Megakaryocytes (MKs) are exposed to shear flow as they migrate from the bone marrow hematopoietic compartment into circulation to release pro/preplatelets into circulating blood. Shear forces promote DNA synthesis, polyploidization, and maturation in MKs, and platelet biogenesis. To investigate mechanisms underlying these MK responses to shear, we carried out transcriptional analysis on immature and mature stem cell-derived MKs exposed to physiological shear. In immature (day (d)9) MKs, shear exposure up-regulated genes related to growth and MK maturation, whereas in mature (d12) MKs, it up-regulated genes involved in apoptosis and intracellular transport. Following shear-flow exposure, six activator protein 1 (AP-1) transcripts (ATF4, JUNB, JUN, FOSB, FOS, and JUND) were up-regulated at d9 and two AP-1 proteins (JunD and c-Fos) were up-regulated both at d9 and d12. We show that mitogen-activated protein kinase (MAPK) signaling is linked to both the shear stress response and AP-1 up-regulation. c-Jun C-terminal kinase (JNK) phosphorylation increased significantly following shear stimulation, whereas JNK inhibition reduced shear-induced JunD expression. Although p38 phosphorylation did not increase following shear flow, its inhibition reduced shear-induced JunD and c-Fos expression. JNK inhibition reduced fibrinogen binding and P-selectin expression of d12 platelet-like particles (PLPs), whereas p38 inhibition reduced fibrinogen binding of d12 PLPs. AP-1 expression correlated with increased MK DNA synthesis and polyploidization, which might explain the observed impact of shear on MKs. To summarize, we show that MK exposure to shear forces results in JNK activation, AP-1 up-regulation, and downstream transcriptional changes that promote maturation of immature MKs and platelet biogenesis in mature MKs.

Megakaryocytes (MKs) are large, polyploid cells that reside in the bone marrow and differentiate from the CD34+ hematopoietic stem cell. MKs are responsible for producing platelets, the small anuclear blood cells that regulate thrombosis, vascular repair, and immune responses (1, 2). Factors that play a role in megakaryocytic differentiation include cytokine composition (3), oxygen concentration (4), matrix elasticity (5), and most recently acknowledged, shear flow (6–8). MKs are subjected to shear flow at different locations during their differentiation and maturation. Mature MKs may squeeze through the incomplete endothelial lining of bone marrow capillaries to shed pro/preplatelets (PPT) into circulation under shear forces generated by the blood flow (6–8). Less mature MKs may enter circulation and mature and shed PPT in the lung vasculature, thus being exposed to a wide spectrum of shear and other biomechanical forces (8). The evidence for the physiological importance of shear forces in platelet biogenesis has been established based on a series of studies and notably in Refs. 6 and 7, as reviewed in our 2014 article (8). Briefly, MKs are exposed to shear forces of different intensity and duration depending on the location in the body. MKs will experience shear forces as they cross the endothelial lining of the bone marrow sinusoids to enter circulation. The level of shear varies from 1.3 to 4.1 dyn/cm² and the duration is about 30 min, but can vary from 10 to 60 min. In circulation, MKs might experience different levels of biomechanical forces. Notably, MKs may experience higher levels of shear and for longer periods of time in the lung vasculature as detailed in our 2014 article (8). It has been shown that shear stress promotes MK morphological maturation, increased DNA synthesis, and extensive platelet-like particle (PLP), PPT, and MK-microparticle production (7, 8). However, the underlying mechanotransduction pathways engaged by MK cells remain unexplored.

Better understanding of megakaryopoiesis and thrombopoiesis has several important implications in human health. The demand for donor-derived platelets for transfusion continues to grow (9). PLPs produced ex vivo would bypass the dependence on blood donors, significantly decrease the risk of contamination with blood-borne pathogens, and prevent alloimmunization to transfused platelets. Current ex vivo PLP pro-
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duction methods are expensive and have low yield (10). Understanding how MKs produce platelets in response to shear flow in vivo would help to replicate the process for affordable, high-yield ex vivo PLP generation. Furthermore, elucidating the transcriptional and molecular processes that ultimately lead to platelet biogenesis in vivo is necessary to develop safe and effective therapies for thrombocytopenic diseases, such as aplastic anemia, hereditary syndromes with decreased platelet production, and medicine-induced myelosuppression.

Mechanotransduction, the process in which cells convert biomechanical stimuli into molecular signaling, has been extensively studied in endothelial cells (11–15), but also in other cell types such as platelets (16, 17), lymphocytes (18), bone cells (19, 20), vascular smooth muscle cells (21–23), chondrocytes (21), and tumor cells (24, 25). In endothelial cells, the MAPK pathways p38, ERK, and JNK are implicated in transducing shear-flow stimuli (26–28). p38 was linked to endothelial cell morphological changes (26), ERK is thought to play a role in cell growth (28), and JNK is hypothesized to mediate apoptotic processes (28). In vascular smooth muscle cells, p38 was responsible for mechanotransduction, but JNK was not involved (29). In osteoblasts, both ERK and p38 were involved in transducing mechanical stimuli and up-regulating osteoblast-specific genes (30).

Mechanotransduction is much less understood in MKs. Previous studies on the impact of shear stress on MK differentiation focus on cytoskeletal and morphological changes (7, 31, 32), but no signaling pathways have been investigated. MAPK pathways are active in MKs, but they have not yet been linked to mechanotransduction (33). In static MK cultures, ERK was linked to growth and CD41 expression, p38 activity had variable effects (both increased and decreased CD41 expression), and JNK has not been extensively studied (33). Also, there exists no reports detailing the impact of shear stress on the MK transcriptional program. Such data could have been engaged to pose hypotheses on mechanotransduction signaling in MK cells.

Here we report genome-wide transcriptional analysis of how MKs respond to shear flow, aiming to elucidate the specific transcriptional changes that occur in MKs following exposure to shear flow. Our data implicate both the MAPK pathway and its downstream transcription factors, notably AP-1. We show significant up-regulation of AP-1 transcripts and proteins in immature and mature MKs following exposure to shear. We also found that the JNK and p38 pathways were necessary for the shear stress response in both immature and mature MKs and was linked to the observed AP-1 up-regulation in response to shear-flow exposure. Beyond the recent demonstration in MKs that fluid shear rapidly activates myosin-II (MYH9) through de-phosphorylation (31), these data are among the first reported on mechanotransduction in MK cells.

Experimental Procedures

Megakaryocyte Cultures and Exposure of MK Cells to Shear Flow—Frozen human G-SCF-mobilized peripheral blood CD34+ cells (Fred Hutchinson Cancer Research Center) were cultured as described (3). On day 8, MKs were then seeded into von Willebrand factor-coated (Hematologic Technologies) rectangular flow slides (μ-Slide IV Luer, Ibidi) (to be used for exposing MK cells to shear in "fluidic" flow chambers (Ibidi)) at 300,000 cells per slide (8). On day 9, cells were subjected to shear flow that results in shear stress levels of either 1.0 dyn/cm² for 2 h or 2.5 dyn/cm² for 30 min using a syringe pump system (Dual NE-4000 Double Syringe Pump; New Era Pump Systems) established by Jiang et al. (8). The remaining MKs were cultured to day 12 using the current condition at day 9.

Immunofluorescence and Flow Cytometric Assays to Quantitate Protein Expression Levels—Immunofluorescence staining was used for protein quantification in place of Western blot analysis due to the low numbers of MKs retrieved after shear flow that result in submicrogram total protein lysate, and scaling up the system was not practical. Thus, immunofluorescence coupled to mean fluorescent intensity quantification was chosen for quantitating the levels of several proteins, a well established method for quantitating protein expression on a single-cell basis (34–36). Immunofluorescence staining was performed as described by Kodih et al. (37) using option 2. Cells were fixed and stained within the flow chamber to retain morphological structures. Primary antibodies used were: anti-JunD (Santa Cruz number sc-74), anti-c-Jun (Santa Cruz number sc-74543), anti-c-Fos (Santa Cruz number sc-52), anti-ATF4 (Santa Cruz number sc-200), anti-JunB (Santa Cruz number sc-73), anti-RGS16 (Santa Cruz number sc-23859), anti-Runx1 (Santa Cruz number sc-8563), anti-p-p38 (Santa Cruz number sc-101759), anti-p-JNK (Santa Cruz number sc-6254), anti-p-ERK (Abcam number ab32538), normal mouse IgG (Santa Cruz number sc-3879), normal rabbit IgG (Santa Cruz number sc-3888), and normal goat IgG (Santa Cruz number sc-2028). Secondary antibodies were anti-mouse Alexa Fluor 488 (Life Technologies number A11017), anti-rabbit Alexa Fluor 647 (Life Technologies number A21245), and anti-mouse Alexa Fluor 647 (Life Technologies number A21235). Confocal microscopy was performed along the lines of what has been previously reported (38–42), as well as from our own laboratory (43–45). Quantification was performed within the cell perimeter only; to delineate the cell borders, 5 μM Syto13 (Life Technologies number S7575) or Syto40 (Life Technologies number S11351) was used.

Fluidic chambers were visualized using a Zeiss LSM 780 microscope with Plan-Apochromat ×40/1.4 oil DIC objective and Zeiss Zen software. Control and treated images were acquired using the same laser settings. Relative immunofluorescence between flow and static samples were quantified using Velocity software (PerkinElmer Life Sciences). All samples were normalized to IgG isotype controls. For cells harvested from the fluidic chambers, analysis for protein quantification was performed on the FACS Aria II flow cytometer.

Microarray-based Gene Expression Analysis—Gene expression microarray analysis was performed according to the pipeline developed in our laboratory (44–49). RNA from 3 biological replicates of unstressed (static) and shear-stressed (flow) MKs were isolated using the ReliaPrep RNA Cell Miniprep System (Promega). Bioanalyzer RNA 6000 Pico chips (Agilent) or fragmentation analysis (Advanced Analytical Technologies, Inc., Fragment Analyzer) was performed to ensure RNA was not degraded (no lower than an RNA integrity number of 7).
The Low Input Quick Amp Labeling Kit (Agilent) was used to generate cDNA and labeled cRNA, along with the Spike-In Kit (Agilent) to detect an amplification bias. RNA was hybridized to Agilent Human GE 4 × 44K v2 Microarray slides (catalog number 427 G2519F-026652) and washing was completed following the manufacturer’s instructions (catalog number 5188-5327). Slides were scanned on an Agilent Microarray scanner (G2565BA) and its Feature Extraction Software was used to extract the data. Raw microarray data were deposited in the Gene Expression Omnibus (number GSE75766). The 3 biological replicates were analyzed each with dye-swap experiments to correct for dye incorporation bias. Lowess normalization between dye-swap experiments was performed in R software (3) using the limma package (45) and the SNN-LERM (segmental nearest neighbor of logarithmic expression ratio method) algorithm (50). The MultiExperiment Viewer (MeV) (51) was used to visualize the data, as well as compare results from days 9 and 12. Significance Analysis of Microarray (52) was performed with a 5% false discovery rate to determine genes that were statistically differentially expressed. DAVID (Database for Annotation, Visualization, and Integrated Discovery) was used for gene ontology analysis (53).

MAPK Inhibitor Studies—MKs were cultured as described to d9 for immature MK cells and d12 for mature MK cells. On each of days 9 or 12, MAPK inhibitors were applied to the MKs 30 min prior to shear flow exposure. The media within each fluidic chamber was exchanged with media containing 0 (control), 1, 10, or 100 μM JNK inhibitor SP600125 (Santa Cruz number sc-200635) (54) or 0 (control), 1, 10, or 30 μM p38 inhibitor SB203580 (Santa Cruz number sc-3533) (55). Inhibitor strengths were chosen based on what others have described in the literature (56–59), in addition to a lower and higher concentration. Optimal inhibitor concentrations were determined by optimizing MAPK inhibition (strongest inhibition of desired MAPK and weakest inhibition of undesired MAPK) while limiting cell death (data not shown). Two fluidic chambers were used for each inhibitor and control, one for a static control and the other for shear flow exposure.

PLP Particle Analysis—PLPs were harvested from d12 MKs as previously described (8). PLPs were obtained from MKs following 2.5 h (30 min of MAPK inhibition in static culture followed by 120 min of shear flow or static culture with MAPK inhibitor) of MAPK inhibition or vehicle control (dimethyl sulfoxide).

Results

The Transcriptome Underlying the Response of Megakaryocytes to Shear Flow Displays Maturation Patterns at D9 and Platelet-biogenesis Markers at D12—We have previously found that immature (d9) and mature (d12) MKs respond differently to shear flow (8). In immature MKs, shear flow increased DNA synthesis and phosphatidylserine expression, whereas in mature MKs, shear flow increased phosphatidylserine expression, caspase activation, and the biogenesis of PLPs, PPTs, and microparticles (8). Therefore, we hypothesized that the transcriptional changes that occur following shear flow exposure reflect these findings. To test this hypothesis, we performed gene expression analysis on three biological replicates of immature and mature MKs. For the first 8 days of culture, MKs were maintained statically, as would be the case in vivo. At the following described time points, MKs were subjected to shear flow for a duration that is consistent with their time at the marrow-vasculature interface in vivo (6). For immature MKs (d9), we examined transcriptional changes at two time points: immediately following 30 min of shear flow (“30 min”), as well as after 30 min of static culture post the 30 min of exposure to shear flow (“60 min”). MKs at the marrow-vasculature interface will release all of their PPTs and PLPs between 10 and 30 min ex vivo and in vivo (6, 7). The former time point was used to capture these molecular changes. The latter time point captured delayed effects of shear-flow exposure that would be seen in vivo if the MK cell entered the vasculature, which is thought to occur with smaller, less mature MKs (60). For mature MKs (d12), transcriptional analysis was performed at only the first time point (immediately following shear flow, 30 min), to focus solely on physiologically relevant changes, as mature MKs shed their cytoplasm within the first 30 min following shear exposure (10). Following shear exposure for 30 min at 2.5 dyn/cm² (MKs are exposed to 1.3–4.1 dyn/cm² in bone marrow sinusoids (6)), there were 293 statistically significant differentially expressed genes at d9: 90 up-regulated at 30 min, 53 up-regulated at 60 min, 49 up-regulated both at 30 and 60 min, and 101 down-regulated at 30 min (Table 1, A-D, and data not shown). At d12, there were 3620 differentially expressed genes: 3573 up-regulated and 83 down-regulated at 30 min (Table 2, A-C, and data not shown). These data are consistent with the notion that MKs modulate their transcriptome in response to shear stress on the time scale of in vivo platelet biogenesis (30 min).

In immature MKs, the three most significantly enriched (p < 0.05) gene ontology (GO) functions were transcription factor (10.3%), metabolism (10.4%), and signaling (18.7%) (data not shown), all of which correlate with changes seen during terminal differentiation of stem cells and MK progenitors (44, 62, 63). In addition, GO clustering detected enrichment of genes important in myeloid differentiation (Table 1E). Furthermore, 10 (3.3%) of the 279 genes that our laboratory has previously identified as transcriptionally up-regulated during megakaryopoiesis (46) were selectively up-regulated following shear flow at d9 (Table 1C). Half of these shear-sensitive MK genes were monomers of the AP-1 transcription factor. AP-1 is active as a dimer and can be composed of c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, Fra-2, or ATF monomer proteins (64). The enriched GO cluster for JNK regulation (Table 1F) complements the up-regulation of AP-1 transcripts (Table 1D), whose expression can be directly induced by JNK (65). Taken together, these data suggest a role for both AP-1 and JNK in the shear stress response of immature MKs.

Although the shear stress response of immature MKs involved strong up-regulation of signaling components and transcription factors, the shear stress response of mature MKs had, instead, a significant enrichment of genes involved in apoptosis and intracellular transport. Compared with d9, the enriched gene functions transcription factor and signaling decreased to 2.8 and 8.0%, respectively, and metabolism increased to 16.7% following shear flow at d12 (data not shown). Although the number of MK maturation genes increased to 46
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Table 1: Gene expression analysis of d9 Mks discovers significantly upregulated genes following shear-flow exposure.

Common categories of significantly upregulated genes in d9 Mks following 30 or 60 min of shear-flow exposure (2.5 dyn/cm²) include cell differentiation (A), cellular biosynthetic processes (B), MK maturation (C), and AP-1 monomers (D), as determined by gene expression microarray analysis. For each official gene symbol (column 1), the respective Agilent microarray probe name (column 2) and average fold-change (FC) of 3 biological replicates at 30 min (column 3) and 60 min (column 4) is provided. Gene ontology (GO) cluster analysis (E, F) detected cellular processes that were enriched in the given data set. Enrichment scores over 1.3 (equivalent to p = 0.05) were considered significant. E, GO processes related to myeloid differentiation. F, GO processes related to the JNK signaling pathway.

Table 2: Gene expression analysis of d12 Mks discovers significantly upregulated genes following shear-flow exposure.

Common categories of significantly upregulated genes in d12 Mks following 30 min of shear-flow exposure (2.5 dyn/cm²) include megakaryopoesis (A), apoptosis (B), and intracellular transport (C), as determined by gene expression microarray analysis. For each official gene symbol (column 1), the respective Agilent microarray probe name (column 2) and average fold-change of 3 biological replicates (column 3) is provided.

The role of AP-1 in the shear stress response, each of these genes was also examined at the protein level. At d9, whereas ATF4 was the most up-regulated transcript, it was down-regulated at the protein level (data not shown). JUN/JUND mRNA was only modestly up-regulated but at the protein level, its expression increased 14-fold following exposure to shear at d9 (Fig. 1, A and E). The other AP-1 proteins were not differentially expressed at d9 (data not shown). Interestingly, c-Fos proteins were concentrated at the nuclear envelope before and after shear flow exposure (Fig. 1E). Others have found that transcription factors, and in particular c-Fos, can be sequestered away from active genes by lamin A/C at the nuclear envelope (49). The strong up-regulation of JunD in combination with c-Fos sequestration suggests that JunD may be acting alone in the shear stress response of immature MKs. Following shear flow at d12, AP-1 transcripts were not differentially expressed compared with statically grown MKs. However, two AP-1 proteins, JunD and c-Fos, were up-regulated following shear flow exposure (Fig. 1, A and F). These data suggest that the active AP-1 dimer in the shear stress response of immature and mature MKs contains JunD, c-Fos, or both.

Each combination of AP-1 monomers binds to its own specific consensus sequence within gene promoters, which explains the diversity in AP-1 cellular responses (70). Bioinfor-
matic analysis showed that a majority of genes up-regulated after shear flow at d9 and a smaller portion at d12 have transcription factor binding sites for the JunD:c-Fos heterodimer (the CRE-1 consensus sequence) or JunD homodimer (the CRE-2 consensus sequence) in their promoter regions (Fig. 1C). Among those, RGS16 and RUNX1, two genes important in MK differentiation, which were both up-regulated transcriptionally (5.2- and 1.2-fold, respectively) and translationally (2.34- and 2.41-fold, respectively) following shear flow at d9 (Table 1C and Fig. 1B and G), have multiple CRE-2 binding sites near their transcriptional start sites (Fig. 1D). This suggests that in the d9 shear stress response, the active AP-1 dimer is a JunD:JunD homodimer. Expression of p21 protein in d9 MKs following shear-flow exposure was also measured due to its significant
mRNA up-regulation (Table 1C), but its protein levels were highly variable across biological donors (data not shown), so no conclusion could be drawn. To summarize, we conclude that of the five AP-1 transcripts up-regulated at d9, only JunD and c-Fos are up-regulated at the protein level. In addition, JunD and c-Fos are up-regulated following exposure to shear flow at
d12. Furthermore, it is possible that following shear flow at d9, JunD forms a homodimer and regulates the MK-relevant genes RUNX1 and RGS16.

Shear Flow Leads to MAPK Phosphorylation That Results in Enhanced AP-1 Expression—We examined the JNK pathway for several reasons. First, JNK proteins are activated in response to mechanical stress in other cell types (e.g. Drosophila s2r+ cells (71) and bovine aortic endothelial cells (28)). Second, AP-1 proteins that are expressed at a basal level are phosphorylated by JNK in response to a stimulus and initiate a forward-feedback loop by further up-regulating additional AP-1 proteins (72), which could explain post-shear up-regulation of the various AP-1 monomers at d9 and d12. Finally, our gene expression analysis shows post-shear up-regulation of genes for JNK pathway transducers and scaffolding proteins (Fig. 2, A and B), which gather the transducers near each other to enhance the JNK signaling cascade (73).

To examine the expression of active JNK proteins, the phosphorylated form was probed before and after exposure to shear. In immature MKs, p-JNK expression was up-regulated 3.3-fold compared with statically grown MK cells (Fig. 2, C and D), whereas in mature MKs, it was up-regulated 2-fold (Fig. 2, C and E). To determine whether ERK was also contributing to the AP-1 induction, p-ERK was examined post-shear and found to be down-regulated 2.1- and 1.9-fold at d9 and d12, respectively (Fig. 2, C-E). Due to the overlap between the p38 and JNK pathways (74–76), p38 phosphorylation was also quantified. Following shear flow at both d9 and d12, p-p38 was either strongly up-regulated or down-regulated, leading to large variations among biological donors (data not shown), and thus, no conclusion about the effect of shear flow on p38 activation could be drawn from these data.

To determine whether there is a link between MAPK phosphorylation and AP-1 up-regulation in the MK shear stress response, we examined the impact of MAPK inhibitors on AP-1-protein expression in immature and mature MKs following exposure to shear flow. In immature MKs (d9), JNK inhibition, but also surprisingly p38 inhibition, abrogated the strong up-regulation of JunD in response to shear flow (Fig. 3A). The impact of p38 inhibition was unexpected given that we were unable to detect a consistent impact of shear stimulation on p38 phosphorylation. c-Fos, which was not differentially expressed following shear flow at d9, was up-regulated with JNK and p38 inhibition (Fig. 3A). In mature d12 MKs, p38 inhibition significantly abrogated post-shear up-regulation of JunD and c-Fos, whereas JNK inhibition impacted only c-Fos up-regulation (Fig. 3B). From these data, we conclude that JNK is phosphorylated in response to shear flow and directly leads to AP-1 up-regulation, whereas p38 plays a role in shear-induced AP-1 expression that is independent of increased p38 phosphorylation.

Higher AP-1 Expression Imparts Increased MK DNA Synthesis and Polyploidization—Given that exposure to shear increases DNA synthesis and polyploidization (8) and, as shown here, up-regulates AP-1 expression, we wanted to examine if higher AP-1 expression might correlate and/or impart the increased DNA synthesis and polyploidization phenotype. To do so, first, the ploidy classes of MKs with either detectable (high) or undetectable (low) AP-1 expression were measured using flow cytometry. In both immature (d9) and mature (d12) MKs, expression of either c-Fos or JunD was associated with higher-ploidy classes (Fig. 4, A and B). It is notable that at d12, AP-1-negative MKs were mostly 2N or 4N, whereas AP-1-positive MKs were mostly 8N or higher ploidy (Fig. 4B). At a single-cell level, AP-1 expression increased one-to-one as the nucleus enlarged (Fig. 4, C and D), thus suggesting that AP-1 is continually needed throughout MK maturation. If AP-1 was an auxiliary protein without a functional role, the enlarging nucleus area would dilute the ratio of AP-1:DNA and the dot plot would...
be more horizontal. These data suggest that higher AP-1 expression might confer an enhanced DNA synthesis and polyploidization phenotype. We pursued this further with experiments involving MAPK inhibitors.

Given that MAPK inhibition reduces AP-1 expression (Fig. 3), and that higher AP-1 expression correlates with higher MK DNA synthesis and polyploidization (Fig. 4), we examined the impact of MAPK inhibitors on MK DNA synthesis and polyploidization with and without shear stimulation. We used the following protocol, which is consistent with prior work in our laboratory (8): 30 min of incubation with the inhibitor, followed by 120-min shear flow exposure, and then 2 h back in culture. Without MAPK inhibition, statically grown AP-1-negative MKs incorporated less BrdU (and thus exhibited less DNA synthesis) compared with the AP-1-positive MKs in all ploidy classes (Fig. 5). With JNK inhibition, statically grown AP-1-positive MKs had higher DNA synthesis than AP-1-negative MKs in the 8N+ ploidy class (Fig. 5). With p38 inhibition, statically grown AP-1-positive MKs had higher DNA synthesis than AP-1-negative MKs in the 2N and 4N ploidy classes (Fig. 5). Also, with p38 inhibition and following shear flow, AP-1-positive MKs had higher DNA synthesis than AP-1-negative MKs in the 2N ploidy class (Fig. 5). In addition, with or without MAPK inhibition, shear flow stimulated DNA synthesis in the AP-1-negative MKs to levels that were found in the AP-1-positive MKs (Fig. 5). Compared with control

FIGURE 4. Increasing AP-1 expression correlates with ploidy class and total DNA content. A, ploidy classes for statically grown d9 MKs that are AP-1-positive (top row) or AP-1-negative (bottom row), where “AP-1” represents either JunD (left) or c-Fos (right). B, ploidy classes for statically grown d12 MKs that are AP-1-positive (top row) or AP-1-negative (bottom row), where “AP-1” represents either JunD (left) or c-Fos (right). At both d9 and d12 (A and B), there are more polyploid (8N+) MKs than low ploidy (2N/4N) MKs in the AP-1-positive population. C, JunD (left) and c-Fos (right) expression (Alexa Fluor 488) versus total DNA content (7-AAD) for d9 MKs. D, JunD (left) and c-Fos (right) expression (Alexa Fluor 488) versus total DNA content (7-AAD) for d12 MKs. Both d9 and d12 MKs (C and D) demonstrate a direct correlation between the strength of AP-1 expression and amount of DNA at a single-cell level.
MKs, JNK inhibition decreased DNA synthesis in shear-stimulated AP-1-positive MKs in 2N and 8N+ ploidy classes (Fig. 5).

In all control MKs and most MAPK-inhibited MK ploidy classes, AP-1-negative MKs in static culture had the least BrdU incorporation, AP-1-positive MKs with shear-flow exposure had the greatest BrdU incorporation, and AP-1-negative MKs with shear-flow exposure were between the two (Fig. 5). This suggests that not only does AP-1 confer a maturation benefit for MKs, but there may also be an additive positive effect with shear flow. Taken together, these data suggest that higher AP-1 expression could stimulate DNA synthesis in a similar manner as shear flow stimulation of AP-1 negative MK cells and, more generally that AP-1 expression correlates with DNA content in immature MKs.

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**FIGURE 5.** AP-1 content correlates with DNA synthesis and JNK inhibition decreases DNA synthesis in immature (d9) MKs. Percent BrdU incorporation of AP-1-positive or AP-1-negative MKs in 2N, 4N, and 8N ploidy classes at d9 following exposure to the JNK or p38 inhibitor according to the protocol of 30 min of JNK or p38 inhibition in static culture, 120 min of JNK or p38 inhibition with shear flow, and 120 min back in culture. $n = 3$, *, $p < 0.05$; **, $p < 0.01$, paired t test, error bars: S.D. In all ploidy classes, there was significantly higher BrdU incorporation of statically grown AP-1-positive control MKs compared with AP-1-negative MKs. Only a fraction of the statistical analysis results is displayed on the graph.

**FIGURE 6.** MAPK inhibition decreases PLP biogenesis and PLP fibrinogen (FGN) binding. A, number of PLPs generated by d12 MKs in static culture (black) and following shear-flow exposure of 2.5 dyn/cm² for 2 h (gray). MKs were incubated with JNK or p38 inhibitors for 30 min prior to 2-h static culture or shear flow. $n = 3$, *, $p < 0.05$, paired t test. B, increase in the number of PLPs bound to fibrinogen after thrombin activation (compared with no-thrombin control). PLPs were generated by d12 MKs in static culture or following shear-flow exposure (2.5 dyn/cm² for 2 h). MKs were incubated with JNK or p38 inhibitors for 30 min prior to a 2-h static culture or shear flow. $n = 3$, *, $p < 0.05$; ***, $p < 0.001$, paired t test, error bars: S.D.
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increased in PLPs derived from MKs following shear-flow exposure (without MAPK inhibition) (Fig. 6B). JNK and p38 inhibition abrogated the increased fibrinogen binding of shear-derived PLPs (Fig. 6B). To summarize, our data here suggest that JNK inhibition decreases PLP quantity and quality, whereas p38 inhibition only decreases PLP quality. This suggests that AP-1 expression, as stimulated by shear flow, impacts both the quantity and quality of generated PLPs.

Discussion

We investigated how shear flow impacts the megakaryocytic transcriptome aiming to identify potential modulators involved in the shear stress response. Our data indicate that shear flow induces transcriptional responses mediated by the AP-1 transcription factor through JNK activation and p38 activity. MK polyploidization initiates in early megakaryopoiesis (77), and is mediated by the c-Fos:JunD heterodimer transcription factor. Eriksson et al. (78) implicated c-Fos and JunD in the megakaryocytic differentiation of K562 cells in static culture. Our work here aimed to understand the role of shear flow in MK transcriptome modulation and through this, we discovered the role of AP-1 in shear-accelerated MK maturation. We discovered that high AP-1 expression correlates with high MK ploidy at d9 (Fig. 4). In addition, AP-1-positive MKs have greater DNA synthesis compared with AP-1-negative MKs (Fig. 5). However, shear flow is capable of increasing DNA synthesis in AP-1-negative MKs to match that found in AP-1-positive MKs (Fig. 5). These data suggest that AP-1 plays a role in increasing DNA synthesis in immature MKs and it may be additive to the maturation benefit from shear flow exposure that we have previously reported (8). Furthermore, JNK inhibition decreased DNA synthesis in shear-stimulated MKs compared with control MKs (Fig. 5). A majority of MKs are AP-1 positive by d9 and many are polyploid (Fig. 4). Further studies are needed to determine whether the MKs that are AP-1 negative and not polyploid at d9 become polyploid by d12. This would inform whether AP-1 expression is absolutely necessary to transition from mitosis into endomitosis (the process in which MKs become polyploid (79)).

AP-1 is up-regulated through MAPK signaling (65, 80, 81) and phosphorylation of basal AP-1 increases its transcriptional activity by improving the binding efficiency of the transcription activator CBP (CREB-binding protein) (82). Furthermore, AP-1 activation initiates a positive feedback loop for further AP-1 up-regulation (70). This could explain the correlation between increased p-JNK (Fig. 2C), the up-regulation of five AP-1 transcripts (JUN, JUNB, JUND, FOS, ATF4) (Table 1D), and the up-regulation of AP-1 proteins (Fig. 1A) following shear flow exposure at d9. Confirming that this relationship between JNK and AP-1 expression also holds true in MKs following shear flow (Fig. 3), we may be able to accelerate MK maturation in vitro without necessitating the use of shear flow (e.g. activating the JNK pathway and AP-1 expression, for which we also demonstrated is correlated with increased DNA content). Although more work is needed to determine how JNK is phosphorylated in the MK shear stress response, the mechanism may resemble what has been observed in endothelial cells. G protein-coupled receptors, Ras, and tyrosine kinases are responsible for activating MAPKs in endothelial cells following shear flow (49). Although there may be multiple receptors responsible for sensing shear flow, G protein-coupled receptors may play the most significant role for activating the JNK pathway. Although p38 phosphorylation did not increase following shear flow, the effects of p38 inhibition show that this kinase is important in transducing shear flow, potentially by collaborating with other factors.

In the in vitro culture of stem cell-derived MKs, mature d12 MKs have extensive PPT formation (8). In addition, shear stress significantly increases biogenesis of functional PLPs (8). Our data (Figs. 5 and 6) show that MAPK inhibition during shear flow exposure of mature MKs impacted both MK ploidy and PLP activation. The negative effects of MAPK inhibitors on fibrinogen binding could be due to a defect in PLP biogenesis caused by the inhibitor interfering with the shear-induced JNK pathway scaffolds MAPK8IP1 (JIP1) and MAPK8IP3 (JIP3) (Fig. 7).
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Author Contributions—E. T. P. and S. L. designed the study; S. L. carried out experiments; S. L. and E. T. P. analyzed and interpreted the data; E. T. P. and S. L. wrote the manuscript.

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