Liver Label Retaining Cancer Cells Are Relatively Resistant to the Reported Anti-Cancer Stem Cell Drug Metformin

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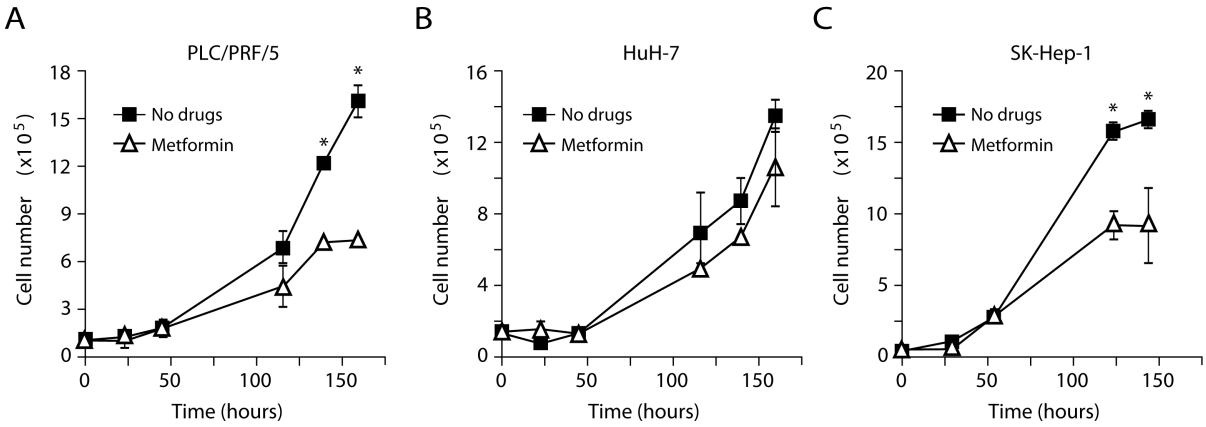
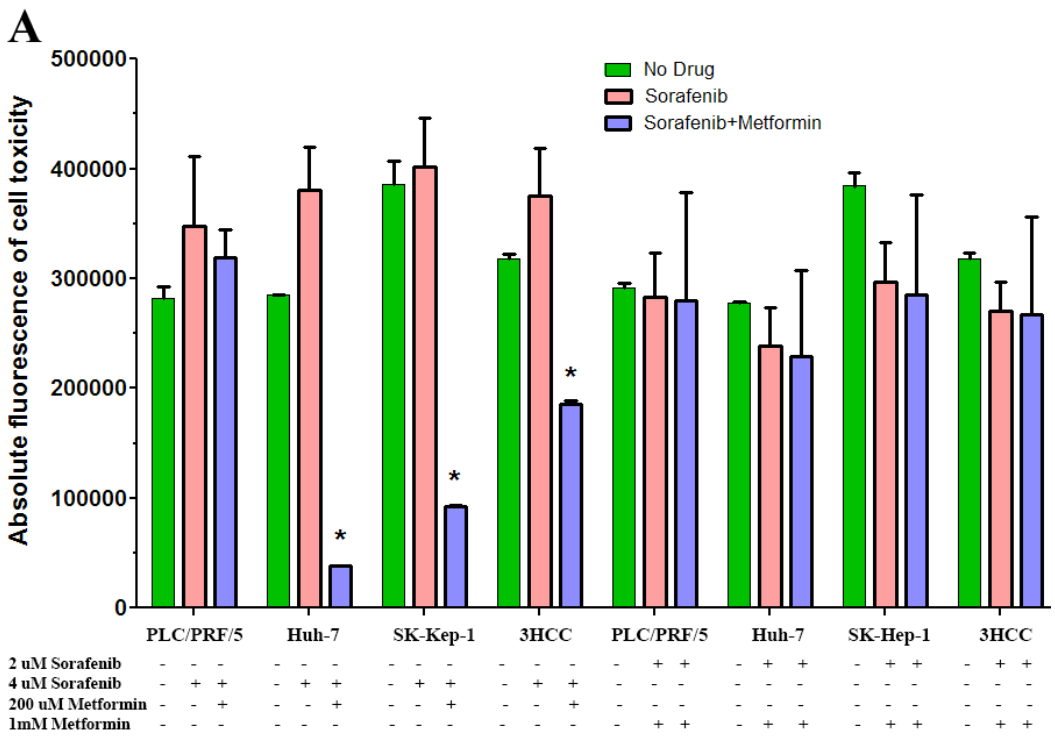
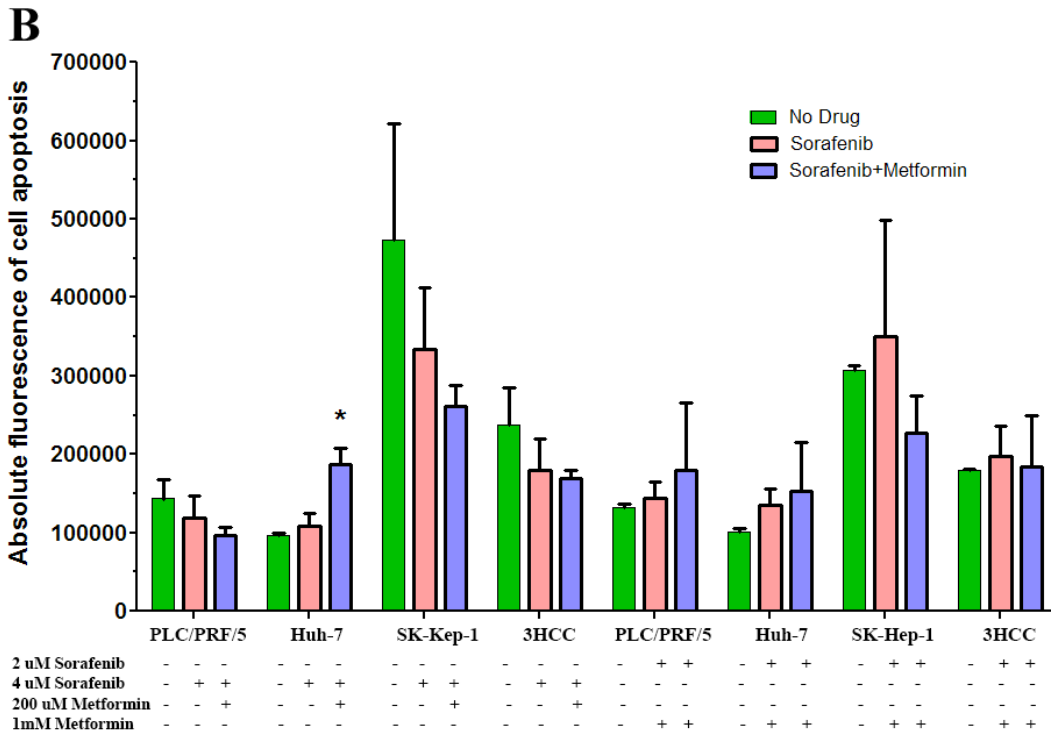
Supplemental Figure legends:



Supplemental Figure S1. Metformin effectively inhibit cell growth of tested two out of three HCC cell lines after 48 hours of treatment. Growth curves of HCC cell lines treated with and without metformin **(A)** PLC/PRF/5 (p=1.11e-2), **(B)** HuH-7 (p=0.32), and **(C)** SK-Hep-1 (p=0.012).

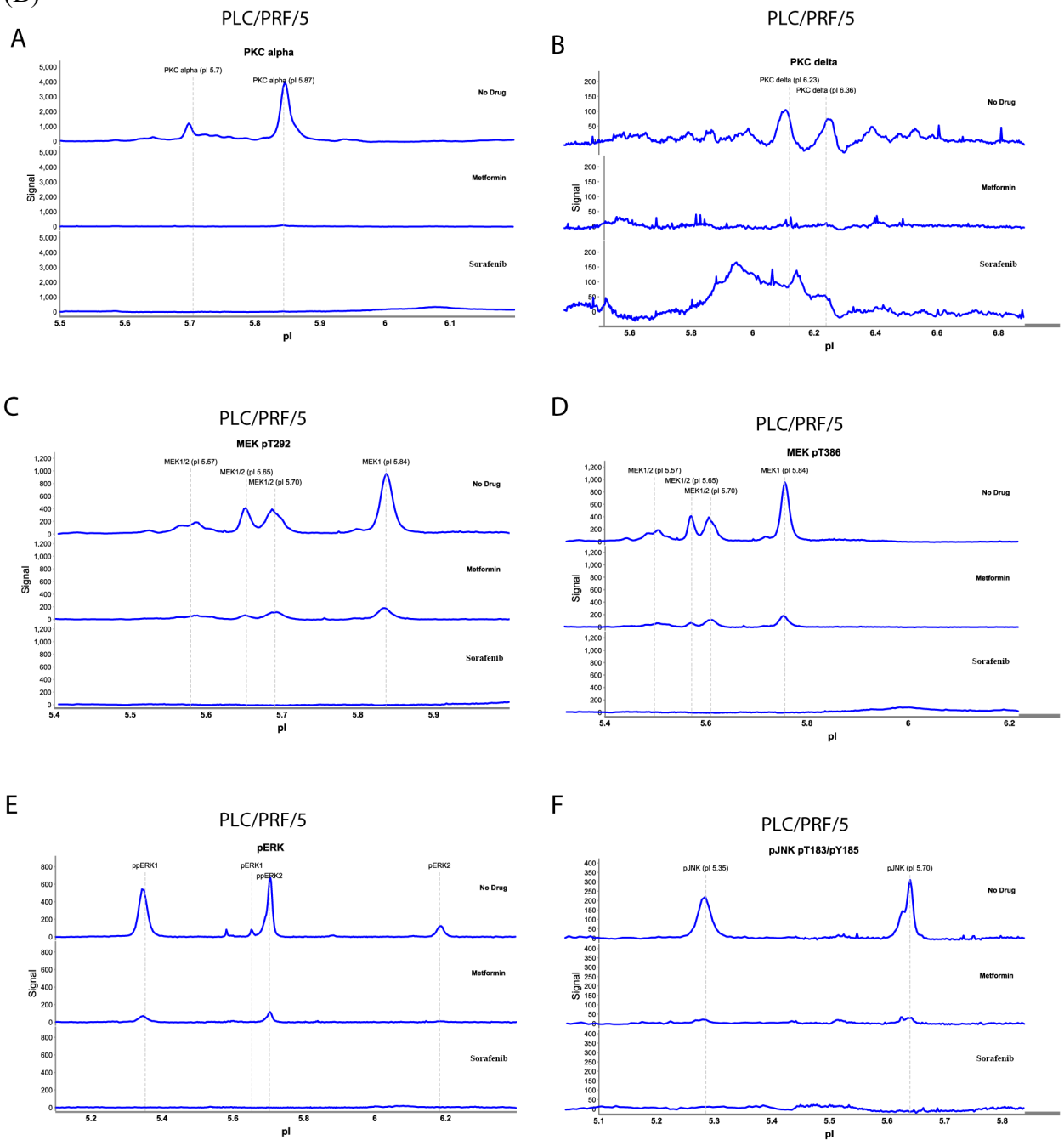




Supplemental Figure S2. Toxicity and apoptosis of HCC cells treated with metformin or/and sorafenib

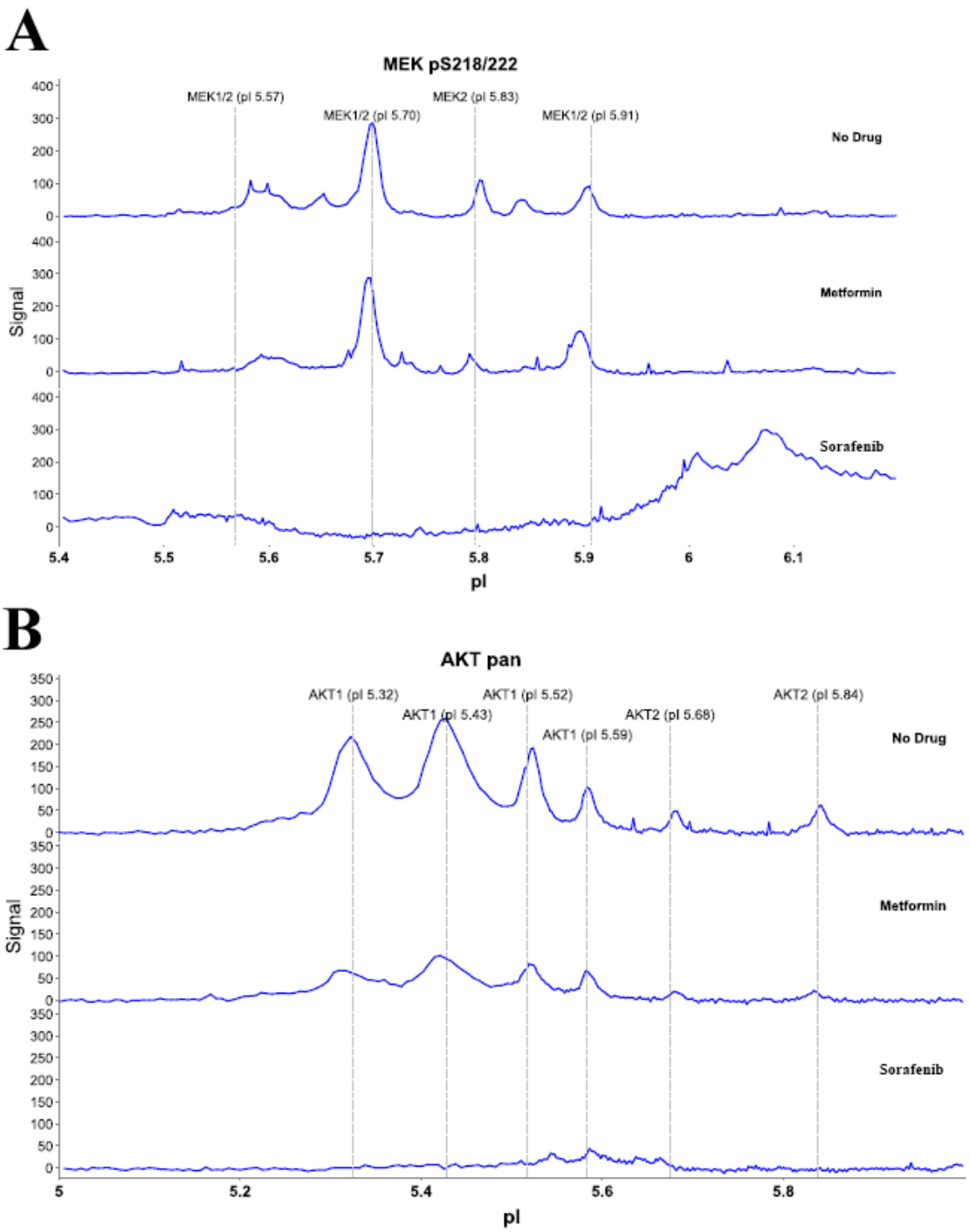
(A) Toxicity and **(B)** apoptosis of HCC cells treated with metformin or/and sorafenib.

(B)



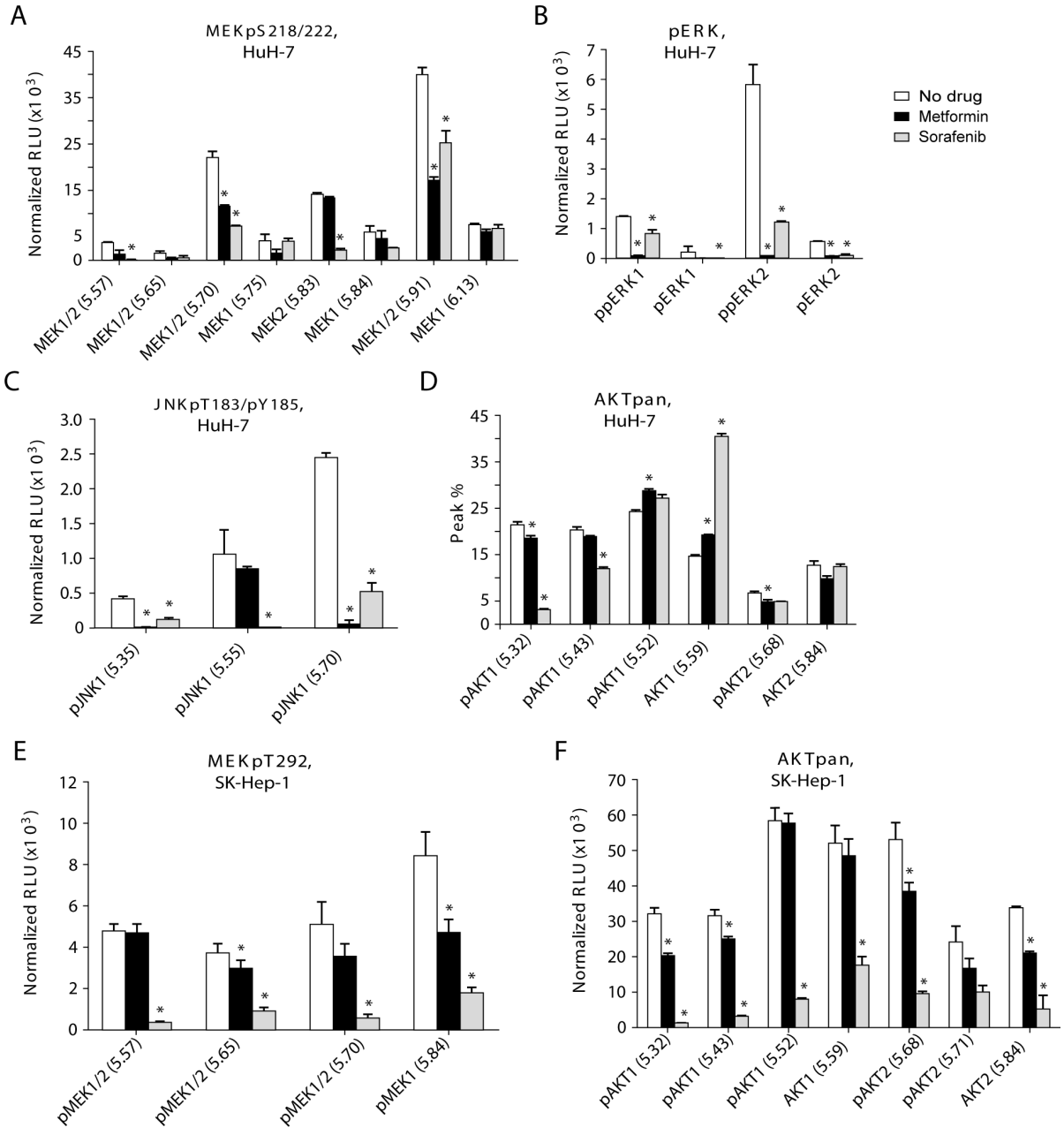
Supplemental Figure S3. Metformin inhibited phosphorylation of PKC-alfal/delta, MEK1/2, ERK1/2 and JNK1/2 in PLC/PRF/5 cells.

After treatment with metformin phosphorylation of multiple protein kinases were inhibited in PLC/PRF/5 cells: **(A)** PKC-alfal, **(B)** PKC-delta, **(C)** MEK1/2 (pT292), **(D)** MEK1/2 (pT386), **(E)** ERK1/2 and **(F)** JNK1/2.



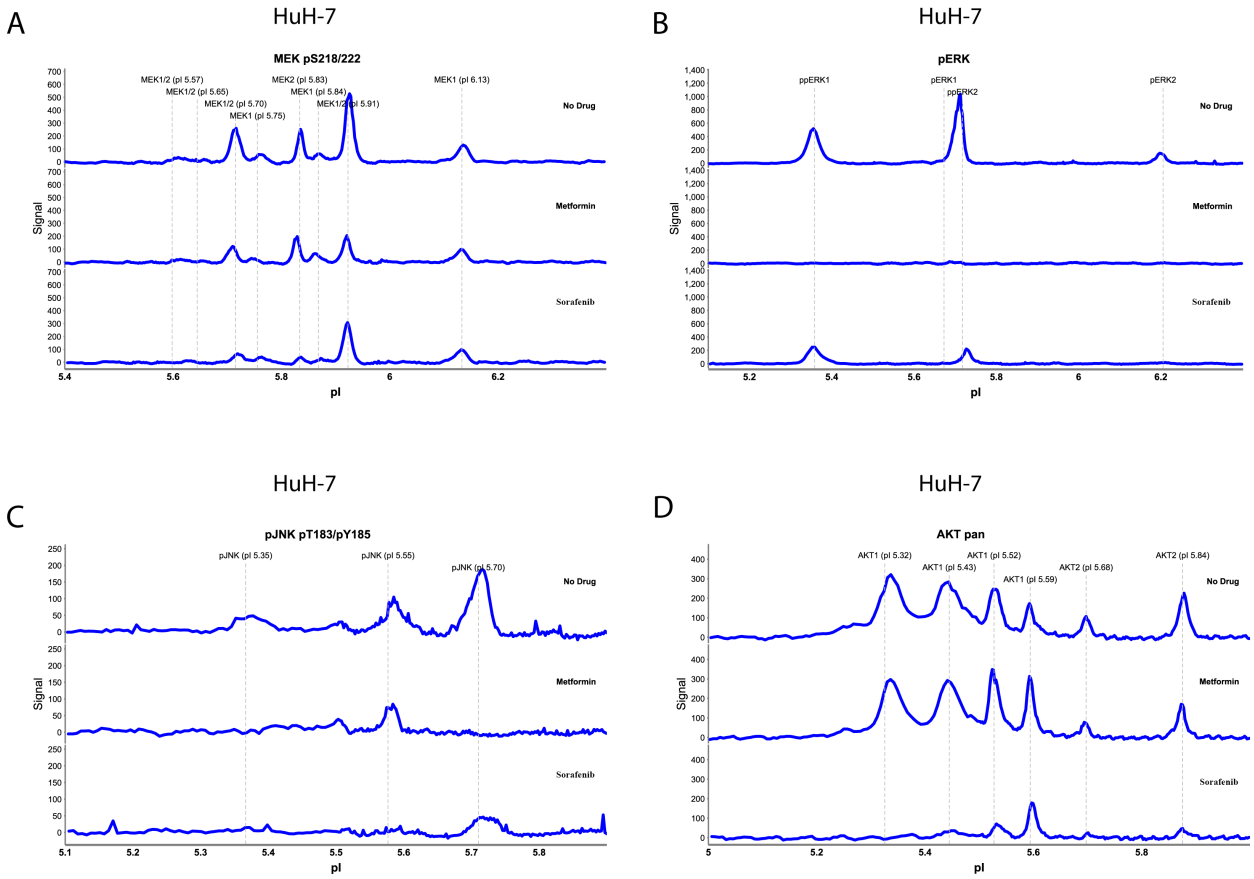
Supplemental Figure S4. Metformin had no significant effects on phosphorylation of MEK1/2 and AKT1/2 in PLC/PRF/5 cells.

After treatment with metformin phosphorylation of multiple protein kinases were inhibited in PLC/PRF/5 cells: **(A)** MEK1/2 (pS218/222) and **(B)** AKT1/2.



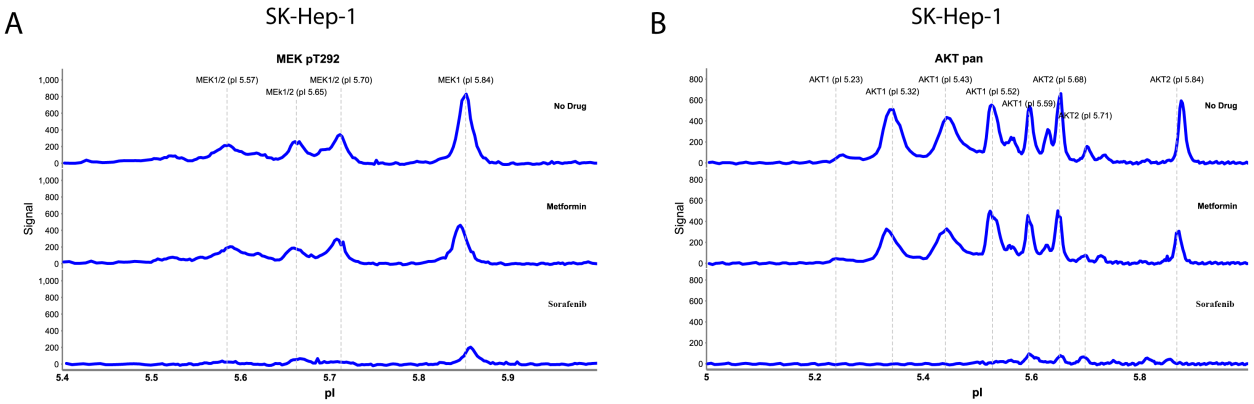
Supplemental Figure S5. MEK1/2, ERK1/2, JNK1/2 and AKT1/2 are inhibited in HuH-7 and SK-Hep-1 cells after treatment with metformin

After treatment with metformin phosphorylation of multiple protein kinases were inhibited in HuH-7 cells: **(A)** MEK1/2 (pS218/222), **(B)** ERK1/2, **(C)** JNK1/2 and **(D)** AKT1/2; and in SK-Hep-1 cells: **(E)** MEK1/2 (pT292) and **(F)** AKT1/2.



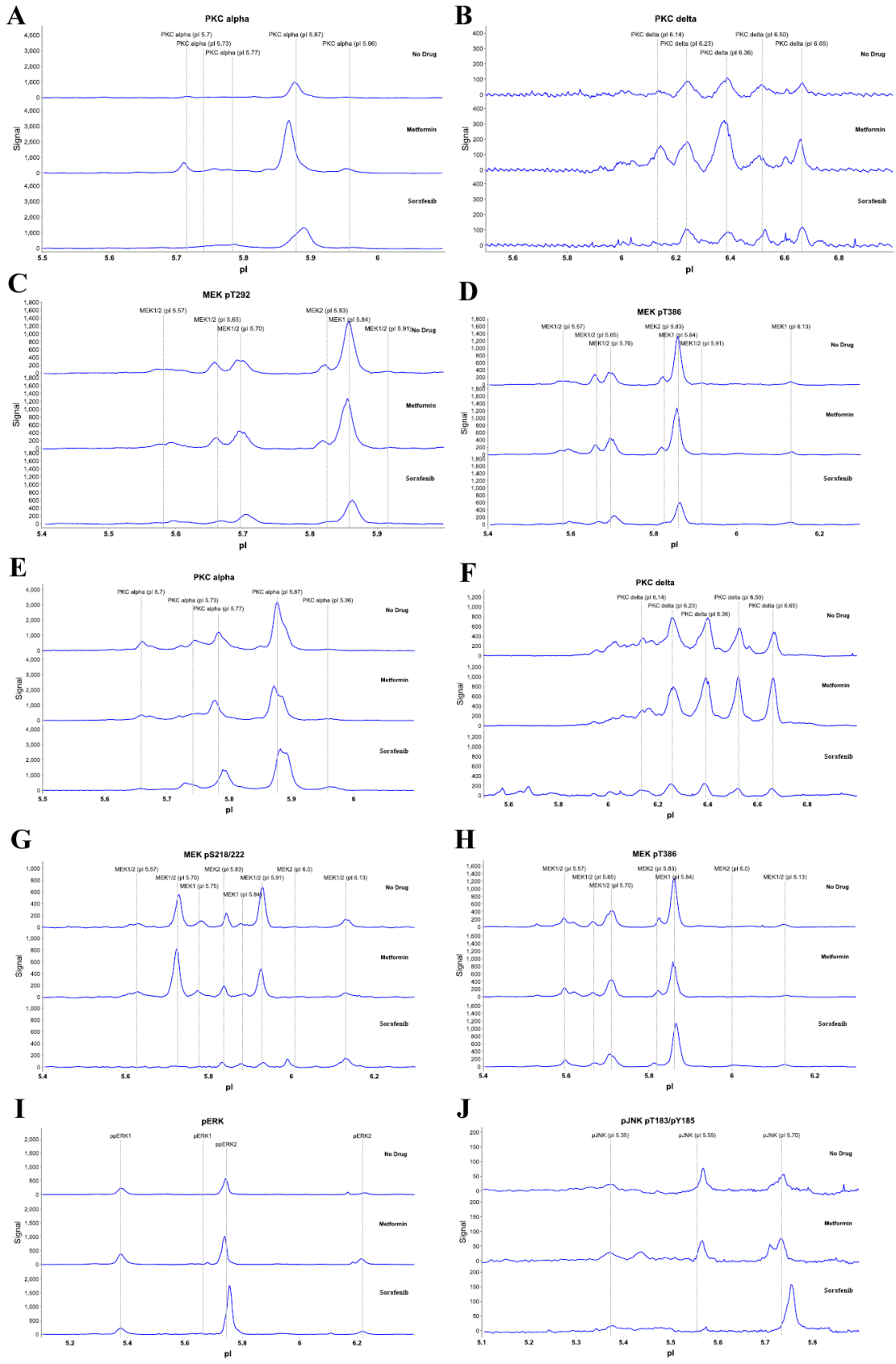
Supplemental Figure S6. Metformin inhibited phosphorylation of MEK1/2, ERK1/2 and JNK1/2 and AKT1/2 in HuH-7 cells.

After treatment with metformin phosphorylation of multiple protein kinases were inhibited in HuH-7 cells: **(A)** MEK1/2 (pS218/222), **(B)** ERK1/2, **(C)** JNK1/2 and **(D)** AKT1/2.



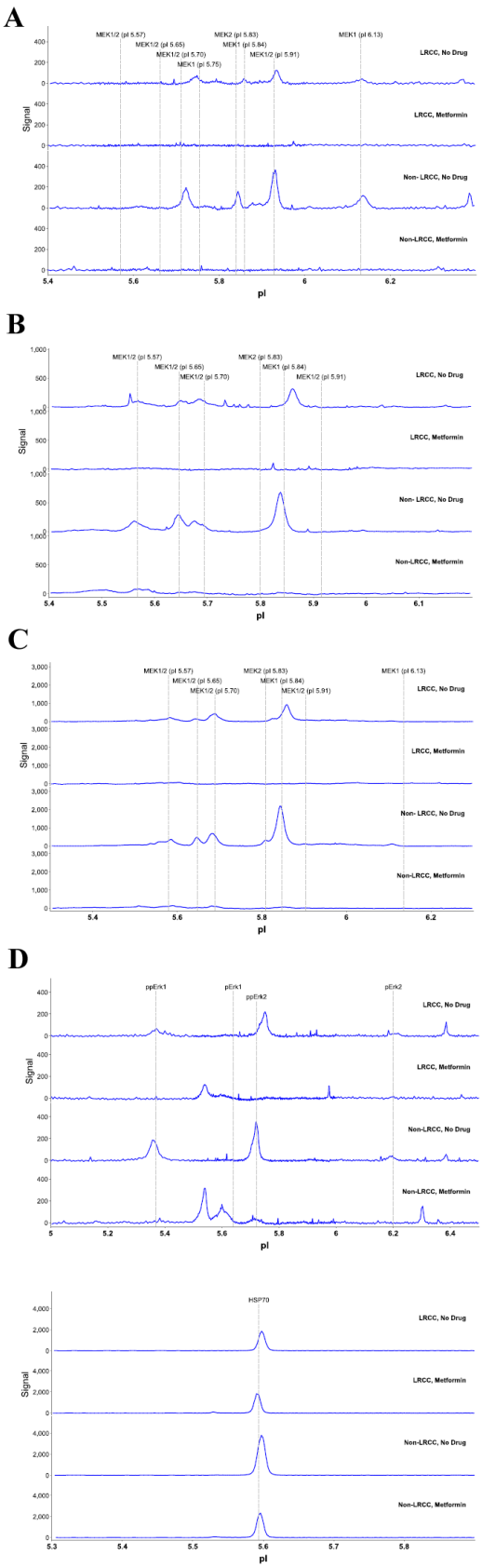
Supplemental Figure S7. Metformin inhibited phosphorylation of MEK1/2 and AKT1/2 in SK-Hep-1 cells.

After treatment with metformin phosphorylation of multiple protein kinases were inhibited in SK-Hep-1 cells: **(A)** MEK1/2 (pT292) and **(B)** AKT1/2.



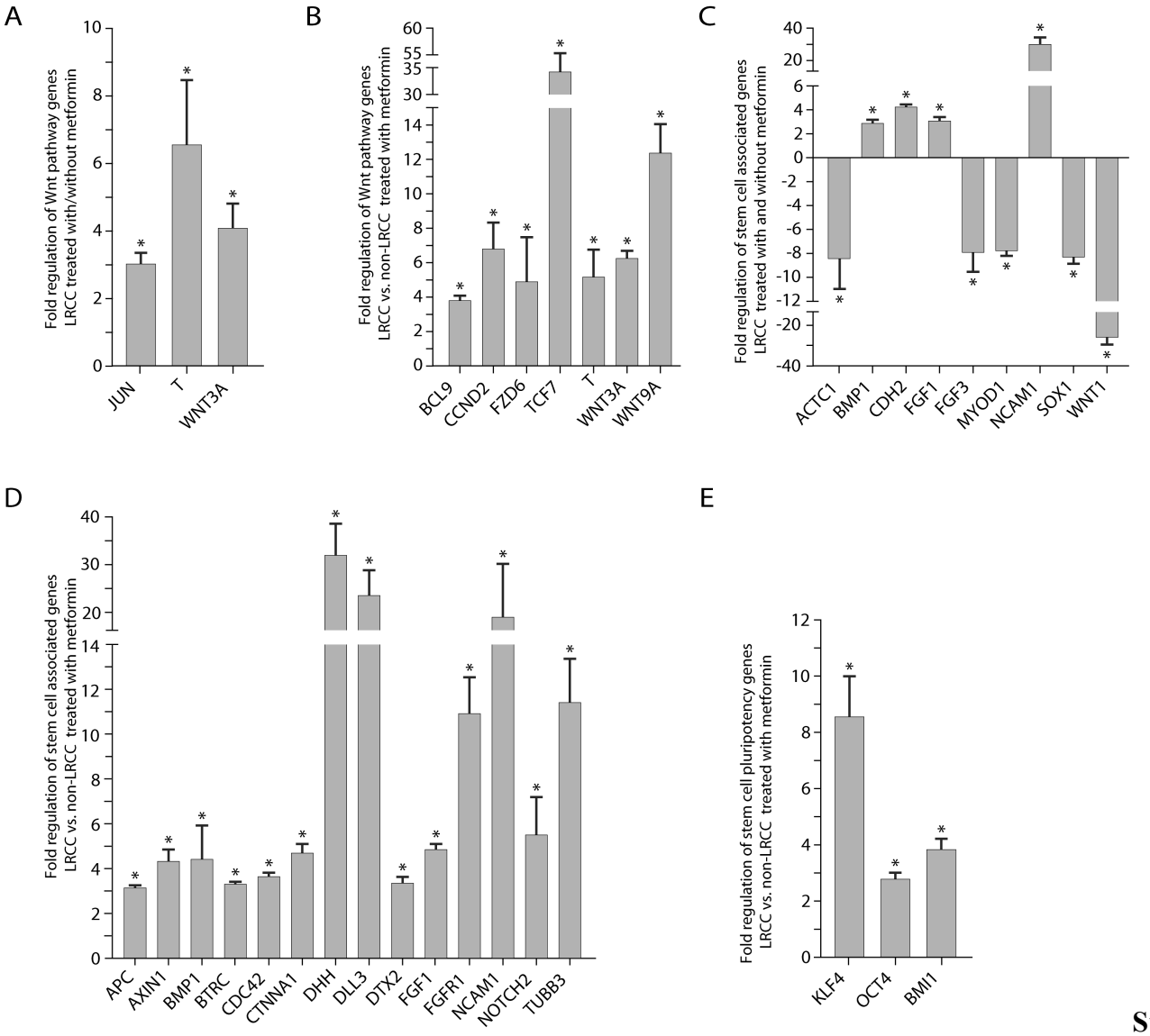
Supplemental Figure S8. Metformin had no significant effects on phosphorylation of some STP in HuH-7 and SK-Hep-1 cells.

After treatment with metformin phosphorylation of multiple protein kinases were not significantly affected in HuH-7 cells: **(A)** PKC-alfal, **(B)** PKC-delta, **(C)** MEK1/2 (pT292), **(D)** MEK1/2 (pT386); and SK-Hep-1 cells: **(E)** PKC-alfal, **(F)** PKC-delta, **(G)** MEK1/2 (pS218/222), **(H)** MEK1/2 (pT386), **(I)** ERK1/2 and **(J)** JNK1/2.



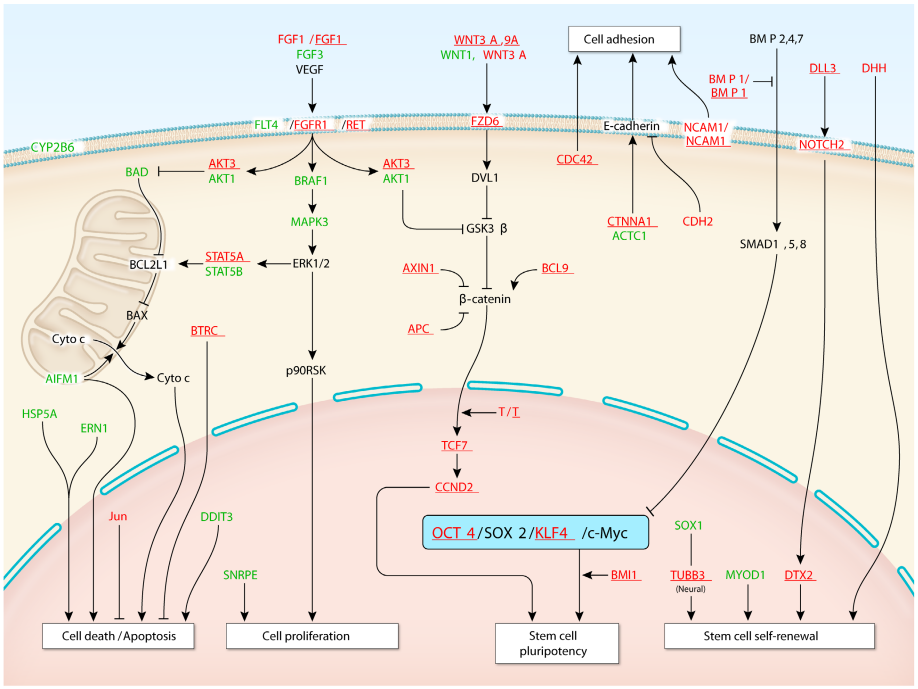
Supplemental Figure S9. Metformin inhibited phosphorylation of MEK1/2 and ERK1/2 in LRCC and non-LRCC of PLC/PRF/5 and HuH-7 cells.

After treatment with metformin phosphorylation of multiple protein kinases were inhibited in both LRCC and non-LRCC: **(A)** MEK1/2 (pS218/222), **(B)** MEK1/2 (pT292), **(C)** MEK1/2 (pT386), **(D)** ERK1/2 in PLC/PRF/5 and HuH-7 cells. **(E)** Head shock protein 70 control.



Supplemental Figure S10. Metformin promoted phosphorylation of the major highly phosphorylated specie of AKT1/2 only in LRCC of PLC/PRF/5 cells.

(A-B) After treatment with metformin phosphorylation of the major highly phosphorylated specie of AKT1/2 was promoted only in LRCC.



Supplemental Figure S11. A pathway map for metformin treated LRCC

Ingenuity Pathway Analysis of gene expression data. Red/green: up-and-down-regulation, respectively; underlines: LRCC vs. non-LRCC; The map suggests a potential less proliferative, more metastatic and less differentiated with less cell death/apoptosis expression profile for metformin treated LRCC.

Supplemental Table

Supplementary Table S1. Gene expression affected by metformin in different HCC cell lines. P value was calculated by two-tailed t test.

Gene group	Comparison	Gene	PLC/PRF/5		HuH-7		SK-Hep-1	
			Fold	p (n=3)	Fold	p (n=3)	Fold	p (n=3)
Sorafenib target genes (STG)	LRCC treated with vs. without metformin	**AIFM1**	-1.5566	0.005484	-28.3518	0.000002	-7.5412	0.000531
		AKT1	-1.5232	0.000125	-13.0878	0.000007	-6.9684	0.000301
		BAD	-1.486	0.009121	-36.4613	0.000008	-14.8511	0.00044
		BRAF	-1.9086	0.000374	-3.3783	0.000639	-4.1417	0.026333
		CYP2B6	-3.7585	0.000005	-7.9119	0.001761	-3.1632	0.031615
		DDIT3	1.2973	0.012283	-7.9021	0.000442	-8.8692	0.005534
		ERN1	-2.5778	0.000006	-121.427	0.000013	-5.5008	0.053132
		FLT4	-1.6225	0.000037	-2.9696	0.000027	-3.6601	0.002009
		HSPA5	-1.6148	0.000305	-2.5155	0.00001	-8.4929	0.00243
		MAPK3	-1.356	0.00573	-35.7244	<0.0001	-9.1404	0.004722
		SNRPE	-1.601	0.001774	-10.0919	0.001488	-6.2677	0.000818
		STAT5B	-1.9913	0.000051	-6.313	0.015942	-6.6857	0.002185
	LRCC vs. non-LRCC treated with metformin	**RET**	-1.2245	0.00573	152.0224	0.000002	2041.346	7E-05
		STAT5A	-2.0447	0.003308	2.7784	0.000081	10150.05	5E-05
		AKT3	1.3922	0.004113	48842.06	<0.0001	8733.125	1E-05
Wnt pathway genes	LRCC treated with vs. without metformin	**JUN**	5.736	0.000038	6.0981	0.001223	-1.2868	0.290592
		T	17.4039	0.000011	14.5775	0.148129	-1.1451	0.718597
		WNT3A	1.8616	0.002701	25.9864	0.002667	1.2233	0.507343
	LRCC vs. non-LRCC treated with metformin	**BCL9**	2.2032	0.00831	21.215	0.01186	1.1564	0.128631
		CCND2	2.1015	0.025836	46.0205	0.185847	2.8943	0.000165
		FZD6	-2.3446	0.584478	56.8878	0.007316	2.2573	0.025851
		T	45.1072	0.000009	78.6364	0.134719	3.3258	0.218266
		TCF7	2.0259	0.022989	41.5275	0.056949	1.2873	0.374777
		WNT3A	1.9412	0.000486	92.3291	0.002359	1.3643	0.36917
		WNT9A	2.697	0.016636	278.1328	0.005638	2.4228	0.163662
Stem cell genes	LRCC treated with vs. without metformin	**ACTC1**	-20.634	0.000122	-2.4106	0.380996	-9.5657	0.00161
		BMP1	1.6534	0.003246	6.7637	0.000536	2.0386	0.009662
		CDH2	1.9097	0.000599	10.7765	0.005712	3.6674	0.000275
		FGF1	1.39	0.007495	6.6334	0.012208	2.9986	0.02268
		FGF3	-26.057	0.045246	-1.7166	0.353731	-9.5657	0.00161
		MYOD1	-3.512	0.002061	-13.531	0.003711	-9.5657	0.00161
		NCAM1	4.0258	0.002352	149.6703	0.005729	41.305	0.000367
		SOX1	-65.939	0.001062	1.1098	0.427311	-9.5657	0.00161
		WNT1	0.00588	-49.3374	0.001274	-35.4335	0.002284	-9.5657
	LRCC vs. non-LRCC treated with metformin	**APC**	3.0711	0.0012	1.5201	0.162344	6.5218	0.0161
		AXIN1	1.3003	0.099888	5.4881	0.000017	10.6449	<0.0001
		BMP1	1.5539	0.076555	6.4285	0.002602	6.5993	0.0002
		BTRC	2.9925	0.000775	-1.3023	0.243131	15.4317	0.0014
		CDC42	4.4185	0.000046	-1.4012	0.079848	14.8405	<0.0001
		CTNNA1	4.4276	0.000103	1.3365	0.112555	16.5218	4E-05
		DHH	2.8455	0.002048	194.384	0.001598	50.0828	<0.0001
		DLL3	2.2096	0.022045	209.2689	0.009388	23.617	2E-06
		DTX2	2.6278	0.00055	-1.3011	0.029051	18.6543	4E-06
		FGF1	1.8868	0.006508	14.3148	0.009008	4.2604	0.0148
		FGFR1	28.7767	0.000048	2.4383	0.125253	17.7309	0.002
		NCAM1	6.7644	0.001555	1.244	0.687532	289.8034	0.0004
		NOTCH2	8.1047	0.000211	1.5142	0.562791	10.7287	0.0002
		TUBB3	6.9841	0.000247	4.5177	0.003547	43.5701	3E-06
		KLF4	9.4146	<0.0001	7.9201	0.000002	12.6198	0.000024
		OCT4	3.1611	0.000146	1.4178	0.087766	7.556	0.000006
		BMI1	1.9002	0.000002	4.3709	0.000196	11.0354	0.000005

Supplementary Note

The LKB1/AMPK/mTOR pathway: The liver kinase B1 (LKB1) regulates the AMP-activated kinase (AMPK). Metformin activates AMPK. Activated AMPK inhibits the mammalian target of rapamycin (mTOR). mTOR controls protein synthesis and cell proliferation.