NOT ALL ROADS LEAD TO SUCCESSFUL REVERTANT CELL THERAPY

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In Chapters 2-5 of this thesis we presented published work on the understanding of revertant mosaicism. Research on the development of revertant cell therapy in the Center of Blistering Diseases in Groningen that we have performed has been much more extensive than the experiments presented in Chapters 2-5. Throughout the process, many hypotheses and possible improvements to expand the revertant area of the skin were investigated. In this chapter I would like to present three selected experiments that were perhaps not a direct step forward on the path to revertant cell therapy but are worth mentioning, as they might have been the beginning of a new approach.
ENRICHMENT OF IN VITRO CULTURE WITH TYPE XVII COLLAGEN REVERTANT CELLS

We would like to acknowledge the participation of Miranda Nijenhuis and Dr. Hendri Pas in this study, both from the Department of Dermatology, University Medical Center Groningen.

Revertant cell therapy based on in vitro cultured skin grafts in patients with mutations in the COL17A1 gene could profit from a technique that allows enrichment of revertant keratinocytes. In vitro enrichment could lead to a skin transplant with a higher percentage of revertant cells than those achieved in Chapters 2 and 3. A method for selection of revertant keratinocytes after isolation from the biopsy would ensure that the revertant cell population had chance to expand. Starting off, we tested different culturing methods, such as 3T3-J2 feeder layer conditions as described by Howard and Green and different defined, serum free media, such as defined-SFM keratinocyte culture medium (Gibco) or CnT-07 and CnT-57 (Cellntec). However, in all cases the percentage of mutant cells relative to the revertant cells increased with each passage as shown in Chapter 3.

Van den Bergh et al. described an interesting method to test cell attachment mediated by type XVII collagen (Col17) and laminin-332 (lam-332) with a method reported earlier by Reyes and Garcia.1,2 In this method, a culturing plate is turned upside down and placed in the centrifuge. Results showed that cells, which do not express Col17, did not attach to the lam-332 coating as strongly as the Col17 expressing cells did. For example, when 55g force was used, 30 - 40% of Col17 expressing cells stayed attached to the plastic, while only 5% of Col17 negative cells was left in the culture wells. The cells tested by Van den Bergh et al, K562 and SK-MEL1, had a very low level of adherence on their own and the Col17 expression and attachment to lam-332 was their strongest attachment mechanism. We attempted to apply this technique to a mixture of COL17A1 revertant and mutant keratinocytes, isolated from a biopsy of the patient described in Chapters 2 and 3. Unfortunately, primary keratinocytes do express many other adhesion molecules and therefore both populations remained strongly attached to different coatings, such as lam-332, type I collagen or even uncoated plastic when the protocol of Van den Bergh was followed. Time given for keratinocytes was shorten and under 5 minutes detachment of all cells was observed, while with longer times no difference before and after application of different g-forces was shown, up to 70g.

Another method to select revertant keratinocytes tested in our laboratory was sorting by flow cytometry. Col17 has an extracellular domain and therefore it is possible to stain living cells, after non-enzymatic detachment from the culture flask, with an antibody against the extracellular domain, such as the 233 monoclonal antibody (Gift from Dr. K. Owaribe, Nagoya, Japan).3 First a staining protocol that allowed the removal of keratinocytes from the culture flask without
enzymatic digestion was established. Briefly, keratinocytes were incubated in a PBS/EDTA (Gibco) solution for 30 minutes and then cells were gently washed from the culture flask. Cells were then placed on ice in the DMEM/10%FCS (Gibco and HyClone, respectively) for 20 minutes. This method of detachment preserved the extracellular domain of Col17. We then established a staining method to label Col17 using the 233 monoclonal antibody with Alexa488-conjugated goat-anti-mouse IgG antibody as a second step (Invitrogen, USA). Cells were first incubated with the 233 monoclonal antibody that recognizes the extracellular domain of Col17 (1118-1143) for 30 minutes on ice and washed twice in PBS. This step was followed by incubation with the Alexa488-conjugated second step antibody for another 30 minutes and two additional washing steps at the end.

The staining protocol was first tested on healthy human keratinocytes and assessed by immunofluorescence microscopy. Green fluorescence indicating Col17 expression could be observed on the cell surface in both, the living cells and air-fixed cells samples stained with the 233 monoclonal (Figure 1 A and B). As a control antibody we used another Col17 monoclonal antibody VK4 (Dr. H.H. Pas, Groningen, the Netherlands), which is directed against an intracellular epitope of Col17. The sample of living cells stained with VK4 showed no fluorescent signal (Figure 1E), while the air fixed one did (Figure 1D). This means that the cell membrane remained stable in the living cells sample during the staining protocol because the VK4 was not able to penetrate the membrane and stain the intracellular epitope of Col17.

Next, keratinocytes were isolated from a revertant biopsy taken from patient EB026-01, described already in Chapters 2 and 3. Side samples of the culture showed 40% of revertant cells after the first passage and 18% after the second passage. For sorting with flow cytometer the same staining protocol was used as for the control cells and living cells were stained (Figure 1 F). Separated cells were further cultured under the same conditions as were used before cell sorting (CnT-07 culture medium, Cellntec).

Sorting of revertant and mutant cells with the flow cytometer facilitates further studies on revertant mosaicism. Enrichment of revertant keratinocytes by flow cytometry using monoclonal antibodies against Col17 is currently not allowed in the process of revertant graft production under good manufacturing practice (GMP). Possibly, different sorting techniques such as magnetic activated cell sorting (MACS) based on magnetic nanoparticles coated with antibodies could be easier certified for GMP conditions.
Figure 1. Immunofluorescence staining for sorting of Col17 positive cells with flow cytometer.

A and D: Air fixed wild-type keratinocytes stained with: A. 233 monoclonal antibody and D. VK4 monoclonal antibody. Col17 is stained in both samples. B and D: living cells after non-enzymatic detachment, without fixation stained with B. 233 monoclonal antibody and E. VK4 monoclonal antibody. Only 233 antibody stains the cells meaning that the cell membrane remained intact. C: Cells after detachment with trypsin stained with 233 monoclonal showed no signal from the cell membrane. This suggested that the extracellular domain of Col17 has been cut off by trypsin. F: Sorting of revertant and mutant cells from patient EB 026-01 after passage 2. 77% of cells were labelled as mutant (R6) and 15% were labelled as revertant (R2).
REVERTANT CELL THERAPY IN A TYPE VII COLLAGEN REVERTANT PATIENT

This study was performed in collaboration with Dr. Marta Garcia, Prof. dr. Marcela Del Rio and Dr. Fernando Larcher from Regenerative Medicine Unit, CIEMAT and Centro de Investigaciones Biomédicas en Red de Enfermedades Raras (CIBERER U714) Madrid, Spain; and Dr. Sara Llames and Dr. Alvaro Meana from Tissue Engineering Laboratory, CCST-PA and Centro de Investigaciones Biomédicas en Red de Enfermedades Raras (CIBERER U714) Oviedo, Spain.

In Chapter 3 we described isolation of cells from a revertant skin biopsy of a Col17 deficient patient, production of skin equivalents and regeneration of human skin on the back of immunodeficient mice. When the first type VII collagen (Col7) revertant patients were identified in 2010,4,5 it was decided to replicate the experiments described in Chapter 3, using the same murine model. This was done in order to test the feasibility of Col7 revertant cell therapy. Patient EB 260-01 was a female, homozygous for a single base pair insertion (c.6527dupC) in the COL7A1 gene and therefore affected by recessive dystrophic EB (RDEB).5 A revertant skin patch was observed on her left arm, where her skin never blistered (Figure 2A). Biopsies from this revertant patch showed levels of Col7 expression similar to the ones seen in the control biopsy and the reversion mechanism was identified by laser dissection microscopy to be a second-site mutation (c.6528delT).5 In the biopsy described in the publication of Pasmooij et al. 85% of the biopsy area was revertant.5

A skin biopsy from the same revertant patch was taken and keratinocytes and fibroblasts were isolated. Cells were expanded in vitro to full thickness skin equivalents with the same technique that was used in the experiments described in Chapter 3 and by Llames et al.6 Mature skin equivalents were transplanted onto the back of nu/nu immunodeficient mice to obtain reconstructed human skin.7,8 Side samples of cultured keratinocytes were analysed for the presence of Col7 revertant cells with immunofluorescence staining with LH 7.2 monoclonal antibody against the NC-1 terminus of Col7. Unfortunately, the percentage of revertant cells in those side samples was lower than 10% and therefore much lower than our expectations based on the biopsy described in 2010. Transplanted grafts after 10 and 16 weeks were analysed for possible changes in the revertant cell population during the in vivo phase of this experiment. Grafts were biopsied and stained with the monoclonal antibody LH 7.2 and a 234192 polyclonal antibody Calbiochem against Col7. Stainings revealed that in parts of the graft, some expression of Col7 was present (Figure 2B). The levels of expression in those parts were substantially lower than in the control graft (Figure 2C). We analysed the sections for presence of the reversion mechanism by first dissecting the fragments with the laser dissection microscopy and then sequencing the DNA in order to assess the presence of the correcting mechanism (c.6528delT). Only the original
homozygous c.6527dupC mutation was identified and the reversion mechanism c.6528delT was not present in the analysed sections.

We concluded that no revertant keratinocytes could be detected in the transplanted graft, because of the low percentage of the revertant keratinocytes in the original cell culture. Col7 seen in the analysed graft might be present as a result of upregulation of the mutant and thus non-functional Col7 expression. Changes in the dermal and/or epidermal homeostasis could result in the changes of Col7 expression in RDEB patients, as it was shown in trials using fibroblast injections. An alternative explanation is the observation that a low level of Col7 expression has been observed in RDEB patients, due to a low level of exon skipping. In the presented study we were not able to isolate and culture a sufficient number of Col7 revertant keratinocytes. We expect, however, that if a biopsy with a satisfactory percentage of revertant keratinocytes is obtained and cells are successfully isolated, a Col7 revertant skin graft could be obtained. This has to be, however, further investigated.

**Figure 2.** Murine model of Col7 revertant cell therapy. Immunofluorescence staining for Col7 of human grafts 10 weeks after transplantation on mice. **A.** Revertant patch on patient’s arm. **B.** Graft 10 weeks after transplantation on the immuno-deficient mice, stained with LH 7.2 monoclonal antibody. Depicted in green Col7 and in blue nuclei. **C.** Wild-type graft (10 weeks) stained with LH 7.2 showing normal levels of Col7 expression.
USAGE OF NON CULTURED EPIDERMAL GRAFTING FOR REVERTANT CELL THERAPY

We would like to acknowledge José Duipmans (Department of Dermatology) and Dr. Mike Ruettermann (Department of Plastic Surgery) from the University Medical Center Groningen for their involvement in this study.

After successful transplantation of revertant keratinocytes described in Chapter 4, we decided to investigate other methods to expand the revertant skin area without an in vitro cell culture. Non-cultured transplantation of epidermal cells has already been widely used in treatment of burn wounds, vitiligo and to some extent as therapy for chronic ulcers. During one of the follow-up visits after the treatment described in Chapter 5, a new ulceration (8 cm²) was found in the affected skin on the foot of patient 029-01 (Figure 3A). This patient had junctional EB, generalized intermediate subtype (JEBe-gen-intermed), due to mutations in the LAMB3 gene with multiple identified revertant skin patches. We decided to treat this ulceration with transplantation of non-cultured epidermal cells in suspension. The ReCell® kit (Avita Medical) was chosen to provide certified equipment allowing preparation of a revertant cell suspension. According to the manufacturer, the ReCell® kit allows an expansion ratio up to 1:80. Because transplantation of non-cultured epidermal cells is an established treatment for chronic ulcers no approval from the ethical committee was necessary.

The patient was admitted to hospital and wound debridement was performed. On the next day, a 1 cm² split thickness skin specimen was harvested with a dermatome from the same revertant skin patch as, used for treatment described in Chapter 4. The skin specimen was digested according to the ReCell® kit manual. Briefly, it was placed in the enzyme mixture provided by Avita Medical for 15 minutes and then scratched with the scalpel to allow epidermal and dermal cell isolation. Enzymes were then blocked by the blocking solution (Avita Medical) and the cell suspension was poured through a strainer to remove cell clumps. The obtained suspension was then placed in the syringe and applied onto the wound (Figure 3B) and onto the donor site. Dressings were placed on both locations and the first inspection took place seven days later during which no clinical improvement of the wound could be seen. The patient reported however, that the pain from the ulceration had substantially decreased.

The patient was seen further at two, four, six and ten weeks after the procedure and each time examination of the wound and photographic documentation were performed. During the follow-up visit at four weeks, signs of re-epithelialisation and reduction of erythema surrounding the ulcer were seen. At six weeks we observed a decrease in the wound area when a fully re-epithelialized bridge, splitting the original ulceration into two smaller wounds, was formed (Figure 3C). At ten weeks the two smaller ulcerations were still present and the patient reported
an increase of pain from the wound region. The decision not to take a follow up biopsy from the treated area to assess if transplantation of revertant cells succeeded was made. To accelerate healing we then chose to perform punch grafting, as was previously done in this patient (Chapter 4). Because of the impaired wound healing, most likely due to advanced age, usage of beta-blockers and arterial obstruction (toe brachial index: 0.71, ankle brachial index: 0.85), the first round of punch grafting was not successful. Within four weeks a slight reduction in size of both ulcers was achieved but no full re-epithelialization occurred. Over the course of the following 4 months, the patient chose a conservative treatment, consisting of dressings changes three times a week, which did not lead to improvement of the ulcers.

Seven months after the application of non-cultured revertant cells in suspension we saw that the fragment, that had re-epithelialized six weeks after the procedure, looked healthy. The two smaller ulcers had unfortunately grown larger in the direction of earlier untreated areas. The second round of revertant punch grafting, taken again from the patient’s right shoulder was applied, which this time led to successful healing of the wounds.

In summary, partial re-epithelialisation after application of revertant cell suspension occurred. Unfortunately, analysis of the healed region for engraftment of lam-332 revertant keratinocytes was impossible, because we did not want to cause an iatrogenic ulceration with a new biopsy in an already affected region. The originally re-epithelialised area stayed blister free for at least 7 months, which might suggest that revertant cells were transplanted during the first procedure (Figure 3D). There was however, an unsuccessful attempt for transplantation of punch grafts in-between, which could have led to the engraftment of additional revertant keratinocytes in the area and positively affected the wound healing.

Transplantation of non-cultured epidermal cells in suspension could lead to successful enlargement of the revertant skin area, if the acceptor site is well prepared, in a patient without impaired wound healing. Further investigation of this technique is necessary in a small clinical trial setting, where the wound bed could be better controlled.
Figure 3. Transplantation of non-cultured epidermal cells in suspension harvested from the revertant skin on a chronic ulcer of JEB-gen-intermed patient. A. Chronic ulcer on patient’s foot. B. Application of cell suspension isolated with ReCell® kit. C. Wound 6 weeks after the procedure. Partial re-epithelialization can be seen in the center of the wound. D. Wound 7 months after the procedure with expansion of the ulcerated area lateral. The part that was healed earlier (in C) remains stable.
REFERENCES


