Activin promotes skin carcinogenesis by attraction and reprogramming of macrophages

Maria Antsiferova[1], Aleksandra Piwko-Czuchra[1], Michael Cangkrama[1], Mateusz Wietecha[1], Dilara Sahin[1], Katharina Birkner[1], Valerie C. Amann[2], Mitchell Levesque[2], Daniel Hohl[3], Reinhard Dummer[3], and Sabine Werner[1*]

Appendix: Supplementary Information

Appendix Table S1. Genes significantly upregulated (FDR≤0.05, $|\log_2 Ratio| \geq 1$,) in FACS-sorted skin macrophages in 2-3 genotype comparisons (wt/Act *vs* wt/wt, HPV8/Act *vs* HPV8/wt and HPV8/Act *vs* wt/wt), grouped in functional categories based on literature search.

Functional category	Gene name	References
Adhesion/migration	*Cx3cr1*	Goda *et al*, 2000
	F11r	Gupta *et al*, 2000
	Itgb5	de Nichilo and Yamada, 1996
	Mmp14	Hara *et al*, 2011
	Itgal	Henderson *et al*, 2001
	Hck	Roach *et al*, 1997 Cougoule *et al*, 2010
Cancer-associated	*Cx3cr1*	Schmall *et al*, 2015
	F11r	Pont *et al*, 2013
	Spp1	Lin *et al*, 2015
	Mmp14	Ager *et al*, 2015
	C3	Markiewski *et al*, 2008; Nunez-Cruz *et al*, 2012
	Tgfbr1	Byrne *et al*, 2008
	Mmp12	Hofmann *et al*, 2005; Qu *et al*, 2011
	Havcr2	Yan *et al*, 2015
Angiogenesis	*F11r*	Rabquer *et al* 2010
	Spp1	Kale, 2014
	Mmp14	Basile *et* al, 2007; Littlepage *et al*, 2010
	C3	Nozaki *et al*, 2006; Nunez-Cruz et al, 2012
Anti-microbial/effector function	*Ass1*	Qualls *et al*, 2012
	Tgtp2	MacMicking, 2004
	Stat1	Spath *et al*, 2009
	C3	Markiewski and Lambris, 2007
	Hck	Nelson *et* al, 2009
	Axl	Seitz *et al*, 2007
	Mmp12	Houghton *et al*, 2009; Marchant *et al*, 2014;
Immunoregulatory	*Klra2*	Gays *et al*, 2006
	Lair1	Colonna *et al*, 1999

	C3	Carroll, 2004
	Clec12a	Lahoud *et al*, 2009
	Hck	English *et* al, 1993
	Ly6e	Xu *et al*, 2014
	Axl	Sharif *et al*, 2006
	Tgfbr1	Takaki *et al*, 2006
	Kcnn4	Kang *et al*, 2014
	Mmp12	Bellac *et al*, 2014
	Havcr2	Monney *et al*, 2002

FDR: false discovery rate

Appendix Table S2. Primers used for RT-PCR or qRT-PCR.

Target gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
HPRT	tgacactggcaaaacaatgca	ggtccttttcaccagcaagct
*INHBA/Inhba**	ggagaacgggtatgtggaga	acaggtcactgccttccttg
Gapdh	tcgtggatctgacgtgccgcctg	caccaccctgttgctgtagccgtat
Fst	agggaaagtgtatcacaaagt	gagttgcaagatccagaatg
E6 of HPV8	gcaggactgttcagtaccgtgcaac	acggcctctcccacagcaatct
Rps29	ggtcaccagcagctctacg	gtccaacttaatgaagcctatgtcc
Cxcl10	gggtctgagtgggactcaagggat	ccatccatcgcagcaccggg
Ccl19	gtcggagcctcggcctctca	ccccagagttggggctggga
Ccl22	ccgcaagcctggcgttgttttg	ggcgtcgttggcaaggctct
Arg1	cttaaagaaacagagtatgacgtgag	ctggttgtcaggggagtgtt
Cx3cr1	cctgcctctgagaaatggag	atctctccagcccctgaaat
Spp1	tgcagttctcctggctgaat	ctttcaccgggagggagga
Ccl17	acagtcagaaacacgatggca	tgctcgagccaccaatgtag
Mpp12	aaagtggggctttaagggaa	gttgcccagttgcttctagc

* the same primers were for used for the detection of human *INHBA* and mouse *Inhba* transcripts.

Appendix Table S3. Antibodies used for immunostaining.

Antibody	**Source**	**Catalog No.**
Primary		
Anti-Activin	R&D Systems, Minneapolis, MN	AF338
Anti-BrdU-FITC	Roche, Rotkreuz, Switzerland	1202693
Anti-CD206	R&D Systems	AF2535
Anti-CD31-biotin	Biolegend, San Diego, CA	102504
Anti-CD68-biotin	Serotec, Raleigh, NC	MCA1957B
Anti-Foxp3-biotin	eBioscience, San Diego, CA	13-5773-80
Anti-Keratin14	BAbCo, Richmond, CA	PRB-155P
Anti-LYVE1	AngioBio, San Diego, CA	11-034
Anti-Osteopontin	R&D Systems, Franklin Lakes, NJ	AF808-SP
Anti-Panendothelial Cell Antigen (MECA-32)	BD Biosciences, San Jose, CA	553849
Anti-TcRβ	eBioscience	14-5961-82
Anti-TcRδ-FITC	BD Biosciences	553177
Secondary		
Anti-goat-Cy2	Jackson Laboratories, Bar Harbor, MN	705-225-003
Anti-goat-HRP	DAKO, Glostrup, Denmark	0449
Anti-hamster-Cy3	Jackson Lab.	127-165-160
Anti-rabbit-Cy2	Jackson Lab.	111-165-003
Anti-rat-Cy3	Jackson Lab.	712-165-150
Streptavidin-Cy3	BioLegend	405215

Appendix Table S4. Antibodies used for flow cytometry.

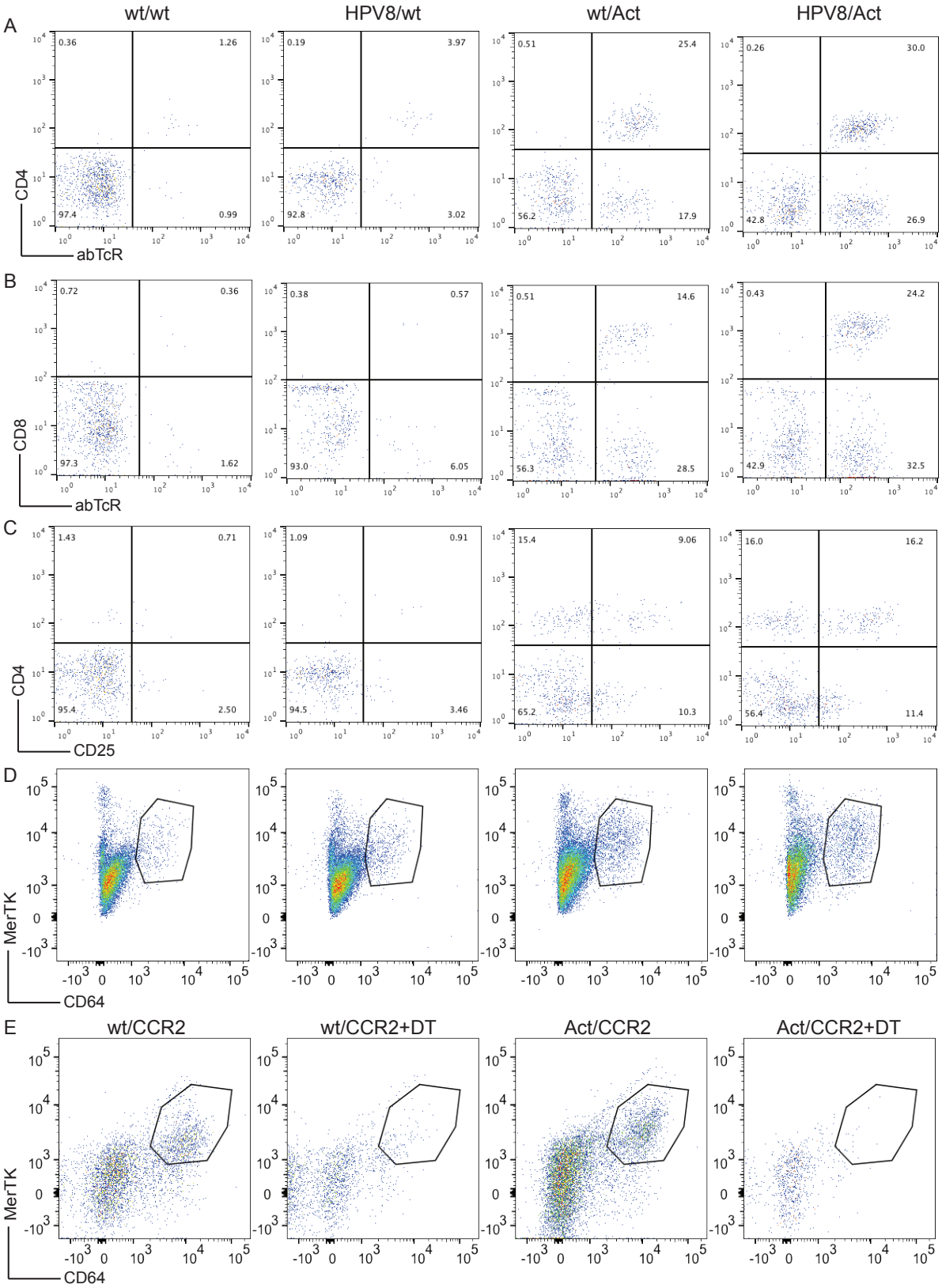
Antibody	**Source**	**Catalog No.**
Anti-CCR3-PE	R&D Systems	FAB729P
Anti-CD11b-BV711	BioLegend	101242
Anti-CD16/CD32	BD Biosciences	553141
Anti-CD25-APC	eBioscience	17-0251
Anti-CD3-APC	BD Biosciences	553066
Anti-CD3-PE	BD Biosciences	553064
Anti-CD3-PE-CF594	BD Biosciences	562332
Anti-CD4-FITC	eBioscience	11-0041
Anti-CD45-AlexaFluor 700	BioLegend	103128
Anti-CD45-APC	eBioscience	17-0451
Anti-CD45-PB	BioLegend	103126
Anti-CD45-PE-Cy7	BioLegend	103114
Anti-CD45R/B220-FITC	BD Biosciences	553088
Anti-CD49b-PE	BD Biosciences	553858
Anti-CD64-APC	BioLegend	139305
Anti-CD64-BV421	BioLegend	139309
Anti-CD69-PE	eBioscience	12-0691
Anti-CD8a-PE	eBioscience	12-0081
Anti-F4/80-AlexaFluor488	BioLegend	123120
Anti-F4/80-BV421	BioLegend	123132
Anti-F4/80-PE	eBioscience	12-4801
Anti-Ly6C-PerCP-Cy5.5	BioLegend	127616
Anti-Ly6G-FITC	BD Biosciences	551460
Anti-MerTK-PE	R&D Systems	FAB5912P
Anti-MHCII-BV510	BD Biosciences	107635
Anti-TcRβ-APC	eBioscience	17-5961
Anti-TcRβ-PE	eBioscience	12-5961
Anti-TcRδ-FITC	BD Biosciences	553177

Appendix Table S5. GEO datasets and sample accession numbers of the published microarrays that were used to generate gene sets for GSEA. Complete gene sets, mapped gene set sizes, ranked gene lists, and GSEA reports can be found in dataset EV4.

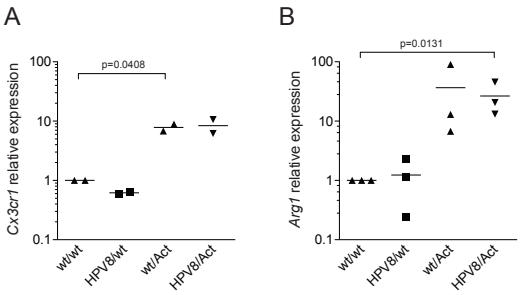
Description	GEO Datasets & Samples	Number of Genes in Sets
Murine mammary tumor-associated macrophages *versus* mammary tissue macrophages	Series: GSE56755 (Franklin *et al*, 2014)	Generated via GEO2Enrichr, Characteristic Direction method
	TAM ("tumor associated macrophages, CD11blo"): GSM1368200, GSM1368201, GSM1368202	Up in TAM: 238 top genes
	Control ("mammary tissue macrophages, CD11bhi"): GSM1368198, GSM1368199	Down in TAM: 262 bottom genes
Murine late-stage breast tumor-associated macrophages *versus* splenic tissue macrophages	Series: GSE18404 (Ojalvo *et al*, 2009)	Generated via GEO2R, Default method using Benjamini & Hochberg p-value adjustment
	TAM ("tumor-associated macrophages"): GSM458919, GSM458920, GSM458921, GSM458922	Up in TAM: 236 top genes
	Control ("splenic macrophages"): GSM458923, GSM458924, GSM458925, GSM458926	Down in TAM: 236 bottom genes
Murine bone marrow tumor-associated macrophages *versus* bone marrow tissue macrophages	Series: GSE57785 (Galletti *et al*, 2016)	Generated via GEO2Enrichr, Characteristic Direction method
	TAM ("Monocytes/macrophages With MEC1 21d"): GSM1388466, GSM1388467, GSM1388468	Up in TAM: 240 top genes
	Control ("Monocytes/macrophages No MEC1 21d"): GSM1388463, GSM1388464, GSM1388465	Down in TAM: 260 bottom genes
Human Actinic Keratosis (AK)	Series: GSE2503 (Nindl *et al*, 2006)	Generated via GEO2R, Default method using Benjamini

versus normal skin (NS)		& Hochberg p-value adjustment
	AK ("cDNA ak"): GSM47612, GSM47613, GSM47614, GSM47615	Up in AK: 234 top genes
	NS ("cDNA no"): GSM47616, GSM47617, GSM47618, GSM47619	Down in AK: 174 bottom genes
Human Actinic Keratosis (AK) *versus* normal skin (NS)	Series: GSE63107	Generated via GEO2R, Default method using Benjamini & Hochberg p-value adjustment
	AK ("involved skin-pretreatment"): GSM1541373, GSM1541378, GSM1541383, GSM1541388, GSM1541393, GSM1541398	Up in AK: 394 top genes
	NS ("uninvolved skin-pretreatment"): GSM1541371, GSM1541376, GSM1541381, GSM1541386, GSM1541391, GSM1541396	Down in AK: 61 bottom genes
Monocytes sorted from human peripheral blood, differentiated into macrophages and differentially activated *in vitro*	Series: GSE46903 (Xue *et al*, 2014)	Calculated from Table S1D: IFNg/control > 1.20: 78 top genes IL4/control > 1.20: 36 top genes TNF/control > 1.20: 79 top genes LPS/control > 1.20: 120 top genes
	Data taken directly from Supplementary Table S1: IFNg: M_GMCSF_IFNγ_72h IL4: M_GMCSF_IL4_72h TNF: M_GMCSF_TNF_72h LPS: M_GMCSF_sLPS_72h Control: M_GMCSF_baseline_72h	
Human macrophage *versus* monocyte, dendritic cell, T cell signatures	Series: GSE46903 (Xue *et al*, 2014)	Generated via GEO2R, Default method using Benjamini & Hochberg p-value adjustment
	Macrophage: GSM numbers associated with "M_GMCSF_baseline_0h"	Monocyte vs macrophage: 95 top genes Dendritic cell vs macrophage: 210 top genes T-cell vs macrophage: 163 top genes Macrophages vs monocyte, dendritic cell, T-cell:
	Monocyte: GSM numbers associated with "Monocyte_CD14+"	
	Dendritic cell: GSM numbers associated with "DC_imm"	

	T cell: GSM numbers associated with “Tconv” and “Treg”	97 top genes (shared by at least two comparisons of macrophage vs other cell type)

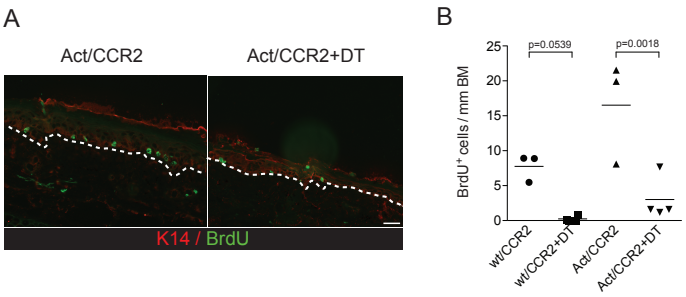


Appendix Fig S1. Original flow cytometry data. Representative flow cytometry scatter plots for quantified data shown in Fig 2D-F (A-C), Fig 3C (D), and Fig 3E (E).



Appendix Fig S2. Activin induces expression of *Cx3c1* and *Arg1* in skin macrophages *in vivo*

(A, B) Expression of *Cx3cr1* (A) and *Arg1* (B) relative to *Rps29* was analyzed by qRT-PCR in $F4/80^{+}CD11b^{+}$ cells, FACS-sorted in 3 additional independent experiments from pools of 2-6 mice. Expression in one of the wt/wt samples was set to 1. N=2 for all groups in (A); N=3 for all groups in (B). One-sample t test was performed after log-transformation of expression data.



Appendix Fig S3. Macrophage depletion in Act/CCR2 mice reduces keratinocyte proliferation

(A) Representative pictures of anti-BrdU (green) and anti-K14 (red) stained cryosections of ear skin from 12 weeks old Act/CCR2 mice, untreated (control) or treated with diphtheria toxin (+DT) 3 times every 48h and analyzed 24h after the last injection. Scale bar: 50µm. Quantification of BrdU+ keratinocytes per mm of basement membrane is shown in (B). N=3 control mice; N=4 DT-injected mice. Statistical significance was performed using 1-way ANOVA and Bonferroni`s Multiple Comparison test.

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