Because more than 80% of murine mature cardiomyocytes in adults are reported to have two nuclei[1, 31], an increase in the number of cardiomyocyte nuclei may not directly reflect the actual increase in the number of cardiomyocytes. To evaluate the effects of the chemicals on the actual cardiomyocyte number, we performed a secondary screen with seven small molecules of independent kinase targets among the nine candidate chemicals (Table S1). We purified mESCMs, re-cultured with chemicals for 5 days, and calculated the actual cardiomyocyte number by cell counting and flow cytometry for αMHC-EGFP (Figure S2d). Three out of the seven chemicals significantly increased mESCM cell number (Figure 1b). BIO (GSK3 inhibitor, 1 μM), SU1498 (Flk1 inhibitor, 5 μM), and KN93 (CaMKII inhibitor, 5 μM) increased cardiomyocyte number to 3.4 ± 1.4, 2.5 ± 0.6, and 2.2 ± 0.4 times over control, respectively.

Next, we compared mESCM number between Flkd6+2 and Flkd6+5 to confirm whether these chemicals could actually increase cardiomyocyte proliferation and whether they did not simply improve the re-plating viability of mESCMs. Whereas re-plated mESCMs showed no increase in cell number with DMSO alone, all three chemicals significantly increased mESCM number during Flkd6+2 to Flkd6+5 (Figure 1c). We further confirmed cardiomyocyte proliferation with time-lapse video recording (Figure 1d, Supplemental Movie 1–4). Whereas a control cardiomyocyte (Movie S1) ceased proliferation and just underwent hypertrophic change, cardiomyocytes treated with these chemicals caused cell division and proliferation (Movie S2–4). These data clearly show that the three chemicals (BIO, SU1498 and KN93) enhance mESCM proliferation and increase in mESCM number accompanying actual cell division but not binucleation.

Induction of Cell Cycle Progression in Cardiomyocytes by Chemicals

Next, we examined changes in mESCM cell cycle. The ratio of S phase in mESCMs, as estimated with 3 hour pulse-labeled EdU incorporation, and the ratio of M phase in mESCMs, as estimated by immunostaining of Phosphorylated Histone H3 (pH3) at Flkd6+3 were increased more than 2.2 times by BIO treatment and increased approximately 1.5–2 times by SU1498 and KN93 (Figure 2a–c). The ratio of 4N cardiomyocytes was examined as doubled DNA contents (4N) and EdU-negative cells in cardiac Troponin T (cTnT)-positive population by flow cytometry. 4N cardiomyocytes, consisting of cells in G2/M phase and cells with binuclei, were not increased by any of chemical treatment but reduced by KN93 treatment compared with DMSO treatment, suggesting binuclear cardiomyocytes were not induced by these chemicals (Figure 2d). Western blotting for cyclins showed that BIO but not SU1498 or KN93 treatment increased Cyclin D2 and Cyclin D3 (Figure 2e) as reported.[32] On the other hand, a CDK inhibitor, *Ink4b* expression was downregulated 30% by treatment of each chemical at Flkd6+2 (Figure 2f). Downregulation of *Ink4b* was sustained until Flkd6+5 in SU1498 or KN93 treatment (Figure 2g). These results indicate that these chemicals actually induce cell cycle progression in cardiomyocytes by regulating Cyclin/CDK activity.

Molecular Targets of the Chemicals

Next we confirmed the molecular targets of these three chemicals. Addition of CHIR99021, another GSK3-specific inhibitor (Figure 3a), or Wnt3a (data not shown) increased cardiomyocyte numbers similar to BIO, indicating that inhibition of GSK3 enhanced mESCM proliferation as reported in rat cardiomyocytes.[32]

SU1498, first reported as a tyrosine kinase inhibitor of vascular endothelial growth factor receptor 2 (VEGFR2; also designated as Flk1)[33], enhanced cardiomyocyte proliferation even though Flk1 is not expressed in mESCMs[25], suggesting that SU1498 exerted its effect through targets other than Flk1. SU1498 was reported to cause accumulation of

phosphorylated extracellular signal-regulated kinase (ERK) via inhibiting phosphatase binding to ERK.[34] We performed Western Blot analysis to show if SU1498 cause accumulation of phosphorylated ERK in mESCMs. Phosphorylation of ERK was increased only when cells were treated by SU1498 but not with BIO or KN93 (Figure 3b). Then we determined whether SU1498-elicited mESCM proliferation is mediated by ERK signaling. Treatment with a MAPK/ERK kinase (MEK) inhibitor PD98059 (PD) that inhibits ERK phosphorylation abolished the increase in SU1498-induced mESCMs (Figure 3c). In addition, ZM336372 (ZM), an activator of Raf1/ERK signaling, similarly increased mESCM number (Figure 3c). Furthermore, an ERK activating growth factor, NRG1β, also increased mESCM numbers by approximately 1.5 times (data not shown) as reported.[9,35] Taken together, these data indicate that SU1498 increase cardiomyocyte proliferation through activation of Raf-MEK-ERK signal cascade.[7,36]

CaMKII was reported to be involved in cardiomyocyte hypertrophy, but little is known about its role in cardiomyocyte proliferation. Two distinct CaMKII inhibitors, KN93 and KN62, similarly increased cardiomyocyte numbers (Figure 3d), suggesting that CaMKII is a novel regulator of cardiomyocyte proliferation. We investigated the role of CaMKII in cardiomyocyte proliferation. CaMKII is a family protein coded by the *Camk2a*, *Camk2b*, *Camk2d* and *Camk2g* isoform genes. Among them, *Camk2a*, *Camk2d* and *Camk2g* mRNA but not *Camk2b* mRNA expressions were detected in purified mESCM at Flkd6 (data not shown). We evaluated the effect of specific small interfering RNA (siRNA) against each CaMKII isoform to identify the main isoform(s) that inhibit mESCM proliferation. Specific knockdown of *Camk2d* and *2g*, the major CaMKII isoforms in cardiomyocytes[37], by siRNA increased mESCM cell numbers by 50% (Figure S3a–b). Conversely, knockdown of *Camk2a* reciprocally decreased mESCM numbers (Figure S3b). These data suggest that KN93 and KN62 increase mESCM by inhibiting CaMKIId/g. Knockdown of *Camk2d* but not *Camk2g* by siRNA resulted in 50% reduction of *Ink4b* expression similar to KN93 treatment (Figure S3c). Furthermore, reduction of *Ink4b* with siRNA significantly increased mESCM numbers (Figure S3d–e). These data suggest that CaMKIId negatively regulates mESCM proliferation through Ink4b.

Our results, thus, show that these chemicals affect cardiomyocyte proliferation through distinct pathways–BIO/GSK3, SU1498/ERK, and KNs/CaMKII.

Combinatory Effects of Chemicals on mESCM Proliferation

Distinct molecular targets of these chemicals prompted us to examine combinations of inhibitors to maximize their proliferative effects. We first examined combinations of two inhibitors. BIO+SU1498 (SU) and BIO+KN93 (KN) significantly increased mESCM proliferation (Figure 4a). Unexpectedly, a combination of SU+KN cancelled their individual effects. Additionally a combination of all three inhibitors, BIO+SU+KN, neither showed any effect on cardiomyocyte proliferation (Figure 4a), suggesting that SU1498 and KN93 could be conflicting in the cardiomyocyte proliferation machinery. A p38 MAPK inhibitor SB203580 (p38i) is known to increase neonatal or adult cardiomyocyte proliferation.[38] In fact, p38i (1 μM) significantly increased the mESCM number by approximately twofold also in our experimental system (Figure 4b). Therefore, we added p38i to each combination, and found that BIO+SU+p38i and BIO+KN+p38i increased in mESCM numbers up to 6–7 times more than control until the mESCMs reached confluency (data not shown). To allow more robust proliferation, we next tested mESCM culture at a lower cell density (5.0×10^4 / well (6-well plate)). In this condition, the BIO+SU+p38i combination increased mESCM up to 14-fold (Figure 4c). We also examined cell cycle of mESCMs treated with each combination (Figure 4d). Both combinations (BIO+SU+p38i and BIO+KN+p38i) significantly increased EdU (3hr pulse) incorporation to approximately 2.5 and 4 times more than DMSO treatment

at Flkd6+3 and Flkd6+5, respectively (Figure 4d). These combinations also significantly increased pH3 in mESCMs up to approximately 2.7 and 2.5 times more than DMSO treatment at Flkd6+3 and Flkd6+5, respectively (Figure 4d). We also evaluated 4N fraction (G2/M and binucleated cardiomyocytes) by flow cytometry, but there were no significant differences (data not shown). Thus, we successfully demonstrated that optimal combinations of the chemicals effectively induced substantial cardiomyocyte proliferation. We, collectively, named these seven chemicals (BIO, CHIR99021, SU1498, ZM336372, KN93, KN62 and p38i) as cardiomyocyte proliferative chemicals (CPCs).

We further confirmed cardiomyocyte features after proliferation. All αMHC-EGFP-positive cells used in our study were also positive for cTnT (Figure S1). These cells were spontaneously beating before and after the chemical treatments. Proliferated cardiomyocytes with combined CPC treatments (BIO+SU1498+p38i and BIO+KN93+p38i) showed similar cardiomyocyte-like action potentials (Figure S4a). The main parameters of action potentials, maximum diastolic potential (MDP), maximum rate of rise of the action potential (dv/dt) and action potential duration (ADP50), were not different among control and CPC-treated mESCMs (Figure S4b). Cardiac myosins (*Myh6*, *Myh7*, *Myl2* and *Myl7*), mature cardiac markers, were increased more than two-eight times during robust proliferation with combined CPCs (BIO+SU1498/KN93+p38i) from Flkd6 to Flkd6+5 (Figure S4c). The data demonstrated that CPCs enhanced proliferation of cardiomyocytes with no apparent bias in cardiomyocyte features.

Developmental Stage Specific Effects of Chemicals on Embryonic, Neonatal and Adult Cardiomyocytes

Next, we examined the effects of CPCs on cardiomyocytes in various developmental stages, such as embryos, neonates and adults.

Cardiomyocytes isolated from the hearts of E9.5 mice, which is in comparable differentiation stage to mESCMs[39], were cultured and treated with each CPC or with the combinations for 3–5 days (E9.5+3 and E9.5+5, respectively). Each CPC treatment increased the S phases and M phases in embryonic cardiomyocytes at E9.5+3 (Figure S5a). Combined CPC treatments (BIO+p38i+SU/KN) increased S phase to approximately 4.5 times and M phase to 3 times more than control at E9.5+3 (Figure S5a). Combined CPC treatments rapidly increased embryonic cardiomyocyte nuclei number up to 8.5 times more than control at E9.5+5 (Figure S5). These data indicated that CPCs efficiently proliferate embryonic cardiomyocytes in a similar manner to those in mESCMs.

Neonatal (P3) rat ventricular cardiomyocytes (NRVCs) were isolated and cultured with CPCs for 2 days. As a baseline, DMSO-treated neonatal cardiomyocytes rarely proliferated (BrdU-positive cells, 3.4±0.3%; pH3-positive cells, 0.37±0.08%; Aurora B-positive cells, 0.36±0.04%). BIO, SU1498 or p38i alone increased in number of BrdU-positive cardiomyocytes by 2–3.5 times (Figure 5a). In contrast to mESCMs and embryonic cardiomyocytes, KN93 failed to increase BrdU incorporation and further progression of the cell cycle in NRVCs (BrdU-positive cells, 4.1±1.4%, Figure 5a). Combination of BIO+p38i synergistically increased BrdU incorporation and BIO+SU+p38i further enhanced the BrdU incorporation (19.4±2.2% and 25.0±2.6%, respectively. $p < 0.05$ *vs.* control. Figure 5a). In BIO+p38i and BIO+p38i+SU1498 condition, the cell cycle progression to M phase (pH3-positive, 3.70±0.20% and 4.03±0.19%, respectively. Figure 5b) and cell division (Aurora B-positive, 3.47±0.21% and 3.76±0.24%, respectively. Figure 5c) were significantly increased compared to the baseline.

Adult rat cardiomyocytes were isolated and cultured with CPCs for 6 days. Less than 0.01% of adult cardiomyocytes were positive for BrdU and none of them were pH3-positive at the

baseline (DMSO treatment). BIO or p38i treatment alone evoked cell cycle reentry in adult cardiomyocytes, that is, BrdU-positive and pH3-positive cells were increased (Figure 5d). Combination of BIO and p38i synergistically and significantly enhanced the cell cycle progression markers in adult cardiomyocytes compared to baseline (BrdU-positive cells, 2.7±0.3%; pH3-positive cells, 0.60±0.05%; Aurora B-positive cells, 0.37±0.07%) (Figure 5d–g). pH3-positive metaphase and anaphase cardiomyocytes were found in BIO+p38i condition (Figure 5f). In contrast, SU1498 and KN93 failed to enhance the effects of BIO +p38i (Figure 5d and e).

These findings suggest that GSK3 and p38 MAPK are broadly and cooperatively regulating various cardiomyocytes including in adults whereas ERK and CaMKII play a novel developmental stage-specific function.

Effects of Chemicals on Cardiomyocytes from Human iPSCs and ESCs

Finally, we evaluated the effects of CPCs on human iPSC- and ESC-derived cardiomyocytes. A human iPSC line 201B6 is one of the most characterized four-factor (Oct3/4, Sox2, c-myc and Klf4)-iPSCs for differentiation properties, including cardiac differentiation.[28,40] Cardiomyocytes (hiPSCMs) were induced with a two-dimensional, defined, serum-free condition-based cardiomyocyte differentiation method (Figure 6a).[22,28] Spontaneously-beating hiPSCMs appeared around d8–10 after Activin A treatment. At day 11, CPC treatment was started. The cell cycle was evaluated by flow cytometry at day 14, and the cardiomyocyte number was calculated at day 16. In agreement with the results from mESCMs, BIO+p38i+SU significantly increased EdU incorporation in hiPSCMs at d14 (Figure 6b–c) and hiPSCM number at d16 (Figure 6d). BIO+p38i+KN had tendencies to increase EdU incorporation and hiPSCM number (Figure 6b–d). Similarly, BIO+p38i+SU increased BrdU incorporation in hESC-derived cardiomyocytes by approximately 1.5 times (data not shown). We purified hiPSCMs with a recently identified cell-surface marker for hiPSCMs, VCAM1[28], and evaluated structural and functional features of CPC-expanded hiPSCMs. CPC-expanded hiPSCMs showed clear sarcomere formation (Figure 6e) and action potentials (Figure 6f), similar to those in control hiPSCMs. These data indicate that CPCs also successfully work in human cardiomyocytes.

Discussion

In the present study, we screened a chemical library with a HCS for effects on the proliferation of mESCMs and succeeded in identifying several distinct chemicals promoting cardiomyocyte proliferation. These findings uncovered common and developmental stage-specific machineries controlling cardiomyocyte proliferation. They also would provide broad and efficient ways of manipulating cardiomyocyte number from embryos to adults and in mouse and human.

We identified chemicals regulating four distinct signaling pathways, namely GSK3 (BIO and CHIR99021), ERK (SU1498 and ZM336372), CaMKII (KN93 and KN62) and p38 MAPK (SB203580) (Fig. 7). Consistent with previous reports[32,38], GSK3 and p38 MAPK signal cascades negatively regulated cardiomyocyte proliferation and inhibitors of these kinases enhanced rodent and human cardiomyocyte proliferation. This is the first report to show synergistic effects of combinatorial inhibition of GSK3 and p38 MAPK, especially on adult cardiomyocyte proliferation.

In contrast to GSK3 and p38 MAPK inhibitors, ERK activators and CaMKII inhibitors have not been reported to be involved in cardiomyocyte proliferation. Raf-MEK-ERK signal cascade positively regulates the proliferation of cardiomyocytes as a downstream target of growth factors.[7,8] Furthermore, phospho-ERK is evident in E8.0 heart, which depends on

the FGF receptor.[41] SU1498 enhances ERK phosphorylation through inhibiting phosphatase (Figure 3b).[34] Phosphatases, such as dual-specificity phosphatase 6 (Dusp6), negatively regulate ERK phosphorylation.[42] Dusp6 null mice showed enhanced ERK phosphorylation and increased cardiomyocyte proliferation during embryonic development and in the early postnatal period.[36] Dusp6 is a possible candidate for a SU1498 target. A Raf activator, ZM336372, also showed a similar pro-proliferative effect. Collectively, all of these results indicate that activation of Raf-MEK-ERK pathway could be a potent molecular target to induce cardiomyocyte proliferation. CaMKII has been reported to regulate cardiac hypertrophy in the later stages of development or in adults.[43,44] Here we showed that in an earlier developmental stage corresponding to E9.5 mouse embryos, CaMKII could inhibit cardiomyocyte proliferation. We confirmed phosphorylation of CaMKII in early mouse embryos (E9.5 and E13.5) and found that CaMKII phosphorylation was drastically increased between E9.5 and E13.5 (Figure S6). CaMKII phosphorylation was more evident in trabecular layer cardiomyocytes than in compact layer counterparts at E13.5. This timing of CaMKII activation in trabecular cardiomyocytes is in accordance with the period when the proliferative ability of trabecular layer cardiomyocytes is reduced[13], suggesting that CaMKII at this differentiation stage could be involved in growth arrest of early cardiomyocytes. CaMKII inhibitors lost its proliferative effect on postnatal and adult cardiomyocytes, suggesting that the growth inhibitory effect of CaMKII has an early developmental stage-specific effect, and switching of its effect to hypertrophy would occur in the later stage. Currently, the precise molecular mechanisms for the developmental stage-specific CaMKII effect are unknown, but this finding is a clue to the precise understanding of CaMKII functions in cardiomyocytes.

Though ERK activators and CaMKII inhibitors did not show sufficient proliferative effects in adult cardiomyocytes, our finding of an effective chemical cocktail for cardiomyocyte proliferation can offer a therapeutic potential for cardiac regeneration. In diseased hearts, cardiomyocytes express embryonic genes[45], and small fraction of cardiomyocytes is de-differentiated and proliferating[3] or newly differentiated from progenitor cells[46], indicating that these cardiomyocytes in diseased hearts are in a more immature stage. Hence, not only will the combination of GSK inhibitors and p38i but also our effective cocktails including three chemicals exert their potent pro-proliferative effects in diseased hearts, facilitating cardiac regeneration.

Recently, PSC-derived cells have drawn considerable interest for use in drug screening, disease modeling[20,21,47] and for regenerative medicine as cell therapies[48] are highlighted. In addition, PSC-derived cells can provide an experimental system for studying cellular physiology especially in cell types difficult to obtain from adults. With the use of our PSC systems, it is likely that other more effective chemicals would be identified from other libraries. Thus, our novel screening system for cardiomyocyte proliferation combining stem cell technology and chemical biology could be a potent tool for investigating efficient molecules and largely contribute to future cardiac regenerative medicine, especially through regeneration with drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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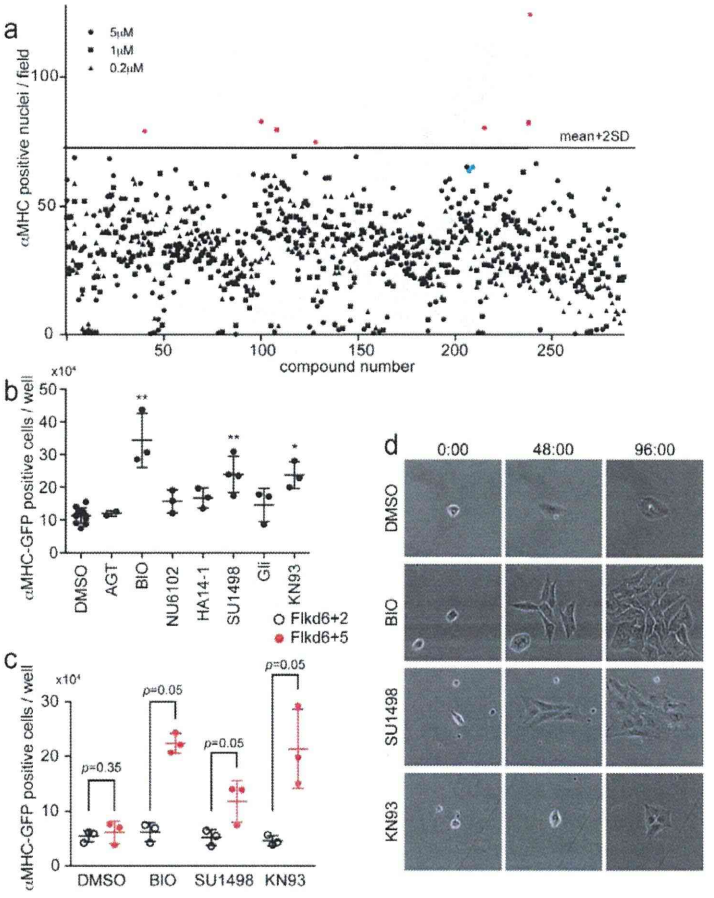

Figure 1.

Three Chemicals were Identified With Chemical Library Screening for mESCM Proliferation. (a) Primary screen with high content screening for nucleus numbers in αMHC-positive cells (mESCMs). Mean nucleus numbers at Flkd6+5 treated with approximately 280 chemicals at three concentrations (● 5μM, ■ 1μM, and ▲ 0.2μM, n = 2). Seven chemicals (red symbols) increased mESCMs more than mean + 2SD of control (control: n = 35). Two CaMKII inhibitors (blue symbols) increased mESCM more than mean + 1SD. (b) Secondary screen for the seven chemicals calculating actual cardiomyocyte numbers with flow cytometry. Abbreviation: Aminoglutethimide, AGT and Glibenclamide, Gli. Concentration: AGT 5μM, BIO 1μM, NU6102 5μM, HA14-1 1μM, SU1498 5μM, Gli 5μM and KN93 5μM. *, $p < 0.05$, **, $p < 0.01$ *vs.* DMSO treatment (Dunn's test for multiple comparisons, n = 3–11). (c) Chemicals increased mESCM numbers during Flkd6+2 to Flkd6+5. p value: Mann-Whitney test, n = 3. (d) Captured images from time-lapse video recording during Flkd6+0.5 (0:00) to 4.5 (96:00).

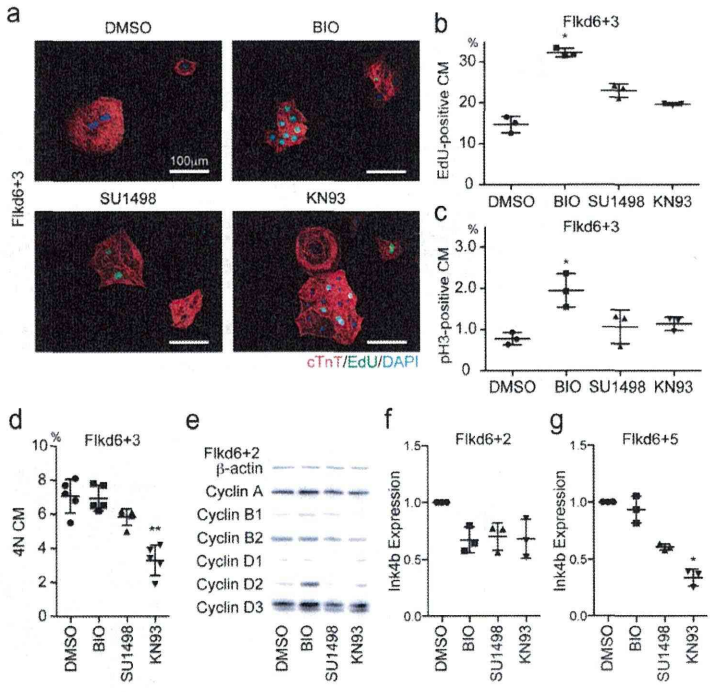

Figure 2.

Cell Cycle in mESCM was Enhanced by Identified Chemicals. (a) Immunostaining of purified mESCMs for cardiac troponin T (red), EdU (Green), and DAPI (Blue) at Flkd6+3. Scale bar = 100μm (b–c) Quantification of mESCMs positive for cell cycle markers (b) S phase (EdU-positive) and (c) M phase (phospho histone H3: pH3-positive) at Flkd6+3. *, $p < 0.05$ vs. DMSO group (Dunn's test, $n = 3$). (d) Quantification of 4N (Binuclear (2×2N) and G2/M) mESCMs by flow cytometry. **, $p < 0.01$ vs. DMSO group (Dunn's test, $n = 5$). (e) Western blotting for cyclins at Flkd6+2. BIO treatment increased Cyclin Ds. (f–g) qRT-PCR for *Ink4b*, a CDK inhibitor at Flkd6+2 (f) and Flkd6+5 (g). The expression of Ink4b was suppressed by all three chemicals at Flkd6+2 and more than 60% by KN93 at Flkd6+5. Relative expression levels compared to DMSO treatment group were shown. *, $p < 0.05$ and **, $p < 0.01$ vs. DMSO group (Dunn's test, $n = 3$).