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Chronic wound healing by fetal cell therapy may be explained by differential gene profiling observed in fetal versus old skin cells

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ABSTRACT

Engineering of fetal tissue has a high potential for the treatment of acute and chronic wounds of the skin in humans as these cells have high expansion capacity under simple culture conditions and one organ donation can produce Master Cell Banks which can fabricate over 900 million biological bandages (9 × 12 cm). In a Phase 1 clinical safety study, cases are presented for the treatment of therapy resistant leg ulcers. All eight patients, representing 13 ulcers, tolerated multiple treatments with fetal biological bandages showing no negative secondary effects and repair processes similar to that seen in 3rd degree burns. Differential gene profiling using Affymetrix gene chips (analyzing 12,500 genes) were accomplished on these banked fetal dermal skin cells compared to banked dermal skin cells of an aged donor in order to point to potential indicators of wound healing. Families of genes involved in cell adhesion and extracellular matrix, cell cycle, cellular signaling, development and immune response show significant differences in regulation between banked fetal and those from banked old skin cells: with approximately 47.0% of genes over-expressed in fetal fibroblasts. It is perhaps these differences which contribute to efficient tissue repair seen in the clinic with fetal cell therapy.

1. Introduction

Considerable interest and research has been dedicated to the understanding of wound healing and the associated process. Whereas adult cutaneous wounds heal more slowly and with scar formation to restore tissue integrity, fetal skin, *in utero*, is observed to have rapid and scar-less tissue repair characterized by regeneration of an organized dermis with normal appendages and by a relative lack of inflammation. (Beanes et al., 2002; Bullard et al., 2003; Cass et al., 1997; Adzick and Lorenz, 1994; Armstrong and Ferguson, 1995; Dang et al., 2003; Lorenz et al., 1995). Fundamental differences between fetal and adult skin and the fetal and adult skin wound environment may be important in inducing efficient tissue repair. Chronic wounds, more specifically leg ulcers, are a major health concern. Patient's suffering has long been underestimated and recent evaluations of quality-of-life reveal that patients with leg ulcers have complaints that are substantial and similar to those previously reported for patients with chronic obstructive pulmonary disease, osteoarthritis or angina (Kahn et al., 2004). Costs are considerable and were calculated to exceed 0.5 billion euros/year in Germany and the direct cost of leg ulcers in the UK were calculated at 400 million euros a year (Hafner et al., 1999). Similarly in the United States, estimations are as high as 3 billion dollars for treatment of all forms of leg ulcers. Important variables for cost differences between countries are the frequency of bandage changes and duration of time for each bandage change (Ragnarson and Hjelmgren, 2005). Etiological treatment of venous ulcers is mandatory in order to prevent relapse. Compression therapy is the cornerstone of venous ulcer treatment and high rates of healing (up to 83% in 6 months) may be obtained by different techniques (Booza et al., 2005). However, such results are not obtained by all teams, and considerable biases of recruitment do exist. In countries with a high standard of health care and a good prevention of venous insufficiency, resistant ulcers are more and more frequent and present as a difficult therapeutic challenge (Chaby et al., 2006; Marklund et al, 2000).

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Local methods for accelerating healing are based on modern wound dressings. However, there is no evidence in the literature that their use really improves the healing rate of venous ulcers (Booza et al., 2005). Therefore, there is an urgent need for new techniques in the treatment of refractory leg ulcers.

New biological therapies for wound healing have significantly advanced including growth factors, skin substitutes, gene and stem cell therapies as well as tissue engineering. The origin of cell choice, their interaction with a biomaterial and the simplicity of preparation is extremely important for eventual therapeutic usage.

Autologous skin fibroblasts and substitutes of various nature have been used on leg ulcers to date (Limat et al., 1996; Uccioli, 2003; Caravaggi et al., 2003; Cavallini, 2007) with different degrees of efficiency. Major drawbacks are that production time is long since patient tissues are necessary for processing. Allogenic skin substitutes using foreskin tissue (Dermagraft®, Apligraf® and Orcel®) have shown decreased healing times for diabetic foot ulcers, other ulcers and non-weight bearing wounds (Falanga et al., 1998; Marston et al., 2003). The production time is long and we have recently shown high differences in gene expression of banked fetal and foreskin cells used in tissue engineering (Hirt-Burri et al., 2008) which could be, in part, responsible for differences of efficiency seen in the clinic to date.

As banked fetal skin fibroblasts have been previously shown to efficiently induce tissue repair in burns and in acute wounds (Hohlfeld et al., 2005; de Buys Roessingh et al., 2006; Quintin et al., 2007), it was of interest to look at the effect on recalcitrant leg ulcers with different etiologies in the elderly to see if a full clinical trial would be of merit. In this Phase I safety clinical study, we particularly wanted to evaluate the tolerance of multiple treatments with fetal cell biological bandages and interaction with the chronic wound environment. With these specifically banked fetal skin cells used in tissue engineering, we performed microarray analysis to identify differences on the molecular level between specific gene clusters compared to old skin cells, banked in the same manner, which could give some insight into significant parameters involved in efficient wound healing.

2. Materials and methods

2.1. Skin donations and cell bank synthesis

Cell lines established in the University Hospital of Lausanne from a fetal skin biopsy at 14 weeks of gestation (14 week male fetal skin;14 wFS) obtained after pregnancy termination with informed and written consent and approval from the local Medical School Ethics Committee. Normal skin from an old male donor (75 yr old skin; 75 yOS) was obtained in the Department of Dermatology in the University Hospital of Lausanne from a non-sun-exposed skin site (buttocks) also with informed consent and approval from the Medical School Ethics Committee.

From 1 cm^2 skin biopsies, three individual cultures were established from each of the biopsies of 14 wFS and 75 yOS to prepare cell banks including all of the vials of cells necessary for the microarray analysis at passage 3 for each of the 6 cell banks (fetal and aged skin cell banks). Dermal tissue was dissected into <0.5 mm^3 fragments and grown in DMEM supplemented with 10% FCS and glutamine and the cells were used for experimentation at passage 3. They were grown to confluence before splitting and rinsed twice with PBS and counted.

The detailed procedure has been described previously (de Buys Roessingh et al., 2006) and specific conditions for the study herein are described briefly. The tissue was divided into three, 10 cm plates with whole tissue fragments ~10 per plate (<0.5 mm^3). Tissue culture dishes were previously scored deeply with a sterile scalpel in a check-board pattern under the laminar flow hood. Tissue fragments were placed into the scored plastic regions mincing gently. A small quantity of nutrient media was placed around each fragment to avoid floating of tissues for the first 24 h. Rare floating tissues could be overlaid with a sterile microscope cover slide for the first 24 h if necessary. Following the first 24 h, 8 ml of culture media was added onto each 10 cm plate and this was changed two times per week before passage. These fragments were grown in DMEM supplemented with only 10% fetal bovine serum (Hyclone) to help insure a consistent dermal skin cell culture. Cell cultures were grown at 37 °C in a humidified atmosphere of 95% air/10% CO_2. It is important to mention that any nutrient component necessary for cell culture for clinical trials should have thorough safety requirements and tracing. All animal derived products, such as for fetal bovine serum and trypsin, specific clinical lots of trypsin and gamma irradiated serum that have been tested for adventitious agents should be employed (Quintin et al., 2007).

When cell growth advanced after approximately 1 week for fetal tissues and 2 weeks for adult, dishes of tissue and cells were trypsinized (0.25% trypsin-0.1% ethylene diaminetetraacetic acid [EDTA]). At this point 20 plates were frozen into individual units in liquid nitrogen for each of the three tissue cultures. Cells were centrifuged at 2000*g* for 15 min and resuspended in a freezing solution of DMEM (5 ml) + FCS (4 ml) + DMSO (1 ml, Fluka) and frozen in 1 ml aliquots (~5 million cells) at –80 °C in Nalgene Cryo 1 °C Freezing Container's (Nalgene) to achieve a −1 °C/min rate of cooling and freezing curve. After 24 h, cells were transferred to liquid nitrogen for longer storage. Three independent cell banks for each biopsy of 14 wFS and 75 yOS were prepared (denominated FCB and OCB hereafter for Fetal Cell Bank and Old Cell Bank). We have seen that cells frozen in this manner are capable of being stored for at least 15 years in our laboratory.

2.1.1. Ethical aspects of working with human fetal cells

Although there is a high medical support for developing cellular based therapies to reach as many patients as possible, there exists a governing political controversy. Scientists and medical doctors have used fetal tissue since the 1930's as a means to understand cell biology and as an essential tool in the development of vaccines. The 1954 Nobel Prize for Medicine was awarded to immunologists who developed the polio vaccine using cultures of human fetal kidney cells. The drastic change in the political environment changed in 1988 when scientists began using fresh fetal tissue and cells for transplantation into patients with Parkinson's disease. Unfortunately, the Reagan administration (US government) declared a moratorium on all federal funding for fetal tissue research. Advances for fetal cell research were then left only to the "private sector" where there is no medical peer-review of adapted therapies. Indeed, if research on whole-cell bio-processing of many fetal tissues could have continued, there certainly would have been advances in the amount of tissue necessary for developing efficient therapies (such as with fetal skin where only one organ donation is necessary to allow for cellular expansion to develop over 900 million fetal skin constructs). In Switzerland and most countries, the fetal skin is considered as an organ donation by law. This process is highly regulated including federal approval for tissue biopsy, stocking and transplantation and ethics committee approval of the procedure and all information for the donor. Even though termination of pregnancy is legally allowed, there will always be strong opponents which will interfere that the laws and practices can remain patient centered (conditions which permit to end an unwanted pregnancy in the best possible way and without unnecessary suffering).

2.2. Fetal dermal skin construct preparation

Preliminary experiments investigating cell seeding density (from 10^2 to 10^5 cells cm^2) and growth periods (from 1 to 6 days) on a 9 × 12 cm equine collagen sheet of 2 mm thickness dry weight (TissueFleece®, Baxter, Switzerland) were performed in order to determine optimal conditions for fetal skin construct preparation. Fetal cells at passages 3 or 4 were placed in 20 ml media (DMEM containing 10% FBS) and seeded on the collagen sheet by making small incisions at 2 cm intervals into the collagen matrix with a sterile, small bored Pasteur pipette. The sheet containing the fetal dermal cells (denominated as fetal skin construct) was placed into a 37° C incubator at 95% relative humidity and 10% CO_2. An additional 30 ml media was added 1 h later. Fetal skin cell constructs remained at a 2 mm thickness as the original dry collagen sheets. A seeding density of 5×10^3 cells/cm^2 with cells in passages 3–4 and a growth period of 3 days were employed for the patients in this study.

2.3. Patient selection, preparation and treatment regimen

Patients were selected on the basis of a history of having refractory chronic leg ulcers, which did not heal using traditional therapies, such as compression (active and passive), hydrocolloids, autografts, among others. A total of 9 patients were admitted in the study including 13 ulcers total (Average age = 73 Yr, 4 males and 5 females). Patients recruited suffered from refractory venous ulcers including ulcers with atrophie blanche. In four of these patients, it was possible to accomplish a thorough investigation, including venous (duplex) and arterial investigations (ABI and duplex). Characteristics of patients are presented in the next section.

At each visit, the chronic ulcers were cleaned with physiological saline. Fibrin and necrotic tissue was removed with a curette. Then, the fetal skin construct was applied to cover the entire wound surface, followed by a gauze impregnated with Vaseline, and, if not contra-indicated (one patient suffered from arterio-venous ulcer), compression therapy with long stretch bandage. Application of constructs was one time a week, followed to 4 days to bandage change. Bandages were then changed every two days (Vaseline gauze). There was no modification of medication, way of living, or compression bandaging throughout the fetal skin construct application.

2.4. Analysis of gene expression

Gene expression for approximately 12,500 probes representing known genes and expressed sequence tag (EST) profiling of cultured fetal and old human primary fibroblasts was analyzed.

The RNA was isolated from cultured cells at passage 1 for each of the three cell banks for 14 wFS and 75 yOS using the NucleoSpin, RNA II kit (Marchery-Nagel, Düren, Germany) as described by the manufacturer. The RNA samples were monitored with the Agilent 2100 Bioanalyser, consistently demonstrating high-quality RNA (28S/18S ratio approximately 2, but always less than 3). Total RNA (7 μg) was converted to biotinylated cRNA as previously described (Mutch et al., 2002), and then hybridized to the Affymetrix human genome U95A chip (Affymetrix UK, High Wycombe) containing about 12,500 probes. The comparison was performed in triplicate with three cell banks established independently from the same biopsies (14 wFS and 75 yOS).

2.5. Data analysis

The statistical analyses were performed as reported by Mansourian et al., 2004 with a classical analysis of variance (ANOVA) followed by The Global Error Assessment (GEA). In short, The GEA approach takes advantage of several inherent characteristics of microarrays that results in a robust estimate of the error and in a slightly increased statistical power. The method is based on the assumption that any observed difference between the error of genes similarly expressed is only due to sampling. Therefore, after estimating from an ANOVA the mean square error (MSE) of each probe on the microarray independently, in a second step, a robust MSE is calculated from average MSE of 200 neighboring genes with similar intensity signals. In a third step, a new F statistic is recalculated replacing the MSE of the current gene with the robust MSE with the appropriate number of degrees of freedom (df). Averaging the MSE results in a more robust statistic and the increased number of df results into a slightly increased power. Genes were selected by this method at α level 0.001. Significant genes, with at least a 1.5-fold change $\log_2$, were classified according to the gene ontology following the criteria of the DAVID (database for annotation, visualization, and integrated discovery) (Dennis et al., 2003). This gene list was subjected to the KEGG database (www.genome.jp/Kegg/tool/colorpathway) for signaling pathways.

2.6. Reverse transcription and real time–PCR (RT–PCR)

The results obtained with the Affymetrix Human GeneChip were confirmed by RT-PCR for 12 genes using the same fetal and old fibroblast cell lines.

One microgram of total RNA was reverse transcribed using the StratScript reverse transcriptase enzyme (Stratagene, San Diego, CA) as described by the manufacturer. The thermocycler Biometra T-1 (Bimedicinisch Analytic GmbH, Göttingen) was programmed as follows: 25 °C 10 min, 37 °C 60 min and 90 °C 5 min.

Real time PCR using 160 ng of cDNA were performed with ABI Prism 7000 Sequence Detection System (Applied Biosystem, Foster City, CA). We measure the gene expression of 10 genes using TaqMan® Gene Expression Assays (Applied Biosytems) (Table 1). Measurements were performed in triplicates and results are presented associated with standard deviation of the mean. Relative gene expressions was expressed as -ΔΔCT ($\log_2$) analyzed with the $2^{-\Delta\Delta CT}$ method by Livak and Schmittgen (2001) and normalized to actin gene.

3. Results

3.1. Preliminary assessment of fetal skin constructs on chronic wounds

In a total of 9 patients treated with fetal cell therapy from 3 to 31 weeks concerning 13 ulcers, we were able to see 8 ulcers closed completely, 4 with significant amelioration in size but not com-

Table 1
Primers for real time confirmation.

Gene symbol	Ref of TaqMan® gene expression assays
GDF10	Hs00192033_m1
MDK	Hs00383235_m1
PTN	Hs00171064_m1
DPT	Hs00170030_m1
INBHH	Hs00173582_m1
CDKN1C	Hs00175938_m1
RBP1	Hs00161252_m1
HLA-DPB1	Hs00157955_m1
FMOD	Hs00157619_m1
BMP6	Hs00233470_m1
MMP14	Hs00237119_m1
MMP1	Hs00233958_m1
MMP3	Hs00233962_m1
TNFRSF11B	Hs00171068_m1
Actin-β	Hs99999903_m1

plete closure and 1 which was lost to follow-up because the patients estimated that there was a substantial improvement. We present the detail of 4 patients herein as we have completed data including venous (Duplex) and arterial investigations (ABI and duplex) described according to CEAP classification (Eklöf et al., 2004).

3.1.1. Patient 1

EF is a very compliant 64 years old female patient, with a history of painful post-thrombotic ulcer for 10 years, consecutive to deep venous system and short saphenous vein insufficiency (CEAP $C_{2,3,4b,6S}$ E_S $A_{S2,3,4\ D13,14}$ P_R) (Fig. 1, Ulcer 1). Previous auto-grafts and different compression therapies including four layers bandages were of no success.

Immediately following the first fetal skin construct, edema diminished, pain was relieved, and fibrin production elimination was evident. This large, deep and painful ulcer healed rapidly with applications of fetal skin constructs one time per week. At 11 weeks, the larger portion of the ulcer was healed. At one year follow-up, patient shows skin that is still atrophic but no presence of scar tissue (Fig. 1, Ulcer 1). A new ulcer presented at the ankle one year later and was also successfully closed rapidly without scar (Fig. 1, Ulcer 2).

3.1.2. Patient 2

LG is a 62 years old female patient presenting with post-thrombotic bilateral lipodermatosclerosis and *atrophie blanche* on the lower legs, with an atypical ulcer on the lateral right lower leg since 20 months (CEAP $C_{4b,6\ s}$, E_S, $A_{S1,2,3,\ D13,14,\ P18,}P_R$) (Fig. 2, Patient 2).

Following fetal cell therapy, the edema and itching immediately disappeared and the original ulcer closed gradually. Because of the fragility of the skin, even though the ulcer of origin was closed a new ulceration occurred at distance from the previous one. For better wound preparation, this patient had the vacuum assisted device (VAC) applied for one week with the intention of applying an autograft. The ulcer and associated minor ulcerations then rapidly

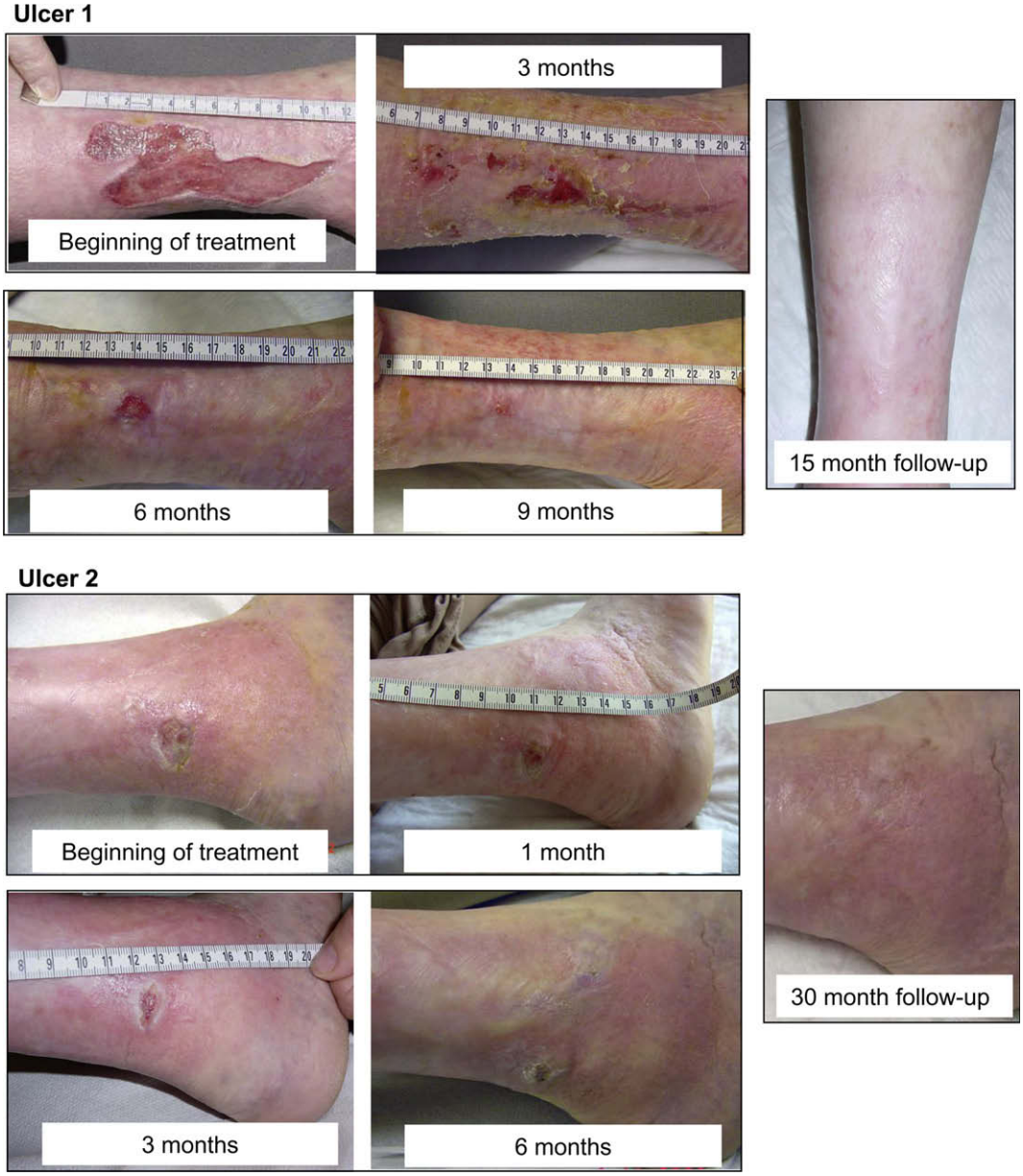

Fig. 1. Ulcers treated with fetal skin constructs one time per week for Patient 1, illustrating the evolution at 1, 3, 6 and 9 months and follow-up at 15 or 30 months for each of two separate ulcers (Ulcer 1 and Ulcer 2).

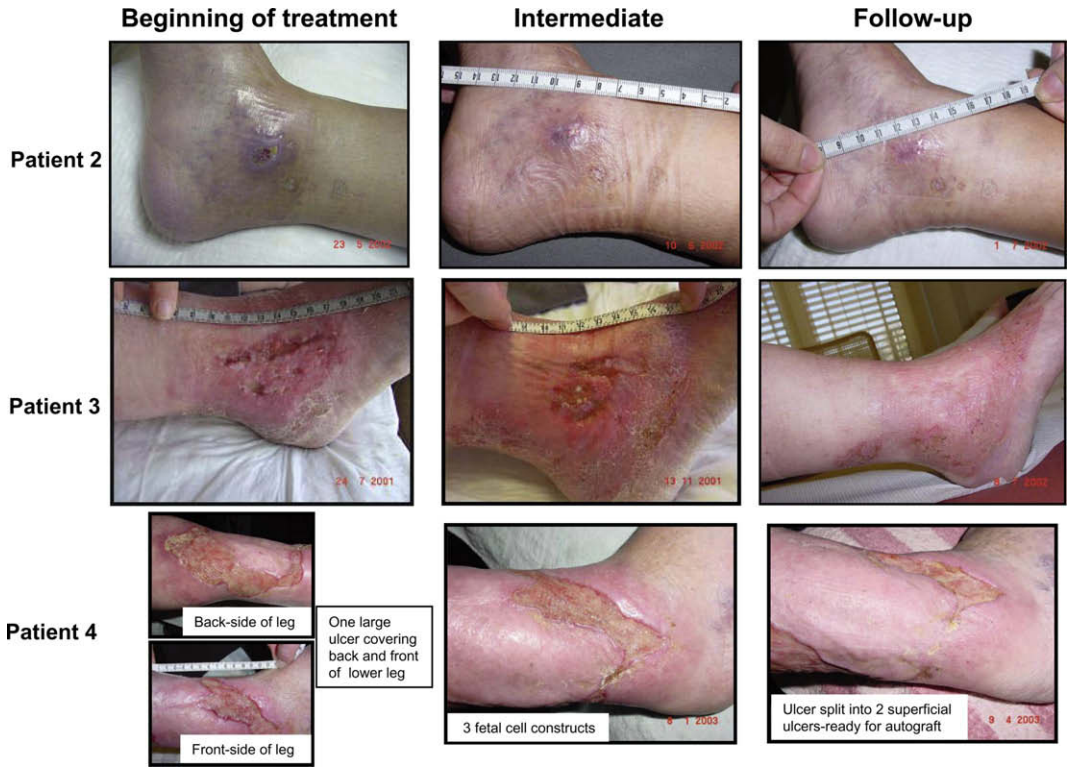

Fig. 2. Patients 2, 3 and 4 at the beginning of fetal skin construct treatment which was applied one time per week. Evolution at 2 w and 1 month for Patient 2 and 3 mo and one year are shown for Patient 3. Patient 4 was treated 3 times with fetal skin constructs to prepare wound bed for auto-graft treatment. These patients show the diversity of sensitivity of the recalcitrant ulcers treated. Fibrous tissue removal remained pivotal for treatment regimen.

healed with fetal cell application and no auto-graft necessary, which is quite an unusual favorable evolution. Atrophie blanche was stable and no new ulceration developed at the one year follow-up.

3.1.3. Patient 3

CC is a 37 years old female patient, suffering since 10 years with a severe post-traumatic post-thrombotic syndrome, with atrophie blanche (Fig. 2, Patient 3). A painful therapy resistant medial malleolar venous leg ulcer developed 2 years ago, consecutive to venous reflux in the deep and superficial venous systems, and leg perforators (CEAP $C_{3,4b,6S}$ E_S $A_{S\ 2,3,4\ D\ 13,14,15\ P\ 18}$ P_R), aggravated by an arthrodesis of the ankle. Local treatment with fetal cell therapy achieved rapid healing and relieving of pain. However, repetitive inflammatory outbreaks of *atrophie blanche* in spite of fetal cell treatment or tacrolimus ointment, new superficial post-traumatic ulcerations developed and healed intermittently.

3.1.4. Patient 4

RG is an 85 year old male patient suffering global heart failure and severe bilateral knee arthritis presenting with bilateral large ankle ulcers with mild edema and pain over the last 10 years. Vascular investigations were not performed in this case (CEAP $C_{3,6\ S}$ $E_N A_N P_N$) (Fig. 2, Patient 4).

Previous auto-grafts associated to lymphatic drainage and compression therapy allowed temporary wound closure, but recurrence appeared after only a few months each time. Following fetal skin construct application used to prepare the wound bed and to eliminate high fibrin content, auto-grafts were performed on the ulcer site.

Rapidly, wounds healed after one auto-graft application whereas in previous treatments, multiple auto-grafts were necessary for closure with this patient. The wound site was stable during the last two years of the patient's life which was a long period for no recurring ulcers compared to previous years in our clinic.

3.2. Microarray analysis

It was of interest to determine gene expression alterations in banked fetal dermal skin cells (used in tissue engineering for burns and wounds to date) and old dermal skin cells that have been banked in the same manner to have a listing of potential gene families from fetal banked cells to compare to old skin cells and other gene profiling studies. To identify differentially expressed genes in banked fetal dermal skin cells, we used cDNA microarray containing approximately 12,500 sequences (U95A human genome chip, Affymetrix UK, High Wycombe). Three arrays were hybridized for banked fetal skin cells and banked old skin cells each. When comparing banked fetal dermal skin cells to banked old fibroblasts with our conditions, 116 genes changed by 1.5-fold log or more. Between those genes 54 were up-regulated in fetal cells and 62 were down-regulated. Gene Ontology of important differentially expressed genes was annotated following the criteria of the DAVID database (http://david.abcc.ncifcrf.gov/) for annotation, visualization, and integrated discovery) (Dennis et al., 2003). Gene ontologies included apoptosis, cell adhesion, cell-cell signaling, extracellular matrix, transcription factor, cytokine activity and immune response. Many of the genes analyzed could be involved in multiple cellular processes so they have been placed in a category for their best representation. In Table 2, an exhaustive listing of all significantly different regulated genes between Banked Fetal Cells and Banked Old Cells are presented. The majority of genes which are up-regulated in banked fetal dermal skin cells are within cell adhesion and extracellular matrix, (Table 3). The gene list was also confronted with the KEGG database for analysis of signaling pathways. Of the 116 genes, 75 were not included in the data base and

Table 2
Regulated genes in fetal banked cells compared to old banked cells.

Entrez gene ID	Gene symbol	Gene name	Fold increase (expressed in $\log_2$)
4232	MEST	Mesoderm specific transcript homolog	4.598
2202	EFEMP-1	EGF-containing fibulin-like extracellular matrix protein 1	4.041
5947	RBP1	Retinol binding protein 1, cellular	3.913
3875	KRT18	Keratin 18	3.533
1191	CLU	Clusterin	3.511
5740	PTGIS	Prostaglandin I2 (Prostacyclin) Synthase	3.209
5764	PTN	Pleiotrophin (Neurite growth-promoting factor 1)	3.050
55816	DOK5	Docking protein 5	2.938
10924	SMPDL3A	Sphingomyelin phosphodiesterase, acid-like 3A	2.833
894	CCND2	Cyclin D2	2.762
11341	SCRG1	Scrapie responsive protein 1	2.718
59	ACTA2	Actin, Alpha 2, Smooth muscle, Aorta	2.714
10234	LRRC17	Leucine rich repeat containing 17	2.676
7052	TGM2	Transglutaminase 2	2.657
4192	MDK	Midkine (Neurite growth-promoting factor 2)	2.579
4781	NFIB	Nuclear factor I/B	2.508
1264	CNN1	Calponin 1, Basic, Smooth muscle	2.490
2331	FMOD	Fibromodulin	2.457
8324	FZD7	Frizzled homolog 7	2.405
23089	PEG10	Paternally expressed 10	2.365
2149	F2R	Coagulation factor II (Thrombin) receptor	2.345
4628	MYH10	Myosin, Heavy polypeptide 10, Non-muscle	2.326
6422	SFRP1	Secreted frizzled-related protein 1	2.226
114882	OSBPL8	Oxysterol binding protein-like 8	2.164
10643	IGF2BP3	Insulin-like growth factor 2 mrna binding protein 3	2.143
8492	PRSS12	Protease, Serine, 12 (Neurotrypsin, Motopsin)	2.124
1909	EDNRA	Endothelin receptor type A	2.095
65108	MARCKSL1	Marcks-like 1	2.068
1000	CDH2	Cadherin 2, Type 1, N-cadherin	2.038
2201	FBN2	Fibrillin 2	2.034
2239	GPC4	Glypican 4	2.034
2719	GPC3	Glypican 3	2.021
23705	IGSF4	Immunoglobulin superfamily, member 4	2.012
7472	WNT2	Wingless-type MMTV integration site family member 2	1.961
22836	RHOBTB3	Rho-related btb domain containing 3	1.881
9249	DHRS3	Dehydrogenase/reductase (SDR family) member 3	1.855
10962	MLLT11	Myeloid/lymphoid or mixed-lineage leukemia	1.795
5179	PENK	Proenkephalin	1.782
1466	CSRP2	Cysteine and glycine-rich protein 2	1.741
586	BCAT1	Branched chain aminotransferase 1, cytosolic	1.736
22795	NID2	Nidogen 2 (Osteonidogen)	1.733
5360	PLTP	Phospholipid transfer protein	1.683
23157	SEPT 6	Septin 6	1.656
23231	KIAA0746	KIAA0746 Protein	1.651
126393	HSPB6	Heat shock protein, alpha-crystallin-related, b6	1.645
1462	CSPG2	Chondroitin sulfate proteoglycan 2 (versican)	1.626
1028	CDKN1C	Cyclin-dependent kinase inhibitor 1c (p57, kip2)	1.596
347733	TUBB2B	Tubulin, beta 2B	1.575
9843	HEPH	Hephaestin	1.570
7373	COL14A1	Collagen, type xiv, alpha 1 (undulin)	1.559
9865	KIAA0644	KIAA0644 gene product	1.550
10154	PLXNC1	Plexin C1	1.546
5157	PDGFRL	Platelet-derived growth factor receptor-like	1.525
5997	RGS2	Regulator of g-protein signalling 2, 24KDA	1.513
10403	KNTC2	Kinetochore associated 2	−1.507
1846	DUSP4	Dual specificity phosphatase 4	−1.521
25945	PVRL3	Poliovirus receptor-related 3	−1.536
1474	CST6	Cystatin E/M	−1.542
202	AIM1	Absent in melanoma 1	−1.549
9975	NR1D2	Nuclear receptor sub-family 1, group d, member 2	−1.551
3600	IL15	Interleukin 15	−1.577
4312	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	−1.626
891	CCNB1	Cyclin B1	−1.626
4907	NT5E	5'-Nucleotidase, ECTO (CD73)	−1.627
133	ADM	Adrenomedullin	−1.654
2922	GRP	Gastrin-releasing peptide	−1.679
9123	SLC16A3	Solute carrier family 16, member 3	−1.680
4603	MYBL1	V-MYB myeloblastosis viral oncogene homolog like 1	−1.699
2199	FBLN2	Fibulin 2	−1.704
1346	COX7A1	Cytochrome c oxidase subunit viia polypeptide 1 (muscle)	−1.704
358	AQP1	Aquaporin 1	−1.725
22885	ABLIM3	Actin binding lim protein family, member 3	−1.782
960	CD44	CD44 antigen (indian blood group)	−1.821
1410	CRYAB	Crystallin, alpha B	−1.824

(*continued on next page*)

Table 2 (*continued*)

Entrez gene ID	Gene symbol	Gene name	Fold increase (expressed in $\log_2$)
5420	PODXL	Podocalyxin-like	−1.830
1805	DPT	Dermatopontin	−1.841
9411	ARHGAP29	RHO gtpase activating protein 29	−1.863
490	ATP2B1	ATPASE, CA++ transporting, plasma membrane 1	−1.880
7035	TFPI	Tissue factor pathway inhibitor	−1.884
1033	CDKN3	Cyclin-dependent kinase inhibitor 3	−1.885
857	CAV1	Caveolin 1, caveolae protein, 22KDA	−1.921
347	APOD	Apolipoprotein D	−1.931
11098	PRSS23	Protease, serine, 23	−1.976
25976	TIPARP	Tcdd-inducible poly(ADP-RIBOSE) polymerase	−1.979
1012	CDH13	Cadherin 13, H-cadheriN	−1.987
1842	ECM2	Extracellular matrix protein 2	−2.004
858	CAV2	Caveolin 2	−2.025
8781	PSPHL	Phosphoserine phosphatase-like	−2.028
3134	HLA-F	Major histocompatibility complex, class I, F	−2.043
3490	IGFBP7	Insulin-like growth factor binding protein 7	−2.062
8714	ABCC3	ATP-binding cassette, sub-family C	−2.080
9404	LPXN	Leupaxin	−2.102
3037	HAS2	Hyaluronan synthase 2	−2.147
4223	MEOX2	Mesenchyme homeobox 2	−2.156
4885	NPTX2	Neuronal pentraxin ii	−2.162
290	ANPEP	Alanyl aminopeptidase	−2.242
10974	C10orf116	Chromosome 10 open reading frame 116	−2.270
26872	STEAP1	Six transmembrane epithelial antigen of the prostate 1	−2.284
3115	HLA-DPB1	Major histocompatibility complex, class ii, dp beta 1	−2.331
1728	NQO1	Nad(P)H dehydrogenase, quinone 1	−2.336
9023	CH25H	Cholesterol 25-hydroxylase	−2.392
1396	CRIP1	Cysteine-rich protein 1 (intestinal)	−2.421
11075	STMN2	Stathmin-like 2	−2.433
5678	PSG9	Pregnancy specific beta-1-glycoprotein 9	−2.569
5730	PTGDS	Prostaglandin d2 synthase 21kda (brain)	−2.620
8839	WISP2	Wnt1 inducible signaling pathway protein 2	−2.825
25907	RIS1	RAS-induced senescence 1	−2.848
8644	AKR1C3	ALDO-keto reductase family 1, member C3	−2.976
4314	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	−3.130
131578	LRRC15	Leucine rich repeat containing 15	−3.163
9976	CLEC2B	C-type lectin domain family 2, member B	−3.258
6275	S100A4	S100 calcium binding protein A4	−3.458
7545	ZIC1	ZIC family member 1	−4.051
3625	INHBB	Inhibin, beta b	−4.292
1311	COMP	Cartilage oligomeric matrix protein	−4.665
4982	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11B	−4.712

50 pathways were obtained. Within these pathways an average of only 1.76 genes were found to be included related to allograft rejection, focal adhesion, TGF-β signaling pathway and cytokine-cytokine receptor interaction. As there were very few genes for any one pathway, it was difficult to interpret the implication.

3.3. Gene expression analysis by real time PCR

RT–PCR was used to verify findings in the gene microarray expression analysis of eight up-regulated and four down-regulated selected genes. Gene expression was assessed relative to the control gene β-Actin in the cells of the microarray analysis. Mean values for the relative expression of selected genes and magnitudes of upward or downward changes detected by RT–PCR are shown in Table 4, as well as the fold differences obtained in the microarray study. The expression levels of these genes were consistent with the two techniques.

4. Discussion

In the clinical setting, we have seen practical advantages of fetal cell therapy applied to preliminary patients with chronic wounds (Ramelet et al., 2001). The cells are able to exert promoting effects on adhesion, proliferation and migration of existing cells as the repaired wounds tend to heal gradually and the skin is much less atrophic. Also, the pain alleviation following fetal construct application was universal. In other studies using other autologous and allogenous grafting techniques (Khachemoune et al., 2002) alleviation of pain was reported after several applications or days when closer to healing was seen whereas in all of our patients it was seen after application of the first fetal construct. Obvious practical advantages of the fetal skin construct technique are that it is non-invasive and therefore surgical facilities are not necessary. The method is easily applied in an ambulatory manor and the cells are available right away instead of 4–6 weeks with traditional auto-graft techniques. Similar to what we have seen in severe burns, fetal skin constructs are showing promising results also for chronic wound management and promoting an increased quality-of-life for these patients. Leg ulcers are multifactor in cause and associated inflammation, fibrosis and pain make the recalcitrant ulcer difficult to manage. There are also differences between males and females as it is well accepted that hormones have a major impact on regulation of wound repair and it has been further shown that estrogen can inhibit local inflammatory responses by down-regulation of macrophage inhibitory factor and thus improved tissue repair seen (Ashcroft et al., 2003; Gulliver et al., in press). Treatment of venous leg ulcers requires commitment and cooperation between the patient and the care provider. It is important to identify health-related quality-of-life factors (i.e. pain, itching, loss of sleep, functional limitations) as many patients feel that related symptoms are overlooked by doctors and thus can delay overall healing (Chaby et al., 2006; Kahn et al., 2004). The impact of this and other complications of venous leg ulcers on overall health and quality-of-life is only beginning to be appreciated. New biolog-

Table 3
Microarray analysis sumary of significantly different genes: fetal vs old.

Gene Ontology	Number of genes differentially expressed (>1.5-fold)	Up-regulated in fetal cells	Percent up-regulated genes in fetal cells (%)
Total	116	54	47
Apoptosis	5	4	80
Cell adhesion	13	5	38
Cell–cell signaling	25	9	28
Extracellular matrix	15	9	60
Transcription factor	5	1	20
Cytokine activity	5	2	40
Immune response	4	0	0

Entrez gene ID	Gene symbol	Gene name	Fold increase
Cell–cell signaling			
5947	RBP1	Retinol binding protein 1, cellular	3.913
4781	NFIB	Nuclear factor I/B	2.508
114882	OSBPL8	Oxysterol binding protein-like 8	2.164
10643	IGF2BP3	Insulin-like growth factor 2 mrna binding protein 3	2.143
8492	PRSS12	Protease, serine, 12 (neurotrypsin, motopsin)	2.124
9249	DHRS3	Dehydrogenase/reductase (sdr family) member 3	1.855
5179	PENK	Proenkephalin	1.782
126393	HSPB6	Heat shock protein, alpha-crystallin-related, B6	1.645
347733	TUBB2B	Tubulin, beta 2B	1.575
9975	NR1D2	Nuclear receptor subfamily 1, group D, member 2	−1.551
3600	IL15	Interleukin 15	−1.577
4312	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	−1.626
4907	NT5E	5′-nucleotidase, ECTO (CD73)	−1.627
133	ADM	Adrenomedullin	−1.654
1346	COX7A1	Cytochrome C oxidase subunit viia polypeptide 1 (muscle)	−1.704
1410	CRYAB	Crystallin, alpha B	−1.824
11098	PRSS23	Protease, serine, 23	−1.976
25976	TIPARP	TCDD-inducible poly(adp-ribose) polymerase	−1.979
4223	MEOX2	Mesenchyme homeobox 2	−2.156
4885	NPTX2	Neuronal pentraxin II	−2.162
1728	NQO1	Nad(P)H dehydrogenase, quinone 1	−2.336
5730	PTGDS	Prostaglandin D2 synthase 21KDA (brain)	−2.620
8644	AKR1C3	Aldo-keto reductase family 1, member C3	−2.976
4314	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	−3.130
3625	INHBB	Inhibin, beta B	−4.292
Apoptosis			
1191	CLU	Clusterin	3.511
5740	PTGIS	Prostaglandin i2 (prostacyclin) synthase	3.209
7052	TGM2	Transglutaminase 2	2.657
2149	F2R	Coagulation factor ii (thrombin) receptor	2.345
4982	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11B	−4.712
Cell adhesion			
1000	CDH2	Cadherin 2, type 1, n-cadherin	2.038
22795	NID2	Nidogen 2 (osteonidogen)	1.733
1462	CSPG2	Chondroitin sulfate proteoglycan 2 (versican)	1.626
7373	COL14A1	Collagen, type xiv, alpha 1 (undulin)	1.559
10154	PLXNC1	Plexin C1	1.546
960	CD44	CD44 antigen (Indian blood group)	−1.821
5420	PODXL	Podocalyxin-like	−1.830
1805	DPT	Dermatopontin	−1.841
1012	CDH13	Cadherin 13, H-cadherin	−1.987
1842	ECM2	Extracellular matrix protein 2	−2.004
9404	LPXN	Leupaxin	−2.102
8839	WISP2	Wnt1 inducible signaling pathway protein 2	−2.825
1311	COMP	Cartilage oligomeric matrix protein	−4.665
Extracellular matrix			
2202	EFEMP-1	EGF-containing fibulin-like extracellular matrix protein 1	4.041
7052	TGM2	Transglutaminase 2	2.657
2331	FMOD	Fibromodulin	2.457
2201	FBN2	Fibrillin 2 (congenital contractural arachnodactyly)	2.034
2239	GPC4	Glypican 4	2.034
2719	GPC3	Glypican 3	2.021
22795	NID2	Nidogen 2 (osteonidogen)	1.733
1462	CSPG2	Chondroitin sulfate proteoglycan 2 (versican)	1.626
7373	COL14A1	Collagen, type xiv, alpha 1 (undulin)	1.559
4312	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	−1.626
2199	FBLN2	Fibulin 2	−1.704
1805	DPT	Dermatopontin	−1.841
1842	ECM2	Extracellular matrix protein 2	−2.004
4314	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	−3.130
1311	COMP	Cartilage oligomeric matrix protein	−4.665

(*continued on next page*)

Table 3 (*continued*)

Entrez gene ID	Gene symbol	Gene name	Fold increase
Transcription factor			
11336	NFIB	Nuclear factor I/B	2.508
5760	NR1D2	Nuclear receptor subfamily 1, group D, member 2	−1.551
673	MYBL1	V-MYB myeloblastosis viral oncogene homolog-like1	−1.699
10496	MEOX2	Mesenchyme homeobox 2	−2.156
Immune response			
3600	IL15	Interleukin 15	−1.577
3134	HLA-F	Major histocompatibility complex, class I, F	−2.043
3115	HLA-DPB1	Major histocompatibility complex, class II, DP beta 1	−2.331
1396	CRIP1	Cysteine-rich protein 1 (intestinal)	−2.421
9976	CLEC2B	C-type lectin domain family 2, member B	−3.258
Cytokine activity			
5764	PTN	Pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)	3.050
4192	MDK	Midkine (neurite growth-promoting factor 2)	2.579
3600	IL15	Interleukin 15	−1.577
3625	INHBB	Inhibin, beta b (activin ab beta polypeptide)	−4.292
4982	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	−4.712

ical techniques may be of high interest if the safety and simplicity can be assured. Nevertheless, a search for improvement of wound healing therapies which can improve inflammation, pain management and long-term closure is necessary for patient management and their quality-of-life. Cell therapies and tissue engineering are showing great promise in wound management. The cell choice is therefore an important factor for simplifying the overall technique and bringing therapy rapidly to the patient.

The precise mechanisms of efficient wound healing remain unknown despite the great increase in knowledge gained over the past decade although fetal wound repair is a tightly regulated process involving many cellular mediators. Chen et al. (2007) have proposed that understanding the "blueprint of fetal skin repair" might allow the manipulation of adult wound healing in order to decrease scarring and fibrosis. There are indeed many genes that are significantly different in the "fetal skin blueprint" when compared to older skin that we have elucidated herein with banked fetal and old dermal skin cells. These factors taken together and presented in a cellular therapy seem to be an interesting approach for chronic wounds.

We have seen major differences in gene families of banked fetal dermal skin cells compared to old skin cells banked in the same manner. Profiles that are predominant are in the families of genes in cell adhesion and extracellular matrix, cell-cell signaling and immune response confirmed that our molecular data obtained by microarray were consistent with the published biochemical and clinical findings related to efficient fetal tissue repair.

Table 4
Comparison of real time pcr to microarray analysis.

	Relative gene expression MA	Relative gene expression real time (-ddcT)
Gene symbol		
RBP1	3.91	7.35 ± 0.97
PTN	3.05	0.12 ± 0.36
MDK	2.57	1.9 ± 0.29
FMOD	2.45	2.86 ± 0.89
CDKN1C	1.59	4.59 ± 0.29
GDF10	0.25	1.45 ± 0.24
BMP6	−0.43	2.21 ± 0.47
MMP14	−0.63	1.64 ± 0.53
MMP1	−1.62	1.41 ± 0.19
DPT	−1.81	−3.82 ± 0.26
HLA-DPB1	−2.33	−16.95 ± 1.82
MMP3	−3.13	−4.36 ± 0.59
INBHH	−4.29	−5.70 ± 0.14
TNFRSF11B	−4.71	−6.73 ± 0.24

In our microarray data, significant increases were seen in matrix proteins such as fibromodulin (FMOD) and collagens compared to old cells and these data correspond to the literature (Mast et al., 1993; Hallock et al., 1993; Soo et al., 2000, 2002). FMOD was also significantly higher in the RT–PCR which was one of the random probes selected for confirmation of the microarray data. Genes above and below the established curve from our statistical analysis were chosen for this conformational purpose only. The most significantly expressed gene in the microarray data, EFEMP-1 or Fibulin 3 (FPLN-3) is homologous to FBLN-5 and are widely expressed ECM proteins known to regulate cell proliferation and have also been shown to antagonize angiogenesis. FBLN-5 has been shown to promote wound healing in vivo using a retroviral gene transfer to dermal wounds of rabbits showing 50% net increase of newly formed granulation tissue and wound closure (Lee et al., 2004; Albig et al., 2006; Soo et al., 2000). Microarray analysis has also shown the importance of ECM glypicans which are proteins that can bind a multitude of GF and ECM molecules which are implicated in signal transduction cascaded that most likely regulate cell proliferation. Glypicans activate or determine activities of morphogens and GF such as FGF, BMPs and IGFs (Berry et al., 1998; De Cat and David, 2001; Hwang et al., 2001). We have seen that glycipans are up-regulated in fetal dermal skin cells (microarray data, GPC4 and GPC3 with 2.02 and 2.03 up-regulated, respectively) and in another recent study by Lener et al. (2006) they had seen the same effect with down-regulation of glycipans in aged skin. Many genes in the ECM family can therefore interact with other gene families to regulate cell growth, angiogenesis and migration. Migration is an important fibroblast response following tissue injury and is crucial to the repair process. Fetal fibroblasts possess more rapid intrinsic rates of migration than that seen in adult skin fibroblasts (Sandulache et al., 2005; Sandulache et al., 2007).

Cell-cell signaling genes are related to cell growth characteristics, differentiation and development of tissues and therefore important for fetal dermal skin cells. In the microarray data, Retinol binding Protein 1 (RBP1, gene encoding the carrier protein for transport of vitamin A) which is necessary for growth and differentiation of epithelial tissues was 3.9-fold increased in fetal dermal skin cells and confirmation with RT–PCR at 6.45-fold higher expression. Others such as Tubulin beta 2B (up-regulated at 1.58) have been shown to be highly present in fetal brain development (Nakamura et al. 2003).

Other studies emphasize that wound healing is regulated by many cytokines, growth factors and their receptors (Constant et al., 1997; Peled et al., 2001; Werner and Grose, 2003; Yang et al., 2003) and it is suggested that efficient wound healing in fetal skin at early gestation is a result of the unique cytokine or growth factor profile. Of these, transforming growth factors-beta (TGF-β) has been most widely studied and there are three highly homologous TGF-β isoforms known in humans: β1, β2 and β3. Published studies have shown that it is the relative proportion of TGF-β isoforms, and not the absolute concentration of any one isoform which determines the wound repair outcome; however, other studies question the efficacy of different isoforms (TGF-β3) in wound healing (Sullivan et al., 1993; Vooijs et al., 2004; Wu et al., 1997; Krummel et al., 1988; Lin et al, 1995) and we have not seen a preponderant expression of these isoforms except for TGF-β2 with 6-fold up-regulation in fetal cells in a previous study analysis with RT–PCR (Hirt-Burri et al., 2008). This suggests that factors other than TGF-β may also be important in efficient repair. In our microarray data, TGF-β superfamily genes such as Inhibin (INHBB) and Cartilage oligomeric matrix protein (COMP) were significantly different in fetal dermal skin cells (INHBB, microarray with −4.292; COMP, microarray with −4.665). Also, known to modulate TGF-β activity, ECM constituents such as fibromodulin or decorin are expressed as a function of gestation age in fetal skin (Beanes et al., 2001). This supports the hypothesis that differential expression of TGF-β isoforms and TGF-β activity modulators, rather than the mere presence or absence of TGF-β has a role in the regulation of efficient repair.

An increased stimulus for angiogenesis and vascular permeability may assist the rapid healing of wounds. Studies have shown evidence for the importance of angiogenesis in wound repair. Pleiotropin (PTN), a cytokine inducing heparin-binding/differentiation, is certain to have a major role in angiogenesis in wound healing and can induce formation of functional neovaculature *in vivo*. Midkine (MDK), which has 50% amino acid sequence identity and striking domain homology to that of PTN are the two members of the *PTN/MDK* developmental gene family (Christman et al., 2005). Both PTN and MDK were shown to be significantly increased in fetal compared to adult skin with fold increase of 3.05 and 2.57, respectively.

Not unexpected, genes involved in immune response were all down-regulated in fetal dermal skin cells with five such genes within the microarray. Among these genes highly implicated in allograft rejection, include transplantation antigen Major Histocompatibility Complex of Class I (HLA-F, microarray with −2.043) and Class II (HLA-DPB1, microarray with −2.331).

In all, fetal skin constructs show promise for treatment of acute and chronic wounds as banked cells can be thoroughly screened for unwanted virus and pathogen. Multiple treatments with fetal cell constructs in an out-patient setting were well tolerated and positive effects of anti-inflammatory and anti-pain were evident. Expression profiling of banked fetal dermal skin cells compared to aged dermal skin cells has provided biological grouping of important gene families implicated in the mechanism of wound repair. Indeed, individual growth factors (TGF-β2, TGF-β3, IL-10, PDGF) have been shown in the clinic to help in different aspects of overall wound healing but it is a very complex process (Adzick and Lorenz, 1994). Most likely, many factors taken together are necessary for complete wound closure which could indeed be offered by a cell-based therapy for chronic wound management.

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