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Dermal lymphatic dilation in a mouse model of alopecia areata

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Abstract

Mouse models of various types of inflammatory skin disease are often accompanied by increased dermal angiogenesis. The C3H/HeJ inbred strain spontaneously develops alopecia areata (AA), a cell mediated autoimmune disorder that can be controllably expanded using full thickness skin grafts to young unaffected mice. This provides a reproducible and progressive model for AA in which the vascularization of the skin can be examined. Mice receiving skin grafts from AA or normal mice were evaluated at 5, 10, 15, and 20 weeks after engraftment. Lymphatics are often overlooked as they are small slit-like structures above the hair follicle that resemble artifact-like separation of collagen bundles with some fixatives. Lymphatics are easily detected using lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) by immunohistochemistry to label their endothelial cells. Using LYVE1, there were no changes in distribution or numbers of lymphatics although they were more prominent (dilated) in the mice with AA. *Lyve1* transcripts were not significantly upregulated except at 10 weeks after skin grafting when clinical signs of AA first become apparent. Other genes involved with vascular growth and dilation or movement of immune cells were dysregulated, mostly upregulated. These findings emphasize aspects of AA not commonly considered and provide potential targets for therapeutic intervention.

Keywords

LYVE1; PECAM1; SMA; lymphatics; inflammation; autoimmune disease

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Disclosure

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1. Introduction

Angiogenesis is a hallmark of inflammation, wound healing, and neoplasia (Patan, 2004). Angiogenesis is also the initial gross and histological finding in two stage cutaneous chemical carcinogenesis or UVB induced skin cancers long before there is any evidence of epidermal hyperplasia or neoplasia (Binder et al., 1998; Konger et al., 2013). Angiogenesis is present in the dermis of various mouse models commonly used to study genetically based inflammatory skin diseases. For example, the chronic proliferative dermatitis mouse (C57BL/KaLawRij-*Sharpin*^{cpdm}/RijSunJ) has an autoinflammatory skin disease where the mice develop progressively worsening inflammation consisting primarily of eosinophils, neutrophils, macrophages, and mast cells that are independent of B and T lymphocytes. These mice develop progressive and prominent neovascularization (HogenEsch et al., 1993). This phenotype is due to a null mutation in the mouse *Sharpin* gene (Seymour et al., 2007) which interacts with a series of pathways, one of which activates the NFκB pathway which regulates inflammation (Ikeda et al., 2011; Liang et al., 2011; Wang et al., 2012a; Wang et al., 2012b). Similarly, the flaky skin mutant mouse (CByJ.A-*Ttc*^{fsn}/J) also has marked dermal neovascularization but less is known about the molecular pathogenesis of this mouse skin disease (Helms et al., 2005; Sundberg et al., 1997).

Although described in detail in various anatomic locations in the skin of mice (Karlsen et al., 2006), lymphatics are largely ignored in the evaluation of mouse models with various types of skin disease, probably because they appear as thin slit-like structures, not unlike separation in the dense irregular collagenous connective tissue that makes up the dermis. LYVE1 is a widely used marker for lymphatic endothelial cells (Jackson, 2004) making it very useful for evaluating these structures in mice and other species. Lymphatics are an essential part of the body's immune defense in which lymph circulates back to the blood circulation by being filtered through lymph nodes where foreign material is taken up by antigen presenting cells which can initiate specific immune responses (Alitalo et al., 2005). Lymphatics, like blood vessels, can proliferate during inflammation (Alitalo et al., 2005; Pullinger and Florey, 1937).

Alopecia areata (AA) is a lymphocyte-dependent autoimmune disease (Carroll et al., 2002). Human AA patients have increased lesional skin temperature that was proposed to be the result of the degree of vascularization (Mijailovi et al., 1997). Conversely, other early studies suggested that angiogenic activity was defective in monocytes from alopecia universalis (the most severe form of AA) patients (Skoutelis et al., 1990). Several inbred strains, most notably C3H/HeJ (McElwee et al., 1999; Sundberg et al., 1994), develop a complex genetic based cellmediated autoimmune disease, AA (Carroll et al., 2002; Sundberg et al., 2003). These mice develop a mixed but primarily lymphocytic inflammation in and around hair follicles without obvious changes in the dermal vasculature. This is a well-established mouse model for AA. C3H/HeJ mice naturally develop AA at a frequency of around 20-25% by one year of age (Sundberg et al., 1994). These mice can be used in experimental studies, although lesions tend to wax and wane. A more consistent model was developed using full thickness skin grafts from old mice with AA onto young mice, 10 weeks of age, who developed disease that repeatedly and consistently progressed from patchy alopecia at 8-10 weeks after grafting to diffuse alopecia by 20 weeks after grafting

(McElwee et al., 1998; Silva and Sundberg, 2013; Sundberg et al., 2004). Using the skin graft model, changes in the dermal vasculature were evaluated at multiple time points from initial development through progression of diffuse AA. Mild to moderate changes in the lymphatics were found but not in blood vessels using histology, immunohistochemistry, and transcriptome analyses.

2. Materials and Methods

2.1 Mice

Graft recipient C3H/HeJ (JR# 659) female mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 10 weeks of age. Donor C3H/HeJ mice were obtained from production colonies as retired breeders, maintained for 6 months, and those that developed spontaneous AA were used as donors. Surgical methods for full thickness skin grafts have been described in detail elsewhere (McElwee et al., 1998; Silva and Sundberg, 2013). All work was approved by The Jackson Laboratory Animal Care and Use Committee (Approval number 07005).

2.2 Histology and Immunohistochemistry

Mice were euthanized by CO₂ asphyxiation. The dorsal skin was surgically removed at the time of necropsy, laid flat on aluminum foil in a cranio-caudal orientation, and fixed overnight by immersion in Fekete's acid-alcohol-formalin solution and then transferred to 70% ethanol (Silva and Sundberg, 2012). The skin was trimmed, processed routinely, embedded in paraffin, sectioned at 6 µm, and stained with hematoxylin and eosin (H&E). Serial sections were immunolabeled using antibodies directed against PECAM1/CD31 (for endothelial cells; Abcam, Cambridge MA, cat# ab14917, 1:100), LYVE1 (for lymphatics, Abcam, Cambridge MA, cat# ab14917, 1:100), and smooth muscle actin isoform (SMA, for vascular smooth muscle; Sigma, St Louis, MO, cat# A2547, 1:200) (<http://tumor.informatics.jax.org/html/antibodies.html>) using a Ventana autostainer (Tuscon, AZ). Diaminobenzidine (DAB; Sigma, St. Louis, MO) was used as the chromogen.

2.3 Transcriptome analyses

RNA samples from 3 AA graft and 3 normal graft-recipient mice were used at each time point and processed using the MOE430v2.0 GeneChip™ (Affymetrix) as described and reported previously (Duncan et al., 2013; McPhee et al., 2012). Briefly, skin and spleen samples were harvested in an RNase free manner and stored in RNeasy Lysis Buffer (Qiagen, Austin, TX) per the manufacturer's instructions and homogenized in TRIzol (Invitrogen, Carlsbad, CA). Total RNA was isolated by standard TRIzol methods, and quality assessed using a 2100 Bioanalyzer instrument and RNA 6000 Nano LabChip assay (Agilent Technologies, Palo Alto, CA). Following reverse transcription with an oligo(dT)-T7 primer (Affymetrix, Santa Clara, CA), double-stranded cDNA was synthesized with the Superscript double-stranded cDNA synthesis custom kit (Invitrogen). In an *in vitro* transcription (IVT) reaction with T7 RNA polymerase, the cDNA was amplified and labeled with biotinylated nucleotides (Enzo Diagnostics, Farmingdale, NY). Fifteen micrograms of biotin-labeled and fragmented cRNA were hybridized onto MOE430v2.0 GeneChip™ arrays (Affymetrix) for 16 hours at 45°C. Post-hybridization staining and washing was done according to the

manufacturer's protocols using a Fluidics Station 450 (Affymetrix). Finally, the arrays were scanned with a GeneChip™ Scanner 3000 laser confocal slide scanner. The images were quantified using GCOS 1.0 software (GeneChip™ Operating Software, Affymetrix). Data were imported into the R software environment and analyzed using the R/MAANOVA package. After graphical diagnostics and appropriate transformations, an analysis of variance (ANOVA) model was applied to the data, and F1, F2, F3, and Fs test statistics constructed along with their permutation p-values.

3. Results

3.1 Lymphatics

Lymphatics in the mouse skin are localized in the dermis and hypodermal fat layer. The lymphatics are uniformly located above the hair bulb and parallel to the hair follicle shaft. As the dense irregular collagenous connective tissue fragments and separates in routine histological sections stained with H&E and fixed in formalin based fixatives, these are usually overlooked. Some of the slit-like structures that resemble collagen bundle separations are lined by endothelial cells. LYVE1 clearly labeled the endothelial cells lining the lymphatics (**Fig. 1**). Lymphatic distribution and numbers did not change between age and gender matched sham grafted and AA grafted C3H/HeJ female mice as alopecia areata developed and progressed. However, lymphatics in the dermis above the hair follicles became progressively larger and more dilated as the disease progressed from normal to patchy to generalized alopecia.

3.2 Blood Vessels

Smooth muscle actin (SMA) labeled vascular smooth muscle and arrector pili muscles in skin sections. Platelet/endothelial cell adhesion molecule 1 (PECAM1) labeled only vascular endothelial cells but not those lining lymphatics. In both cases, blood vessels, not normally prominent in the dermis of the mouse, remained similar in size and distribution between the normal sham-grafted mice and those developing progressive AA (data not shown, available on <http://pathbase.net/>). This is in contrast to changes in the dermis seen in primarily neutrophil (flaky skin mutant mice, *Ttc7^{fsn}*) or a mixture of neutrophil and eosinophil driven (chronic proliferative dermatitis, *Sharpin^{cpdm}*) inflammatory scaly skin disease models in which lymphatics are unchanged, but blood vessels become prominent and tortuous in the dermis and hypodermal fat layer (data not shown).

3.3 Transcriptome results

While there were histologically evident changes in lymphatics in the dermis of mice with developing AA, there were no significant changes in *Lyve1* transcripts over time (**Table 1**). This is consistent with dilation of lymphatics rather than proliferation. Blood vessels were of normal size and distribution between controls and those mice developing progressive AA, although some heavily affected areas had focal areas where the blood vessels were more prominent and larger. Transcripts coding for proteins that regulate blood vessels, lymphatics, endothelial cells, and the immune cells that are carried in these vessels (*End1*, *Flt1*, *Pdpn*, *Ece1* and 2, *Ccr7*, *Cxcl12*, and *Cxcr4*) were significantly upregulated at 10 weeks or longer

after engraftment (q-value < 0.05) (**Table 1**). Only *Kdr* and *Sema3a* transcripts were downregulated.

4. Discussion

Neither lymphangiogenesis nor angiogenesis (the formation of new or disorganized lymphatics or blood vessels) associated with increased infiltration of inflammatory cells, primarily lymphocytes in and around dystrophic hair follicles, were found in the AA mice as their disease progressed. It has been reported that as mice age they experience a decline in cutaneous lymphatic density (Karaman et al., 2015). While the mice in these studies were not considered to be “old”, the only observed vascular change between mice with AA at the end of the study, 30 weeks/7.5 months of age, was lymphatic dilation. Lymphatics were dilated in the AA mouse dermis which may accompany the lymphocyte co-stimulatory cascade pathogenesis of AA (Carroll et al., 2002). Evaluation of transcripts associated with lymphangiogenesis and angiogenesis revealed relatively few were highly dysregulated which supports this observation. Transcripts associated with lymphatic endothelial cell growth, migration, and survival (most of the vascular endothelial growth factors (*Vegfs*) and their receptors) (Alitalo et al., 2005) were not significantly dysregulated. *Vegfa* was significantly upregulated only at 10 weeks after skin engraftment, which is when clinical evidence of AA first appears. This may partially account for the lymphatic dilation as experimental localized overexpression of *Vegfa* in mice caused distention of lymphatics (“giant” lymphatics) and edema (Nagy et al., 2002). Some of the class 3 semaphorins (*Sema3s*) negatively regulate dermal lymphatic network formation (Uchida et al., 2015) but they were either not dysregulated, or barely significantly down regulated.

VEGFC and its receptor (FMS-like tyrosine kinase 1, FLT1) can also regulate lymphatic migration from tissues to draining lymph nodes (Iwami et al., 2015) as appears to be the case with other dysregulated transcripts in the AA mouse model. Podoplanin (*Pdpn*) was significantly upregulated from 10 weeks post engraftment on. Mice genetically engineered to be null for this gene developed congenital lymphedema and dilation of lymphatic vessels (Schacht et al., 2003). While this appears to be counter to the observations here, global deletion of *Pdpn* results in exaggerated T cell responses and spontaneous experimental autoimmune encephalomyelitis (EAE) in mice with a susceptible genetic background (Peters et al., 2015). As such, in the AA mouse model, this may be an attempt to down regulate the T cell autoimmunity having effects beyond the lymphatics. Similarly, *Ccr7*, which was also significantly upregulated from 10 weeks post engraftment on, can have a role in lymphatics; however, it also functions as a key regulator of homeostatic B and T cell trafficking to secondary lymphoid organs as well as an essential mediator for entry of both dermal and epidermal dendritic cells (DC) into the lymphatic vessels within the dermis (Alitalo et al., 2005; Johnson and Jackson, 2013; Ohl et al., 2004). Chemokine (C-X-C motif) ligand 12 (*Cxcl12*) regulates hematopoietic stem cells (HSCs) and their lympho-hematopoietic progeny (Omatsu et al., 2010). Chemokine (X-C motif) receptor 4 (*Cxcr4*) is the receptor for CXCL12 (Rueda et al., 2012). The other upregulated cytokines have similar functions (Johnson and Jackson, 2013). These findings suggest that the dysregulated transcripts can affect both lymphatics directly or the immune cells and other cytokines carried within the lymphatics.

Endothelin 1 is a potent vasoconstrictor peptide produced by vascular endothelial cells. Conditional null mutant mice (*Edn1^{tm1.1Ywa}*) in which the *Edn1* is selectively deleted in endothelial cells have low blood pressure (Kisanuki et al., 2010). *Edn1* was one of the significantly dysregulated transcripts with some of the largest fold changes suggesting that AA mice might have increased blood pressure which would indirectly increase the diameter of lymphatics in the skin. Conversely, the receptor (*Endra*), which regulates vascular smooth muscle function (Seo et al., 1994), was down regulated. Ubiquitously null mice (*Edn1^{tm1Uti}*) die of respiratory failure at birth. Heterozygous mice, which produce lower levels of EDN1 than controls, develop elevated blood pressure (Kurihara et al., 1994) indicating the protein has a very specific localized effect on endothelial cells. VEGFA, which was upregulated at the onset of AA, interacts with FMS-like tyrosine kinase 1 (FLT1) and kinase insert domain receptor (KDR), both of which are transmembrane tyrosine kinases predominantly expressed in vascular endothelial cells (Ho et al., 2012). *Flt1* transcripts were significantly upregulated while *Kdr* transcripts were down regulated at 10, 15, and 20 weeks post engraftment. Work with *Flt1^{tm1Jrt}* null mice demonstrated that the protein is essential for the organization of embryonic vasculature, but is not essential for endothelial cell differentiation (Fong et al., 1995). KDR directly promotes the expansion of the lymphatic network (Dellinger et al., 2013). Endothelin converting enzyme 1 and 2 (*Ece1* and *2*) are involved in heart development (Yanagisawa et al., 2000) and play a role in blood pressure (Kuwaki et al., 1999) and therefore may have a role in lymphatic dilation.

Proteins coded for by the dysregulated transcripts appear to act both on the lymphatics directly, and/or on other organs or cells. Some will affect the diameter and function of the lymphatics directly or indirectly by regulating blood pressure. Several proteins regulate the T, B, and dendritic cells that flow through the lymphatics. All of these factors contribute to the pathogenesis of AA. Taken alone, lymphatic changes are a minor part of the AA pathogenesis. However, by identifying single or groups of transcripts with similar functions that are dysregulated in the AA mouse model it will be possible to target them therapeutically.

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Abbreviations

AA	alopecia areata
Ccr7	chemokine (C-C motif) receptor 7
Cxcl11	chemokine (C-X-C motif) ligand 12
Cxcr4	chemokine (X-X-C motif) receptor 4

Ece1 and 2	endothelin converting enzyme 1 and 2
End1	endothelin 1
Flt1	FMS-like tyrosine kinase 1
H&E	hematoxylin and eosin stain
Kdr	Kinase insert domain receptor, also known as vascular endothelial growth factor 2, <i>VEGF2</i>
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
PECAM1	platelet/endothelial cell adhesion molecule 1, formerly CD31
Pdpn	Podoplanin
Sharpin^{cpdm}	SHANK-associated RH domain interacting protein, chronic proliferative dermatitis allelic mutation
SMA	smooth muscle actin isoform
Ttc7^{fsn}	tetratricopeptide repeat domain 7, flaky skin allelic mutation

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Fig. 1. LYVE1 expression in lymphatics

Immunohistochemical localization of LYVE1 protein defined lymphatics as being located above the hair follicles. The left two columns are skin grafts of normal skin from C3H/HeJ mice to normal C3H/HeJ recipients at 5, 10, 15, and 20 weeks after surgery. The right two columns are skin grafts from C3H/HeJ mice with alopecia areata to normal recipients at the same time intervals. Note the LYVE1 expression (brown) of lymphatic endothelial cells. Lymphatics are equally abundant in both groups; however, they are larger in the mice with alopecia areata. Boxed areas are enlarged in the figures immediately to the right.

Table 1

Vascular gene transcriptome results.

Probe ID	Gene Symbol	RFC 5 wk	FsQvalue 5 wk	RFC 10 wk	FsQvalue 10 wk	RFC 15 wk	FsQvalue 15 wk	RFC 20 wk	FsQvalue 20 wk
1423466_at	<i>Ccr7</i>	1.4507	0.4891	2.2553	0.0006	1.7014	0.0116	2.3895	0.0003
1417574_at	<i>Cxcl12</i>	-1.0570	0.8151	-1.1513	0.2256	0.0000	0.6018	-1.1975	0.1403
1439084_at	<i>Cxcl12</i>	-1.0353	0.8363	-1.2198	0.0636	-1.2058	0.0739	-1.1810	0.1106
1448823_at	<i>Cxcl12</i>	1.2283	0.6545	1.9407	0.0006	2.2346	0.0004	1.8921	0.0008
1448710_at	<i>Cxcr4</i>	1.1122	0.8298	2.5906	0.0039	2.5906	0.0047	3.6723	0.0003
1434177_at	<i>Ece1</i>	1.2058	0.7024	1.4948	0.0263	1.6096	0.0113	1.5656	0.0145
1441423_at	<i>Ece1</i>	0.0000	0.8834	-1.0258	0.5437	-1.0473	0.4444	1.0473	0.4841
1455741_a_at	<i>Ece1</i>	1.2114	0.6437	1.3165	0.0599	1.5123	0.0088	1.3044	0.0665
1423358_at	<i>Ece2</i>	1.2425	0.6203	1.5334	0.0088	1.2687	0.1037	1.4674	0.0158
1424561_at	<i>Ece2</i>	1.0234	0.8636	-1.1277	0.3298	-1.1810	0.2078	1.0210	0.6059
1422586_at	<i>Ecel1</i>	1.0968	0.7008	-1.0093	0.5946	-1.0473	0.4185	1.0023	0.6389
1451924_a_at	<i>Edn1</i>	1.5333	0.6295	2.6027	0.0048	3.3559	0.0013	3.4105	0.0008
1449161_at	<i>Edn2</i>	1.2953	0.7794	1.4607	0.2976	1.8489	0.1216	1.2030	0.4963
1421136_at	<i>Edn3</i>	1.0473	0.8298	-1.2628	0.0719	-1.1647	0.1965	-1.0892	0.4093
1438696_at	<i>Edn3</i>	-1.1070	0.7844	-1.1070	0.4150	-1.3724	0.0599	-1.2142	0.2223
1441923_s_at	<i>Edn3</i>	-1.1892	0.6303	-1.1198	0.2995	-1.2775	0.0506	-1.2599	0.0654
1441924_x_at	<i>Edn3</i>	-1.0595	0.8430	-1.2805	0.1945	-1.1329	0.3891	-1.0843	0.5133
1433525_at	<i>Ednra</i>	-1.0329	0.8523	1.1461	0.2890	-1.0792	0.4257	-1.2142	0.1672
1440093_at	<i>Ednra</i>	-1.0867	0.7983	-1.0425	0.5428	-1.2599	0.1244	-1.4879	0.0163
1451691_at	<i>Ednra</i>	-1.1044	0.7073	1.0186	0.5684	1.0644	0.3829	-1.0693	0.4013
1460513_a_at	<i>Ednra</i>	-1.0047	0.8801	-1.0817	0.4504	-1.0163	0.5722	-1.1173	0.3843
1423594_a_at	<i>Ednrb</i>	-1.0497	0.8636	1.1620	0.4586	1.3851	0.2315	1.3472	0.2854
1426314_at	<i>Ednrb</i>	1.0140	0.8773	1.1783	0.4195	1.2687	0.3012	1.3410	0.2543
1437347_at	<i>Ednrb</i>	-1.1122	0.8385	1.1567	0.4818	1.3348	0.3028	1.3915	0.2848
1438953_at	<i>Figf</i>	-1.0943	0.8360	1.0668	0.5526	-1.3947	0.1882	-1.0093	0.6371
1438954_x_at	<i>Figf</i>	-1.1224	0.8170	1.1199	0.4842	-1.3629	0.2029	-1.0234	0.6226
1449528_at	<i>Figf</i>	-1.0281	0.8685	1.1408	0.4126	-1.1251	0.4176	1.0767	0.5367
1419300_at	<i>Flt1</i>	1.2397	0.7006	2.3512	0.0005	2.2606	0.0011	2.7446	0.0001
1440926_at	<i>Flt1</i>	1.3947	0.6341	3.3019	0.0002	2.4171	0.0023	3.8906	0.0001
1451756_at	<i>Flt1</i>	1.4845	0.5184	3.2192	0.0001	2.6146	0.0008	3.7409	0.0000
1454037_a_at	<i>Flt1</i>	1.6780	0.5178	2.5026	0.0046	2.7256	0.0032	3.5064	0.0004
1421442_at	<i>Flt4</i>	-1.0892	0.7940	-1.1513	0.2974	-1.1199	0.3453	1.1487	0.3149
1444052_at	<i>Flt4</i>	-1.0918	0.8162	-1.3349	0.1385	-1.1865	0.3029	-1.4274	0.0755
1449379_at	<i>Kdr</i>	-1.3851	0.5996	-1.7092	0.0185	-1.9231	0.0065	-2.0279	0.0032
1429379_at	<i>Lyve1</i>	1.1540	0.8181	1.5765	0.1599	-1.0023	0.6009	1.1947	0.4631
1453128_at	<i>Lyve1</i>	1.1647	0.7948	1.4811	0.1440	-1.0401	0.5613	1.0792	0.5615

Probe ID	Gene Symbol	RFC 5 wk	FsQvalue 5 wk	RFC 10 wk	FsQvalue 10 wk	RFC 15 wk	FsQvalue 15 wk	RFC 20 wk	FsQvalue 20 wk
1418084_at	<i>Nrp1</i>	-1.2003	0.7052	-1.1096	0.4161	-1.1674	0.2892	-1.1070	0.4401
1448943_at	<i>Nrp1</i>	-1.3165	0.6395	-1.1837	0.3314	-1.0401	0.5457	-1.2311	0.2711
1448944_at	<i>Nrp1</i>	-1.1674	0.7255	-1.0843	0.4637	-1.1702	0.2769	-1.0993	0.4501
1457198_at	<i>Nrp1</i>	-1.1355	0.7416	-1.0473	0.5298	-1.1975	0.2000	-1.1173	0.3859
1426528_at	<i>Nrp2</i>	1.1173	0.7684	1.1198	0.3794	-1.0353	0.5389	1.0401	0.5732
1435349_at	<i>Nrp2</i>	1.0376	0.8405	1.2805	0.0499	-1.0281	0.5346	1.0843	0.4126
1447343_at	<i>Nrp2</i>	-1.0546	0.8415	1.0917	0.4613	-1.0473	0.5242	1.0093	0.6324
1419309_at	<i>Pdpr</i>	1.1302	0.7483	1.8404	0.0010	1.5764	0.0083	1.7941	0.0013
1416846_a_at	<i>Pdpr3</i>	-1.0234	0.8703	-1.0116	0.6094	1.5334	0.0368	1.0817	0.5174
1443309_at	<i>Pdpr3</i>	1.0377	0.8481	-1.0668	0.4753	1.0742	0.4364	1.0281	0.5903
1420416_at	<i>Sema3a</i>	-1.2924	0.6088	-1.4406	0.0409	-1.4110	0.0486	-1.2599	0.1676
1420417_at	<i>Sema3a</i>	-1.0497	0.8208	0.0000	0.6241	-1.1919	0.1173	-1.0644	0.4607
1449865_at	<i>Sema3a</i>	1.0093	0.8797	-1.0918	0.5238	-1.4011	0.1887	-1.2003	0.4121
1420909_at	<i>Vegfa</i>	1.1303	0.7650	1.4507	0.0390	1.1728	0.2831	1.2599	0.1709
1451959_a_at	<i>Vegfa</i>	1.0817	0.8024	1.1975	0.2106	1.1621	0.2616	1.2924	0.0926
1419417_at	<i>Vegfc</i>	-1.1837	0.7653	1.1329	0.4506	-1.1070	0.4664	1.1356	0.4660
1439766_x_at	<i>Vegfc</i>	-1.2513	0.7253	1.1728	0.3953	-1.0497	0.5435	1.1594	0.4331
1440739_at	<i>Vegfc</i>	-1.1756	0.7898	1.1251	0.4869	-1.1461	0.4418	1.0570	0.5887

Key	Significant for at least 1 contrast	Relative Fold Change (RFC) upregulated RFC < 0	Relative Fold Change (RFC) down regulated RFC > 0	q-value < 0.05
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Relative fold change is indicated for skin grafts at 5, 10, 15, and 20 weeks after full thickness skin grafts for alopecia areata mice compared to controls. *Ccr7*, *Cxcl12*, *Cxcr4*, *Ece1* and *2*, *End1*, *Ftl1*, and *Pdpr* were significantly (q-value < 0.05) upregulated at 10, 15, and 20 week time points while others were transiently dysregulated. Only *Kdr* and *Sema3a* were significantly down regulated.