CHAPTER 9

Generation of growth hormone expressing cells in a vascularised bioengineered microchamber

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Summary

Growth hormone, secreted by the somatotrope cells of the pituitary gland, is essential for key physiological processes. Deficiency in growth hormone is a significant clinical problem in both children and adults. Current treatment by injection therapy does not mimic physiological secretion, which is pulsatile and responds to growth hormone regulatory factors. A cell implant that secretes growth hormone and is sensitive to physiological cues would be the ideal long-term treatment. This chapter discusses the potential for development of a growth hormone secreting organoid from a bioengineered vascularised microchamber. We show that pituitary hormone precursor cells transplanted in vivo into microchambers survive and differentiate into growth hormone expressing cells.

KEYWORDS: Pituitary, Precursor cell, Growth hormone, Bioengineering, Vascularisation.

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1. INTRODUCTION - GROWTH HORMONE DEFICIENCY

Growth hormone (GH) secreted by the somatotrope cells of the pituitary gland is essential throughout life, regulating growth, bone density, the cardiovascular system and the metabolism of proteins, carbohydrates and lipids (1). GH deficiency is a significant clinical problem in both children and adults. It is commonly idiopathic in children and can be triggered at any age by conditions including genetic abnormalities, immune or infectious diseases, injury, trauma, intracranial tumours, radiotherapy, chemotherapy, obesity and ageing (2, 3).

The current treatment for GH deficiency is by daily subcutaneous injection of recombinant GH. Injections cannot mimic the sensitive pulsatile physiological secretion of GH which is finely controlled by opposing regulatory factors (1, 4). Treatment by GH injection is therefore difficult to control, requires meticulous monitoring and the majority of young patients on such treatment do not achieve the average height and weight range for their age (1). The long term effects of GH treatment are also unclear, posing complications associated with GH excess (5). When injection therapy is used to treat GH deficiency resulting from irradiation of an intracranial tumour, it carries the theoretical risk of predisposition to a secondary tumour (6). The current GH injection therapy is in need of replacement with an improved treatment strategy. Ideally the most effective method of treatment is a cell based therapy replenishing GH cells that has the capacity to respond to physiologically released regulatory factors.

2. TISSUE ENGINEERED MICROCHAMBERS FOR THE TREATMENT OF GROWTH HORMONE DEFICIENCY

A cell based therapy for GH deficiency could be achieved by the development of a new GH-secreting "organoid" using vascularised, tissue-engineered microchambers that have an adjustable microenvironment (7). The microchamber model, first developed by our laboratory, features a chamber that can be sited on any suitable blood vessels, such as the epigastric or femoral vessels in the groin, the splenic vessels in the pancreatic bed, the portal vein, the mesenteric vessels, or the aorta. The microchamber can be seeded with cells or tissues of various types and the vessels supply oxygen and nutrition to the developing graft (8).

The microchamber model has been developed in species including rats, mice and pigs (9). In mice this model specifically takes advantage of transgenic and knockout models, for

example in the case of GH deficiency it can be used in GH-deficient mice (*lit/lit* or *dwarf* mice). Figure 1 shows the mouse microchamber model which involves an arteriovenous pedicle enclosed in a subcutaneously-inserted cylindrical silicone chamber (7). Angiogenesis and spontaneous outgrowth of new fibrovascular tissue occurs in the hypoxic space within the chamber (10). The new, three-dimensional, vascularised tissue substantially fills the chamber within 2 to 4 weeks. During weeks 4 to 12 there is further expansion and maturation of the graft tissue, with progressively greater collagen fibril alignment. The new vascularised tissue will grow even in the absence of added extracellular matrix, factors or cells. However, the rate of new tissue growth within the chamber can be influenced by the addition of various growth factor-containing extracellular matrix scaffolds.

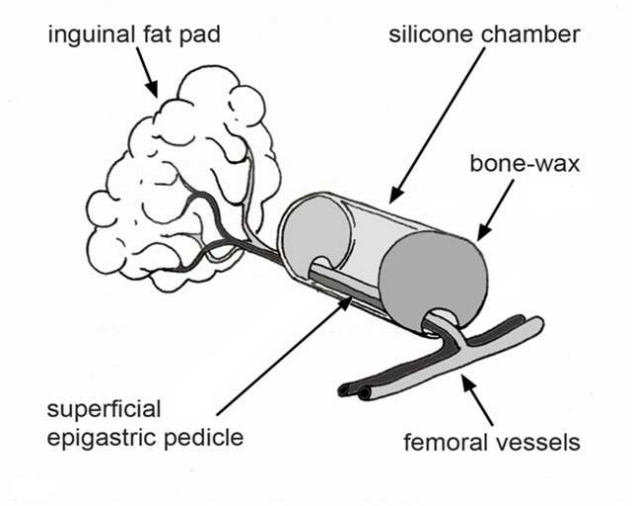

Figure 1. Schematic diagram of the vascularised microchamber: A silicone tube (5 x 3.3 mm) envelops the isolated epigastric artery and vein originating from the femoral vessels which supply the inguinal fat pad. Firstly, one end of the chamber is sealed with bone wax, then the donor cells are injected suspended in a supporting extracellular matrix (Matrigel), which sets at body temperature thereby immobilising the cells within the chamber. Secondly, the other end of the chamber is then sealed. The circulating blood that flows through the chamber supports the growth and survival of the chamber contents through blood vessels that sprout by angiogenesis from the pedicle. This figure was adapted from our original publication (35). Figure published with consent from Stem Cells (AlphaMed Press).

Importantly the microchamber can be seeded with mature adult cells, immature precursor/stem cells or engineered cells. The donor cells are usually suspended/delivered in a compatible extracellular matrix scaffold in the form of a hydrogel or sponge. Laminin-containing matrices such as Matrigel are known to form cell-extracellular matrix interactions with, for example, pancreatic islets; these interactions are essential for their signalling, growth and

development (11). The extracellular matrix may also be supplemented with exogenous growth/survival factors such as basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) or vascular endothelial growth factor (VEGF) for improved angiogenesis (12,13) and growth hormone releasing hormone (GHRH), Ghrelin or somatostatin (regulators of GH synthesis/secretion) (14).

3. THE GENERATION OF SOFT TISSUE AND ENDOCRINE TISSUE IN MICROCHAMBERS

Microchambers in mice were initially used to grow new soft tissues. When seeded with adipose-derived precursor cells (preadipocytes), adipose tissue formed (15). Myoblasts seeded into vascularised chambers in rats survived and developed into desmin- and dystrophin-positive myotubes (16). More dramatically neonatal rat cardiomyocytes seeded into vascularised chambers developed in four weeks into a spontaneously beating three-dimensional mass of cardiac tissue (17). Immature pancreatic precursor cells from the adult pancreas seeded into chambers in immunodeficient SCID mice survived and differentiated into cells that secreted insulin, glucagon and somatostatin (18).

The functional potential of the microchamber was elegantly demonstrated by an insulin-secreting "organoid" developed to treat diabetic mice. Hyperglycaemic, streptozotocin (STZ)-induced diabetic mice were treated by seeding ~350 islets from syngeneic donors into vascularised microchambers. The aim was to show that, in this environment, islets remain viable and normalize blood glucose (i.e. achieve euglycaemia). There was a significant reduction in blood glucose levels compared with untreated diabetic mice within three weeks. This was sustained for five weeks, in 6 out of 7 chambers sited around the splenic vessels and in 4 in 7 chambers sited in the groin. These results were corroborated by positive intraperitoneal glucose tolerance tests in the presence of the islet-containing chambers (at week 8), a return of hyperglycaemia after the chambers were surgically removed (week 10) and the immunochemical detection of islets in the microchamber (19). Similarly, a GH-secreting organoid could potentially restore GH levels in GH-deficient mice.

4. PITUITARY PRECURSOR CELLS (PCFCS) GENERATE GROWTH HORMONE CELLS

In order to develop a long-term cell-based treatment for GH deficiency using the microchamber model, we first sought to identify a cell type for transplant that possessed both long-term potential (the ability to self-renew) and the ability to produce GH-secreting somatotrope cells. We were the first to report the identification and characterisation of a resident pituitary precursor cell: **P**ituitary **C**olony **F**orming **C**ell (PCFC) (Figure 2). PCFCs share characteristics with progenitor/stem-like

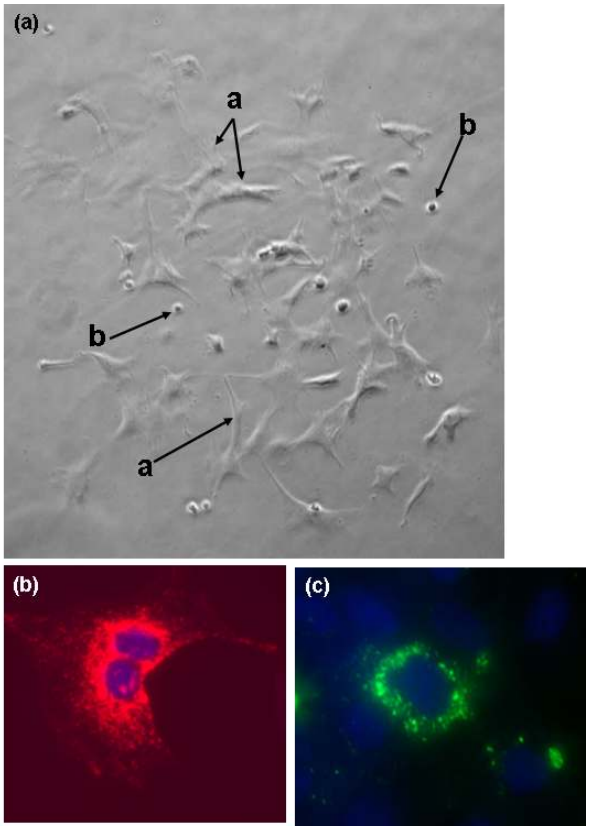

Fig. 2. (a) A colony of cells that has arisen from a single Pituitary Colony Forming Cell (PCFC). Cells are heterogenous in nature, either stellate in shape with long cytoplasmic processes (arrow a) or round in shape (arrow b). Rare GH(+) cells found within colonies were also either stellate or round in shape. (x200 magnification). **(b)** Stellate GH(+) cell showing red GH granules immunostained with rabbit anti-rat GH detected with goat anti-rabbit Alexa 595. (x600 magnification). (c) Round cell showing green GH granules immunostained with rabbit anti-rat GH detected with goat anti-rabbit Alexa 488. Nuclei are counterstained in blue with DAPI (4,6-diamidino-2-phenylindole, dihydrochloride). (x600 magnification). This figure was adapted from our original publication (21). Published with consent from Exp Cell Res (Elsevier).

cells (20, 21). PCFCs are rare, they have clonogenic properties in that they form a heterogenous colony from a single cell, have high proliferative potential forming colonies as large as 5000 cells, express cell surface stem cell-related antigens and have the capacity to differentiate into somatotropes. These characteristics make PCFCs an attractive long-term cell-based treatment strategy for GH deficiency.

5. ENRICHMENT OF PCFCS

We have developed a protocol to obtain enriched preparations of PCFCs that could be used for implantation into microchambers. PCFCs belong exclusively to a sub-population of pituitary cells that have the cellular cytoplasmic machinery (PepT2 transporter) to import large di-peptides (22). Utilising this mechanism we can enrich for PCFCs (21). Fluorescent-tagged di-peptide, β-Ala-Lys-Nε-7-amino-4-methylcoumarin-3-acetic acid (AMCA) is actively imported by single cell digests of the pituitary gland (Figure 3). PCFCs which are normally 0.2% of the whole pituitary population are exclusively found in the AMCA(+) sub-population and are enriched 94-fold to 18% by the selection of AMCA(+) cells using Fluorescence-Activated Cell Sorting (FACS).

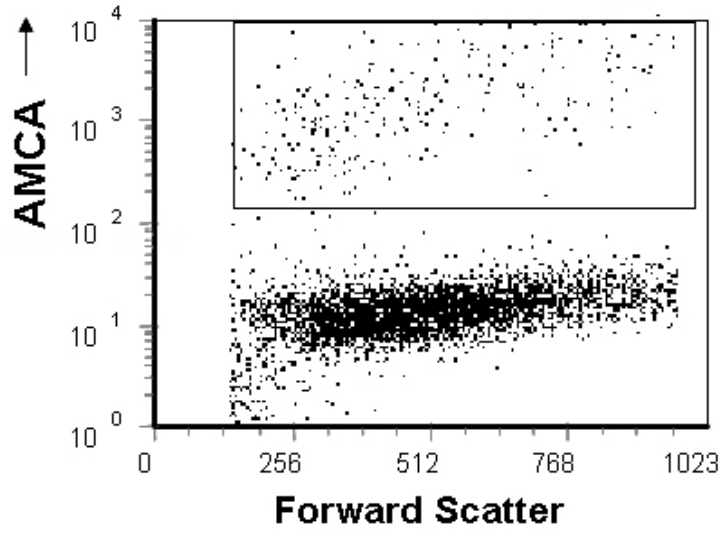

Fig. 3. Flow cytometric analysis of pituitary single cell suspensions that have imported β-Ala-Lys-N_ε-7-amino-4-methylcoumarin-3-acetic acid (AMCA). AMCA(+) cells representing 4% of pituitary cells can be seen on the vertical axis $>10^2$. The physical characteristics of the cells in terms of size (forward scatter) can be seen along the horizontal axis.

Further enrichment of PCFCs with high recovery can be achieved by refining the FACS selection to AMCA(+) cells that also express stem cell-related markers: Angiotensin Converting Enzyme (ACE) and Stem Cell Antigen-1 (Sca-1) (Figure 4) (20). The selection of AMCA(+)ACE(+) cells results in a 170-fold enrichment of PCFCs (a clonogenicity of 1 PCFC for every 3 cultured cells). When both ACE and Sca-1 selection are combined with AMCA(+) cells there is even further enrichment of PCFCs to 195-fold (a clonogenicity of 1 in every 2 cultured cells). This excludes endothelial and hemopoietic cells which are themselves clonogenic. It is therefore possible to efficiently isolate highly enriched populations of PCFCs (20, 21).

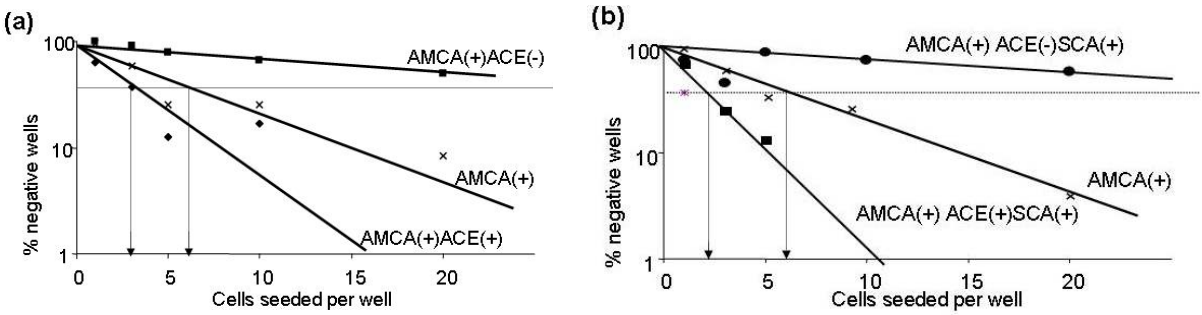

Fig. 4. Limiting dilution analysis shows enrichment of PCFCs using cell surface expression of Angiotensin Converting Enzyme (ACE) and Stem Cell Antigen-1 (Sca-1). The clonogenic activity of AMCA(+) cells that also expressed cell surface ACE and/or Sca-1 was compared with that of the control AMCA(+) population. Cells were sorted using Fluorescence Activated Cell Sorting (FACS) into 96-well plates (n = 24 wells per cell concentration of 1, 3, 5, 10 and 20 cells per culture well) and the clonogenicity was determined using the Poisson distribution analysis method (45). This plot represents data gathered from three independent experiments. **(a)** One in three ACE(+)AMCA(+) cells formed a colony compared with one in six for the AMCA(+) population. There was no detectable clonogenic activity in the ACE(-) sub-fraction of AMCA(+) cells. **(b)** One in two cells of the Sca-1(+)ACE(+)AMCA(+) population gave rise to a colony compared with one in six for the AMCA(+) population. This figure was adapted from our original publication (20). Published with consent from Stem Cells (AlphaMed Press).

6. MIMICKING THE PCFC MICROENVIRONMENT IS THE KEY TO THEIR SURVIVAL AND GROWTH IN VIVO

The key to optimal survival and growth of PCFCs transplanted *in vivo* into the microchamber is to provide them with the microenvironment in which they normally reside. In order to unravel the PCFC microenvironment, we investigated the location of PCFCs within the pituitary gland. We found that PCFCs are located in the pituitary luminal zone (20). This region has been proposed to harbour pituitary stem/progenitor cells, although the identity of these cells has never

been elucidated (23-32). The research and speculation that surrounds putative stem cells of the pituitary has been reviewed and discussed in detail by Vankelecom (33). Interestingly, the pituitary luminal zone is the "undifferentiated" remnant of the pituitary embryonic primordium often termed the marginal epithelial cell (MEC) zone (31). We have shown the MEC zone actively imports AMCA (Figure 5a). Cells with a PCFC-rich phenotype, ACE(+)AMCA(+), are found in the MEC lining of the pituitary gland and occasionally in the subluminal region (Figures 5b,c,d) (20). Interestingly in the brain, this same zone harbours Neural Stem Cells (NSCs) (34). PCFCs are analogous to the NSCs found in the brain luminal zone and share location, behavioural characteristics and phenotype.

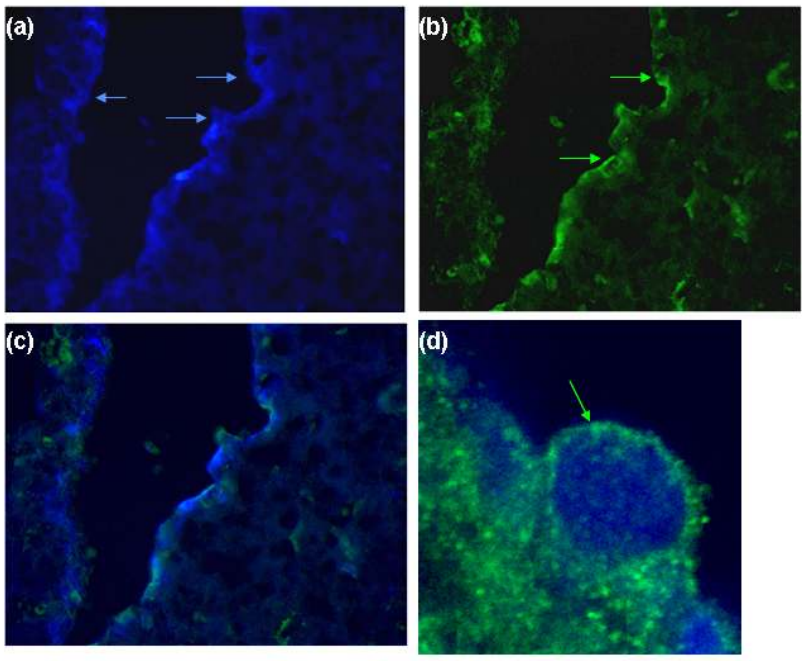

Fig. 5. (a) AMCA-loaded pituitary showing AMCA(+) cells in blue. The pituitary luminal lining is indicated by the arrows (x400 magnification). **(b)** The same view showing expression of ACE (green) detected with Alexa 488 bound to rabbit immunoglobulins to cell surface ACE. **(c)** Merge of image (a) plus (b). **(d)** A confocal micrograph showing a dual positive AMCA(+)ACE(+) cell located in the pituitary luminal lining (x600 magnification). This figure was adapted from our original publication (20). Published with consent from Stem Cells (AlphaMed Press).

Further characterisation of PCFC identity and location (Lepore *et al*., unpublished data) has revealed that PCFCs can be distinguished from their neighbouring cells using a combination of AMCA uptake, expression of the cell surface marker, Alpha-6-Integrin, together with Heat Stable

Antigen (HSA) or ACE expression. The PCFCs belong to the HSA(+) sub-population of the ACE(+)AMCA(+) population. PCFCs are located in the Alpha-6-Integrin(+) epithelial lining of the pituitary gland. Now that PCFCs can be clearly distinguished from their neighbours, the details of their environmental niche can be investigated, helping to elucidate the optimal environment, factors and signals required to sustain, propagate and differentiate PCFCs.

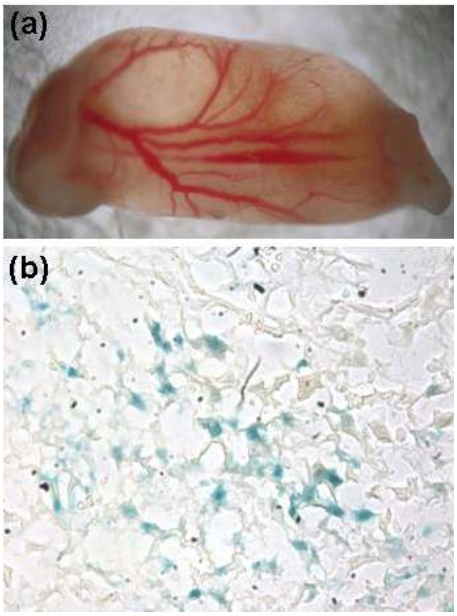

Fig. 6. (a) A graft harvested at six weeks showing the well-formed vascular tree that has sprouted to fill the graft from the central supplying epigastric vessels. **(b)** Beta-galactosidase stain to detect LacZ(+) cells (blue) within cross sections of the 6 week old grafts (x200 magnification). These are the surviving donor cells. This figure was adapted from our original publication (35). Published with consent from Stem Cells (AlphaMed Press).

7. PCFCS TRANSPLANTED INTO MICROCHAMBERS GROW AND DIFFERENTIATE IN VIVO

The potential of PCFCs *in vivo* has been tested by their implantation into the microchamber model described above (Figure 1). This model offers the advantage of both vascularisation and containment of the implanted cells within the microchamber. The implanted cells are exposed to both blood-borne nutrients and locally released factors. Figure 6a shows the complete vascularisation of the graft at six weeks post-implant. Donor cells that survived in the grafts were distinguishable in grafts by their expression of beta-galactosidase linked to the ubiquitous LacZ

gene (Figure 6b) (35). On average the surviving cells were 30% of the original implanted population.

The differentiation capacity of PCFCs was tested using donor PCFCs from a mouse that enables tracking of GH. This transgenic mouse expresses enhanced Green Fluorescent Protein (eGFP) controlled by the GH promoter (36). PCFC-rich populations of cells were pre-selected by FACS as not expressing GH, in other words were eGFP(-) at the time of transplant into the microchamber but were AMCA(+) (Figure 7, gate 1). These donor cells survived and differentiated into GH(+) cells. GH expression was characterised by eGFP expression in grafts detected six weeks after implant of donor cells. GH(+) cells were consistently located near blood vessels in grafts and contained GH vesicles in their cytoplasm (Figure 8). This result was confirmed using an alternative method for detection of GH: the FACS detection of eGFP(+) cells derived from grafts that had been digested enzymatically into single cell suspensions. The percentage differentiation of PCFCs under the current conditions was modest, only 3.3% of implanted cells.

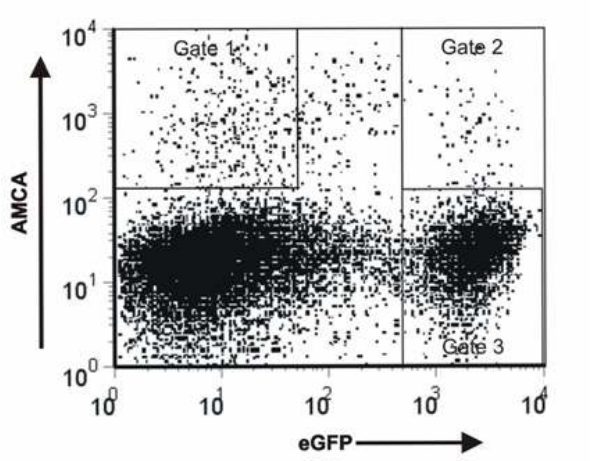

Fig. 7. Fluorescence Activated Cell Sorting (FACS) analysis of single cell suspensions of the pituitary gland, taken from transgenic mice that express eGFP under control of the GH promoter, that have imported AMCA (β-Ala-Lys-Nε-7-amino-4-methylcoumarin-3-acetic acid). AMCA(+) cells can be seen on the vertical axis at $>10^2$ and eGFP(+) on the horizontal at $>10^2$. Gate 1 shows the AMCA(+)eGFP(-) cells that were selected as donor cells for transplanting into the microchambers. Gate 2 shows AMCA(+)eGFP(+) cells that may be differentiating GH-intermediate cells. These cells were selected for functional studies. Gate 3 shows typical somatotropes (GH cells) that were selected as positive controls for use in functional studies to test differentiating GH-intermediate cells. Figure published with consent from Stem Cells (AlphaMed Press).

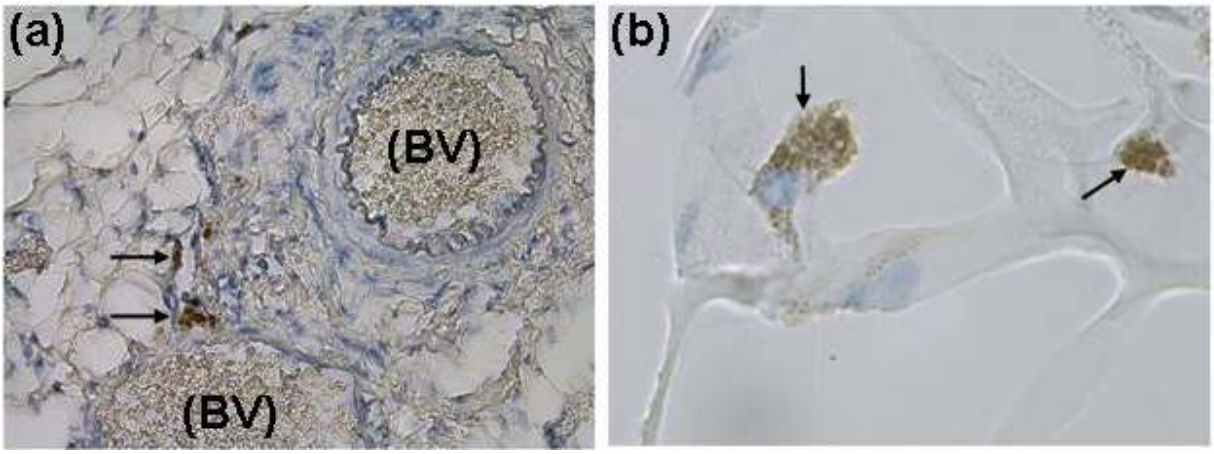

Fig. 8. Detection of GH(+) cells in grafts at six weeks. Immunoperoxidase detection of eGFP using rabbit anti-mouse eGFP with goat anti-rabbit immunoglobulins conjugated to horse radish peroxidase. **(a)** GH(+) cells (brown) were detected in groups near blood vessels (BV). (x 400 magnification). **(b)** At high magnification, cytoplasmic vesicles containing growth hormone granules (brown) can be seen around the nucleus counterstained with hematoxylin in blue. (x600 magnification). This figure was adapted from our original publication (35). Published with consent from Stem Cells (AlphaMed Press).

8. THE MICROCHAMBER AVOIDS THE NEED FOR DONOR CELL IMPLANTATION DIRECTLY INTO THE PITUITARY GLAND

The differentiation of PCFCs into GH cells in the microchamber, albeit at modest levels, indicates that the appropriate signals are received by the cells, despite their remoteness from the pituitary gland. Implanted cells are exposed to both circulating and locally released factors that influence GH synthesis and secretion, that can reach the cells in the graft via the vasculature. PCFCs would therefore be exposed to the circulating hypothalamic regulatory hormones, GH-releasing hormone (GHRH) and somatostatin, and to locally released gherlin, GHRH, somatostatin, gastrointestinal peptide, vasointestinal peptide, insulin-like growth factor or glucagon (1, 37).

9. CONSIDERATIONS FOR THE DEVELOPMENT OF THE MICROCHAMBER MODEL INTO A GROWTH HORMONE SECRETING ORGANOID

For the microchamber to meet the requirements of a fully functional GH secreting organoid in a

patient with GH deficiency there are two key requisites: firstly, the sufficient generation of GH, and secondly, the functional secretion of GH. In our current mouse model, 30% of implanted donor cells survive and only 3.3% differentiate (35). This necessitates the implant of close to one million donor cells to simulate the number of somatotropes found in a normal mouse pituitary gland. Although the microchamber permits millions of cells to be implanted (total volume 45µl), the lowest possible donor cell number is preferred due to the complexities of obtaining donor cells. On-going studies of the PCFC micro-environment are designed to shed light on the factors and signalling mechanisms that could be added to the microchamber at the time of implant to maximise PCFC survival, growth, and differentiation.

With regard to the functionality of the GH cells formed in the graft, we have two models for testing whether graft-derived GH cells secrete GH. First, it is well known that normal GH secreting cells (somatotropes) respond to GH regulating factors with characteristics changes in their membrane electrical properties that relate to the control of GH release (14, 38, 39). We have already compared the response of normal somatotropes (Figure 7, gate 3) with that of putative differentiating PCFCs termed "GH-intermediate cells" (Figure 7, gate 2). Both cell types responded to GHRH, gherlin and somatostatin with the well documented expected changes in membrane current and potential measured *in vitro* (Lepore *et al*., unpublished data). If these studies can be replicated with graft-derived GH cells it will support the microchamber device as a GH-secreting organoid. Secondly, the ability of the microchamber to simulate pituitary function will be demonstrated by testing the donor PCFCs in a GH-deficient mouse *in vivo*. The *dwarf* mouse strain has a genetic anomaly resembling the human disease, manifesting as GH deficiency despite having all the normal hypothalamic signalling mechanisms (40). If graft-derived GH cells are indeed functional they will secrete sufficient GH to restore normal GH levels in these mice reflected by normalised levels of insulin-like growth factor, growth factor binding protein, bone, muscle and fat density, lipids, electrolytes and glucose (41, 42).

10. SUMMARY AND CONCLUSIONS

It has been shown that the vascularised microchamber model could be used to generate a GH-secreting organoid. Any combination of cells, extracellular matrix and growth or differentiation factors may be added to the chamber in order to optimise the survival, differentiation and GH

production of the seeded PCFCs. In experimental animals, or clinically in humans, this microchamber could be grown *in situ*, transplanted microsurgically to a remote site or even into another individual. The optimised chamber has the potential to provide more efficient delivery of GH than the current alternative. This model allows the growth and differentiation of *autologous* PCFCs, thus avoiding the need for immunosuppressants. However, given the limited number of PCFCs in a GH-deficient individual, it is more likely that allografted PCFCs from healthy human donors or xenografted porcine donors would be required for transplantation. In these latter circumstances an alternative immune protection strategy that has minimal interference with stem cell behaviour or survival, as currently promoted by transplantation immunologists, would be the preferred option (43). This combined therapy uses anti-CD154 (CD40L) and CTLA4-Ig, which block activated CD4+ lymphocyte binding to macrophages and T-cell activation, respectively. These agents can be used alone or in combination with calcineurin inhibitors such as cyclosporin and tacrolimus (44). Testing the PCFC–containing organoid in GH-deficient mice, relative to the current gold standard of daily GH injections, is likely to be the next challenge for this novel model.

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