days
BM, human	inhibition	1 %; 50-10,000; 10 days
BM, human	inhibition	1-5 %; 6,250; 7 days
BM, human	promotion	1-7 %; 50; 2-7 passages
AT, murine	promotion	2 %; 10,000 ; 13 days
AT, human	promotion	2 %; 3,000; 14 days
BM & AT, canine	inhibition	1%, 5%; 5,000; 7 to 14 days
WJ, human	promotion	2%; 5,000; 10 passages
PDB, human	inhibition	1 %; 10,000; 2-4 days

BM=bone marrow; AT=adipose tissue; PDB=placental decidua basalis; WJ=Wharton's jelly

Figure 12 Effect of oxygen tension on MSCs proliferation.

INTRODUCTION

AIM OF THE WORK

Our group recently described and characterised a specific population of AFSCs inside the amniotic fluid retrievable from amniocentesis samples obtainable during the mid-gestational period (second trimester of pregnancy). This specific subset of amniocytes is characterised by expression of the surface marker c-Kit (or CD117, the Mast/stem cell growth factor receptor) (De Coppi *et al.*, 2007).

It was tried to standardise a protocol of isolation and expansion *in vitro* of this population, in hope that our technique will allow to obtain more comparable results among other groups working on the same subject.

Another important aim pursued in this thesis project was trying to isolate and characterise amniocytes expressing c-Kit also from AF samples of the third trimester, collecting them at term during caesarean section. This was done not only to compensate the lack of information about this potential source of AFSCs (as already told, in literature there are few and quite ambiguous works on this subject) but also because obtaining pluripotent-like stem cells from a natural discarded tissue of the body would be extremely interesting and will avoid all the problematic related to donors and ethical concerns.

After characterisation of third trimester AFSCs and making a comparison with the cells already known from the second trimester, the effect of hypoxic culture methods was also tested to see if there was an influence in the behaviour of our cells.

Notably our approach is to expand in large scale the original population obtaining a great amount of cells usable for regenerative medicine approaches.

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MATERIALS AND METHODS

Isolation of AFSCs

Samples

AF samples were obtained by two different gestational periods after informed consent of pregnant women:

- Second trimester AF: samples were collected during routine amniocentesis performed at mid-gestation (16-18 weeks) for genetic screening purposes; one aliquot (usually 0.2 up to 2 millilitres) of the total aspirate were retrieved for research purposes.
- Third trimester AF: from women undergoing eligible caesarean section, after cutting through the abdominal wall and the uterus, the amniotic sac was punctured with a syringe needle and AF was taken before rupturing the membrane.

Seeding

Pre-selection medium (or Chang B+C): 10 mL of sterile distilled water were used to resuspend Chang Medium C Lyophilized Supplement (IrvineScientific©) and this was let to equilibrate until no powder was visible inside the vial. Reconstituted Chang Medium C Supplement was added to the bottle of Chang Medium B Basal (IrvineScientific©) making a 100 mL final volume of medium called "Chang B+C". This medium was completed with antibiotics (Penicillin and Streptomycin, Gibco®) and also with L-Glutamine (2mM final) (Gibco®, P.N. 25030) at the moment of use.

Samples were filtered through a 70 µm nylon mesh filter (BD©) and volume were adjusted with Phosphate Buffered Saline (PBS, Gibco®) for centrifugation.

AF samples were centrifuged at 300 x *g* for 5 minutes. Supernatant were removed without disturbing the pellet. If erythrocyte contamination was observable, the pellet was then incubated with Auto Lyse PLUS (BioSource©) for lysing red cells following manufacturer's specifications and washing steps.

Cells were then resuspended in "Chang B+C" medium and seeded over a glass coverslip of the size suitable for the inside of a 35 mm x 10 mm petri dish (BD Falcon©).

The morphology of the cells was checked daily and medium change was performed every 5-7 days until clusters of cells with mesenchymal-like morphology started to grow.

Immune-selection

After taking out the supernatant from the petri dish and wash twice with PBS, cells were detached with Trypsin 0.05% with EDTA and Phenol Red (Gibco®). Trypsin action was blocked adding 4 volumes of MEM Alpha (Gibco®) containing 25% of FBS Fetal Bovine Serum (FBS, Gibco®), and cells were pelleted with a centrifuge at 300 x *g* for 5 minutes.

Isolation was performed positively selection CD117 positive (C-Kit positive) cells using the MACS® CD117 MicroBead Kit and the other required instruments from Miltenyi Biotec©. Briefly:

1. After removing the supernatant, cell pellet was resuspended in 300 µL of buffer (buffer composition as specified in manufacturer's instructions).
2. 100 µL of FcR Blocking Reagent and 100 µL of CD117 MicroBeads were added and mixed well.
3. Incubation for 15-20 minutes at 4°C.
4. An MS Column was placed on the Separator attached to the MultiStand and washed once with buffer.
5. Cells were washed once adding 2 mL of buffer to the cell suspension and centrifuging at 300 x *g* for 10 minutes.
6. After resuspension in 500 µL of buffer, immune-labelled cells were passed through the MS Column.
7. Three washes of the column with 500 µL of buffer.
8. Column was removed from the magnetic support and placed on a 15 mL tube for collection: 1 mL of buffer was added onto the column and the plunger was used to flush out the content.
9. Repeating points 4 to 9 reduced the contamination by unwanted cells (with one new column).

10.

Cells expansion

Expansion medium (or Chang Complete): MEM Alpha containing 20% Chang B+C, 15% FBS, Penicillin, Streptomycin and L-Glutamine.

Selected cells were initially grown in a well of a 24-multiwell plate (BD Falcon©) and then – before confluence – they were split in another appropriate surfaces to maintain subconfluence (< 70-80%).

Note: for both pre-selection passages and expansion process it was always used multiwell plates or petri dishes that were not “tissue culture” treated.

Freezing of cells were performed using FBS containing 10% Dimethyl sulfoxide (DMSO, Sigma©).

Culture conditions and oxygen tension

Cells were cultured under standard 20% O₂, 5% CO₂ and relative humidity of 95% level, referred as “normoxia” (using a Thermo Scientific Heraeus® CO₂ incubator). Cells were also cultured under “hypoxia” with the environment at 5% O₂ (using a CO₂/O₂ controller connected to a hypoxic chamber (Biospherix©) inside the above mentioned incubator).

***In vitro* experiments**

Differentiation protocols

Osteogenic

Cells were seeded at a density of 4000 cells/cm² on wells of 24-multiwell plates in Chang Complete. After 12 hours the cells were checked for adhesion and confluence (best ~80%), cells were washed once with PBS and medium was switched with the osteogenic one, composed by:

- Dulbecco's Modified Eagle Medium (DMEM) Low Glucose
- Penicillin and Streptomycin
- FBS 10 %
- Dexamethasone (Sigma©) 100 nM
- Beta-Glycerol Phosphate (STEMCELL©) 10 mM
- Ascorbic acid 2-Phosphate (Sigma©) 50 μM

Medium was changed every 4-5 days.

Adipogenic

Cells were seeded at a density of 4000 cells/cm² on wells of 24-multiwell plates in Chang Complete. After 12 hours the cells were checked for adhesion and confluence (best ~80%), cells were washed once with PBS and medium was switched with the osteogenic one, composed by:

- Dulbecco's Modified Eagle Medium (DMEM) Low Glucose
- Penicillin and Streptomycin
- FBS 10 %
- Insulin (Lilly©) 10 μg/mL
- IBMX (3-isobutyl-1-methylxanthine, Sigma©) 1 mM
- Dexamethasone 1 nM
- Indomethacin (Sigma©) 60 μM

Medium was changed every 4-5 days.

Endothelial

Wells of 96- or 24-multiwell plates were coated with undiluted Matrigel™ Basement Membrane Matrix (BD Biosciences ©), respectively 50 or 200 µl, and then let stand in the incubator for one up to two hours.

Cells were detached from the original expansion culture, resuspended and seeded over the solidified coating in EGM-2 (Endothelial Growth Medium-2, Lonza©). 30.000 cells were seeded for 24-multiwell plates and 5.000 cells were seeded for 96-multiwell plates.

In vivo experiments**Mice**

BALB/c strain Rag2^{-/-} γc^{-/-} immunodeficient mice were used In the experiments in order to avoid the possible rejection of our cells after xenotransplant.

Matrigel plug

A constant amount of cells were resuspended in 500 µl of Matrigel added with:

Heparin (Pharmatex Italia©)	0.75	mg/mL final concentration
Mouse recombinant FGFb (PeproTech©)	50	ng/mL final concentration
Human recombinant VEGF (PeproTech©)	100	ng/mL final concentration

Mice were anesthetised with isoflurane, via nose cone, and cold liquid Matrigel containing cells (or just PBS for controls) was injected in the back lumbar region of the animals (resulting in *quasi* immediate solidification).

Mice were euthanized after 14 days by cervical dislocation and plugs were harvested for obtaining cryosections or measuring haemoglobin levels (after being snap-frozen in liquid nitrogen and stored at -80°C).

Teratoma test

Cells were injected either in the subcapsular space of the kidney after opening the animal on the abdominal side, or inside the *tibialis anterior* (TA) muscle of the hindlimb. 1 x 10⁶ cells were injected in each test and organs were harvested nine weeks later.

Imaging

Phase Contrast (PhC) and Bright Field (BF) pictures were taken using an Olympus© IX71 inverted microscope.

Immunofluorescence (IF) pictures were acquired using a Leica© DMI 6000B inverted microscope.

Spectrophotometry

We used a SpectraMax Plus Spectrophotometer (Molecular Devices©) for measuring absorbance (or Optical Density, O.D.) of specific reactions (see later).

Immunofluorescence

For specific antibodies concentrations and conditions see APPENDIX A.

IF protocol for cells grown in monolayer:

1. Fix in para-formaldehyde (PFA, Sigma©) 4% in PBS, 10 minutes at room temperature.
2. Wash briefly in PBS.
3. Permeabilisation with Triton X-100 (Fluka©) 0.5% in PBS for 10 minutes.
4. Wash in PBS two times for 5 minutes.
5. Blocking of non-specific binding with PBS containing 3% Bovine Serum Albumin (BSA, Sigma©) for 30 minutes.
6. Wash briefly in PBS.
7. Incubate with primary antibody(-ies) in PBS containing 1% BSA.
8. Wash in PBS three times for 5 minutes.
9. Incubate with secondary antibody(-ies) in PBS containing 1% BSA.
10. Wash in PBS three times for 5 minutes.
11. Cells nuclei were counterstained with a mounting medium containing 49,6-diamidino-2-phenylindole (DAPI) (Sigma-Fluoroshield™, Sigma-Aldrich©) or with Hoechst 33342 (Invitrogen©).

IF protocol for cryosections:

Tissue samples were processed in two different ways:

- Frozen in liquid nitrogen: for entire organs or muscle samples (with isopentane cooling instead of submerging muscles inside liquid nitrogen). Fixation step before immunostaining as for cells grown in monolayer.
- Fixed and dehydrated: for Matrigel plugs.
Fixation for 1-2 hours in PFA 4% in PBS, dehydration at 4°C with sucrose gradient method: sucrose 10% in PBS for 1 hour, 15% for 1 hour and 30% overnight (O.N.). Then pieces were frozen in liquid nitrogen.

Subsequently cryosections were obtained after embedding samples in OCT (Kalttek©) and using a cryostat (Leica©) to produce transverse sections.

1. Wash briefly in PBS.
2. Permeabilisation with Triton X-100 0.5% in PBS for 10 minutes.
3. Wash in PBS two times for 5 minutes.
4. Blocking of non-specific binding with PBS containing 10% Horse Serum (HS, Gibco©) for 30 minutes.
5. Wash briefly in PBS.
6. Incubate with primary antibody(-ies) in PBS containing 1% BSA.
7. Wash in PBS three times for 5 minutes.
8. Incubate with secondary antibody(-ies) in PBS containing 1% BSA.
9. Wash in PBS three times for 5 minutes.
10. Cells nuclei were counterstained with a mounting medium containing DAPI (Sigma-Fluoroshield™, Sigma-Aldrich©).

Immunocytochemistry

Von Kossa

To demonstrate calcium or calcium salts deposit inside wells after osteogenic induction Von Kossa staining was carried out.

1. 24-multiwell plates well were fixed with PFA 4% for 30 minutes.
2. Wash in distilled water twice for 5 minutes.
3. Incubation for 1-2 hours under UV light after adding a 1% silver nitrate (Sigma©) aqueous solution.
4. Wash in distilled water at least twice for 5 minutes.
5. Remove of unreacted silver adding a 5% aqueous solution of sodium thiosulfate (Sigma©) for 5 minutes.
6. Wash in distilled water for 5 minutes.
7. Counterstain with Hematoxylin QS (Vector®) for 45 seconds and then rinse with tap water until the wash is colourless.
8. Mount with aqueous mounting medium.

Calcium deposits are substituted with observable brown/black precipitates of silver.

Oil Red O

To demonstrate accumulation of lipid droplets inside cells induced towards adipogenesis a coloration with Oil Red O (Sigma©), a lysochrome (fat-soluble dye), was used.

- Stock solution: Oil Red O 0.5% solution in isopropanol.
 - Working solution: 3/5 of Oil Red O Stock solution and 2/5 of distilled water.
1. 24-multiwell plates well were fixed with PFA 4% for 30 minutes.
 2. Wash in distilled water twice for 5 minutes.
 3. Incubation for 30 minutes with Oil Red O working solution.
 4. Wash in distilled water at least twice for 5 minutes.
 5. Counterstain with Hematoxylin QS (Vector®) for 45 seconds and then rinse with tap water until the wash is colourless.
 6. Wells covered with distilled water until observation at brightfield microscope.

Quantification

Stained wells were washed 10 minutes with a Triton X-100 0.5% solution and then Oil Red O pigment was extracted from the stained lipids incubating 15 minutes in agitation with isopropanol.

The O.D. of the extracted dye was measured at 518 nm.

Alkaline Phosphatase

Following manufacturer's instructions, Sigma-Aldrich© Alkaline Phosphatase kit was used for showing presence of Alkaline Phosphatase (AP) on surface of cells after osteogenic differentiation.

Cells were fixed on bottom of the wells with Fixative Solution (2 volumes of Citrate Working Solution and 3 volumes of acetone) for 30 seconds and then rinsed in distilled water for 45 seconds.

Fixed cells were incubated with Alkaline-dye mixture (FastBlue RR Salt solution plus Naphthol AS-MX Phosphate Alkaline Solution) for 30 minutes at Room Temperature (R.T.).

After rinsing several times in distilled water nuclear counterstain was performed covering the bottom of the wells with Mayer's Hematoxylin Solution for 10 minutes.

After rinsing in distilled water an aqueous mounting medium was used for later observation.

Quantifications

Calcium quantification

After rinsing 2 times with distilled water, wells with cells in osteogenic differentiation were incubated overnight R.T. in agitation with 250ul of HCl 0.6N. Supernatant were collected and stored at -20°C (at least for 24 hours before quantification).

Calcium was quantified using Chema diagnostica© calcium kit and following manufacturer's protocol.

Haemoglobin quantification

Matrigel Plugs were smashed with liquid nitrogen in a mortar and then the resulting powder was lysed in distilled water.

Cells residue were pelleted and supernatants were used for haemoglobin (Hb) quantification using Drabkin's Reagent (Sigma©).

Lysates were incubated with Drabkin's Reagent for 20 minutes R.T.

Reaction product absorbance was read with spectrophotometer at 540 nm.

Protein quantification

Protein content of Matrigel plugs was obtained using Bradford Reagent (Sigma©) and following manufacturer's instructions. Briefly: after incubating an aliquot of lysate (the same used for Hb quantification) with the reagent and incubating for 30 minutes at R.T., proteins absorbance was read at 595 nm.

Ac-LDL

Human low-density lipoprotein (LDL) was used as a marker of endothelial differentiation as the acetylated (Ac) form of this apoprotein is taken up by microglial and endothelial cells that possess receptors specific for this modified LDL. This complex is also coupled with an Alexa Fluor® 488 dye that permits recognition by fluorescence.

EGM-2 medium was taken out and replaced from the wells with an appropriate amount of fresh medium containing Alexa Fluor® 488 AcLDL (Molecular Probes®) at 10 µg/mL. After 6 hours of incubation medium was removed, cells were washed in PBS and then fixed for later observation with PFA 4% for 15 minutes. Cells nuclei were counterstained with Hoechst 33342.

Proliferation curves and doubling time

An equal amount of cells were seeded on multiwall plates at the same time for later counting the increased number of cells. At determined time points the cells were:

1. fixed with PFA 4% for 15 minutes
2. washed in PBS twice for 5 minutes
3. a brief permeabilisation was carried out with Triton X-100 0.5% in PBS for 5 minutes

4. after a brief wash cells nuclei were stained with Hoechst 33342.

Cells numbers were evaluated counting the nuclei on a series of randomised fields for the different samples and for each time point.

Flow Cytometry

Cells in culture were detached by trypsinisation and then incubated with specific antibodies following manufacturer's indications on quantities.

Cell suspensions were incubated 20' at 4°C in the dark.

After a wash step the cells were resuspended in a suitable volume of PBS and fluorescence emission was detected using a FACSCalibur™ (BD Biosciences©) flow cytometer.

Acquired data were analysed using FlowJo software (TreeStar Inc.©) after gating on viable cells.

Cells analysed starting from freshly retrieved AF samples were not trypsinised but were instead filtered through 70 µm nylon mash filter and then stained as described above.

Cell cycle analysis

PI staining

Propidium Iodide (PI) was used to stain the genetic material inside each single cell and discriminate the cell cycle phase in base of the amount of DNA, relative fluorescence of the bound molecule was acquired by flow cytometry and this indirectly gave information on DNA content.

PI staining solution:

- PBS
 - Triton X-100 0.1 %
 - DNase-free RNase A (Sigma©) 0.2 mg/mL
 - PI (Sigma©) 1 mg/mL
1. Cells were detached by trypsinisation and then centrifuged in PBS at 200 x g for 5 minutes at 4°C.
 2. Pellets were resuspended in 1 mL of cold PBS per billion of cells.
 3. Cell suspension was added dropwise while stirring a tube with an equal volume of absolute cold ethanol; tubes were stored at -20°C for at least one day before proceeding with the staining.
 4. Cells were washed once with cold PBS at 200 x g for 10 minutes at 4°C.
 5. Staining was performed adding 300 µl of staining solution per billion of cells.
 6. After incubation at 37°C for 15 minutes cells were analysed at the flow cytometer.

Gene expression

RNA extraction

Total RNA was extracted using RNeasy “Micro” or “Mini” Kit (Qiagen©) in base of calculated number of starting material, procedures was conducted following the manufacturer’s instructions.

RT-PCR

RNA extracted was retrotranscribed (RT) using:

- SuperScript® II Reverse Transcriptase (Invitrogen™).
- Oligo(dT)12-18 as primer for reaction (Invitrogen™).
- RNase H (Invitrogen™) for eliminating DNA-RNA hybrids after reaction.

All procedures were carried out based on manufacturer’s instructions and using GeneAmp® 2720 thermal cycler (Applied Biosystems®).

Synthesized cDNA was quantified for downstream application using a Nanodrop ND-2000 Spectrophotometer (Thermo Scientific©).

Polymerase chain reaction (PCR) for target genes were performed using recombinant *Thermus aquaticus* (*Taq*) DNA Polymerase system (Invitrogen™) with specific primers (see APPENDIX B).

PCR products were analysed by electrophoresis in agarose gel 1-1.5% in TBE 1× Buffer (54 g of Tris base, 27.5 g of boric acid, 20 ml of 0.5 M EDTA) containing SYBR® Safe DNA gel stain (Invitrogen™).

RealTime PCR

Quantitative RealTime PCR (qPCR) was performed using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen™) and reactions have been carried out in a LightCycler II instrument (Roche©), using 5 ng of cDNA and a 300 nM mix of specific forward and reverse primers (final concentrations).

Results have been expressed in Arbitrary Units (A.U.) considering the ratio of targeted gene mRNA content and a housekeeping gene (human β 2 microglobulin, abbreviated “B2M”) mRNA content.

Angiogenesis

Matrigel network formation was analysed and quantified with ImageJ software (Rasband, 1997) coupled with Carpentier G. Angiogenesis Analyzer (2012) available online

(<http://imagej.nih.gov/ij/macros/toolsets/Angiogenesis%20Analyzer.txt>).

RESULTS

Second trimester AFSCs

Here is a brief scheme illustrating the procedure followed to isolate CD117⁺ AFSCs from AF primary cultures (Figure 13).

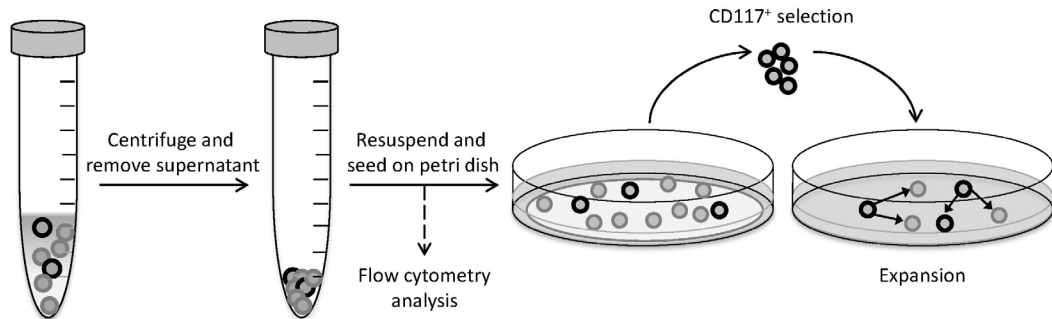


Figure 13

As already confirmed in previous works (Gosden, 1983) the number of cells inside AF samples is very variable and non-constant, without a clear correlation between retrieved volume and number of cells (see Figure 14).

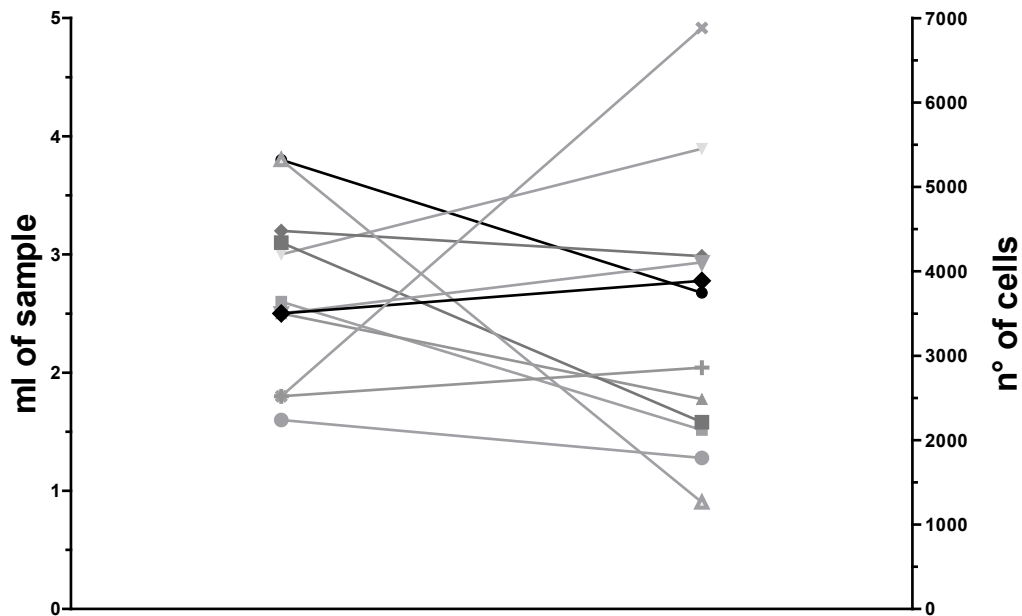


Figure 14 Correlation study between AF retrieved volumes and cells content.

Cells isolated from AF samples showed also a very heterogeneous positivity for some surface markers and numbers of viable cells (negative for the cell viability stain with 7-Aminoactinomycin D (7AAD)) (see Figure 15). In particular high variability was observed for our marker of interest CD117 (see Figure 16). A mean of 5.3 % of CD117⁺ cells were present among the samples, with variations

RESULTS

ranging from almost zero to a fifth of the total number of viable cells. Interestingly this percentage even if not from a very large statistical group is higher compared to those reported in literature (Klemmt *et al.*, 2011).

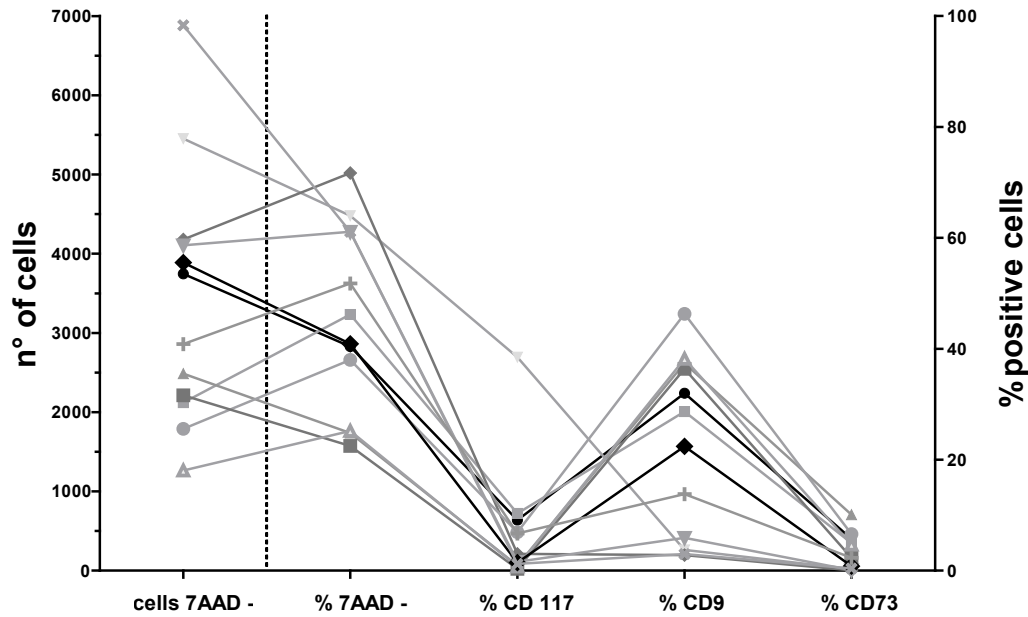


Figure 15 Percentage of cells stained positive for some markers and viability (7AAD -) by flow citometry. Each line connecting the values represent a single analysis for that sample.

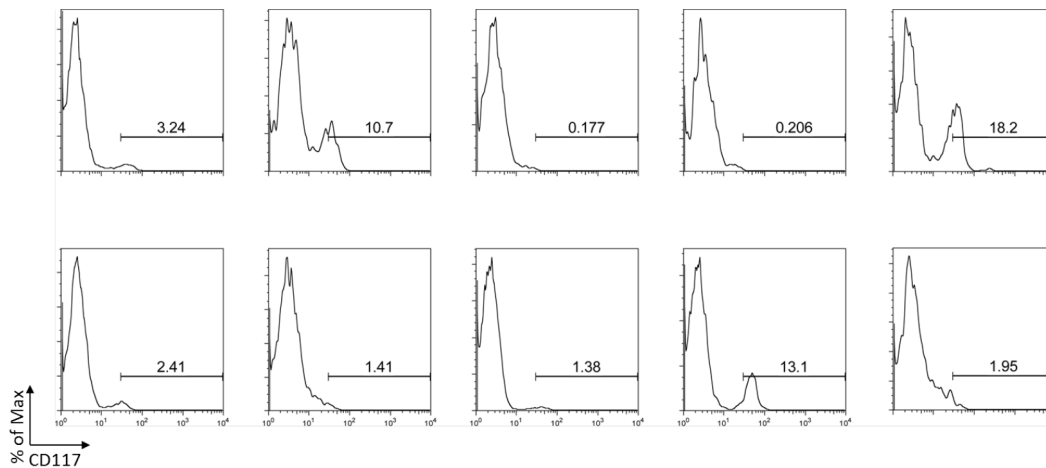


Figure 16 Flow cytometry histograms showing the percentage of CD117 positive expression in freshly retrieved AF.

After plating the cells over glass coverslips and culturing them for some days, the resulting morphology was different among samples: some presented a majority of floating round- or cuboidal-shaped aspect and no adhesion was observed (Figure 17 A), others presented a mixed morphology with few adherent cells low-proliferating and some floating (Figure 17 B), in some cases the percentage of adherent and well proliferating cells were higher (Figure 17 C). In some cases an

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epithelial-like morphology was observed, these cells did not yield AFSCs after positive selection for CD117 (Figure 17 D).

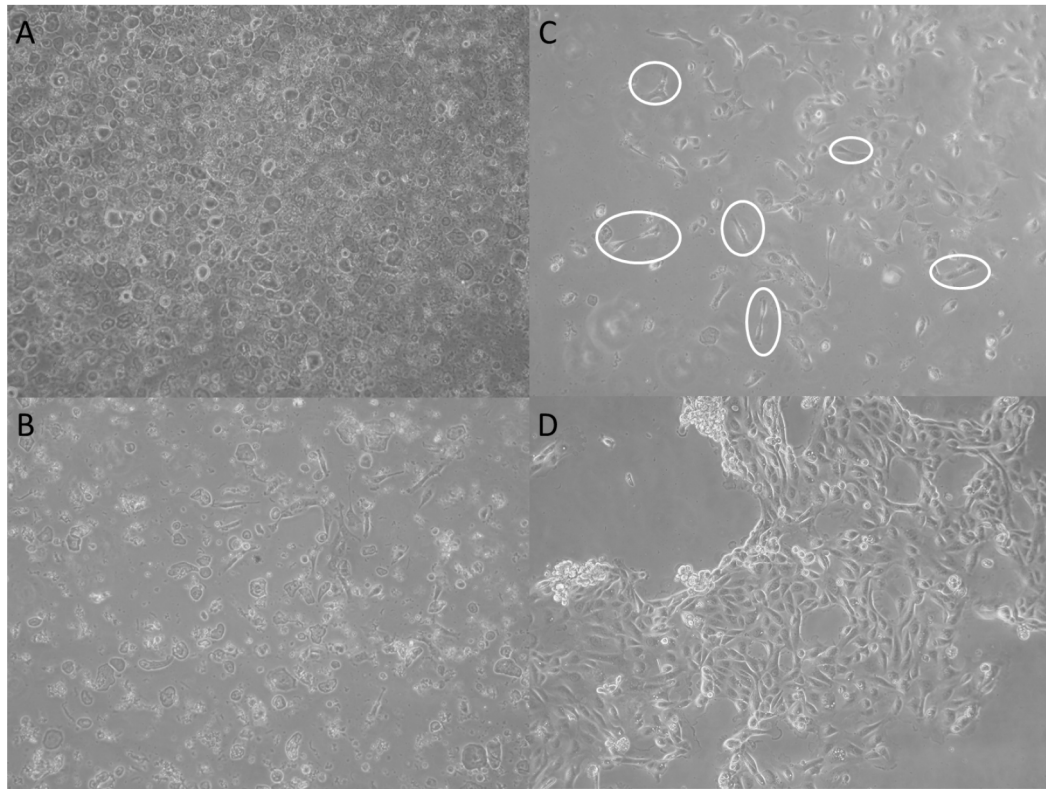


Figure 17 Morphology of primary culture of AF samples showing different populations of amniocytes in PhC (original magnification 10X).

Cells selected for CD117 maintained proliferative potential and the same morphology after selection, in Figure 18 it can be seen a typical morphology after immune-selection compared to a primary culture showing same-aspect cells (Figure 17 C, white circles).

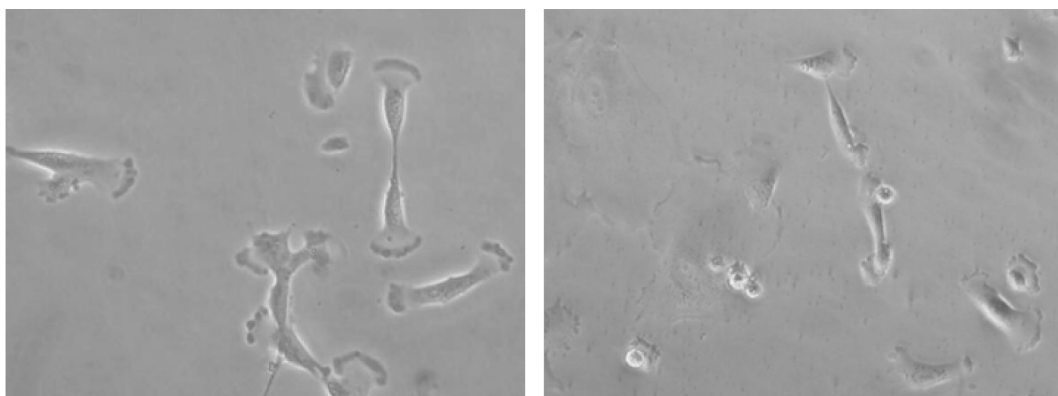


Figure 18 PhC images of AFSCs after selection for CD117 and in expansion medium (original magnification 20X).

RESULTS

Expression of CD117 (as already confirmed in our experience and also from other authors) was diminished after expanding cells in culture and after few passages no detectable protein was found either via flow cytometry (Figure 19 A) or IF (Figure 19 B). Interestingly at passage 2 (P2) the protein was not detectable on the surface and it rather seems to localise in the cytoplasm, later on (P4-P5) only few rare cells were still positive. It is known that CD117 positive cells also express Oct4 (a nuclear transcription factor known to identify pluripotent stem cells, also one of the factors used by Yamanaka first to generate iPS cells) as already demonstrated in previous works (De Coppi *et al.*, 2007) in expanded AFSCs there is no detection of the protein. Among other marker for pluripotency the only two found after expansion were SSEA4 and KLF4 (see later, Figure 22).

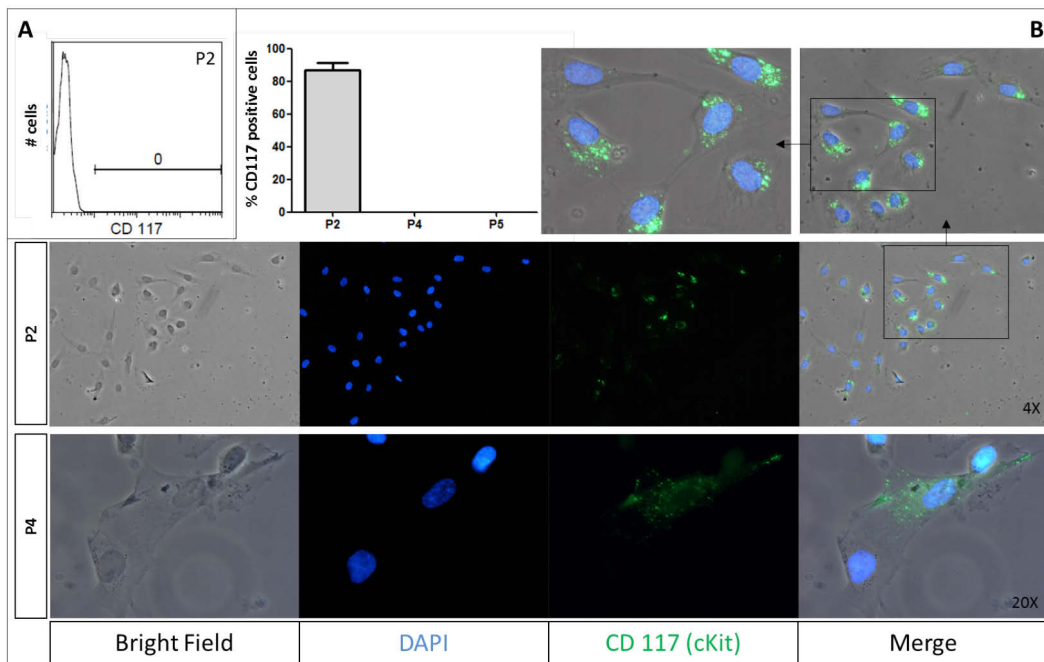


Figure 19 CD117 expression in expanded AFSCs. A: expression of surface marker by flow cytometry. B: IF staining at different passages (n=3) (original magnification 4X and 20X).

Third trimester AFSCs (and comparison with 2nd trimester)

AFSCs cells from the third (3rd) trimester contain CD117⁺ cells (Figure 20 A) that could be identified on primary culture of AF. Morphology resembled that of second (2nd) trimester counterpart (Figure 20 B, right columns), staining for CD117 revealed positivity before selection (Figure 20 B, right column) and expanded culture yielded same morphology as well.

RESULTS

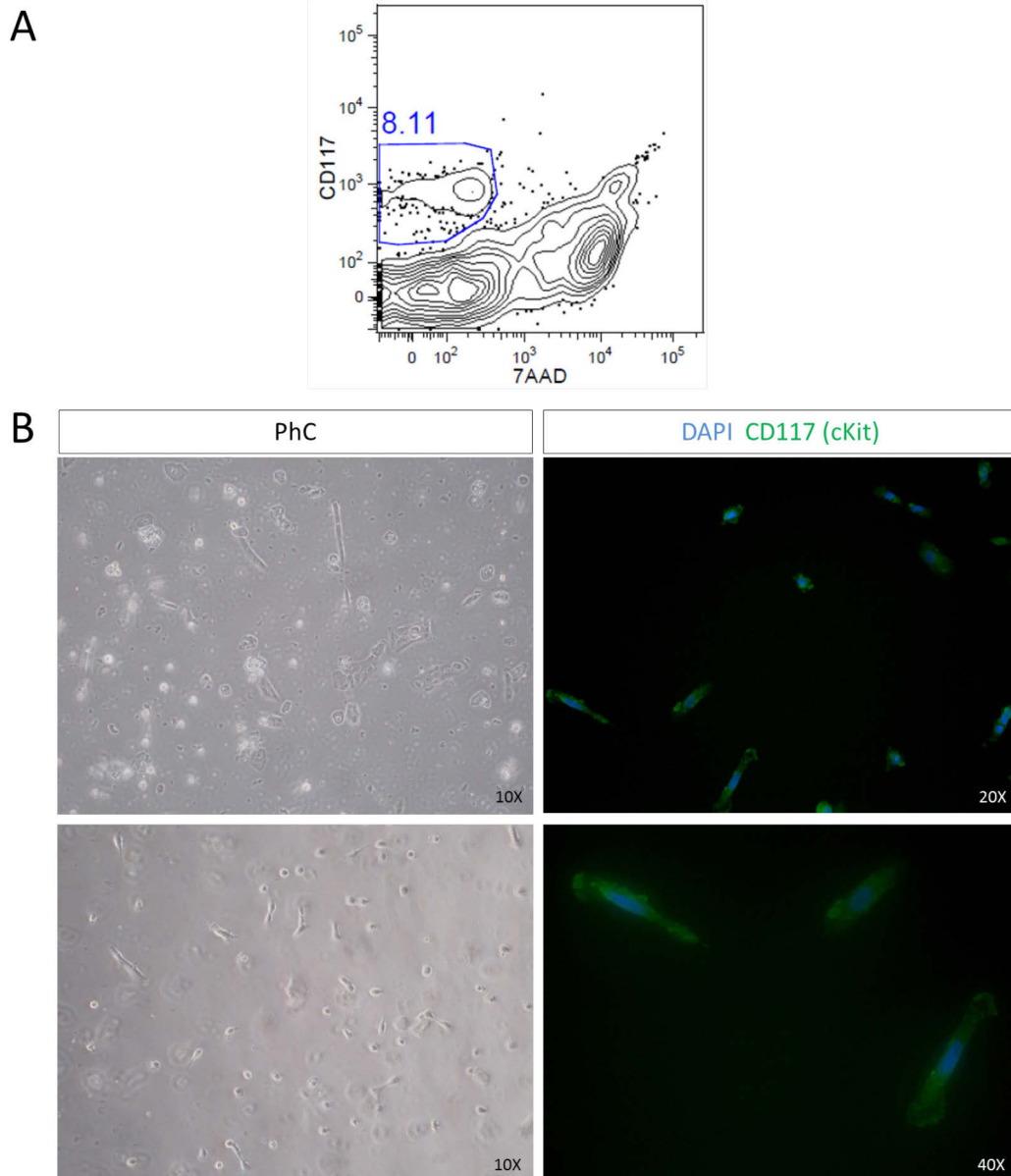


Figure 20 A: flow cytometry of fresh 3rd trimester AF cells after gating on viable cells (7AAD). B: images of unselected cells in primary culture of 3rd trimester. Left panel: PhC of AF cells (original magnification 20X). Right panel: IF staining for CD117 (original magnification 40X).

Fresh samples of AF from 2nd and 3rd trimester were analysed for surface markers just after collection, half sample was seeded in culture and the other half was stained for specific antigens (Figure 21).

Cells from both trimesters are in the majority negative for CD29, CD31 and CD271, except for some CD117⁺ cells of the 3rd trimester which are positive for those three markers. CD45, CD34, CD166, CD56 and HLA-DR are not expressed in both trimesters (except a little population CD117⁻ positive for CD56). CD117⁺ cells from both trimesters are slightly positive for CD184 and HLA-ABC. CD117⁺ fraction is also positive for mesenchymal markers as CD105, CD90, CD73, the

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receptor for hyaluronic acid CD44 and CD146. CD117⁺ fraction also presented positivity for CD73. The main difference noticeable in freshly collected samples from the two trimesters is about the expression of CD9: both present expression of this molecule but 2nd trimester cells seems to not co-express CD117 and CD9 whereas in 3rd trimester the cells positive for CD117 are always double-positive CD9⁺CD117⁺. CD9 is a molecule known to act in processes like motility, activation of cells (especially in platelet activation and aggregation, also cells releasing histamine are CD9⁺), growth and development (Mazzocca *et al.*, 2002; Le Naour *et al.*, 2000; Runge *et al.*, 2007). More interestingly CD9 is known to associate at the membrane with other molecules at some extent and between those there is also CD117 (Anzai, 2002).

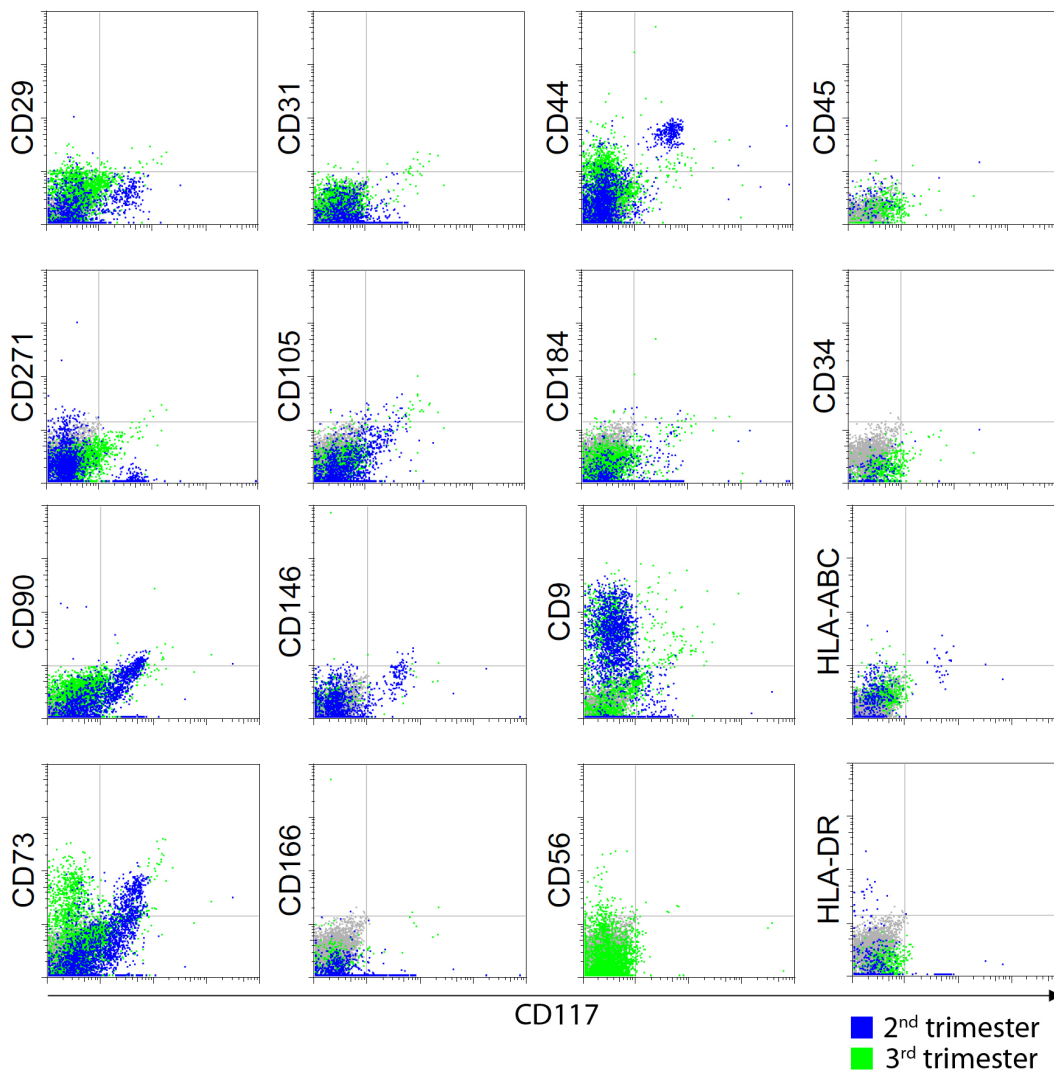


Figure 21 Flow cytometry characterisation of freshly collected AF cells of 2nd and 3rd trimester. Plots representing specific markers (Y axis) versus CD117 expression (X axis) after gating for excluding dead cells (7AAD⁺). Isotype controls and threshold axes in grey.

RESULTS

As already investigated for 2nd trimester AFSCs, expression of pluripotency marker SSEA4 and KLF4 was confirmed, the first showed a reduced percentage of expression in expanded cells whereas the second is expressed in trimesters' AFSCs at similar levels (Figure 22).

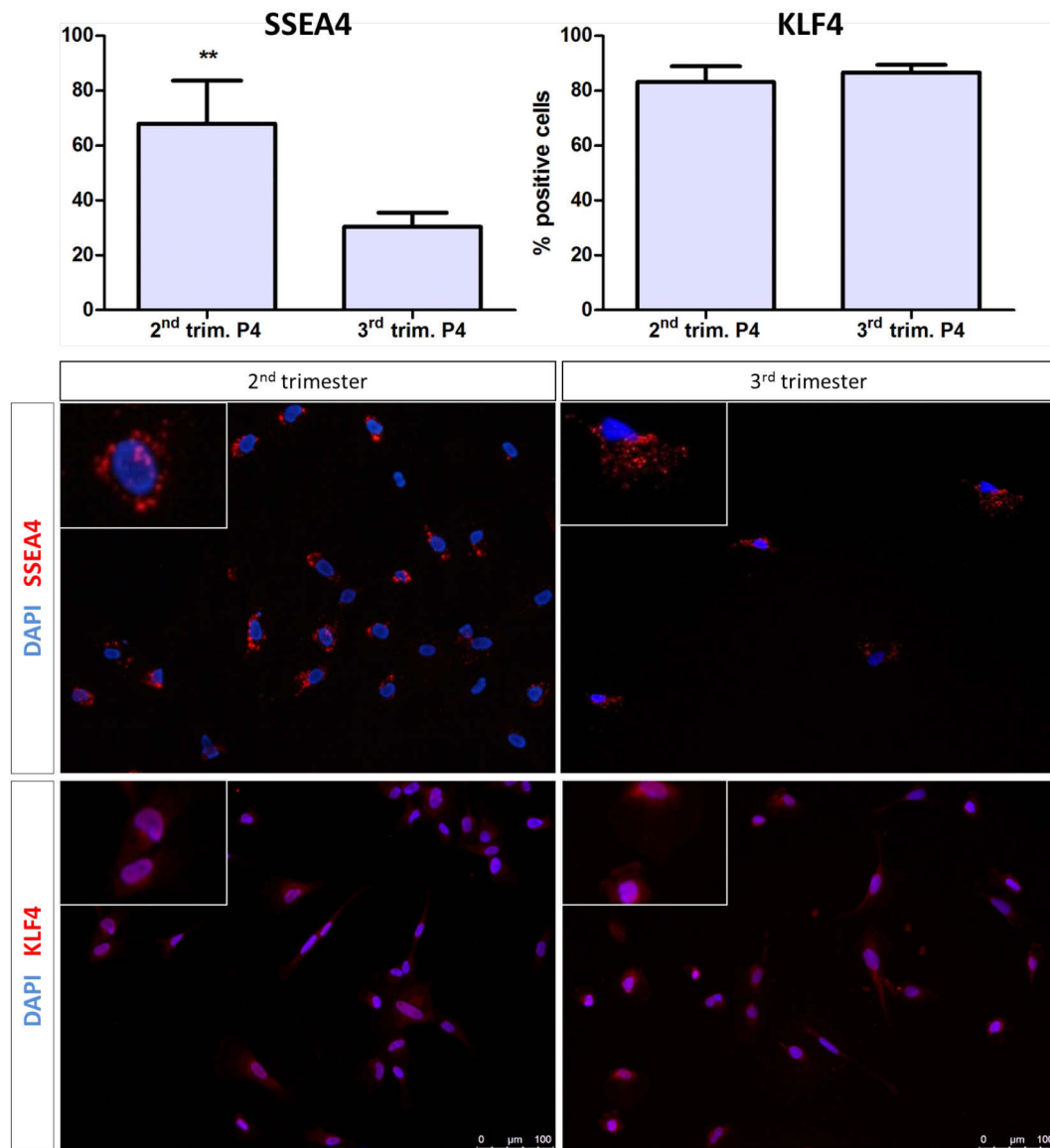


Figure 22 Counts and relative sample images for cells stained by IF for presence of pluripotency markers SSEA4 and KLF4 (counts on randomised fields, % positive cells = positive cells / total DAPI positive cells) (n=3; **p < 0.01).

Hypoxia

Two different hypoxic concentrations were tried: 2% O₂ and 5% O₂. 2% was first used because in a recent study of our group we demonstrated the effect of this oxygen tension on satellite cells (small progenitor cells found in the muscle) (Urbani *et al.*, 2012). No growing or attaching cells were observable with this condition so 5% of oxygen was tried and with successful isolation of AFSCs (see Figure 23).

RESULTS

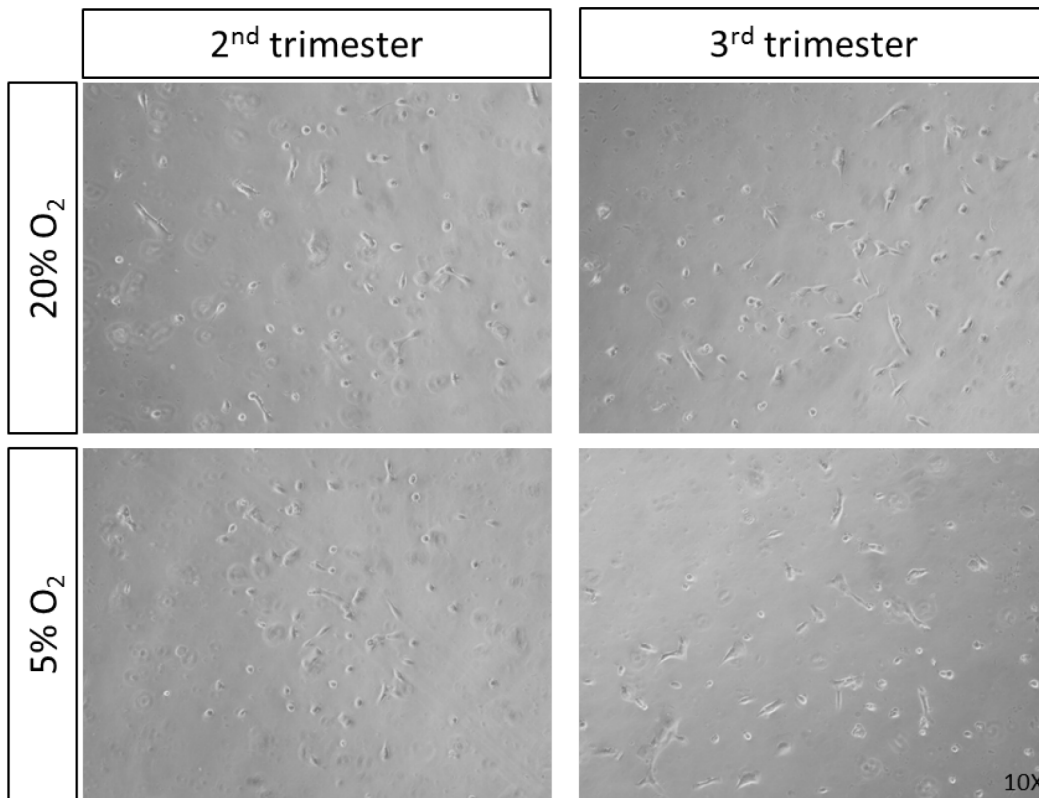


Figure 23 PhC images of AF cells primary culture showing typical morphology at different concentration of oxygen and between the two trimesters (original magnification 10X).

After growing and selecting primary AFSCs cultures in one or the other oxygen concentration, it was also tried to switch (simply by moving the culture petri/multiwell) to the other condition but cells did not survive this change and stopped proliferating undergoing senescence after some time.

A comparison of the proliferation rate between the two conditions of oxygen and the two trimesters was performed: a defined number of cells were plated at “day 0” and then at determined time-points cells on the bottom of the wells were fixed and DAPI positive cells were counted. The proliferation was expressed in terms of fold-change compared to the day of seeding (see Figure 24 A). After that it was calculated also the doubling time in those culture conditions (see Figure 24 B). Hypoxia greatly influences cell proliferation, augmenting the yields of cells of almost 7 times, confirmed by doubling time reduction. Between trimesters there was no observable differences in terms of proliferation when cultured at the same oxygen tension.

RESULTS

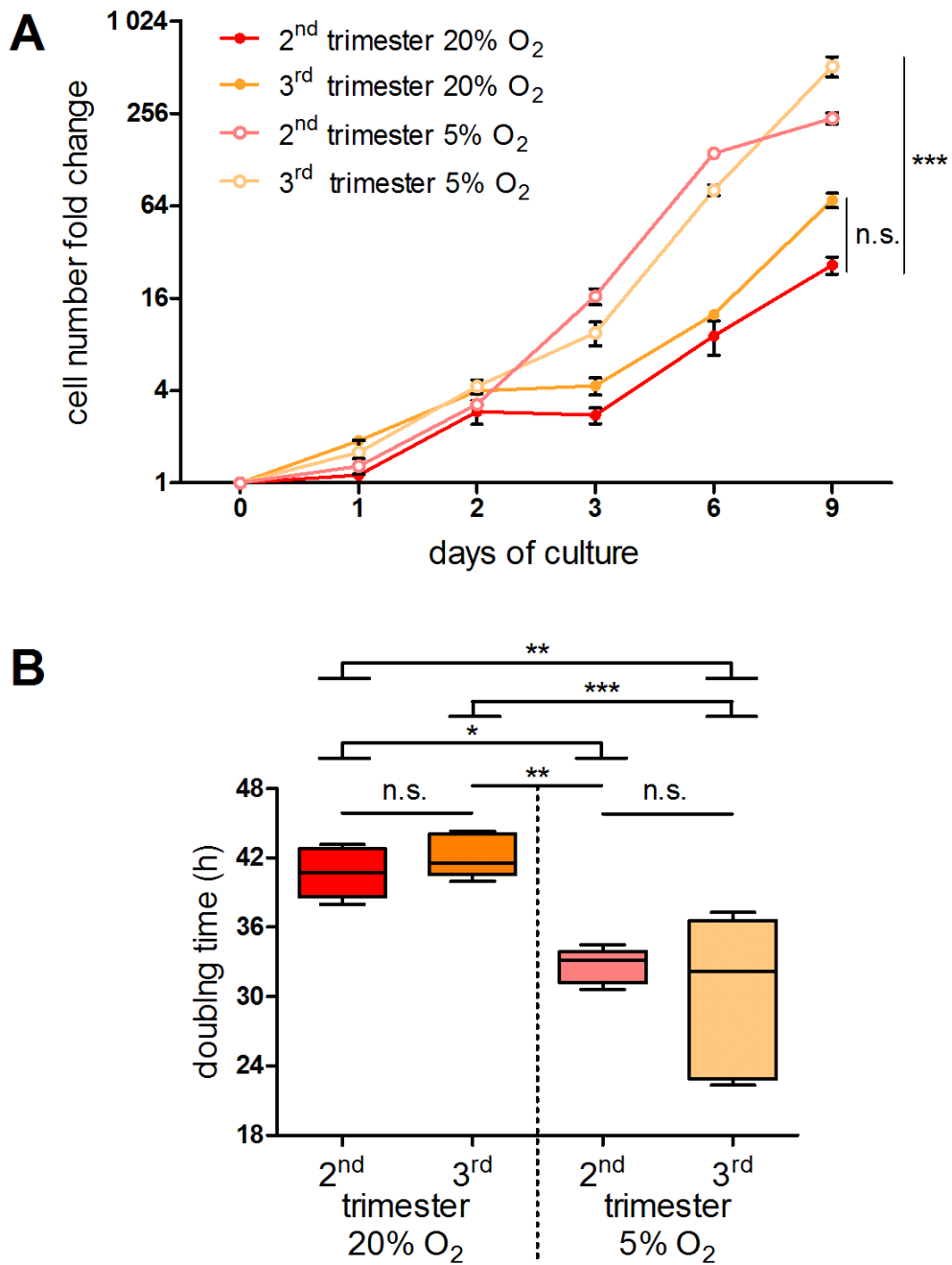


Figure 24 A: counts of cells during culture at time-points for proliferation rate. B: calculated doubling time based on cell number at different time-points (n=5; *p < 0.05, **p < 0.01, ***p < 0.001).

Also the cell cycle were analysed at the same moment of proliferation, using PI to stain genetic material and discriminate between phases in relation to the amount of DNA present in each cell (Figure 25). Surprisingly no appreciable differences among trimesters or oxygen conditions were observed.

RESULTS

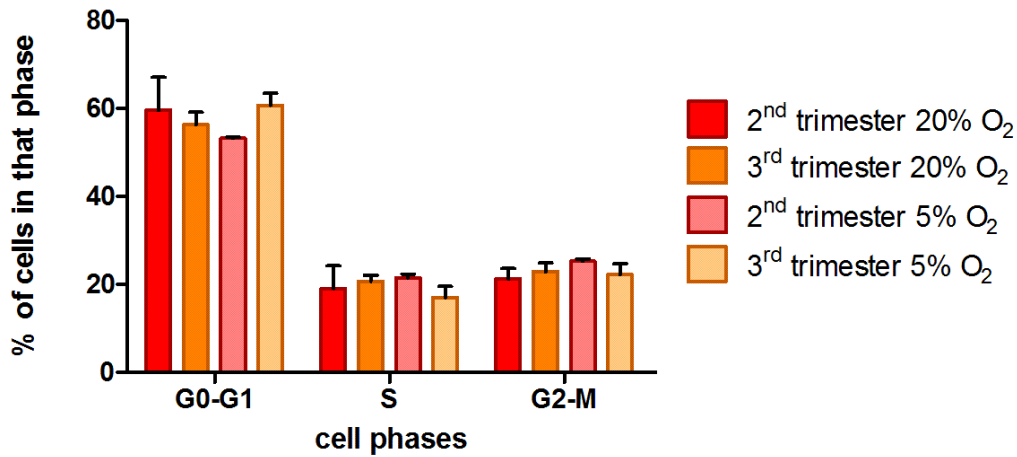


Figure 25 Cell cycle results between trimesters and conditions of oxygen (n=5).

KLF4 pluripotency marker was investigated and again found expressed in AFSCs cultured in 5% oxygen tension with similar levels to those expanded under normoxia for both trimesters (Figure 26).

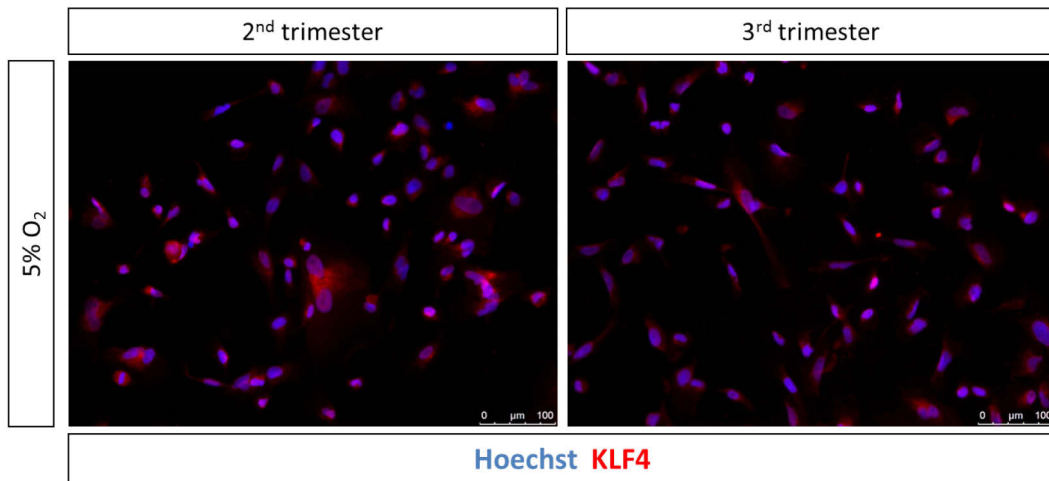


Figure 26 Images of cells stained by IF for presence of pluripotency marker KLF4.

A comparison between hypoxia and normoxia was also conducted on the same markers for flow cytometry used in freshly collected samples. Here there are summarised the results grouped by trimesters showing overlay between 20 and 5% of oxygen (Figure 27). Primarily this was done to see the difference of markers expression after expansion and compare them to freshly collected AF samples (Figure 21). A summarised table of the results for each trimester is found in Table 2.

RESULTS

The phenotype is changed compared to freshly collected samples, concentrating on the original CD117+ population the differences are related to upregulation of some molecule:

- CD29: some cells were already positive in 3rd trimester.
- CD271: some cells are positive in both trimesters, before only in 3rd trimester.
- CD90 and CD73: increased positivity in hypoxic-expanded 2nd trimester cells.
- CD105: increased positivity in hypoxic-expanded cells.
- CD166: upregulated in expanded cells (more in hypoxic-expanded 2nd trimester).
- CD9: 2nd trimester cells became also positive after expansion.
- CD56: slight upregulation, hypoxia seems to reduce expression in 3rd trimester.

Marker	2 nd trimester			3 rd trimester		
	AFTER EXPANSION	COMPARISON TO FRESH	HYPOXIA EFFECT	AFTER EXPANSION	COMPARISON TO FRESH	HYPOXIA EFFECT
CD29	+	>	>	+	>=	=
CD271	+/-	=	=	+/-	=	=
CD90	+	=	>	+	>=	=
CD73	+	>=	>	+	>=	=
CD31	-	=	=	-	=	=
CD105	+	>	>	+	=	>
CD146	+	>=	=	+	>=	=
CD166	+	>	>	+	>	=
CD44	+	=	=	+	>=	=
CD184	+/-	=	=	+/-	=	=
CD9	+	>	=	+	=	=
CD56	+/-	>	=	+	>	<=
CD45	-	=	=	-	=	=
CD34	-	=	=	-	=	=
HLA-ABC	+	>=	=	+	>=	=
HLA-DR	-	=	=	-	=	=

Table 2 results and comparison of AFSCs phenotyping after expansion. + and -: positive and negative; +/- around 50% of positivity; > and <: increased and reduced positivity. Grey cells represent major observable differences.

RESULTS

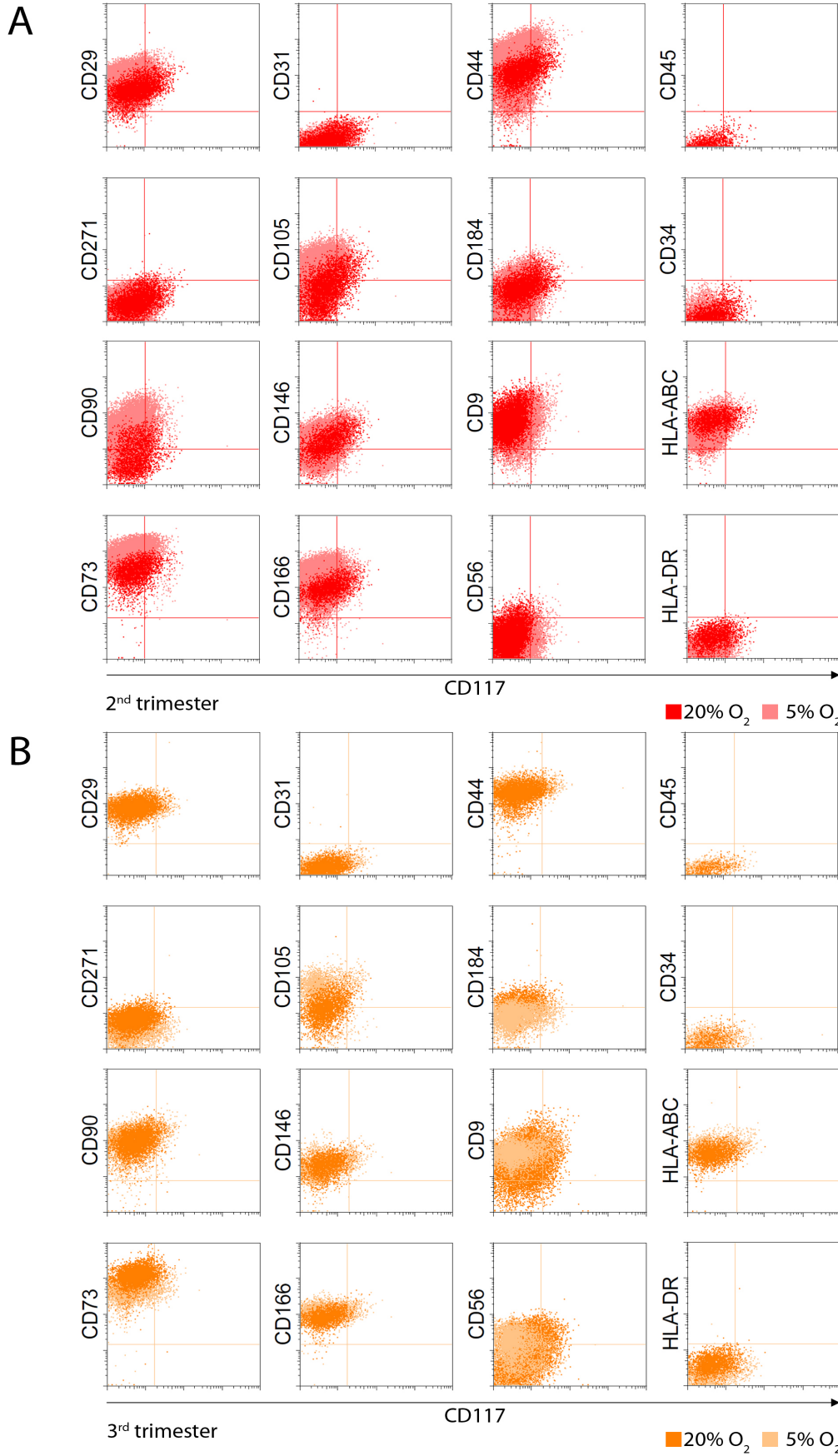


Figure 27 flow cytometry phenotype of 2nd trimester (A) and 3rd trimester (B) AFSCs between 20 and 5% of oxygen culture.

Osteogenic differentiation

To evaluate osteogenic differentiation Von Kossa staining was performed to show calcium deposit in culture (Figure 28), then detection of Alkaline Phosphatase enzyme (ALP, Figure 29) was carried out: both marker of occurring osteogenesis. It results that cells can differentiate to a stage where they can deposit calcium deposits that are found in culture and are appreciable both by specific stainings but also the macroscopic appearance of the bottom of the well looked like some deposit were present (Figure 30 A). Calcium present in each well at different time-points was then quantified (Figure 30 B), 3rd trimester cells present higher quantities of calcium at the final day of the culture (day 21). Also some osteogenic genes have been investigated by RT-PCR (Figure 30 C) showing that differentiated cells express the late osteogenic marker Osteocalcin and still express RUNX2 (involved in osteogenic lineage maturation), this last one was interestingly found also on the undifferentiated cells, meaning that maybe they are prone to differentiate into osteoblasts having this gene already expressed.

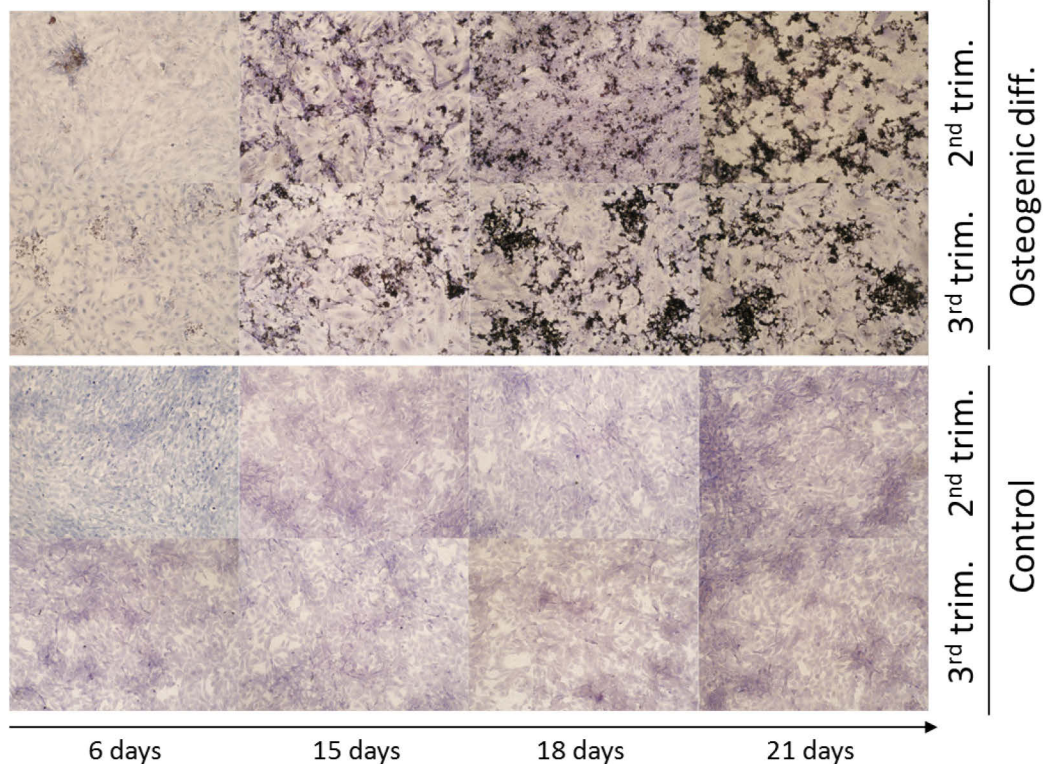


Figure 28 top rows: Von Kossa staining on 2nd and 3rd trimester AFSCs differentiated in osteogenic lineage; bottom rows: controls, undifferentiated cells (original magnification 10X).

RESULTS

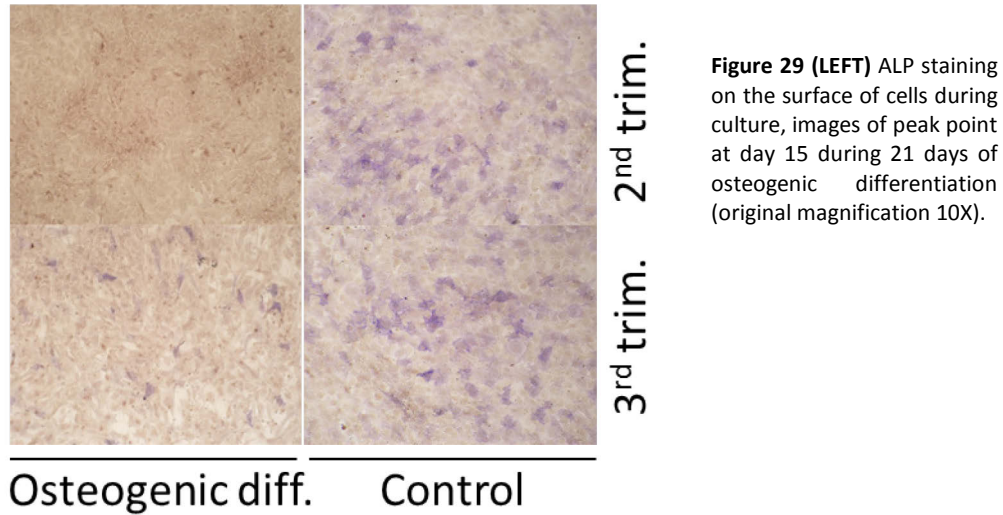
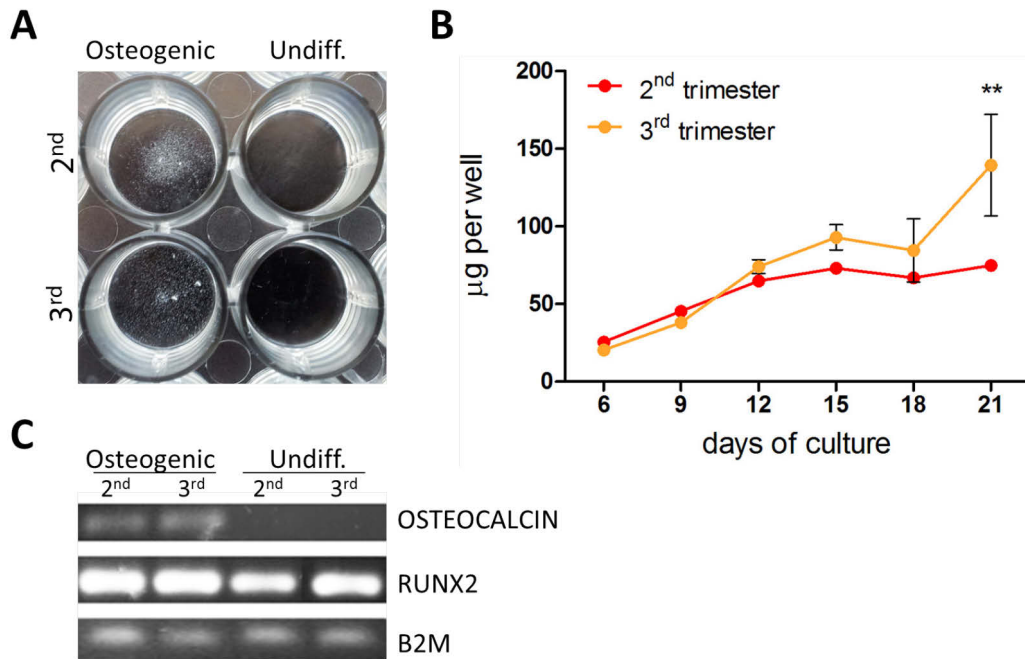


Figure 29 (LEFT) ALP staining on the surface of cells during culture, images of peak point at day 15 during 21 days of osteogenic differentiation (original magnification 10X).

Figure 30 (BOTTOM) A: macroscopic aspect of differentiated and undifferentiated AFSCs towards osteogenic lineage at day 21. B: calcium quantification during osteogenic differentiation in a 24-multiwell plate (n=3; **p < 0.01). C: specific RT-PCR products after electrophoresis in gel of agarose.



Adipogenic differentiation

Adipogenic differentiation was tried both in normoxia and in hypoxia, after 21 days Oil Red O staining was performed to mark lipid droplets inside what seemed to be pre-adipocytes (Figure 31).

Then the dye was extracted from lipids and the absorbance quantified by spectrophotometry (Figure 32). As it seems, adipogenesis is achieved in both oxygen tensions but with contrasting results: at 5% more cells (or more mature lipid vacuoles) are bounded to the specific dye (yielding a higher absorbance after extraction) for 2nd trimester cells, on the contrary AFSCs from the 3rd trimester show less accumulation of Oil Red O.

RESULTS

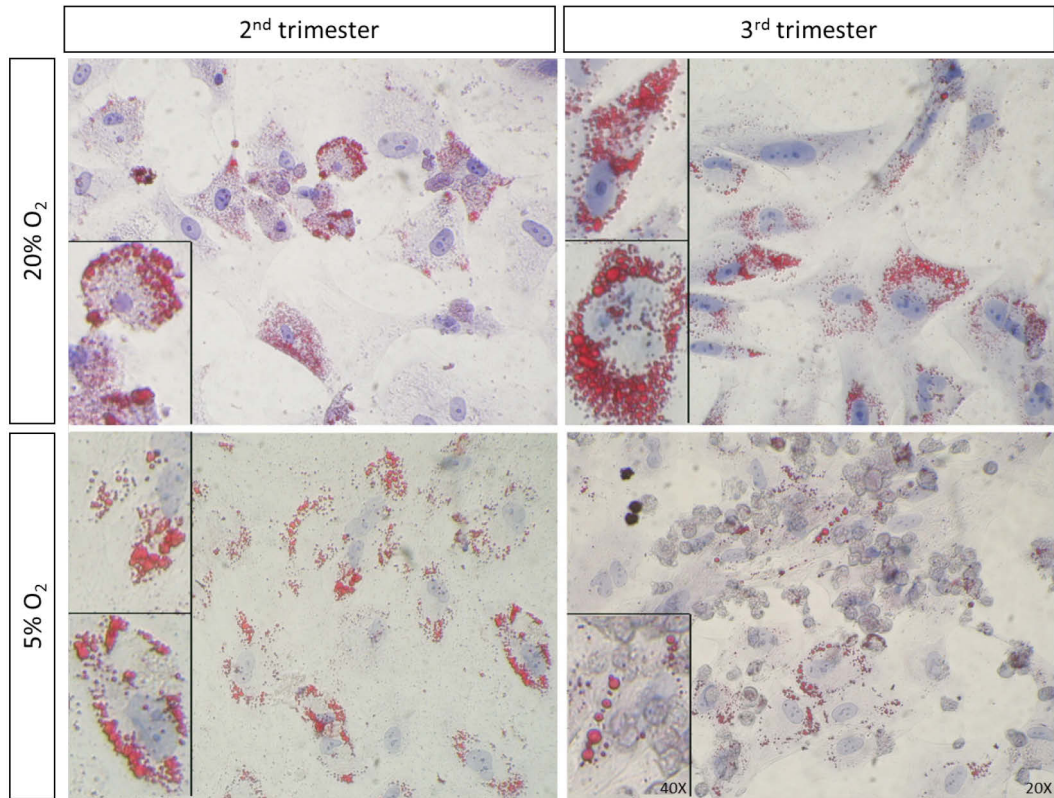


Figure 31 Oil Red O staining on differentiated 2nd and 3rd trimester AFSCs in different oxygen tensions at day 21 (original magnification 20X, 40X for inserts).

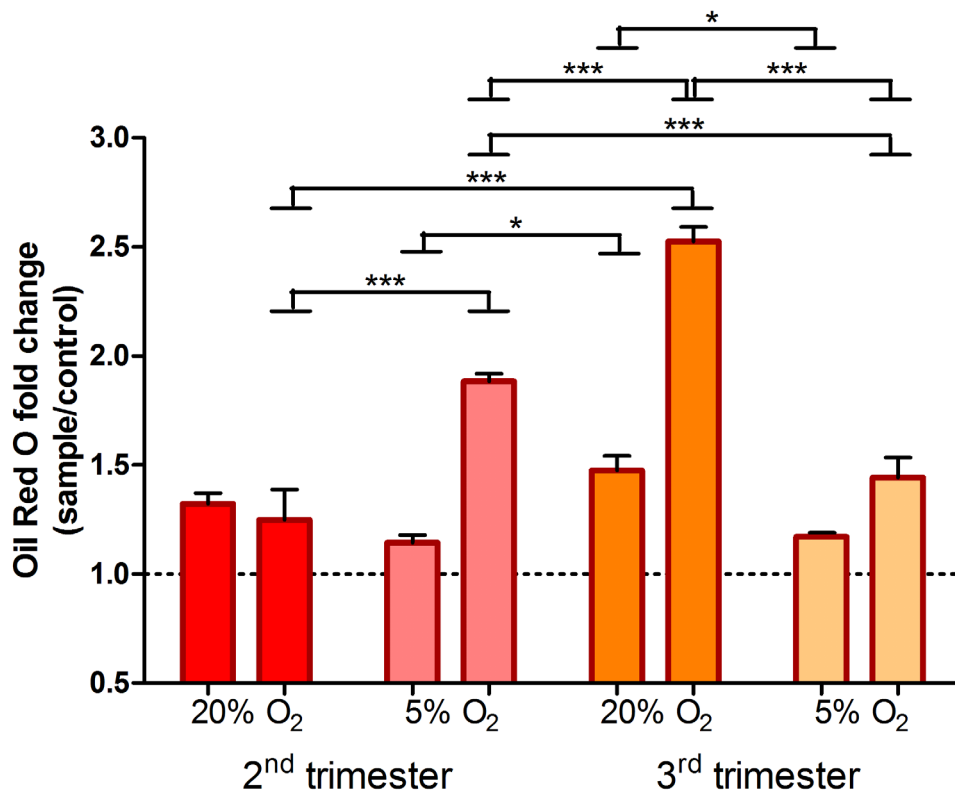


Figure 32 Ratio sample/control (undifferentiated) of extracted Oil Red O absorbance at 518 nm between trimesters and oxygen concentrations (n=3; *p < 0.05, **p < 0.01, ***p < 0.001).

Endothelial differentiation

In vitro

Capillary like structures were formed after using AFSCs in the endothelial cell tube formation assay on basement membrane (Arnaoutova *et al.*, 2009). These meshes were photographed in PhC microscopy (Figure 33) and then analysed. As positive control for the assay, and to obtain a comparable standard, the assay was performed with HUVEC (Figure 34) (Park *et al.*, 2006).

Imaging analysis revealed that the average area of meshes were comparable to those of control cells (Figure 34, "Tot. meshes area") whereas the total number or branches left outside of closed meshes seemed reduced in hypoxia (Figure 34, "Branching interval"). Also, considering the mean size and the abundance of the meshes in each condition the hypoxic condition seemed to favour a higher number of complete structures compared to normoxia (Figure 34, "Mesh index" and "Mean Mesh Size"). For a complete view of analysed parameters see Appendix C.

To test if those structures were possessing endothelial markers, IF for von Willebrand Factor (vWF) was performed (Figure 35) and also verified the ability of those cells to uptake AcLDL in culture (recognizable later because of a fluorophore attached to the molecule) (Figure 36). Cells from both trimesters were showing positive staining for vWF both in normoxia and hypoxia. AcLDL uptake was observable as fluorescent vacuoles after 6 hours of incubation in both trimesters and concentrations of oxygen.

Gene expression analysis by RealTime PCR was also performed after endothelial induction on tube formation assay on Matrigel (Figure 37). HUVEC and CB-derived mononuclear cells were used as positive controls.

These genes were chosen because of a recent published article describing their implication on commitment and role in endothelial differentiation of progenitors and reprogrammed cells (Ginsberg *et al.*, 2012).

Basal levels of ETV2 were detectable in all conditions, after endothelial differentiation ETV2 was downregulated for hypoxic 3rd trimester and instead upregulated for 2nd trimester hypoxic.

FLI1 was present in small quantity on basal normoxic condition, whereas in hypoxia it was more expressed, after differentiation FLI1 decreased in expression on hypoxic conditions reaching levels similar to that of basal normoxic conditions. Almost no expression was detectable in normoxia after endothelial induction.

ERG1 was almost no detectable in hypoxia cultivated and differentiated cells, also basal level in normoxia expanded cells is very low but and upregulation is observed after endothelial differentiation.

RESULTS

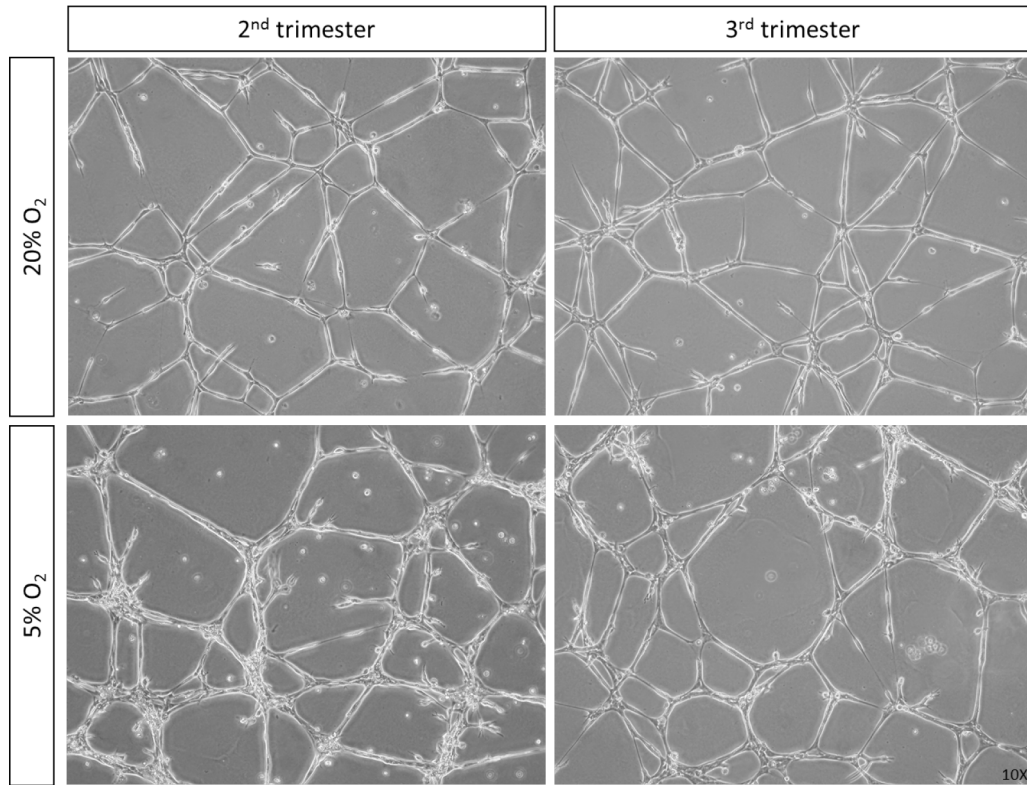


Figure 33 PhC images of AFSCs from 2nd and 3rd trimester after tube formation assay on Matrigel 18h after seeding (original magnification 10X).

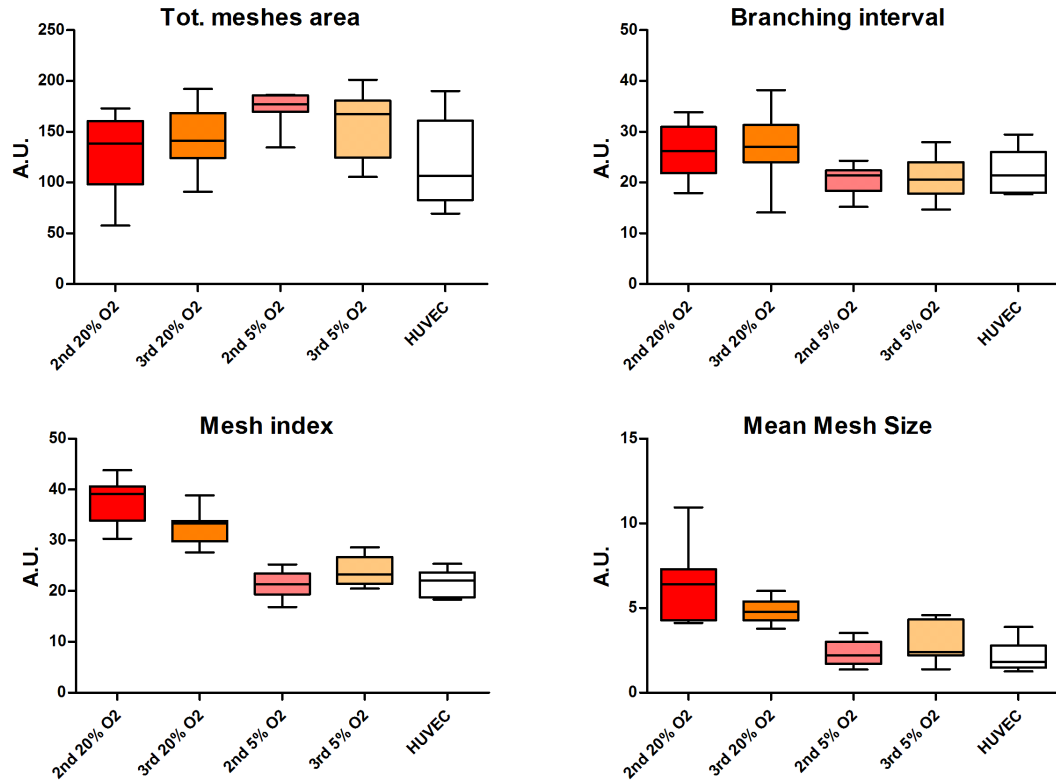


Figure 34 Calculated values by interpolation of PhC images of AFSCs after tube formation assay on Matrigel.

RESULTS

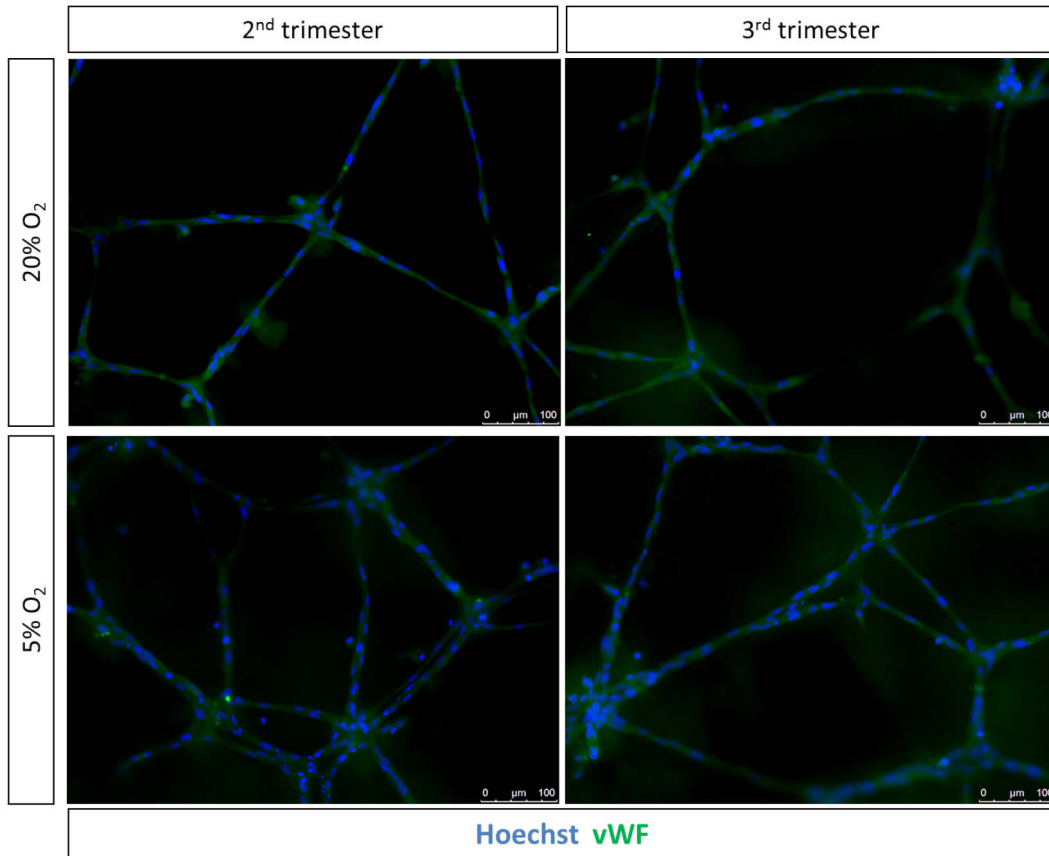


Figure 35 IF showing expression of vWF on differentiated AFSCs (Hoechst as nuclear counterstain).

RESULTS

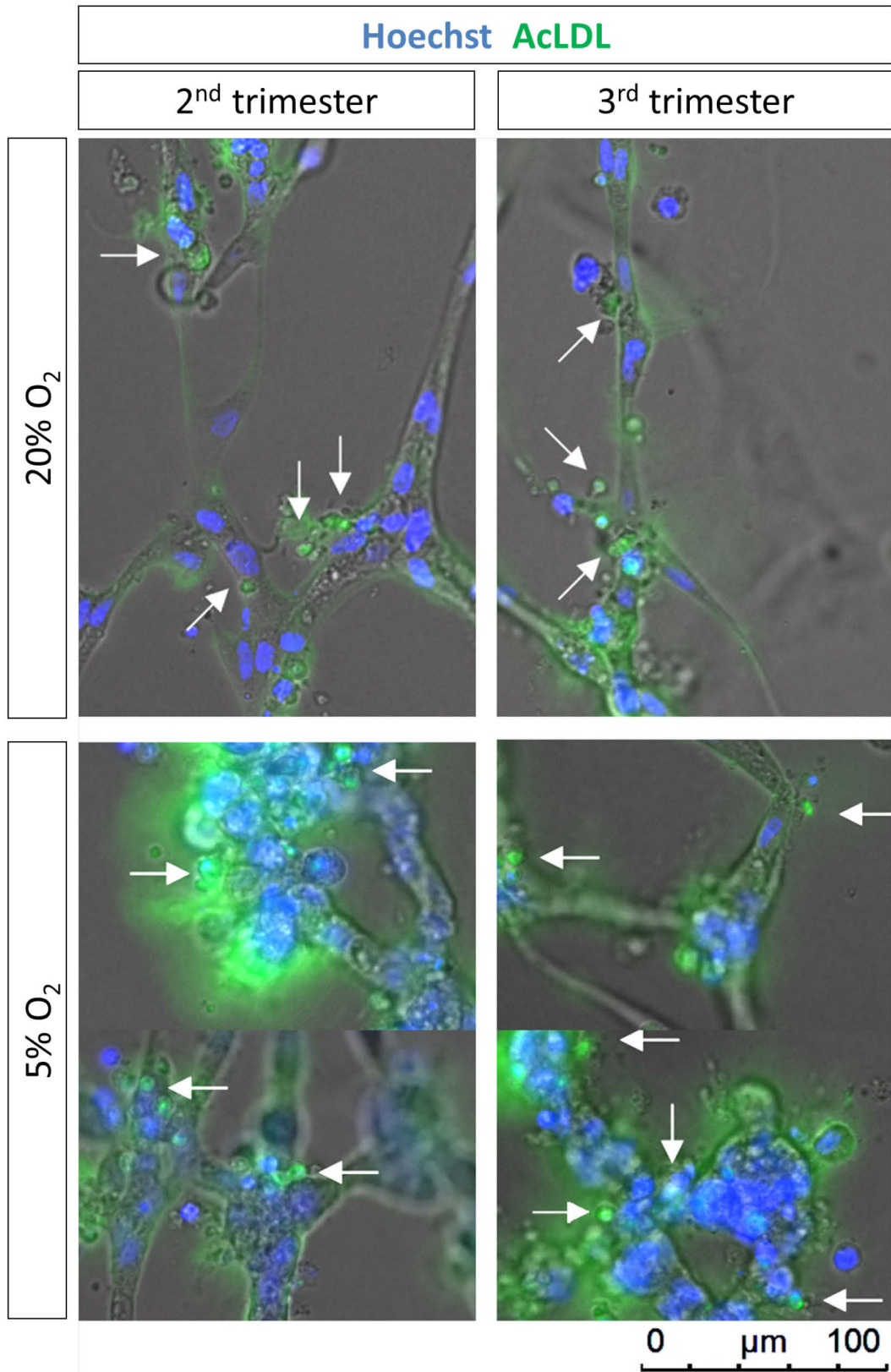


Figure 36 PhC and fluorescence images showing AcLDL (green) uptake by tubular structures formed by AFSCs (Hoechst as nuclear counterstain).

RESULTS

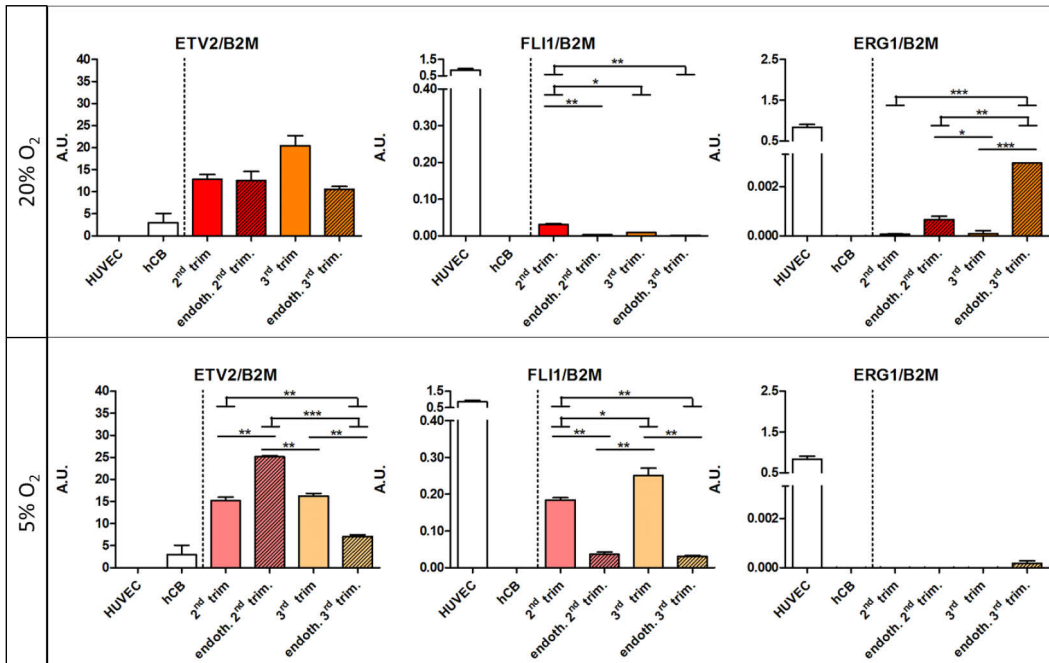


Figure 37 RealTime PCR analysis on AFSCs after tube formation assay on Matrigel. Starting from the left on the histograms: HUVEC (FLI1 and ERG1) or hCB (ETV2) as positive controls, 2nd trimester before and after endothelial differentiation assay, 3rd trimester before and after endothelial differentiation assay (plots show relative expression normalised to housekeeping gene B2M; *p < 0.05, **p < 0.01, ***p < 0.001).

In vivo

Matrigel plugs were harvested from subcutaneous areas of injection after two weeks. Pictures were taken (Figure 38 Aii and Aiii) and then the plugs were either fixed in PFA for immunostaining of snap-frozen for haemoglobin quantification. Evident formation of new vases was observable inside plugs injected with cells (Figure 38 Ai).

Haemoglobin content was then measured for each condition and normalised for the quantity of total proteins in each sample (Figure 38 B). Cells from the 2nd trimester expanded in 5% of oxygen gave the highest Hb content compared to the others. Haemoglobin content gives a relative functionality measure of new vases formed inside the Matrigel plugs, meaning that new circulation was brought inside the implanted material, this do not exclude that newly formed vases derive from the host instead of the injected cells so immunostainings to confirm the contribute of AFSCs to angiogenesis was performed.

Sectioned plugs containing cells presented vases staining positively using antibodies against specific human von Willebrand Factor protein; to be sure in identifying the injected cells, sections were stained also with an antibody against human mitochondria. In Figure 38 C there is a representative image of a circular structure co-expressing the two antigens.

RESULTS

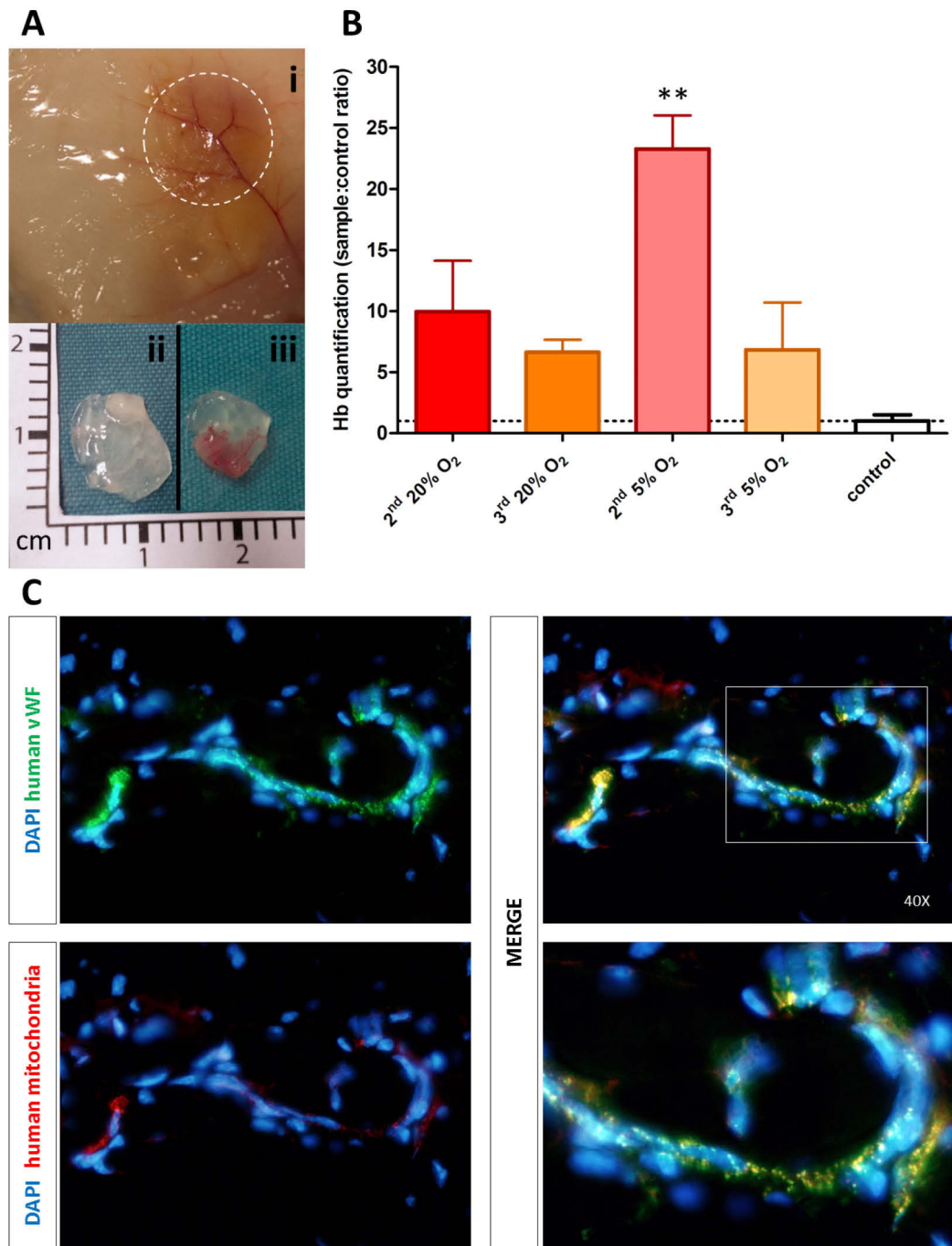


Figure 38 *In vivo* angiogenesis of AFSCs. A: representative image of Matrigel plug appearance at subcutaneous injection site (i) and pictures taken after excision of a control (ii) and a plugs with cells (iii). B: haemoglobin quantification showing fold-change of Hb normalised to total protein content and control plugs (without cells) (** $p < 0.01$, $n=3$). C: IF on sectioned Matrigel plug containing AFSCs (original magnification 40X).

Teratoma test

No evident teratoma formation was found by morphological and histological analysis.

RESULTS

DISCUSSION

In this work it was successfully standardised a method for isolating AFSCs starting from freshly collected samples. This technique isolate specifically CD117⁺ cells in primary AF cultures and then expand them in great scale maintaining their original mesenchymal-like morphology and potential.

Samples from 3rd trimester gestational age were also able to give rise to the same population when cultured with this method.

A phenotypic characterisation of cells deriving from 2nd or 3rd trimester showed great similarity between the two sources of cells but also some differences have been noticed.

The main phenotypic differences are about the expression of CD271 which is present in the CD117⁺ cells from the 3rd trimester but not in the 2nd trimester. 3rd trimester whole population also shows higher level of CD73 in both CD117⁺ and CD117⁻ fractions. Some HLA-DR positive cells are detectable in the CD117⁻ fraction of 3rd trimester AF cells.

But the most remarkable difference – as already told – is about the expression of CD9. The presence of two distinct population positive for one or the other marker (CD9⁺ or CD117⁺) in 2nd trimester AFSCs may indicate an intermediate stage where the CD117 positive population has yet to express CD9, maybe later during the gestation period (like in 3rd trimester AFSCs) they will start to express this marker; this is based also on the fact that cells are presenting the antigen after expansion without compromising some of their differentiation characteristics. Almost certainly a double-positive CD9⁺CD117⁺ population is still of interest, thus CD9 should not be included in some lineage depletion procedure with specific antibodies (even if it is a common marker of adult cells (*e.g.* mastocytes)); this is particularly important for 3rd trimester AFSCs where eliminating CD9⁺ cells would mean also to remove the CD117 fraction.

The same phenotyping, performed after selection and identification of wanted cells, revealed a change in the markers present at the surface of expanded cells. In contrast to fresh samples the expanded cells levelled the differences between trimesters, just for the exception of CD56 which remained not expressed in 2nd trimester whereas became expressed in 3rd trimester AFSCs.

The effect of hypoxia on surface marker expression was most appreciable on CD105 that was nearly present in all cells when cultured at 5% of oxygen, in contrast to normoxia where positivity percentage was variable.

DISCUSSION

The already told difference in CD56 expression was diminished under hypoxic condition: 3rd trimester CD56⁺ cells were about half compared to normoxia. Still, 2nd trimester AFSCs express very low percentage of CD56⁺ cells.

2nd trimester cells also upregulated more CD29, CD90, CD73 and CD166 when cultured in hypoxia than in normoxia.

Interestingly CD9 profile after expansion resulted quasi identical (both expressed this surface molecule) despite the initial differences in fresh samples.

Pluripotency markers are expressed in both trimesters after expansion also at different oxygen concentration, notably KLF4 is counted for the majority of AFSCs cells whereas SSEA4 is more present in 2nd trimester cells at initial passages and equalise with 3rd trimester later in culture.

Also the proliferation rate among the two is similar during normoxic condition, instead when placed in in 5% of oxygen an increase of proliferation for both is noticed. A reduced doubling time was not observed to couple with a variation in percentage of cells into different phases of the cell cycle, so the cells still maintain the proportions of the different phases during mitosis but probably the cells are cycling more rapidly. Time-lapse microscopy is being performed to answer to that question with more certainty, still it remains a fact that expanding cells in hypoxia greatly increase the number of cells that can be obtained after expansion of a single sample of AF. The later experiments on phenotype characterisation and potential of differentiation were made to verify the potency between trimesters and also see if a difference was observable when culturing the cells in 20% or 5% oxygen tension.

Osteogenesis is achieved from cells of both trimesters, quantification of calcium deposition show a higher capacity of mineralisation by 3rd trimester AFSCs, both presented Osteocalcin expression (a marker of late osteogenesis) and still expressed RUNX2 after 21 days of differentiation. Macroscopic appearance also seems to confirm those results.

Osteogenic differentiation was not performed in 5% of oxygen because it is known that hypoxic environment inhibit this type of induction with a well-known method: during hypoxic condition the HIF-1 α subunit of the Hypoxia-inducible factor-1 (HIF-1) factor is upregulated and among other effects in cell metabolism it results in the activation of the HIF-TWIST pathway that ultimately leads to downregulation of the osteogenic gene RUNX2. Without RUNX2 cells are not able to underwent osteogenic differentiation also because RUNX2 is necessary to activate others osteogenic specific genes like Osteopontin and also Alkaline Phosphatase activity is reduced (Tsai *et al.*, 2012).

Adipogenic potential is also conserved among cells of both trimesters but with inverted tendencies. 3rd trimester show a higher rate of differentiation

(comparing the size of lipid droplets and the number of differentiated cells) in normoxia, whereas if adipogenesis is performed at 5% of oxygen there are few cells showing positive staining. On the other hand the hypoxic condition seems to increase the quantity of cells differentiated from 2nd trimester AFSCs, where instead 20% of oxygen results are comparable to those of 3rd trimester at 5% (low differentiation). In literature there are contrasting opinions about the benefits of oxygen conditions in adipogenesis, it has to be noticed that in some work on reduced adipocyte differentiation is described but they also use a very low concentration of oxygen (1%) (Carriere *et al.*, 2004), other authors instead describe a potent adipogenic effect exerted by hypoxic conditions (Chung *et al.*, 2009a) so it is probable that oxygen levels are important for adipocytes formation but maybe a dynamic combination of O₂ tensions and specific differentiation pathways are not easily reproducible during *in vitro* assays.

About angiogenic potential it was demonstrated that 5% of oxygen permits angiogenesis. Considering the well characterised HUVEC population as a control for endothelial committed cells, it seems that during cell tube formation assay the AFSCs cultivated in hypoxia form structures more similar to HUVEC than the counterpart cultivated in normoxia. Interestingly it can be postulated that AFSCs are plastic enough to have the angiogenic program opened and ready to act, considering the low amount of time needed to generate endothelial-like cells also from the functional point of view (AcLDL uptake). Gene expression reveals that AFSCs have very high levels of ETV2 present constitutively, this is a key regulator for commitment into endothelial lineage (whereas HUVEC do not present this transcript natively because they are fully committed, Cord Blood endothelial progenitors show instead very little expression of ETV2 compared to AFSCs). Also FLI1, which is typical of a more mature endothelial cell, is expressed in AFSCs: very low levels are expressed in normoxia-cultivated cells and it is downregulated after tubule formation assay, hypoxic-cultivated cells show higher expression of FLI1 before induction and they also present down regulation after. These two genes and their specific levels of expression may be enough to maintain AFSCs in a state of pre-endothelial cells (but as already demonstrated they are not fully committed because of their ability to differentiate also in other lineages). Notably ERG1 is instead not expressed in AFSCs cultivated and differentiated in hypoxia, also normoxic-cultivated cells do not express ERG1 but they present an upregulation after endothelial differentiation. This gene has been demonstrated not to be expansible for endothelial differentiation (Ginsberg, Cell 2012), it is instead required for cell commitment; considering that cells were analysed at day 0 and day 3 after induction it should be interesting to evaluate the presence of this gene in between the 72 hours of differentiation for

DISCUSSION

the hypoxic condition and see if the upregulation in normoxia is instead the residue of a transient expression in early stages of culture.

When comparing the *in vivo* results of AFSCs it is clear the difference on *de novo* angiogenesis of cells compared to controls. A striking formation of new vases is encountered when injecting cells from the 2nd trimester expanded in hypoxia, this could be related to the specific presence of ERG2 and FLI1 in these cells, strangely 3rd trimester expanded at 5% of oxygen did not yield a similar result even sharing the same pattern of expression for these three genes; considering the high upregulation of ERG2 on 2nd trimester AFSCs during *in vitro* assays, one can hypothesise that induced progenitors proliferate and develop more when compared to those of the hypoxic-cultivated 3rd trimester cells (which has a downregulation of ERG2 *in vitro* after induction).

About safety on perspective use of these cells in regenerative medicine purposes, no teratoma was found when these cells were injected into immunodeficient recipients.

Concluding, it was demonstrated that AFSCs can be isolated from AF samples not only from mid gestational period after amniocentesis but also from samples of the final period of pregnancy. This is very important because amniocentesis is becoming more rare and so here is reported a method for collecting and obtaining AFSCs during childbirth, without particular effort or ethical concern considering that AF is usually discarded during delivery.

AFSCs deriving from both trimesters shows similarity about phenotype and potency of differentiation.

Moreover some beneficial effect of hypoxia in cultivating AFSCs have been described. Cells expanded in 5% of oxygen also showed a pronounced effect on angiogenesis *in vivo*, more evident in 2nd trimester cells.

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APPENDIX A

Antibodies used for flow cytometry and relative manufacturer, concentrations and application following original specifications.

ANTIBODY	MANUFACTURER
CD29	Biolegend©
CD271	Miltenyi Biotec©
CD90	Biolegend©
CD73	Biolegend©
CD31	Biolegend©
CD105	Beckman Coulter©
CD146	BioCytex™
CD166	Beckman Coulter©
CD44	eBioscience©
CD184	Beckman Coulter©
CD9	Biolegend©
CD56	Beckman Coulter©
CD45	BD Pharmingen©
CD34	Miltenyi Biotec©
HLA-ABC	Beckman Coulter©
HLA-DR	Beckman Coulter©

Antibodies used for immunofluorescence, relative manufacturers, dilutions and conditions in which they were used.

	MANUFACTURER	DILUTION	INCUBATION TIME - TEMPERATURE
PRIMARY ANTIBODIES			
CD117	Santa Cruz Biotechnology©	1:80	1 h – 37°C
SSEA4	Abcam©	1:80	1 h – 37°C
KLF4	Santa Cruz Biotechnology©	1:50	O.N. – 4°C
von Willebrand Factor	Dako©	1:100	1 h – 37°C
Human mitochondria	Abcam©	1:80	1 h – 37°C
SECONDARY ANTIBODIES			
Alexa Fluor® 594 Goat Anti-Mouse	Molecular Probes®	1:150	1 h – 37°C
Alexa Fluor® 488 Goat Anti-Mouse			
Alexa Fluor® 594 Chicken Anti-Rabbit			
Alexa Fluor® 488 Chicken Anti-Rabbit			

APPENDIX B

Primers forward (Fw) and reverse (Rv) used for RT-PCR and/or RealTime PCR:

TARGET GENE	SEQUENCE (5'→3')	
RUNX2	Fw	AGAGGTACCAGATGGGACTGTGGTT
	Rv	GGTAGCTACTTGGGGAGGATTTGTG
Osteocalcin	Fw	TGAAGAGACCCAGGCGCTA
	Rv	GATGTGGTCAGCCAACTCGTC
β2 Microglobulin	Fw	GCTGTGCTCGCGCTACTCT
	Rv	CAACTTCAATGTCGGATGGATG
ETV2	Fw	CCGACGGCGATACCTACTG
	Rv	GTTCGGAGCAAACGGTGAG
FLI1	Fw	GAGGAGCTTGGGGCAATAAC
	Rv	AGAGCAGCTCCAGGAGGAAT
ERG1	Fw	TGCTCAACCATCTCCTTCCA
	Rv	TGGGTTTGCTCTTCCGCTCT

APPENDIX C

