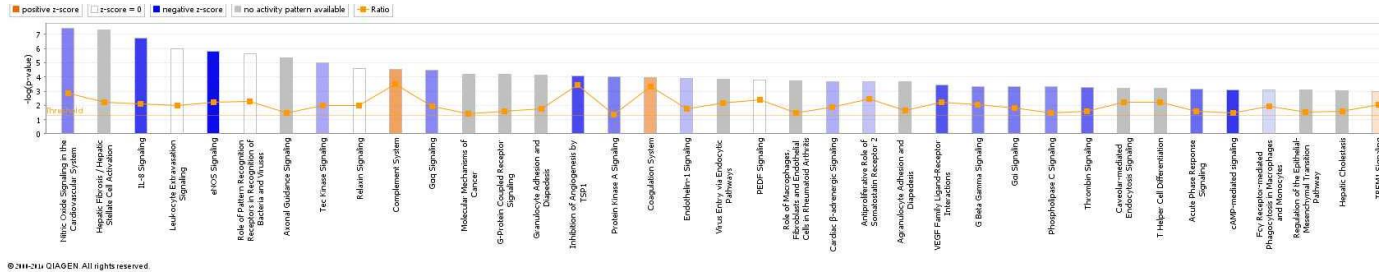


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Supplemental Information

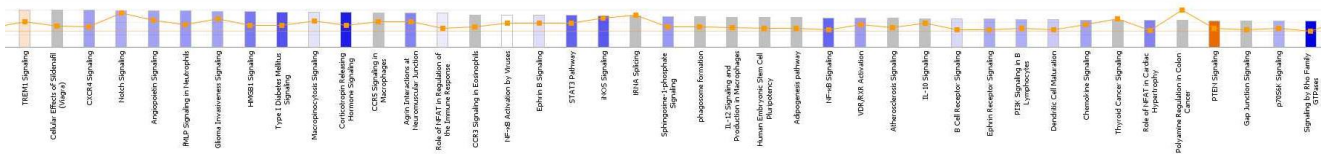
High Expression of CD200 and CD200R1 Distinguishes Stem and Progenitor Cell Populations within Mammary Repopulating Units

Gat Rauner, Tania Kudinov, Shlomit Gilad, Gil Hornung, and Itamar Barash

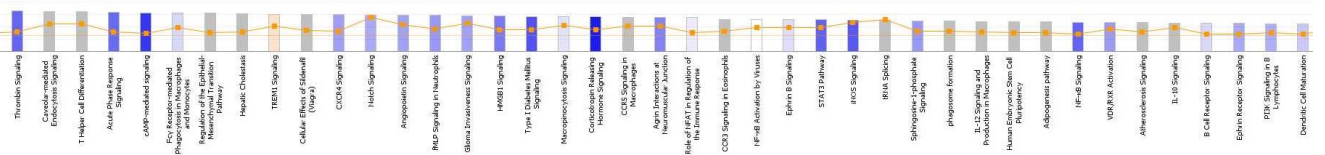


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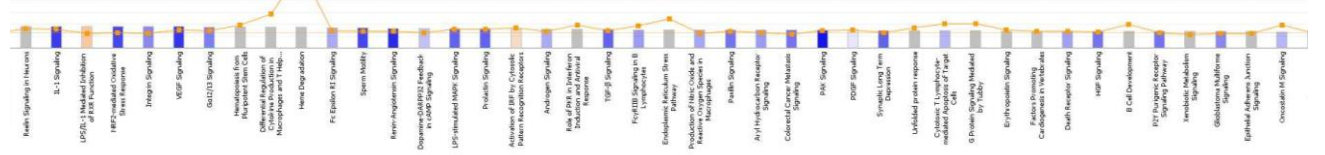
Cont.



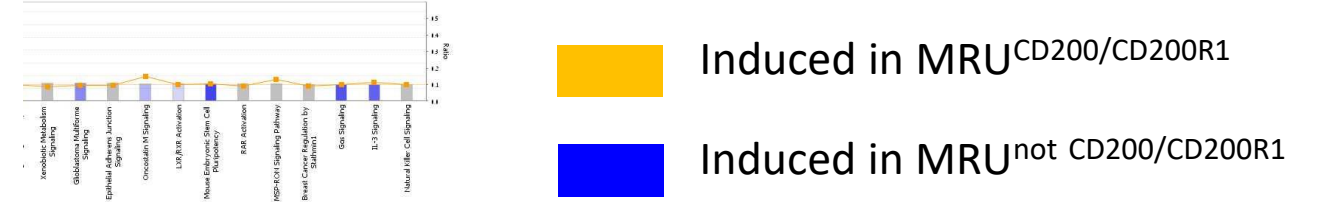
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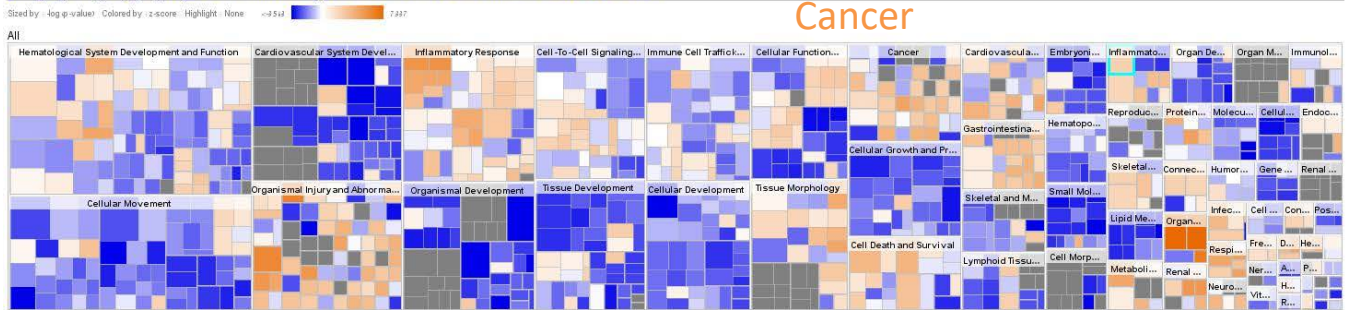


Induced in MRU^{CD200/CD200R1}
 Induced in MRU^{not CD200/CD200R1}

Figure S1. Canonical Pathway Analyzed by IPA. Pathways that are Highly Induced in each of the MRU Subpopulations Relative to their Counterpart Are Marked. Related to Figure 2, and Table 1.

Inflammatory response

both_vs_none_only_for_IPA_270116 fold 1.5 adj p 0.07- 2016-01-27 01:56 PM - Diseases & Functions



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Cellular movement

Organismal injury

Tissue devel.
Cellular devel.

Cell growth and prolif.
Cell death

Highly activated in MRU^{CD200/CD200R1}

Highly activated in MRU^{not CD200/CD200R1}

Figure S2. IPA Analysis of Genes with Divergent Expression

between MRU^{CD200/CD200R1} and MRU^{not CD200/CD200R1} that Maintain

Different Diseases and Functions. Related to Figure 2 and Table 1.

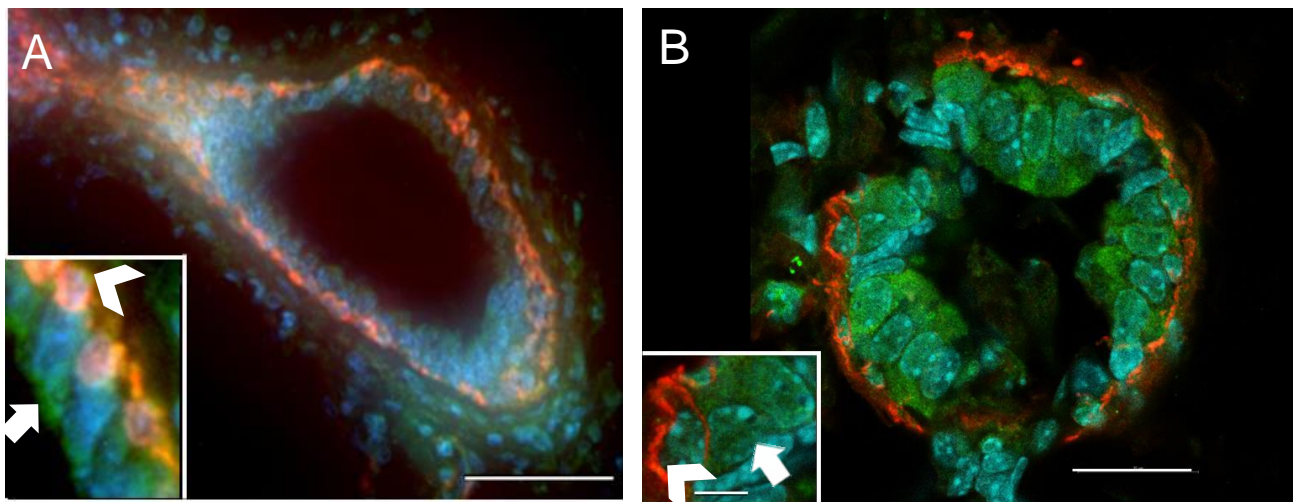


FIGURE S3. CD200 and CD200R1 are Ubiquitously Expressed in Basal and Luminal Cells of the Mammary Gland.

(A, B) Immunofluorescence analysis of FITC-labeled CD200/CD200R1 (green) and Cy3-labeled α SMA (red) expression in ductal structures of the virgin gland.

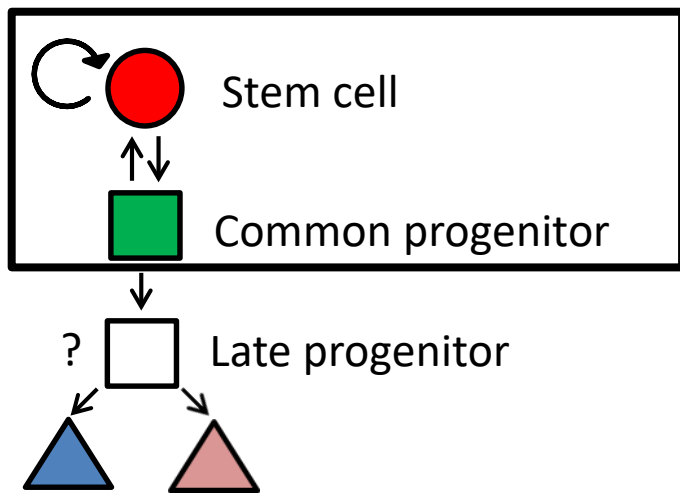
(A) CD200 is expressed at different levels in luminal and basal cells as well in the stroma. Bar = 50 μ m. Inset x10 magnification. CD200-expressing basal cells are marked by arrowheads.

CD200-expressing luminal cells are marked by arrow.

(B) CD200R1 expression is detected in both luminal and α SMA-expressing basal cells. Bar in main figure = 20 μ m. Bar in inset = 5 μ m. CD200R1-expressing luminal cells are marked by arrow.

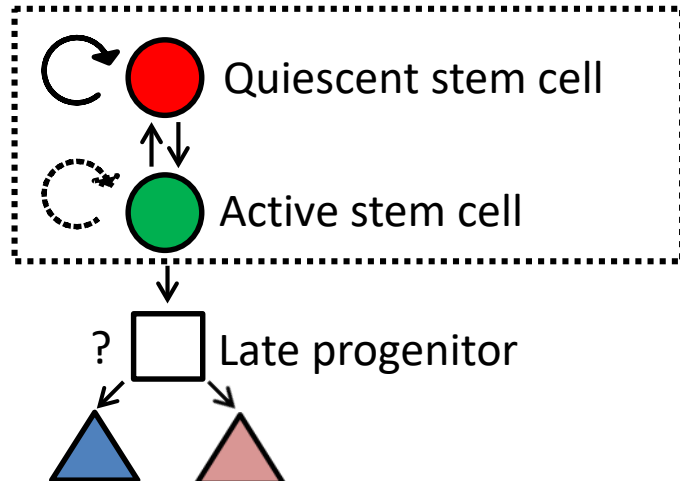
CD200R1-expressing basal cells are marked by arrowheads. Related to Figure 1.

A Stem cell and common progenitor



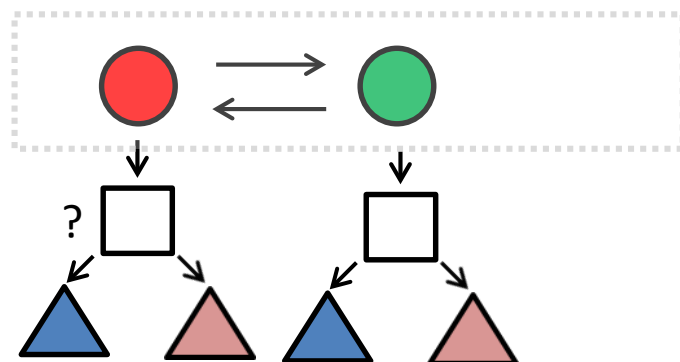
$MRU^{CD200/CD200R1}$ (stem cells, red) maintain self-renewal capability and multipotency, have higher mammosphere generation and are more basally oriented than $MRU^{not\ CD200/CD200R1}$ (common progenitors, green). They share stem cell markers. $MRU^{not\ CD200/CD200R1}$ (common progenitors, green) are multipotent but lack self-renewal capability. They are present in higher numbers after transplantation and have better differentiation capability than $MRU^{CD200/CD200R1}$. Supported by earlier studies (Rios et al., 2014).

B Quiescent and active stem cell



Quiescent and active stem cells have been characterized by dos Santos et al. (2013). The “active stem cells” (here, $MRU^{not\ CD200/CD200R1}$, green) do not share stem cell markers and do not maintain self-renewal capability. They differ in their basal origin from the quiescent stem cells (here, $MRU^{CD200/CD200R1}$, red).

C Two active stem cells



The two active stem cells should maintain comparable characteristics. However, self-renewal capability and stem cell markers are not shared by both active stem cells [here, $MRU^{CD200/CD200R1}$ (red) and $MRU^{not\ CD200/CD200R1}$ (green)]. There is a significant difference in the expression of genes leading to differentiation. The stem cells differ in basal origin and in mammosphere generation.

Figure S4. A Scheme of the Proposed Relationship Between $MRU^{CD200/CD200R1}$ and $MRU^{not\ CD200/CD200R1}$ in Composing the MRU Population.

A. Evidence supporting our view of a stem cell-common progenitor relationship within the MRU is in black font.

B,C. Evidence negating the alternative possibilities is in gray font. Dashed arrow indicates that this possibility cannot be formally ruled out. Related to all Figures and Tables.

Table S1. Outgrowth Development in the Host Cleared Fat Pad after Serial Transplantations^a. Related to Figure 1.

Transplantation	Number of cells transplanted	Type of cells transplanted	Number of MRUs	Number of outgrowth-developing glands		P value
				MRU ^{CD200/CD200R1}	MRU ^{not CD200/CD200R1}	
1st	40	MRU	40	6/16 (38%)	4/14 (29%)	0.6
2nd	1000	Total mammary cell population	20 (estimated)	2/18 (11%)	0/16 (0%)	< 0.0001

^aInitial transplantation was performed with sorted MRUs. The subsequent transplantation included dispersed mammary cells. These cells originated from small sections of outgrowth-containing tissue that were isolated under the binocular. The estimated number of MRUs in the total mammary cell populations is based on flow cytometry analyses indicating $57.0 \pm 1.6\%$ ($n = 3$) of epithelial cells in the total dispersed cells, of which $3.5 \pm 1.0\%$ ($n = 3$) are MRUs. *P* value was determined by Chi square (Pearson) analysis.

Table S2. Genes with Hierarchical-Dependent Expression in this Study^a.
Related to Figure 3 and Table 2.

Gene	Gene ID
<i>Nalcn</i>	ENSMUSG00000000197
<i>Grik3</i>	ENSMUSG00000001985
<i>Ltbp2</i>	ENSMUSG00000002020
<i>Mrv1</i>	ENSMUSG00000005611
<i>Fblim1</i>	ENSMUSG00000006219
<i>Dll1</i>	ENSMUSG00000014773
<i>list</i>	ENSMUSG00000015143
<i>Matn4</i>	ENSMUSG00000016995
<i>Stac2</i>	ENSMUSG00000017400
<i>Jph2</i>	ENSMUSG00000017817
<i>Ctgf</i>	ENSMUSG00000019997
<i>Hal</i>	ENSMUSG00000020017
<i>Kcnmb1</i>	ENSMUSG00000020155
<i>Smtn</i>	ENSMUSG00000020439
<i>Id4</i>	ENSMUSG00000021379
<i>Irx4</i>	ENSMUSG00000021604
<i>Ctnnd2</i>	ENSMUSG00000022240
<i>Trp63</i>	ENSMUSG00000022510
<i>Popdc2</i>	ENSMUSG00000022803
<i>Mylk</i>	ENSMUSG00000022836
<i>Il17b</i>	ENSMUSG00000024578
<i>Col17a1</i>	ENSMUSG00000025064
<i>Vwa2</i>	ENSMUSG00000025082
<i>Gfra1</i>	ENSMUSG00000025089
<i>1500015O10Rik</i>	ENSMUSG00000026051
<i>Wnt10a</i>	ENSMUSG00000026167
<i>Pkp1</i>	ENSMUSG00000026413
<i>Csrp1</i>	ENSMUSG00000026421
<i>Fcna</i>	ENSMUSG00000026938
<i>Itga6</i>	ENSMUSG00000027111
<i>Fermt1</i>	ENSMUSG00000027356
<i>Col9a3</i>	ENSMUSG00000027570
<i>Col11a1</i>	ENSMUSG00000027966
<i>Pdgfc</i>	ENSMUSG00000028019
<i>Pappa</i>	ENSMUSG00000028370
<i>Tpm2</i>	ENSMUSG00000028464
<i>Col9a2</i>	ENSMUSG00000028626
<i>Tfap2c</i>	ENSMUSG00000028640
<i>Cda</i>	ENSMUSG00000028755
<i>Plch2</i>	ENSMUSG00000029055
<i>Bst1</i>	ENSMUSG00000029082

<i>Shroom3</i>	ENSMUSG00000029381
<i>Sym</i>	ENSMUSG00000030554
<i>Micalcl</i>	ENSMUSG00000030771
<i>Col4a6</i>	ENSMUSG00000031273
<i>Col4a5</i>	ENSMUSG00000031274
<i>Trim29</i>	ENSMUSG00000032013
<i>Tagln</i>	ENSMUSG00000032085
<i>Elovl4</i>	ENSMUSG00000032262
<i>Ephb1</i>	ENSMUSG00000032537
<i>Folr2</i>	ENSMUSG00000032725
<i>Lama1</i>	ENSMUSG00000032796
<i>Fhod3</i>	ENSMUSG00000034295
<i>Ccdc106</i>	ENSMUSG00000035228
<i>Kank4</i>	ENSMUSG00000035407
<i>Fgf1</i>	ENSMUSG00000036585
<i>Arhgef26</i>	ENSMUSG00000036885
<i>Frem2</i>	ENSMUSG00000037016
<i>Zfp365</i>	ENSMUSG00000037855
<i>Trpm4</i>	ENSMUSG00000038260
<i>Scube3</i>	ENSMUSG00000038677
<i>Nexn</i>	ENSMUSG00000039103
<i>Apoc1</i>	ENSMUSG00000040564
<i>Fzd7</i>	ENSMUSG00000041075
<i>Sdk2</i>	ENSMUSG00000041592
<i>Ptpre</i>	ENSMUSG00000041836
<i>Lgr6</i>	ENSMUSG00000042793
<i>Tceal3</i>	ENSMUSG00000044550
<i>Defb1</i>	ENSMUSG00000044748
<i>Plekha7</i>	ENSMUSG00000045659
<i>Lrrc75b</i>	ENSMUSG00000046807
<i>Atxn1</i>	ENSMUSG00000046876
<i>Lmod1</i>	ENSMUSG00000048096
<i>Oxtr</i>	ENSMUSG00000049112
<i>Lrp1b</i>	ENSMUSG00000049252
<i>Tenm2</i>	ENSMUSG00000049336
<i>Tmem200a</i>	ENSMUSG00000049420
<i>Lgr4</i>	ENSMUSG00000050199
<i>Cd209f</i>	ENSMUSG00000051906
<i>Nav2</i>	ENSMUSG00000052512
<i>Cntn2</i>	ENSMUSG00000053024
<i>Hunk</i>	ENSMUSG00000053414
<i>Lgals7</i>	ENSMUSG00000053522
<i>Plxnb1</i>	ENSMUSG00000053646
<i>1600014C10Rik</i>	ENSMUSG00000054676
<i>Dsp</i>	ENSMUSG00000054889
<i>Fat2</i>	ENSMUSG00000055333
<i>Timd4</i>	ENSMUSG00000055546

<i>Trnp1</i>	ENSMUSG00000056596
<i>Cdh3</i>	ENSMUSG00000061048
<i>Retnla</i>	ENSMUSG00000061100
<i>Krt5</i>	ENSMUSG00000061527
<i>Hs6st2</i>	ENSMUSG00000062184
<i>Nrg1</i>	ENSMUSG00000062991
<i>Kcnma1</i>	ENSMUSG00000063142
<i>Luzp2</i>	ENSMUSG00000063297
<i>Chil1</i>	ENSMUSG00000064246
<i>Cd209b</i>	ENSMUSG00000065987
<i>Hs3st3b1</i>	ENSMUSG00000070407
<i>Npw</i>	ENSMUSG00000071230
<i>Tmem88b</i>	ENSMUSG00000073680
<i>Ntf5</i>	ENSMUSG00000074121
<i>Cd209g</i>	ENSMUSG00000079168
<i>Frpm1os</i>	ENSMUSG00000086284
<i>Mia</i>	ENSMUSG00000089661
<i>Gm15867</i>	ENSMUSG00000089812
<i>Gm21655</i>	ENSMUSG00000096878
<i>Gm26615</i>	ENSMUSG00000097088
<i>A330048O09Rik</i>	ENSMUSG00000097326
<i>Gm26813</i>	ENSMUSG00000097819
<i>4631405K08Rik</i>	ENSMUSG00000097850
<i>Gm8579</i>	ENSMUSG00000099762
<i>Gm29157</i>	ENSMUSG00000100635
<i>Gm21190</i>	ENSMUSG00000106445

^aGene expression decreased from MRU^{CD200/CD200R1} via MRU^{not CD200/CD200R1} toward CD200⁺- and CD200R1⁺-expressing cells.

Table S3. Genes with Hierarchical-Dependent Pattern of Expression that also Belong to the Previously Published Mammary/Breast Stem Cell Subset¹, or Mark Stem Cells in Other Tissues. Related to Figure 4 and Table 2.

Gene ID	Gene name	Symbol	Log ₂ fold change MRU ^{CD200/CD200R1} / MRU ^{not CD200/CD200R1}
ENSMUSG00000061527	keratin 5	<i>Krt5</i>	0.54863591
ENSMUSG00000034295	formin homology 2 domain containing 3	<i>Fhod3</i>	0.75778299
ENSMUSG00000022510	tumor protein p63	<i>Trp63</i>	0.7799422
ENSMUSG00000025064	collagen, type XVII, alpha 1	<i>Col17a1</i>	0.61716078
ENSMUSG00000027470	myosin light chain kinase	<i>Mylk</i>	0.95655246
ENSMUSG00000028464	tropomyosin 2 (beta)	<i>Tpm2</i>	0.50264019
ENSMUSG00000032085	transgelin	<i>Tagln</i>	0.53413686
ENSMUSG00000048096	leiomodulin 1 (smooth muscle)	<i>Lmod1</i>	0.58008322
ENSMUSG00000005611	murine retrovirus integration site 1 homolog	<i>Mrvi1</i>	0.42691702
ENSMUSG00000030554	synemin, intermediate filament protein	<i>Synm</i>	0.56189161
ENSMUSG00000030554	smoothelin	<i>Smtn</i>	0.56189161
ENSMUSG00000032013	tripartite motif containing 29	<i>Trim29</i>	0.63381269
ENSMUSG00000061048	cadherin 3, type 1, P-cadherin (placental)	<i>Cdh3</i>	0.83388473
ENSMUSG00000021604	iroquois homeobox 4	<i>Irx4</i>	0.70640527
ENSMUSG00000026413	plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)	<i>Pkp1</i>	0.81727681
ENSMUSG00000021379	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	<i>Id4</i>	0.70828064
ENSMUSG00000026051	chromosome 2 open reading frame 40	<i>1500015O1ORik</i>	0.8787596
ENSMUSG00000063142	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	<i>Kcnma1</i>	0.79965976
ENSMUSG00000062991	neuregulin 1	<i>Nrg1</i>	0.40250429
ENSMUSG00000019997	connective tissue growth factor	<i>Ctgf</i>	0.55736619
ENSMUSG00000028626	collagen, type IX, alpha 2	<i>Col9a2</i>	0.59256227
ENSMUSG00000032796	laminin, alpha 1	<i>Lama1</i>	0.80467718
	lectin, galactoside-binding, soluble, 7	<i>Lgals7</i>	0.52174104
ENSMUSG00000024578	interleukin 17B	<i>Il17b</i>	0.57630163
ENSMUSG00000089661	melanoma inhibitory activity	<i>Mia</i>	0.7526307
ENSMUSG00000027111	integrin, alpha 6	<i>Itga6</i>	0.47572854
ENSMUSG00000041836	protein tyrosine phosphatase, receptor type E	<i>Ptpre</i>	0.27070909
ENSMUSG00000032262	ELOVL fatty acid elongase 4	<i>Elovl4</i>	0.55469019
ENSMUSG00000049112	oxytocin receptor	<i>Oxtr</i>	0.70664276
ENSMUSG00000032537	EPH receptor B1	<i>Ephb1</i>	0.60780788
ENSMUSG00000017400	SH3 and cysteine rich	<i>Stac2</i>	0.48402756

	domain 2				
ENSMUSG00000035407	KN motif and ankyrin repeat domains 4	<i>Kank4</i>	0.70168944		
ENSMUSG00000014773	delta-like 1 (<i>Drosophila</i>)	<i>Dll1</i>	0.57855187		
ENSMUSG00000020155	potassium large conductance calcium-activated channel, subfamily M, beta member 1	<i>Kcnmb1</i>	0.73793513		
ENSMUSG00000042793	leucine-rich repeat containing G protein-coupled receptor 6	<i>Lgr6</i>	0.50525771		
ENSMUSG00000054676	chromosome 19 open reading frame 12	<i>1600014C1</i> <i>ORik</i>	0.61677566		
ENSMUSG00000029055	phospholipase C, eta 2	<i>Plch2</i>	0.71703952		
ENSMUSG00000041592	sidekick homolog 2 (chicken)	<i>Sdk2</i>	0.56928968		
ENSMUSG00000022803	Popeye domain containing 2	<i>Popdc2</i>	0.68476		
Gene ID	Gene name	Symbol	Log ₂ fold change: MRU ^{CD200/} CD200R1/ MRU ^{not} CD200/CD200R1	Tissue	Reference
ENSMUSG00000002020	latent transforming growth factor beta binding protein 2	<i>Ltbp2</i>	0.76	Chondrocytes	Goessler et al., 2005
ENSMUSG00000020017	histidine ammonia-lyase	<i>Hal</i>	2.29	Xenopus laevis intestine	Luu et al., 2013
ENSMUSG00000026167	Wnt family member 10a	<i>Wnt10A</i>	0.63	Esophageal squamous cell carcinoma	Long et al., 2015
ENSMUSG00000029082	bone marrow stromal cell antigen 1	<i>Bst1</i>	0.86	Mesenchyma	Aumatuso et al., 2014
ENSMUSG00000041075	frizzled class receptor 7	<i>Fzd7</i>	0.98	Intestinal epithelial Lgr5 ⁺ cells	Flanagan et al., 2015
ENSMUSG00000050199	leucine-rich repeat-containing G-protein-coupled receptor 4	<i>Lgr4</i>	0.63	Embryonic intestine	Kinzel et al., 2014
ENSMUSG00000056596	TMF1-regulated nuclear protein 1	<i>Tmp1</i>	0.56	Cerebral cortex	Sthal et al., 2013

¹Lim et al., 2010

Table S4. Validation of Proposed Cell Hierarchy^a. Related to Figures 3 and to Table 2.

#	Source of genes in set 1	Number of genes in set 1	Source of genes in set 2	Number of genes in set 2	Number of common genes	Significance (in set of 20,000 genes)
1	Current analysis: Stem cell subset	114	Stem cell subset (Lim et al., 2010)	489	39	$P = 2.75 \times 10^{-34}$
2	Current analysis: progenitor subset	262	Stem cell subset (Lim et al., 2010)	489	20	$P = 5 \times 10^{-6}$
3	Current analysis: differentiated cell subset	97	Stem cell subset (Lim et al., 2010)	489	10	$P = 1 \times 10^{-4}$

^aThe gene set identifying stem cells is much more compatible with the previously published stem cell list (Lim et al., 2010) than the progenitor and differentiated cell subsets.

Table S5. Transplantation of MRU^{CD200/CD200R1} population results in outgrowths containing MRU^{not CD200/CD200R1} and vice versa. Related to Figures 1,3 and 5.

Population	MRU composition in the resulting outgrowths (%)	
	MRU ^{CD200/CD200R1}	MRU ^{not CD200/CD200R1}
Control	17.0	83.0
Transplanted MRU ^{CD200/CD200R1}	14.2	85.8
Transplanted MRU ^{not CD200/CD200R1}	28.1	71.9

MRU^{CD200/CD200R1} and MRU^{not CD200/CD200R1} populations were sorted from depressed mammary epithelial cells collected from 36 #4 mammary glands as described in Experimental procedures. From each population, 100 cells were transplanted into each one of 10 de-epithelized mammary fat pads of recipient virgin females. Eight weeks after transplantation, fat pads of each group were collected and digested for dispersed epithelial cells. These were sorted for the presence of MRU^{CD200/CD200R1} and MRU^{not CD200/CD200R1} together with cells extracted and pooled from six #4 mammary glands of 6-week-old control virgin mice.

Table S6. List of Antibodies Used in this Study.

Antigen/ Purpose	1 st Antibody and dilution	Manufacturer	2 nd antibody and dilution	Manufacturer
CD24 FACS	PE-conjugated, rat monoclonal, clone M1/69 (1:30)	StemCell Technologies		
CD49f FACS	FITC-conjugated, rat monoclonal, clone GoH3 (1:30)	StemCell Technologies		
CD200 FACS	APC-conjugated, rat monoclonal, clone OX-90 (1:30)	BioLegend, San Diego, CA		
CD200R1 FACS	Alexa Fluor 700- conjugated, rat monoclonal, clone OX-110 (1:30)	Novus Biologicals, Littleton, CO		
CD200 IP	FITC-conjugated rat monoclonal LS- C534810 (1:50)	LSBio, Seattle, WA		
CD200R1 IP	Rabbit polyclonal LS-C378947 (1:50)	LSBio	Alexa Flour 488 goat anti rabbit A1108 (1:500)	Life Technology Paisley, UK
α SMA IP	Mouse monoclonal SC-32251 (1:75)	Santa Cruz, Dallas, TX	Cy3 goat anti mouse 115-165- 003 (1:100)	Jackson ImmunoResearch Baltimore Pike, PA
α SMA IHC	Mouse monoclonal SC-32251 (1:75)	Santa Cruz	EnVision- labeled HRP polymer	Dako Cytomation, Glostrup, Denmark
β -casein IHC	Rabbit monoclonal (1:100)	Barash's Lab.	EnVision- labeled HRP polymer	Dako Cytomation,
CK18 IP	Mouse monoclonal (ab668) (1:100)	Abcam, Cambridge, UK	Cy3 goat anti mouse 115-165- 003 (1:100)	Jackson ImmunoResearch
CK14 IP	Mouse monoclonal clone LL002 (1:50)	Biorad, Perth, UK	Cy3 goat anti mouse 115-165- 003 (1:100)	Jackson ImmunoResearch

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Dissociation of Mammary Tissue into Single-Cell Suspension and Flow Cytometry

Mouse mammary cells were dissociated from the fourth inguinal mammary glands of 6- to 8-week-old FVB/N virgin females. The dissociation procedure was as previously described (Rauner and Barash, 2012), except that enzymatic digestion was shortened from 3 h to 1 h. Lin⁻ cell suspension was prepared using the EasySep mouse mammary enrichment kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer's protocol, as previously described (Rauner and Barash, 2012). Epithelial cells were resuspended in HF solution containing Hank's balanced salt solution (Biological Industries, Bet-Haemek, Israel) supplemented with 0.1% (v/v) Hepes (Biological Industries) and 2% (v/v) fetal bovine serum (Biological Industries) at a concentration of 10⁷ cell/ml, and incubated for 90 min on ice with fluorescent conjugated antibodies. The antibodies and their dilutions are listed in Table S6. Cell viability was tested by staining with BD Horizon Fixable Viability Stain 450 (FVS450, BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Cell sorting and cell analyses were performed in a FACS Aria II or III cell sorter and LSR II cell analyzer (BD Biosciences) at the Department of Biological Services of the Weizmann Institute of Science (Rehovot, Israel). Resulting data were visualized and analyzed by FACSDiva (BD Biosciences) and WinMDI 2.9 software (Scripps Research Institute, La Jolla, CA).

Sorting MRU^{CD200/CD200R1} and MRU^{not CD200/CD200R1} populations:

Live MRU^{CD200/CD200R1} and MRU^{not CD200/CD200R1} cells were sorted from Lin⁻ mammary epithelial cells that were simultaneously analyzed for CD49f, CD24, CD200, CD200R1 and FVS450 (which separates live from dead cells). CD200^{high}CD200R1^{high} cells which express both proteins at high levels were gated. The gated CD200^{high}CD200R1^{high} population was projected onto the plot depicting expression of CD24 and CD49f, to collect only those CD200^{high}CD200R1^{high} cells that were also MRU (MRU^{CD200/CD200R1}). The rest of the MRUs are referred as MRU^{not CD200/CD200R1}.

Some of the CD200^{high}CD200R1^{high} cells were not MRU (and were not collected), but rather demonstrate a CD49f^{high}/CD24^{low} phenotype, suggesting that they may be myoepithelial cells (Stingl et al. 2006, Nature 23:439). We did not identify CD200^{high}CD200R1^{high} cells expressing CD49f^{med/low}/CD24^{med/high} as would be expected from luminal cells or luminal progenitors (Stingl et al. 2006, Nature 23:439).

Cell Transplantation

The endogenous mammary epithelium was surgically removed bilaterally from #4 mammary glands of 21-day-old female mice weighing 10–12 g (i.e., "clearing"). Sorted mammary epithelial cell populations were resuspended in 1:1 (v/v) HF solution:Matrigel (BD Biosciences) and the cell suspension (20 µl) was injected into the cleared mammary fat pad using a 50-µl Hamilton syringe (Hamilton Company, Reno, NV) equipped with a 21-gauge needle. Outgrowths were allowed to develop for 8.5 weeks before the transplanted fat pad was removed.

Serial Transplantation

A similar transplantation procedure was performed for serial transplantations of CD200^{high}/CD200R1^{high} MRUs. Host mice were C57BL and donor mice were C57/GFP [ubiquitin-EGFP mice (Reichenstein et al., 2016)] that express enhanced GFP under the direction of the human ubiquitin C promoter in all tissues; the expression is uniform within a cell-type lineage and remains constant throughout development. GFP-expressing primary outgrowths were visualized in whole mounts of host mammary fat pad using a binocular (Olympus SZX16, Tokyo, Japan) equipped with CellSens standard 1.4 software (Olympus). The observed outgrowths were dissected from the host fat pad and dissociated into single cells as described above. The single-cell suspension inevitably included non-GFP-expressing stromal cells from the host mouse. Cells (n = 1000) were transplanted as described above into the cleared fat pad of C57BL secondary hosts, and developed outgrowths were visualized after 7 weeks using a binocular equipped with CellSens standard 1.4 software.

Outgrowth Analysis

For whole-mount examination, transplanted mammary fat pads were excised from sacrificed mice, fixed and stained with Carmine Red as previously described (Rauner et al., 2013). Stained whole mounts were visualized and photographed using the binocular equipped with CellSens standard 1.4 software.

Histological Analysis and Immunostaining

Immunostaining of paraformaldehyde-fixed cells in culture was performed as previously described (Rauner and Barash, 2012). Tissue immunostaining was performed on paraffin-embedded (Rauner and Barash, 2012) or frozen tissue sections. For the latter, tissue biopsies were fixed in 4% paraformaldehyde for 24 h at 4°C and then transferred to 15% and 30% sucrose solution for 6 h and 24 h, respectively. Tissues were submerged in OCT compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and stored at -80°C. Immunostaining was performed on 5-mm paraffin-embedded or frozen sections. Antigen retrieval for paraffin-embedded sections was performed by boiling in 0.01 M citrate buffer for 10 min. For frozen sections, it was performed by submerging the slides in 1% SDS solution pH 7.4. The reactions with primary and secondary fluorescence-labeled antibodies (Table S6) followed the protocol described in (Rauner and Barash, 2012). For immunohistochemistry, paraffin-embedded sections were treated with 3% hydrogen

peroxide for 30 min and boiled for 10 min in 0.01 M citrate. Sections were incubated with the primary antibody (Table S6), followed by incubation with EnVision-labeled HRP polymer (Dako Cytomation, Glostrup, Denmark) for 1 h at room temperature. Signal was generated with 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol.

Clonal and Mammosphere Assays

For clonal assay, sorted or unsorted cells were seeded in a 24-well culture plate (Corning, Lowell, MA) at a density of 2500 cell/well and cultured for 7 days, in mammary medium (Rauner and Barash, 2012). Developing colonies were fixed in 4% paraformaldehyde supplemented with 0.03 M sucrose, permeabilized with 0.5% Triton X-100 and stained with antibodies to CK14 or CK18 as described in the Histological Analysis and Immunostaining section. Clones consisting of at least three individual adjacent cells were counted using an inverted fluorescence microscope (Eclipse Ti, Nikon Instruments, Melville, NY) and characterized as luminal or basal.

For mammosphere assay, sorted populations were individually seeded in 96-well ultra-low-attachment plates (Corning) at a density of 100 cell/well and supplemented with mammary medium (Rauner and Barash, 2012) for 8 days. Developing mammospheres were analyzed and counted as described above.