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Heterogeneity in proliferative potential of ovine mesenchymal stem cell colonies

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Bone marrow biopsies were taken from the iliac crest of 28 individual sheep from three different breeds, ranging in age from 4 months to 8 years and mesenchymal stem cells (MSCs) isolated using selection due to plastic adherence. Cells were cultured in medium that had been selected for its effect on observed MSC proliferation, until populations of greater than 50 million had been obtained from each biopsy. The identity of the isolated cell populations as progenitors of the mesenchymal lineage was verified by deriving both osteoblastic and chondrocytic phenotypes when cultured in osteogenic and chondrogenic medium supplements, respectively. The rate of cell proliferation for each marrow biopsy was measured at each passage and the number of initial stem cells in each sample estimated. There was no statistically significant correlation between the age of the sheep and MSC proliferative potential, or age and estimated initial MSC number. There was no apparent significant difference between proliferation rate and sheep breed and colonies established from frozen cells grew at similar rates to pre-frozen cells. Counter intuitively, there appeared to be a negatively correlated trend between proliferation rate and MSC concentration in the samples. It is concluded that no initial descriptive statistics of the marrow biopsies can assist in estimating the proliferative potential, and therefore the timing of future surgeries, of MSCs sampled for the purposes of tissue engineering.
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Introduction

Tissue engineering has been heralded as a technology of great promise in the area of regenerative medicine, but relies on the creation of *de novo* tissue of the correct phenotype or use of progenitor cells which can be encouraged or directed to differentiate into the correct tissue for the required application. Stem cells are generally described as being uncommitted with the ability to be derived into a number of different cell types [1] given the correct biological and/or mechanical cues. Mesenchymal stem cells (MSCs), which are termed multipotent, can differentiate into bone, cartilage, adipose and fibroblastic cell types, such as those comprising tendons and ligaments [2–4]. There is evidence that a degree of phenotypic plasticity renders the conventional hierarchical ''pathways'' of cell differentiation to some extent outmoded [5, 6], but methods to control this plasticity are not well understood. For this reason, MSCs are often regarded as essential tools to create constructs representing the replacement skeletal tissue, but the practicalities of their use cannot be assessed without understanding how qualitative and quantitative differences in cell sources will influence the final outcome.

The ability to control or predict the rate of proliferation of cells taken from a donor for autologous transplantation within a tissue-engineered construct is essential for their clinical use. The number and ability of stem cells to differentiate or proliferate is frequently described as declining with age [7, 8], suggesting that older individuals may be unsuitable for this type of clinical approach. Conversely, very young individuals would be expected to yield high numbers of cells that are extremely proliferative.

Using the sheep as a model for donor variability and bone marrow stromal cells as a source of MSCs, the variation in proliferation rate was studied to measure the correlation between donor age and donor suitability in

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tissue engineering, the major objective being to calculate the spread of time required for each donor sample to proliferate to the 45 million cells required to populate a construct for use in the replacement of an anterior cruciate ligament (ACL).

Materials and methods

Sheep ranging in age from 4 months to 8 years were used as donors of bone marrow. This spans the age most sheep will reach in their adult size until their natural death. For sheep, however, their natural death occurs largely as a result of a reduction in their ability to eat due to tooth loss rather than as a result of large scale cardiovascular or other tissue degeneration. Sheep were sourced from the following breeds: Merino, South prealp and Cambridge-cross from Germany, France and the United Kingdom. The area around the hip was shaved then treated with surgical iodine. Bone marrow was extracted immediately after either lethal injection or stable anaesthesia with O_2–N_2O–halothane from the iliac crest either percutaneously or after surgical exposure of the crest. Marrow was extracted using a disposable seven-gauge trephine biopsy needle (Rocket Medical, Watford, UK) by suction in to a 30-ml syringe containing 3000 IU sterile heparin. Marrow samples collected in Germany and France were initially stored at 4 °C then transferred to bespoke transportation vessels complying with IATA650 shipping regulations. They consisting of a sterile 50 ml tube with rubber sealing insert, pre-cooled ''ice'' packs which prevented sample freezing, all packed in polystyrene containers which allowed cooled transportation for up to 48 h.

MSCs were isolated largely as described by Solchaga [9]. The total number of mononuclear cells in each marrow sample was calculated after first removing aggregates and debris by careful but firm aspiration through a sterile 19-gauge needle, then washing in Dulbecco's minimum essential medium (DMEM, Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (FCS). Counting was achieved after destruction of red blood cells using 2.7% (v/v) final concentration acetic acid. Samples of FCS from different suppliers had already been batch tested for their ability to allow MSC proliferation on identical cell populations. To allow quantitative data to be compared, a large quantity of the best batch (Australian origin from Invitrogen, Paisley, UK) was purchased, frozen and stored until required.

Washed stromal cells were plated-out at a concentration of $3.6 \times 10^5/\mathrm{cm}^2$ in T-75 culture flasks and maintained at 37 °C in 5% CO_2. Medium was changed subsequently twice per week with DMEM with 10% FCS. Non-adherent mononuclear and red blood cells were removed during the first few medium changes. Adherent cells were passaged just prior to confluency by detachment using 0.25% trypsin in EDTA (Sigma, Poole, UK). Numbers of mononuclear cells were counted at each stage of isolation/passage in a haemocytometer.

The identity of the cells as MSCs was verified by testing the ability of cell colonies to express both osteoblastic and chondrocytic functionality after culture with osteogenic and chondrogenic supplements respectively [9, 10]. In each case, each supplemented sample was compared with cells from the same sample but without the differentiating supplement. The osteogenic supplement comprised 100 μM ascorbate-2 phosphate, 10^{-7} M dexamethasone and 10 mM β-glycerophosphate as final concentrations, all purchased from Sigma (Poole, UK). The chondrogenic medium was DMEM-HG (high glucose) supplemented with 1% ITS + Premix (standard supplement containing insulin, transferrin and selenous acid, purchased from BD Biosciences, Oxford, UK), 100 μM ascorbate-2 phosphate, 10^{-7} M dexamethasone and 10 ng/ml TGF-β1 (all three from Sigma Poole, UK). Chondrogenesis was achieved by spinning 2.5×10^5 MSCs at 1000 g for 5 min in a 15 ml polypropylene conical tubes (Falcon, Oxford, UK) and culturing the resultant pellet in the chondrogenic medium at 37 °C in 5% CO_2. Medium was changed twice a week for the osteogenic samples, and every other day for the chondrogenic samples.

Functionality of the differentiated cells was assessed by staining the osteogenic cultures and controls with von Kossa after three weeks in culture. The chondrogenic samples were fixed in 10% neutral buffered formalin, embedded in paraffin and 6-μm serial sections stained with Toluidine Blue.

Results

Isolation of MSCs from a heterogenous mixture as occurs in bone marrow stroma using conventional plastic adherence relies on the loss of the majority of the mononuclear cells during medium changes. The MSCs initially contained within the sample proliferate exponentially whilst sub-confluent, so long as a satisfactory cell seeding density is initially selected. A graph of the log (MSC number) over time should therefore be a straight line, as long as the growth conditions (nutrient supply, temperature, CO_2 concentration, sub-confluency), re-seeding concentration and counting accuracy are maintained. The square of the correlation coefficient (r^2) of this curve was used as a guide to the quality of the quantitative cell data and culturing accuracy and efficacy, and ranged from 0.908 to 0.9999 (Fig. 1). The number of days required for the different colonies to reach the desired 45 million covered a vast range, from 13 to 230 days. When colonies were established from frozen cells, they grew at similar rates to pre-frozen cells. A comparison of the slope of the log growth curve, Fig. 1 (proliferation rate) and age of sheep is shown in Fig. 2, but with no clear correlation or trend.

There was variability in each bone marrow sample, from the volume of marrow collected, the total number of mononuclear cells and the percentage of MSCs making up the stroma. The initial number of MSCs in each sample was estimated by extrapolating the growth curve back to zero days, ignoring the inevitable short lag period, and giving us what we have termed the notional initial MSC number. This allowed us to plot the age of the sheep against this initial MSC number (Fig. 3). Furthermore, we could also estimate the ratio of MSCs to mononuclear cells, i.e. the MSC concentration, and this is plotted against age of sheep (Fig. 4) and MSC colony proliferation rate.

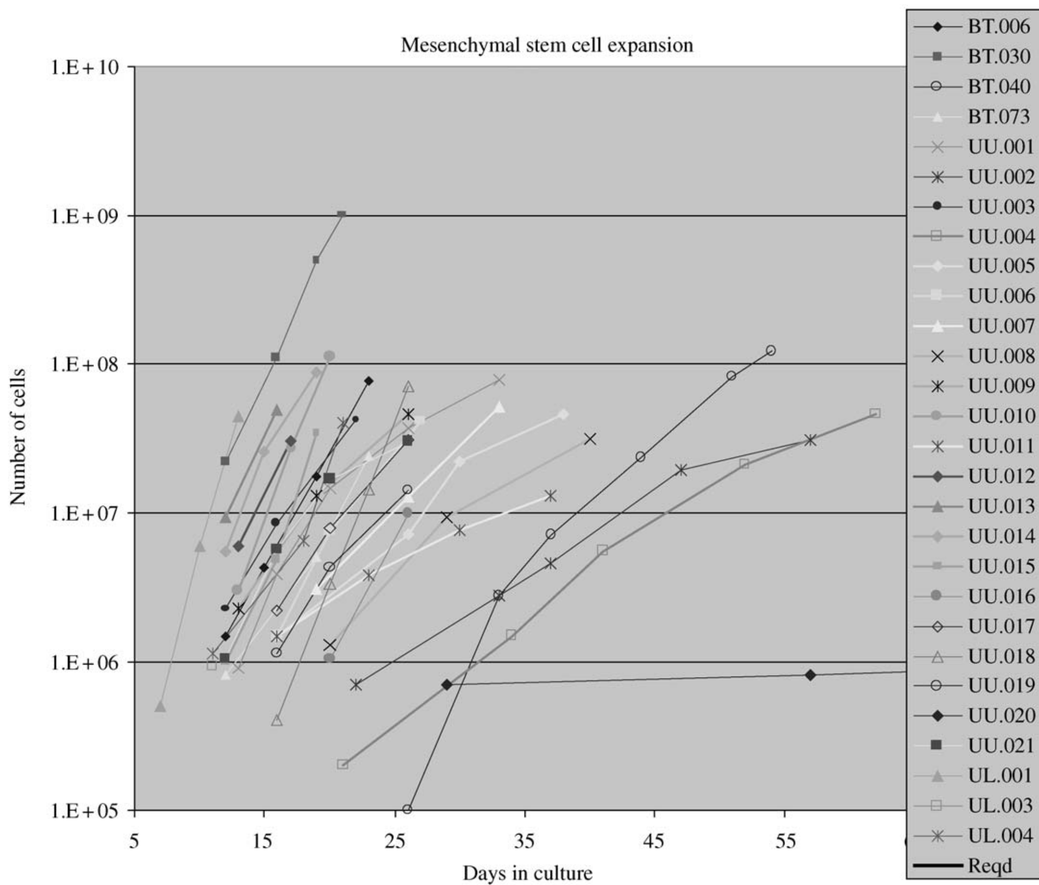

Figure 1 Expansion of ovine MSCs in culture for 28 separate bone marrow biopsies.

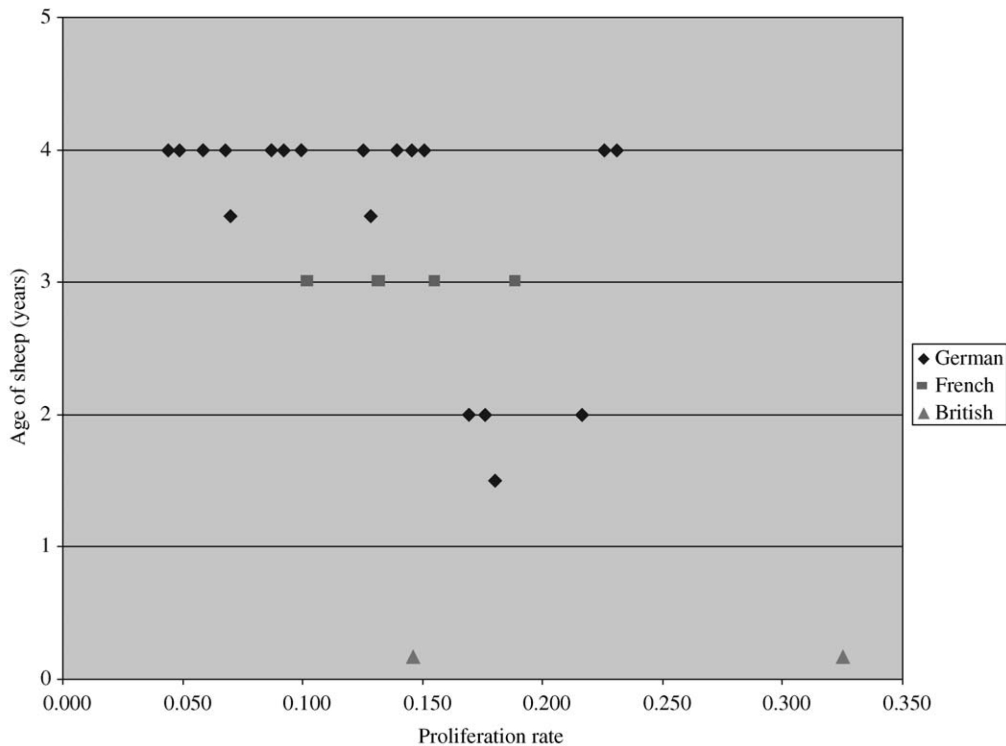

Figure 2 Comparison of sheep age against MSC proliferation rate.

There was no statistically significant correlation between age of sheep and growth rate (correlation $\rho = -0.57$) or age and initial MSC number ($\rho = 0.25$). The significance of differences in growth rate between different breeds was assessed, and found not to be significant ($p = 0.64$ for Merino [German] versus South prealp [French]).

There was a large variation in total number of mononuclear cells (5×10^6–530×10^6) despite a relatively narrow range of biopsy volumes (4–17 ml).

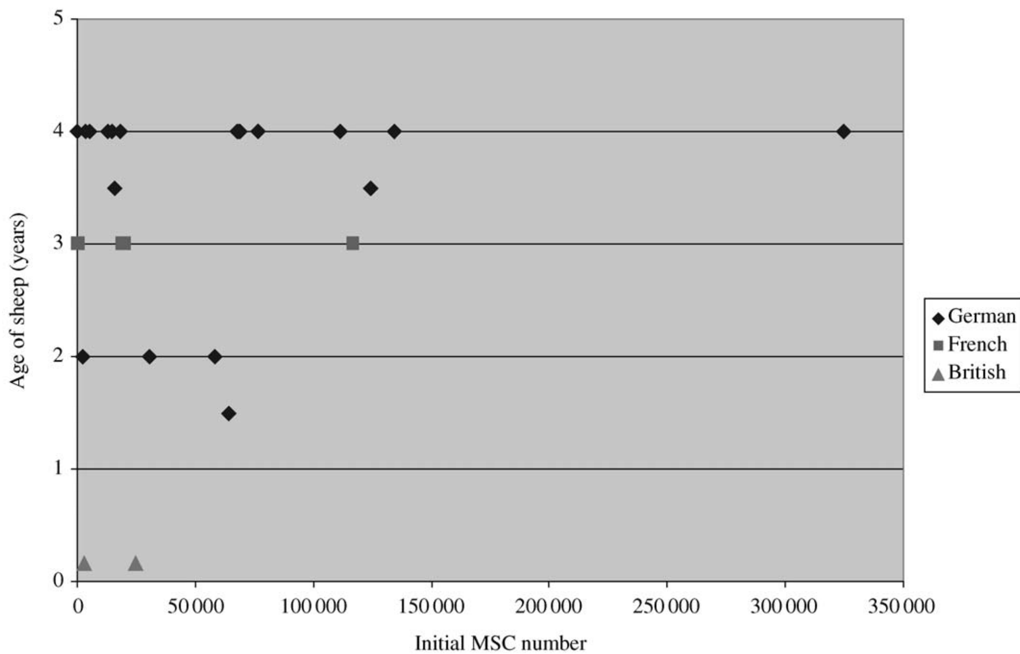

Figure 3 Comparison of sheep age against notional initial MSC number.

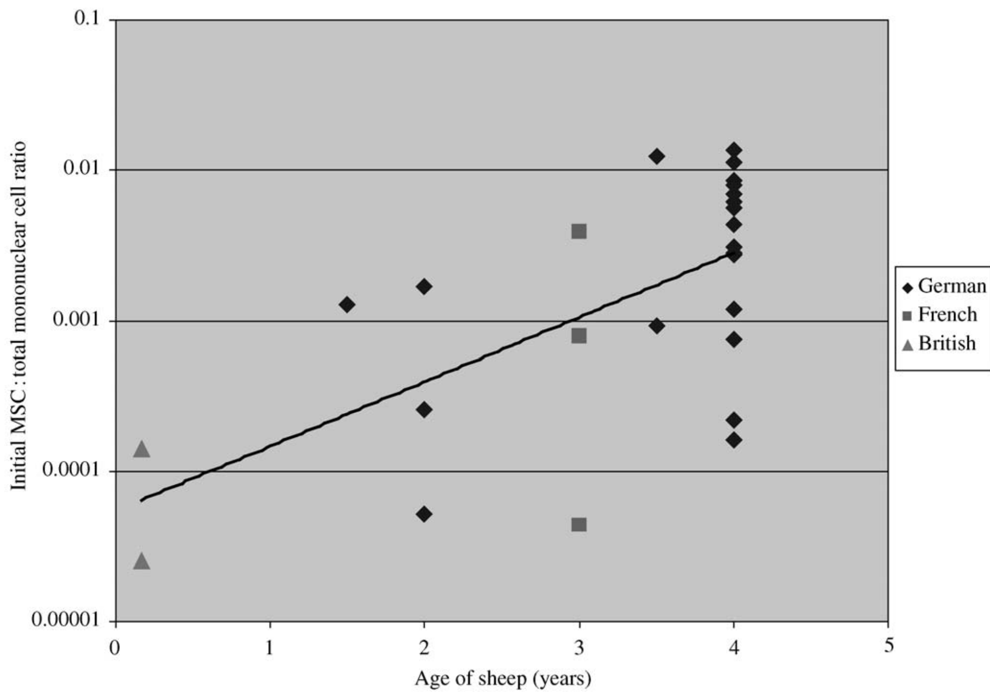

Figure 4 Comparison of the ratio of initial MSC number to total mononuclear cells with age.

Consequently, there was also a large range in notional initial MSC number (0.6×10^3–260×10^3). However, when expressed as a ratio of initial MSC number to total number of mononuclear cells (i.e. the percentage of MSCs in the mononuclear cell population), the percentage of MSCs appeared, counter intuitively, to become larger with animal age, although with a sample of only 28, not significantly so, given the distribution of values, with $r^2 = 0.4$. When plotted against proliferation rate, the percentage of MSCs in the mononuclear cell population also appeared to correlate inversely, again with $r^2 = 0.4$.

Discussion

There is uncertainty expressed in published literature regarding the ability of clinicians to use patients' regenerative potential when considering autologous tissue engineering principles in the treatment of degenerated tissues [11]. Notwithstanding these doubts, many proof of principle concepts are developed using animal models or human tissue which is either in a healthy state and/or from individuals that are still quite young. For companies wishing to exploit these tissue engineering concepts, the question of how to determine the suitability of the patient's cells and tissues and how to

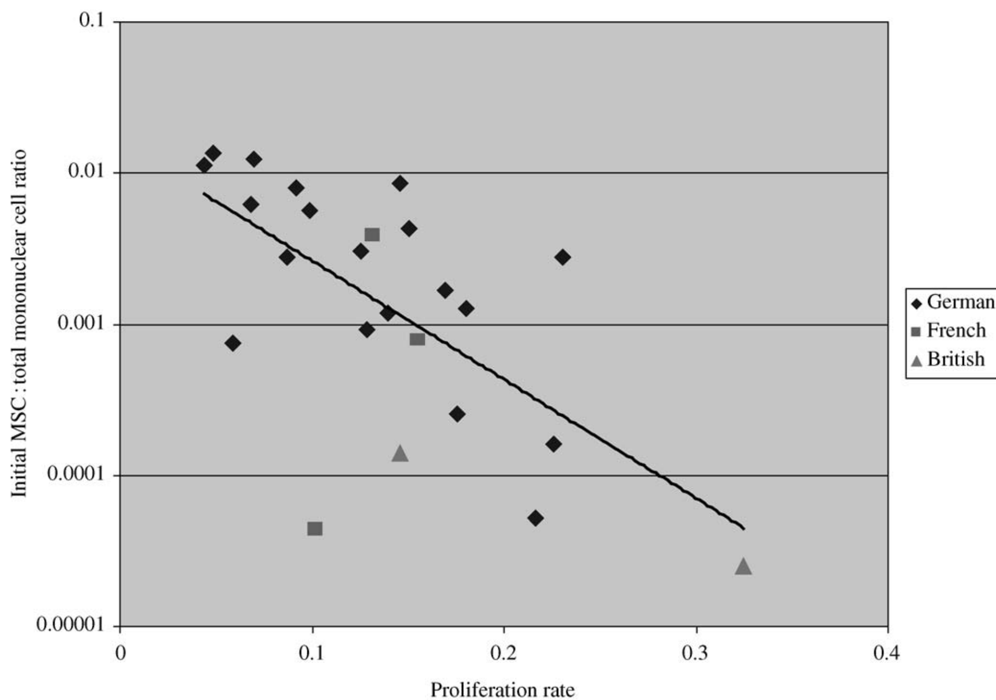

Figure 5 Comparison of the ratio of initial MSC number to total mononuclear cells with MSC proliferation rate.

phase the biopsies with ultimate surgery are extremely important. Yet there appears to be little published data regarding the distribution of progenitor cell content in the marrow of human or model systems and how quickly these cells proliferate.

The clinical application, therefore determines to some extent, the timescale that the patient will be able withstand before treatment, since critically burned patients require immediate attention. For conditions which are not life-threatening, a delay in final surgery of several weeks may be of little consequence to the ultimate health of the patient. Nevertheless, lengthy *in vitro* culture is not in the best interests of either the company or the patient, either in terms of cost or risks associated with data management. Additionally, there is evidence that prolonged *in vitro* culture has an adverse effect on cellular phenotype which is a cause of increased immunogenicity [12].

It is recognised, of course, that individuals vary considerably in the numbers of cells and their rates of cellular reactions, including cell cycling and proliferation. Indeed, it has been reported that there is considerable heterogeneity in colonies derived from rabbit bone marrow [9] which is not observed in other species [13, 14]. It is not clear from the literature, however, how these variations are inter-related and if it is possible from the outset of marrow biopsy to determine potential culturing timescales.

The results of this study do, indeed, indicate there is variation in proliferation rate from animal to animal, but that it is more than one might have expected. Of interest is the fact that the difference in time to achieve the required number of cells ranged 13–230 days, a situation which would be clinically untenable in the latter animal. This sheep imparted 9 ml marrow with a total of 38×10^6 mononuclear cells, figures which might lead one to expect the future proliferation of the cells to be quite good. Sheep UU.018, for example, was almost identical in every respect: the same age, same breed, had the same number of mononuclear cells in a volume of marrow only 10% different, yet reached the scaffold seeding number in approximately one-tenth of the time.

The notional initial MSC number also varied enormously although this parameter must be treated with caution since it is derived from extrapolated data. However, other literature does suggest that a wide range of MSC numbers are present between individuals [11].

It might be assumed that an important parameter applicable to successful MSC isolation would be the ratio of MSCs to total mononuclear cells, i.e. the MSC concentration. However, not only did this ratio appear to increase with sheep age, but the proliferation rate also appeared to be negatively correlated with this parameter, although not demonstrably significantly so. It is clear that the very young sheep had many times more total mononuclear cells than the older sheep ($115–530 \times 10^6$ for 4-month-old sheep compared to $5–25 \times 10^6$ for 4-year-old sheep), suggesting that the most profound decline as the animals age may be in the total number of mononuclear cells, not MSC number. This runs contrary to experience in humans and rats [11]. Again counter intuitively, the notional initial MSC number appeared to correlate negatively with proliferation rate.

In all other respects, the cell cultures did not differ: cell seeding density, culture medium, FCS, temperature, CO_2 concentration and seeding density after passage. One must conclude, therefore, that the proliferation rate is not indicated by other features within the marrow sample, but is either intrinsic to the biochemical and molecular biological features of the cell or due to a high number of the isolated cells residing dormantly in G_0/G_1 phase. One possible indicator may be telomere length, since it is well known that large scale removal of telomere bases is an indicator of cell senescence [15]. It

is proposed that a comparison of length of telomeres in a cell population may provide an indication of patient suitability for a particular tissue engineering treatment. Alternatively, factors which stimulate cells into cell cycling may also be used to more quickly expand the MSC population.

Conclusions

Age of individual in the sheep model was demonstrated to be a poor indicator of potential for mesenchymal stem cells derived from marrow stroma to proliferate to useful population size for use in tissue engineering. Neither was the number of mononuclear cells nor the extrapolated initial number of MSCs in the sample. Proliferation rate was shown to vary widely, covering a factor of more than 17-fold from slowest to fastest. This may indicate that some individuals will be unsuitable for autologous tissue engineering therapies.

Acknowledgments

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