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Full Length Research Paper

Simulation of Living Intra-Epidermal Micro Patterns to Facilitate Guided Tissue Regeneration of the Human Skin

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The methodology for isolating living intra-epidermal micro patterns using a tissue culture technique is detailed. The disassembly of the stripped epidermis (after Dispase digestion at 37°C) into epidermal brown rosettes (observed under a phase contrast microscope) forms intra-epidermal micro patterns. The stepwise reasoning for identifying epidermal basal layer rosettes and the distinction between attached upright and inverted brown rosettes spreading their epidermal cell contents onto a prepared extracellular matrix is explained. Mimicking the circular shape of brown rosette layers using a microscopic sterile cornstarch granule (appearing donut-shaped under the microscope) as a biodegradable nutritional scaffold is proposed. A lightweight antibiotic ointment combined with the cornstarch granules was utilized, and the significance of this mixture in guided tissue regeneration of the epidermis after Mohs surgery for basal cell carcinomas, as a treatment for second-degree burns, and for potential healing of donor sites after skin biopsies is discussed.

Key words: Natural micro patterns, Mohs surgery, basal layer, tissue repair, epidermal-melanin unit, human epidermis, Cellular Potts Model, microscopic nutritional cell scaffold.

INTRODUCTION

The human skin, the largest organ in the human body, has little of the dramatic colouring of animals, butterfly wings or peacock feathers. This has led to skin envy on the part of many humans, and in turn to colourful skin tattoos. However a dermatological problem, allergic eczema against henna dye allergens can arise and in steps the dermatologist whose job it is to recognize skin patterns. These can be referred to as macro patterns, like the pattern of human male *androgenic alopecia*, more commonly known as, 'male pattern baldness'. Living natural micro patterns inside the human body have not been described *in vitro*. There is one example of cellular mimicry and intriguingly, it also involves the skin and its cells, but ones that had undergone some unknown kind of molecular perturbation. Aggressive melanoma cells can form part of tumour vasculature; a process described as 'vasculogenic mimicry'; thought to be driven by expression of endothelial specific genes in melanoma cells such as ESM-1 and VE-Cathedrin (Maniotis et al., 1999; Hendrix et al., 2001; Gaggioli and Sahai, 2007). The epidermal brown rosette, which I described in 2002, looks like a brown micro mass under the phase contrast microscope. If upright or inverted, only the top **or** bottom is being shown in cellular photomicrographs. Likewise, a stack of coins in its sleeve wrapper, if photographed from above, only shows a single coin (with an edging of wrapper), but we do know there are hidden 'layers' of coins out of sight. Hence, the top coin might show 'heads'. On inverting the stack of coins, does the possibility exist that we will see 'tails'? Yes, it does. I will explain that there are normal brown rosettes which do not preferentially attach to an extracellular matrix and those

which do attach. If inverted, their cell content is exposed and can be seen to be morphologically different to upright attached ones. Basal layer cells, like melanocytes and the epidermal-melanin unit, which are important in diagnostic tests for basal cell carcinomas can be now morphologically recognized *in situ*. A full explanation of factual occurrences leading to the elucidation of these facts will be described in stepwise fashion. Using a similar shape and size, in the form of self-aggregating granules comprising a nutritional source and a microscopic cell scaffold, I will show how this simple set of 'imitation' principles can be guided towards tissue repair of the human epidermis by describing their use in postoperative treatments. Along the way, I will question the standard practices of both medical doctors and cell culture scientists, like myself.

METHODOLOGY

Methodology was previously described (Solomon, 2002). Briefly, it consisted of trimming the subcutaneous fat from a skin biopsy sample, washing the trimmed biopsy sample with Dulbecco's phosphate buffered saline (DPBS) pH 7.4 and antibiotics, laying epidermis side down in a small pond of 2% Dispase solution, incubating at 37°C, 5% CO_2/ 95% O_2 until separation of the epidermis from the dermis could be achieved. Rinsing in complete culture medium (Medium 199 or IMDM) Iscove's modified Dulbecco's medium plus 20% (FBS) foetal bovine serum, followed by DPBS was done. The epidermis was stripped using a pair of forceps. A cellular photomicrograph (phase contrast microscopy) of the epidermal-dermal junction is shown in Figure 5. The stripped epidermis was inverted in DPBS for 30 min until disassembly into brown rosettes.

Basal layer brown rosettes

If brown rosettes are transferred to an extracellular matrix substrate (ECM), either autologous dermal fibroblast (secreted by both papillary and reticular fibroblasts) or (HUVECs), human umbilical endothelial cells ECM, some preferentially attach. These are basal layer rosettes. The attached brown rosettes are fed initially with complete medium then only 'topped up' with culture medium until they shed their epidermal cells onto the underlying extracellular matrix substrate. If the brown rosettes are inverted, the spilled cells will display morphologically, recognizable melanocytes and melanosomes and the epidermal-melanin unit (Figure 4). Specific cell markers will identify other basal layer epidermal cells, for example, the Merkel cells.

First Aid antibiotic ointment - Net weight ½ oz. (14g).

Bacitracin zinc 400 units, Neomycin 3.5 mg, Polymyxin B sulfate, 5000 units. No name of manufacturer was listed on tube...just Distributor....Walgreen Company, Deerfield, Illinois 60015-4616.

Sterile cornstarch granules

A cornstarch granule under the microscope has the morphology of a donut. Aggregates of cornstarch occur in solution or in a cell culture medium allowing multi-directional cell attachment (Type of cell could vary). Wound fluid will determine type and size of aggregates occurring. Particle size was a consideration (see www.engineeringtoolbox.com). Red blood cells are 5-10 microns in size, whereas cornstarch is of the order of 0.1-0.8 microns, but the latter do form aggregates of different sizes. Published reports of the invasive use of cornstarch granules as a lone tool in wound healing or as glove powder inadvertently contaminating wound beds resulted in tissue inflammation. A Medline search for published material containing the concomitant use of cornstarch granules and an antibiotic (or antibiotics) yielded no results. These granules are being put forward for use as biodegradable, nutritional, cell scaffolds with the lightweight triple antibiotic ointment.

Guided tissue regeneration

The primary scaffold of wound healing is the fibrin blood clot and the cascade of subsequent events results in scar tissue (Broughton et al., 2006). Interference with the formation and amount of the fibrin clot by application of the nutritional cell scaffold and triple antibiotic ointment does cause subcutaneous tissue repair with little or no scar tissue. Three 'case reports' described below will serve to illustrate this tentative conclusion:

(1) An 88 year-old man had undergone a procedure for skin cancer on his right cheek. Post-operatively, he was medically advised to use Vaseline and Hydrogen peroxide for home wound care management. He and his elderly wife were upset with the lack of post-operative care and distressed with the amount of dribbling seepage. Within 6 weeks, with use of the cornstarch granules/antibiotic mixture applied after every two days with a change of normal dressing by his wife on home premises, the dermis was rebuilt flush with his cheek, the epidermis was also regenerated and only close examination could reveal two thin scalpel lines. There was no skin crater or scarring.

(2) A 60 year old man, obese and diabetic developed a basal cell carcinoma about an inch and a half distal to his left shin bone. After Mohs surgery, wherein the blood vessels were cauterized, he was prescribed Mupirocin (Bactobran) and Gentamicin sulphate ointment. Use of the nutritional scaffold and antibiotic ointment resulted in tissue repair after three weeks. I was advised that there had been no inflammation and looking at the skin site now, no one could tell it had been the focal point of a medical procedure.

(3) A second degree burn on the first web space of the dorsal surface of the author's right hand was obtained through accidental contact with the pre-heated filament of a domestic kitchen oven. No scar tissue resulted after concomitant repeated use of the nutritional scaffold and the triple antibiotic ointment with a simple gauze dressing. Too much of the cornstarch granules were used. A basket weave pattern of raised healing epidermis, similar to that described by Hoath and Leahy (2003) in their Figure 1 (a photomicrograph of human skin following transplantation to a mouse with severe combined immunodeficiency) was observed. This was naturally discarded eventually to leave an unblemished surface area. Since 1999, untreated burns on the dorsal surface of my right hand leave behind an area of perturbed discoloured skin, unlike the hard-to-perceive cosmetic result after treatment with cornstarch granules and the lightweight antibiotic ointment.

RESULTS AND DISCUSSION

Pattern formation in nature is best thought of as a

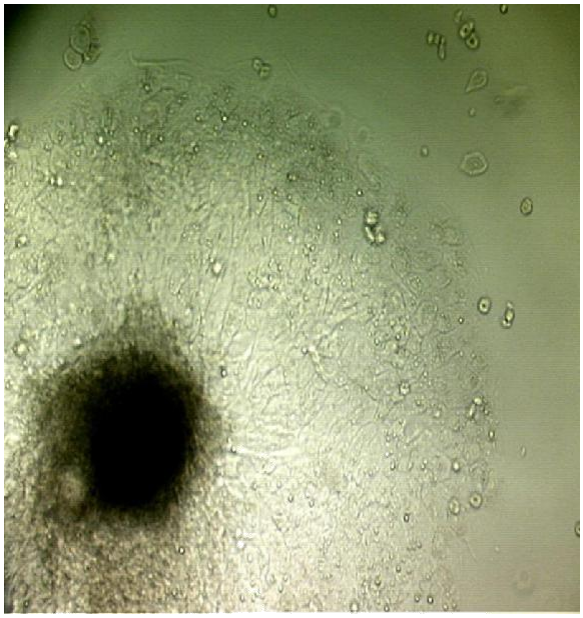

Figure 1. An *upright* attached brown rosette in an upright position displaying its intact micro pattern while shedding its cell load onto a prepared extracellular matrix and creating another micro pattern Magnification: x 100.

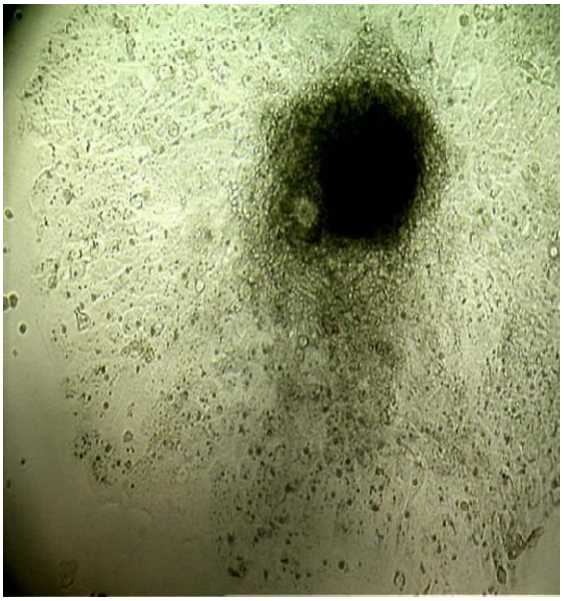

Figure 2. An *inverted* attached brown rosette shedding its cell load of epidermal cells. Magnification: x 200

process of symmetry breaking, that is, an initially homogeneous system becomes spatially, and sometimes temporally, inhomogeneous. Examples include the wind-dependent generation of sand dunes (Chuong et al., 2006). The essential reference book for understanding patterns is 'The Self-Made Tapestry: Pattern Formation in Nature', by Philip Ball, Oxford University Press, 1999. There is no reference material on natural 'live' micro patterns in the human body's living tissues. Five are presented here; the attached basal epidermal brown rosette spilling its cell content, being upright or inverted and the epidermal-dermal junction.

Published after my 2002 report, Hoath and Leahy (2003) theorized the concept of functional epidermal units centred around a phi (1.618034) proportionality, providing a central organizing principle. The melanocyte: keratinocyte ratio was given as 1:36 (Frenck and Schellhorn, 1969) and the Langerhans cell: epidermal cell as 1:53 (Bauer et al., 2001). These strikingly constant ratios allied with the fact that the epidermal brown rosette is circular do give this theory some credence.

Fifty years after Turing (1952) proposed a reaction-diffusion mechanism for biological pattern formation providing a biological explanation for animal coats, feather buds and fish skin patterns, an attempt was made to expand his framework by proposing a developmental mechanism. The two-dimensional (CPM) Cellular Potts Model (Zeng et al., 2004) speaks to enhanced local cell-cell adhesion and preferential cell-extracellular matrix (ECM) adhesion in 'condensation' (*in vitro* biological cell clustering), depending only on biological mechanisms and chemicals shown experimentally to be significant during patterning. Their experimental images at *low* density condensations, in their Figure 2, particularly (E) and (F), show a passing pattern resemblance to the epidermal brown rosette, which under the phase contrast microscope appears to be a wholly amorphous micro mass.

Of note, is their commentary that condensing precartilage cells employ transmembrane adhesion molecules, N-CAMs and N-Cathedrins (see my earlier comment on vasculogenic mimicry) and the time of maximal expression of N-Cathedrin (a calcium-dependent integrin of neural origin) corresponds to the period of active precartilage mesenchymal 'condensation' (also see Crosby et al., 2005).

Cross talk between the integrins (Monier-Gavell and Duband, 1997) was not considered in the CPM simulations, for example, control of N-Cadherin activity by intracellular signals elicited by beta1 and beta3 integrins in migrating neural crest cells. Different migrating cells, e.g. basal keratinocytes and neural crest cells have the bet1 integrins as a common denominator. Interestingly, Merkel cells of the basal layer of the epidermis are believed to function as sensory mechanoreceptors and are thought to be derived from either neural crest cells or basal keratinocytes. Whereas the basal keratinocytes may use the epithelial beta1 integrins as ECM receptors, the possibility exists that the neural crest cells may use

Figure 3. An *inverted* attached basal brown rosette. There appears to be an encapsulated basal cell lesion showing a cellular configuration within. To the left are the edges of the plastic tissue culture dish. Magnification: x 200.

them for embryonic neural cell motility exhibiting the versatility of integrin useage.

The CPM can be adapted to explain basal brown rosette parameters using the preferential attachment to an ECM which can be of embryonic origin, human umbilical vein endothelial cells (HUVECs) or an ECM secreted by dermal fibroblasts, thus exhibiting a dual capability in terms of their ECM requirement; neonatal or mesenchymal. Interestingly, the majority of cells in the epidermis, the keratinocytes, are affected by calcium concentration which could be a reflection of inherent integrin needs for proper functioning.

In published reports, epidermis is separated from the underlying dermis after enzymatic digestion with 0.25 % Trypsin- 1 mM EDTA (Ethylene diamine tetra-acetic acid) (Sorrell et al., 2004) or Thermolysin (Germain et al., 1993) or Dispase (Stenn et al., 1989). Trypsin continues to be used in spite of Barton and Marks' (1981) report of changes (invagination of desmosomes, vacuolation, and redistribution of tonofibrils) in suspensions of human keratinocytes directly attributable to use of this enzyme. Dispase, on the other hand, is a neutral protease which is both a fibronectinase and type IV collagenase (Stenn et al., 1989), dissolving the attachments between the basal keratinocytes and the basement membrane, without disturbing the desmosomal intercellular junctions between adjacent cells (Green, 1991). It had been reported that Dispase causes the internalisation of basal cell adhesion dependent domains containing the α6β4 integrin (Poumay et al., 1992), a receptor for laminin-5, an ECM component. This finding was based on Dispase-detached *cultured* human keratinocytes. It must be emphasized that caution should be exercised in extrapolating previous research reports on individual epidermal cells to the brown rosettes. 2% Dispase was used not at 12°C (Normand and Karasek, 1995), but at an incubator temperature of 37°C because it was privately thought that both cadaver and fresh skin tissue segments would better keep their normal constitutive properties at an ambient temperature that approximated *in vivo* conditions of the human body. The incubation time of 16 h can be shortened, depending on type of skin tissue sample (thin skin, wrinkly skin from knees and elbows et cetera). To neutralise Dispase, the enzyme manufacturers in their catalogues recommend using 5-10 mM EDTA. It has not been realised that leaving the enzyme Dispase in contact with human epidermis layer on an overnight basis has two consequences. The enzyme will lose its potency; hence no need to use 5-10 mM EDTA to neutralise and furthermore, the stripped epidermis layer disassembles (after immersion in DPBS) into brown micro masses (under a phase contrast microscope), I had christened as 'brown rosettes' in my 2002 paper. These cellular structures have not been previously described because in so- called 'established techniques' researchers have persistently used a double enzyme digestion (e.g. Dispase at various temperatures other than 37°C, followed by Trypsin-EDTA on the stripped off layer of epidermis) which yields single keratinocytes.

My experience with the burn on the dorsal surface of the first web space of my right hand provided nagging thoughts that I was overlooking a scientific concept. I could not understand or explain scientifically why there was no scar tissue or darkening of the burn area and why a spur of the moment remedy had worked so well. After 2002, I guessed that the appropriate cells had filled in the hole in the donut (the cornstarch granule) and that it represented a microscopic cell scaffold. I also realised that all cultured skin substitutes and (CEAs), cultured epithelial autografts (Green, 1991) had an inbuilt flaw. There was no inbuilt source of nutrition. Why had a lightweight triple antibiotic ointment sold over the counter at an American drugstore done its job so proficiently?

Writing a Scientific American article (March, 2008) 'Regrowing limbs: Can people regenerate body parts?' Professor Ken Muneoka had made the point that medical treatment *inhibits* tissue regeneration because of its focus on preventing infection. Medical management of subcutaneous wound healing has not been practised by

placing a quantity of a microscopic absorbent scaffold composed of a nutritional binding agent together with an antibiotic ointment within the wound bed to allow epithelial-mesenchymal crosstalk to dictate the architecture and regeneration of damaged tissue at its own pace. In essence, the microscopic scaffold sets up the sub sequential evolution of its own two-dimensional coordinates within the wound bed. Wound seepage will cause the manifestation of the binding, aggregation and absorbent properties of the scaffold. Body heat will cause the antibiotic ointment to degrade to a semi-liquid form and bind to the scaffold resulting in something approaching, a 'powdery filler'. Cells within the damaged subcutaneous tissue will be directly fed by the carbohydrate nutritional agent.

The question then arose as a result of my friends' post operative experiences whether a full-strength prescriptive medical antibiotic ointment was really necessary to help regenerate an avascular tissue layer that had a thickness measured in millimetres. The thickness of human epidermis and the papillary dermis layer is 0.3 mm; a further 0.7 mm down lies the reticular dermis (Sorrell et al., 2004), shows a 'keratinocyte mass' formed in the presence of papillary fibroblasts. It was not fully understood that the well being of the epidermal layer of human skin was influenced by both papillary and reticular fibroblasts (contained in the living dermis) acting in consent with the dermal-epidermal junction possibly acting as a traffic policeman directing and/or monitoring the cross-talk between the epithelial and mesenchymal cell layers with their inherent autocrine, paracrine loops plus the release of diffusible growth factors and cytokines.

Looking back at my own paper with the benefit of hindsight and with a much clearer understanding of these matters, I can now fully understand why the brown rosettes when co-cultured with autologous human dermal microvascular endothelial cells and dermal fibroblasts (papillary and reticular fibroblasts) lifted off from all substrates. A major epidermal player was completely out of its normal, accustomed milieu. Back then, I (and others) thought a proliferative limit had been reached and I reported it in those terms.

It was simply not understood at the time that (1) the attached brown rosettes originated from the basal layer of the epidermis and (2) the spread of cells emanating from an attached brown rosette on the dermal ECM was composed of the *full complement* of basal epidermal cells. They were misidentified. Also not properly recognised was that the cellular photomicrograph showed only the *top layer* of a possible series of epidermal layers hidden and hence out of view, underneath or within. Secondly, there was no published identity test or specific cell marker for human basal keratinocytes. A single report appeared years after (Spichkina et al., 2006) describing the selective adhesion of human basal keratinocytes to ECM proteins. A simple observation made while idly fingering the smooth surface of a green leaf and inverting it to see a configuration of veins provided a 'Eureka' moment. Chlorophyll makes the green leaves, green but its porphyrin rings which act as photoreceptors are hidden within.

I dashed off to find and examine old cellular photomicrographs to scrutinise the now understood, attached 'inverted' brown rosettes and their spread of epidermal cells onto the ECM. This led me to a literature search for integrin receptors (in basal epidermal cells) with a similarity in apical and basal polarity. Only one report (Bishop et al., 1998) was found, stating that keratinocyte beta1 integrins in the basal layer of the epidermis did not display an intrinsic polarity with regard to their ligand-binding capacity. Therefore, it was concluded that upright or *inverted* 'brown rosettes' would adhere to the dermal fibroblast or HUVECs ECM in identical fashion and the *full complement of epidermal cells* would spread out from the attached brown rosette. If the attached *inverted* 'brown rosettes' were truly basal layer epidermal cells, the cell-spread, away from the micro mass, should expose resident keratinocytes together with other *in situ* epidermal cells within the micro pattern, for example, those described by Quevedo (1969, 1972) as *the epidermal-melanin unit*. Quevedo (1972) described this concept wherein 'a structural and functional organization of melanocytes and keratinocytes exists at levels of biological organisation that transcend those characterizing the individual component cells'. The refractive index of the melanosomes provided persuasive signposts in the positive identification of the melanocyte, which are not found as contiguous cells in the stratum basale of human epidermis, but within the epidermal-melanin unit. Hindsight is the only exact science.

The analogy I drew in my Introduction, of a stack of coins in its wrapper does have some relevance. It will be noted that the perimeter of an attached, *inverted* brown rosette (Figure 2 to 4) shows a lighter colour to the main body of the brown micro mass, indicative perhaps of the migration of a 'concentric circle layer of epidermal cells' away from the main body onto the ECM. On close examination of Figure 2 to 4, tiny circles are seen migrating away in a 'V' shape from the main body of the micro mass, reminiscent of nascent tiny buds arrayed within a floral pattern. Perhaps my fingering of a leaf was not far off the mark; in other words, a resonant botanical pattern/symmetry might be applicable here. The realisation that a cornstarch granule with its inherent attributes of being a microscopic cell scaffold was providing a form of mimicry was verified by me, and my friends' medical experiences with postoperative tissue regeneration.

Taken together, these facts question the standard

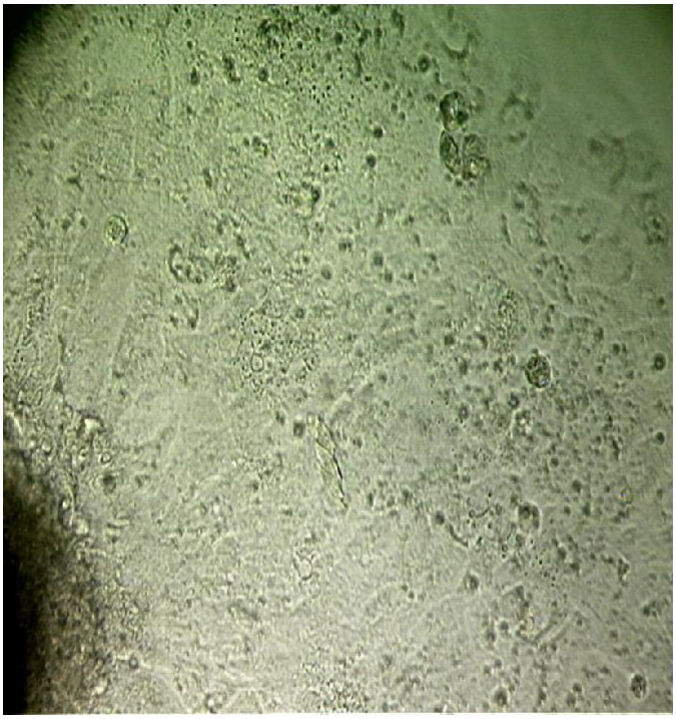

Figure 4. An *inverted* attached basal brown rosette shedding its cell load of epidermal cells. The *epidermal-melanin unit* can be seen. The refractive index of the melanosomes provided persuasive signposts in the positive identity of the melanocyte, which are not found as contiguous cells in the stratum basale of human epidermis. Magnification: x 100. .

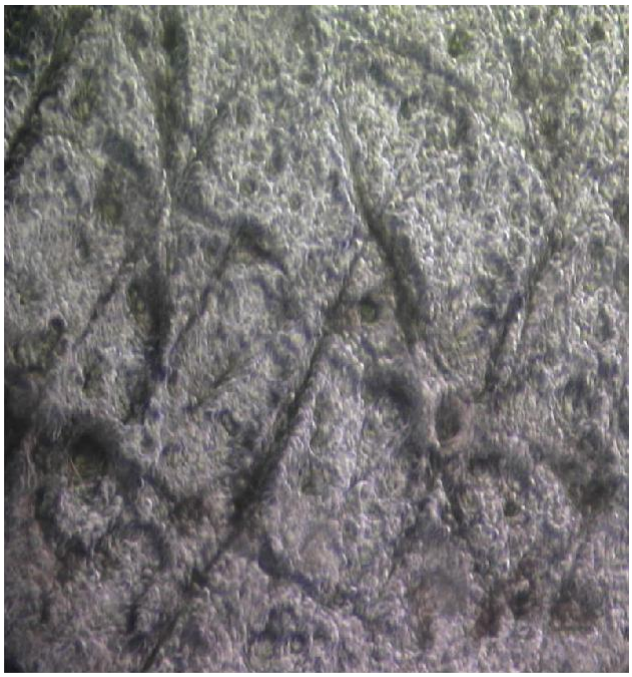

Figure 5. Another micro pattern. The epidermal-dermal junction (after stripping off the Dispase-digested epidermis) displaying dermal papillae and sweat ducts (the 'round holes'). Magnification: x 200.

medical practice of covering wounds with CEAs, a sheet material, only containing one type of epidermal cell, the keratinocyte. This also might explain why on occasion, they do not 'take', that is become vascularized and in certain instances are rejected. This is the routine '*Outside-In'* of medical science. Perhaps, this needs to be reconsidered and as I am describing in this paper, an '*Inside-Out'* technique might have its merits with respect to a better cosmetic outcome.

Surgical treatment for basal cell carcinomas (Rubin et al., 2005) includes curettage and electrodessication, cryosurgery, surgical excision, and Mohs micrographic surgery, for which there is no standardized post-operative treatment.

My friend's father deemed the medical advice of hydrogen peroxide and Vaseline, not fit for purpose. With my other friend, the medical prescription of Gentamicin ointment was curious and puzzling. I had stopped using this antibiotic in the tissue culture laboratory, after a report (Goetz et al., 1979) that it inhibited proliferation of human diploid fibroblasts, had become widely accepted. What was the explanation behind the prescription? I could not pursue this matter because of medical records confidentiality. My own medical doctor told me I probably had a skin quality with a propensity for normal healing, when eventually I sought him out, on my return home. The intricacies of inter-cellular tissue repair are more or less unknown to family doctors.

It would be easy to prove or disprove this methodology by clinically employing it to treat donor sites after skin biopsies are taken. The whole dermis would now become subject to this tissue repair method. A similar principle would apply to treatment of second degree burns. Further work is needed to determine the cellular architecture of the epidermal brown rosettes and what role they play in melanomas. If it can be shown that allogenic brown rosettes are non-antigenic like dermal fibroblasts, it will provide an improvement to the techniques described by Gerlach et al. (2011).

Finally, little do the tattoo artists know that they are superimposing their colourful patterns over an invisible micro pattern that Nature has so brilliantly devised.

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