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Maternal serum, an isolation and expansion tool for umbilical cord matrix mesenchymal stromal cells.

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Abstract

The umbilical cord offers a source of readily available mesenchymal stromal cells for use in research and ultimately therapeutic application. However, methods of isolating these cells vary between investigators, and no standard method has been adopted. The aims of this work were to i) develop a methodology for the isolation of umbilical cord matrix cells without the use of enzymatic digestion or complicated dissection; ii) investigate the use of pooled maternal serum as a media supplement; and iii) to demonstrate that the cells isolated were mesenchymal stromal cells.

We have demonstrated that incubating tissue explants of less than $2mm^3$ in serum for an hour, followed by the gradual addition of serum containing culture medium can increase cell yield compared to incubation in serum containing culture medium alone. More importantly, our method demonstrated that the use of pooled serum from women > 37 weeks pregnant (pooled maternal serum) yields higher cell numbers than the use of fetal bovine serum or pooled umbilical cord serum. Irrespective of the type of serum used the isolated cells were mesenchymal stromal cells according to the minimal criteria set out by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy.

In conclusion, maternal serum has the potential to be used as an alternative to FBS for isolation and expansion of umbilical cord MSCs for clinical purposes.

Impact Statement

The umbilical cord is a source of readily available MSCs for use in research and ultimately therapeutic application. However, alternative serum sources to FBS are needed to maximise the use of MSCs for clinical purposes. This paper demonstrates that pooled serum from women > 37 weeks pregnant (pooled maternal serum) could be used as an alternative for isolation and expansion of UCMSCs, as it avoids the issues surrounding the use of FBS. It is also shown to be more effective than FBS and cord serum in producing a larger yield of MSC's, which is promising for future stem cell therapy.

1 Introduction

The umbilical cord matrix or Wharton's Jelly which surrounds the two umbilical arteries and one umbilical vein, is known to contain mesenchymal stromal cells (MSCs) [1,2]. MSCs are spindle shaped cells that have the ability to self-renew, and to undergo osteogenic, adipogenic, chondrogenic, myogenic, and stromal cell differentiation, demonstrating a high degree of plasticity [3-5]. Given the interest in the use of MSCs in regenerative medicine and the ethical challenges imposed by embryonic stem cells, the umbilical cord offers a readily available source of MSCs for use in both basic research to better understand human MSC biology, and in therapeutic applications.

Many different methods of isolating MSCs from umbilical cord have been investigated, and no standard method has been adopted. The main differences in methods used are associated with whether or not the blood vessels were drained and/or removed, whether enzymatic treatment was used, the methods of harvesting Wharton's jelly, and the culture approach used (either culture of the tissue or of the isolated cells). There is some variation in the culture media used but Dulbecco's Modified Eagle's Medium (DMEM) is typical.

Compounding the variation in methods used to isolate the cells are the different MSC characterisation methods used, especially prior to 2006. In 2006 the minimal criteria to define human MSCs were set out by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) [6]. The three minimal criteria are: 1. plastic adherence; 2. expression of certain surface markers - at least 95% of the cell population must express CD73, CD90, and CD105, and less than 2% of the cell population can express CD14 or CD11b, CD34, CD45, CD79α or CD19, and HLA class II (HLA-DR);

and 3. differentiation potential – as a minimum the cells must be able to undergo osteogenic, adipogenic, and chondrogenic differentiation. Since 2006 a number of investigators refer to these minimal criteria to define umbilical cord MSCs [7-11].

Although different methods of isolating and characterising MSCs from umbilical cord have been investigated, more recently, isolation methods have been referred to generally as either "enzymatic" or "explant" [8, 12, 13]. Still, no standard method has been adopted as being the most effective. Different groups have adapted their own methods to suit their style of working. Some of these methods involve quick isolation of cells, but complicated methods of digestion [1, 7, 9, 14]. Others involve dissection of the umbilical cord by removal of blood vessels [10, 12, 15, 16]. Two important factors to consider when choosing an isolation method are cell yield and the time taken to achieve a desirable cell yield; these must be well balanced for therapeutic gain.

Recently, it has been highlighted that there is a need to find alternatives to FBS for use as a media supplement for MSC culture, due to the risk of transmitting pathogens that may cause viral infections and bacterial infections, transmission of prions, inflammatory reactions due to contamination with bovine proteins, and ethical issues with collection of animal products [11, 12, 15, 17]. Alternatives have been considered: MSCs have been cultured successfully in autologous [18, 19] and non-autologous [20, 21] human serum, human platelet lysate [12, 17, 20], autologous cord blood serum [20, 22], and autologous cord blood platelet rich plasma [23, 24]. Serum is collected from whole blood after it is left for a specific set time to clot and is then subsequently centrifuged [25, 26]. Special blood tubes are available for serum collection containing no anticoagulants, and plasma collection containing anticoagulants [26].

Plasma is collected from whole blood by centrifugation, which can be followed by a further centrifugation to remove platelets [26, 27]. Platelet rich plasma is produced when the platelet pellet is re-suspended in the plasma after centrifugation [27, 24]. Platelet lysate is produced when the platelet rich plasma is taken through a number of freeze thaw cycles [25, 28, 29]. To our knowledge serum taken from the blood of the Mother has not been investigated as a culture supplement for umbilical cord MSCs.

Here, the aims were to: i) optimise a protocol for the isolation of umbilical cord matrix MSCs without the use of complicated digestion or dissection methods; ii) investigate the use of pooled maternal serum as a media supplement for isolation and culture of umbilical cord matrix MSCs; and iii) to demonstrate that the isolated cells are MSCs as defined by the Mesenchymal and Tissue Stem Cell Committee of the ISCT.

2 Materials and Methods

2.1 Ethics and Samples

Umbilical cords and umbilical cord blood were collected from women undergoing elective caesarean section at full-term (>37 weeks of gestation) at Singleton Hospital, Swansea. Women undergoing elective section for fetal or maternal anomalies were not recruited therefore samples were typically from women scheduled for caesarean section due to breech presentation, cephalo-pelvic disproportion or emergency caesarean section at previous delivery. Maternal blood was collected from women in the 1-3 days leading up to their elective caesarean section. All samples were collected with informed written consent and

ethical approval was obtained from the South West Wales Research Ethics Committee (REC No. 11/WA/0040). Serum was prepared by collecting blood into serum separation tubes (Greiner Bio-one, Gloucestershire, UK), allowing clotting for 90 minutes and centrifugation to collect serum which was aliquoted and stored frozen at -80ºC. After centrifugation, samples were processed aseptically in a class II tissue culture cabinet.

2.2 Umbilical Cord Collection and Preparation

The placenta was collected at elective caesarean section and a 10 - 12cm long piece of umbilical cord proximal to the placenta was excised within two hours of delivery. This was washed in phosphate buffered saline (PBS; Life Technologies, Paisley, UK) two to four times to remove excess blood. The piece of cord was then placed in Hank's balanced salt solution (HBSS; Life Technologies) and kept in the refrigerator until processing (two hours).

2.3 Optimising MSC Isolation Protocol

2.3.1 Size of Umbilical Cord Explants

An explant approach was taken to isolate MSCs from the umbilical cord. The effect on MSC yield of explant size at the initiation of culture was investigated first. Segments of cord measuring 2cm were cut in half. Each pair of halves were then cut into (i) six pieces of equal size, (ii) ten pieces of equal size, or (iii) multiple small pieces measuring $<2mm^3$. Pieces generated from each half were placed into individual T25 tissue culture flasks (2 flasks/tissue size; Greiner Bio-One) with 4ml low glucose, phenol red free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% glutamax, 10% Fetal Bovine Serum (FBS), 1% penicillin streptomycin fungisone (PSF) (100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B), and 0.1% non-essential amino acids (NEAA) (all Life

Technologies), hereafter called complete DMEM. This was designated as day 0. On day 2 an extra 2ml of the same medium was added to each flask.

In parallel, another 2cm segment of umbilical cord was cut in half, each half was chopped into small pieces (<2mm^3), placed into T25 flasks, and 500µl of FBS was added. An hour later 1ml complete DMEM medium was added to each flask (designated day 0). A further 1ml of medium was added to the flasks every day until day 5.

On day 8 the tissue and medium were removed from all flasks, the flasks were rinsed with PBS (Life Technologies), and fresh medium was added. On day 15 the cells were harvested and counted (see below).

2.3.2 Serum Used

FBS was compared with umbilical cord blood serum (CS) and pooled serum prepared from peripheral blood of women >37 weeks pregnant (pooled maternal serum; MS). Umbilical cord serum [20, 22], umbilical cord platelet rich plasma [23, 24] and human serum (not from pregnant donors) [18, 20, 21] have been reported elsewhere as a supplement in MSC isolation media. Segments of cord measuring 1cm were cut in half, each half was chopped into small pieces (<2mm^3), and the small pieces were placed into 6 well plates. FBS, CS, or MS was then added 250µl/well in duplicate (not paired cord halves). After 1hr, 500µl low glucose phenol red free DMEM supplemented with 10% FBS, CS, or MS (+ glutamax, PSF, and NEAA as above) was added and then 500µl every day until day 3, to give a final volume of 2ml in each well. On day 8 the tissue and medium were removed, each well was rinsed with

PBS, and fresh corresponding medium was added. On day 15 the cells were harvested and re-seeded in T25 culture flasks (one well to one T25 flask). On day 18 the cells were harvested and counted.

2.4 Cell Harvesting and Counting

For harvesting, the cells were first rinsed with PBS to remove any remaining medium and then detached using 0.05% Trypsin/EDTA (Life Technologies) at 37°C for 8 to 10 minutes. Trypsin was neutralised by the addition of FBS containing medium. The cells were pelleted by centrifugation at 400 x g for 5 minutes. Cells were re-suspended in complete medium and counted using a C-Chip disposable haemocytometer (Labtech International, East Sussex, UK).

2.5 Cell Cryopreservation and Resuscitation

Cells were cryopreserved in 1ml of 10% DMSO (Sigma-Aldrich, Dorset, UK) in complete DMEM with $1x10^6$ cells per vial. Cryopreservation vials (2ml, Greiner Bio-One) were placed in a Nalgene cryopreservation 1ºC freezing container (Thermo-Scientific, Leicestershire, UK) containing 250ml of 2-propanol (Sigma-Aldrich) at -80°C for 24 hours with a decrease in temperature of 1ºC/min. The vials were then transferred to liquid nitrogen storage.

For cell resuscitation, vials were removed from liquid nitrogen and warmed media was added to the vials gradually to thaw the cell suspension. Once thawed, the cell suspensions were transferred to larger vessels containing warmed medium. The cells were then pelleted by centrifugation at 400 x g for 5 minutes to remove any DMSO.

2.6 Cell Characterisation

2.6.1 Cell Surface Phenotype by Flow Cytometry

The phenotype of previously cryopreserved cells isolated using FBS, CS, and MS were investigated by incubating $1x10^5$ cells with antibodies shown in Table 1 (all from eBioscience, Hatfield, UK) including markers used for phenotyping as set out by the ISCT [6] and an unstained control. The cells were incubated with pre-optimised concentrations of antibody in the dark on ice for 30 minutes. After washing the cells with 0.2% BSA/0.05% sodium azide in PBS, the cells were fixed (BD Cytofix; BD Biosciences, Oxford, UK) and acquired on a flow cytometer (FACS Aria I; BD Biosciences) within 24 hours. Compensation was conducted using single stained cells for each fluorochrome used. Analysis of acquired data was undertaken using Kaluza Flow Cytometry Analysis software (version 1.2, Beckman Coulter). The signal indices were calculated by dividing the median fluorescence of the stained cells by the median fluorescence of the unstained cells.

2.6.2 Differentiation

2.6.2.1 Adipogenesis

Cells were seeded at $3x10^3$ cells per cm^2 in a 24 well plate. After 5 days of culture the media was removed and replaced with 500µl/well adipogenesis differentiation medium (Adipocyte Differentiation Basal Medium supplemented with 10% Adipogenesis supplement, 1% PSF Life Technologies). The media was replaced every 3 to 4 days. On day 16 the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and stained with Oil Red O staining solution (300mg Oil Red O powder (Sigma-Aldrich) in 100ml 99% isopropanol (Fisher Scientific); 6ml of this solution was added to 4ml of distilled water, incubated for 10 minutes

at room temperature then filtered through Whatman filter paper (Whatman International, Maidstone, UK)).

2.6.2.2 Chondrogenesis

Cells were seeded at $1x10^5$ cells in 5µl droplets of low glucose phenol red free complete DMEM in the centre of a well in a 24 well plate. After 2 hours, 500µl/well chondrogenesis differentiation medium (Chondrocyte Differentiation Basal Medium supplemented with 10% Chondrogenesis supplement, 1% PSF; Life Technologies) was added. The media was replaced every 2 to 3 days. On day 23 the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and stained with Alcian Blue staining solution (1mg Alcian Blue powder (Sigma-Aldrich) in 99ml 0.1N hydrochloric acid, HCl (Sigma-Aldrich)).

2.6.2.3 Osteogenesis

Cells were seeded at $6x10^3$ cells per cm^2 in a 24 well plate. After 4 days of culture the media was removed and replaced with 500µl/well osteogenesis differentiation medium (Osteocyte Differentiation Basal Medium supplemented with 10% Osteogenesis supplement, 1% PSF; Life Technologies). The media was replaced every 3 to 4 days. On day 25 the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and stained with Alizarin Red S staining solution (2g Alizarin Red S powder (Sigma-Aldrich) in 100ml distilled water and the pH was adjusted to 4.2).

2.7 Statistical Analysis

Paired t-tests and ANOVA were performed on the results of the MSC isolation experiments using the SPSS statistical software package (version 19, IBM). A p-value <0.05 was considered significant.

3 Results

3.1 Optimising the Isolation Protocol – Size of Umbilical Cord Explants

The number of putative MSCs isolated from the umbilical cord increases with decreasing size of the tissue explants (Figure 1). However, the only significant difference (for pieces + media) was between 10 pieces of tissue and 6 pieces of tissue ($p=0.041$; $n = 3$). Seeding < $2mm^3$ explants of tissue onto 500µl FBS and then increasing the total volume of media in the flask gradually over the next 5 days yielded significantly more putative MSCs than seeding explants of the same size directly with 4ml media ($p=0.011$; $n = 3$; Figure 1).

3.2 Optimizing the Isolation Protocol – Serum Used

Having shown that seeding small explants onto a bed of FBS rather than into 4 ml complete medium increased the yield of putative MSCs, the possibility that different serum substrates might further improve cell yield was investigated. The substrates chosen were umbilical cord serum and pooled maternal serum prepared from women who were > 37 weeks pregnant. The use of pooled maternal serum gave the greatest yield of cells (Figure 2); significantly more than FBS ($p = 0.004$; $n = 3$) and more than cord serum but not significantly so ($p = 0.065$; $n = 3$).

3.3 Characterisation of Umbilical Cord Matrix-Derived Cells

3.3.1 Plastic Adherence

Irrespective of the substrate used for seeding and culture (FBS, cord serum, pooled maternal serum) the cells derived from the umbilical cord matrix explants were plastic adherent and had a fibroblast-like structure on day 18 (Figure 3).

3.3.2 Phenotype Marker Panel by Flow Cytometry

To confirm that irrespective of the serum used the cells isolated were MSCs, cell phenotypes were studied using flow cytometry. The cells isolated using FBS, umbilical cord serum, or pooled maternal serum were all negative for CD14, CD19, CD34, CD45, and HLA-DR (Figure 4a) and were all positive for CD73, CD90, and CD105 (Figure 4b). Overall there were no differences in the level of expression of these markers on MSCs isolated using different sera with the exception of CD105 which was significantly higher on cells isolated using umbilical cord serum ($p=0.047$; ANOVA; Figure 4c).

3.3.3 Differentiation

Cells isolated using FBS, umbilical cord serum, and pooled maternal serum were all able to undergo adipogenesis, chondrogenesis, and osteogenesis (Figure 5).

Table 2 summarises the characterisation results for the cells isolated using each of the three sera confirming that they match the minimal criteria for MSCs as set out by the Mesenchymal and Tissue Stem Cell Committee of the ISCT [6].

4 Discussion

Here we describe an explant method for isolation of MSCs from human umbilical cord matrix. The smaller the explant the greater the yield and seeding the explants on serum and gradually increasing the culture volume also enhanced yield. On comparing serum sources – fetal bovine serum, umbilical cord blood serum, or serum from women > 37 weeks pregnant – the latter gave the greatest yield of MSCs. Irrespective of the serum source used for seeding and media supplementation, the cells isolated from small explants of umbilical cord met the minimal criteria for MSCs based on surface marker expression and differentiation potential [6].

There is a belief that the minimal criteria set out by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy are no longer a robust enough method of determining MSC authenticity [30], as they could be used to characterise fibroblastic cell strains [31]. Moreover, there are reports in the literature supporting a theory that transplanted MSCs themselves do not differentiate to form new tissue *in vivo*, but rather migrate to a site of injury or disease and secrete factors which stimulate the resident stem cells to construct new tissue [32]. This is clearly an important area for further research.

Nevertheless, the minimum criteria of the ISCT are still being used to define MSCs [33] as there have been no new guidelines set out for MSC characterisation. It is important to note, therefore, that even though our cells have met the minimal criteria, the true *in vivo*

differentiation potential of these isolated cells remains unknown and is worth further investigation.

We have also demonstrated here that the cells met the minimum criteria through identification as being CD34 negative. There have been reports stating that MSCs are in fact CD34 positive [34, 35]. However, these reports also state that it has been demonstrated that any tissue resident CD34 positive cells lose their expression after expansion in culture, which would account for our CD34 negative result. There may be CD34 positive MSCs within the umbilical cord matrix, but so far none have been identified.

An explant approach was chosen to avoid the limitations of introducing tissue digestion enzymes into the isolation procedure. The number of cells isolated from the umbilical cord increased with decreasing size of the tissue explants so that small pieces measuring $< 2mm^3$ yielded the most. This approach likely increases the tissue surface area and enables more cells to migrate out of the tissue and onto the plastic surface of the culture flasks. Other authors similarly minced or cut the tissue into small pieces or fragments measuring around 1-$5mm^2$ [5, 12, 16]. We found that cell yield could be increased further when the explants were first seeded onto a small volume of FBS for an hour, followed by the gradual addition of 1ml of medium each day rather than immediate seeding into culture media. The most likely explanation for this is exposure to increased concentrations of key factors, such as chemokines or growth factors, in the serum.

Given the need for alternative supplements to FBS for MSC culture, FBS was compared to cord serum and serum prepared from pregnant women (> 37 weeks pregnant; full-term). After 18 days of culture, explants seeded on and cultured in pooled maternal serum yielded more cells than either FBS or cord serum. This would suggest that pooled maternal serum draws more cells from the explant and/or has a greater proliferative effect on the cells than FBS or cord serum, at least in the short term. While there are no reports comparing relevant mediator levels in FBS, cord, and serum from pregnant women a number of studies highlight differences between serum from pregnant and not pregnant women. For example, TGFß1 is elevated in pregnant versus not pregnant women [36] and it would be worthwhile extending this phenotype to growth factors and cytokines with a role in MSC proliferation.

Alternative serum sources to FBS are needed to maximise the use of MSCs for clinical purposes, due to the risk of contamination, inflammatory reactions, and ethical issues [11, 12, 15, 17]. Alternative supplements have been used successfully, such as human serum [18-21], human platelet lysate [12, 17, 20], cord blood serum [20, 22], and cord blood platelet rich plasma [23, 24]. The difference between serum and platelet rich plasma is that serum is collected from centrifuged whole blood which was left to clot [25, 26], whereas platelet rich plasma is collected when un-clotted whole blood is centrifuged, the plasma is removed, and the platelet pellet is re-suspended in the plasma [26, 27, 24]. To our knowledge, the use of maternal blood serum has not been investigated. We now show that serum prepared from the blood of full-term pregnant women, with further investigation, has the potential to be an alternative to FBS for isolation and expansion of umbilical cord MSCs for clinical purposes. The use of serum prepared from the blood of full-term pregnant women would be more convenient than cord serum, especially where delayed cord clamping is employed. Its use does, however, pose other potential problems because the invasive nature of collection limits

the volumes available from any one pregnant woman. Pooling of samples could overcome this, however, any immunological effects on the cells of pooling samples from different women would have to be investigated. Also, investigation of why serum from pregnant women had this effect is warranted and might identify a growth factor mix that could be used instead.

In conclusion, we have developed an umbilical cord matrix MSC isolation approach using small explants first seeded on pooled maternal serum and then cultured in medium containing the same serum, which yields cells that meet the criteria of MSCs. While pooled maternal serum gives the greatest yields, further work is required including detailed investigation of how this affects MSC phenotype and function, especially the immunomodulatory properties of these cells. Further investigation is also needed into the characterising of these cells, including by means of an *in vivo* transplantation.

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Author Disclosure Statement

No competing financial interests exist.

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Table 1: Antibodies used for flow cytometry

Surface antigen	Fluorochrome	Clone
CD19	PC7	-
CD34	eFluor450	4H11
CD45	APC	2DI
CD73	FITC	AD2
CD90 Thy-1	PE	eBio5E10
CD14	APC eFluor780	-
CD105	PE	SN6
HLA-DR	eFluor450	L243

Table 2: Effect of different sera selection on the minimal criteria for defining mesenchymal stromal cells as set out by the ISCT

	Minimal Criteria	**Fetal Bovine Serum**	**Cord Serum**	**Pooled Maternal Serum**
Plastic Adherence	+	+	+	+
CD73	+	+	+	+
CD90	+	+	+	+
CD105	+	+	+	+
CD14	-	-	-	-
CD19	-	-	-	-
CD34	-	-	-	-
CD45	-	-	-	-
HLA-DR	-	-	-	-
Adipogenesis	+	+	+	+
Chondrogenesis	+	+	+	+
Osteogenesis	+	+	+	+

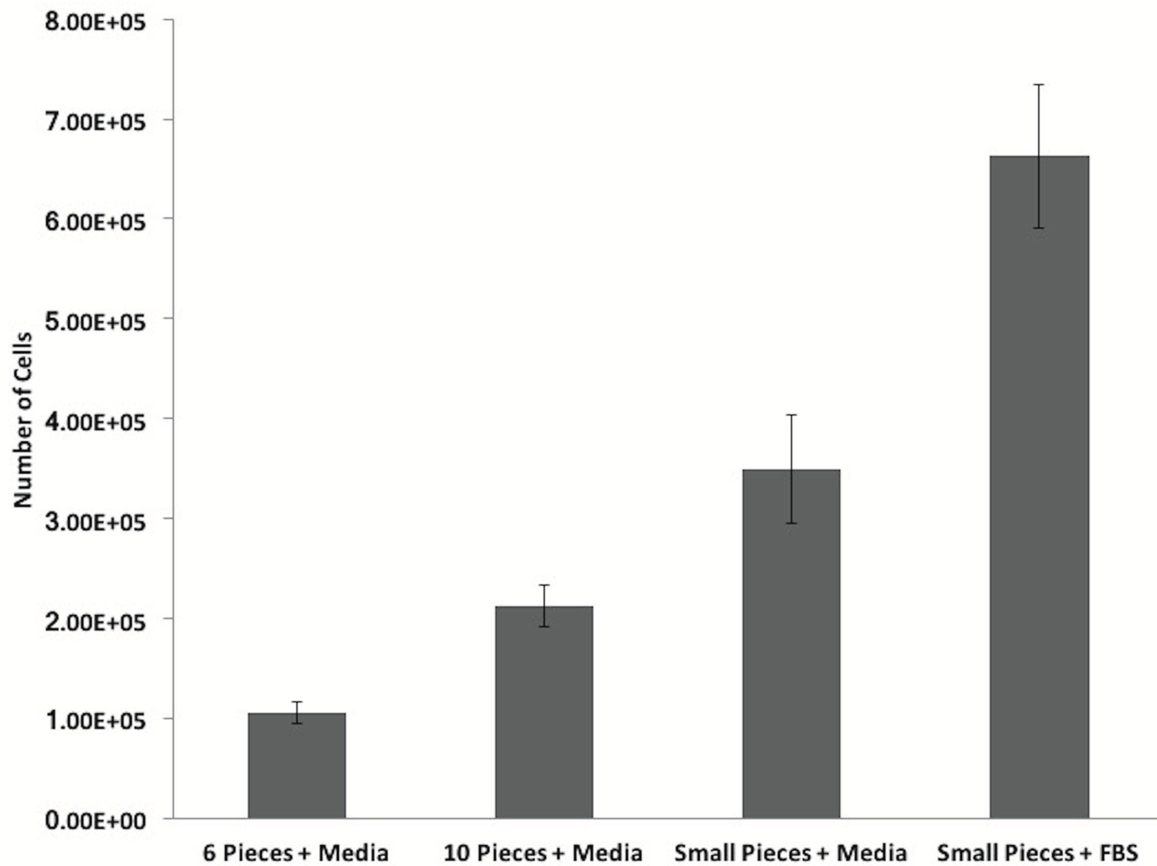


Figure 1: The effect of umbilical cord explant size on the yield of putative mesenchymal stromal cells

Explants of umbilical cord of varying size were cut from the cord as detailed in the *Materials and Methods*. Pieces were seeded into 4 ml complete DMEM with 2 ml additional media on day 2 or, in the case of small (<2mm^3) explants + FBS, pieces were seeded onto 500 µl FBS then 1 ml media was added after 1 hour and every day until day 5. On day 8 the tissue was removed, the media was replaced, and the putative MSCs were cultured for a further 7 days before harvesting by trypsinisation and counting. All experiments were conducted in T25 culture flasks. Cell counts are shown as mean ± SEM. n=3 different umbilical cords, each in duplicate.

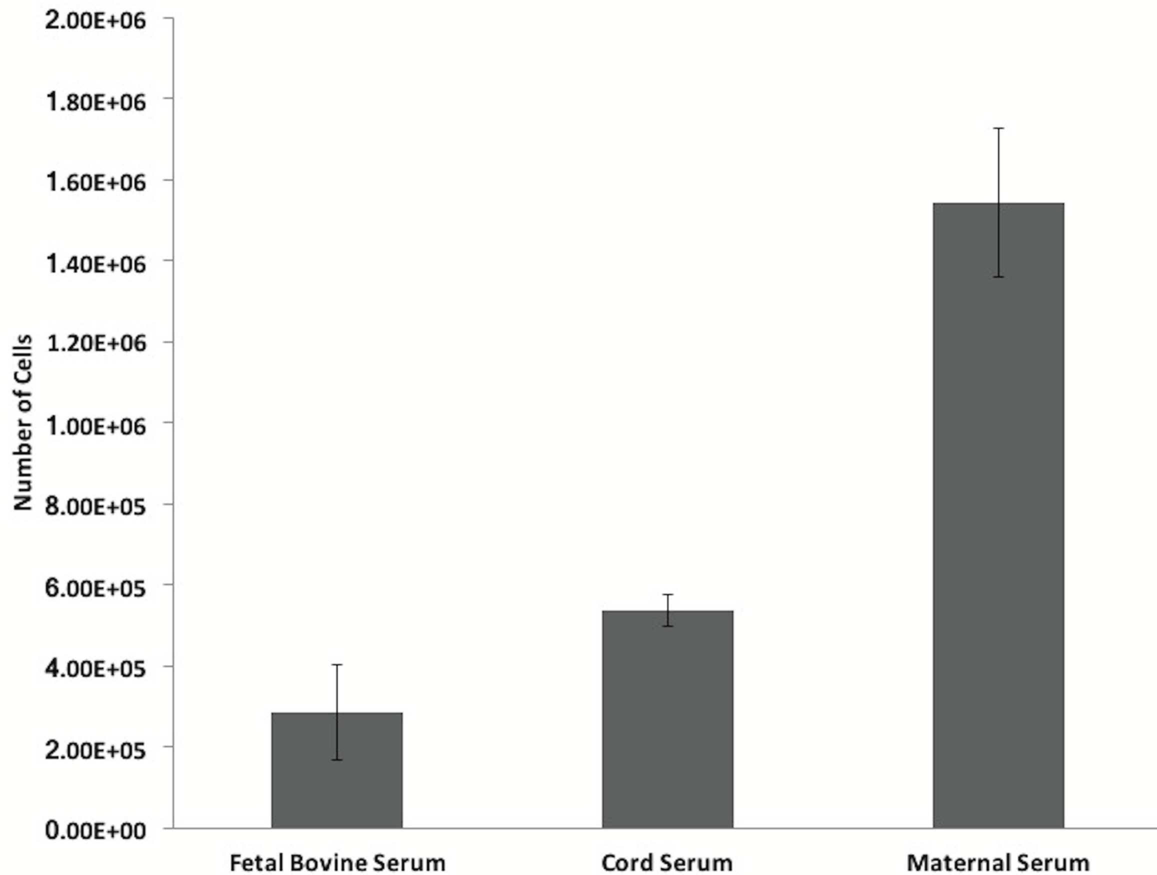
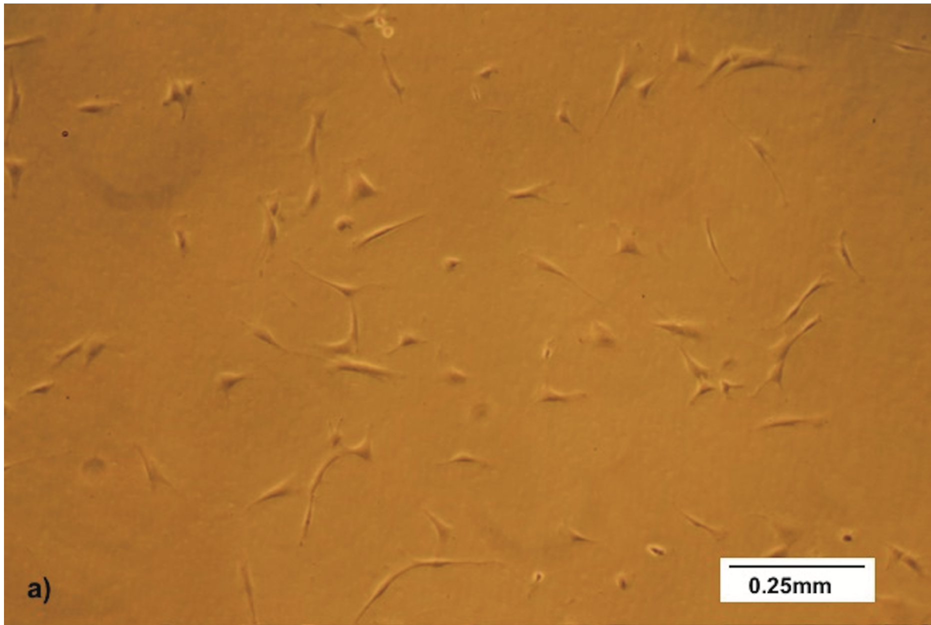
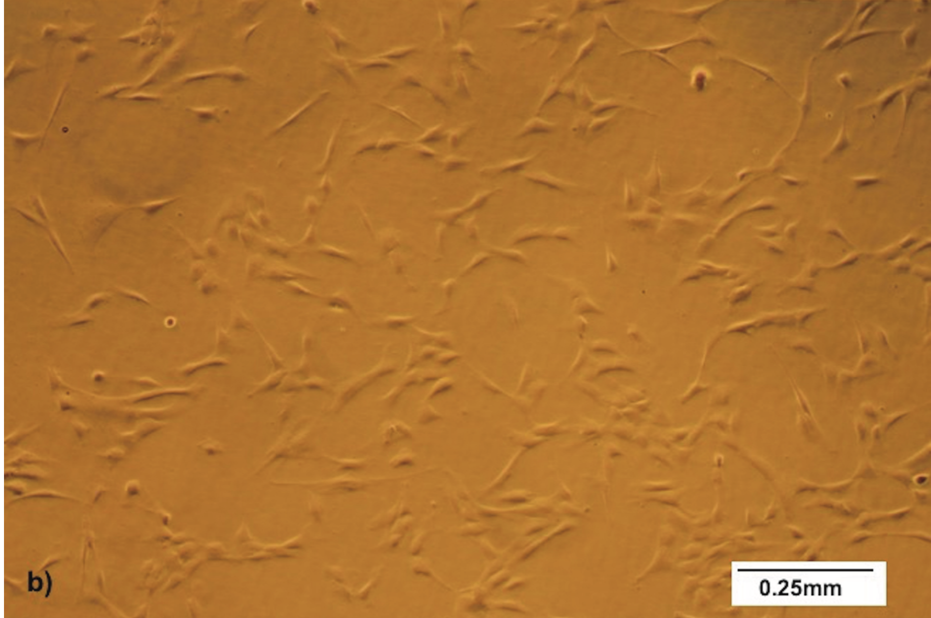


Figure 2: The effect of different sera used at seeding and as a medium supplement throughout culture on the yield of putative mesenchymal stromal cells from umbilical cord explants

Small (<2mm^3) explants of umbilical cord were seeded onto 250µl of FBS, pooled umbilical cord serum from full-term non-laboured deliveries, or pooled serum from women > 37 weeks pregnant. After 1 hour and then each day until day 3, 500µl of media containing the same serum as used for seeding was added to each flask. On day 8 the tissue was removed and the media was replaced with fresh corresponding media. On day 15 the cells were harvested and re-seeded before harvesting and counting on day 18. All experiments were conducted in 6 well culture plates until day 15 and in T25 culture flasks from day 15 – 18. Cell counts are shown as mean ± SEM. n = 3 different umbilical cords, each in duplicate.


a)
0.25mm


b)
0.25mm

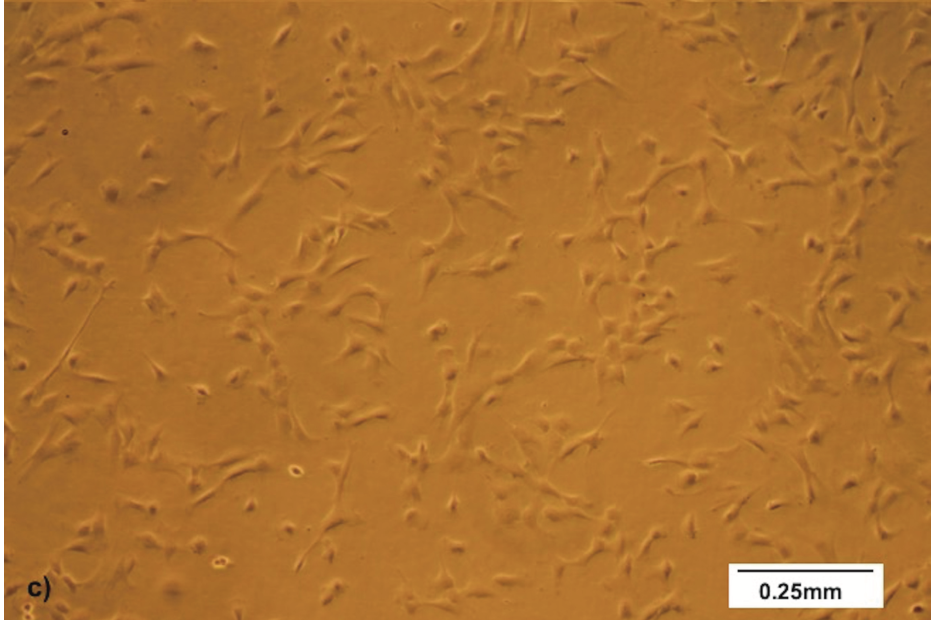
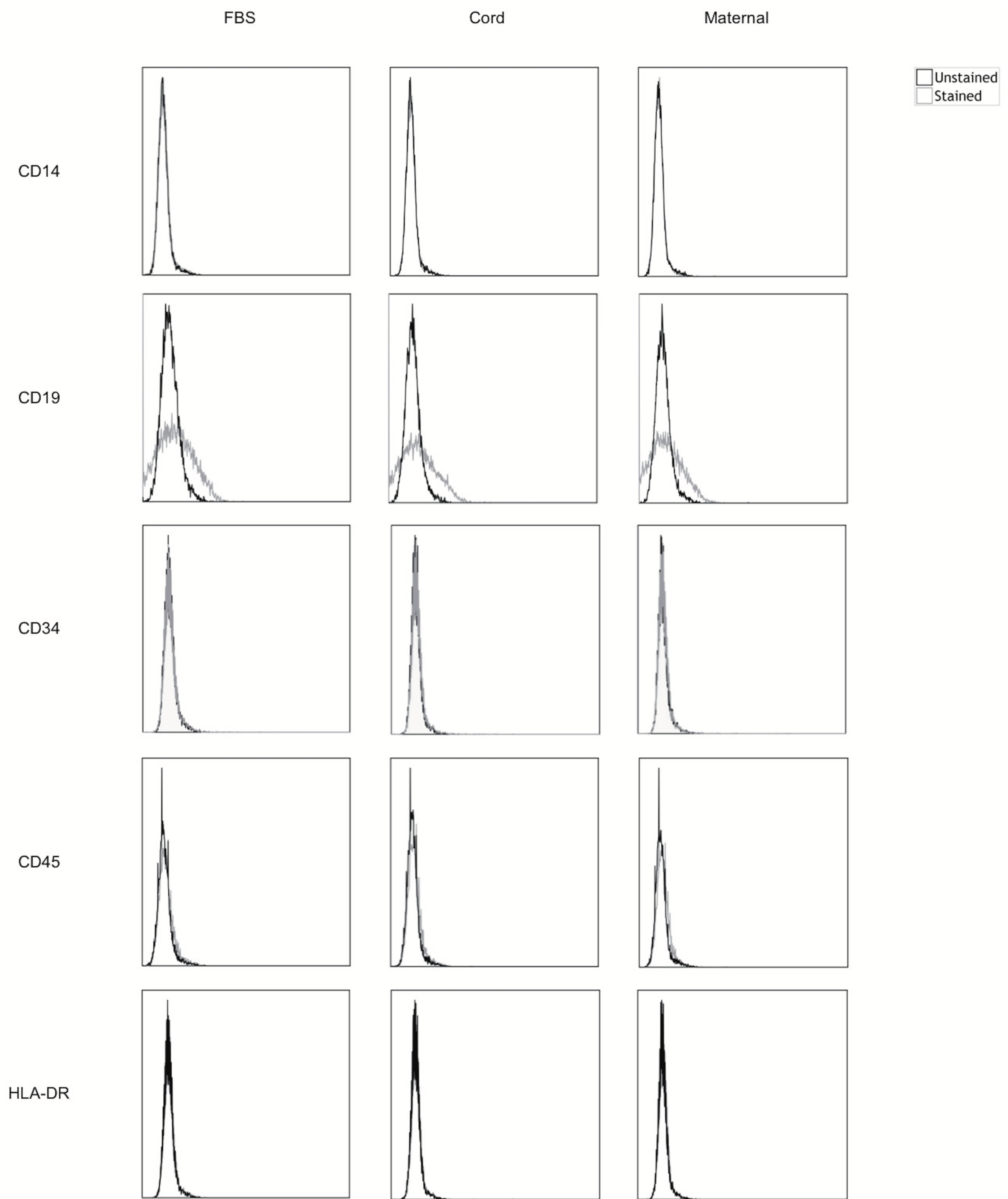
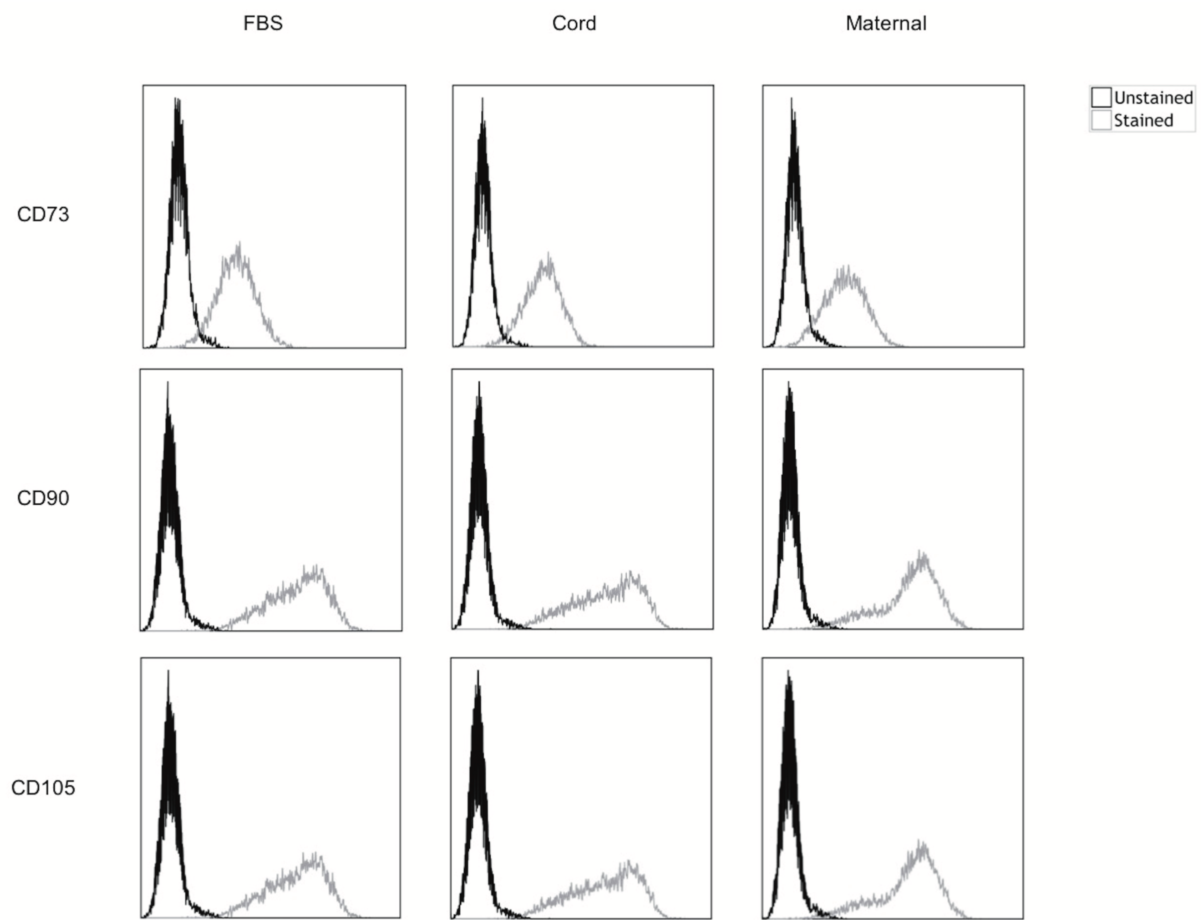

c)
0.25mm

Figure 3: Plastic adherence of putative mesenchymal stromal cells isolated from umbilical cord explants cultured in three different sera

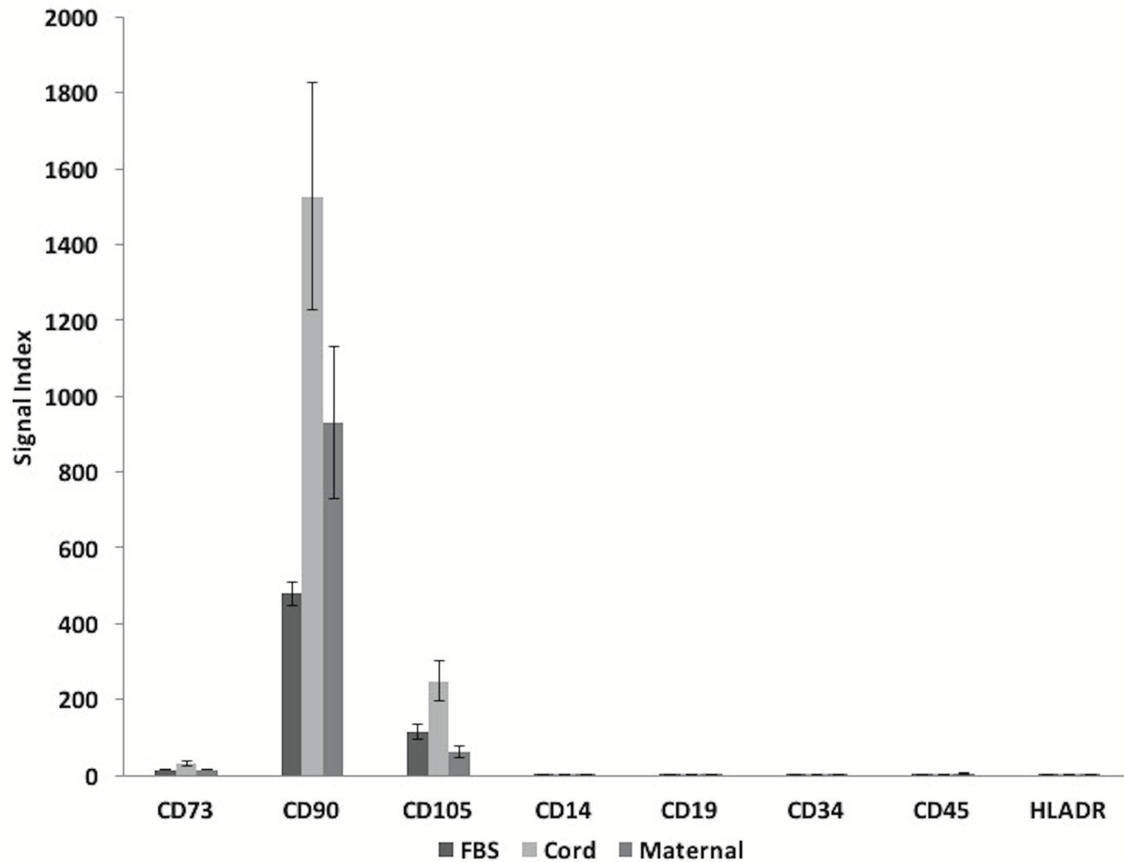
Small ($<2mm^3$) explants of umbilical cord were seeded onto different sera and then cultured in medium supplemented with the same sera as described in the *Materials and Methods.* On day 18, photomicrogrpahs were taken prior to harvesting and cryopreservation of the cells. Representative examples of 6 preparations of cells isolated using a) fetal bovine serum, b) umbilical cord blood serum, and c) pooled maternal blood serum are shown. 0.25mm scale bars are shown. Images were taken at room temperature using a Zeis Axiovert 40 microscope at x5 magnification and a Canon 1100D camera with the Canon EOS Utility software



a)



b)



c)

Figure 4: Phenotype of putative mesenchymal stromal cells isolated from umbilical cord explants cultured in three different sera

Small (<2mm^3) explants of umbilical cord were seeded onto different sera and then cultured in medium supplemented with the same sera as described in the *Materials and Methods.* On day 18, cells were harvested and cryopreserved; the cells were then resuscitated and stained for flow cytometry with fluorochrome conjugated antibodies to a) CD14, CD19, CD34, CD45, or HLA-DR and b) CD73, CD90, or CD105. The median fluorescent intensity was used to calculate the signal index (calculated by dividing the median fluorescence of the stained cells by the median fluorescence of the unstained cells) and the mean ± SEM is shown (n=3/group) (c).

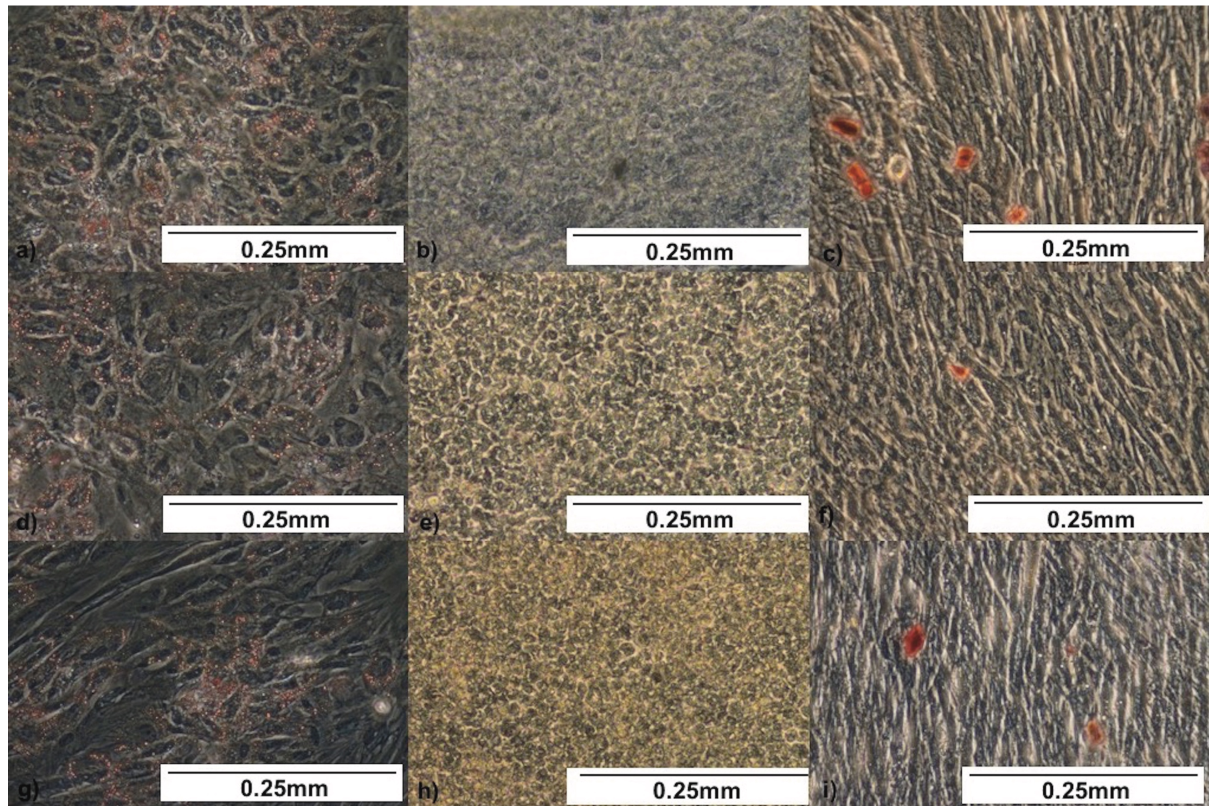


Figure 5: Differentiation of putative mesenchymal stromal cells isolated from umbilical cord explants cultured in three different sera

Small (<2mm^3) explants of umbilical cord were seeded onto different sera and then cultured in medium supplemented with the same sera as described in the *Materials and Methods.* On day 18, cells were harvested and cryopreserved; the cells were then resuscitated and adipogenesis, chondrogenesis, or osteogenesis was induced in the cells. After 16 days in adipogenesis differentiation medium the cells were fixed and stained with Oil Red O, shown in a), d), and g); after 23 days in chondrogenesis differentiation medium the cells were fixed and stained with Alcian Blue, shown in b), e), and h); after 25 days in osteogenesis differentiation medium the cells were fixed and stained with Alizarin Red S, shown in c), f), and i). Representative examples of preparations of cells isolated using fetal bovine serum (a, b, and c), umbilical cord blood serum (d, e, and f), and pooled maternal blood serum (g, h, and i) are shown. 0.25mm scale bars are shown. Images were taken at room temperature using a Zeis Axiovert 40 microscope at x10 magnification and a Canon 1100D camera with the Canon EOS Utility software.