Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells

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Inventory of Supplemental Information

Supplemental data consists of 4 figures, 3 tables, and 1 video. Supplemental Experimental Procedures is included in the supplemental materials.

Figure S1 and Table S1 relate to Figure 1.
Figure S2 relates to Figure 2.
Figure S3 relates to Figure 3.
Figure S4 relates to Figure 4.
Table S2 relates to Figures 2, 3 and 4.
Table S3 relates to Figures 2, 3 and 4.
Video S1 relates to Figure 1 and Figure S1.

Table S1. Cloning Efficiency of human ES cells and iPS cells
Table S2. siRNA duplexes used for gene knockdown.
Table S3. Primer pairs used for quantitative RT-PCR.

Video S1A. Individualized cells survived by re-establishing multi-cellular colonies.
Video S1B. Blebbistatin facilitated colony formation through proliferation from a single cell.
Video S1C. E-cadherin antibody suppressed colony formation and survival.

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Supplemental Figures

Supplemental Figure 1

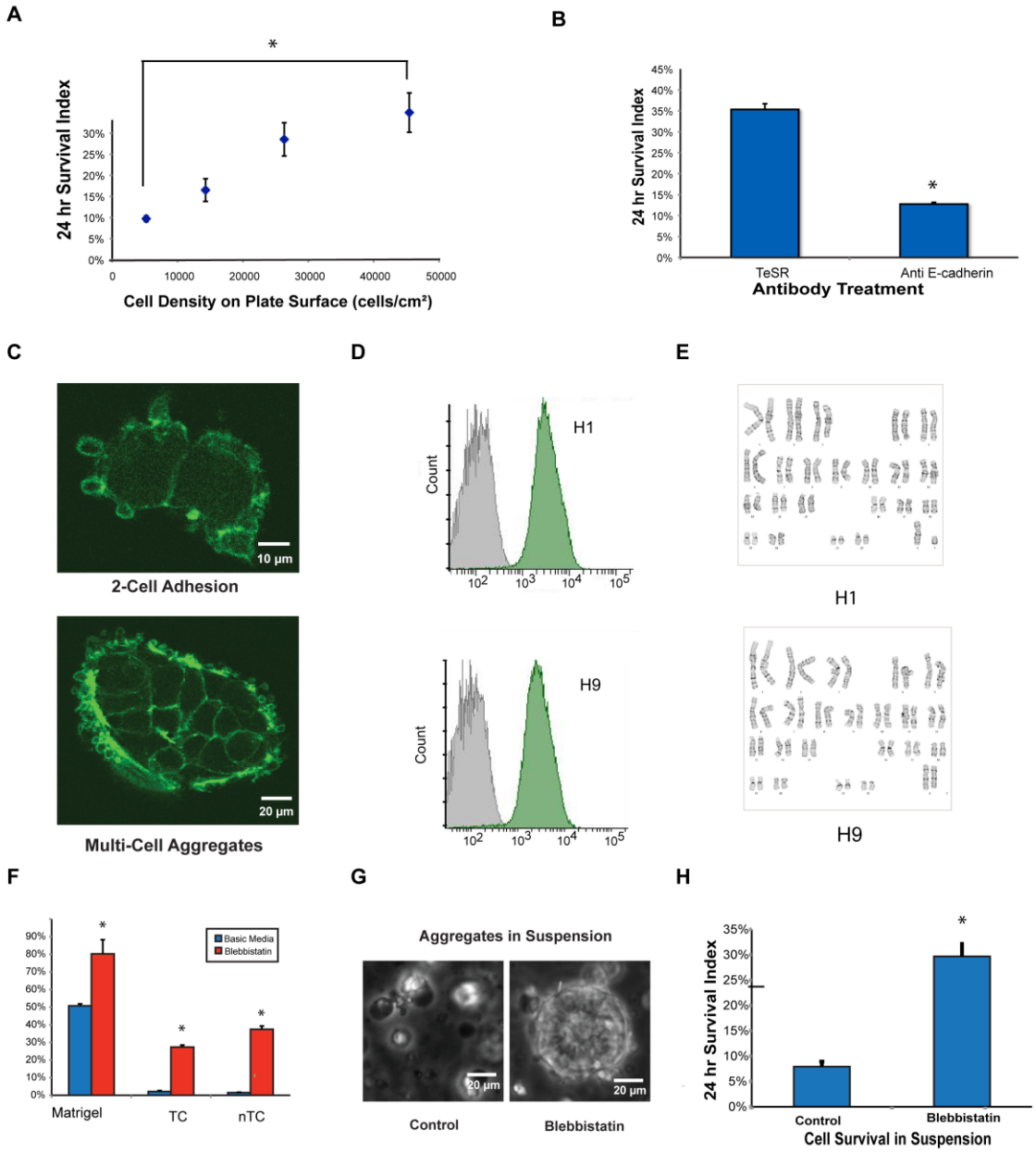

Figure S1. Human ES cell survival after dissociation.

Human ES cells survive through direct cell-cell interaction at high density after dissociation.

(A) Human ES cell survival improves significantly with increased cell density per unit of surface area. The same number of cells was seeded onto different surface areas with

equal volume of media. (*p< 0.05 between the highest and the lowest densities). It suggests that surface density, not secreted growth factors, is a major contributing factor in cell survival after dissociation.
(B) A neutralizing antibody to E-cadherin reduces human ES cell survival (*p < 0.05 at 24 hr). See also Video S2. It indicates the necessity of direct cell-cell contact through E-cadherin in the survival of dissociated cells.
(C) In a reformed colony, blebbing phenotype was maintained at the edge of colonies, but was suppressed at the site of cell adhesion junctions. (The green color represents actin filaments stained with Alexa-488 conjugated phalloidin.) See also Video S3.

Normal pluripotency markers and karyotypes were maintained after blebbistatin treatment
(D) H1 and H9 cells were plated at clonal density with the presence of Blebbistatin for 24 hours, cells were harvested 5 days after plating for flow cytometry analysis. Oct4 protein was stained with anti-Oct4 antibody and Alexa 488 secondary antibody (Gray peak: IgG control, Green peak: anti-Oct4 antibody). Oct4 staining is maintained in more than 95% positive.
(E) Normal karyotypes were observed in H1 and H9 cells that were treated with blebbistatin in cloning assay. Cells were examined 5 passages after the treatment.

Blebbistatin improves cell survival on uncoated plates and in suspension
(F) Individualized human ES cells were plated onto matrigel coated tissue culture plates (matrigel), uncoated tissue culture plates (TC) or uncoated non-tissue culture plates (nTC) in the presence or absence of 10 μm Blebbistatin. Blebbistatin treatment significantly improved human ES cell survival on matrigel, non-tissue culture (nTC) and tissue culture (TC) plate surfaces at 24 hours (*p < 0.05). Comparable experiments were repeated (n=2).
(G and H) Blebbistatin treatment significantly improved human ES cell survival in suspension. Human ES cells were cultured in suspension in the presence or absence of 10 μM Blebbistatin for 24 hours. (G) Representative cells or aggregates for each condition were photographed, and (H) a cell Survival Index calculated (*p <0.05). Comparable experiments were repeated (n=2).

Supplemental Figure 2

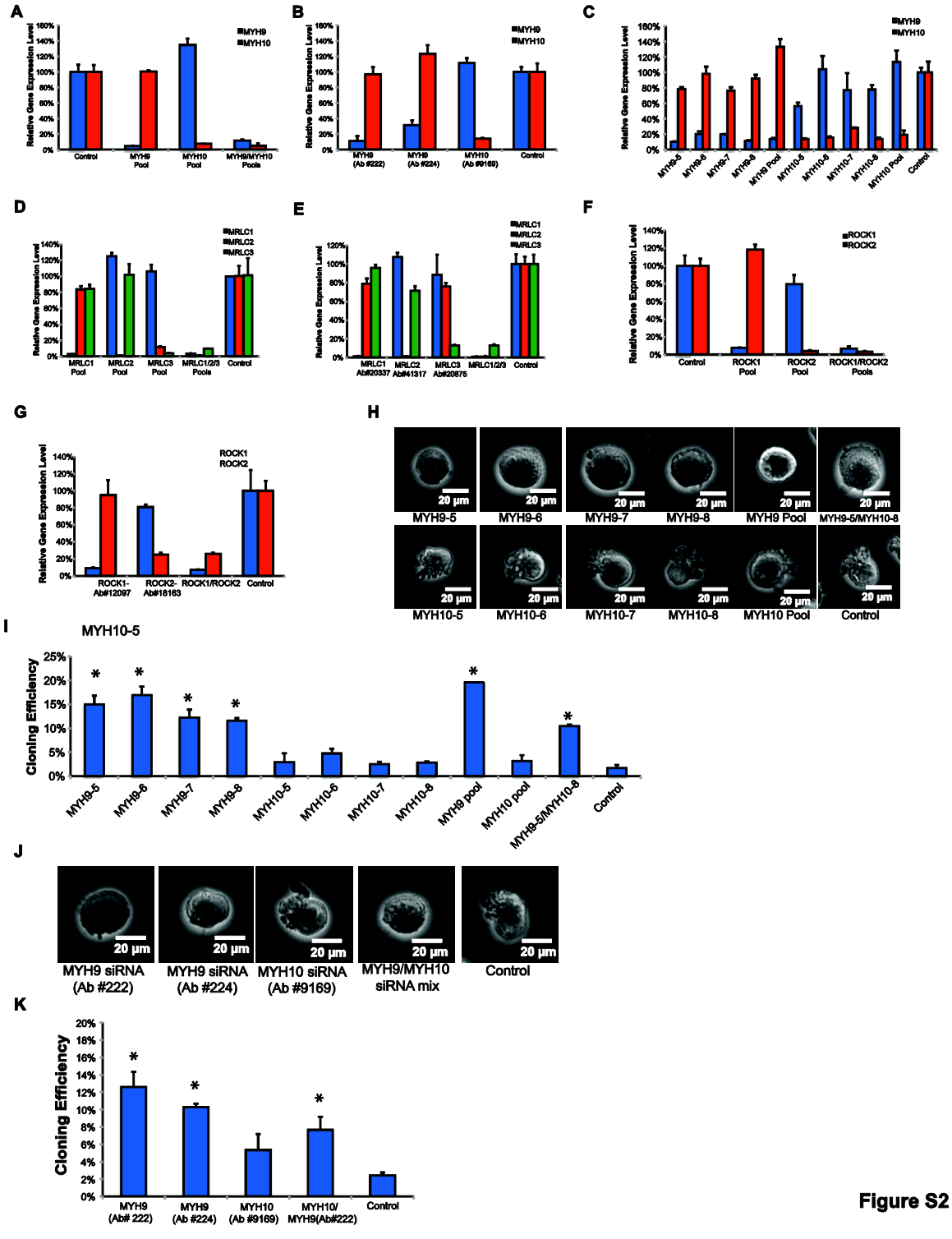

Figure S2

Figure S2. MYH contributes to cell death after dissociation.

Specific gene's mRNA level was measured by quantitative RT-PCR, and was then normalized with GAPDH expression. The knockdown efficiency of a named gene was calculated by comparing its expression level to the expression level in control cells. When gene expression was reduced to around 30% of its normal expression, or below, it was considered efficient. Knockdown was considered non-specific, if off-target homolog was not knocked down below 70%.

(A) *MYH9* or *MYH10* was knocked down with Dharmacon on-target SMART pools. Both *MYH9* and *MYH10* siRNA pools were efficient and specific. (Red columns indicate *MYH9* gene expression, and blue columns indicate *MYH10* gene expression.)

(B) *MYH9* or *MYH10* was knocked down with Ambion Select siRNAs. All three *MYH9* and *MYH10* siRNA were efficient and specific. (Red columns indicate *MYH9* gene expression, and blue columns indicate *MYH10* gene expression.)

(C) *MYH9* or *MYH10* was knocked down with individual duplex in Dharmacon on-target SMART pools. Most *MYH9* and *MYH10* siRNA duplexes were efficient and specific except MYH10-5. (Red columns indicate *MYH9* gene expression, and blue columns indicate *MYH10* gene expression.)

(D) Three *MRLC* genes were knocked down with Dharmacon on-target SMART pools. All three pools were efficient. Only *MRLC1* and *MRLC2* pools were specific, while *MRLC3* pools had off-target effect on *MRLC2*. (Red columns indicate *MRLC1* gene expression, blue columns indicate *MRLC2* gene expression, green columns indicate *MRLC3* gene expression.)

(E) Three *MRLC* genes were knocked down with Ambion Select siRNAs. All three duplexes were efficient and specific. (Red columns indicate *MRLC1* gene expression, blue columns indicate *MRLC2* gene expression, green columns indicate *MRLC3* gene expression.)

(F) *ROCK1* or *ROCK2* was knocked down with Dharmacon on-target SMART pools. Both *ROCK1* and *ROCK2* siRNA pools were efficient and specific. (Red columns indicate *ROCK1* gene expression, and blue columns indicate *ROCK2* gene expression.)

(G) *ROCK1* or *ROCK2* was knocked down with Ambion Select siRNAs. Both *ROCK1* and *ROCK2* siRNA pools were efficient and specific. (Red columns indicate *ROCK1* gene expression, and blue columns indicate *ROCK2* gene expression.)

***MYH* effect on blebbing phenotype and cloning efficiency**

(H) MYH genes were knocked down with Dharmacon siRNA duplexes. Membrane blebbing was suppressed when *MYH9* was knocked down alone or with *MYH10,* while decrease in expression of *MYH10* did not significantly inhibit blebbing.

(I) When MYH9 was knocked down by Dharmacon siRNA duplexes, cloning efficiency was significantly improved (*$p<0.05$), while MYH10 siRNAs did not lead to significant improvement.

(J and K) MYH genes were knocked down with Ambion siRNAs. (J) *MYH9* not *MYH10* siRNA inhibited blebbing phenotype, and (K) also significantly improved cloning efficiency (*$p<0.05$)

Supplemental Figure 3

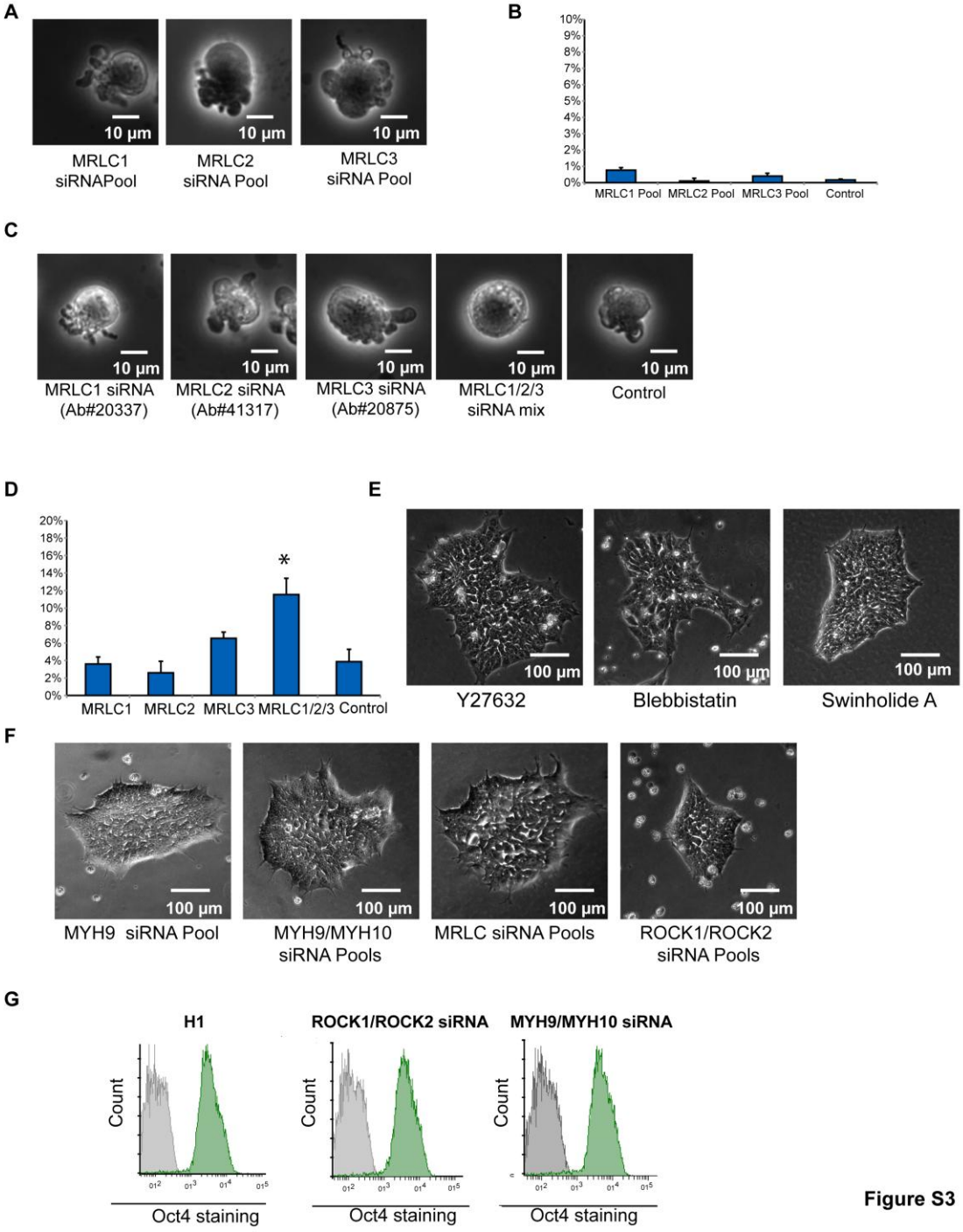

Figure S3

Figure S3. MLC and actin affect blebbing and cloning efficiency

(A) When a single MLC was knocked down by Dharmacon siRNA pool, blebbing was not inhibited, and **(B)** cloning efficiency was not significantly improved.

(C and D) Ambion Select siRNAs were used to knockdown and confirm MLC function. (C) Only when three MLC members were knocked down simultaneously, membrane

blebbing was suppressed, and (D) cloning efficiency was significantly improved (*$p<0.05$).

(E) Y27632, blebbistatin and Swinholide A improved cloning efficiency, and colonies displayed normal ES cell morphology 5 days after plating.

(F) Human ES cell colonies were derived with each treatment and they resembled normal ES cell phenotype 5 days after plating.

(G) Oct4 expression in H1 cells was maintained at high level after *MYH9/MYH10* or *ROCK1/ROCK2* siRNA knockdown. Oct4 protein was stained with anti-Oct4 antibody and Alexa 488 secondary antibody (Gray peak: IgG control, Green peak: anti-Oct4 antibody), and cells were analyzed by flow cytometry. Cells were harvested 5 days after initial plating.

Supplemental Figure 4

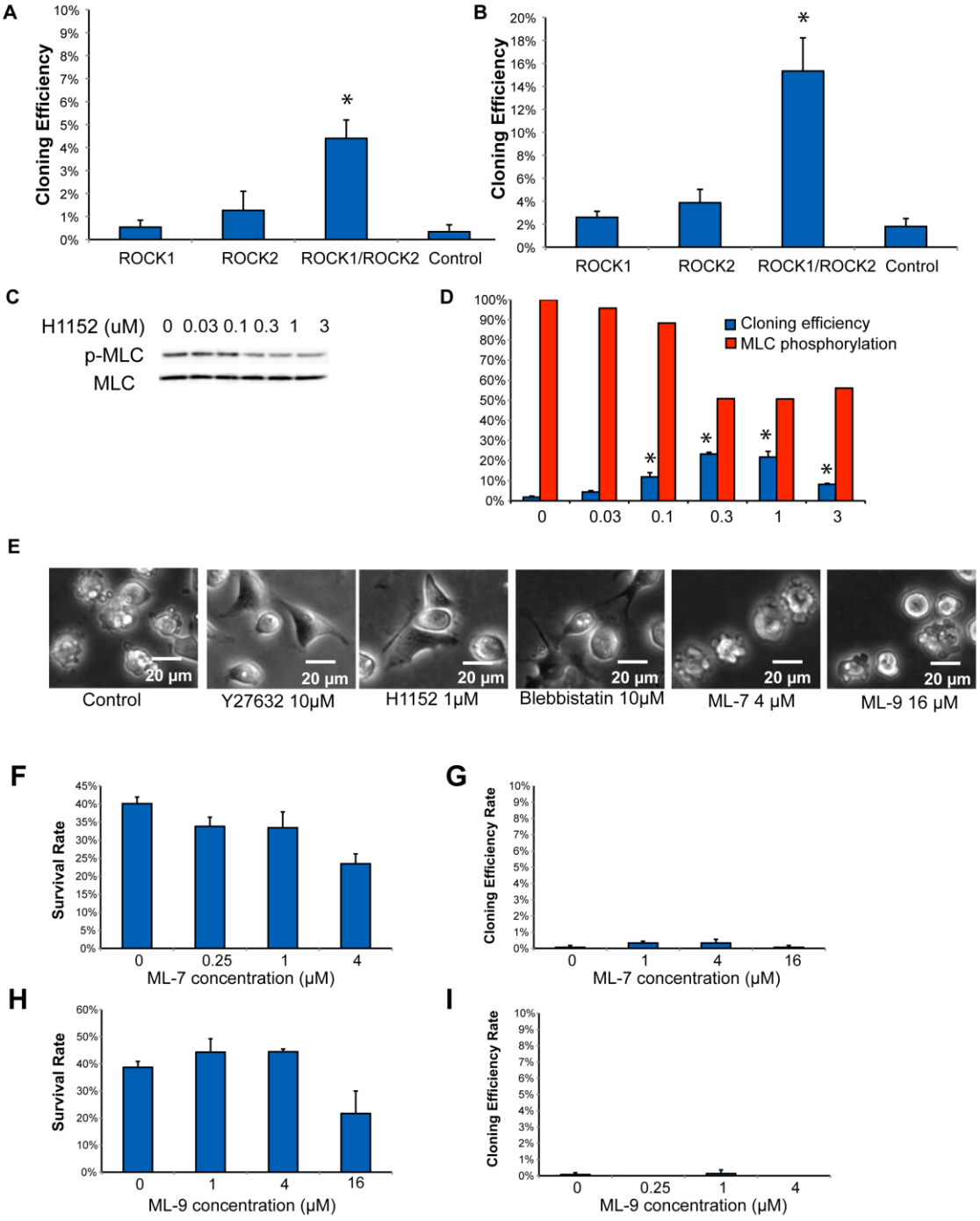

Figure S4. ROCK inhibition and cloning efficiency

(A) Dharmacon siRNA pools were used to knockdown ROCK1 and/or ROCK2. Only when both genes were silenced, the cloning efficiency was significantly improved (*$p<0.05$).

(B) Ambion Select siRNAs were used to knockdown ROCK1/ROCK2. Only when both genes were silenced, the cloning efficiency was significantly improved (*$p<0.05$).

(C) Western blot of MLC phosphorylation showed that H1152, another ROCK inhibitor, also inhibited phosphorylation with increased concentration at 5 hours.
(D) A decrease in MLC phosphorylation after H1152 exposure coincided with an increase in cloning efficiency (*$p<0.05$). (Blue columns represent cloning efficiency, and red columns represent MLC phosphorylation level). Note: Cells treated with H1152 at 3 μM started to have toxic effect.
(E) One hour after plating, Y27632, H1152 and Blebbistatin inhibited blebbing phenotype, but MLCK inhibitor ML-7 and ML-9 failed to do so.
(F) ML-7 did not improve cell survival, and **(G)** cloning efficiency.
(H) ML-9 did not improve cell survival, and **(I)** cloning efficiency.

Supplemental Tables

Table S1. Cloning Efficiency of human ES cells and iPS cells under blebbistatin treatment

Cell lines	Mock experiments	Blebbistatin treatments
H1 (n>20)	<2%	~25% to ~60%
H9 (n>6)	<2%	~25% to 50%
iPS-foreskin (n>10)	<2%	~30% to 50%
iPS-IMR90 (n>6)	<2%	~30% to 60%

Table S2. Duplexes used for specific siRNA gene knockdown

Gene	Duplex name	Company	Target sequence	Specificity *	Blebbing **
MYH9	MYH9 on-target SMART pool	Dharmacon	MYH9-5, 9-6, 9-7, 9-8 pool	Good	+
MYH9	MYH9-5	Dharmacon	GUAUCAAUGUGACCGAUUU	Good	+
MYH9	MYH9-6	Dharmacon	CAAAGGAGCCCUGGCGUUA	Good	+
MYH9	MYH9-7	Dharmacon	GGAGGAACGCCGAGCAGUA	Good	++
MYH9	MYH9-8	Dharmacon	CGAAGCGGGUGAAAGCAAA	Good	+
MYH9	MYH9 (Ab #222)	Ambion	GGGUAUCAAUGUGACCGAUUU	Good	+
MYH9	MYH9 (Ab #224)	Ambion	CGGCAAGGUGGAUUACAAAUU	Good	++
MYH10	MYH10 on-target SMART pool	Dharmacon	MYH10-5, 10-6, 10-7, 10-8 pool	Good	+++++
MYH10	MYH10-5	Dharmacon	CCAAUUUACUCUGAGAAUA	Nonspecific for MYH9	+++++
MYH10	MYH10-6	Dharmacon	GGGCAACUCUACAAAGAAU	Good	+++++
MYH10	MYH10-7	Dharmacon	GAGCAGCCGCCAACAAAUU	Good	+++++
MYH10	MYH10-8	Dharmacon	GGAAGAAGCUCGACGCGCA	Good	+++++
MYH10	MYH10 (Ab #9169)	Ambion	CCCUAACUUUGUUCGUUGUUU	Good	+++++
ROCK1	ROCK1 on-target SMART pool	Dharmacon		Good	+++++
ROCK1	ROCK1 (Ab #12097)	Ambion	GGUUAGAACAAGAGGUAAAUU	Good	+++++
ROCK2	ROCK2 on-target SMART pool	Dharmacon		Good	+++++
ROCK2	ROCK2 (Ab #18163)	Ambion	GGAUCGAACCCAUGGAUCAUU	Good	+++++
MRLC1	MRLC1 on-target SMART pool	Dharmacon		Good	+++++
MRLC1	MRLC1 (Ab #20337)	Ambion	CAAUGUCUUUCGCAAUGUUUUU	Good	+++++
MRLC2	MRLC2 on-target SMART pool	Dharmacon		Good	+++++
MRLC2	MRLC2 (Ab #41317)	Ambion	GCCUCUUCUUUUUGAUGUAUU	Good	+++++
MRLC3	MRLC3 on-target SMART pool	Dharmacon		Nonspecific for MRLC2	+++++
MRLC3	MRLC3 (Ab #20875)	Ambion	GGUCUAUACAGAGUCAAUAUU	Good	+++++

* Specificity of each siRNA was evaluated by the combination of the on-target knockdown efficiency and off-target effect (Figure S2). A duplex was considered with good specificity, if on-target gene product knockdown was less than 30% of control cells, while off-target control gene product level was maintained at higher than 70% of control cells.

** The prevalence of blebbing cells in the population was visually examined under microscope after dissociation with TrypLE. The severity was represented by the number of "+", with "+++++" for most severe blebbing that usually happened to the control cells after dissociation.

Table S3. qRT-PCR primer pairs used in this paper. They were designed with the on-line program Primer3.

Gene	Forward	Reverse
GAPDH	gtggacctgacctgccgtct	ggaggagtgggtgtcgctgt
MYH9	gaagagctagaggcgctgaa	ctttgccttctcgaggtttg
MYH10	gtaccttgcccatgttgctt	ttttgcttgacgaacagcac
ROCK1	gaagctcgagagaaggctga	ttgtctgcctcaaatgcttg
ROCK2	tgaagcctgacaacatgctc	tctcgcccatagaaaccatc
MRLC1	accccacagacgaatacctg	ccggtacatctcgtccactt
MRLC2	caggcaccattcaggaagat	gtgggggaagtgtctgagaa
MRLC3	ttttagcggctctctgggta	ctgaatctgcgactggtcaa

Video S1A. Cell survival by re-establishing multi-cellular colonies

Cells were plated on matrigel-coated plates, and the time-lapse microscopy experiment was performed for 24 hours with 5-minute intervals. Propidium iodide was added to TeSR media to demonstrate the emergence of dead cells.

Video S1B. Blebbistatin facilitated colony formation through proliferation from a single cell

Cells were plated on matrigel-coated plates, with the 10 μM Blebbistatin in TeSR media for the initial 20 hours. The whole time-lapse experiment lasted for 2 days with 10-minute intervals.

Video S1C. E-cadherin antibody suppressed colony formation and survival

Cells were plated on matrigel-coated plates, with or without E-cadherin antibody in TeSR media, and the time-lapse microscopy experiment was performed for 24 hours. The left panel indicates the absence of E-cadherin antibody; the right panel indicates the presence of E-cadherin antibody.

Supplemental Experimental Procedures

Human ES Cell Culture

Human ES cells were maintained in mTeSR medium on matrigel-coated tissue culture plates (Ludwig et al., 2006). Cells were passaged routinely with EDTA. Briefly, cells were washed twice with PBS/EDTA medium (0.5mM EDTA in PBS, osmolarity 340 mOsm), then incubated with PBS/EDTA for 5 minutes at 37^{o}C. PBS/EDTA was removed, and cells were washed off swiftly with a small volume of corresponding media. Human ES basic media was optimized for cloning and survival assays. Media contained DMEM/F12, L-ascorbic acid (64 mg/L), sodium selenium (14 μg/L), FGF2 (100 μg/L), insulin (19.4 mg/L), $NaHCO_3$ (543 mg/L) and transferrin (10.7 mg/L).

Reagents:

Chemicals: Blebbistatin (-) (Sigma), Cytochalasin D (Biomol), Swinholide A (Biomol), Mycalolide B (Biomol), H1152 (Tocris), Y27632 (Tocris), ML-9 (Tocris), and ML-7 (Sigma).

Antibodies: anti-E-cadherin (R&D), anti-MLC (Cell signaling), anti-phospho-MLC (Ser-19) (Sigma), anti-Oct4 (Santa Cruz), anti-SSEA4 (Millipore) and Alexa-488 anti-mouse IgG (Invitrogen).

Alexa-633 conjugated Phalloidin and Alexa-488 conjugated Phalloidin (Invitrogen)

Survival Assay

Unless specified, all the experiments were done on 12-well plates. Usually triplicates were prepared for each treatment. Prior to the addition of cells, 500 μl media was loaded into each well. Cells were dissociated with TrypLE (Invitrogen) for 5 minutes or until

fully detached from the plate, neutralized with equal volumes of media, counted, washed, and diluted to 300,000 to 1,000,000 cells/ml, and 100 μl of cells were added into each well. Plates were then placed into 5% O_2 and 10% CO_2 37°C incubator. Small chemical compounds or proteins were added or washed away according to the specified procedure. Cells were again dissociated at specific time point with 0.4 ml TrypLE, neutralized with equal volumes of 10% FBS in DMEM, harvested with pipettes, and counted by flow cytometry. As an internal control, 5000 count bright beads were added to each sample, and usually ~200 beads were counted for each sample.

Immunostaining and Actin Staining

At each time point, cells were fixed with 4% Paraformaldehyde in PBS with Calcium and Magnesium at room temperature for 5 minutes, neutralized with 0.1M Glycine for 15 minutes at 37^{o}C, rinsed with PBS, and stored at 4^{o}C before staining. Cells were permeabilized with 0.5% Triton-X100 PBS and stained with phalloidin. Nucleus was counterstained with DNA dye DAPI.

Supplemental References

Ludwig, T.E., Bergendahl, V., Levenstein, M.E., Yu, J.Y., Probasco, M.D., and Thomson, J.A. (2006). Feeder-independent culture of human embryonic stem cells. Nature Methods *3*, 637-646.