Mammalian Target of Rapamycin Regulates Cell Differentiation through STAT3-p63-Jagged-Notch Cascade

Supplemental Data

Plasmid generation: pLXIN-hyg-*Jagged1* was made by the insertion of a *Jagged1* PCR fragment, amplified from MEFs cDNA using primers of 5'-aatctcgagatgcggtccccacggacgc-3' and 5'-tatgcggccgcctatacgatgtattccatccg-3', digested by Xho I and Not I, into a modified pLXIN retroviral vector with a hygromycin resistance gene (pLXIN-hyg) (1). Similarly, pLXIN-hyg-*PTEN* was created with insertion of a Xho I/Not I digested PCR product of human *PTEN* cDNA using primers 5'-tgacctcgagatgacagccatcatc- 3' and 5'- tcatgcggccgctcagacttttgtaat-3' into pLXIN-hyg.

E17K *AKT1* mutagenesis: A retroviral plasmid expressing human AKT1 (E17K mutant) (2) was generated first by overlapping PCR amplification of *AKT1* from pLNCX-HA-AKT1 with PfuUltra™ High-Fidelity DNA Polymerase (Stratagene). Primers P1 (5'-gcgtggatagcggtttgactc-3') and P2 (5'-gatgtacttccctcgtttg-3') were used to amplify a DNA fragment covering the fragment of pLNCX sequences upstream of 5' *AKT1* and *AKT1* (1-57). P3 (5'-caaacgagggaagtacatc-3') and P4 (5'-tgaatcgattcaggccgtgccgctggc-3') were for the remaining *AKT1* sequences (33–1433). The two PCR products were then mixed as templates and amplified with P5 (5'-tcaggatccatgagcgacgtggctattgtg-3') and P4 to generate the full-length *AKT1E17K* mutant. The mutant *AKT1* digested with Bam H I and Cla I was inserted into pLXIN-hyg to create the pLXIN-hyg-*AKT1*(E17K) plasmid.

Virus production. pLXIN-hyg, pLXIN-hyg-*AKT1E17K*, pLXIN-hyg-*PTEN*, pLXIN-hyg-*Jagged1*, pMSCV-*PPARγ*, pMSCV-*MyoD*, or pMSCV plasmids were transfected into the retroviral packaging cell line PT67 (Clontech) using Lipofectamine 2000. Conditioned culture medium containing viruses was filtered through a 0.45-μm filter and then used to transduce MEF cells. The transduced cells were selected with 100 μg/ml hygromycin B or 2 μg/mL puromycin for stably expressing cells. The lentivirus expressing a dominant negative form of MAML1 for blocking Notch signaling (3) was generated as follows: 293FT cells in 10-cm plates were co-transfected with pMIG-DNL1 or control pMIG vector in addition to help plasmids delta 8.9 and VSVG in a ratio of 4:3:1. Conditioned culture medium containing viruses was harvested and then filtered through a 0.45-μm filter for transduction of MEF cells. High efficiency of transduction was achieved by repeatedly infecting the targeted cells with these lentiviruses.

Muscle differentiation. MEF cells were first transduced with pMSCV-*MyoD* retroviruses or pMSCV control viruses and selected for stably expressing cells with 2 μg/mL puromycin for 2 weeks. The MyoD expressing MEF cells seeded in 6-well plates were induced for muscle differentiation by switching to differentiation medium (DMEM containing 2% horse serum and 10μg/ml insulin), with or without rapamycin treatment, for up to 6 days. The differentiation medium was changed every two days. Cells were photographed for documentation and then harvested for immunoblotting.

Adipocyte differentiation. MEF cells were first transduced with pMSCV-*PPARγ*

retroviruses or pMSCV control viruses and then selected for stably expressing clones with 2 μg/mL puromycin. The cells with PPARγ expression were induced to differentiate into adipocytes (defined as day 0) by incubation in DMEM with 10% FBS, 10 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μg/ml insulin. At day 2, the induction medium was replaced by DMEM with 10% FBS plus 10 μg/ml insulin only, and cells were then fed every 2 days with the same medium. For the rapamycin group, cells were treated with 1 nM rapamycin throughout the entire differentiation process. For the Notch signaling study, the PPARγ stably expressing cells were further transduced either with pMIG-*DNL1* or pMSCV IRES-*GFP* (pMIG vector) viruses prior to undergoing adipogenic differentiation. The γ-secretase inhibitor, Compound E, was also used to block Notch signaling. Cells were pretreated with 100 nM Compound E 16 hours prior to induction of differentiation. For *p63* knockdown experiments, the cells were split 24 hours post-siRNA *p63* transfection and then switched to differentiation medium after the cells settled down. To determine the extent of adipocyte differentiation, the cells were either harvested for immunoblotting analysis or Oil Red O staining for lipid droplets after 6–8 days of differentiation. For Oil Red O staining, cells were first fixed in 10% formaldehyde for 1 hour. Oil Red O stock solution (0.5% Oil Red O in isopropanol) was diluted with an equal volume of distilled water and filtered, then added to the fixed cells for 1 hour. After washing briefly with 60% isopropanol and distilled water twice, these cells were photographed under a microscope.

Notch luciferase reporter assay. Cells seeded in 6-well dishes were co-transfected with *Hes1-luc* reporter plasmid encoding firefly luciferase (500 ng/well) and an internal nonspecific control pRL-*TK* plasmid encoding *Renilla luciferase* (25 ng/well) in DMEM containing Lipofectamine 2000. Twenty-four hours later, the cells were treated with or without 10 nM rapamycin (Sigma) for 24 hours. Cells were lysed with 250 μl lysis buffer from the Dual-Glo™ Luciferase Assay System (Promega). Lysates collected in 1.5 ml tubes were frozen and thawed twice. The luciferase activities of the supernatants from the spun lysates were measured with a Berthold luminometer (Lumat LB9507) using the Dual-Glo™ Luciferase Assay System. The specific *Hes1* transcription activity was determined by normalizing the relative *Renilla reniformis* luciferase expression to firefly luciferase expression.

Quantitative real-time RT-PCR. Total RNA was extracted from cells using Trizol (Invitrogen) following the manufacturer's protocol. 0.5 microgram RNA was reversely transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). After 20-fold dilution, 4 μl of cDNA was used as template in a quantitative realtime PCR reaction. Amplification was done for 40 cycles using iQ SYBR Green Supermix on an iCycler (Bio-Rad). Oligonucleotide primers were synthesized to detect *Jagged1*, *Jagged2*, *Dll1*, *Dll3*, *Dll4*, and *Hes1*, with *β-actin* as internal control. Primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services. The primer sequences were as follows:

Jagged1 Forward, 5'-cgcggatccagccgccactgcagccatgaag-3', Reverse, 5'-ccgctcgaggagagctgcagtctgctttggacc-3'; *Jagged2* Forward,

5’-aatgacaccactccagatgag-3’, Reverse 5’-ggccaaagaagtcgttgcg-3’; *Dll1* Forward, 5’-gacctcgcaacagaaaaccca-3’, Reverse, 5'-tccgtagtagtgctcgtcaca-3'; *Dll3* Forward, 5’-ctggtgtcttcgagctacaaat-3’, Reverse, 5’-tgctccgtatagaccgggac-3’; *Dll4* Forward, 5’-cttgctgtgggtaagatttggc-3’, Reverse, 5’-cttcttgcataggcgagaaca-3’; *Hes1* Forward, 5’-gagcacagaaagtcatcaaagcctatc-3’, Reverse, 5’-gccgggagctatctttcttaagtgc-3’; *β-actin* Forward, 5’-agagggaaatcgtgcgtgac-3’, Reverse, 5’-caatagtgatgacctggccgt-3’

Small interfering RNA (siRNA) knockdown:

The siRNA sequences and their targets are as follows:

p63 (all isoforms), 5’-cacagaccacgcacagaaudTdT-3’, 5’-uccagaugacuuccaucaadTdT-3’ (1:1 ratio mixture) (4); *STAT3*, 5’-ctggataacttcattagca-3’; *Notch1* 5’-uugaugucgaucucgcagg-3’; *Rictor*, 5’-caaggaaauuaccgauaaadTdT-3’; *mTOR*, 5’-gaactcgctgatccagatg-3’; *p65*, 5’-ggaccuaugagaccuucaadTdT 3’

All the siRNA above and the scrambled siRNAs were synthesized by GuangZhou RiboBio. Cells seeded in 12-well plates were transfected with 100 nM siRNA using Lipofectamine 2000 following the manufacturer's instructions. Forty-eight hours post-transfection, the cells were used for immunoblotting or differentiation experiments.

Cell proliferation assay. Cell proliferation was measured using an MTT Assay Kit (BioDev-Tech). Five thousand cells per well were seeded in a 96-well plate for 1 day prior to addition of DAPT or DMSO. Cell proliferation was monitored for up to 3 days. On the day of detection, cells were incubated with 100 μl of fresh medium containing 10 μl MTT reagent (0.5 mg/mL) at 37℃ for 4 hours. Then, 100 μl DMSO

was added to each well and the plates were shaken for 10 minutes in the dark. The absorbance values measured at 490 nm on Day 0 were set as 100%, and the rest of measurements were then normalized relative to the value on Day 0.

Bioinformatic analysis of human tumor microarray data for mTOR regulation on Notch signaling: Transcript profiling of Lymphangioleiomyomatosis (LAM) nodules from TSC human patients (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE12027) (Table S1) was analyzed with version 2.8.1 of the statistical programming language R (http://www.R-project.org) and packages from Bioconductor (5) on the Red Hat Linux platform (http://www.redht.com). The sigPathway algorithm (6) was used to compute T-Statistics and *P*-Values for the identification of corresponding significantly changing gene sets ($P<0.01$). All the significantly changing gene sets were listed in Table S2 which may be opened with Notepad or Editplus 3. The relative fold changes of the selected genes were calculated with a cutoff of 1.5 (7). R package of Affymetrix Human Genome U133 Set annotation data (chip hgu133a) was used to assign gene symbol to each probe set. The relevant genes for mTOR and Notch pathways were further analyzed (Table S3, Table 1).

Table S1 GSE12027_series_matrix in excel file and Table S2 Changing gene sets in TXT file are listed in separated files.

Table S3. Expression changes of the gene sets correlated to mTOR-Notch pathway

Probe	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Average	Fold Change LAM *vs.* PASM
VEGFD	LAM	4881.3	9041.8	3172.4	2843.8	2071.1	2940.2	2370.9	4356.4	7447.5	5031.6	2231.2	1698.6	2387.4	7930.7	4171.8	30.2
	PASM	75.9	93.7	69.1	128.9	287.5	236	75.2								138.0	
Jagged21	LAM	682.1	450.5	277	724.3	656.1	649	662.7	700.7	451.6	795.4	249.3	277.5	667.2	266.3	536.4	2.8
	PASM	297.2	149.2	172.3	105.1	200.8	299.4	116.9								191.6	
Jagged22	LAM	1185	780.3	682.6	1429.1	1475.2	1330.9	1153.2	1441.7	878.2	1020.8	542.2	601.6	927.2	538.9	999.1	1.8
	PASM	808.2	513.6	548.8	437.5	646.2	564.2	418.4								562.4	
Hes11	LAM	674.8	520.3	534.1	604.5	444.4	556.3	628.4	527.1	1099.1	1247.6	324.6	357.6	233.7	521.3	591.0	2.2
	PASM	587.4	379.1	225.9	146.4	182.2	226.9	125.6								267.6	
Hes12	LAM	1379.4	960.1	1233.8	1279.9	1177.3	1550.3	1717.3	1637.2	2083	3173.8	1191.4	1299.6	923.6	1157.7	1483.2	3.4
	PASM	656.3	665.3	296.4	186.7	360.7	672.9	214.5								436.1	

Note:

1. The gene sets were generated from the R script calculation.
2. The fold changes were calculated as described in Supplemental Data.
3. The R script is as follows:

```
.tar.gz
R CMD INSTALL ....tar.gz
# Download and Install sigPathway_1.12.1.tar.gz and hgu133a_1.12.0.tar.gz

R
library(sigPathway)
library(hgu133a)
a<-read.table("GSE12027_series_matrix_data.txt",sep="\t",header=T)
# As the data had been normalized with Microarray Suite Normalization 5.1 by the series_contributors, no additional normalization here.
exp<-a[,2:ncol(a)]
rownames(exp)<-a[,1]

exp1<-cbind(exp[,1:14],exp[,19:25])
# LAM group 1:14    0      PASM group 19:25    1
p<-c(0,0,0,0,0,0,0,0,0,0,0,0,0,0,1,1,1,1,1,1,1)

statList <- calcTStatFast(exp1, p, ngroups = 2)
# Calculate t-stat and P-value
names<-rownames(exp1)
pval<-cbind(statList$pval,statList[[2]])
rownames(pval)<-names
want<-pval[which(pval[,1]<0.01),]
symbol<-as.list(hgu133aSYMBOL)
# Assign gene symbol to probe
probeset<-rownames(want)
nrow(want)
for (i in 1:nrow(want))
{
      rownames(want)[i]<-symbol[rownames(want)[i]]
}
want<-cbind(probeset,rownames(want),want)
write.table(want,file="dif_GSE12027_LAM_PASM_0.01.txt",sep="\t",row.names=F,
col.names=F)
```

Table S4. Clinical characteristics of the human breast cancer cohort and the quantified expression levels of the mTOR-STAT3-p63-Jagged1-Notch cascade.

Patient Number	Age	Differentiation	p-S6	p-STAT3	p63	Jagged1	Hes1
1	45	M	119886	91514	301262	81742	55411
2	52	M	71799	127787	227772	130972	71203
3	47	L	122992	257422	354261	188077	108933
4	34	M	40072	182890	305457	74202	127461
5	51	M	62836	108756	200259	100304	58608
6	53	M/L	186873	40999	189036	46688	150539
7	54	L	248054	269713	262470	136278	168520
8	37	L	117080	124407	257995	113486	63187
9	54	M/L	639683	436851	279617	143246	276693
10	59	M/H	9656	57739	234882	43681	122024
11	72	M/L	33188	52639	163912	73772	74674
12	41	M	77275	101933	166387	64147	100753
13	63	M	9688	139376	169771	45322	125990
14	31	L	55824	241105	190476	61881	97761
15	42	L	36560	123469	206747	51830	99709
16	45	M	8741	52787	213123	138770	121815
17	65	M/L	2489	30112	233421	106342	78557
18	61	M	28324	39347	220733	65615	70673
19	57	M	6285	86617	207671	65852	126637
20	54	L	285685	233358	441215	227546	92753
21	48	H	164818	167537	253504	175212	241279
22	48	H	181340	162612	258538	174854	136618
23	46	M	270352	174873	214189	172190	UD
24	51	L	305220	220824	321624	211190	247933
25	55	L	205432	204103	235883	207176	182255
26	65	M	162827	177619	139912	171666	200364
27	55	H	491868	179646	157321	201481	163017
28	53	M	287871	209501	239078	199545	196293
29	71	M	123797	182825	111722	184364	109243
30	52	M	125875	186019	74936	192232	145321

H: high, L: low, M: medium, UD: undetected.

Table S5. OLS Regression analysis of the cascade effect of mTOR-STAT3-p63-Jagged1-Notch signaling in human breast cancers

	p-STAT3	p63		Jagged1			Hes1			
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
p-S6 Coeff.	0.509	0.188		0.227			0.313			
	(0.083)***	(0.096)*		(0.073)***			(0.057)***			
p-STAT3 Coeff.			0.303		0.337			0.409		
			(0.147)*		(0.113)***			(0.099)***		
p63 Coeff.						0.346			0.072	
						(0.156)**			(0.173)	
Jagged1 Coeff.										0.443
										(0.182)**
samples #	26	25	25	26	26	25	25	25	24	25
R-squared	0.61	0.14	0.16	0.29	0.27	0.18	0.57	0.42	0.01	0.20

1. Column 1, the effect of mTOR as indicated by p-S6 levels on p-STAT3; column 2 and 3, the effect of mTOR and p-STAT3 on p63, respectively; column 4-6, the effects of mTOR, p-STAT3, and p63 on Jagged1, respectively; column 7-10, the effects of mTOR, p-STAT3, p63, and Jagged1 on Hes1, respectively.
2. Four high differentiation observations (H and M/H as shown in Table S4) were excluded from analysis.
3. One outlier observation of p63 was dropped (#30 in Table S4).
4. p63 levels reported in this table are the lower band of p63 (assumed ΔNp63) from the Western blot as shown in Figure 8C.
5. *, **, *** denote significance at 10%, 5% and 1%, respectively.

References

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