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Expansion of adherent cells for cell therapy

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Table of contents

1 Summary

Stem cell based therapy is a relatively new and rapidly expanding strategy within the medical area. It can be used for treatment of numerous disorders and possibly even replacement of whole damaged organs in the future. Examples of such disorders are stroke, diabetes, cancer, cardiac failure and age related diseases. These diseases are common around the world and cell therapy has been proposed to be a promising field to focus on in order to develop efficient treatment to the patients. However, there are two major issues that have to be solved to make the cell therapy technique successful. Firstly, an efficient cell expansion (i.e. cell proliferation) method has to be developed. Secondly, this method has to be reliable and cost effective. The WAVE Bioreactor is a relatively new device, which was initially designed for suspension cultures. Nowadays the WAVE Bioreactor can also be combined with microcarriers (spherical supportive matrix), which has generated a new market for the bioreactor. This combination is a promising alternative when culturing adherent cells. Microcarriers offer several advantages when culturing anchorage-dependent cells. Compared to conventional methods the microcarriers provide growth in several dimensions and at high densities, which makes it possible to grow several million cells/ml. This means that the laboratory and storage space can be reduced markedly, which will lower the costs and also save time.

In this project the main focus was to optimise the expansion of adherent cells in the WAVE Bioreactor combined with microcarriers (i.e. Cytodex). I also studied a degradable microcarrier that hopefully will improve the cell release and simplify the workflow for cell therapy.

The expansion of cells was measured with two different quantification methods. In the first method the nuclei of the cells were stained with crystal violet that lyses the cells and stains the nuclei. In the other method the cells were counted after trypsination. In both methods a light microscope was used to count the cells. Cell quantification by nuclear counting was shown to be more reproducible. However, if the cells grow too close to each other, both methods will become less reliable since there will be difficulties to release the cells or nuclei. Both methods for cell counting and detachment of cells from the microcarriers have to be improved to be successful.

The cultivation of cells on Cytodex 1 and 3 (different types of Cytodex) in the WAVE Bioreactor was enhanced during the project. The existing method, where cells are released from the microcarrier before new carriers are added to the cultivation, was improved. It was shown that carriers could be added several times throughout the cultivation. The migration of human mesenchymal stem cells was demonstrated and found to be an important feature for the cell expansion in the WAVE Bioreactor. A new way of expanding cells in the WAVE Bioreactor was developed and confirmed to be successful. The cells were allowed to migrate to a gradually increased number of microcarriers. Several degradable microcarriers with different surface layers were tested to see if some variants were comparable to Cytodex, primarily in terms of cell growth. Degradation of the microcarriers was also tested to see whether this could improve the cell release from the microcarriers in an efficient way. However, the difficulty remains to identify a carrier that the cells like to grow on and that still has the ability to degrade.

2 Introduction

Stem cell based therapy is a relatively new and rapidly expanding strategy within the medical area. It can be used for treatment of numerous disorders and possibly even replacement of whole damaged organs in the future (Gojo et al. 2011). Examples of such disorders are stroke, diabetes, cancer, cardiac failure and age related diseases (e.g. osteoarthritis and Alzheimer's disease) (Vemuri et al. 2011). These diseases are common around the world and cell therapy has been proposed to be promising to focus on in order to develop efficient treatment to the patients. However, there are two major issues, which have to be solved to make the cell therapy technique successful. Firstly, an efficient cell expansion (i.e. cell proliferation) method has to be developed. Secondly, this method has to be reliable and cost effective. Since organs (e.g. heart, liver) are composed of several cell types, it would be ideal to use pluripotent stem cells (PSCs). PSCs have the ability to mature into all three germ lineages (i.e. mesoderm, ectoderm and endoderm), and also the capacity to regenerate (Ratajczak et al. 2008). However, the use of PSCs remains controversial since they are extracted from developing embryos (Stem Cell Transplant, 2011). Current research aims to find an alternative source of PSCs, preferably from adult tissues, or alternatively from more differentiated adult stem cells (oligopotent or multipotent), which still have the potential to regenerate. Recently, stem cells closely related to PSCs were identified in bone marrow (BM) and cord blood (CB) in adults. They can potentially differentiate into cells that are able to form more than one germ layer (Ratajczak et al. 2008). Mesenchymal stem cells (MSCs) may be an attractive alternative compared to PSCs in cell-based regenerative medicine (Ratajcak et al. 2008). They are easily accessible, straightforward to isolate, and can be bio-preserved without loss of potency (Malgieri et al. 2010).

2.1 The WAVE Bioreactor™ System

The WAVE Bioreactor is a relatively new device that was developed in 1996 by Wave Biotech (GE Healthcare, 2012). A few years ago Wave Biotech was acquired by GE Healthcare and the WAVE Bioreactor instruments are now manufactured in Umeå. Originally the WAVE Bioreactor was designed for suspension cultures of human, animal, insect and plant cells, but also bacteria and fungi can be cultured (Mikola et al. 2007; Terrier et al. 2007). Nowadays the WAVE Bioreactor, combined with a spherical supportive matrix (microcarrier) has generated a new market for the bioreactor. This combination has been a successful alternative when culturing adherent cells.

Intensive laboratory work is required when adherent cells are cultured using conventional equipment (e.g. T-flasks and spinner flasks). The volume is the limiting factor when a T-flask is used since adherent cells only grow in the bottom. The spinner flask permits larger culture volumes but requires cleaning and sterilization after each use. Moreover, systems like these are not closed systems and the culture volume is often restricted to less than 1 litre (Singh 1999). In a closed system one should be able to take sterile samples and add medium without any risk of contamination throughout the cultivation. Cell therapy requires a large number of cells and none of the above-mentioned methods can culture enough cells.

The WAVE Bioreactor (Figure 1) provides a closed system with disposable bags, called cellbags, which are sterilized in advanced. One can easily add medium and take sterile samples throughout the cultivation. The bioreactor is produced in four sizes that hold cell culture volumes from 0.2 to 500 litres. The WAVE Bioreactor 2/10 is the smallest system available. The working volumes are 0.2 to 1 litre or 0.5 to 5 litres depending on the bag size,

which is either 2 litres or 10 litres. The cellbag is placed on a rocking platform, which is also part of the WAVE Bioreactor system. The platform provides a rocking motion that induces waves in the culture, which leads to a natural mixing of the cells combined with an increased uptake of oxygen. The rocking can be adjusted in the range of 1-40 rocks per minute to reach the most favourable level of oxygen and mixing of the cells. The platform also provides heating to the cellbag (for temperature control) to keep the cells at a constant temperature of 37 ºC. A CO_2-mixer is coupled to the incoming air that supplies carbon dioxide and oxygen to adjust the carbon dioxide level. The air enters via a 0.2 µm inlet filter that sterilizes the air, and is transferred out via an exhaust filter that is heated to 56 ºC to prevent condensation from the culture blocking the filter (Sing 1999). Moreover, the WAVE Bioreactor 2/10 system can be combined with a perfusion system to exchange medium continuously during the process. All these properties result in an optimised condition for cell growth that easily can support growth to more than 10^7 cells/ml. Another advantage of the WAVE Bioreactor is that the cellbag is disposable, eliminating all cleaning and sterilization steps necessary for other devices, which simplifies the handling and protects the cultures from cross-contamination.

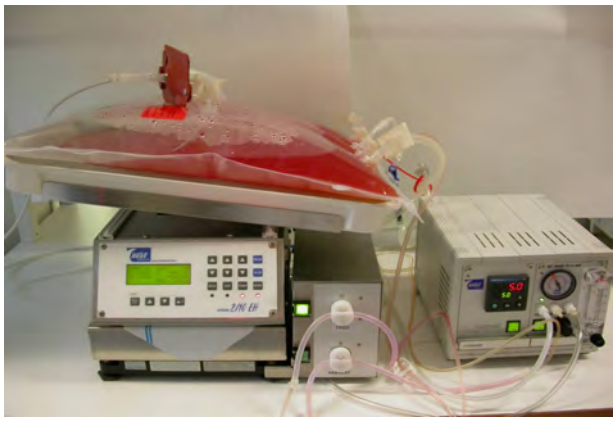

Figure 1. The WAVE Bioreactor™ 2/10 system combined with a perfusion system to exchange medium continuously during the process. Photo kindly provided by Linnea Pauler.

Apart from the air inlet and outlet ports there are two other ports connected to the cellbag, the sample port and a port with a tube connector. They are connected to the top of the cellbag and have different functions. The tube connector consists of tubing with a luer lock, which permits a sterile connection inside a laminar air flow (LAF) hood when medium, cells and microcarriers are added to the cellbag. Samples can be withdrawn from the self-sealed sample port in a sterile way during culturing without the use of a LAF hood.

2.2 Cell culturing on microcarriers

When microcarriers are used in culture, the cells are grown in monolayer on the surface of small carriers, such as beads, scaffolds or discs, suspended in culture medium and gently stirred. Microcarriers are available in a variety of materials (e.g. plastics, glass, dextran, cellulose and collagen). The ideal microcarrier should be non-toxic, biocompatible, promote favourable cellular interactions and tissue development and at the same time fulfil adequate physical and mechanical properties (Mano et al. 2007). Non-toxic microcarriers are required to obtain good cell growth, but also when the cell culture products are used clinically. The following criteria are essential for microcarriers: 1) They must have surface properties that allow the cells to adhere and spread in order for the cells to proliferate. 2) The density of the

microcarriers should be slightly greater than that of the surrounding medium so that cells and medium can be separated easily, but should allow for complete suspension of the microcarriers with only gentle stirring. 3) To make the cells reach confluence at approximately the same time the size distribution should be narrow. The size of the microcarriers should also allow easy sampling and handling. 4) The optical properties should permit studying the cells, throughout the culture, using standard microscopy techniques and without having to remove the cells from the carriers. 5) The stirring by waving of the microcarriers during cultivation require a microcarrier that is not too rigid to avoid damage when a collision occurs.

Microcarriers offer several advantages when culturing anchorage-dependent cells. Compared to conventional methods the microcarriers provide growth in several dimensions and at high densities, which makes it possible to grow several million cells/ml (Boo et al. 2011). This means that the laboratory and storage space can be reduced markedly, which will lower the costs and also save time. The cell culture techniques where microcarriers are used have become very important when large amounts of biological materials are produced, such as vaccines, antibodies, enzymes, hormones, nucleic acids and interferons (Mano et al. 2007). Even though the advantages are predominant using microcarriers, there are some drawbacks to consider. The microcarriers might not promote as good cell growth as tissue culture plastics and it can be difficult to detach the cells from the carriers. Detachment can be problematic if the cells have to be recovered with good viability at the end of cell culturing. Tough and long trypsination of the cells may be needed, which might affect the cell viability and recovery negatively. It may also be difficult to separate the carriers from the cells once they have been detached (Melero-Martin et al. 2006).

2.2.1 Cytodex microcarriers

The Cytodex microcarriers are produced in Uppsala at GE Healthcare. All the Cytodex microcarriers fulfil the physiological criteria's mentioned above. There are two types of Cytodex microcarriers, Cytodex 1 and Cytodex 3 (Table 1). Both of them are based on a spherical matrix of cross-linked dextran that is non-toxic and provides the microcarriers with suitable physical properties. Another advantage is that the Cytodex microcarriers are transparent which makes it easy to observe the cells throughout the culturing using a light microscope (Bluml. 2005).

Cytodex 1 has a surface of N,N-diethylaminoethyl (DEAE) groups which makes the microcarriers positively charged throughout the entire matrix. The negatively charged cells can bind to the positively charged molecules on the Cytodex 1 microcarriers. Cytodex 3 microcarriers are coated with a surface layer of denatured collagen. Most normal epithelial cells will attach more efficiently to collagen than to other surfaces. However the denatured collagen is derived from pig skin type I collagen, which is not beneficial when the cells or the cell products are aimed to be used clinically (Bluml. 2005).

Table 1. Physical characteristics of Cytodex microcarriers.

Microcarrier	Approx. area[a] (cm^2/g dry weight)	Approx. no. microcarriers/g dry weight	Swelling[a] (ml/g dry weight)	Density[a] (g/ml)	Size d50 [a, b] (µm)
Cytodex 1	4.4	6.8×10^6	18	1.03	180
Cytodex 2	2.7	4.0×10^6	14	1.04	175

[a] In 0.9% NaCl
[b] d50 is the mean value of the particle diameter

2.2.2 Degradable microcarriers

In the microcarrier based cell expansion area there are different needs. The microcarrier should be animal-free and fully synthetic. Also technologies to enable easier detachment (like soluble or biodegradable microcarriers) and isolation of cells are becoming more important (Fernandes-Platzgummer et al. 2011). In addition to improved release and simplified workflow, degradable carriers might also be used as injectable scaffolds for tissue engineering to enable cell therapy at the site of injury (Figure 2). One great advantage with such a system would be the minimal invasive surgical procedure required. The challenge would be to design an appropriate microcarrier suitable for this system. A degradable microcarrier could permit separation of cells from the microcarrier either *in vivo* at the site of injury or *in vitro* right before the injection of cells. A possible customer workflow for degradable microcarriers is shown in Figure 2.

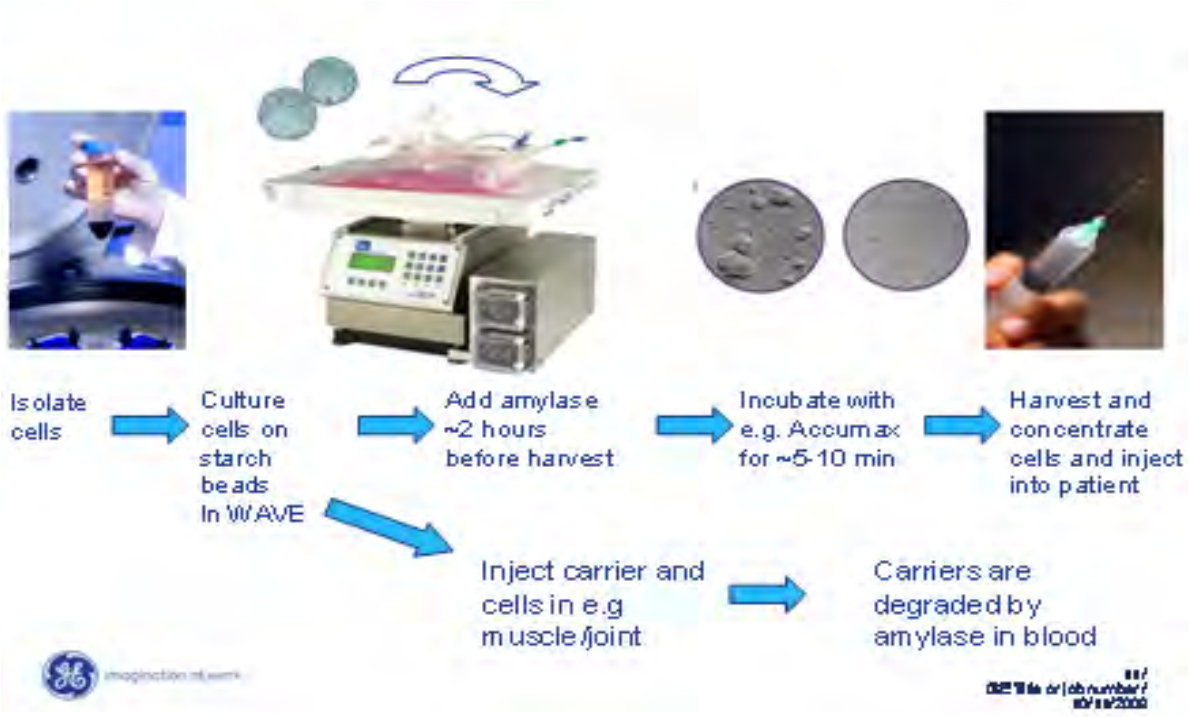

Figure 2. Possible customer workflow for the degradable microcarriers in cell therapy, kindly provided by Cecilia Annerén.

The base matrix for the degradable microcarrier is starch. Company X (confidential information) developed this starch microcarrier whereas the coupling chemistry has been carried out in house at GE Healthcare. Tuzlakoglu et al (2005) created a nano- and microfibre combined scaffold from starch, which shows unique architecture and provides an ideal structure for cell deposition and organisation in bone tissue engineering. The degradable microcarriers have a higher density compared to the Cytodex microcarriers. The diameter of the degradable carriers varies from 180 to 200 µm, slightly larger than Cytodex 1 and 3, table 1. The carriers can be degraded by amylase, an enzyme that is naturally found in the saliva. Amylase is also present at low level in serum in the cell culture medium.

2.3 Anchorage-dependent cells and cell lines

2.3.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into a variety of cell types including bone, fat, cartilage, tendon and muscle cells (Pittenger et al. 1999). MSCs are adherent cells that can be isolated primarily from the bone marrow, but more recently also from other tissues like adipose tissue, fetal liver, peripheral blood and cord blood (Książek 2009). Bone marrow is currently viewed as the richest and most reliable reservoir for MSCs (Wexler et al. 2003). When grown in culture they look much like fibroblasts, long and thin stretched out on the surface they grow on (Javason et al. 2001). In Figure 3, human MSCs (hMSCs) were grown in a monolayer. Extensive studies of MCSs have resulted in advanced knowledge in how to grow these cells in culture (Frauenschuh et al. 2007). The advantages with MSCs are that they can be maintained and propagated in 5-7 passages without losing their multipotency and reach cell quantities appropriate for clinical applications. Another reason why MSCs are suitable for human applications is the possibility to derive MSCs from a relatively small bone marrow sample from a given patient. The cells can then be expanded in culture and transferred back to the patient (i.e. autologous transplantation). This means that the issues regarding immune rejections can be easily avoided.

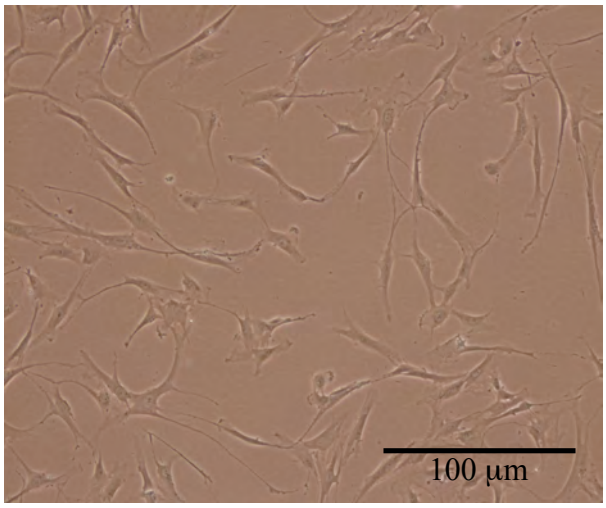

Figure 3. Human mesenchymal stem cells (hMSCs) grown in T-flask. 5000 cells/ml were seeded out and cultivated for two days (Light micrograph: Hanna Åkerström).

2.3.2 Skeletal muscle stem cells

The body consists of three different muscle types: smooth, cardiac and skeletal muscles. The skeletal muscles, also called cross-striated muscles, consist of multinucleated striated myofibers, so called skeletal muscle cells (SkMCs). SkMCs form the major muscles and are held together by connective tissue. During the embryonic development multinucleated skeletal muscle fibers arise from the fusion of mononuclear myogenic cells that migrate from the somites and form primary myofibers. These cells later mature and grow in size by fusion of additional myogenic cells (Cerletti et al. 2006). SkMCs are highly complex since they are coordinated directly or indirectly by the nervous system. If a skeletal muscle gets injured, new skeletal muscle cells will be produced and the fiber composition of the original muscle will be re-established.

2.4 Aims

This degree project was divided into two major parts. The focus was to improve the cell expansion of adherent cells in the WAVE Bioreactor combined with microcarriers (Cytodex). The other goal was to examine a new type of microcarrier that is degradable and hopefully will improve the cell release and simplify the workflow for cell therapy.

A degradable carrier that exhibited adequate degradation ability plus the ability to support cell growth comparable to Cytodex was difficult to identify. Therefore, the focus changed during the project to disregard the degradation properties and instead find a degradable carrier that supported superior cell growth compared to Cytodex.

3 Results

3.1 Comparison of different methods for quantification of cells

A reliable method for quantification of cells is desirable to be able to make comparison of cultivation techniques. Two methods with light microscopy were used to monitor cell growth, a qualitative method by visual observation and quantitative methods by cell counting in a hemacytometer. The cell type MSC was chosen to be grown either in monolayer or on Cytodex 1 in six-well plates. This will also give a comparison of growth of cells in monolayer or on microcarriers, which is important for the following experiments with the WAVE Bioreactor. The two studied quantification methods were nuclear counting and counting in a hemacytometer after trypsination of cells. In the nuclear counting method the cells were treated with crystal violet, which lyses the cells and colour the nucleus. In the second method, the cells were treated with trypsin, which released the cells from the monolayer and the microcarriers. The cells were then filtered through a 70 µm filter in order to get rid of the microcarriers and other waste products. A hemacytometer was used to count the cells or nuclei in both methods.

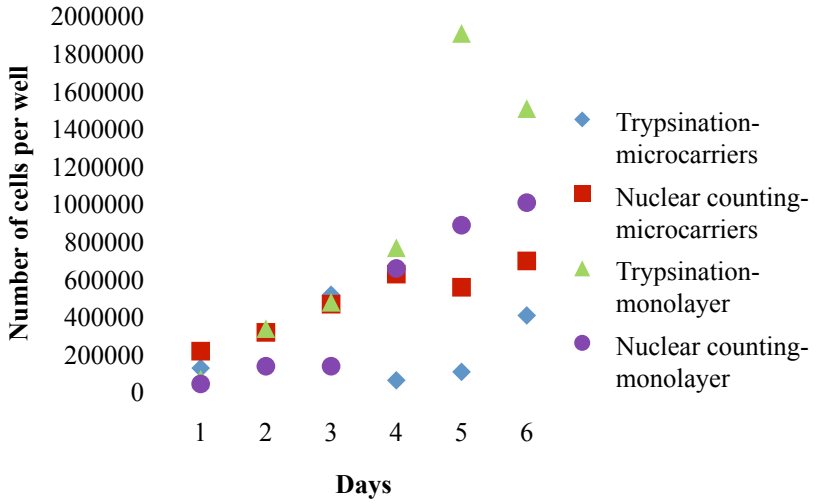

Figure 4. Comparison of different quantification methods. Trypsination and cell counting in a hemacytometer and nuclear counting were used to estimate the growth of hMSCs in a monolayer (T-flask) or on microcarriers (Cytodex 1). In the monolayer experiments, 5000 cells/cm^2 were seeded as recommended by the supplier of the cells. Cells grown on carriers, 100 µl sedimented Cytodex 1 was added to each well and eight cells/carrier were seeded. Cells were grown during a six day period and counted each day. Trysination method A was used with 0.25 gram trypsin per litre (Sciencell).

The results from the comparison of the two quantification methods are shown in Figure 4. The nuclear counting method demonstrated an increased growth over time, both together with microcarriers and when grown on a monolayer (violet and red line in Figure 4). This indicates that this method is reproducible and stable. The value at day five in the trypsination of monolayer looks like an outlier. If this value was omitted the growth curve would look more similar to the nuclear counting growth curves. The growth curve for the method using trypsination on microcarriers is displayed in pale blue in Figure 4. The number of living cells dropped at day four. The microcarriers were trypsinised using a 70 µm filter to separate the cells from the carriers after the cells were detached (see materials and methods). No cells were observed left on the carriers after the separation in the filter. However, when counting

the cells before and after the filtration step, some cell loss was noticed. Since there were no cells on the carriers, one explanation might be that the cells got stuck in the filter while passing. Hence, the filtration step only partly explains why the cell counts after trypsination of microcarriers were so low.

3.2 Migration of human mesenchymal stem cells on Cytodex 3 in spinner flasks

The migration of human mesenchymal stem cells (hMSCs), in this case movement of the hMSCs on or between the microcarriers is not yet fully understood. To investigate the migration in more detail wild-type (wt) hMSCs and hMSCs expressing the green fluorescent protein (hMSCs-GFP) were used. The experiment was set up in two spinner flasks with medium and Cytodex 3, which were incubated over night to adjust to the temperature and absorb the medium. hMCSs and hMSCs-GFP were then added to separate spinner flasks. The cells were cultured in separate spinner flasks during one day and then pooled together into one spinner flask and cultured for three extra days. Three days after the cells were pooled they started to migrate between carriers, as shown in Figure 5C. The arrows in Figure 5A and B points out corresponding microcarriers in the respective panel showing one carrier with almost only hMSCs-GFP and the other one with almost only wt-hMSC. A migrating cell is shown in the carrier marked by the right arrow in both panels, which contains one green cell and many wild-type cells suggesting that one hMSCs-GFP had migrated to a carrier that originally came from the wt-hMSCs subpopulation.

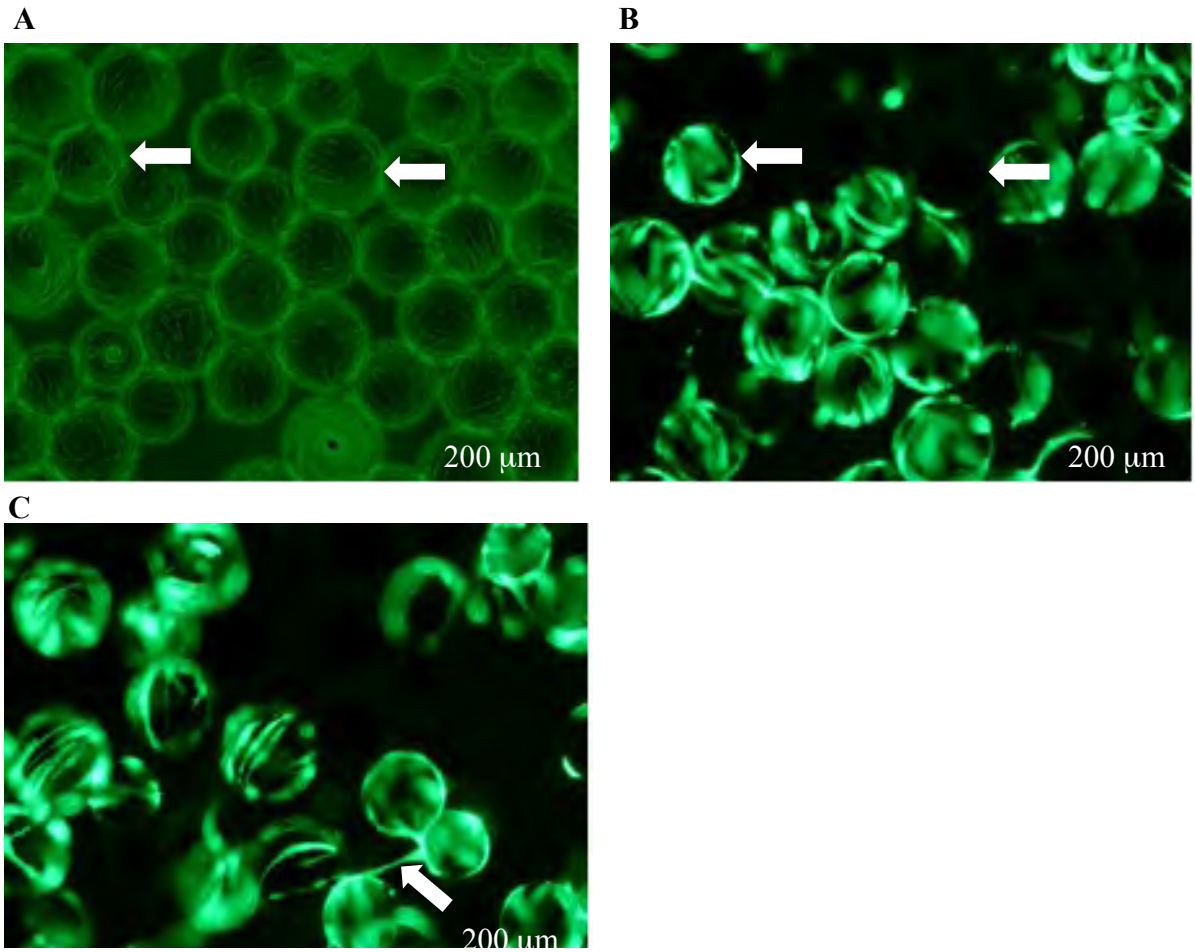

Figure 5. Migration of human mesenchymal stem cells on Cytodex 3. Wild-type ($3x10^6$ cells) and green fluorescent protein (GFP) expressing ($2.5x10^6$ cells) human mesenchymal stem cells (hMSCs) were added to separate spinner flasks containing 40 ml DMEM, with10% FBS (Gibco®) and 1.5 ml sedimented Cytodex 3 that had been equilibrated overnight at 37 °C. The cells were cultured for 24 hours and then pooled into one spinner flask. Images were obtained after 3 days of culture. Panel A shows light microscopy with a green filter, while panel B shows the same field in fluorescence microscopy where GFP-expressing cells are bright green. Arrows show the same microcarrier in both panels. Panel C shows hMSC-GFP migrating between two Cytodex 3 microcarriers. Photos were taken in an immunofluorescent microscope.

In Figure 5C the arrow points to an hMSC-GFP that has migrated between two Cytodex 3 microcarriers three days after the subpopulations were pooled together. Eight days after cultivation in the spinner flask the cells had become confluent on the carriers and an additional 0.75 ml sedimented Cytodex 3 was added, i.e. the Cytodex 3 was sedimented for approximately 30 minutes prior to addition. At the same time the medium volume was increased from 40 ml to 50 ml. The culture was stirred immediately after new carriers were added. This was done to prevent the cells from clumping together. Seven days later the culture was trypsinated. At this point the cells had formed similarly shaped clusters with a lot of carriers and cells grown together. Throughout the cultivation one millilitre cell suspensions were sampled daily for nuclear counting. The cells were counted using both the nuclear counting method and the trypsination method. The experiment started with $5.5x10^6$ cells and the final count was $21.4x10^6$ cells when the trypsination method was used and $27.4x10^6$ cells when the nuclear counting method was used. During the cultivation one hundred microcarriers were scored and divided into three different groups dependent on cell density. The results are presented in Figure 6, which shows that the cells have migrated from the

confluent and semi confluent carriers to the empty carriers. When 0.75 ml of sedimented microcarriers were added to the culture (already containing 1.5 ml sedimented microcarriers) one can assume that 33 % of the carriers were empty. After one day, only 16% of the carriers had no cells attached (left blue bar). Whilst, after four days only 1% of the carriers had no cells attached (right blue bar).

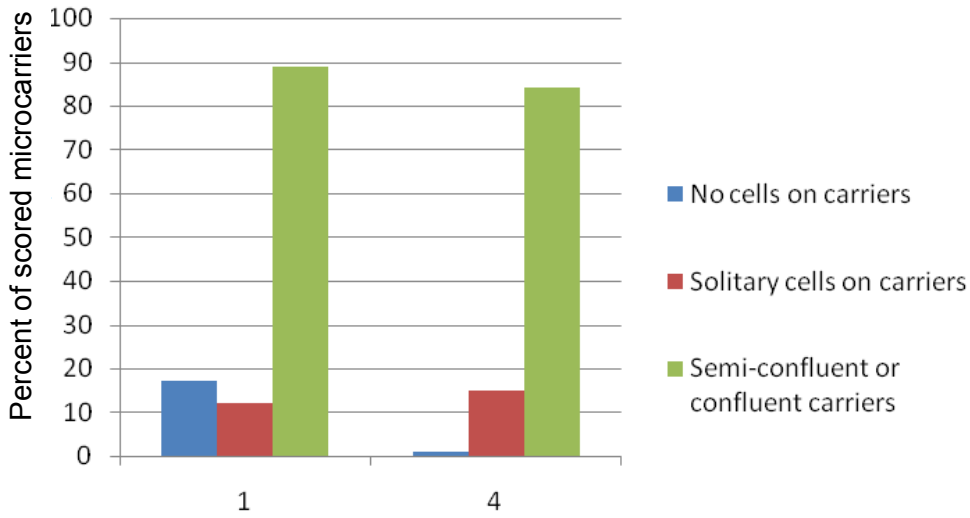

Figure 6. Cells migrating to empty Cytodex 3 carriers. The experiment was performed as explained in Figure 5. When growth on the carriers was semi-confluent or confluent an additional 0.75 ml sedimented Cytodex 3 was added and after one respectively four days one hundred microcarriers were scored dependent on cell density and divided into three groups. (i) no cells on carriers, (ii) solitary cells on carriers, (iii) semi-confluent or confluent carriers.

3.3 WAVE Bioreactor system

3.3.1 Skeletal muscle cells on Cytodex 1 in WAVE Bioreactor

Previous efforts to cultivate cells on microcarriers in the WAVE Bioreactor (Linnea Pauler, GE Healthcare, personal communication) only included one passage, e.g. only one trypsination and addition of new microcarriers. Consequently it would be of interest to grow cells for several passages, each time adding more microcarriers, to determine the maximal cell yield. This was done in the first experiment with the WAVE Bioreactor system in which SkMCs were grown on Cytodex 1. All adjustments on the WAVE Bioreactor were taken from previous experience (Linnea Pauler, GE Healthcare, personal communication) performed at GE Healthcare.

Cytodex 1 microcarriers were added to a T-flask. When the cells were added to the T-flask it was important to wiggle the T-flask carefully to make sure that as many cells as possible were attached to the carriers, approximately after 30 minutes. The attachment procedure continued overnight followed by transfer of carriers with cells to a cellbag with pre-conditioned medium via a transfer bottle and the tube connector of the cellbag. After five days, the cells were approximately 90% confluent on the microcarriers and the first trypsination, using trypsination method B with 2.5 gram trypsin per litre (Gibco®), was performed (Figure 7).

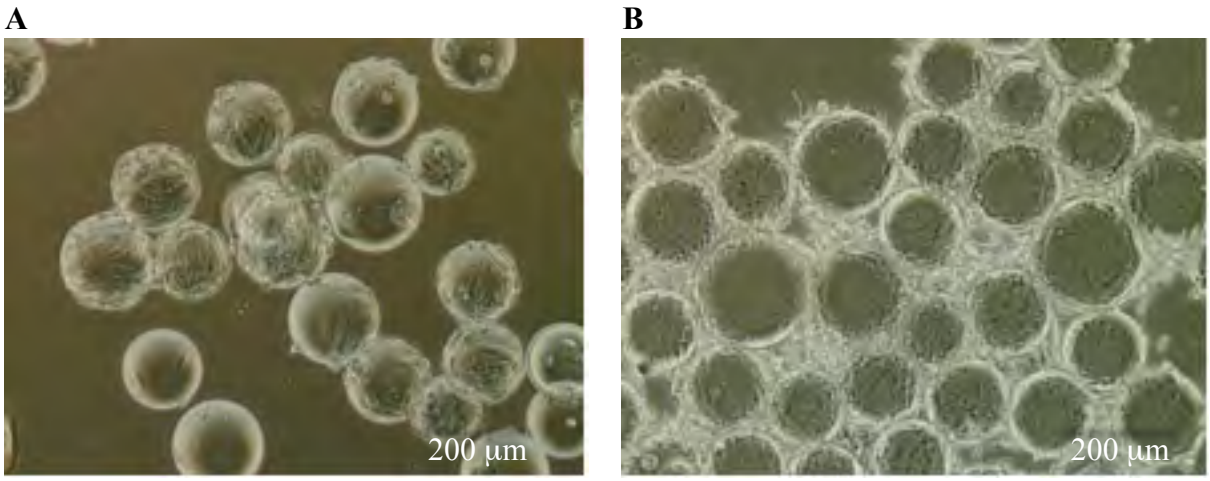

Figure 7. SkMCs grown on Cytodex 1 in the WAVE Bioreactor. Cells were transferred to the cellbag and attached overnight to the carriers in two T-flasks with a cultivation area of 175 cm^2. T-flasks were carefully wiggled to make sure that as many cells as possible were attached to the carriers. The recommended dose of 0.01 ml sedimented carrier/cm^2 (Linnea Pauler, GE Healthcare, personal communication) in the two 175 cm^2 T-flask ((0.01x175)x2) resulted in a starting volume of 3.5 ml sedimented Cytodex 1. The final cell culture volume was 100 ml. Approximately eight cells per carrier were seeded out, which resulted in a cell number of ~$11x10^6$. After attachment, carriers with cells were transferred to a cellbag with 200 ml pre-conditioned medium via a transfer bottle and the tube connector of the cellbag. The WAVE settings during the pre-condition step were 37ºC, angle 4º and carbon dioxide 5%. Once the cells were added, the rocking speed was set to 8 rocks per min the first three days and then decreased to 4 rocks per min. After five days of cultivation in WAVE the cells were approximately 90% confluent on the microcarriers and the first trypsination was performed using trypsination method B with 2.5 gram trypsin per litre and 0.38 gram EDTA per litre (Gibco®). Panel A shows the SkMCs after two days in the WAVE Bioreactor and the same culture after four days are shown in panel B where most of the cells had reached confluence, one day before trypsination.

At the first trypsination 20% of the culture was subjected to trypsination method B. This was performed to enable a more accurate cell count, in order to estimate the number of microcarriers to be added to the culture. The cells had grown to a total cell number of ~$25x10^6$ when additional 5 ml sedimented microcarriers were added to the culture. The rocking was started immediately after the cells and carriers were added. After an additional 11 days of WAVE culture, 16 days in total, the cells had reached confluence and were trypsinated with trypsination method B. The cells were counted and had tripled to ~$77x10^6$ cells. An additional 14 ml of sedimented microcarriers were added and the medium volume was adjusted to approximately 500 ml. Three days after the second trypsination I could observe some white slimy things floating around. I suspected a bacterial contamination and took out samples to study in the light microscope. However, no bacterial growth was found, and the cultivation was continued. Eight days after the second trypsination the cells looked messy and there were a lot of aggregates of cells and carriers. When the culture was observed in the cellbag, cells and carriers were stuck together creating large clumps floating around in the upper part of the medium. 14 days after the second trypsination, 31 days total in WAVE, the cells had started to create large cocoons of cells whereas other carriers were practically empty (Figure 8).

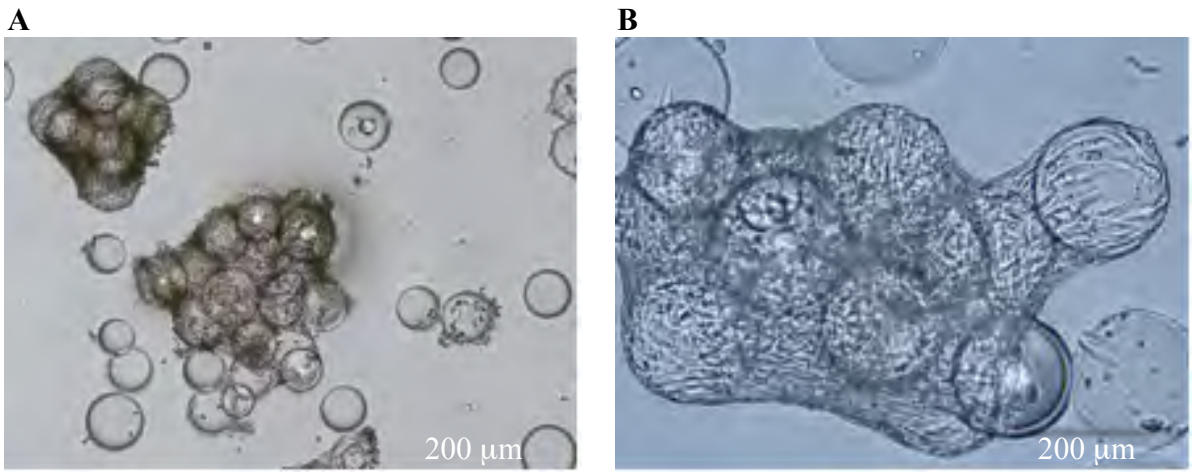

Figure 8. SkMCs grown on Cytodex 1. The experiment was setup as described in Figure 7. When cells had grown to a total cell number of ~25x10^6 an additional 5 ml sedimented microcarriers were added to the culture. The rocking was started immediately after cells and carriers were added. After an additional 11 days, 16 days in total, the cells had reach confluence and next trypsination was performed, using the trypsination method B. The cell number was then counted to ~77x10^6 cells. An additional 14 ml of sedimented microcarriers was added and the medium volume was adjusted to ~500 ml. Photos were taken after 31 days in the WAVE Bioreactor, 14 days after the second trypsination. Panel A is less zoomed and panel B shows a close up of cells and carriers. Photos were taken in a light microscope.

The empty carriers remained empty throughout the experiment whereas the cell density inside the clusters seemed to increase. To prevent cells from getting too dense and difficult to trypsinate I decided to perform a final trypsination to end the experiment. The cells were trypsinised with the trypsination method B. The cells and carriers were transferred from the T-flask via the inlet tubing to a Separator in order to separate the cells from the carriers. Cells were counted both before and after the separation to ensure that no cells were lost in the separation step. The cell number before the separation was 172x10^6 cells and the cell number after the separation was 152x10^6. Viability of the cells was also assessed and was found to be 95%. The documented cell numbers for SkMCs during cultivation in the WAVE Bioreactor is shown in Figure 9.

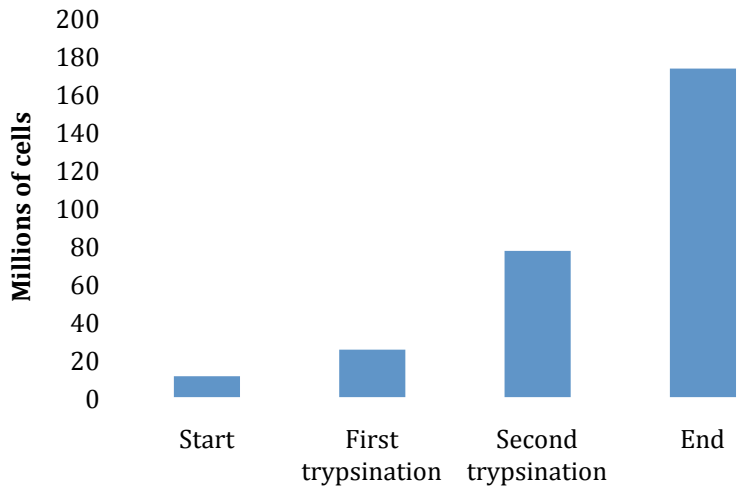

Figure 9. Number of skeletal muscle cells on Cytodex 1 in the WAVE Bioreactor. The experiment setup is described in Figure 7 and 8. The cells were counted from the start throughout the culture and passed two trypsinations using trypsination method B.

After the two trypsination steps the cell density on the carriers was scored. One hundred microcarriers were scored and divided into four groups depending on their cell density (Figure 10). Generally the distribution of cells changed over time after the trypsination steps.
The fraction of carriers with no cells and carriers with solitary cells were higher at the initial measurement and got lower over time. In contrast, the fraktion of confluent carriers and semi-confluent carriers increased over time. This indicated that the cells had no problem to attach to the carriers and continued to grow after trypsination.

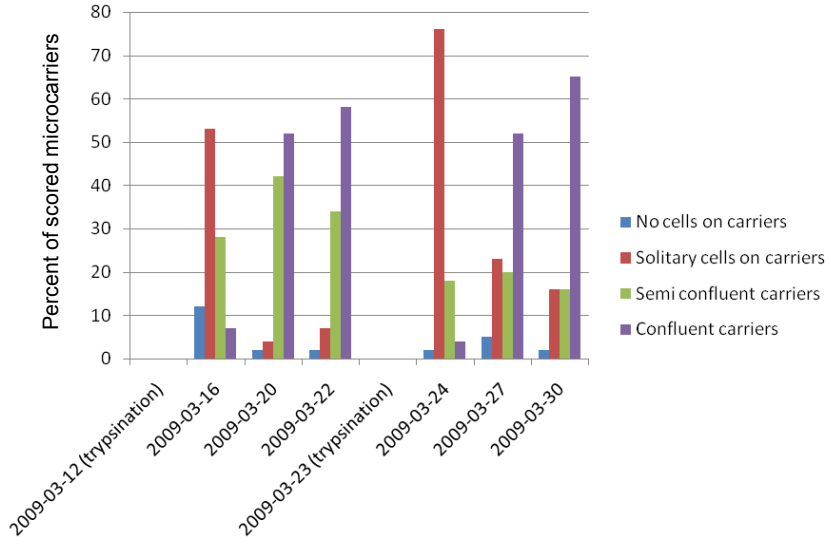

Figure 10. Distribution of SkMCs on Cytodex 1 in the WAVE Bioreactor. The experiment setup is described in Figure 7 and 8. After trypsination using method B the cell density on the microcarriers was observed. One hundred microcarriers were scored and divided into four groups dependent on the cell density: (i) no cells on carriers, (ii) solitary cells on carriers, (iii) semi-confluent carriers, (iv) confluent carriers.

3.3.2 Migration of hMSCs on Cytodex 3 in WAVE Bioreactor

The second WAVE Bioreactor experiment had the same setup that shown in Figure 7, except the starting volume was 2.2 ml of sedimented Cytodex 3. $5.0x10^6$ wt-hMSCs and $3.0x10^6$ hMSCs-GFP were pre-incubated during 1 hour in 37 °C in separate T-flasks, which allowed the cells to attach to Cytodex 3. These two cultures were then pooled together in the cellbag. The total cultivation time in the WAVE was 18 days and additional Cytodex 3 was added at day 11 and 13 (Figure 11). Samples were taken through the sample port for nuclear counting, growth estimation and observation of distribution of wt-hMSCs and hMSCs-GFP on Cytodex 3. This was observed in a light microscope. The growth varied throughout the experiment and the cell number decreased after 14 days, although the cells looked good in the light microscope. This could be explained by the difficulties in taking out representative samples from the WAVE Bioreactor. However, the correct cell number couldn't be estimated until the final trypsination when the cells were counted.

The distribution of wt-hMSCs and hMSCs-GFP on Cytodex 3 during cultivation in the WAVE Bioreactor was observed in a fluorescent microscope. To estimate the distribution of cells, the carriers were first observed in blue light where all carriers with green shining light were counted as carriers with hMSCs-GFP. Then the blue light was turned off and the carriers

with only wt-hMSCs and carriers that were empty were counted. The results are shown in Figure 11, where the black arrows indicate the time when new carriers were added.
The distribution of wt-hMSCs and hMSCs-GFP changed throughout the experiment. When new carriers were added the fraction of empty carriers increased as expected. Carriers with hMSCs-GFP increased over time while carriers with only wt-hMSC decreased.

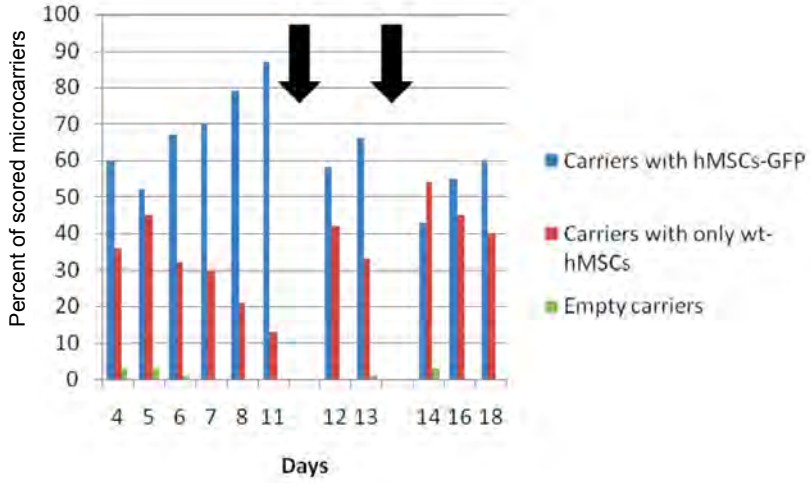

Figure 11. Distribution of wt-hMSCs and hMSCs-GFP on Cytodex 3 in the WAVE Bioreactor. The experiment had the same setup as described in Figure 7 except for the starting volume of sedimented Cytodex 3 that was 2.2 ml. $5.0x10^6$ wt-hMSCs and $3.0x10^6$ hMSCs-GFP were pre-incubated in separate T-flasks to allow attachment to Cytodex 3 and were then pooled together in the cellbag. The total cultivation time in the WAVE was 18 days and daily samples were taken for nuclear counting, growth estimation and observation of the distribution of wt-hMSCs and hMSCs-GFP on Cytodex 3 in a fluorescent microscope. After 11 and 13 days in culture additional 4.4 ml sedimented Cytodex 3 was added, respectively. The black arrows point at the adding of new carriers.

The distribution of cells on Cytodex 3 during the cultivation in the WAVE Bioreactor is documented in Figure 12. A change in the distribution right after the start and after the addition of new carriers was observed. After the addition of new carriers the fraction of empty carriers and carriers with solitary cells was increased while the fraction of semi-confluent carriers and confluent carriers were decreased. This relationship was inversed after approximately two days.

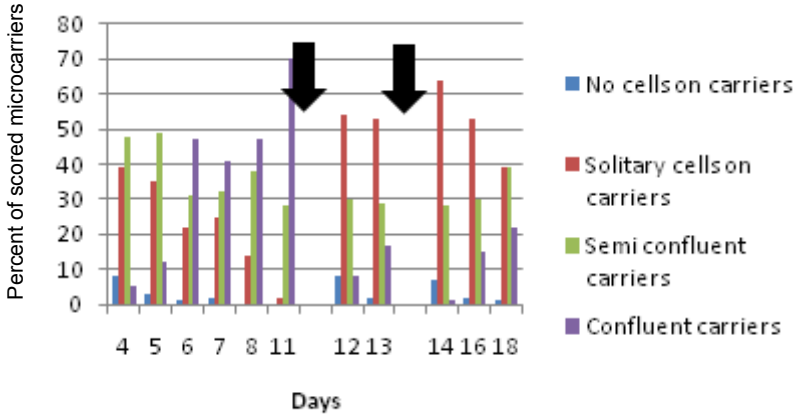

Figure 12. Distribution of hMSCs on Cytodex 3 in the WAVE Bioreactor. The experiment had the same setup as described in Figure 11. One hundred microcarriers were scored dependent on cell density and were divided into four groups. (i) no cells on carriers, (ii) solitary cells on carriers, (iii) semi-confluent carriers, (iv) confluent carriers. The black arrows point at the addition of new carriers.

After the final trypsination, after 18 days in the WAVE, the Separator was used to separate the cells from the carriers. The cells were counted both before and after the cells had been separated to verify that the cells were not lost in the Separator. Before the final trypsination, 20% of the cell suspension was removed in order to test trypsination method A with 0.25 gram trypsin per litre (Sciencell). This was done to get a better knowledge how different trypsinations affected the cells and if all cells were detached with a more gentle trypsination. The results are presented in table 2. To ascertain that the hMSCs remained undifferentiated after the cultivation in the WAVE, the cells were collected for further growth in a T-flask. The characteristic features for hMSCs were studied in a light microscope and no differentiation could be observed.

Table 2. The cell numbers of hMSCs on Cytodex 3 in the WAVE Bioreactor.

	Before Separator[a]	Before Separator[b]	After "Separator"
Number of cells	$20x10^6$	$17x10^6$	$9x10^6$

[a] Trypsination method A.
[b] Trypsination method B.

3.4 Degradable microcarriers

3.4.1 Degradation study

To find a degradable microcarrier with optimal degradation, which was degraded neither too fast nor too slow, the first focus was to test different concentrations of amylase combined with different time exposures. The first degradable carriers to be tested had been developed using a new type of coupling chemistry (GE Heathcare, Uppsala). The prototypes are referred to as 2278033 A and B, and 2278035 A and B. The carriers were exposed to 3.1 and 102.5 units of amylase for 24 hours, respectively. Dulbecco's Phosphate Buffered Saline (DPBS) buffer was included as a control. The result for prototype 2278033A is shown in Figure 13. It seems like the carriers were degraded from the outside and in and left a core that was reduced in size until it disappeared completely. Only transparent shells ("ghost carriers") of the carriers remained.

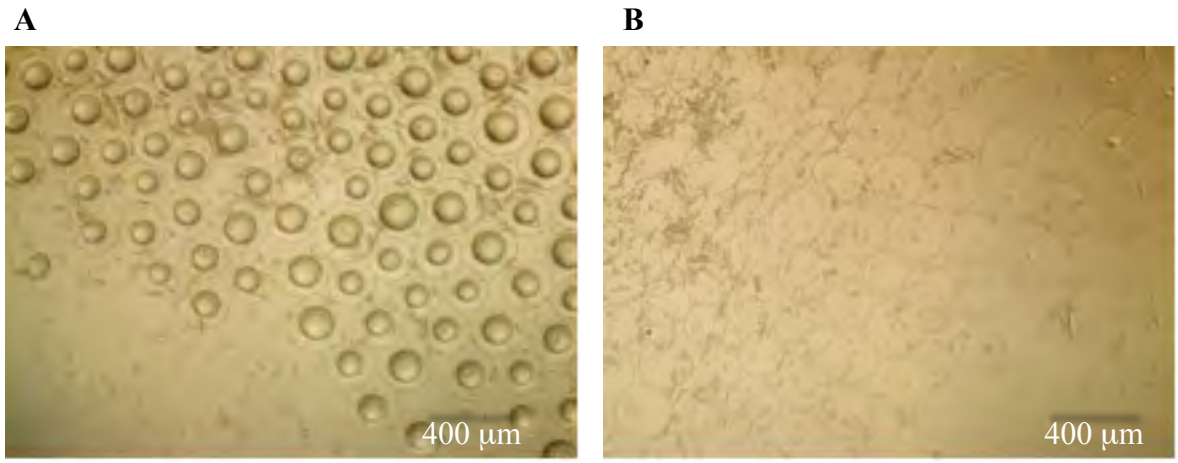

Figure 13. Degradation of prototype 2278033A with 102.5 units of amylase. The degradable microcarriers (20 µl sedimented carrier) were placed in six well plates (area one well, 9.6 cm^2) with 700 µl Dulbecco's Phosphate Buffered Saline and photographed in a light microscope. A) after one hour. B) after two hours.

The other prototype with a different coupling chemistry, 2278035, was included in the same experiment. The results with 102.5 units amylase are shown in Figure 14. For this prototype it seems as if the carriers were degraded from the inside and out, leaving an outer shell that after additional time also was degraded. As shown in Figure 14, prototype 2278035B was more easily degraded than prototype 2278035A. Prototype 2278033 was practically degraded after two hours. Prototype 227835A on the other hand was not degraded after 22 hours of exposure even when higher concentration of amylase was used (data not shown). In conclusion, prototype 2278035 seemed less degradable than prototype 2278033.

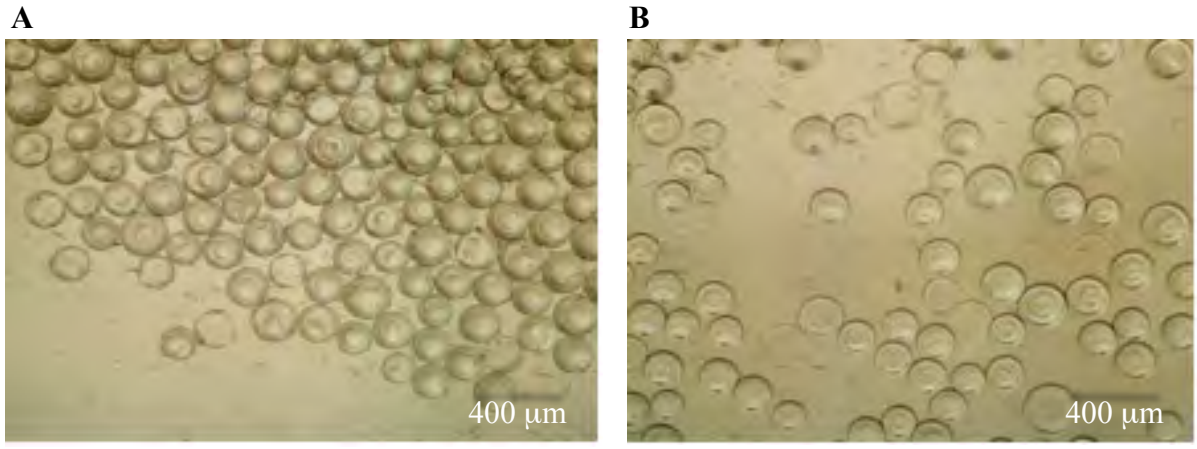

Figure 14. Degradation of prototype 2278035 A and B with 102.5 units of amylase. The experiment had the same setup as described in Figure 13 and photographed in a light microscope. A) after four hours for prototype 2278035A. B) after four hours for prototype 2278035 B.

3.4.2 SkMCs grown on degradable microcarriers

The prototypes (2278033 and 2278035) used in the degradation study were tested for growth of SkMCs. The cell growth was observed and evaluated in a light microscope. To better visualize the SkMCs on the carriers the cells were stained with hematoxylin. Cytodex 1 was used as a control.

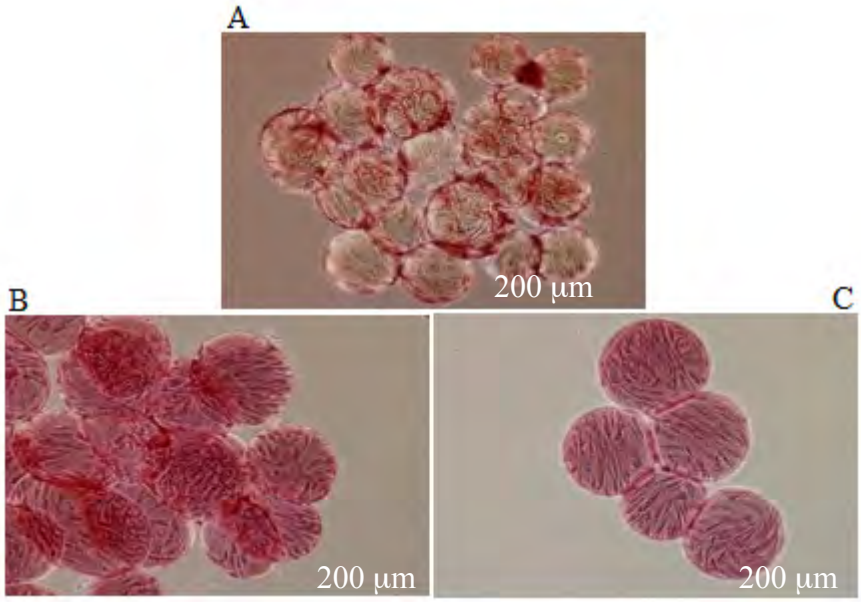

Figure 15. Hematoxylin stained SkMCs grown in a 6 well plate. The microcarriers (20 µl 50:50 slurry of carriers and Dulbecco's Phosphate Buffered Saline) were placed in a six well plate (area one well, 9.6 cm^2) with 700 µl SkMCM (3H Biomedicals) and $0.2x10^5$ cells per well. A) cells grown on Cytodex 1 (control) for 24 hours. B) cells grown on prototype 2278033A for 72 hours. C) cells grown on prototype 227833B for 72 hours. Photos were taken in a light microscope.

Prototypes 2278035A and B didn't yield as good cell growth as Cytodex 1 and are therefore not shown. Prototypes 2278033A and B, on the other hand, yielded cell growth comparable to that on Cytodex 1, as illustrated in Figure 15B and C. Photos of SkMCs grown on Cytodex 1 (control) are shown in Figure 15A. The cells on prototypes 2278033A and B grew more smoothly around the carriers in a very organized manner whereas cells on Cytodex 1 grew more in-between carriers in cocoon-like structures. The way cells grow on the microcarrier may also be an important issue since shear forces in the WAVE Bioreactor might tear the cells apart and detach them from the microcarrier. Carriers that allow cells to grow more tightly might prevent this from happening. When degradable microcarriers were exposed to amylase in the serum-containing medium, the carriers might have been affected. As shown in Figure 15B and C the prototypes 2278033A and B had swollen a little and had become oval-shaped after 72 hours in the medium. As conclusion one can say that the prototypes 2278033A and B permitted cell growth comparable to that on Cytodex 1, if not better.

3.4.3 hMSCs on degradable microcarriers

The best degradable prototype, 2278033A and another prototype 2287036, with slightly different coupling chemistry, were used for cultivation of hMSCs in a six well plate. This time visual observation in a light microscope was complemented with nuclear counting of the cells to receive a more precise number of the cell growth. The results for the nuclear counting are summarized in Figure 16.

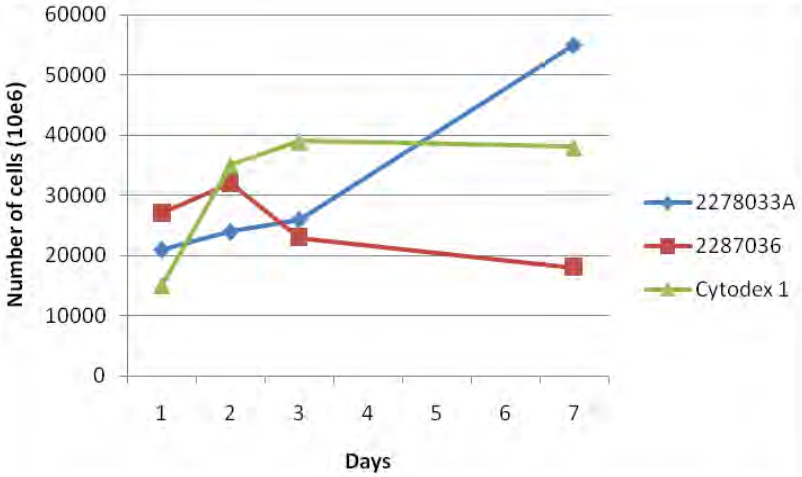

Figure 16. Growth of human mesenchymal stem cells (hMSCs) on degradable microcarriers in a six well plate. The experiment had the same setup as described in Figure 15 although another medium was used (MSCBM^{TM}). The degradable prototypes and Cytodex 1 (control) were cultivated for seven days. The number of cells was estimated by nuclear counting.

The results demonstrated better final cell growth on prototype 2278033A than on Cytodex 1. Prototype 2287036 and Cytodex 1 had a peak in their growth at day two and three where they both stabilized and decreased in cell number. The cell growth on prototype 2278033A on the other hand had a slow start but increased in cell number towards the end of the cultivation.

3.4.4 Degradable microcarriers in spinner flask

To elucidate how the degradable microcarriers behaved in the WAVE Bioreactor or in stirred cultures, hMSCs were cultivated in spinner flasks. The most promising prototype 2278033A was selected and also prototype 2287040, which had still another type of coupling chemistry. Cytodex 1 was used as control. Nuclear counting and observations in a light microscope estimated the growth. The results are shown in Figures 17 and 18.

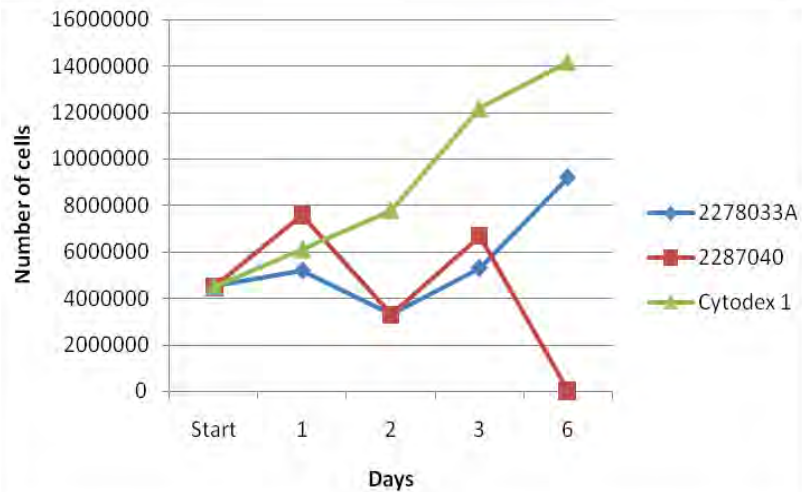

Figure 17. Growth of human mesenchymal stem cells (hMSCs) on 2278033A and 2287040 in spinner flasks. hMSCs ($4.5x10^6$ cells) were added to separate spinner flasks containing 40 ml DMEM, with10% FBS (Gibco®) and 1.5 ml of sedimented microcarrier that had been equilibrated overnight at 37 °C. Cytodex 1 was used as a control. During six days of culture, the cell number was obtained by nuclear counting.

Regarding prototype 2287040 (Figure 17 and 18A) all cells were detached and floated around as single cells, probably dead. Prototype 2278033A (Figure 17 and 18C) and Cytodex 1 (Figure 17 and 18B) seemed to have comparable cell growth. The cells on Cytodex 1 were a bit more clustered together while the hMSCs on prototype 2278033A grew more closely around the carriers.

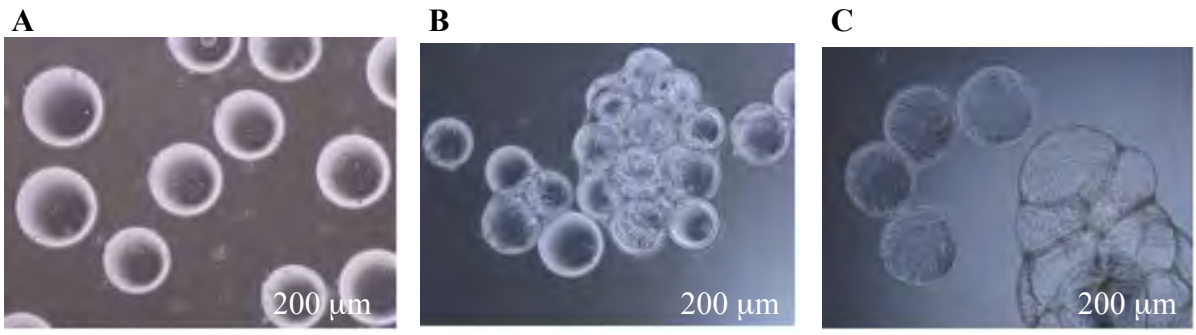

Figure 18. hMSC grown on prototype 2287040 (A), Cytodex 1 (B) and 2278033A (C) in spinner flasks for seven days. The experiment had the same setup as described in Figure 17. Photos were taken in a light microscope.

4 Discussion

Stem cell therapy is generating great interest both scientifically and medically. The importance of finding large-scale cell expansion methods is obvious for effective treatment (Rodrigues et al. 2011, Cormier et al. 2006). The WAVE Bioreactor has the advantage of being a closed system with the potential of monitoring the culture parameters such as carbon dioxide level and temperature. In the WAVE Bioreactor cells are expanded during continues wagging for optimal growth and cultures can be scaled-up more easily compared to other bioreactor systems.

4.1 Quantifying cells

All interpretations of how well cells were growing during this project were based on both cell counting and observation in light microscope. The nuclear counting resulted in nice growth estimations for both microcarriers and monolayers, which confirms its reproducibility and stability as a quantification method. The method using trypsination on microcarriers proved to be less suitable when the cultures were very dense. A repetition of this experiment would be of interest to improve and scale up the experiment to verify these results further.

When the microcarriers were trypsinized a 70 μm filter was used to separate the cells from the carriers. Although no cells were observed left on the carriers after the separation in the filter, the counting of cells before and after the filtration step demonstrated a cell loss. Since the carriers were free from cells one explanation might be that the cells got stuck in the filter while passing. However, further controls if any cells were stuck in the filter are recommended. This quantitative method ought to be improved by counting the cells without removal of the carriers. It can also be complemented using a qualitative method, e.g. observation in light microscope.

It was shown that the nuclear counting method gave questionable results when samples were collected from the WAVE Bioreactor. This could be explained by the difficulty to take representative fractions from the cellbag. Samples were taken from the sample port, which is on the upper part of the cellbag. The purpose was to get a representative sample from the culture that is not perfectly homogenous. This is tricky to perform and can also harm the cells due to the forces from possible collisions of carriers and cells. Therefore, a more suitable sample port at another position could improve the cellbag.

4.2 WAVE Bioreactor

One problem with the WAVE Bioreactor has been to increase the number of cells in the cultures after the cells have reached confluence on the carriers. Consequently it was of interest to grow cells for several passages including addition of more microcarriers. SkMCs were grown on Cytodex 1 where the cell number increased twentyfold when cultured over 30 days, including two trysinations, in the WAVE Bioreactor (Figure 9). Although the viability was 95% I noticed that the cells looked messy and that there were a lot of aggregates of cells and carriers whereas some carriers were empty. One explanation might be the repeated trypsinations that can be both tough and long for the cells in order to detach them from the carriers. This might affect the cells in a negative way with a decreased cell growth and

recovery (Fernandes et al. 2007). I ascertained that this was not the optimal way to expand cells in the WAVE Bioreactor.

My hypothesis that a culture could be scaled up by successive adding of the microcarriers to the WAVE Bioreactor was tested. The cells grew better on the carriers and migrated from carrier to carrier without problems when new carriers were added (Melero-Martin et al. 2006). This was shown in the experiment (Figure 11 and 12) where wild-type and GFP-expressing human mesenchymal stem cells (hMSCs) were cultured on Cytodex 3 in the WAVE Bioreactor. When the culture reached the desired cell number the challenge remained to detach the cells from the microcarriers. Since the usage of trypsin has not been successful so far this requires further development.

4.3 Migration of hMSC

It was shown that hMSCs efficiently could migrate between the microcarriers (e.g. Cytodex 3) during cultivation in spinner flask and that continuous addition of carriers was an excellent method to expand the cell culture. This was therefore implemented for the WAVE Bioreactor. Microscopy of the culture in the WAVE Bioreactor suggested this as a promising way of culturing the cells. The cells grew better around the carriers and did not clump together as they did when the culture was expanded and the cell number increased by trypsination and addition of carriers. However, difficulties in quantification yielded much lower cell numbers than expected and the hypothesis that this was a good way of growing cells could not be confirmed.

4.4 Degradable microcarriers

The three-dimensional expansion of MSCs on the Cytodex microcarriers has already been found to be a useful alternative to the conventional monolayer cultivation methods (Frauenschuh et al. 2007). In my experiments the cells on the Cytodex microcarriers grew in between the carriers in cocoon-like structures while the cells on the degradable microcarrier 2278033 grew more smoothly around the carriers in a very organized way. This can be an advantage when cells are grown in the WAVE Bioreactor since the mixing in the bioreactor might tear the cells apart and detach them from the microcarrier.

It is still a problem to detach the cells from the carriers and further improvements have to be made. The goal for the degradable microcarrier to have superior way of cell release to improve and simplify the workflow during cell therapy was not met. It can be concluded that the way cells are growing on the microcarrier may play a fundamental role when cultivating cells in the WAVE Bioreactor since shear forces might tear the cells apart and detach them from the microcarrier. Another interesting discovery was the alternative way of cultivating adherent cells on Cytodex 3 in the WAVE Bioreactor. Adding microcarriers without any trypsination seem to have potential of working in larger scale. In future experiments it would be of interest to combine the advantage of cultivating cells in the WAVE Bioreactor with other advantages such as the migration of cells and the superior cell growth on the degradable microcarrier 2278033.

4.5 The field today

The WAVE Bioreactor is under continues development and is used widely today in the pharmaceutical industry. A new improvement is the new software, which has improved the process control and understanding of the WAVE Bioreactor. Also a new control unit called WAVEPOD II has been introduced and it is now possible to monitor the pH optically with a light- and proton-sensitive luminophore (GE Healthcare, 2011). Dalton et al (2012) have used the WAVE Bioreactor for the large scale in vitro culturing in suspension of blood-stage *Plasmodium falciparum* parasites. They could conclude that the bioreactor provides low-shear and favorable hydrodynamic conditions for cell cultivation and very efficient gas transfer. They also showed that they could obtain healthy parasites that retain synchronicity over at least three cycles of invasion and development. This could revolutionize the culturing and propagation of malaria parasites for future basic studies. More relevant to my studies are the work of Timmins et al (2012) who have cultivated human placenta MSC on microcarriers in the WAVE Bioreactor. The most widely researched source of MSC is bone marrow and little attention has been given to isolation from complex tissues such as the placenta. Timmins et al (2012) have estimated that a single placenta may be sufficient to produce over 7000 doses of therapeutic MSC using the WAVE Bioreactor combined with microcarriers.

5 Materials and Methods

5.1 Cell lines

Skeletal muscle cells (SkMCs) were cultured in skeletal muscle cell medium (SkMCM) (3H Biomedicals). Cells cultured in monolayer were seeded at a density of 5000 cells/cm^2. Confluent cells were washed with DPBS (Dulbecco`s phosphate buffered saline-0.0095 M phosphate (PO4), calcium- and magnesium-free) (Lonza) at least two times. Two trypsination methods were used. Trypsination method A with 0.25 gram trypsin per litre and pH range 7.2 - 8.0 (Sciencell). Trypsination method B with 2.5 gram trypsin per litre, 0.38 gram EDTA per litre and pH range 7.2 - 8.0 (Gibco®). The cells were then detached from the surface using trypsination method A or B during incubation at 37ºC for less than 5 min.

Mesenchymal stem cells (MSC) were obtained from either 3H Biomedicals or Lonza. MSCs from 3H Biomedicals were cultured in Mesenchymal stem cell medium (MSCM) (Sciencell) and the MSCs from Lonza were cultured in Mesenchymal stem cell basal medium (MSCBM^{TM}) (Cambrex BioScience). MSCs expressing green fluorescent protein (MSCs-GFP) cells were a kind gift from Dr Massimo Dominici, University of Modena. In this report these cells will be referred to as hMSCs-GFP. These cells exhibit bright green fluorescence when exposed to blue light. After the cells were defrosted these cells were seeded at 10000 cells/cm^2, and cultured in Dulbecco's Modified Eagle Medium (DMEM), with 10% fetal bovine serum (FBS) (Gibco®). After two days the cells were split and seeded at 5000-6000 cells/cm^2 and cultured in Quantum 333 Complete Fibroblast Medium (A&E Scientific (PAA)) for fibroblasts with L-glutamine (292 mg/l) for cell growth. Streptomycin was added to all media to a concentration of 1%. Wild-type bone marrow-derived MSCs were purchased from Lonza.

5.2 Cell culture equipment

Several different equipments were used to culture the cells T-flasks (Thermo Scientific) of different sizes were used (25, 75 and 175 ml) were used for cultivation and scale up. In experiments where many different parameters were investigated, e.g. different prototypes of the degradable microcarriers, multidishes (6 or 24 well plates) (Thermo Scientific) were used. In this thesis referred to as 6 or 24 well plates (Thermo Scientific). The spinner flasks (Sigma-Aldrich) were used to mimic a smaller bioreactor.

5.3 Microcarriers

1 g Cytodex 1 or Cytodex 3 (GE Healthcare) microcarriers were swollen in 50 ml DPBS for at least eight hours (but preferably overnight). The carriers were then autoclaved and washed three times with ~50 ml of DPBS. The day before use, the carriers were washed three times, or until the medium retained its proper colour, with the medium intended to be used for the cells. A couple of hours before seeding of cells, the carriers were allowed to settle and the supernatant was decanted and replaced with fresh medium. The carriers were equilibrated in the incubator at 37ºC and pH 6.9-7.5. If the carriers were to be used in the WAVE Bioreactor, the equilibration was implemented inside the bioreactor instead. When cells were growing on microcarriers statically (T-flasks or six well plates), there might be problems with cells attaching to the bottom instead of to the carriers. Consequently, an in-house coating method

was used, where the T-flask or plate (6 or 24 well) was covered with phenyl dextran with a working solution of 10 mg/ml for approximately 5 min. This was done to prevent the cells to attach to the bottom of the flask or plate used.

5.4 The WAVE Bioreactor

In the WAVE Bioreactor medium and carriers were pre-conditioned inside the cellbag while connected to the CO_2-mixer set to 5%, a temperature of 37 ºC, angle of 4º and agitation of 4 rocks per minute. The settings were adjusted approximately one hour before the cells were added and used throughout the complete cultivation. When cells were attached to the carriers outside the cellbag, only the medium was equilibrated inside the bag. Medium, cells and carriers were added with a 50 ml syringe or a 500 ml transfer bottle via the tube connector to the cellbag. The inlet and outlet filters were clamped off and the bag was placed inside a LAF hood to maintain sterility during the transfer. When the experiment was started 200-300 ml medium was used, this amount was increased as the cells grew. A concentration of 0.01 ml of sedimented microcarriers/cm^2 was maintained throughout the experiment. 5-10 cells/microcarrier were seeded when an experiment was started.

5.5 Cell counting

5.5.1 Cell detachment and counting using trypsin

Cells on microcarriers were trypsinized when 80-90% of the carriers were covered by confluent cells. For smaller volumes, such as those from the six well plates, everything from the well was transferred to a test tube. The carriers were then allowed to sediment and the supernatant was decanted. The carriers were then allowed to sediment again and washed with DPBS three times and once with 0,02% EDTA before 0.5 ml Trypsin/EDTA (Gibco®) were added to the cell pellet (trypsination method B). After trypsination everything was transferred to a new well and incubated for 5-10 minutes at 37 ºC. After the cells detached from the carriers everything was transferred through a 70 µm filter (BD Biosciences) and the filtrate was transferred to another test tube. The filter was washed with DPBS so that all cells could go through. The filter was then examined in the light microscope to ensure that no cells were left in the filter. The cells in the test tube were then counted in a hemacytometer.

For larger volumes, such as those from the WAVE Bioreactor, the trypsination was performed inside T-flasks or T-flasks combined with the Separator. The Separator was obtained from a co-worker at GE Healthcare in Cardiff.

5.5.2 Nuclear counting

Upon staining with crystal violet cells will burst and the nucleus of cells will be set free and coloured to enable cell counting. For smaller volumes the cells, if grown on microcarriers, were transferred together with the carriers from the well to a test tube. The carriers were allowed to sediment and the supernatant was removed. 500 µl crystal violet (with a final concentration of 0.1 %) was added and the sample was mixed in a vortex before being incubated for 1 hour at 37ºC. After incubation, the test tube containing the cell suspension was carefully mixed with a vortex once more before the cell nuclei were counted in a hemacytometer. For cells grown in monolayer, the medium was removed from the well and 500 µl crystal violet was added and flushed up and down with a pipette. The suspension was then incubated for 1 hour at 37 ºC. After incubation the nuclei were counted in a hemacytometer.

5.5.3 Microscopy

All microscopy was performed with an Eclipse TE2000-U (Nikon) that both could use light and immunofluorescence. The camera (Nikon) connected to the microscope was used to take all photos on cells in this thesis.

5.6 Hematoxylin Staining

The content of a well in a six well plate was transferred to a test tube. The carriers were allowed to sediment and the supernatant was removed. Cells and carriers were washed with approximately 2 ml DPBS and then 200 µl hematoxylin (1%) was added. After incubation at room temperature for ~30 minutes, 500 µl distilled water was added and the sample was incubated for an additional 1.5 hours at room temperature. The cells were then counted in the light microscope.

5.7 Cell detachment and expansion of cells in the WAVE bioreactor system

When approximately 90% of the microcarriers were confluent with cells and the heat and rocking were turned off. The inlet and outlet filters were then clamped off and the cellbag was placed inside a LAF hood in an upright position. All carriers were allowed to sediment so that the superfluous medium could be removed via the tube connector port. The remaining medium with cells and carriers were then transferred into 50 ml Falcon tubes and cells and carriers were allowed to sediment without any centrifugation. Cells and carriers were washed at least six times with DPBS, or until all colour was gone. Then one additional wash was performed with 0.02% EDTA solution (pH 7.4) at room temperature. Then approximately 5 ml Trypsin/EDTA (Gibco®) were added and transferred to one or several T-flasks, depending on the amount of cells and carriers (trypsination method B). The cells and carriers were incubated 5-15 minutes at 37ºC. It might be necessary to flush with a pipette to detach all cells and then stop the trypsination by adding medium. The medium used for respective cell line is described in section 5.1. Cells and carriers were then put inside the additive bottle together with new carriers. The amount was dependent on each experiment and are described in the result section, and transferred back to the cellbag.

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