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Novel Differences in the Expression of Inflammation-Associated Genes Between Mid- and Late-Gestational Dermal Fibroblasts

Brian C. Wulff, Ph.D.¹, Lianbo Yu, Ph.D.², Allison E. Parent, B.S.¹, and Traci A. Wilgus, Ph.D.¹

¹Department of Pathology, The Ohio State University, Columbus, Ohio, USA

²Center for Biostatistics, The Ohio State University, Columbus, Ohio, USA

Abstract

While cutaneous wounds of late-gestational fetuses and on through adulthood result in scar formation, wounds incurred early in gestation have been shown to heal scarlessly. Unique properties of fetal fibroblasts are believed to mediate this scarless healing process. In this study, microarray analysis was used to identify differences in the gene expression profiles of cultured fibroblasts from embryonic day 15 (E15; mid-gestation) and embryonic day 18 (E18; late-gestation) skin. Sixty-two genes were differentially expressed and twelve of those genes are associated with inflammation, a process that correlates with scar formation in fetal wounds. One of the differentially expressed inflammatory genes was cyclooxygenase-1 (COX-1). COX-1 was more highly expressed in E18 fibroblasts than in E15 fibroblasts, and these differences were confirmed at the gene and protein level. Differences in COX-1 protein expression were also observed in fetal skin by immunohistochemical and immunofluorescent staining. The baseline differences in gene expression found in mid- and late-gestational fetal fibroblasts suggest that developmental alterations in fibroblasts could be involved in the transition from scarless to fibrotic fetal wound healing. Furthermore, baseline differences in the expression of inflammatory genes by fibroblasts in E15 and E18 skin may contribute to inflammation and scar formation late in gestation.

Keywords

Fetal; scar; scarless; microarray; skin

Introduction

The response to injury is a complicated and highly regulated cascade of events that quickly restores homeostasis after injury; however, this process results in nonfunctional scar tissue that can have detrimental effects. Tissue repair after injury to normal adult skin can be described as having three phases: inflammation, proliferation, and scar formation/remodeling. In contrast to adult wound healing, fetal skin wounds heal without scarring and do not mount an inflammatory response after injury until late in gestation (1, 2)

Many studies have shown that stimulating inflammation enhances the extent of scarring in fetal wounds, and a more substantial inflammatory response to injury is seen in late-gestational fetal skin that heals with a scar. Several pro-inflammatory mediators are produced at higher levels in fibrotic wounds, and exposing wounds that would otherwise

heal scarlessly to inflammatory stimuli results in scar formation. These molecules include interleukin-6, interleukin-8, transforming growth factor- β (TGF- β), platelet-derived growth factor, and prostaglandins (3–7). There are also gestational differences in the number and/or activity of several cell types which contribute to inflammation, including endothelial cells which aid in inflammatory cell trafficking, as well as platelets, neutrophils, macrophages, and mast cells (8–12).

In addition to differences in inflammation, changes in the behavior of fibroblasts have been suggested to play a role in regulating scar formation in fetal wounds. Because fibroblasts produce and maintain the extracellular matrix and are responsible for producing collagen, these cells are a key target in understanding the biology of scarring. Studies by Lorenz *et al.* showed that fetal fibroblasts differ from adult fibroblasts in their ability to mediate scarless repair (13). A model was used in which human fetal skin healed with a scar when grafted cutaneously onto nude mice, but healed scarlessly when grafted subcutaneously. Using species-specific antibodies to detect human and mouse collagen, they showed that adult murine fibroblasts were responsible for producing scar tissue in normal grafts, but fetal human fibroblasts produced normal collagen in the scarless subcutaneous grafts (13). Studies have also demonstrated differences between fibroblasts from skin that heals with or without scarring (14–21). In the current study we cultured fibroblasts from embryonic day 15 (E15) and embryonic day 18 (E18) skin, ages that correspond to scarless and fibrotic healing phenotypes, respectively (7, 22). To better understand the mechanisms involved in scarless repair, microarray analysis was used to compare the baseline transcriptomes of E15 and E18 fibroblasts. Differences in genes associated with proliferation, cell signaling, and cell-cell interactions as well as the inflammatory response were identified.

Materials and Methods

Generation of fetal skin

FVB mice were purchased from Taconic Farms (Germantown, NY) and Col3.6-GFP^{tpz} mice, which express green fluorescent protein (GFP) under the control of the collagen 1a1 (col 1a1) promoter, were obtained from Dr. David Rowe (University of Connecticut). Protocols approved by the Ohio State University Institutional Animal Care and Use Committee were followed for all animal studies. Dorsal fetal skin was collected at E15 or E18 from time-mated mice as described previously (12). These time points were chosen based on earlier studies indicating that wounds made at E15 heal without scarring while those made at E18 heal with a scar (7). Skin samples were used for cell culture as described below or fixed in 10% formalin. Formalin-fixed tissue was embedded in paraffin for standard immunohistochemistry or incubated in phosphate buffered saline (PBS) with 30% sucrose (Fisher Scientific, Fair Lawn, NJ) for 24 hours then frozen in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) for immunofluorescence.

Cell culture

Freshly harvested E15 or E18 dorsal skin was cut into small pieces and allowed to adhere, dermal side down, to the bottom of a 6-well tissue culture plate. Skin from individual fetuses was cultured in separate wells. Dulbecco's Modified Eagles Medium (high glucose) supplemented with non-essential amino acids, sodium pyruvate, L-glutamine, penicillin/streptomycin, and 20% fetal bovine serum (all reagents from Invitrogen, Grand Island, NY) was added as described previously (7). The fibroblasts were allowed to grow for 5 days at 37°C and 5% carbon dioxide, during which time the culture medium was changed daily (passage 1). On day 5, the tissue pieces were removed using sterile forceps. Adherent cells were trypsinized and transferred to a T75 flask for 24 hours (passage 2). These cells were then lysed either in RIPA (radioimmunoprecipitation assay) lysis buffer (Thermo Scientific,

Rockford, IL) for protein isolation or Trizol (Invitrogen) for RNA isolation. After isolation, RNA from multiple fetuses within the same litter was pooled. A total of three pooled samples from independent litters at each time point (E15 and E18) were used. To confirm that the cultured cells were fibroblasts, Western blotting was performed for the intermediate filament vimentin, a commonly used marker of fibroblasts. All fibroblast samples expressed vimentin. Microarray results confirmed that the cells expressed high levels of vimentin, but keratin expression was below the background threshold level (data not shown).

Microarray gene expression analysis

Pooled RNA isolated from cultured E15 or E18 fetal fibroblasts by Trizol (Invitrogen) extraction was treated with DNase (Qiagen Sciences, Germantown, MD) and purified with the RNeasy kit (Qiagen) according to manufacturers' instructions. RNA quality and concentration were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and Nanodrop (Nanodrop technologies, Wilmington, DE), respectively. Single stranded complementary DNA (cDNA) was generated using the Ambion WT expression kit (Affymetrix, Santa Clara, CA). cDNA was then fragmented and labeled with the Ambion WT terminal labeling kit (Affymetrix). Labeled cDNA was hybridized to GeneChip Mouse Gene 1.0 ST Array chips (Affymetrix) for 16 hours at 45° C. Chips were scanned with the GeneChip Scanner 3000 7G system. Signal intensities were quantified using Affymetrix software. Background correction and quantile normalization was performed to adjust for technical bias, and gene expression levels were summarized using the Robust Multichip Average method (23). A filtering method based on percentage of samples above noise cutoff was applied to filter out low expression genes (this included a minimum of two genes with gene expression levels above six). A linear model was employed to detect differentially expressed genes. In order to improve the estimates of expression variability and statistical tests for differential expression, a moderate t-statistic with variance smoothing was employed for this study (24). The significance level was set at 0.05. A volcano plot of log2 fold and log10 p-value and heatmap with hierarchical clustering dendrogram using Euclidean distance and average linkage were prepared to visualize the test results and the expression data.

Real-Time reverse transcription-polymerase chain reaction (RT-PCR) Gene Expression Assays

Taqman (Applied Biosystems Inc, Foster City, CA) gene expression assays were used to confirm gene expression differences for cyclooxygenase-1 (COX-1), cluster of differentiation 109 (CD109), and suppressor of cytokine signaling 2 (SOCS2) by real-time RT-PCR. All reagents, primers, and probes (pre-designed, pre-optimized) were obtained from Applied Biosystems (Applied Biosystems). Three hundred ng of total RNA was used for each reverse transcription reaction. All reverse transcription reactions, including no-template controls and controls without reverse transcriptase, were prepared using the high capacity RNA to cDNA kit (Applied Biosystems) and run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems). Gene expression levels were quantified using the ABI Prism 7900HT Sequence detection system (Applied Biosystems). Comparative real-time RT-PCR was performed in triplicate, including no-template controls. The housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used for normalization. Relative expression was calculated using the comparative Ct method.

Western blot analysis

COX-1 protein levels were determined by Western blot using standard methods. Total protein (20 µg) was separated on 12% sodium dodecyl sulfate-polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were cut at the 50 kilo-Dalton mark, blocked with 5% milk in tris

buffered saline with 0.1% tween 20 (TBST), and antibodies were used to probe for COX-1 (Cayman Chemical, Ann Arbor, MI) or β -actin (Cell Signaling Technology, Danvers, MA) overnight at 4°C. The membrane was washed in TBST and incubated for 1 hour with horse radish peroxidase-conjugated goat anti-rabbit antibody (Cell Signaling). Proteins were visualized using Immun-Star Western C chemiluminescence reagents (Bio-Rad) and digital images were captured using the Chemidoc XRS imaging system (Bio-Rad). Densitometry was performed using Quantity One software (Bio-Rad) and data are presented as a ratio of the density of the bands for COX-1 to β -actin.

Immunohistochemical staining for COX-1 in fetal skin

Formalin fixed, paraffin embedded tissue sections (4 μ m) were deparaffinized in Clear-rite 3 (Richard-Allan Scientific, Kalamazoo, MI) and rehydrated in a series of ethanol washes ending in phosphate buffered saline (PBS). Antigen unmasking was performed by steaming for 10 minutes in Dako Target Retrieval Solution (DakoCytomation, Carpinteria, CA) followed by 10 minutes of cooling at room temperature. Endogenous peroxidase activity was blocked by incubation for 15 minutes with 0.3% hydrogen peroxide in methanol. The tissue was blocked with 10% normal goat serum for 30 minutes. Anti-COX-1 antibody (Cayman Chemical) was allowed to react with the tissue overnight at 4°C. Sections were washed with PBS and a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) was added for 30 minutes. After washing in PBS, avidin-biotin-horseradish peroxidase complex (ABC-HRP kit; Vector Laboratories) was allowed to bind for 30 minutes and colorimetric development was performed by reaction with 3,3'-diaminobenzidine for 10 minutes. Samples were counterstained briefly with hematoxylin then dehydrated in a series of ethanol washes ending in Clear-rite 3 (Richard-Allan). Sections were coverslipped with Permount (Fisher Scientific) and digital pictures were taken with a Zeiss AxioCam digital camera (Carl Zeiss, Thornwood, NY) mounted on an Axioscope 40 microscope (Carl Zeiss).

Immunofluorescent staining for COX-1 in dermal fibroblasts

Cryosections (10 μ m) of formalin-fixed tissue were placed in PBS. Non-specific antibody binding was blocked with 10% normal goat serum for 30 minutes. Anti-COX-1 antibody (Cayman Chemical) was allowed to react with the tissue overnight at 4°C. Sections were washed with PBS and Alexa fluor 594 conjugated goat anti-rabbit antibodies (Life Technologies, Eugene, OR) was added for 30 minutes. Samples were washed in PBS then incubated for 30 minutes at room temperature in PBS containing 1 mM magnesium chloride (Sigma, St. Louis, MO). Samples were counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen) then coverslipped using Prolong Gold (Invitrogen). Digital images were taken on an Olympus FV1000 spectral confocal microscope (Olympus America Inc., Melville, NY).

Results

Microarray analysis of gene expression in cultured fetal fibroblasts

Affymetrix GeneChip mouse gene 1.0 ST arrays were used to analyze the expression profile of 28,853 genes in cultured E15 and E18 dermal fibroblasts. Using a cutoff threshold of a 1.5-fold difference in mean $\log_2$ expression or greater and a p-value < 0.05, sixty-two unique genes showed differential expression between E15 and E18 fibroblasts as shown by volcano plot (FIG. 1). Of these genes, twenty-two were up-regulated in E18 fibroblasts compared to E15 fibroblasts (TABLE 1), while forty genes were down-regulated in E18 fibroblasts compared to E15 fibroblasts (TABLE 2). Hierarchical clustering of genes with similar expression levels and clustering of samples can be seen in the heatmap shown (FIG.

- 2). Clustering distinctly stratified E15 fibroblast samples from E18 fibroblast samples (FIG. 2).

Functional grouping of differentially expressed genes

The Ingenuity Pathways Knowledge Database was used to categorize differentially expressed genes into functional groups. The top five “molecular and cellular function” groups were: cellular growth and proliferation, cellular movement, cell-to-cell signaling and interaction, cell signaling, and small molecule biochemistry (TABLE 3). Interestingly, many of the genes (twenty-four) were included in two or more of these functional groups. Five of these genes were in all five functional groups. Examination of the “diseases and disorders” functional groups showed that many of the differentially expressed genes fell into the inflammatory response category. The twelve differentially expressed genes involved in the inflammatory response were: Rho GDP dissociation inhibitor (GDI) beta, caveolin 1, cluster of differentiation 59a, gremlin 1, interleukin 11, lysozyme 2, cyclooxygenase-1, S100 calcium binding protein A4, serine peptidase inhibitor clade G member 1, suppressor of cytokine signaling 2, thymus cell antigen 1 theta, and thrombomodulin. The other top “diseases and disorders” functional groups were not as relevant to our model and included: cancer, cardiovascular disease, renal/urological disease, and gastrointestinal disease. Since the inflammatory response was one of the most significant groups highlighted by the Ingenuity Pathways Knowledge Database and inflammation regulates healing outcomes in fetal wounds, further examination focused on inflammatory genes.

Real-time RT-PCR confirmation of selected genes

Differential gene expression was confirmed by real-time RT-PCR for three genes of interest: COX-1, SOCS2, and CD109 (FIG. 3). All three genes have been implicated in inflammation, which is known to be involved in the transition from scarless (E15 wound) to fibrotic (E18 wound) healing in fetal skin. Significant differences in mRNA expression were detected for all three genes, confirming the differences in gene expression seen by microarray analysis. Both CD109 and SOCS2, which have been shown to have anti-inflammatory roles, were down-regulated in E18 fibroblasts compared to E15 fibroblasts. Conversely, the pro-inflammatory prostaglandin-producing enzyme COX-1 was up-regulated in E18 fibroblasts, consistent with the increased inflammation seen in scar-forming E18 wounds.

Analysis of COX-1 protein expression in fetal fibroblasts and fetal skin

Based on the documented importance of the cyclooxygenase enzymes and their prostaglandin products in wound healing and scar formation, further analysis was focused on COX-1. Western blot analysis showed that COX-1 protein levels were higher in cultured E18 fibroblasts compared to E15 fibroblasts (FIG. 4), confirming the differences seen in mRNA levels by microarray and real-time RT-PCR. Immunohistochemical and immunofluorescent staining for COX-1 was also performed in normal E15 and E18 skin (FIG. 5). Overall, more COX-1 staining was seen in E18 skin compared to E15 skin by immunohistochemistry. Interestingly, there were striking differences in COX-1 staining in the epidermal layer. Minimal COX-1 staining was seen in interfollicular epidermal keratinocytes in E15 skin, whereas keratinocytes in E18 skin displayed intense staining for COX-1. Positive staining was apparent in dermal cells and in endothelial cells lining blood vessels in both E15 and E18 skin. Using skin from mice expressing GFP under the control of the Col 1a1 promoter (Col-GFP), immunofluorescent staining and confocal microscopy demonstrated the presence of COX-1-positive/GFP-positive dermal fibroblasts *in vivo*.

Discussion

Many studies have shown that inflammation is involved in the transition from scarless to fibrotic healing during skin development (3, 6, 7, 25, 26). In addition to differences in inflammation, changes in intrinsic characteristics of fetal fibroblasts may also be involved in this transition. Important experiments by Lorenz, *et al* suggested that fetal fibroblasts have a unique ability to mediate scarless healing (13). Several studies have also demonstrated that cultured mid-gestational fibroblasts and fibroblasts from more developed skin display basal differences in proliferation and migration rates, contractile ability and gene expression, and that they respond differently when stimulated by mediators such as TGF- β and prostaglandin E₂ (PGE₂) (14–21, 27, 28). Despite these observations, global changes in basal dermal fibroblast gene expression at different stages of fetal skin development have not been assessed. While several other studies have used a global approach to study scarless repair, these studies examined normal skin or wound samples (29–31). Because whole tissue was used, the specific contribution of the fibroblasts to any alterations in gene expression detected could not be determined.

In the current study, microarray analysis was used to compare gene expression profiles of cultured fetal fibroblasts generated from explants of E15 (scarless healing) and E18 (fibrotic healing) murine skin. Skin from these ages have been shown to undergo scarless (E15) and fibrotic (E18) healing (7, 22); therefore, microarray analysis of E15 and E18 fibroblasts was used to screen for differences in basal gene expression that might contribute to scar formation. Cells derived from explant cultures are often used to study dermal fibroblast function and this method offers a way to study fibroblasts separately from other cell types within the skin. However, there are limitations to studying fibroblasts *in vitro*, as they are not likely to behave exactly the same as fibroblasts within the skin *in vivo*. The cells used here were grown on plastic tissue culture plates in media supplemented with 20% serum. Although these conditions are often used for growing fibroblasts from skin explants, there is evidence that the conditions under which fibroblasts are grown, including the substrate used for fibroblast adherence, can alter cellular behavior and gene expression (32). Murine cells were used in these studies because the gestational ages corresponding to scarless and fibrotic healing are well defined in the mouse and murine fetal skin is relatively easy to generate, but the gene expression differences observed here should be confirmed in other animal models and human cells before drawing broad conclusions about the results.

In the present study, sixty-two genes were found to be differentially expressed between E15 and E18 fibroblasts. Although some studies compare fibroblasts from mid-gestation and adult skin to help understand scarless repair, a comparison of fibroblasts from mid-gestation (E15) and late-gestation (E18) fetal skin was used in the present studies. The narrow developmental timeframe examined here may partially explain why a relatively small number of genes were differentially expressed. However, the advantage of comparing cells from fetal skin at two different gestational ages is that all of the skin samples were obtained *in utero*. Therefore, the gene expression differences identified are more likely to be involved in the transition from scarless to fibrotic healing rather than simply being a result of comparing skin from two distinct environmental conditions. Several interesting genes were found to be differentially expressed. For example, tenascin showed higher expression in E15 fibroblasts compared to E18 fibroblasts and this extracellular matrix molecule has been shown previously to be elevated during the rapid reepithelialization of early fetal wounds (33). There were also a number of genes that suggested differences in cell growth and differentiation such as NADH proteins and ATP synthases. Analysis of differentially expressed genes using the Ingenuity Knowledge Database showed that the functional group with the most differentially expressed genes was “cell growth and proliferation” and the majority of those genes were up-regulated in E15 fibroblasts. These results are in line with

previous studies demonstrating that mid-gestational fibroblasts proliferate rapidly in culture (27). Interestingly, the inflammatory response functional group also contained several differentially regulated genes. Further analysis focused on genes from this group due to the demonstrated importance of inflammation in the transition from scarless to fibrotic healing. SOCS2 (34, 35) and CD109 (36, 37) are two genes with anti-inflammatory functions that were up-regulated in E15 fibroblasts compared to E18 fibroblasts. In addition to limiting inflammation, CD109 can also be anti-fibrotic, which may be explained by its ability to alter TGF- β signaling by facilitating TGF- β receptor degradation (38) and/or sequestering TGF- β 1 (39).

Another gene of interest identified by microarray was COX-1, which was up-regulated in E18 fibroblasts. In addition to higher COX-1 levels in cultured E18 fibroblasts and enhanced dermal COX-1 staining in E18 skin, keratinocytes in E18 skin also stained more intensely for COX-1 compared to keratinocytes in E15 skin. Cyclooxygenase enzymes (COX-1 and COX-2) are important during inflammation, as they convert arachidonic acid into pro-inflammatory prostaglandins, such as PGE₂. The COX-2 isoform is induced after skin injury and thus has been the focus of most wound healing studies, including studies by our lab showing that COX-2 is expressed at higher levels in scar-forming fetal wounds and that PGE₂ levels correlate with inflammation and scar formation in both fetal and adult wound healing models (7, 40). However, several studies have suggested that COX-1 also contributes to PGE₂ production during wound healing, as the selective COX-1 inhibitor SC-560 reduces PGE₂ levels in healing wounds (41, 42). Because COX-1 present at the time of wounding can quickly convert arachidonic acid to pro-inflammatory prostaglandins, the increased basal levels of COX-1 observed in fibroblasts and other cells in E18 skin could impact inflammation at early stages after injury.

In the current study, novel differences in gene expression between E15 and E18 fibroblasts were identified which may be important to the transition from scarless to fibrotic healing in fetal wounds. Among these genes were those associated with proliferation, cell signaling, and the inflammatory response. Alterations in the expression of inflammatory genes, in particular, may contribute to the transition from non-inflammatory, scarless healing early in development to inflammatory, fibrotic healing at later stages of development in fetal skin.

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FIG. 1.

Volcano plot of differentially expressed genes between E15 and E18 fibroblasts. Log₂ of the fold change in gene expression was plotted against Log₁₀ of the p-value from microarray samples. For each age group, n=3 pooled samples from separate litters of mice. Genes were identified as having a significant change in expression if the fold change was 1.5 or greater and the p-value less than 0.05 (red dots).

FIG. 2.

Heatmap of gene expression patterns. The expression levels ($\log_2$) of E15 fibroblast (left 3 columns) and E18 fibroblast (right 3 columns) genes from each array which had a fold change ≥ 1.5 and a p-value < 0.05 are shown. Red denotes higher expression levels and green denotes lower expression levels. Hierarchical clustering was used to group both the samples and the genes. For each age group, n=3 pooled samples from separate litters of mice.

FIG. 3.

Real-time RT-PCR confirmation of microarray data. Real-time RT-PCR was performed on RNA isolated from E15 or E18 fibroblast cultures to determine COX-1 (left), CD109 (middle), and SOCS2 (right) mRNA expression. For each age group, n=3 pooled samples from separate litters of mice. The mean ratio of target gene expression compared to the housekeeping gene HPRT $\pm$ standard error of the mean (SEM) is shown. *p-value <0.05, student's two-tailed t-test.

FIG. 4.

Western blot analysis for COX-1 protein in cultured fetal fibroblasts. COX-1 protein levels were compared in cultured E15 and E18 fibroblasts using Western blot. COX-1 and β -actin bands from two representative samples are shown (top). For each age group, n=4 fibroblast protein samples from individual fetuses. Densitometry was used to determine the ratio of COX-1/ β -actin (bottom). Bars represent the mean ratio $\pm$ SEM. * p-value < 0.05, student's two-tailed t-test.

FIG. 5. COX-1 protein expression in fetal skin. Immunohistochemistry was used to determine COX-1 protein expression in E15 and E18 skin samples. For each age group, n=5 fetal skin samples from individual fetuses. Representative photomicrographs are shown for E15 (top row left) and E18 (top row right) fetal skin. Enlarged insets (lower left corners) show localization of COX-1 in dermal cells (brown staining). Black scale bars represent 50 μ m. Immunofluorescent staining for COX-1 was performed on E15 (middle row) and E18 (bottom row) skin from Col-GFP expressing mice. Images showing dermal fibroblasts (white arrows) staining positive for both COX-1 (left column, red) and Col-GFP (center column, green) are shown, with merged images in the right column. Samples were counterstained with DAPI (blue) to visualize cell nuclei. The white scale bars in the bottom panels represent 10 μ m.

TABLE 1

List of genes with higher expression in E18 fetal fibroblasts compared to E15 fetal fibroblasts.

Gene Symbol	Gene Description	fold ↑ in E18	p-value
AY036118	cDNA sequence AY036118	2.46	<0.001
ND6	NADH dehydrogenase subunit 6	2.38	<0.001
Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1	2.04	0.001
Slc38a1	solute carrier family 38, member 1	1.94	0.008
Cck	cholecystokinin	1.85	0.005
Slc7a11	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	1.80	0.013
Il11	interleukin 11	1.74	0.010
Ptgs1 (COX-1)	prostaglandin-endoperoxide synthase 1	1.72	<0.001
Thbd	thrombomodulin	1.69	0.005
Gm5070	predicted gene 5070	1.66	<0.001
Npr3	natriuretic peptide receptor 3	1.66	0.007
Il1rl2	interleukin 1 receptor-like 2	1.66	0.015
ND3	NADH dehydrogenase subunit 3	1.65	0.015
Gm5921	predicted gene 5921	1.59	0.007
Lrrc32	leucine rich repeat containing 32	1.58	0.012
LOC100047986	similar to eukaryotic translation elongation factor 1 gamma	1.57	0.007
Rps6	ribosomal protein S6	1.55	0.001
Rpl17	ribosomal protein L17	1.55	0.001
ATP6	ATP synthase F0 subunit 6	1.54	0.001
ND4L	NADH dehydrogenase subunit 4L	1.54	0.003
Usmg5	upregulated during skeletal muscle growth 5	1.53	0.013
4932438A13Rik	RIKEN cDNA 4932438A13 gene	1.51	0.012

TABLE 2

List of genes with lower expression in E18 fetal fibroblasts compared to E15 fetal fibroblasts.

Gene Symbol	Gene_Description	fold ↓ in E18	p-value
Lyz2	lysozyme 2	19.04	<0.001
Tnn	tenascin N	6.45	<0.001
Actc1	actin, alpha, cardiac muscle 1	4.46	<0.001
Igfbp5	insulin-like growth factor binding protein 5	2.55	<0.001
Thy1	thymus cell antigen 1, theta	2.52	0.003
C1qtnf3	C1q and tumor necrosis factor related protein 3	2.45	<0.001
Crabp1	cellular retinoic acid binding protein I	2.24	0.001
Itga11	integrin alpha 11	1.94	0.012
S100a4	S100 calcium binding protein A4	1.78	0.011
Avpr1a	arginine vasopressin receptor 1A	1.77	0.003
Cspg4	chondroitin sulfate proteoglycan 4	1.70	0.011
Col8a1	collagen, type VIII, alpha 1	1.70	0.001
Cd109	CD109 antigen	1.67	<0.001
Chst2	carbohydrate sulfotransferase 2	1.66	0.006
Fxyd5	FXYD domain-containing ion transport regulator 5	1.66	0.028
Fbln5	fibulin 5	1.65	<0.001
Rpl36	ribosomal protein L36	1.65	0.032
Gas6	growth arrest specific 6	1.63	0.015
Arhgdib	Rho, GDP dissociation inhibitor (GDI) beta	1.63	0.01
Flrt2	fibronectin leucine rich transmembrane protein 2	1.62	0.022
Crispld2	cysteine-rich secretory protein LCCL domain containing 2	1.62	0.016
Ckb	creatine kinase, brain	1.59	0.049
Socs2	suppressor of cytokine signaling 2	1.58	0.049
Ephx1	epoxide hydrolase 1, microsomal	1.58	0.009
Prrx2	paired related homeobox 2	1.56	0.035
Cd59a	CD59a antigen	1.56	0.040
Imp3	IMP3, U3 small nucleolar ribonucleoprotein, homolog (yeast)	1.56	0.004
Smoc2	SPARC related modular calcium binding 2	1.56	0.003
Plxdc2	plexin domain containing 2	1.55	0.003
Grem1	gremlin 1	1.55	0.019
Cav1	caveolin 1, caveolae protein	1.55	0.039
Ecm1	extracellular matrix protein 1	1.55	0.017
Crip1	cysteine-rich protein 1 (intestinal)	1.55	0.027
1110032E23Rik	RIKEN cDNA 1110032E23 gene	1.55	0.005
2700023E23Rik	RIKEN cDNA 2700023E23 gene	1.54	0.002
Fhl1	Four and a half LIM domains 1	1.53	0.043
Ankrd28	ankyrin repeat domain 28	1.53	0.004
Rgs4	regulator of G-protein signaling 4	1.51	0.021
Lrrc17	leucine rich repeat containing 17	1.51	0.032

Gene Symbol	Gene_Description	fold ↓ in E18	p-value
Rnpep	arginyl aminopeptidase (aminopeptidase B)	1.50	0.036

TABLE 3

Functional groups of differentially expressed genes

	Gene Functional Group				
	Cellular Growth and Proliferation	Cellular Movement	Signaling and Interaction	Cell Signaling	Small Molecule Biochemistry
CAV1	●	●	●	●	●
CKK	●	●	●	●	●
GAS6	●	●	●	●	●
RGS4	●	●	●	●	●
THBD	●	●	●	●	●
CSPG4	●	●	●	●	●
IL11	●	●	●	●	●
S100A4	●	●	●	●	●
THY1	●	●	●	●	●
ARHGDIB	●	●	●	●	●
AVPR1A	●	●	●	●	●
FBLN5	●	●	●	●	●
GREM1	●	●	●	●	●
IGFBP5	●	●	●	●	●
Lyz1/Lyz2	●	●	●	●	●
COX-1	●	●	●	●	●
SERPINC1	●	●	●	●	●
SOCS2	●	●	●	●	●
TNN	●	●	●	●	●
FHL1	●	●	●	●	●
FXYD5	●	●	●	●	●
ITGA11	●	●	●	●	●
PRRX2	●	●	●	●	●
SLC7A11	●	●	●	●	●
ANKRD28	●	●	●	●	●
AY036118	●	●	●	●	●

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	Gene Functional Group				
	Cellular Growth and Proliferation	Cellular Movement	Signaling and Interaction	Cell Signaling	Small Molecule Biochemistry
C1qmf3	●				
Cd59a		●			
CHST2		●			
CKB					●
COL8A1	●				
CRIP1	●				
ECM1	●				
EPHX1					●
IL1RL2				●	
LRRC32	●				
NPR3	●				
RPS6	●				
SLC38A1					●