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In Vitro Culture Methods of Skin Cells for Optimal Skin Reconstruction by Tissue Engineering

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1. Introduction

Replacement of a wounded or destroyed tissue is now technically possible using an in vitro method of tissue engineering. This method involves isolating and culturing human cells (autologous or not) in optimal conditions to form a reconstructed tissue with similar characteristics to its in vivo counterpart. Burn victims were the first patients to benefit from this method in 1981, wherein reconstituted human epidermal tissues were used to treat the burns (O'Connor et al., 1981). The pioneered technique comprises epidermal cell isolation, culturing and grafting as a unique layer of cells organized in a sheet. The differentiation of the cells into a pluristratified epidermis is performed in vivo after grafting.

Numerous improvements, most notably the addition of a dermal part in skin tissue reconstruction have been made since the technique's inception. However, the dermis is more difficult to reconstitute because of the complexity of its organization. Briefly, the dermis is a mix of matrix and cells, which are primarily fibroblasts. Moreover, the dermis is also composed of several structures such as the epidermal annexes (e.g., hair and sebaceous and sweat glands) and a network of capillaries. Several others cells like lymphocytes, or neurons are also more difficult to add on dermis despite their crucial roles in acquiring all the functions of the skin. Furthermore, the third part of the skin, the hypodermis is vital in vivo and addition of this tissue will increase the function of the grafted skin.

In classical cutaneous grafting with split- or full-thickness skin, the amount of dermis that is grafted to a wound bed inversely correlates with the degree of scarring and wound contracture which impacts the functional and cosmetic outcome (Bombaro et al., 2003). Similarly, dermal cells have been fundamental in the efficacy and quality of cultured keratinocyte grafts (Moulin et al., 2000) (El Ghalbzouri et al., 2002) (Kirfel and Herzog, 2004; Gallant-Behm et al., 2011) (Robert et al., 1997).

Currently clinics widely use dermis that is constituted of matrix and fibroblasts. The methods used to obtain cultured living dermis, which can be grouped into three different categories (Table 1) represent the three main methods of tissue engineering. These categories are based on the materials used to originate the matrix, such as biomaterials, biological materials and dermal fibroblasts. Biomaterials are materials that are not present in skin but

that are compatible with cells. Biological materials such as collagens are primarily derived from non-human sources. Lastly, the matrix may be created by human dermal fibroblasts themselves during culture. We refer to this last method as the “self-assembly method” in our lab. The cell source is primarily allogeneic, which allows for a high quantity production of tissues with a quick delay before grafting. Using this method, the cells are available to stimulate healing and are replaced by the patient’s cells with time. In a few cases (Hyalograft 3D™ or “LOEX” skin), cells are isolated directly from the patient’s skin. Using the approach, reconstituted tissues comprise a true graft and do not disappear with time. However, this method is time consuming and costly.

Matrix protein origin	Description
Biomaterial	
Biodegradable polyglactin mesh scaffold	Dermagraft™: Cryopreserved allogeneic fibroblasts are cultured on the mesh
Biological	
Esterified hyaluronic acid matrix	Hyalograft 3D™: Autologous fibroblasts are cultured in the scaffold
Bovine collagen sponge	Orcel™: Allogenic keratinocytes are seeded over a dermal scaffold containing allogeneic fibroblasts.
Bovine collagen sponge	Apligraf™: Allogenic keratinocytes are seeded over a dermal scaffold containing allogeneic fibroblasts.
Bovine collagen matrix	Permaderm™: Autologous keratinocytes are seeded on collagen gels made with autologous fibroblasts
Cellular	
Fibrin matrix at the beginning replaced by fibroblast-secreted matrix	ICX-SKN™: Matrix is freeze dried prior to repopulation with allogeneic fibroblasts
Fibroblast-secreted matrix	VCT01™: Allogenic keratinocytes are seeded on top of a dermis made of allogenic fibroblasts secreting their own ECM
Fibroblast-secreted matrix	LOEX: Autologous keratinocytes are seeded on top of a dermis made of autologous fibroblasts secreting their own ECM

Table 1. Matrix types in commercial and pre-commercial tissue-engineered dermis or skins

2. Fibroblast functionality

While simplified dermis comprised of matrix and fibroblasts are presently used to treat patients, several improvements in this tissue reconstruction method are necessary to obtain a skin with better functional acceptability.

The capacity of the skin fibroblasts to grow, remodel the matrix, secrete and degrade proteins, and produce numerous growth factors or cytokines is crucial for the regulation of the tissue structure and its cellular microenvironment. During reconstitution of dermal substitutes, the cells must retain their capacity to regulate their environment. Several parameters must be monitored to ensure proper fibroblast functions in growth capacity, matrix deposition and remodeling or differentiation state maintenance, all of which are inherent to these cells in normal human skin. There is a paucity of published studies dedicated to the analysis of the cell phenotype changes in vitro versus in vivo. This phenotype can change depending on several parameters, such as the cell duration of the cell culture (Endt et al., 2011), the location of the skin biopsies (Chang et al., 2002) and the age of the donor (Dumas et al., 1994). However, these results are highly controversial due to a lack of reproductibility by other groups (Ng et al., 2009) (Falanga et al., 1991).

2.1 Cell passage number

The replicative potential of a human cell population must be considered when those cells are used to reconstruct an engineered tissue. Indeed, because the replicative potential of most cells is limited, tissues reconstructed from cells at lower passage yield tissues of better quality. However, using cells promptly after their isolation and limited expansion is not always possible due to the requirement for several passages to obtain the quantity of cells needed. Therefore, knowing the cell passage range that yields the optimal results when using fibroblasts in tissue reconstruction is important.

We studied three fibroblast populations isolated from human skin biopsies at different times of culture, which were assigned by passage number. Fibroblasts were isolated using a collagenase solution (Rompré et al., 1990) and cultured with DME+10% fetal calf serum. When cells reached confluency, they were trypsinized and seeded until they reached the twelfth passage. Population doubling times, which were indicative of the cell growth capacity, the diameter of the cells, and the contractile capacity were evaluated from the first passage until the 12th passage.

Doubling times were calculated using the following formula: $DT = \ln 2 \cdot t / (\ln C1 - \ln C0)$, where t is the culture duration, $C1$ is the number of cells at the end of the culture and $C0$ is the number of seeded cells. Doubling times of three cell populations isolated from human skin biopsies were studied, and no statistical difference was detected from Passage 1 to 12 (Figure 1A). This is in agreement with results reported by Endt et al. (2011), wherein analysis of only one fibroblast population did not reveal any doubling time differences before the 15th passage. The cell diameter variation has also been investigated (Figure 1B) as a marker of a cell phenotype modification. This parameter remained constant with time ($17 \mu\text{m} \pm 0.83$) and lacked any apparent modifications.

Cell senescence has been shown to increase with time and has been detected in fibroblasts from the 30th passage (Endt et al., 2011). However, a higher sensitivity to apoptosis is also indicative of cellular aging. We monitored the apoptotic rate of cells using propidium iodide incorporation (Moulin et al., 2004) in the presence of 10% FBS, an additive that induces cell growth. A very low rate of apoptotic cells can be detected (<4%) at all passages and cell

populations (Figure 2A). When FBS was removed from the culture medium, the absence of a growth factor induced a slight increase in apoptosis (<4%) during the initial passages. However, passages 10 and 12 exhibited a marked increase in the apoptotic rate, which is indicative of a change in the cell's response to apoptotic stimuli (Figure 2B).

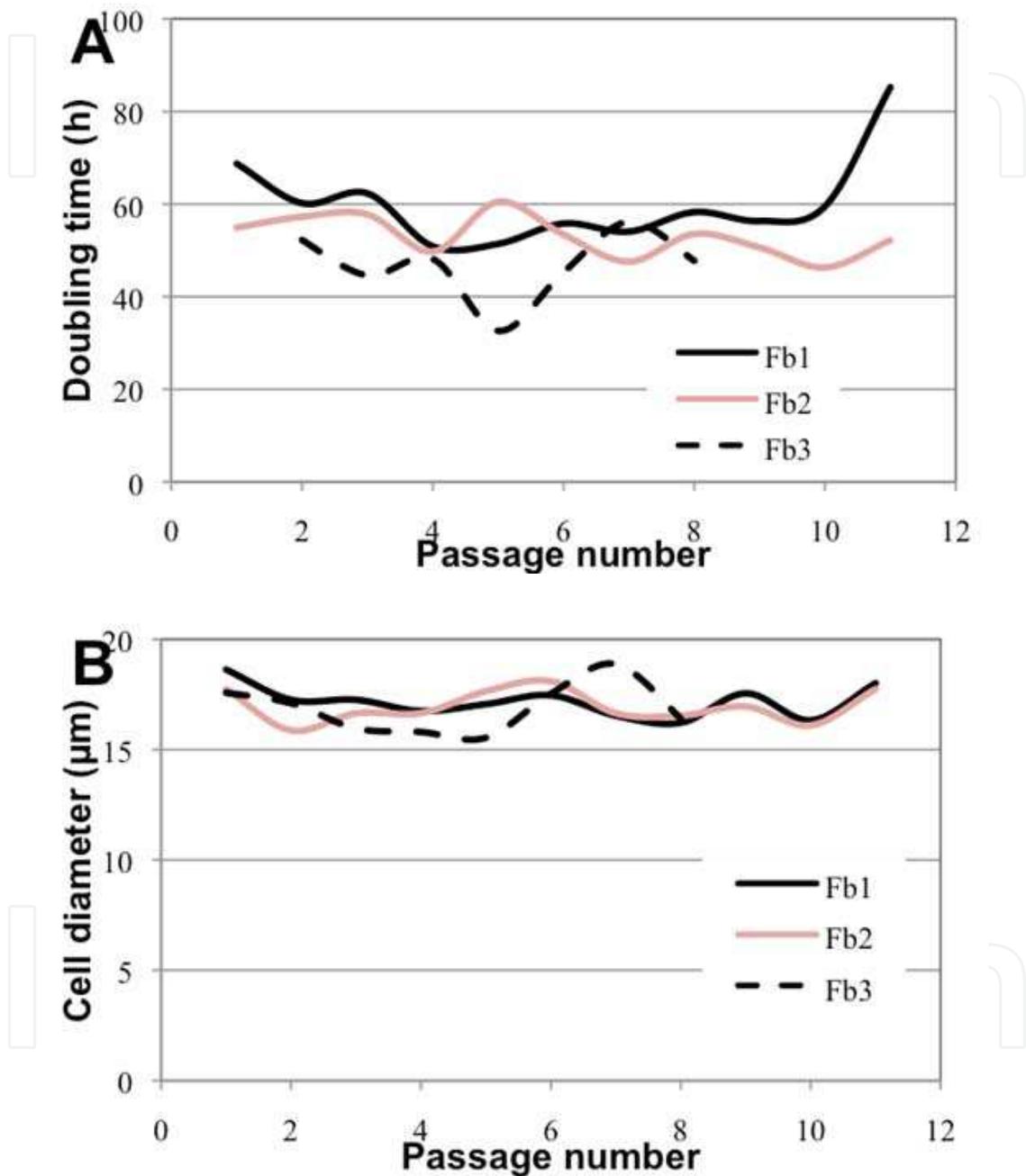


Fig. 1. The doubling times and diameters of fibroblasts cultured for different durations

The contractile capacity of cells has been studied to evaluate one of their function during healing (Finesmith et al., 1990; Delvoye et al., 1991). Cells were seeded into a collagen gel according to Moulin et al. (1998), and the gel diameter was evaluated daily (Figure 3). Differences in the gel contraction speed, which reflects the contractile capacity of the cells, were not observed when cells were cultured from passages 3 to 12.

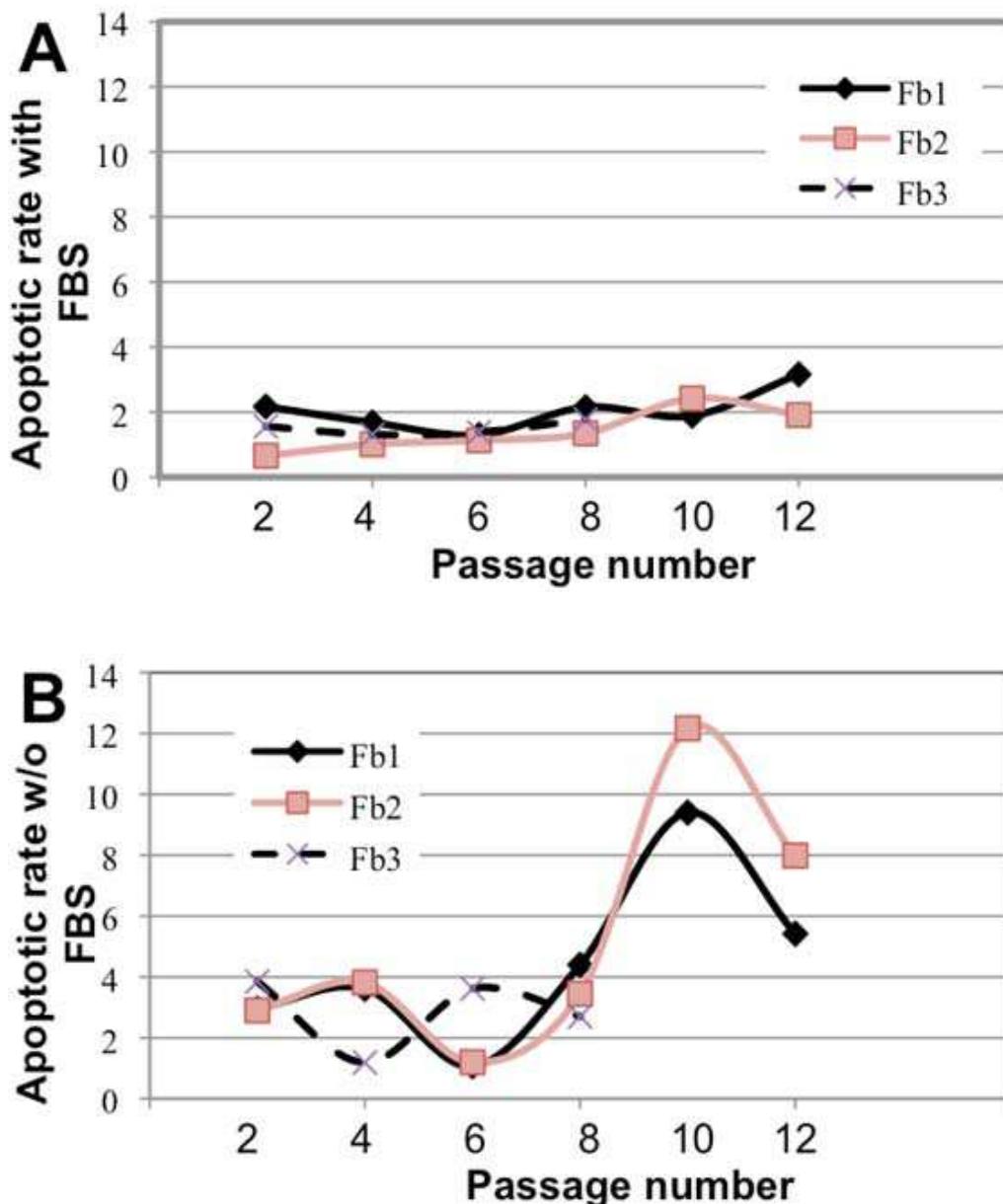


Fig. 2. The apoptotic rate of fibroblasts (A) in presence of 10% FBS or (B) without FBS. The rate of apoptosis was estimated from particle numbers containing less than 2n of DNA after propidium iodide incorporation

In summary, fibroblasts isolated from the dermis can be cultured without any major changes for at least 8 passages. After this time, the occurrence of several minor stresses, such as a transient lack of nutrients, can interfere with the phenotype of the cells and, thus, their response to stress in vivo. After grafting, cells placed in an engineered, reconstructed tissue must respond to several stresses, such as an absence of nutrients before revascularization, trauma, or infection, for the entirety of the patient's life. Thus, cells must be as similar to the original cells as possible. For a skin graft, sub-culturing of fibroblasts for 8 passages, corresponding to at least 25 doubling populations where one isolated cell will generate 8.4 million cells, permits the accumulation of enough cells to reconstruct a large surface area of the dermis.

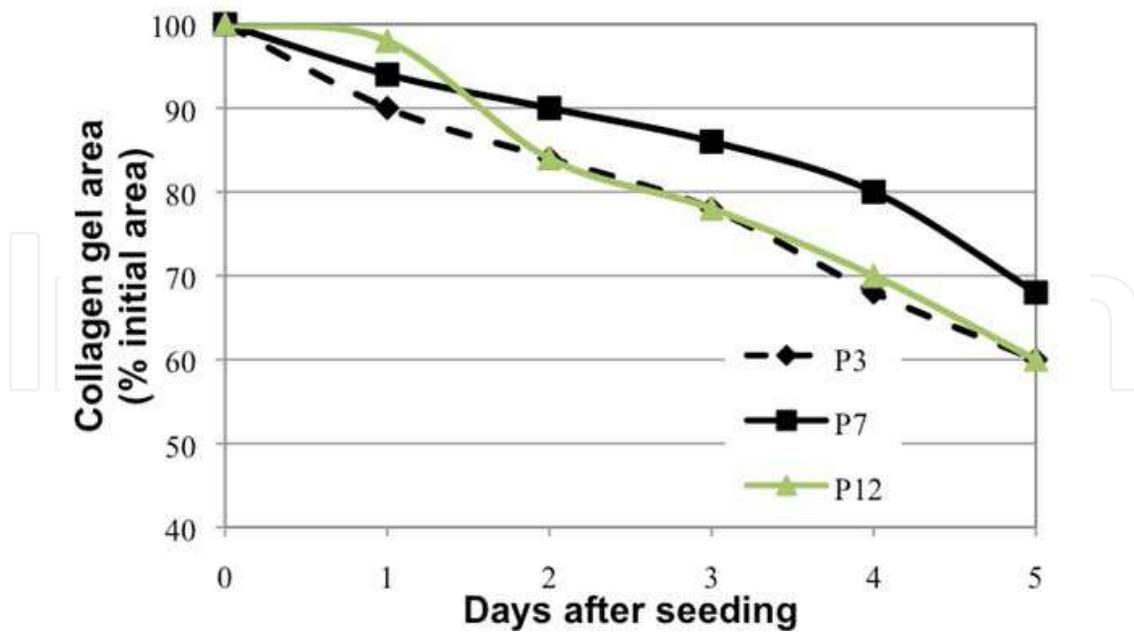


Fig. 3. The contractile capacity of fibroblasts (Fb8) at different passages. Representative results obtained with 3 different populations are presented

2.2 Age and sex of the donor

For autologous grafting, the age of the donor should not change. However, many tissue-engineered derma are produced using allogeneic cells. Therefore, the age of the donors' cells is a valuable parameter to estimate. We have calculated the doubling times for 17 different cell populations isolated from the human dermis at passage 5. Biopsies were taken from 20- to 64-year-old donors. Variations in the doubling time were not detected with donor age. In our experimental conditions, the doubling time mean of the fibroblasts was 47.2 ± 7.5 hours (Figure 4).

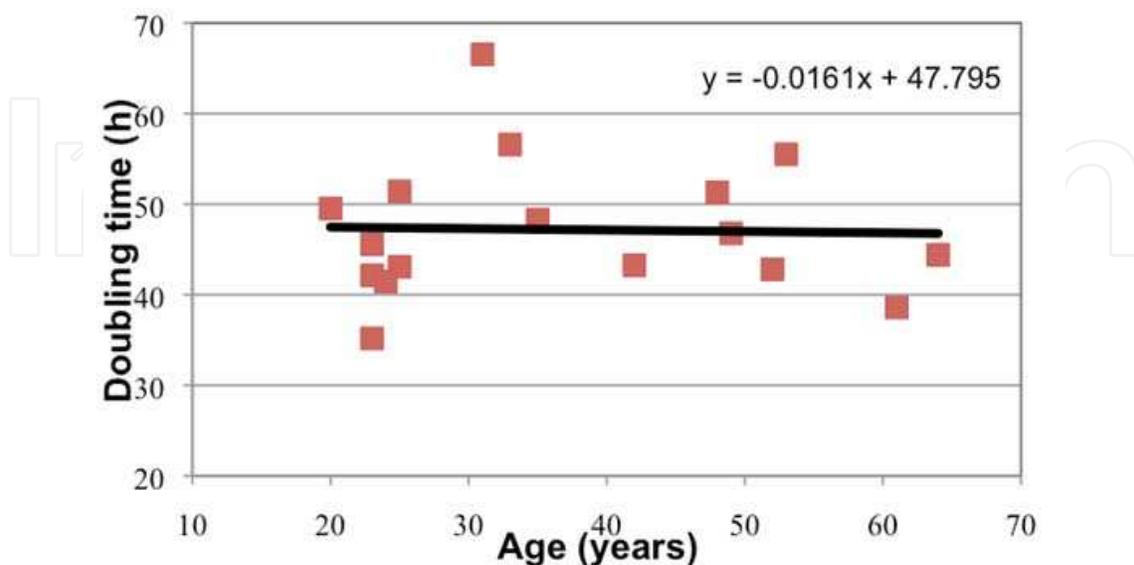


Fig. 4. The doubling time of fibroblasts from skin donors of different ages. The formula of the line was calculated using linear regression analysis

A comparison of cell populations from female and male donors of similar age and biopsy location did not reveal a statistically different doubling time (Student's t-test, $p=0.63$, $N=3$ for each category) (data not shown).

2.3 Location of the biopsy

The importance of the location of the biopsy at the origin of the fibroblasts in modulating in vitro cellular phenotypes has been addressed with mixed results (Chang et al., 2002; Chipev and Simon, 2002; Falanga et al., 1991). We did not find a statistical difference in the doubling time of fibroblasts from different biopsy locations, including the forearm, scalp, abdomen, and breast (Figure 5).

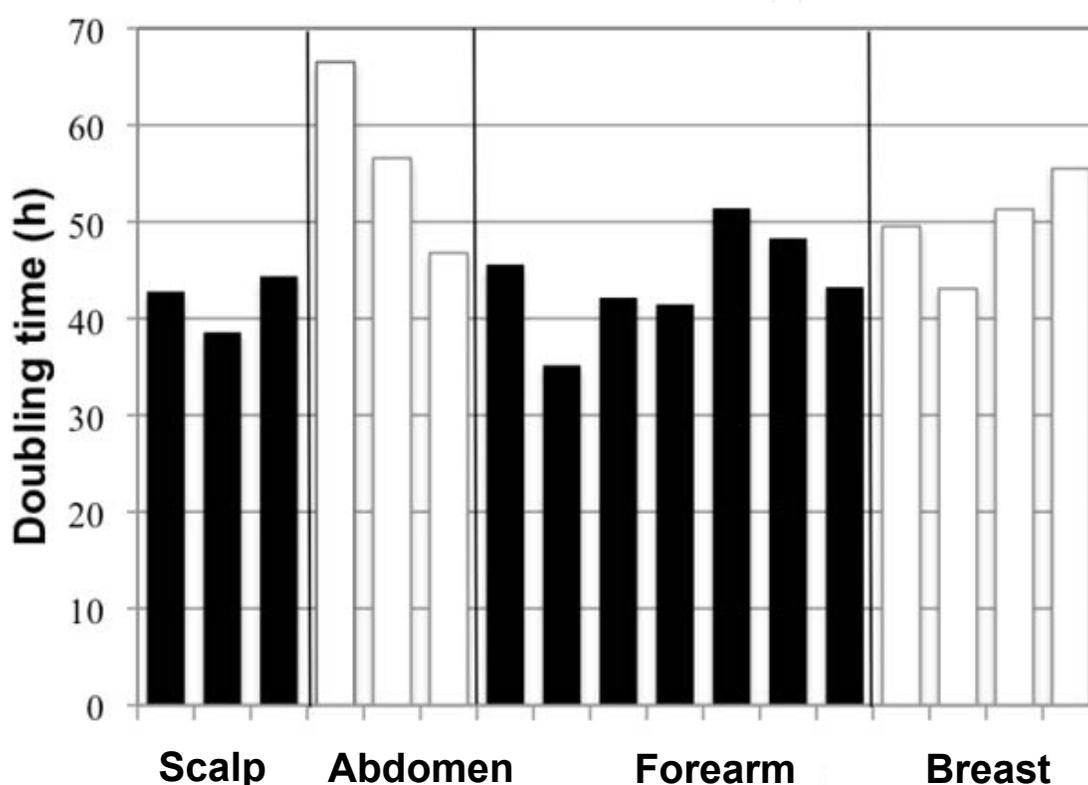


Fig. 5. The doubling time of the fibroblasts from different biopsy locations. Student-Newman-Keul multiple range test: *, $p<0.05$

One of the most important properties of fibroblasts is to secrete extracellular matrix to create the dermis. To evaluate this parameter, we used the self-assembly approach developed at the LOEX (Michel et al., 1999). Fibroblasts that are cultured for 4 weeks in the presence of ascorbate secrete and remodel extracellular proteins, a measure of the matrix remodeling capacity of the cells (Bellemare et al., 2005). A statistical difference in the thickness of the reconstructed dermis was observed for the various biopsy locations tested. When cells were isolated from the scalp or abdomen, the dermis was thicker than when fibroblasts were isolated from the breast or forearm (Figure 6). However, the thickness of the dermis was not dependent on the age of the patient (data not shown). Because the biopsy site may dictate the quality of the reconstructed dermis and, thus, the viability of its graft, the need for accurately choosing the biopsy site from which to collect cells to reconstruct a tissue is crucial.

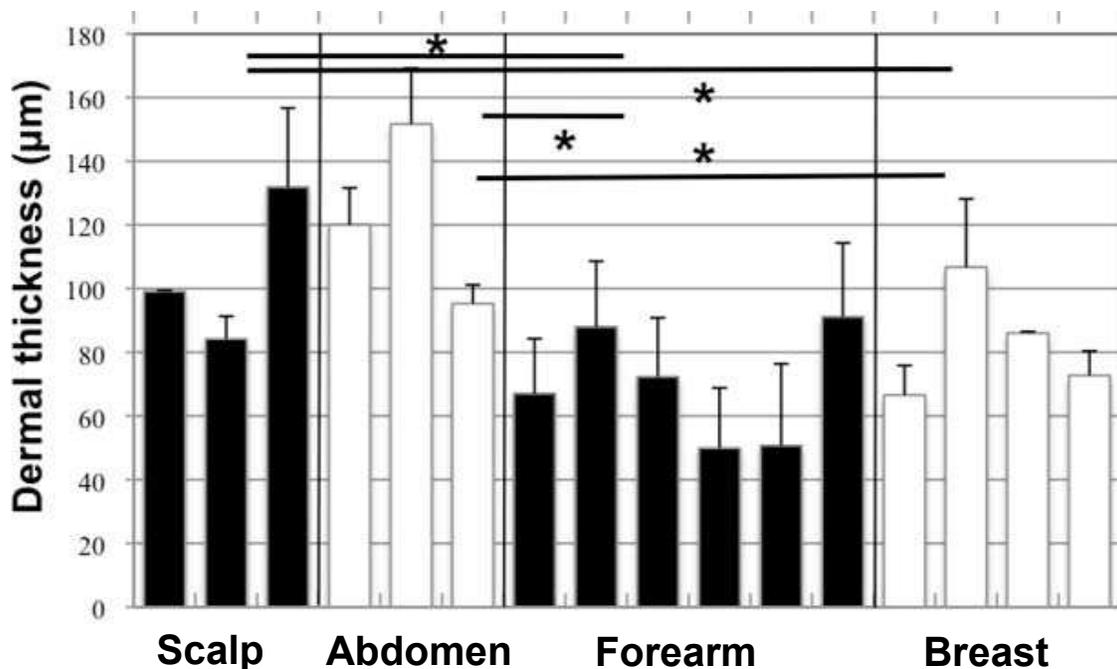


Fig. 6. The thickness of the dermis reconstructed using the self-assembly approach with fibroblasts isolated from different location of the body. Student-Newman-Keul multiple range test: *: $p < 0.05$

3. Capillary network

The graft of thick tissues is always a challenge due to the absence of a capillary network, which impedes nourishment of the tissue during the first days after grafting. Malnourishment is often responsible for the increase in the percentage of rejected grafts observed with these types of tissues. Different approaches are currently used to improve skin substitute vascularization before or after transplantation. The addition of growth factors, including VEGF and FGF2, via absorption or incorporation into scaffolds has been shown to trigger a localized and sustained delivery that promotes vascularization (Bouhadir and Mooney, 2001). Transplanted cells can also be genetically modified to produce angiogenic proteins that promote better and faster vascularization (Supp et al., 2000). Forming a capillary network into the tissues before grafting has been reported as an effective method to bypass this problem. Of several described techniques, seeding endothelial cells in biomaterial or on a dermal sheet that is then superimposed has been used most often (Black et al., 1998).

3.1 Isolation of human capillary endothelial cells

The isolation of the needed capillary cells can be performed from the same small tissue biopsy (less than 1cm^2) used to isolate the keratinocytes and the fibroblasts, that allows for the reconstitution of an autologous skin with a differentiated epithelium and a vascularized dermis.

The skin biopsy was cut into 0.5 cm^2 pieces and transferred into a thermolysin solution ($500\mu\text{g/ml}$) in HEPES buffer (Germain et al., 1993) then incubated at 4°C overnight. The epidermis was gently peeled from the dermis with forceps and constantly agitated in a trypsin/EDTA (Valeant Canada Limited) solution for 15 min. The epidermal cells were

centrifuged, and plated in the presence of irradiated 3T3 feeder layer cells as previously described (Moulin et al., 2000).

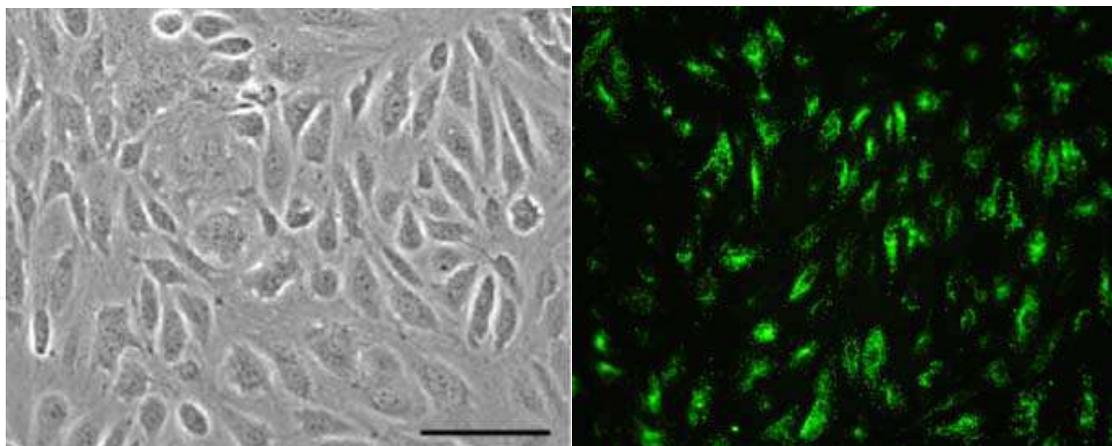


Fig. 7. Microvascular endothelial cells were isolated from human dermis; (left) phase contrast and (right) von Willebrand factor immunostaining (bar: 200 μ m)

The capillaries were extruded by pressing the dermal layer and were plated on gelatin-coated tissue culture flasks with EGM-2 medium (Cambrex Bio Science Baltimore, Inc., Baltimore, MD). After 24h, the medium was replaced to eliminate non-adherent cells and the cells were cultured for one week. The cells were removed from the culture flask by trypsin/EDTA and processed with CD31 antibody-coupled magnetic bead (Dynabeads, Invitrogen) for 30 min, allowing for further purification of the cells (Richard et al., 1998). Immunostaining was performed to validate the presence of the von Willebrand factor, a specific marker of endothelial cells, in the whole population (Figure 7). After the extrusion of the capillaries, the dermis was incubated in a collagenase H (0.125U/ml) solution for 4-5 hours at 37°C then plated and cultured as in (Moulin et al., 2001).

3.2 Reconstruction of a tissue-engineered endothelialized skin

The self-assembly approach is a tissue engineering method based on the *in vitro* production of mesenchymal sheets devoid of biomaterial or exogenous matrix proteins that were previously described for the production of human skin and blood vessels (L'Heureux et al., 1998; Michel et al., 1999). Dermal fibroblasts are cultured on petri dishes with the fibroblast medium supplemented with ascorbic acid (50 μ g/ml). Cells secrete and remodel matrix that form a manipulable sheet after 4 weeks. Endothelial cells are then seeded on the sheet and cultured for an additional week in EGM-2 medium with ascorbic acid. Two of these sheets are then superimposed to form an endothelialized tissue-engineered dermal substitute. Subsequently, keratinocytes can then be added onto the dermis to form an epidermis after differentiation at the air-liquid interface (Rochon et al., 2010) (Figure 8).

We have evaluated the formation of the capillary structure with time after endothelial cell seeding on dermal sheets and we observed an increase of capillary like structure over time. The formation was evaluated using CD31, a protein that localizes to junctions between endothelial cells. The formation of capillary-like structures reached a maximum at 21 days and remained stable until at least day 28 (Figures 9 and 10).

This endothelialized tissue-engineered skin has been grafted onto mice. Complete revascularization of non-vascularized skin substitutes usually takes 14 days. Authors have

demonstrate that endothelial network inoscules with the host's own vascular system within 4 days allowing to a quick revascularization of the tissue (Gibot et al., 2010). Furthermore, an active invasion of the dermis by the sprouting of host capillaries from the wound bed has been detected. The authors conclude that the microvascular network constructed in vitro is an interesting method to quickly vascularize a thick tissue. This finding will facilitate the graft take of thick engineered-tissues that, currently, die due to a lack of oxygen and nutriment.

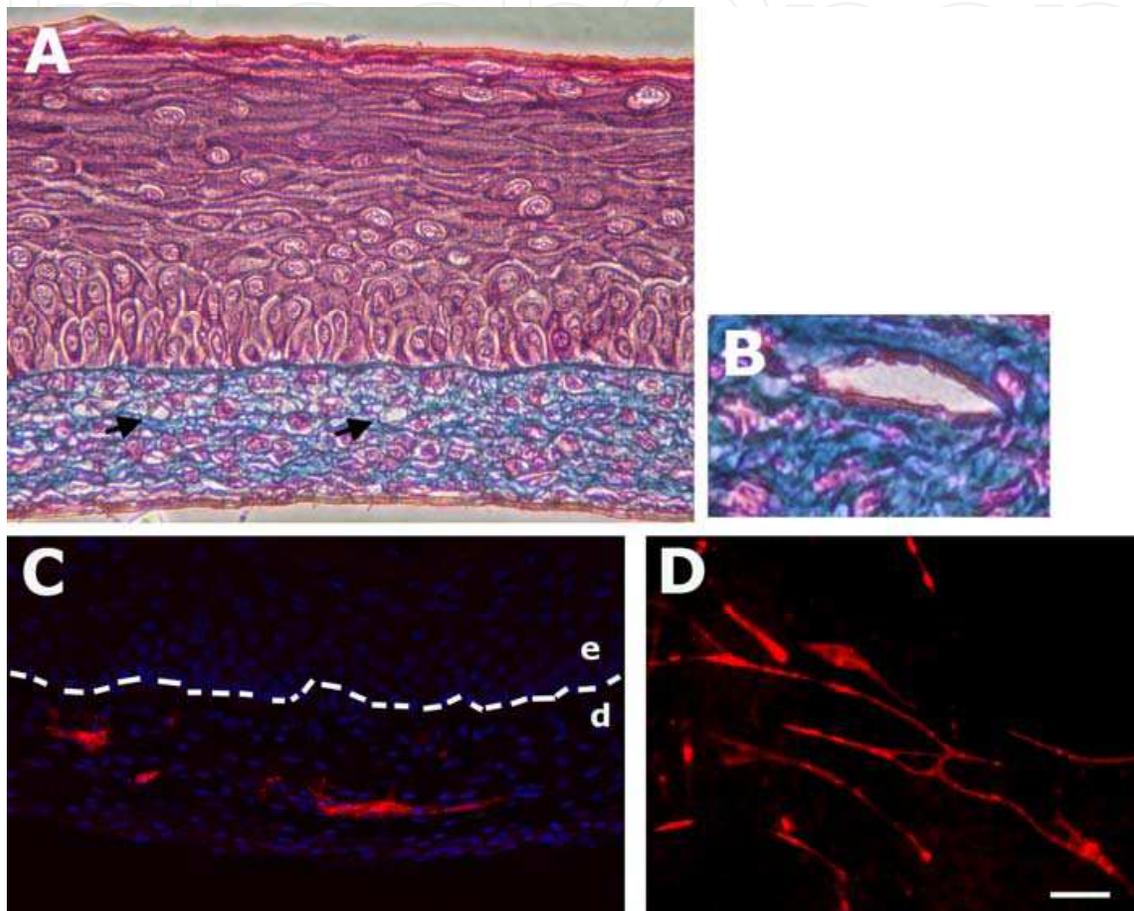


Fig. 8. Endothelialized tissue-engineered skin performed with three cell populations (i.e., keratinocytes, fibroblasts and endothelial cells) isolated from the same skin biopsy (A) Masson's Trichrome staining. Arrows indicate capillary-like structures. (B) Higher magnification of a capillary-like structure. (C) Transversal section of CD31-immunolabeled endothelialized tissue-engineered skin (dotted line: basal membrane between epidermis (e) and dermis (d)). (D) Bird's-eye view of CD-31-labeled endothelialized tissue-engineered dermis. Bar: 50 μ m for A, C and D and 7 μ m for B

4. Other possible improvements

The skin is a complex organ that cannot be pared down to a bilayer tissue comprised simply of an epidermis and a dermis. The third part of the skin, the hypodermis, is often ignored; however, it is crucial in controlling the temperature and nutriment storage, as well as secreting important hormones such as leptin that are important during healing (Frank et al., 2000). The addition of this skin moiety to a tissue-engineered reconstructed skin should

increase the functions of the grafted skin. Researchers can now reconstruct an autologous hypodermis using human adipose-derived stem/stromal cells and incorporate it to a reconstructed skin (Trottier et al., 2008).

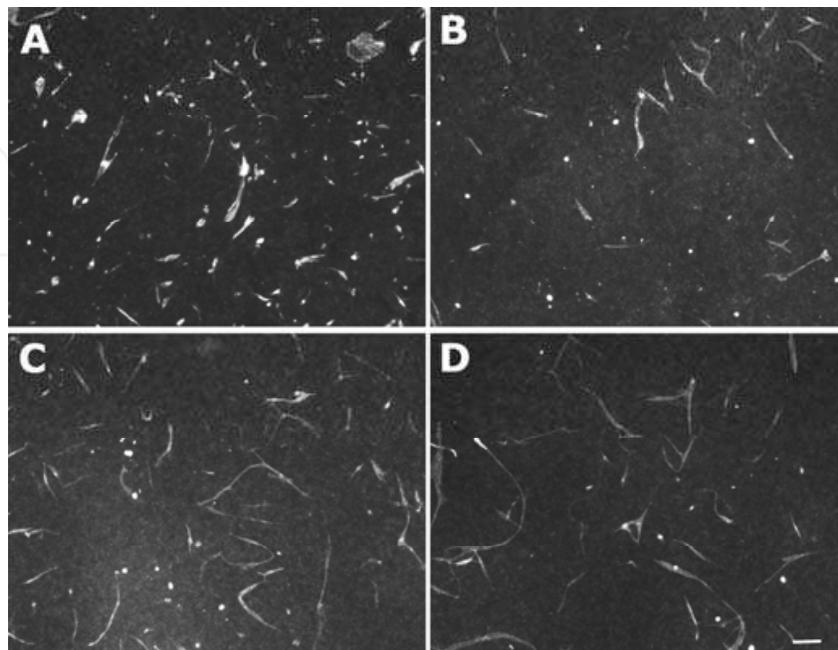


Fig. 9. Immunohistochemistry with CD31 antibodies on endothelialized-tissues after 7 (A), 14 (B), 21 (C) and 28 (D) days of culture. Bar: 200 μ m

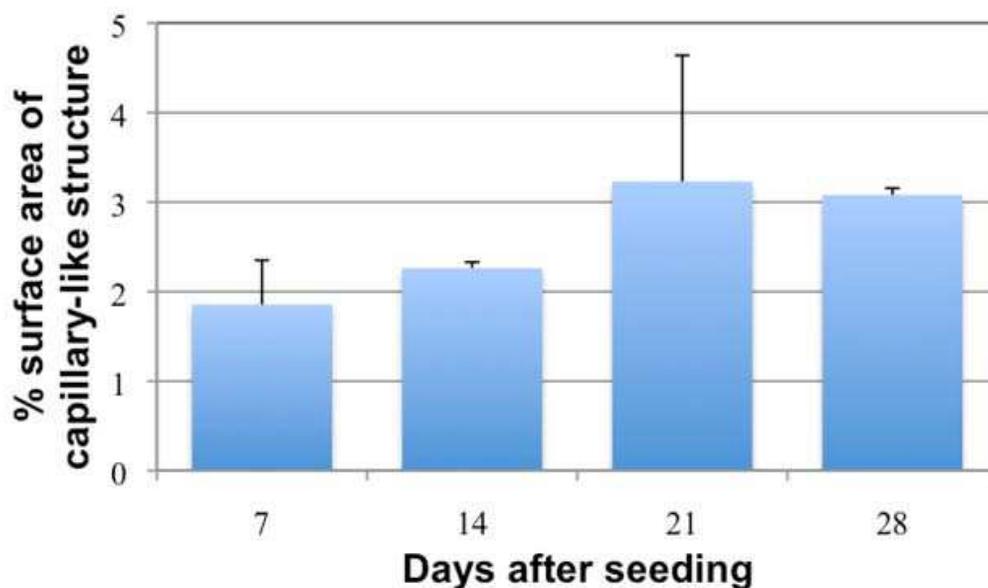


Fig. 10. The percent surface area of capillary-like structure evaluated from pictures of immunohistostaining with CD31-antibodies as in Figure 9

The absence of sebaceous and sweat glands in the reconstructed tissue is a recurrent problem for patients with large burn areas that have been grafted with tissue-engineered skin. This absence induces important thermoregulation problems and causes a dry skin that

needs to be constantly moisturized. The addition of these glands to a tissue-engineered skin is not trivial but recent results offer promise (Huang et al., 2010).

Hair follicle regeneration was thought to be impossible during adult life. Wound stimulus and wnt pathway activation have been recently shown to trigger de novo hair follicle formation from epidermal stem cells (Ito et al., 2007). This observation suggests that tissue-engineered skin with normal hair follicles is plausible.

If added, these complex skin structures can improve the functionality of the tissue. Furthermore, several other cell populations have also been added such as melanocytes, improving UV protection of the skin (Scuderi et al., 2008); Langerhans and dendritic cells, immunological cells present in the skin (Bechetoille et al., 2007) or nerves to improve skin sensation (Blais et al., 2009). These additions, if possible in laboratories for experiments, have now to be added in tissue-engineered skin for routine use as human skin replacement on patient.

5. Conclusion

The possibilities of the tissue engineering method are wide and an increase in the functionality of the grafted tissues will enhance their use. The skin has been the first tissue to be reconstructed and used in clinics to cure patients including large burn victims and for ulcer therapy. Techniques that have been developed for the skin reconstitution are currently used to reconstitute other tissues with more complex structures. However, numerous improvements are needed before obtaining an identical tissue.

6. Acknowledgments

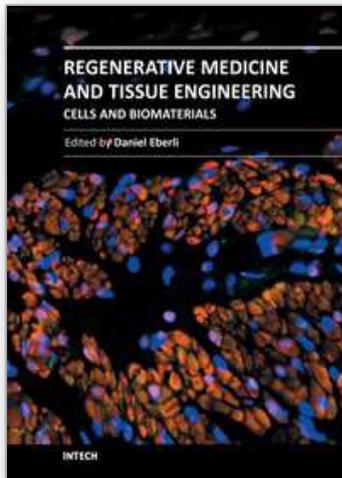
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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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