Pathway Analysis of Gene Expression of E14 Versus E18 Fetal Fibroblasts

Michael S. Hu,^{1,2} Mimi R. Borrelli,¹ Michael Januszyk,¹ Anna Luan,¹ Samir Malhotra,¹ Graham G. Walmsley,¹ Wan Xing Hong,¹ Ruth Tevlin,¹ Geoffrey C. Gurtner,¹ Michael T. Longaker,^{1,2} and Hermann P. Lorenz^{1,*}

¹Hagey Laboratory for Pediatric Regenerative Medicine, Division of Plastic Surgery, Department of Surgery, Stanford University School of Medicine, Stanford, California.

²Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California.

Objective: Fetuses early in gestation heal skin wounds without forming scars. The biological mechanisms behind this process are largely unknown. Fibroblasts, however, are cells known to be intimately involved in wound healing and scar formation. We examined fibroblasts in different stages of development to characterize differences in gene expression that may result in the switch from regenerative wound repair to repair with scarring.

Approach: Fibroblasts were isolated and cultured from the back skin of BALB/c wild-type mouse fetuses at embryonic day (E)14 and E18 ($n = 10$). The fibroblast total RNA was extracted, and microarray analysis was conducted using chips containing 42,000 genes. Significance analysis of microarrays was performed to identify genes with greater than twofold expression difference and a false discovery rate of less than two. Identified genes subsequently underwent enrichment analysis to detect differentially expressed pathways.

Results: Two hundred seventy-five genes were differentially expressed between E14 and E18 in fetal fibroblasts. Thirty genes were significantly downregulated and 245 genes were significantly upregulated at E18 compared with E14. Ingenuity pathway analysis identified the top 20 signaling pathways differentially activated in fetal fibroblasts between the E18 and E14 time points.

Innovation: To our knowledge, this work represents the first instance where differentially expressed genes and signaling pathways between fetal fibroblasts at E14 and E18 have been studied.

Conclusion: The genes and pathways identified here potentially underlie the mechanism behind the transition from fetal wound healing via regeneration to wound healing by repair, and may prove to be key targets for future therapeutics.

Keywords: wound healing, scarless repair, regeneration, microarray

Hermann P. Lorenz, MD

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*Correspondence: Hagey Laboratory for Pediatric Regenerative Medicine, Division of Plastic Surgery, Department of Surgery, Stanford University School of Medicine, 257 Campus Drive, Stanford, CA 94305-5148 (e-mail: plorenz@stanford.edu).

INTRODUCTION

THE PHYSIOLOGICAL RESPONSE to cutaneous injury is a complex and tightly regulated process. Cutaneous injury initiates an acute inflammatory response, followed by a rapid migration of progenitor cells to the wound site. Multipotent cells differentiate into

numerous cell types that rapidly form new blood vessels and epithelium. Concurrently, fibroblasts proliferate at the wound site, synthesize collagen, and differentiate into myofibroblasts, which act to contract the wounded skin. Remodeling of the wounded tissue continues far beyond the initial

injury, and scarring is characterized by the excess accumulation of extracellular matrix (ECM). The organization of the newly synthesized ECM never recapitulates that found in uninjured skin, resulting in a 20–30% decrease in overall tensile strength in the repaired, compared to normal, skin tissue.¹

Interestingly, Rowlatt described the ability of the human fetus, early in gestation, to repair cutaneous wounds without forming a scar.² Further work has subsequently confirmed these findings in both animal models and in human fetuses.³ The finding of scarless wound healing in human skin suggests there is potential that adult skin can be stimulated to function as fetal skin, and heal via regeneration rather than fibrosis. The mechanisms of scarless healing, however, require further characterization before such knowledge can be applied for therapeutic benefit.

There are numerous differences documented between fetal and adult wound healing phenotypes. Scarless wound healing is age dependent; there is a distinct switch from regenerative to scarring healing around 24 weeks of gestation in human embryos⁴ and around gestational age 18 days (embryonic day [E]18) in mice.⁵ Scar-free healing is also dependent on the size of the wound, with larger wounds healing via scarring at earlier gestational ages.⁴ *In vitro*, fetal skin fibroblasts are able to simultaneously proliferate and synthesize collagen, whereas adult skin fibroblasts proliferate before they synthesize collagen, suggesting a central role of fibroblasts in the mechanism of scarless wound healing.⁶ This study focused on the difference in fibroblast function at different ages of gestation. A microarray transcriptional profiling comparison was conducted on fetal fibroblasts harvested from mouse fetuses at E14 and E18 to detect any deviations in transcriptomes between scarless and scarring repair. The aim was to identify novel pathways involving fibroblasts that promote regenerative repair and scarless healing.

CLINICAL PROBLEM ADDRESSED

Scarring is the expected outcome of the human adult wound healing process and is a significant medical issue that can substantially reduce patients' quality of life. Numerous physiological and psychological consequences result from scarring, either the result of trauma or surgery.⁷ Facial scars are often cosmetically displeasing and result in substantial psychosocial distress. Keloid scars can cause immense pain and severe itching, while hypertrophic scars can lead to contracture, erosion of skeletal structure, and potentially lifelong disabili-

ty.⁸ Scars and their associated consequences surmount to enormous economic cost estimated in the tens of billions of dollars.⁹

MATERIALS AND METHODS

Animals

BALB/c wild-type mice at 6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). After acclimation for at least 1 week, male and female mice were bred overnight. Every day, the female mice were checked and the day of vaginal plug was determined to be E0.5 for gestational timing. All animal procedures were performed in accordance with National Institutes of Health (NIH) guidelines according to university-approved protocols. Mice were closely monitored by the Stanford Administrative Panel on Laboratory Animal Care (APLAC).

Primary cell culture

Gestational age E14 and E18 pregnant mice were sacrificed with CO₂ and cervical dislocation. Fetal mice were surgically removed using sterile technique. Under a dissecting microscope, E14 ($n=10$) and E18 ($n=10$) fetal mouse dorsal skin was collected and pooled for fibroblast isolation and primary cell culture under sterile conditions.

Fibroblast primary cell culture was conducted by mincing tissue and treating it with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) in 37°C with mild agitation for 10 min. Mouse embryonic fibroblast culture medium consisting of Dulbecco's modified Eagle's medium, GlutaMAX supplement (Thermo Fisher Scientific, Waltham, MA), 10% fetal bovine serum (Omega Scientific, Tarzana, CA), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO), and 1% penicillin/streptomycin (Thermo Fisher Scientific) was used for cell culture. Cells were cultured at 37°C in a humidified incubator with 5% CO₂. All experiments used passage one fibroblasts from male and female fetuses.

RNA extraction and amplification

RNA was extracted using the TRIzol protocol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Subsequently, the Messa-gAmp antisense RNA (aRNA) kit (Ambion, Austin, TX) was used to amplify 1 µg of extracted RNA from each group. To compare the different arrays between gestational time points, universal mouse RNA was amplified in individual reaction mixtures of 1 µg aliquots simultaneously, with amplifications in the experimental sample and utilized as an internal amplification control.

Preparation of fluorescent complementary DNA probes

Two micrograms of random hexamer and 4 μ g of RNA were heated at 65°C for 10 min. Samples were then reverse transcribed with 2 mM of each deoxyribose nucleotide triphosphate, 1 $\times$ first-strand buffer, 0.5 μ L RNase inhibitor, 200 U superscript II, 10 mM dithiothreitol, and either 3 μ L Cy3-deoxyribose uridine triphosphate (dUTP) (for experimental samples) or Cy5-dUTP (for universal mouse control samples) at 42°C for 1 h in a 30 μ L total reaction volume. To increase the reaction, 200 U superscript II was added to the mixture and samples were incubated at 42°C for 1 h. Fluorescent Cy3- or Cy5-labeled probes were washed with Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA) through a microcon mini column (Millipore, Billerica, MA), treated with 450 μ L TE buffer, and the inverted mini column was spun into a new tube. Microarray chips were immediately hybridized with probes.

Pretreatment of microarray chips

The Stanford Microarray Database Center was used to print microarray chips with 42,000 specific complementary DNAs (cDNAs) printed onto lysine-coated slides. Sequences and accession numbers can be accessed at <http://genome-www5.stanford.edu/index.shtml>, where single accession numbers from GenBank represent chosen cDNAs. Microarray chips were rehydrated before hybridization by being held down quickly over distilled water. Microarray chips were then quickly snap-dried over a 100°C heating block and DNA was crosslinked using a UV crosslinker (300 mJ).

Microarray hybridization

Fluorescent-labeled probes were heated at 100°C for 2 min, denatured, and then incubated at 37°C for 20 min. The recovered probe hybridization mixture with a volume of 32 μ L, 6.8 μ L of 20 $\times$ saline sodium citrate (SSC), and 1.2 μ L of 10% sodium dodecyl sulfate (SDS) was placed onto microarray slides that were prewarmed. Coverslips were applied and slides were placed into a sealed moisture chamber at 65°C for 16 h to hybridize. Slides were then immediately washed with 1 $\times$ SSC in 0.03% SDS, with 0.5% SSC twice, and with 0.06% SSC twice. After the washes, slides were centrifuged at 84 g for 2 min and immediately scanned. An Axon microarray scanner (Molecular Devices, Sunnyvale, CA) was used for scanning slides.

Microarray data analysis

GenePix Pro 4.0 software (Molecular Devices) was used to analyze scanned slides. Densitometry data for gene identification and analysis were up-

loaded into the Stanford Microarray Database. The log (base 2) of red/green normalized ratio (mean) was found and filtered based on a regression correlation of 0.6. Each gene was centered to the median and was only included in the final analysis if genes passed >80% good data filter criterion. Pearson correlation was used to cluster genes. Subsequently, genes between E14 and E18 with significant differences were selected using significance analysis of microarrays (SAM). By utilizing a set of gene-specific *t* tests, SAM is able to identify gene expression changes that are statistically significant. The analysis assigns a score to each individual gene dependent on the expression change based on the standard deviation of the gene repeated measurements. Permutations of repeated measurements are used to determine the false discovery rate (FDR) for genes equivalent to chance. Only genes that had both an FDR less than two and at least twofold expression difference were selected.

Functional analysis of differentially expressed genes

As previously described by Jovov *et al.*,¹⁰ network and pathway analyses of probes were performed using Ingenuity Pathway Analysis (IPA; www.ingenuity.com, Ingenuity Systems, Redwood City, CA) between significantly regulated genes to identify functional connections. The significance of networks was calculated by IPA's integrated Ingenuity algorithm, which calculates *p*-values using the right-tailed Fisher's exact test for each canonical pathway. The association between a subset of genes from the whole experimental data set and a related function/pathway is evaluated to be due to random association.

RESULTS

Differential gene expression between scarless E14 and scarring E18 fibroblasts

SAM identified a total of 275 genes that were differentially expressed, with at least a twofold difference, in fetal fibroblasts between E14 and E18. Of these genes, 30 were significantly down-regulated (Table 1) and 245 genes were significantly upregulated at the E18 compared with the E14 time point (Table 2).

Functional pathway analysis

Of the 245 genes upregulated in E18 compared with E14 fetal fibroblasts identified using microarray analysis (Fig. 1A), IPA identified 20 functional pathways represented by these genes (Fig. 1B). The top five pathways identified were associated with

Table 1. Genes downregulated in E14 fibroblasts

Symbol	Gene Name
Arf4	ADP-ribosylation factor 4
Mfge8	Milk fat globule-EGF factor 8 protein
Tmem147	Transmembrane protein 147
Mtg1	Mitochondrial GTPase 1 homologue (<i>Saccharomyces cerevisiae</i>)
Galnt10	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10
Crmp1	Collapsin response mediator protein 1
Rpl27	Ribosomal protein L27
Timp3	Tissue inhibitor of metalloproteinase 3
1500003003Rik	RIKEN cDNA 1500003003 gene
Dpm1	Dolichol-phosphate (beta-D) mannosyltransferase 1
Csnk1a1	Casein kinase 1, alpha 1
Pgk1	Phosphoglycerate kinase 1
Pdcd6	Programmed cell death 6
Hdgf	Hepatoma-derived growth factor
Lama2	Laminin, alpha 2
Rab10	RAB10, member RAS oncogene family
Tmem206	Transmembrane protein 206
Ubqln1	Ubiquilin 1
Myadm	Myeloid-associated differentiation marker
Mat2a	Methionine adenosyltransferase II, alpha
Parn	poly(A)-specific ribonuclease (deadenylation nuclease)
Rae1	RAE1 RNA export 1 homologue (<i>Schizosaccharomyces pombe</i>)
Seh1l	SEH1-like (<i>S. cerevisiae</i>)
Rpl3	Ribosomal protein L3
Sub1	SUB1 homologue (<i>S. cerevisiae</i>)
Nrbp2	Nuclear receptor binding protein 2
Ppp3ca	Protein phosphatase 3, catalytic subunit, alpha isoform
Mtmr6	Myotubularin-related protein 6
Odc1	Ornithine decarboxylase, structural 1
Ttc12	Tetratricopeptide repeat domain 12

colorectal cancer (CRC) metastasis signaling, hepatic fibrosis/hepatic stellate cell activation, pancreatic adenocarcinoma signaling, platelet-derived growth factor (PDGF) signaling, and endometrial cancer signaling. From the 30 genes downregulated in E18 compared with E14 fibroblasts, IPA identified 20 functional pathways represented by these genes (Fig. 1C). The five most significant functional pathways identified were putrescine biosynthesis III, s-adenosyl-l-methionine biosynthesis, d-myo-inositol (1,4,5,6)-tetrakisphosphate biosynthesis, d-myo-inositol (3,4,5,6)-tetrakisphosphate biosynthesis, and dolichyl-diphosphooligosaccharide biosynthesis.

DISCUSSION

Previous work conducted by our laboratory analyzed the differences in the transcriptomes of fetal keratinocytes and fibroblasts between E16 and E18. At the time of this research, however, it was difficult to efficiently culture E14 fetal fibroblasts.¹¹ Using a larger number of E14 fetal mice and a smaller culture plating surface in this study, we were able to successfully culture E14 fetal fibroblasts. The microarray analysis and signal pathway analysis

conducted at the E14 time point in this study revealed a number of significant gene expression changes that occur during the transition period from scarless healing to healing with scarring. A recent investigation by our laboratory compared gene expression in fetal (E17) and adult mouse wounds and identified 178 genes upregulated and 13 genes downregulated in fetal compared with adult wounds. A selection of down- and upregulated pathways was identified (unpublished observations). Together with the findings presented here, this research contributes to unraveling the mechanism of fetal regenerative cutaneous wound repair. In the following sections, we characterize in greater detail some of the notable pathways differentially activated in E14 fibroblasts versus E18 fibroblasts.

CRC metastasis signaling

SAM revealed a significant increase in CRC metastasis signaling in E18 compared with E14 fetal fibroblasts. CRC tumorigenesis is well characterized and results from the accumulation of genetic mutations. Inflammation is a known environmental factor strongly associated with genetic mutations in cells of the colonic mucosa, which predisposes it to developing CRC. The association between CRC tumorigenesis and inflammation is demonstrated by the increased risk of CRC in patients with extensive and long-term colitis.¹² A number of proinflammatory factors are activated by the network of CRC signaling,¹³ including TNF α , interleukin (IL)-8, IL-6, and VEGF, which are all found to be elevated in the serum of patients with CRC.¹⁴ Strong evidence identifies transforming growth factor- β (TGF β) as one of the key factors promoting inflammation during tumorigenesis.¹⁵ Interestingly, inflammation and TGF β have also been demonstrated to play an immediate role in the fibrotic response to cutaneous injury, and TGF β expression is significantly elevated in the fibroblasts present in keloid and hypertrophic scars.¹⁶ It is highly likely, therefore, that the observed upregulation of CRC metastasis signaling in fetal fibroblasts at E18 compared with E14 is associated with increased levels of proinflammatory factors, including TGF β , which ultimately contribute to scar formation. Consequently, targeted inhibition of one or more proinflammatory factors upregulated in scar-forming fetal fibroblasts could significantly improve the wound repair process in adult cutaneous wounds.

Hepatic fibrosis/hepatic stellate cell activation

A significant upregulation of hepatic fibrosis/hepatic stellate cell activation pathway in fetal fibroblasts at E18 compared with E14 was found.

Table 2. Genes upregulated in E14 fibroblasts

<i>Symbol</i>	<i>Gene Name</i>	<i>Symbol</i>	<i>Gene Name</i>
Lpar4	Lysophosphatidic acid receptor 4	Pex19	Peroxisomal biogenesis factor 19
Ankrd17	Ankyrin repeat domain 17	Pikfyve	Phosphoinositide kinase, FYVE finger containing
C330019G07Rik	RIKEN cDNA C330019G07 gene	Plac9	Placenta specific 9
Cstf2t	Cleavage stimulation factor, 3' pre-RNA subunit 2, tau	Dstyk	Dual serine/threonine and tyrosine protein kinase
Golt1b	Golgi transport 1 homologue B (<i>S. cerevisiae</i>)	Fcgbp	Fc fragment of IgG binding protein
Adprh	ADP-ribosylarginine hydrolase	Pdgfa	Platelet-derived growth factor, alpha
lfrg15	Interferon alpha responsive gene	Txndc16	Thioredoxin domain containing 16
4632428 N05Rik	RIKEN cDNA 4632428 N05 gene	Gatm	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)
Slc35a1	Solute carrier family 35 (CMP-sialic acid transporter), member 1	Foxn2	Forkhead box N2
Gsk3b	Glycogen synthase kinase 3 beta	Mrpl49	Mitochondrial ribosomal protein L49
Mepce	Methylphosphate capping enzyme	Rbm34	RNA binding motif protein 34
Ficd	FIC domain containing	Bfar	Bifunctional apoptosis regulator
Ptges	Prostaglandin E synthase	Mdh2	Malate dehydrogenase 2, NAD (mitochondrial)
BC016495	cDNA sequence BC016495	Wrnp1	Werner helicase interacting protein 1
Myo10	Myosin X	Thap7	THAP domain containing 7
Pbk	PDZ binding kinase	Map1lc3b	Microtubule-associated protein 1 light chain 3 beta
Tex19.1	Testis expressed gene 19.1	Hoxb2	Homeobox B2
Mocs1	Molybdenum cofactor synthesis 1	Mphosph10	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)
Snap25	Synaptosomal-associated protein 25	Cyba	Cytochrome b-245, alpha polypeptide
Adam10	A disintegrin and metallopeptidase domain 10	Slc9a6	Solute carrier family 9 (sodium/hydrogen exchanger), member 6
Idh3a	Isocitrate dehydrogenase 3 (NAD+) alpha	Pepd	Peptidase D
Phtf2	Putative homeodomain transcription factor 2	Tmsb15l	Thymosin beta 15b like
Hist1 h2ae	Histone cluster 1, H2ae	Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma
Stmn3	Stathmin-like 3	Cct6a	Chaperonin containing Tcp1, subunit 6a (zeta)
Ift74	Intraflagellar transport 74 homologue (Chlamydomonas)	Smurf2	SMAD-specific E3 ubiquitin protein ligase 2
Mettl21a	Methyltransferase-like 21A	Nol4	Nucleolar protein 4
Il2	Interleukin 2	Ppp1r2	Protein phosphatase 1, regulatory (inhibitor) subunit 2
Ppp3cc	Protein phosphatase 3, catalytic subunit, gamma isoform	4833439 L19Rik	RIKEN cDNA 4833439 L19 gene
Ndufa13	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a
Gzmb	Granzyme B	Spire1	Spire homologue 1 (Drosophila)
Ccr5	Chemokine (C-C motif) receptor 5	Acadslb	Acyl-coenzyme A dehydrogenase, short/branched chain
Mbd1	Methyl-CpG binding domain protein 1	Stat1	Signal transducer and activator of transcription 1
Atp7a	ATPase, Cu++ transporting, alpha polypeptide	Notch4	Notch gene homologue 4 (Drosophila)
Psmc2	Proteasome (prosome, macropain) 26S subunit, ATPase 2	Cdc42ep5	CDC42 effector protein (Rho GTPase binding) 5
Cenph	Centromere protein H	Coro1a	Coronin, actin binding protein 1A
Mllt3	Translocated to, 3	Map2k2	Mitogen-activated protein kinase kinase 2
Zfp868	Zinc finger protein 868	Wnt4	Wingless-related MMTV integration site 4
4930528F23Rik	RIKEN cDNA 4930528F23 gene	Agpat3	1-acylglycerol-3-phosphate O-acyltransferase 3
Khdc1b	KH domain containing 1B	Sh3d19	SH3 domain protein D19
Tm4sf4	Transmembrane 4 superfamily member 4	Wdr54	WD repeat domain 54
Mcm5	Minichromosome maintenance-deficient 5, cell division cycle 46 (<i>S. cerevisiae</i>)	Adal	Adenosine deaminase-like
Fank1	Fibronectin type 3 and ankyrin repeat domains 1	Rpl19	Ribosomal protein L19
Vegfc	Vascular endothelial growth factor C	Aasdhpt	Amino adipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase
Parp16	Poly (ADP-ribose) polymerase family, member 16	Cdh3	Cadherin 3
Ubxn8	UBX domain protein 8	Rsph9	Radial spoke head 9 homologue (Chlamydomonas)
Dll1	Delta-like 1 (Drosophila)	Hao2	Hydroxyacid oxidase 2
Eef1b2	Eukaryotic translation elongation factor 1 beta 2	Syf2	SYF2 homologue, RNA splicing factor (<i>S. cerevisiae</i>)
Abhd14a	Abhydrolase domain containing 14A	Pcp4l1	Purkinje cell protein 4-like 1
Ergic2	ERGIC and golgi 2	Appl1	Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1
Josd2	Josephin domain containing 2	Cdk5rap3	CDK5 regulatory subunit associated protein 3
Cyth1	Cytohesin 1	Tmem41b	Transmembrane protein 41B
Atp4a	ATPase, H+/K+ exchanging, gastric, alpha polypeptide	G3bp2	GTPase activating protein (SH3 domain) binding protein 2
Unkl	Unkempt-like (Drosophila)	Slc35b2	Solute carrier family 35, member B2
Ndutfaf1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1	Lmbrd1	LMBR1 domain containing 1
Fam57a	Family with sequence similarity 57, member A	Uck1l	Uridine-cytidine kinase 1-like 1
Prkag1	Protein kinase, AMP-activated, gamma 1 noncatalytic subunit	Scnm1	Sodium channel modifier 1

(continued)

Table 2. (Continued)

Symbol	Gene Name	Symbol	Gene Name
Phip	Pleckstrin homology domain interacting protein	Hspbp1	HSPA (heat shock 70 kDa) binding protein, cytoplasmic cochaperone 1
Akap13	A kinase (PRKA) anchor protein 13	1810009J06Rik	RIKEN cDNA 1810009J06 gene
Odz3	Odd Oz/ten-m homologue 3 (Drosophila)	Ilk	Integrin linked kinase
Lrat	Lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)	Cyp26a1	cytochrome P450, family 26, subfamily a, polypeptide 1
Fam20c	Family with sequence similarity 20, member C	Fdx1l	Ferredoxin 1-like
Zfp821	Zinc finger protein 821	Ndufa11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11
8030474K03Rik	RIKEN cDNA 8030474K03 gene	Cct8l1	Chaperonin containing TCP1, subunit 8 (theta)-like 1
Dock7	Dedicator of cytokinesis 7	Rbbp6	Retinoblastoma binding protein 6
Rab3a	RAB3A, member RAS oncogene family	Nppb	Natriuretic peptide type B
5430437P03Rik	RIKEN cDNA 5430437P03 gene	Ppat	Phosphoribosyl pyrophosphate amidotransferase
Lysmd4	LysM, putative peptidoglycan-binding, domain containing 4	Lrch2	Leucine-rich repeats and calponin homology (CH) domain containing 2
Zchc3	Zinc finger, CCHC domain containing 3	Paqr5	Progesterin and adipoQ receptor family member V
Ucp1	Uncoupling protein 1 (mitochondrial, proton carrier)	Csnk2b	Casein kinase 2, beta polypeptide
Ckap2l	Cytoskeleton-associated protein 2-like	Egf	Epidermal growth factor
Atp7a	ATPase, Cu++ transporting, alpha polypeptide	Acs16	Acyl-CoA synthetase long-chain family member 6
BC016423	cDNA sequence BC016423	Gosr2	Golgi SNAP receptor complex member 2
Tipin	Timeless interacting protein	Myo16	Myosin XVI
Cnot1	CCR4-NOT transcription complex, subunit 1	Sult1d1	Sulfotransferase family 1D, member 1
Al314180	Expressed sequence Al314180	Setd3	SET domain containing 3
Hspb7	Heat shock protein family, member 7 (cardiovascular)	Slc30a4	Solute carrier family 30 (zinc transporter), member 4
Prpsap1	Phosphoribosyl pyrophosphate synthetase-associated protein 1	Plekfh2	Pleckstrin homology domain containing, family F (with FYVE domain) member 2
Sft2d1	SFT2 domain containing 1	Tgif2	TGFB-induced factor homeobox 2
Crym	Crystallin, mu	Dynll2	Dynein light chain LC8-type 2
Edn2	Endothelin 2	Ccs	Copper chaperone for superoxide dismutase
Senp6	SUMO/sentrin-specific peptidase 6	Car10	Carbonic anhydrase 10
Mbd4	Methyl-CpG binding domain protein 4	5-Sep	Septin 6
Rpl3	Ribosomal protein L3	Mycbp2	MYC binding protein 2
1700001J03Rik	RIKEN cDNA 1700001J03 gene	Vps41	Vacuolar protein sorting 41 (yeast)
Angptl1	Angiopoietin-like 1	Lefty1	Left right determination factor 1
Camsap1	Calmodulin-regulated spectrin-associated protein 1	Dcbl1	Discoidin, CUB and LCCL domain containing 1
Dhx16	DEAH (Asp-Glu-Ala-His) box polypeptide 16	Mpp1	Membrane protein, palmitoylated
1190007F08Rik	RIKEN cDNA 1190007F08 gene	Kcnab3	Potassium voltage-gated channel, shaker-related subfamily, beta member 3
Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	Stoml2	Stomatin (Epb7.2)-like 2
Fam195b	Family with sequence similarity 195, member B	Steap3	STEAP family member 3
Muc2	Mucin 2	Tspan31	Tetraspanin 31
Stat6	Signal transducer and activator of transcription 6	Ankrd1	Ankyrin repeat domain 1 (cardiac muscle)
Med19	Mediator of RNA polymerase II transcription, subunit 19 homologue (yeast)	Ormdl1	ORM1-like 1 (<i>S. cerevisiae</i>)
Gas5	Growth arrest-specific 5	1110008F13Rik	RIKEN cDNA 1110008F13 gene
Tbl2	Transducin (beta)-like 2	Oma1	OMA1 homologue, zinc metallopeptidase (<i>S. cerevisiae</i>)
Ehd3	EH-domain containing 3	Cda	Cytidine deaminase
Cntln	Centlein, centrosomal protein	Slc6a1	Solute carrier family 6 (neurotransmitter transporter, GABA), member 1
Myh8	Myosin, heavy polypeptide 8, skeletal muscle, perinatal	Ccdc17	Coiled-coil domain containing 17
Srp14	Signal recognition particle 14	Lsm6	LSM6 homologue, U6 small nuclear RNA associated (<i>S. cerevisiae</i>)
Asnsd1	Asparagine synthetase domain containing 1	D2Erd750e	DNA segment, Chr 2, ERATO Doi 750, expressed
Zfp692	Zinc finger protein 692	Tgm1	Transglutaminase 1, K polypeptide
Fam187b	Family with sequence similarity 187, member B	Galnt15	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetyl-galactosaminyltransferase-like 5
Efna2	Ephrin A2	Mobkl2a	MOB1, Mps one binder kinase activator-like 2A (yeast)
Prpf40a	PRP40 pre-mRNA processing factor 40 homologue A (yeast)	Il10ra	Interleukin 10 receptor, alpha
Zyx	Zyxin	Pja1	Praja1, RING-H2 motif containing
Calb1	Calbindin 1	Cd83	CD83 antigen
Asb1	Ankyrin repeat and SOCS box-containing 1	Stra13	Stimulated by retinoic acid 13
Zfp105	Zinc finger protein 105	1700028P14Rik	RIKEN cDNA 1700028P14 gene
Trmt2a	TRM2 tRNA methyltransferase 2 homologue A (<i>S. cerevisiae</i>)	1700012B15Rik	RIKEN cDNA 1700012B15 gene

(continued)

Table 2. (Continued)

Symbol	Gene Name	Symbol	Gene Name
Celf4	CUGBP, Elav-like family member 4	Chid1	Chitinase domain containing 1
1110034A24Rik	RIKEN cDNA 1110034A24 gene	Chgb	Chromogranin B
Acdb5	Acyl-Coenzyme A binding domain containing 5	Kctd14	Potassium channel tetramerization domain containing 14
Gtf3c6	General transcription factor IIIC, polypeptide 6, alpha	Rab11fip1	RAB11 family interacting protein 1 (class I)
Myc	Myelocytomatosis oncogene	Aldh3b1	Aldehyde dehydrogenase 3 family, member B1
Rrp12	Ribosomal RNA processing 12 homologue (<i>S. cerevisiae</i>)	Tpd52l1	Tumor protein D52-like 1
Mapk10	Mitogen-activated protein kinase 10	2210012G02Rik	RIKEN cDNA 2210012G02 gene
Pde6d	Phosphodiesterase 6D, cGMP-specific, rod, delta	Tspan3	Tetraspanin 3
Mtus2	Microtubule-associated tumor suppressor candidate 2	Rbm39	RNA binding motif protein 39
Pon1	Paraoxonase 1	Bin1	Bridging integrator 1
Sfxn1	Sideroflexin 1	Smc3	Structural maintenance of chromosomes 3
Pak2	p21 protein (Cdc42/Rac)-activated kinase 2	Dnttip1	Deoxynucleotidyltransferase, terminal, interacting protein 1
Etv6	ETS variant gene 6 (TEL oncogene)	Dcaf4	DDB1- and CUL4-associated factor 4

This pathway involves activation of the hepatic stellate cell following liver injury, the principal effector of hepatic fibrogenesis, which becomes highly proliferative and synthesizes a fibrotic matrix that is rich in type I collagen.¹⁷ Hepatic fibrosis can arise secondary to a number of factors causing liver injury, including viral hepatitis or alcohol abuse. Regardless of the precise etiology, hepatic fibrosis is ultimately characterized by an increase in ECM deposition and formation of a hepatic scar. Immediately following liver injury, there is a marked transition of hepatic stellate cells from a quiescent to a robust, activated state. This response is elicited by neighboring Kupffer, endothelial, and injured hepatocyte cells, which release potent reactive oxygen intermediates (ROI) that exert paracrine stimulation of stellate cells.¹⁸ Excessive accumulation of reactive oxygen species (ROS) in wounds significantly impairs wound healing and causes substantial tissue damage.¹⁹ Our findings here suggest a differential stellate cell activation pathway between the E14 and E18 time points in fetal fibroblasts, and this could be partially caused by increased ROS in the wound bed at E18. Accordingly, methods that eliminate accumulated ROS in wound beds may enhance the regenerative capability of skin.

In addition to activating stellate cells, endothelial cells also activate plasmin. Plasmin stimulates the conversion of latent TGF β 1 to an active fibrogenic form.²⁰ TGF β , as mentioned, plays a major role in the fibrotic response to injury and the formation of scars. Therefore, targeted inhibition of the conversion of TGF β 1 into its more active fibrogenic form will likely enhance the regenerative properties of skin once beyond the E14 time point.

PDGF signaling

Our microarray analysis demonstrated significantly increased expression of PDGF in E18 com-

pared with E14 fetal fibroblasts. Higher levels of PDGF have been documented both in adult wounds compared with embryonic wounds, and in uninjured adult skin compared with uninjured embryonic skin.^{21,22} No previous work has specifically compared the differential activation of PDGF signaling between early scar-free fetal skin and later scar-forming fetal skin. The functional pathway analysis here suggests that increased PDGF signaling contributes to the loss of the regenerative healing capability between the E14 and E18 time points. This finding is further supported by the role of PDGF in mediating proliferation and differentiation of fibroblasts. PDGF expression also leads to subsequent upregulation of TGF β 1 receptors, which have a major role in the fibrotic response to tissue injury.

Putrescine biosynthesis III

Microarray analyses showed greater downregulation of putrescine biosynthesis in E18 compared with E14 fetal fibroblasts. Putrescine has been identified to play a significant role in cell proliferation in response to injury, and is required for the completion of DNA synthesis.²³ Putrescine is also suggested to be an integral precursor for the development of complex polyamines, which are major regulatory factors of mammalian tissues and influence both growth and signal transduction.²⁴ The formation of new tissue, through cellular proliferation and migration of cells to the wound site, is a key part of wound healing. Downregulation of the putrescine biosynthesis III pathway at E18 may impair signaling, impact the regulation of cellular proliferation, and ultimately increase the amount of new tissue formed, leading to the formation of a scar. Further exploration of the putrescine biosynthesis III pathway could reveal a target for drug therapy that could potentially rescue regenerative capability at scar-forming time points.

Figure 1. Microarray analysis of E14 and E18 fibroblasts. **(A)** Hierarchical clustering of upregulated and downregulated genes from fetal fibroblasts at E14 versus E18. Individual genes cluster according to the dendrogram on the left and are visually represented in the heat map on the right; blue indicates downregulation and yellow indicates upregulation. **(B)** Canonical pathways identified via IPA that were significantly enriched for among genes observed to be substantially upregulated. **(C)** Canonical pathways identified via IPA that were significantly enriched for among genes observed to be substantially downregulated. E14, embryonic day 14; E18, embryonic day 18; IPA, Ingenuity Pathway Analysis.

D-myo-inositol (3,4,5,6)-tetrakisphosphate biosynthesis

Microarray analysis revealed that d-myo-inositol (3,4,5,6)-tetrakisphosphate biosynthesis was downregulated in E18 compared with E14 fetal fibroblasts. D-myo-inositol (3,4,5,6)-tetrakisphosphate is part of the inositol phosphate family, which are intracellular signaling molecules regulating the crucial functions of cell growth, apoptosis, and cell differentiation. Previous work has tentatively identified the target of d-myo-inositol (3,4,5,6)-tetrakisphosphate as a plasma membrane Ca^{2+} -activated chloride channel.²⁵ Interestingly, blockage of chloride channels severely reduces the rate of wound healing.²⁶ Downregulation of chloride channel function at E18 may reduce healing capability in fetal wounds and prolong the formation of new tissue and scars. D-myo-inositol (3,4,5,6)-tetrakisphosphate is also involved in mediating the

ability of remodeling complexes to induce transcription in phosphate-responsive genes.²⁷ This finding suggests that reduction in d-myo-inositol (3,4,5,6)-tetrakisphosphate activity may contribute to epigenetic silencing and consequently the loss of regenerative healing capability observed at E18. Further work with d-myo-inositol (3,4,5,6)-tetrakisphosphate biosynthesis could reveal a novel target to induce regenerative healing mechanisms.

INNOVATION

Previous work has identified the key time point during fetal development when the ability to heal without scar is lost. However, the mechanism driving the transition from scarless, regenerative fetal wound healing to scar-forming adult wound repair is still largely unknown. Our work significantly narrows the search to a set of key genes and

KEY FINDINGS

- Thirty genes were significantly downregulated in E18 fetal fibroblasts compared with E14 fetal fibroblasts.
- Enrichment analysis revealed over 20 pathways were downregulated in E18 fetal fibroblasts compared with E14 fetal fibroblasts, including putrescine biosynthesis III, s-adenosyl-L-methionine biosynthesis, d-myo-inositol (1,4,5,6)-tetrakisphosphate biosynthesis, d-myo-inositol (3,4,5,6)-tetrakisphosphate biosynthesis, and dolichyl-diphosphooligosaccharide biosynthesis.
- Two hundred forty-five genes were significantly upregulated in E18 fetal fibroblasts compared with E14 fetal fibroblasts.
- Enrichment analysis revealed over 20 pathways upregulated in E18 fetal fibroblasts compared with E14 fetal fibroblasts, including CRC metastasis signaling, hepatic fibrosis/hepatic stellate cell activation, pancreatic adenocarcinoma signaling, PDGF signaling, and endometrial cancer signaling.

pathways that drive the scar-free healing mechanism. Further detailed examination of these genes and signaling pathways will give us a better understanding of the mechanism behind scarless wound healing and potentially identify targets for future therapies.

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ABOUT THE AUTHORS

Michael S. Hu, MD, MPH, MS, is a postdoctoral fellow at Stanford interested in stem cell biology and regenerative medicine. He is pursuing a career in craniofacial plastic surgery. **Mimi R. Borrelli, MBBS, MSs, BSc**, is a postdoctoral scholar at Stanford. **Michael Januszyk, MD, PhD**, is a resident in plastic surgery at UCLA. **Anna Luan, MD, MS**, is a resident in plastic surgery at Stanford. **Samir Malhotra, BS**, is a premedical undergraduate student at Stanford. **Graham G. Walmsley, MD, PhD**, is a stem cell scientist working in venture capital. **Wan Xing Hong, MD**, is a resident in general surgery at Stanford. **Ruth Terlin, MB, BCh, BAO, MRCSI, MD**, is a resident in plastic surgery at Stanford. **Geoffrey C. Gurtner, MD**, is a Professor of Surgery and Vice Chairman for Research in the Department of Surgery at Stanford. **Michael T. Longaker, MD, MBA**, is a Professor of Surgery and Bioengineering at Stanford. He is the Director of Research for the Program in Regenerative Medicine, Children's Surgical Research, and Division of Plastic and Reconstructive Surgery. His extensive research experience includes wound healing, tissue engineering, and developmental/stem cell biology. **H. Peter Lorenz, MD**, is a Professor and Chief of Plastic Surgery at the Lucile Packard Children's Hospital at Stanford. His clinical interests are in craniofacial surgery, pediatric plastic surgery, and reconstructive and cosmetic surgery. His laboratory group is studying mechanisms underlying scarless skin healing and the function of progenitor cells during wound repair/regeneration.

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Abbreviations and Acronyms

aRNA	= antisense RNA
cDNA	= complementary DNA
CRC	= colorectal cancer
dUTP	= deoxyribose uridine triphosphate
E14	= embryonic day 14
E18	= embryonic day 18
ECM	= extracellular matrix
EDTA	= ethylenediaminetetraacetic acid
FDR	= false discovery rate
IL	= interleukin
IPA	= Ingenuity Pathway Analysis
NIH	= National Institutes of Health
PDGF	= platelet-derived growth factor
ROI	= reactive oxygen intermediates
ROS	= reactive oxygen species
SAM	= significance analysis of microarrays
SDS	= sodium dodecyl sulfate
SSC	= saline sodium citrate
TE	= Tris-EDTA
TGF β	= transforming growth factor- β