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ERK, JNK, and p38 MAP Kinases Differentially Regulate Proliferation and Migration of Phenotypically Distinct Smooth Muscle Cell Subtypes

Mary M. Kavurma[1,2] and Levon M. Khachigian[1,2]*

[1]Centre for Vascular Research, The University of New South Wales, Australia
[2]Department of Haematology, The Prince of Wales Hospital, Sydney, Australia

Abstract Proliferation and migration of vascular smooth muscle cells (SMCs) are important processes involved in the pathogenesis of vascular disorders such as atherosclerosis and post-angioplasty restenosis. Here we demonstrate that proliferation and migration of specific SMC subtypes is mitogen-activated protein (MAP) kinase-dependent. WKY12-22 SMCs derived from the aortae of 12 day-old pup rats proliferate and migrate faster than WKY3M-22 SMCs derived from the aortae of adult rats. WKY12-22 and WKY3M-22 cells equally expressed the active forms of phospho (Thr^{183}/Tyr^{185})-c-Jun N-terminal kinase (JNK) and phospho (Tyr^{182})-p38, whereas the activity of extracellular signal-regulated kinase (ERK) was greater in WKY12-22 cells compared with WKY3M-22 cells. Proliferation of both SMC subtypes was attenuated by PD98059, SP600125 and SB202190, inhibitors of ERK, JNK, and p38, respectively. However, inhibition of PD98059 had a more profound effect on WKY12-22 SMCs. Furthermore, migration of WKY12-22 and WKY3M-22 cells was inhibited by SP600125 and SB202190, however, PD98059 failed to influence migration of either SMC subtype. Hence, migration of both SMC subtypes is JNK- and p38-dependent, but not ERK-dependent. These findings demonstrate that SMC heterogeneity is mediated, at least in part, by the activity of specific MAP kinase subtypes. J. Cell. Biochem. 89: 289–300, 2003.

Key words: smooth muscle cell; heterogeneity; proliferation; migration; phenotype

It is now well accepted that smooth muscle cell (SMC) heterogeneity is a distinct feature in the pathogenesis of vascular disorders [Majesky and Schwartz, 1990; Ross, 1993]. In the vascular response-to-injury, SMC from the normally quiescent media migrate to the inner surface (intima), where they proliferate and accumulate, thickening the arterial wall. The fact that SMC can undergo such dramatic changes in cell movement and growth, has led to the hypothesis that differences in phenotype between SMC subtypes may contribute to arterial remodeling during development, repair, and disease [Majesky and Schwartz, 1990].

SMC heterogeneity has been the focus of considerable investigation over many years [Majesky et al., 1988, 1992; Lemire et al., 1994, 1996; Adams et al., 1999]. Based on morphological differences, growth properties, and gene expression, SMC are known to exist in at least two distinct phenotypes. SMC restricted to the medial compartment of the artery wall express proteins involved in contraction including isoforms of myosin and actin, and are, accordingly, classed as the 'contractile' phenotype [Campbell and Campbell, 1990]. In contrast, SMC from the intima of diseased vessels are immature in phenotype and express growth factors and extracellular matrix proteins [Majesky and Schwartz, 1990; Glukhova et al., 1991; Ross, 1993], and are thus termed 'synthetic.' The precise basis of SMC modulation and heterogeneity within the arterial wall is still unclear.

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*Correspondence to: Levon M. Khachigian, PhD, Centre for Vascular Research, The University of New South Wales, Sydney NSW 2052, Australia.
E-mail: L.Khachigian@unsw.edu.au

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The existence of two distinct SMC subtypes, has provided a useful comparative model to investigate differences in phenotype. SMC derived from the aortae of pup (2-week old, WKY12-22 cells) and adult (3-month old, WKY3M-22 cells) rats correlate with the 'synthetic' and 'contractile' phenotypes of SMC in atherosclerotic lesions in both human [Glukhova et al., 1991] and animal models [Frid et al., 1994; Hao et al., 2002]. Like the 'synthetic' phenotypes, WKY12-22 SMC are cobblestone in morphology, proliferate in plasma-derived serum, express mRNA for PDGF-A, PDGF-B [Majesky et al., 1988; Majesky and Schwartz, 1990; Lemire et al., 1994], and are deficient in PDGF-αR expression [Lemire et al., 1994]. These cells also strongly express the extracellular matrix proteins tropoelastin, α_1 procollagen (type I) and osteopontin [Majesky et al., 1992; Lemire et al., 1994]. In contrast, WKY3M-22 cells are elongated spindle-like in morphology, proliferate poorly in plasma-derived serum [Lemire et al., 1994], strongly express PDGF-αR [Lemire et al., 1994] and versican [Lemire et al., 1996], but poorly express PDGF [Lemire et al., 1994]. The signaling pathways differentiating one phenotype from another is extremely limited.

We previously demonstrated that strong basal PDGF-B expression in WKY12-22 cells is mediated, at least in part, by zinc finger transcription factors [Rafty and Khachigian, 1998]. The mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase, have been linked with a diverse range of biological activities such as cell attachment [Chen et al., 1994; Komaki et al., 2000], DNA synthesis [Seger et al., 1994], proliferation [Seger et al., 1994; Lai et al., 1996], differentiation [Schonhoff et al., 2001; Vaudry et al., 2002], SMC contraction [Adam et al., 1995; Meloche et al., 2000] and cell death [Ono and Han, 2000]. ERK1/2, for example, has been shown to regulate genes involved in SMC heterogeneity, including PDGF [Deguchi et al., 1999], tropoelastin [DiCamillo et al., 2002], osteopontin [Moses et al., 2001; Xie et al., 2001], and versican, indirectly through upregulation of angiotensin II [Shimizu-Hirota et al., 2001]. Understanding the signaling pathways controlling SMC phenotype could provide an important basis for targeted intervention or even forced pathway shifts in pathologies involving SMC heterogeneity such as, atherosclerosis and post-angioplasty restenosis. Here we examined the role played by these MAP kinase subtypes in the regulation of WKY3M-22 and WKY12-22 cell phenotype.

MATERIALS AND METHODS

Cell Culture

WKY12-22, WKY3M-22, or primary rat aortic SMC (RASMC) used between passage 3–8, were maintained in Waymouth's medium (Life Technologies, Inc.) pH 7.4, supplemented with 10% fetal bovine serum, 1 mM L-glutamine (Gibco BRL), penicillin 10 U/ml and streptomycin 10 μg/ml, at 37°C in a humidified atmosphere of 5% CO_2.

Proliferation Assay

Three thousand cells per well of WKY12-22, WKY3M-22, and RASMCs were seeded into 96WTP. Cells were trypsinized at indicated time points and placed into 10 ml vials of Isoton II. Total cell counts were performed using a Coulter Counter. Various indicated concentrations of PD98059 (MAPKK inhibitor, Calbiochem), SB202190 (p38 inhibitor, Calbiochem), and the JNK inhibitor (SP600125 [Bennett et al., 2001], Calbiochem) were added 10 and 34 h prior to counting. For PDGF-BB induced proliferation, RASMC were starved for 48 h in serum-free Waymouth's medium, followed by pre-treatment of cells with MAP kinase inhibitors. Ten hours post treatment with inhibitors, PDGF-BB (25 ng/ml) was added. Cells were counted after 48 and 62 h following PDGF-BB addition.

Flow Cytometry

DNA analysis of single cell suspension was performed by flow cytometry. Briefly, exponentially growing WKY12-22 and WKY3M-22 cells were lifted by trypsin/EDTA and washed in 1 × phosphate buffered saline (PBS). Cells were pelleted to 1×10^6 cells/100 μl. To 1×10^6 cells, 50 μl of 10 mg/ml RNase A, 200 μl of 100 μg/ml propidium iodide solution (in 0.9% NaCl), 200 μl of 2% Triton X-100, and 1 ml 1 × PBS was added. Cells were stained at room temperature for 30 min, prior to analysis by FacStar.

Western Blot Analysis

WKY12-22 and WKY3M-22 cells were lysed and freeze thawed in 1× RIPA buffer (150 mM

NaCl; 50 mM Tris-HCl, pH 7.5; 1% deoxycholate; 0.1% SDS; 1% Triton X-100) together with protease inhibitors (1% aprotinin; 2 mM PMSF; 10 μg/ml leupeptin; 5 mM EDTA). Proteins were resolved by 12% SDS–polyacrylamide gel electrophoresis and transferred onto Immobilon-P transfer membranes (Millipore). The membranes were blocked overnight in PBS containing 5% skim milk and 0.05% Tween 20. Phospho-JNK and p38 were detected with polyclonal antibodies (1:500, Santa Cruz Biotechnology) and visualized by chemiluminescence.

ERK Activity

The ERK assay (p42/44 MAP kinase enzyme assay system, Amersham) was performed in direction with the manufacturer's requirements. Briefly, SMC exposed to serum were lysed, and the supernatant was collected. The incorporation of ^{32}P into peptide was performed at 30°C for a period of 30 min. The reaction was terminated by the addition of stop reagent. For separation of phosphorylated peptides, 30 μl of terminated reaction mixture was blotted onto paper discs, washed with 75 mM orthophosphoric acid or 1% acetic acid. Paper discs were washed for a second time, followed by washing with distilled water. The discs were then placed in a scintillation vial and assayed for radioactivity. Calculations were performed to determine total phosphate transferred to peptide.

For MAP kinase-induced phosphorylation of Elk-1, the p44/42 MAP kinase assay kit (Cell signaling Technology) was used. Briefly, p44/42 MAP kinase was selectively immunoprecipitated from SMC lysates treated with or without PD98059. The resulting immunoprecipitate was incubated with an Elk-1 fusion protein in the presence of ATP and kinase buffer (supplied). Phosphorylation of Elk-1 was executed at 30°C for 30 min. The reaction was stopped by the addition of 3 × SDS sample buffer (187.5 mM Tris, pH 6.8; 6% w/v SDS; 30% glycerol; 150 mM DTT; 0.3% w/v bromophenol blue). Phosphorylation of Ser^{383} was measured by Western blot using the phospho-Elk-1 antibody provided.

SMC Migration Assay

Trypsinized and washed cells were resuspended in 2 ml of binding medium (Waymouth's, 1% bovine serum albumin, 25 mM HEPES) at a final density of 2×10^5 cells/ml. Cells were plated on duplicate polycarbonate membranes (Neuro Probe, Inc.) that were previously treated with 30 μg/ml Collagen Type IV (Sigma), Waymouth's medium and 75 mM HEPES in a total volume of 10 ml. Cells were allowed to migrate for 24 h at 37°C in a humidified atmosphere of 5% CO_2. Following incubation, cells that had migrated through the membrane and attached to the underside were fixed in methanol (cells present on the top of the membrane were removed). Conversely, cells that did not migrate through and left on topside of the duplicate membrane were also fixed (cells present on the bottom of the membrane were scraped off). This was followed by hemotoxylin staining for 1 min. Membranes were mounted on glass slides with immersion oil. Percentage of migrated cells were quantified by counting the number of cells in 3–6 random 40× high power fields on both the underside and topsides of the membranes. For PDGF-BB induced chemotaxis of RASMCs, PDGF-BB (25 ng/ml) was added to the binding medium in the lower chamber. Cells in the top chambers were also treated with indicated concentrations of PD98059 and SB202190 and SP600125 10 h prior to fixing the membrane.

SMC In Vitro Injury Assay

WKY12-22 and WKY3M-22 cells were seeded and grown to confluence in eight well chamber slides (Nunc, Copenhagen Denmark). Injury was performed using a single scratch by a sterile toothpick in 5% FBS Waymouth's for 24 h. Prior to photomicroscopy, cells were washed in PBS, fixed in 4% formaldehyde (v/v) and stained with hemotoxylin and eosin. Percentage of cellular growth in the denuded zone was calculated for both cell types. Area occupied by re-growth was determined by morphometric analysis.

RESULTS AND DISCUSSION

ERK Activity, but not JNK or p38, is Greater in WKY12-22 Cells Than WKY3M-22 Cells

To begin investigating a potential link between MAP kinase activity and distinct phenotypic differences observed between WKY12-22 and WKY3M-22 SMC subtypes, we evaluated ERK activity using an assay in which a synthetic peptide is specifically phosphorylated with ^{32}P by the presence of ERK in the cell lysate. Figure 1A demonstrates that WKY12-22 SMC have higher basal levels of ERK1/2 activity compared with WKY3M-22 cells under normal

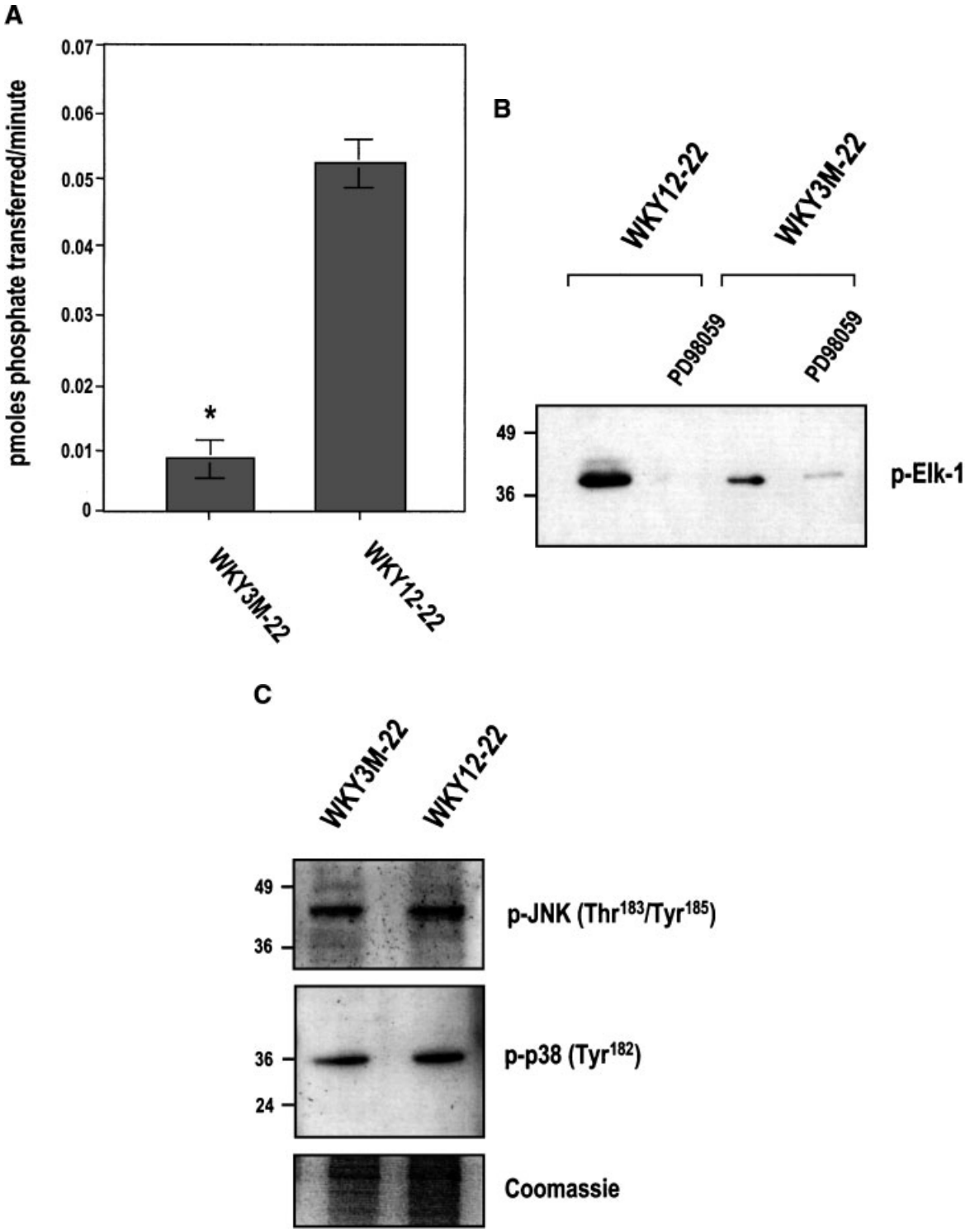

Fig. 1. WKY12-22 and WKY3M-22 SMC express active forms of ERK, JNK, and p38 MAP kinases. **A**: ERK1/2 phosphorylation activity. **B**: Elk-1 phosphorylation by immunoprecipitated phospho-ERK1/2. PD98059 (10 μM) is used. **C**: p-JNK and p-p38 expression. Western blot analysis was performed using the phospho-specific antibodies indicated. The data are representative of two independent determinations. Asterisk (*) demonstrates significance at $P < 0.05$ (Student's *t*-test).

conditions (10% fetal bovine serum). ERK activity was also higher in WKY12-22 than WKY3M-22 cells under a serum-free environment (data not shown). To confirm these observations, we performed a second assay that detects ERK1/2-induced phosphorylation of Elk-1. Figure 1B demonstrates that WKY12-22 cells have higher levels of phosphorylated Elk-1 in comparison to WKY3M-22 cells. The ERK1/2 inhibitor (PD98059) blocked phosphorylation of Elk-1 by ERK1/2 in both SMC subtypes (Fig. 1B) however, PD98059 inhibition was more profound in WKY12-22 cells than WKY3M-22 cells (Fig. 1B).

To determine whether the greater activity of ERK1/2 in WKY12-22 cells was a general feature of other MAP kinases in this SMC subtype, we next compared levels of active JNK and p38 kinase. Western blot analysis revealed that JNK ($pThr^{183}/pTyr^{185}$) and p38 ($pTyr^{182}$) levels did not differ between WKY12-22 and WKY3M-22 cells (Fig. 1C). Additionally, specific inhibitors to these MAP kinases (SP600125 and SB202190) blocked active phospho-JNK and phospho-p38, respectively (data not shown). MAP kinase activity in WKY12-22 and WKY3M-22 cells up till now has not been examined.

WKY12-22 Cells Proliferate and Migrate More Rapidly Than WKY3M-22 Cells

Proliferation and migration of SMC are key cellular processes underlying intimal thickening and progression of atherosclerotic lesions. We next compared the rate of growth and migration of WKY12-22 and WKY3M-22 cells. Quantitation of total cell numbers over 6, 24, and 48 h (after initial plating) by Coulter Counter revealed that WKY12-22 proliferation was higher than WKY3M-22 proliferation (Fig. 2A). To confirm these data, we performed flow cytometry comparing levels of DNA synthesis between the two SMC subtypes. Figure 2B illustrates that WKY12-22 cells have a greater proportion of cells in S-phase compared with WKY3M-22 cells, irrespective of the absence or presence of serum (Fig. 2B), consistent with the capacity of WKY12-22 cells to produce their own growth factors [Majesky et al., 1992; Lai et al., 1996].

To evaluate whether these cells differed in their capacity to migrate, we performed a response to in vitro injury study. The population of cells in the denuded zone 24 h after mechanical injury was higher in the WKY12-22 group than in the WKY3M-22 group (Fig. 3A). Similar results were obtained if the cells were scraped in the absence of serum (data not shown).

Since re-growth after injury can involve both migration and proliferation, we next assessed the migration component alone using a modified Boyden chamber. Over half the population of WKY12-22 cells added to the upper chamber migrated onto the underside, following 24 h incubation (Fig. 3B). In contrast, only 20% of the WKY3M-22 cells spontaneously migrated to the underside of the membrane (Fig. 3B). These findings, thus demonstrate significant differences in rates of migration and proliferation between the two SMC subtypes.

MAP Kinase Subtypes are Required for SMC Proliferation

The preceding data demonstrates that while WKY12-22 and WKY3M-22 cells proliferate and migrate at different rates, they share similarities and differences in the activities of certain MAP kinases. We next evaluated the regulatory role of these MAP kinases on proliferation and migration in both SMC subtypes. WKY12-22 cells, as previously observed (Fig. 2A), proliferated faster than WKY3M-22 cells (Fig. 4A, compare *column 5* and *column 6* with *column 1* and *column 2*). Proliferation of both WKY12-22 and WKY3M-22 cells was inhibited 48 h after plating (and 34 h continuous incubation with the MEK/ERK inhibitor PD98059 at a concentration of 10 μM) (Fig. 4A, *closed columns*), but not after 24 h (Fig. 4A, *open columns*). Consistent with comparative ERK activities between these SMC subtypes (Fig. 1B), inhibition of proliferation by PD98059 was greater in WKY12-22 cells than WKY3M-22 cells (Fig. 4A, compare *column 8* to *column 6* for WKY12-22 cells with *column 4* to *column 2* for WKY3M-22 cells).

We next evaluated the anti-proliferative effect of inhibitors targeting JNK and p38. SP600125 (JNK inhibitor) inhibited WKY12-22 and WKY3M-22 cell proliferation. Inhibition by SP600125 was apparent within 24 h (Fig. 4B, *open columns*). The p38 inhibitor, SB202190 also suppressed SMC proliferation by this time (Fig. 4C, *open columns*). The extent of inhibition of cell proliferation was even greater after 48 h (Fig. 4B,C, *closed columns*). These findings demonstrate that JNK and p38 are essential for proliferation of both SMC subtypes. ERK activation however, appears to contribute more

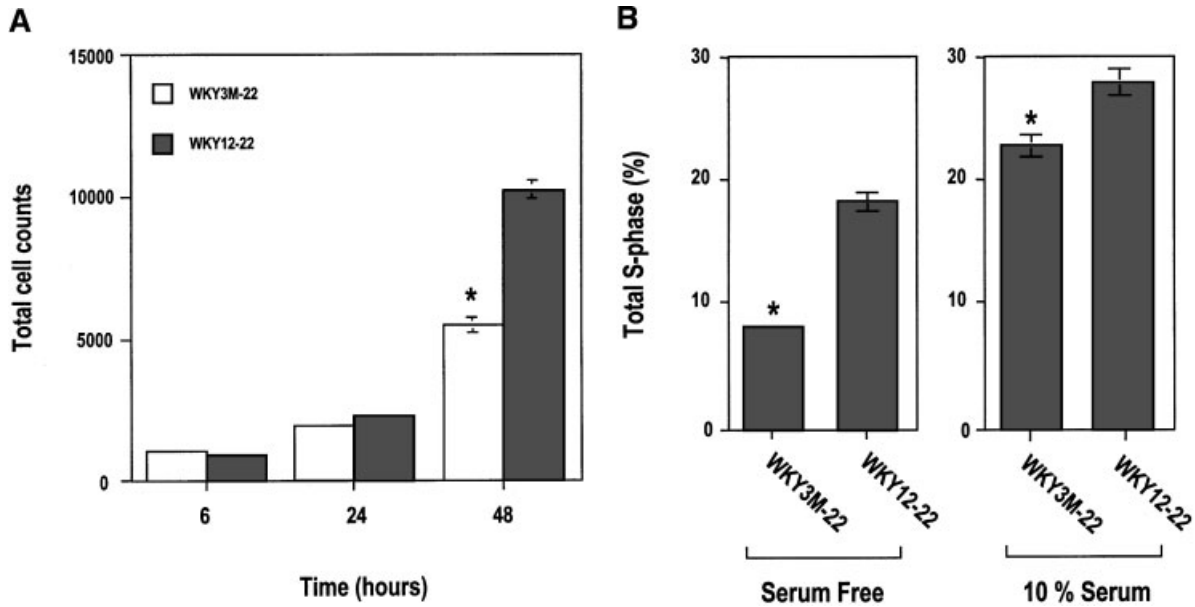

Fig. 2. WKY12-22 cells proliferate faster than WKY3M-22 cells. **A**: Total cell counts performed using a Coulter Counter over a 48 h period. **B**: Cell cycle (S-phase) analysis. The data are representative of two independent determinations. Asterisk (*) demonstrates significance at $P < 0.05$ (Student's *t*-test).

significantly to the signaling mitogenesis observed in WKY12-22 cells.

JNK and p38, but not ERK, are Required for WKY12-22 and WKY3M-22 Migration

We next evaluated the impact of ERK, JNK, and p38 kinase inhibition on migration by these SMC subtypes. PD98059 (10 μM) failed to inhibit WKY12-22 and WKY3M-22 cell migration in modified Boyden chambers (Fig. 5, compare *column 2* to *column 1*, and *column 6* to *column 5*). In contrast, JNK inhibition (10 μM SP600125) significantly blocked migration of WKY12-22 and WKY3M-22 cells (Fig. 5, compare *column 3* to *column 1*, and *column 7* to *column 5*). The p38 kinase inhibitor (1 μM SB202190) similarly, suppressed migration of the SMC subtypes (Fig. 5, compare *column 4* to column 1, and *column 8* to *column 5*). These data, taken together, demonstrate that JNK and p38 signaling, but not ERK, is essential for migration of WKY12-22 and WKY3M-22 cells. The effect of MAP kinase inhibition on SMC migration in the absence of added growth factor has, to our knowledge, not previously been evaluated.

Requirement of MAP Kinase Subtypes in RASMC Migration and Proliferation

Finally, we performed experiments to reconcile our present data in WKY3M-22 and WKY12-22 cells with the control of migration and proliferation in primary RASMC. We found that ERK was not required for spontaneous RASMC migration in modified Boyden chambers (Fig. 6A, compare *column 2* to *column 1*) nor did it mediate chemotaxis inducible by PDGF-BB (40% stimulation by 25 ng/ml after 24 h) (Fig. 6A, compare *column 4* to *column 3*). In contrast, inhibitors of JNK and p38 blocked RASMC migration (Fig. 6B, compare *columns 3* and *4* to *column 1*). Figure 6C demonstrates the effect of PD98059, SP600125, and SB202190 on RASMC proliferation after 24 h (*open columns*) or 48 h (*closed columns*). Each MAP kinase inhibitor suppressed RASMC proliferation in a time-dependant manner (Fig. 6C). PDGF-BB-inducible RASMC proliferation was also

Fig. 3. WKY12-22 cells migrate faster than WKY3M-22 cells. **A**: Regrowth of SMCs into the denuded zone following in vitro injury. Photomicrographs were taken 24 h after injury. **B**: Modified Boyden chamber assay. Cells on the underside of the filter were quantitated 24 h after inoculation of the chambers. Migration was calculated as a percentage of the total number of cells, as described under Materials and Methods The data are representative of two independent determinations. Asterisk (*) demonstrates significance at $P < 0.05$ (Student's *t*-test). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

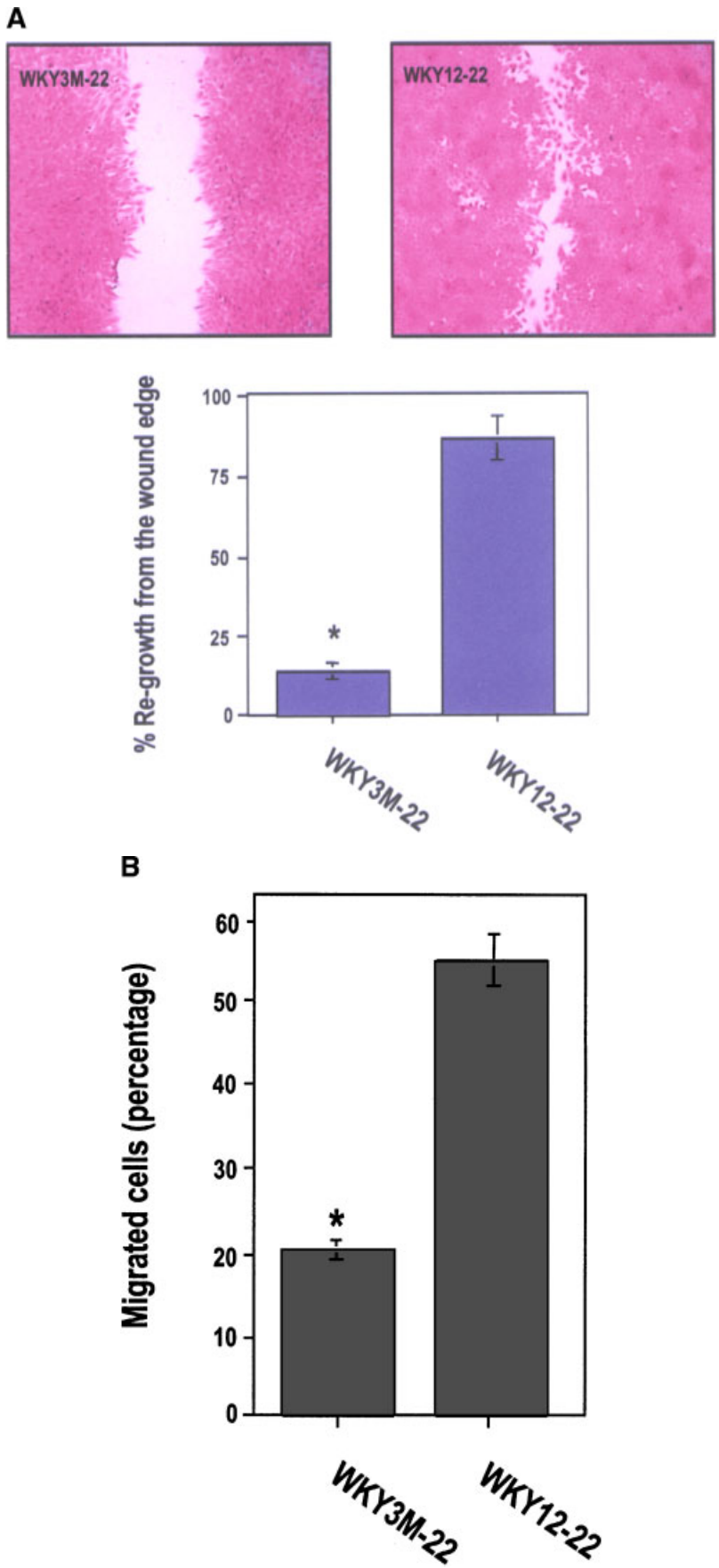
A
WKY3M-22
WKY12-22
% Re-growth from the wound edge
100
75
50
25
0
*
WKY3M-22
WKY12-22
B
Migrated cells (percentage)
60
50
40
30
20
10
0
*
WKY3M-22
WKY12-22

Fig. 3.

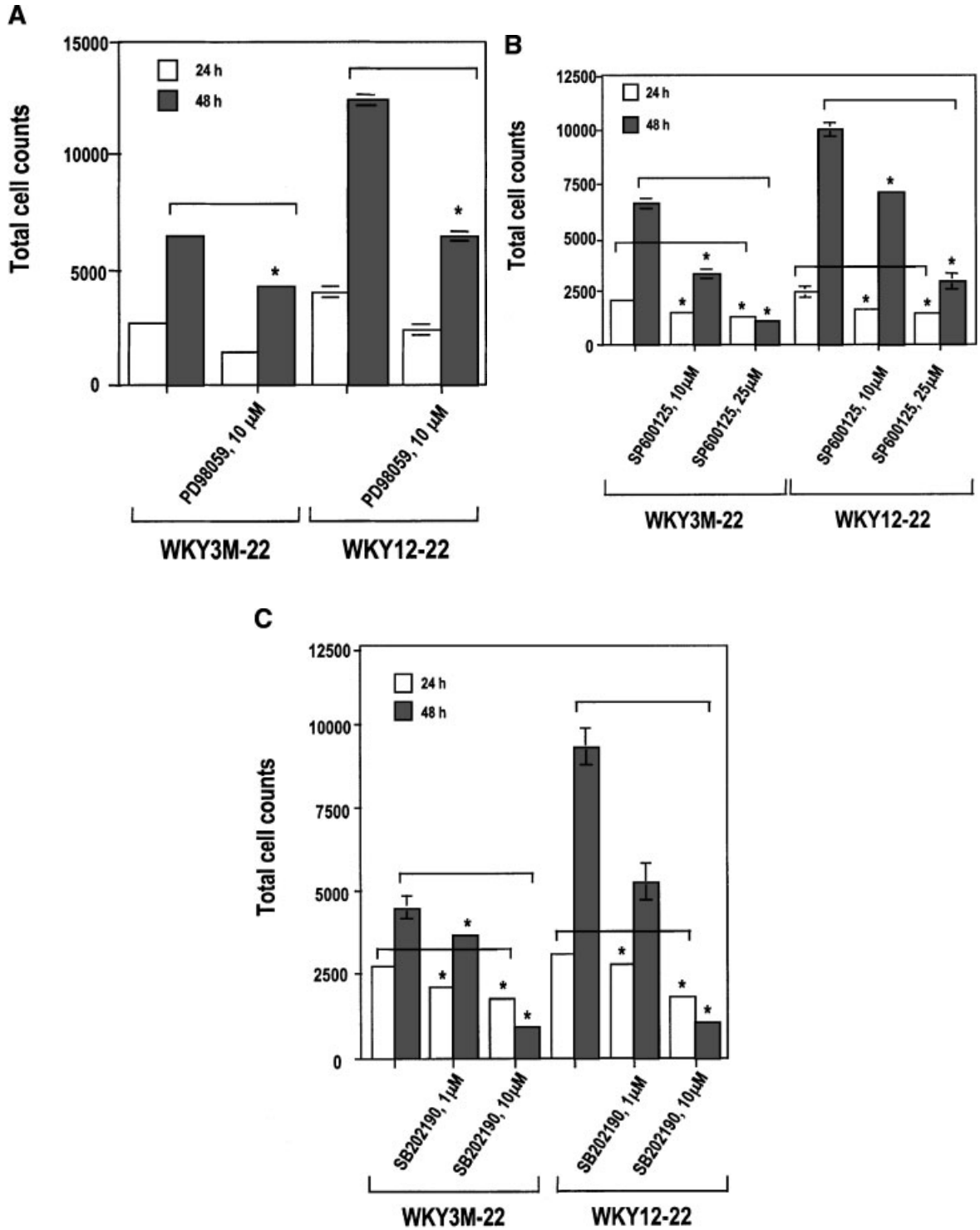

Fig. 4. MAP kinase subtypes differentially regulate vascular SMC proliferation. Assessment of the effect of inhibitors of **A**: ERK, **B**: JNK, and **C**: p38, on total cell counts 24 and 48 h after seeding. Concentration of inhibitors are indicated. Cells were quantified as described under Materials and Methods. Data are representative of two independent determinations. Statistical analysis was conducted between groups assigned by the horizontal line bars. Asterisk (*) demonstrates significance at $P < 0.05$ (Student's *t*-test).

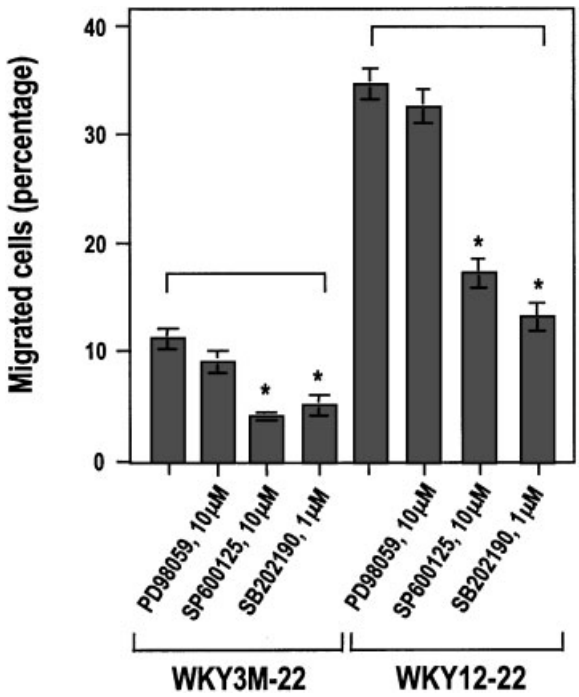

Fig. 5. MAP kinase subtypes differentially regulate WKY12-22 and WKY3M-22 SMC migration. Assessment of the effect of inhibitors of ERK, JNK, and p38, on cell migration 24 h after seeding. Migration was assessed in Boyden chambers as described under Materials and Methods. Data are representative of two independent determinations. Statistical analysis was conducted between groups assigned by the horizontal line bars. Asterisk (*) demonstrates significance at $P < 0.05$ (Student's *t*-test).

blocked by PD98059, SP600125, and SB202190 after 48 h (Fig. 6D, *open columns*) or 62 h (Fig. 6D, *closed columns*). These data, taken together, show for the first time that JNK and p38 are essential for both spontaneous and growth factor-directed SMC migration and proliferation. ERK is required for SMC proliferation, but is not necessary for SMC migration. Thus, SMC heterogeneity in proliferation and migration is a function of the activity and requirement of specific MAP kinase subtypes.

In this study, we have shown that subtypes of vascular SMC share similarities, but also have important differences in the pathophysiologically relevant processes of migration and proliferation. SMC derived from the aortae of pup rats (WKY12-22) proliferate and migrate faster than SMC derived from aortae of adult rats (WKY3M-22). WKY12-22 and WKY3M-22 cells expressed equivalent levels of the active forms of phospho-JNK (Thr^{183}/Tyr^{185}) and phospho-p38 (Tyr^{182}), whereas ERK activity was greater in WKY12-22 cells. Inhibitors of JNK and p38 kinase suppressed proliferation of both cell types, while more profound suppression of WKY12-22 cell growth was observed using the ERK inhibitor. Migration of SMC subtypes was also blocked by specific MAPK inhibitors directed to active JNK and p38. The ERK inhibitor, however, did not affect migration of any SMC subtype. These findings demonstrate that SMC heterogeneity is mediated, at least in part, by the regulatory influence of distinct MAP kinase subtypes. Moreover, these data build on the long held belief that SMC derived from the artery wall are heterogeneous [Bochaton-Piallat et al., 1994]. For example, only a subpopulation of SMC in injured rat arteries express PDGF B-chain mRNA [Lindner et al., 1994].

Unlike JNK and p38, significant differences were observed in ERK activity between WKY12-22 and WKY3M-22 cells. More abundant ERK activity in WKY12-22 cells was supported by the higher mitogenic index of this SMC subtype. ERK inhibition attenuated WKY12-22 SMC proliferation, but did not influence migration of any SMC subtype. Presently there is no absolute consensus on the exact role played by ERK in vascular SMC proliferation and migration. This may be accounted by insufficient inhibition of target kinases, or the specific differential states of SMC populations under investigation. In the present study inhibitors were incubated with cells of stable phenotype for periods of up to 34 h (rather than just 30 min), and ERK inhibition was confirmed using 10 μM PD98059. The present study demonstrates that WKY12-22 cells proliferate and migrate faster than WKY3M-22 cells, possess greater ERK activity, and are more amenable to growth-inhibition by PD98059.

The monoclonal origin of human atherosclerotic lesions provided the first suggestive evidence for the existence of SMC subpopulations [Benditt and Benditt, 1973]. It is now widely accepted that SMC are heterogeneous in human atherosclerotic plaques. SMC in middle/advanced-stage atherosclerotic lesions, for example, express fibroblast surface protein (FSP) whereas FSP is not expressed in early lesions [Martinez-Gonzalez et al., 2001]. Hao et al. [2002] showed that SMC heterogeneity is also a feature of porcine coronary arteries, a widely used model of human restenosis. Bochaton-Piallat et al. [1994] recently reported that SMC phenotypic heterogeneity can be maintained in vivo by seeding two

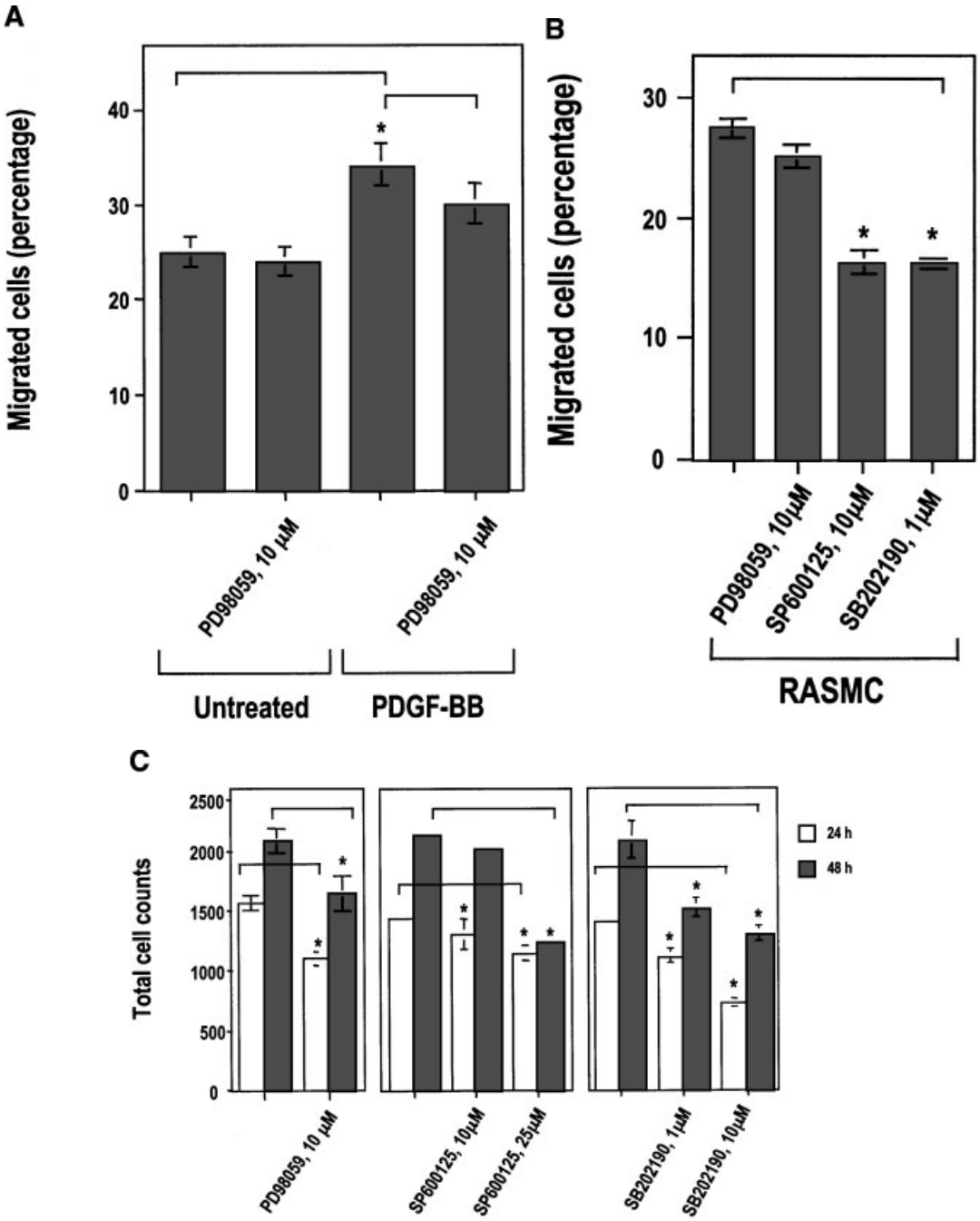

Fig. 6. Effect of MAP kinase inhibitors on primary RASMC growth. **A**: Effect of PDGF-BB (25 ng/ml) on RASMC migration. **B**: Effect of inhibitors of ERK, JNK, and p38 on RASMC migration. **C**: Effect of inhibitors of ERK, JNK, and p38 on RASMC proliferation. **D**: Effect of inhibitors of ERK, JNK, and p38 on PDGF-BB-inducible RASMC proliferation. Concentrations of inhibitors used are indicated. Statistical analysis was conducted between groups assigned by the horizontal line bars. Asterisk (*) demonstrates significance at $P < 0.05$ (Student's *t*-test).

phenotypically separate SMC populations (from aortic media of newborn or old rats) into the intima of denuded rat carotid arteries. They demonstrated that each population maintained their distinct biochemical features, suggesting that SMC phenotypic heterogeneity is controlled genetically and not necessarily by the local microenvironment. The present study illustrates both similarities and differences in signaling pathways controlling migration and proliferation in distinct SMC subtypes. As such, it broadens our understanding of molecular

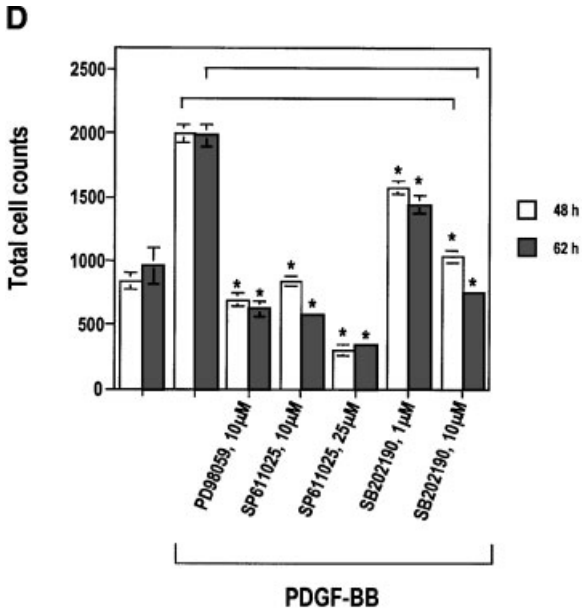

Fig. 6 (*Continued*)

mechanisms steering SMC phenotype and helps define potential targets for the treatment and prevention of vascular pathologies.

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