p21\textsuperscript{Cip1} modulates arterial wound repair through the stromal cell–derived factor-1/CXCR4 axis in mice

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Cyclin-dependent kinase inhibitors, including p21\textsuperscript{Cip1}, are implicated in cell turnover and are active players in cardiovascular wound repair. Here, we show that p21\textsuperscript{Cip1} orchestrates the complex interactions between local vascular and circulating immune cells during vascular wound repair. In response to femoral artery mechanical injury, mice with homozygous deletion of p21\textsuperscript{Cip1} displayed accelerated proliferation of VSMCs and increased immune cell infiltration. BM transplantation experiments indicated that local p21\textsuperscript{Cip1} plays a pivotal role in restraining excessive proliferation during vascular wound repair. Increased local vascular stromal cell–derived factor-1 (SDF-1) levels were observed after femoral artery injury in p21\textsuperscript{Cip1}\textsuperscript{+/–} and p21\textsuperscript{Cip1}\textsuperscript{–/–} mice, although this was significantly greater in p21\textsuperscript{Cip1}\textsuperscript{–/–} animals. In addition, disruption of SDF-1/CXCR4 signaling inhibited the proliferative response during vascular remodeling in both p21\textsuperscript{Cip1}\textsuperscript{+/–} and p21\textsuperscript{Cip1}\textsuperscript{–/–} mice. We provide evidence that the JAK/STAT signaling pathway is an important regulator of vascular SDF-1 levels and that p21\textsuperscript{Cip1} inhibits STAT3 binding to the STAT-binding site within the murine SDF-1 promoter. Collectively, these results suggest that p21\textsuperscript{Cip1} activity is essential for the regulation of cell proliferation and inflammation after arterial injury in local vascular cells and that the SDF-1/CXCR4 signaling system is a key mediator of vascular proliferation in response to injury.

Introduction
Vascular wound repair is controlled by the interaction of local vascular cells (endothelial and smooth muscle) and infiltrating inflammatory cells (macrophages, neutrophils, and lymphocytes). Particularly during arterial wound healing, a balanced control of vascular cell growth and death critically regulates the determination of both the composition of the healed arterial wall and luminal patency. Normally, during vascular homeostasis there is a low turnover rate of endothelial and smooth muscle cells. However, following arterial injury there is disruption of vessel architecture, triggering the early release of growth factors and inflammatory modulators that initiate a further cascade of downstream events (1, 2). Circulating inflammatory and progenitor cells are recruited to the site of injury and infiltrate the damaged vessel via the vessel lumen or the vasa vasorum, while previously quiescent local vascular cells also enter the cell cycle and proliferate (3). Although many cells participate in this early response to vascular injury, monocytes/macrophages have been noted as being particularly abundant (4). The recruitment of monocytes/macrophages is mediated by the chemokine stromal cell–derived factor-1 (SDF-1), which is upregulated at the site of tissue injury (5). SDF-1 is selectively bound by the chemokine receptor CXCR4, which is expressed on macrophages (6) and a wide range of other cells, including VSMCs (7, 8). CXCR4 signaling is mediated by G protein–dependent PI3K signal transduction pathways and the G protein–independent JAK/STAT pathway (9, 10).

Nonstandard abbreviations used: LIF, leukemia inhibitory factor; SDF-1, stromal cell–derived factor-1.

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The present study was undertaken to delineate the functions of p21$^{Cip1}$ in vascular and circulating inflammatory cells during arterial wound repair. Our results indicate that SDF-1/CXCR4 signaling mediates the local inflammatory and cellular proliferative response after arterial injury in WT mice and that blockade of this pathway leads to decreased neointimal formation. We provide evidence that p21$^{Cip1}$ has a particularly important role in restraining local levels of VSMC-derived SDF-1 and that after vascular injury, p21$^{-/}$ mice show an increase in both SDF-1 expression and neointimal formation as compared with WT animals. In addition, we show that vascular SDF-1 is transcriptionally regulated through the JAK/STAT pathway as part of a positive feedback loop and that p21$^{Cip1}$ represses this process by binding to STAT3 and decreasing the STAT3-dependent transcriptional activation of the SDF-1.

Defining the roles of p21$^{Cip1}$ and SDF-1 in the vascular repair process provides essential information regarding their function in the development of cardiovascular disease and may assist in the development of novel strategies to ameliorate this condition.

**Figure 1**
Accelerated proliferation and apoptosis in vitro in p21$^{-/}$ VSMCs. (A) Increased growth rate in p21$^{-/}$ VSMCs (blue triangles) compared with p21$^{+/}$ VSMCs (yellow squares) ($n = 3$; *$P < 0.05$, **$P < 0.001$). (B) Increased percentage of apoptotic cells in p21$^{-/}$ VSMCs (blue bars) induced with 0, 375, or 750 μM of H$_2$O$_2$ compared with p21$^{+/}$ VSMCs (yellow bars) ($n = 3$; ***$P < 0.001$ versus corresponding p21$^{+/}$ group).

**Figure 2**
p21$^{Cip1}$ and p53 are induced after vascular injury. Vascular wire injury was performed on p21$^{+/}$ mice, and femoral arteries were harvested 3 and 7 days following injury. (A) Upper panel: Total RNA was isolated from femoral arteries, and endogenous p21$^{Cip1}$ mRNA was quantified by quantitative PCR and normalized to levels of 18S RNA. Levels of p21$^{Cip1}$ mRNA at 3 (yellow bar) and 7 days (blue bar) are expressed relative to that measured in uninjured control (Co) arteries ($n = 3$; *$P < 0.05$ versus Co; **$P < 0.01$ versus Co). Lower panel: Western blot analysis of p21$^{Cip1}$ levels in femoral arteries at 3 and 7 days after injury compared with uninjured arteries. (B) Levels of p53 mRNA at 3 (yellow bar) and 7 days (blue bar) are expressed relative to that measured in uninjured arteries ($n = 3$; *$P < 0.05$ versus Co; **$P < 0.001$ versus Co). p21$^{Cip1}$ (C) and p53 (D) staining were detected by confocal microscopy in VSMCs arteries 3 days after injury. VSMCs were identified by smooth muscle α-actin (green), p21$^{Cip1}$ (red), p53 (D, red), and nuclear counterstaining by DAPI (blue). p21$^{Cip1}$ and p53 appear pink due to the overlay with the blue DAPI staining.
Results

Increased proliferation and apoptosis in p21⁻/⁻ VSMCs. p21⁰/° is known to modulate apoptosis in several cell types, including endothelial cells, VSMCs, cardiomyocytes, and tumor cells (41–44). Therefore, we hypothesized that p21⁰/° may modulate cellular proliferation and susceptibility to apoptosis during vascular wound repair, and we initially examined these cellular functions of p21⁰/° in VSMCs. Low-passage p21⁺/⁺ and p21⁻/⁻ VSMCs were seeded in equal number, and cellular growth was recorded. After 96 hours, there were 2.5-fold more p21⁻/⁻ than p21⁺/⁺ VSMCs (Figure 1A), indicating that p21⁻/⁻ VSMCs had a higher growth rate than p21⁺/⁺ VSMCs. Next, cellular stress and apoptosis were induced by adding increasing concentrations of either H₂O₂ or FasL to the culture medium. In the absence of H₂O₂ or FasL, the percentage of apoptotic p21⁺/⁺ and p21⁻/⁻ VSMCs was similar (Figure 1B and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI31244DS1). However, upon induction of cellular stress with increasing doses of H₂O₂ or FasL, the percentage of apoptotic cells increased significantly in the p21⁻/⁻ group compared with the p21⁺/⁺ group (Figure 1B and Supplemental Figure 1). These results confirm that p21⁰/° supports cell viability during cellular stress and also inhibits VSMC proliferation.

p21⁰/° inhibits neointimal formation during arterial wound repair. We used an established model of femoral artery wire injury to study the role of p21⁰/° in the vascular repair process (45). First, we explored p21⁰/° mRNA and protein levels in p21⁺/⁺ mice during arterial wound repair. Quantitative PCR and western blot analyses revealed that both p21⁰/° protein and mRNA levels were low in uninjured femoral arteries (Figure 2A). However, 3 days after vascular injury, p21⁰/° protein and mRNA levels were markedly upregulated, the latter more than 90-fold (Figure 2A). p21⁰/° mRNA subsequently decreased at later stages of the arterial repair process (Figure 2A). Since p21⁰/° transcription is activated by p53, we explored p53 expression in the context of the vascular injury. We observed that p53 mRNA levels followed the same pattern as p21⁰/° mRNA. Thus, p53 mRNA was near the limits of detection in uninjured vessels, but was elevated 20-fold 3 days after injury and subsequently decreased by 7 days after injury (Figure 2B). Confocal microscopic imaging confirmed the induction of nuclear p21⁰/° and p53 expression in VSMCs within the medial arterial layer (Figure 2, C and D). These results suggest that p21⁰/° may be at least partially regulated by p53 in the early phase of the vascular remodeling.

Next, we explored whether p21⁰/° modulates arterial wound repair. We performed vascular injury in p21⁻/⁻ and p21⁺/⁺ mice and examined cell proliferation and lesion formation. Cell proliferation was determined by in vivo BrdU pulsing and was significantly increased within the neointima of p21⁻/⁻ arteries compared with p21⁺/⁺ arteries at 1 and 2 weeks after injury (Figure 3, A and C). Correspondingly, there was significantly greater neointimal for-
mation in \(p21^{-/-}\) mice when compared with \(p21^{+/+}\) mice at both the 1- and 2-week time points (Figure 3, B and C). These observations confirm the role of \(p21^{\text{Cip1}}\) in vascular wound repair and demonstrate that this process is greatly compromised in \(p21^{-/-}\) mice compared with \(p21^{+/+}\) mice.

\(p21^{\text{Cip1}}\) modulates the neointimal inflammatory response after vascular injury. We noted a significant accumulation of mononuclear cells within the adventitia, media, and neointima after arterial injury and undertook further characterization of these cells. Histological sections were stained with antibodies specific for macrophages, neutrophils, and T lymphocytes at 1 and 2 weeks after injury (Figure 4). Compared with \(p21^{+/+}\) mice, in \(p21^{-/-}\) mice 1 week after injury, the number of macrophages in the adventitia, media, and neointima was 3.8-fold greater. However, there was no difference in macrophage counts 2 weeks after injury (Figure 4A). There were twice as many neutrophils in the lesions of \(p21^{-/-}\) mice compared with \(p21^{+/+}\) mice 1 week after arterial injury, but interestingly, this trend was reversed in the later phase of arterial wound repair (Figure 4B). In contrast, the number of T lymphocytes was increased in the lesions of \(p21^{-/-}\) mice compared with those of \(p21^{+/+}\) mice at both time points (Figure 4C). In summary, at the 1-week time point after arterial injury, all 3 inflammatory cells (macrophages, neutrophils, and T lymphocytes) were more abundant in the lesions of \(p21^{-/-}\) mice than in those of \(p21^{+/+}\) mice. However, \(p21^{\text{Cip1}}\) deletion generally failed to sustain this enhanced inflammatory response in the later phase of arterial wound repair. Interestingly, we found no difference between \(p21^{-/-}\) and \(p21^{+/+}\) mice in the number of circulating inflammatory cells or the percentage of monocyte subtypes, at baseline and after wire injury (Supplemental Table 1 and Supplemental Figure 2, A and B). These data show that, particularly in the early phase of arterial wound repair, \(p21^{\text{Cip1}}\) acts to decrease the migration and trafficking of inflammatory cells into areas of vascular damage.

Vascular \(p21^{\text{Cip1}}\) restrains excessive proliferation and decreases apoptosis during arterial wound repair. To delineate the specific function of \(p21^{\text{Cip1}}\) in local vascular cells versus that in circulating immune cells, we performed BM transplantation from \(p21^{-/-}\) or \(p21^{+/+}\) donors into \(p21^{-/-}\) or \(p21^{+/+}\) recipient mice. Successful engraftment was confirmed by the detection of the SRY gene in the blood of female recipients that received male BM by quantitative PCR (data not shown). Mice underwent vascular injury, and neointimal formation was determined 2 weeks later. When \(p21^{-/-}\) mice received BM from \(p21^{+/+}\) mice, neointimal formation was decreased compared to \(p21^{-/-}\) mice that received \(p21^{-/-}\) BM (Figure 5A). However, when \(p21^{+/+}\) mice received BM from \(p21^{-/-}\) mice, arterial wound repair was not altered, and neointimal formation was identical to that seen in \(p21^{-/-}\) mice that received \(p21^{+/+}\) BM (Figure 5A). These experiments indicate that

Figure 4
\(p21^{-/-}\) mice develop acute arterial inflammation after vascular injury. Accumulation of (A) macrophages, (B) neutrophils, and (C) T lymphocytes in the femoral artery (neointima, media, and adventitia) of \(p21^{+/+}\) (yellow bars) and \(p21^{-/-}\) (blue bars) at 1 and 2 weeks after vascular injury (\(n = 8\); *\(P < 0.05\) and **\(P < 0.01\) versus \(p21^{+/+}\) at same time point). (D) Representative photomicrographs of cross-sections of \(p21^{+/+}\) (left) and \(p21^{-/-}\) (right) arteries immunostained for macrophages, neutrophils, and T lymphocytes (brown). Arrows indicate the internal elastic lamina. Scale bars: 20 \(\mu\m).
local vascular p21<sup>Cip1</sup> is sufficient to restrain excessive neointimal formation. In addition, p21<sup>Cip1</sup> deletion in local vascular cells caused increased infiltration of monocytes/macrophages (Figure 5B).

We then determined the influence of p21<sup>Cip1</sup> expression on apoptosis during arterial wound repair. Using a TUNEL assay, we found that at 1 week after arterial injury the percentage of apoptotic cells was significantly increased in p21<sup>−/−</sup> mice compared with p21<sup>+/+</sup> mice, although this difference was not sustained at 2 weeks after injury (Supplemental Figure 3A). In BM transplantation experiments 1 week after injury, transplantation of p21<sup>−/−</sup> BM into p21<sup>−/−</sup> recipients did not significantly increase the percentage of vascular apoptotic cells compared with p21<sup>+/+</sup> mice receiving p21<sup>+/+</sup> BM. Also, transplantation of p21<sup>−/−</sup> BM into p21<sup>−/−</sup> recipient mice did not significantly decrease this process compared with transplantation of p21<sup>−/−</sup> BM into p21<sup>−/−</sup> recipient mice (Supplemental Figure 3B). Thus, p21<sup>Cip1</sup> within local vascular cells appears to influence apoptosis after vascular injury, supporting the importance of local p21<sup>Cip1</sup> function in vascular wound repair.

As a whole, these observations led us to question what distinguishes local p21<sup>−/−</sup> from p21<sup>−/−</sup> vascular cells, and in particular, what factors might mediate the interactions of these resident vascular cells with circulating immune and inflammatory cells.

Local vascular p21<sup>Cip1</sup> modulates SDF-1 during vascular wound repair. As SDF-1 is known to play an important role in recruiting and detaining inflammatory cells at the site of vascular injury (5, 46), we speculated that local vascular SDF-1 may be differentially regulated in p21<sup>−/−</sup> versus p21<sup>+/+</sup> mice. Initial in vitro experiments confirmed increased protein levels of SDF-1 in p21<sup>−/−</sup> versus p21<sup>+/+</sup> VSMCs. After the induction of oxidative stress with H<sub>2</sub>O<sub>2</sub>, SDF-1 levels were significantly reduced in both p21<sup>−/−</sup> and p21<sup>+/+</sup> VSMCs (Supplemental Figure 4). Moving to our in vivo models, we first established that serum SDF-1 levels did not differ, before or 3 and 7 days after vascular injury, between p21<sup>−/−</sup> and p21<sup>−/−</sup> mice (data not shown). We then proceeded to investigate local vascular SDF-1 levels at these time points. In the absence of arterial injury, there was no difference in SDF-1 tissue levels between p21<sup>−/−</sup> and p21<sup>−/−</sup> mice. After injury, both p21<sup>−/−</sup> and p21<sup>−/−</sup> mice exhibited increased local SDF-1 levels compared with uninjured controls. However, in p21<sup>−/−</sup> mice this increase both was of a greater magnitude and also, unlike p21<sup>−/−</sup> mice, was sustained until at least 7 days after injury (Figure 6, A and B). In order to further explore this observation, local vascular SDF-1 levels were also measured in p21<sup>−/−</sup> and p21<sup>−/−</sup> mice that had undergone BM transplantation. Local SDF-1 was upregulated in p21<sup>−/−</sup> mice that received p21<sup>−/−</sup> BM compared with p21<sup>−/−</sup> mice that received p21<sup>−/−</sup> BM. In addition, p21<sup>−/−</sup> mice receiving p21<sup>−/−</sup> BM had similar levels of SDF-1 compared with p21<sup>−/−</sup> mice that received p21<sup>−/−</sup> BM (Figure 6C). These results indicate that p21<sup>Cip1</sup> expression (or lack thereof) by BM-derived cells has minimal influence on vascular SDF-1 levels after injury, and that it is local p21<sup>Cip1</sup> expression that modulates vascular SDF-1 levels.

Inhibition of SDF-1/CXCR4 signaling restrains excessive proliferation during vascular wound repair in p21<sup>−/−</sup> mice. Our results presented above suggest an important role for local vascular p21<sup>Cip1</sup> in the modulation of vascular SDF-1 levels. However, while SDF-1 has been implicated in the regulation of cellular migration in this (femoral wire injury) and other situations (47), the precise anatomical localization of SDF-1 during wound repair, at a cellular level, is not well described. We explored SDF-1 expression by immunohistochemistry in uninjured vessels and during the early phase of vascular wound repair. As indicated by double staining for VSMC-specific α-actin and SDF-1, in uninjured p21<sup>−/−</sup> and p21<sup>+/+</sup> murine femoral arteries, SDF-1 was predominantly expressed by VSMCs (Figure 6A). We did not observe significant staining in the endothelial cell layer (double staining for CD31 and SDF-1) or in the adventitia. This pattern of vascular SDF-1 expression was not altered by arterial injury. In addition, we did not detect obvious SDF-1 staining in either CD45-positive inflammatory cells, or F4/80-positive macrophages (Figure 6A and data not shown).

SDF-1 signaling is mediated via the CXCR4 receptor, which is commonly expressed on immune and vascular cells. We hypothesized that apart from playing an important role in WT animals, inhibition of SDF-1 signaling would reverse the hyperproliferative phenotype in p21<sup>−/−</sup> mice. We utilized 2 independent approaches to inhibit SDF-1/CXCR4 function. First, we administered AMD3100, a specific CXCR4 inhibitor, to p21<sup>−/−</sup> and p21<sup>+/+</sup> mice via osmotic minipumps and examined the inflammatory response and neointimal formation 1 and 2 weeks after vascular injury. Next, we inhibited the SDF-1/CXCR4 signaling pathway by treatment with a neutralizing monoclonal antibody against CXCR4 (48). As shown in Figure 7, A and B, the inhibition of SDF-1/CXCR4 signaling by either approach significantly reduced neointimal formation after vascular injury in p21<sup>−/−</sup> mice. CXCR4 blocking antibody also significantly inhibited neointimal formation in p21<sup>−/−</sup> mice (Figure 7B). However, possibly due to the additional early BM cell–mobilizing effect of AMD3100 and consistent with other reports (49), we observed that in p21<sup>−/−</sup> mice this reduction was only significant at the 2-week time point (Figure 7A). Cellular proliferation, as assessed by BrdU incorporation,
was significantly decreased by both CXCR4 blocking antibody and AMD3100 in p21−/− and p21+/+ mice at 1 and 2 weeks after vascular injury (Figure 7C and Supplemental Figure 5A). We further identified that either approach to inhibiting SDF-1/CXCR4 signaling was associated with reduced macrophage infiltration at 1 and 2 weeks after vascular injury in both p21−/− and p21+/+ mice (Figure 7D and Supplemental Figure 5B). In contrast, neither CXCR4 blocking antibody nor AMD3100 administration altered cellular apoptosis in p21−/− or p21+/+ mice after vascular injury (Figure 7E and Supplemental Figure 5C). Interestingly, 1 week after vascular injury, p21−/− mice displayed a marginal increase in local vascular SDF-1 levels after treatment with AMD3100. However, this treatment did not influence vascular SDF-1 levels in p21+/+ mice (Supplemental Figure SD). Overall, these data confirmed the important influence of p21Cip1 on the SDF-1/CXCR4 signaling pathway during vascular remodeling.
Cip1 regulates SDF-1 via the JAK-STAT signaling pathway. We then investigated the mechanism whereby p21<sup>Cip1</sup> modulates SDF-1 levels. Recent publications suggest that p53 represses SDF-1 and its receptor CXCR4 as part of a negative regulatory loop (50, 51). We hypothesized that p21<sup>Cip1</sup> might also play an important role in this pathway and connect the cellular stress repair program to the inflammatory response. We screened the murine SDF-1 promoter region for possible transcription factors associated with p21<sup>Cip1</sup> and were able to localize a putative STAT binding site (−516/−527) in the murine SDF-1 promoter region. This was consistent with previous reports that JAK/STAT is an important mediator of SDF-1/CXCR4 signaling (9) and that STAT3 is a major transcriptional activator of SDF-1 and may also act upstream of SDF-1 by modulating HIF-1α activity (52). We initially explored the potential p21<sup>Cip1</sup>/STAT3/SDF-1 interactions in primary murine VSMCs. A luciferase construct containing the −2,200-bp SDF-1 promoter sequence was transfected into p21<sup>+/+</sup> and p21<sup>−/−</sup> VSMCs. As shown in Figure 8A, this transfection resulted in a significant increase in SDF-1 promoter activity in p21<sup>−/−</sup> compared with p21<sup>+/+</sup> VSMCs.

Next, we explored whether activation or inhibition of the STAT3 binding site within the SDF-1 promoter serves to modulate the expression of SDF-1 in VSMCs. After serum starvation of VSMCs for 24 hours, p21<sup>−/−</sup> VSMCs exhibited a 3-fold increase in SDF-1 mRNA expression compared with p21<sup>+/+</sup> VSMCs (Figure 8B). Subsequently, the addition of leukemia inhibitory factor (LIF), which is known to activate STAT3 (53), to the culture medium caused a further increase in SDF-1 mRNA expression (Figure 8B). This activation was blocked by the cell membrane-permeable STAT3 inhibitor PpYLKT-mts (Figure 8B). We then mutated the STAT3 binding site of our luciferase construct of the SDF-1 promoter sequence by a 4-bp substitution-mutation of the STAT3 binding site and transfected it into p21<sup>+/+</sup> and p21<sup>−/−</sup> cells. As shown in Figure 8C, this caused a significant decrease in luciferase activity in both p21<sup>+/+</sup> and p21<sup>−/−</sup> cells compared with transfection with the nonmutated SDF-1 promoter construct. Further experiments also demonstrated that
increased STAT3 activation through Tyr705 phosphorylation was present in p21\(^{-/-}\) VSMCs compared with p21\(^{+/+}\) VSMCs, and reciprocal coimmunoprecipitation experiments in VSMCs revealed an interaction between p21\(^{Cip1}\) and STAT3 (Figure 8D).

Finally, we investigated whether STAT3 occupancy of the SDF-1 promoter is increased in p21\(^{-/-}\) compared with p21\(^{+/+}\) VSMCs. This was achieved by analyzing the chromatin of human VSMCs by ChIP using primers that flank the –576/–372 region of the SDF-1 promoter that we previously identified as being likely to contain a STAT3 binding site (see above). PCR analysis of the chromatin immunoprecipitates, isolated with anti-STAT3 antibodies, showed increased STAT3 occupancy of the SDF-1 promoter in p21\(^{-/-}\) compared with p21\(^{+/+}\) VSMCs (Figure 8E). These findings indicate that, at least in vitro, p21\(^{Cip1}\) is likely to influence SDF-1 levels in VSMCs by modulating STAT3 transcriptional activity. To further explore this possibility in vivo, we performed a ChIP assay on femoral arteries from p21\(^{-/-}\) and p21\(^{+/+}\) mice at both 3 and 7 days after vascular injury. We observed an enhanced and prolonged STAT3 occupancy of the SDF-1 promoter in vessels from p21\(^{-/-}\) mice compared with vessels from p21\(^{+/+}\) mice (Figure 9, A and B). In sum, these data suggest that p21\(^{Cip1}\) modulates the inflammatory response and SDF-1 through the JAK/STAT signal transduction pathway in vitro and in vivo during vascular wound repair.

**Discussion**

Arterial wound repair is a complex process involving predominantly local vascular cells and circulating inflammatory cells. Despite the fact that this process is directly implicated in the etiology of clinical ischemic heart disease, the precise functioning of the relevant inter- and intracellular signaling pathways is not entirely understood (54, 55). Here, we provide direct evidence that p21\(^{Cip1}\) plays a central role in vascular repair after injury. In brief, the major new findings to
arise from this work are as follows: (a) Deletion of p21\textsuperscript{Cip1} enhances the inflammatory response during vascular wound repair. (b) Within medial VSMCs, p21\textsuperscript{Cip1} restrains SDF-1 levels in the early phase of vascular remodeling. (c) Inhibition of SDF-1/CXCR4 signaling by the CXCR4-specific receptor antagonist AMD3100, or a CXCR4-blocking monoclonal antibody, acts to limit inflammation and neointimal formation in p21\textsuperscript{+/+} and p21\textsuperscript{−/−} mice during vascular wound repair. (d) VSMC-derived SDF-1 is transcriptionally regulated by the JAK/STAT signaling pathway. (e) p21\textsuperscript{Cip1} modulates the occupancy of STAT3 on the SDF-1 promoter in VSMCs both in vitro and in vivo during vascular remodeling.

p21\textsuperscript{Cip1} is acknowledged as being a key inhibitory regulator of cellular proliferation and a mediator of p53-dependent cellular apoptosis (35) and is considered to be a fundamental player in vascular wound repair (21, 23, 24, 26). To date, supporting evidence has included the observation that overexpression of recombinant p21\textsuperscript{Cip1} decreases VSMC proliferation and attenuates the progression of vascular proliferative diseases (21, 23, 24, 26) and that, conversely, reduced p21\textsuperscript{Cip1} expression leads to increased neointimal formation following arterial injury (28). This has led to the evaluation of novel treatment strategies for these diseases that are at least partially mediated through p21\textsuperscript{Cip1} upregulation (25, 56, 57). Our results further extend this work and serve to reinforce the important role of p21\textsuperscript{Cip1} in modulating vascular wound repair. We now additionally show that the hyperproliferative phenotype seen in p21\textsuperscript{−/−} mice is associated with an increased number of inflammatory macrophages in the early phase of vascular wound repair. While other researchers have investigated this phenomenon in different settings, our findings are broadly consistent with the fact that p21\textsuperscript{Cip1} plays an important role in regulating cell proliferation and monocyte/macrophage differentiation (21, 22). However, BM transplantation experiments demonstrated that the enhanced neointimal formation that was observed in p21\textsuperscript{−/−} mice could not be recapitulated by transplanting p21\textsuperscript{−/−} BM into p21\textsuperscript{−/−} mice. Collectively, our experiments suggest that the hyperproliferative vascular phenotype of p21\textsuperscript{−/−} mice is largely independent of BM-derived cells, but rather arises from the effects of p21\textsuperscript{Cip1} on the local vascular milieu. Interestingly, this latter finding is in direct opposition to the situation with p27\textsuperscript{kip1}, which primarily exerts its effects on arterial wound repair through infiltrating BM-derived immune cells (4).

Vascular wound repair is orchestrated by various cytokines and inflammatory mediators. While local SDF-1 levels were increased after vascular injury in p21\textsuperscript{−/−} mice, we observed that p21\textsuperscript{−/−} mice exhibit a significantly greater increase in local SDF-1 levels after arterial injury. This finding was not recapitulated by transplanting p21\textsuperscript{−/−} BM into p21\textsuperscript{−/−} mice, again suggesting that the effect of p21\textsuperscript{Cip1} is at the local vascular level. These results also implicated p21\textsuperscript{Cip1} in the regulation of SDF-1, and we therefore explored these potential interrelationships within the context of vascular wound repair. This was achieved by abrogating SDF-1/CXCR4 signaling using either the selective CXCR4 receptor antagonist AMD3100 (58) or a monoclonal antibody directed against CXCR4. Strengthening our hypothesis that p21\textsuperscript{Cip1} is involved with SDF-1 regulation, we found that either approach led to decreased neointimal formation in both p21\textsuperscript{+/+} and p21\textsuperscript{−/−} mice. However, perhaps due to their higher degree of neointimal formation, we found that p21\textsuperscript{−/−} mice exhibited a greater reduction in neointimal formation after SDF-1/CXCR4 blockade than did p21\textsuperscript{−/−} animals.

Numerous publications have described a central role for the SDF-1/CXCR4 axis in new vessel formation, vascular wound repair, and VSMC proliferation (5, 48, 59–63). Zernecke et al. (63) found that the transplantation of CXCR4\textsuperscript{+/+} fetal liver cells into ApoE\textsuperscript{−/−} mice resulted in a greater than 50% reduction in neointimal area after vascular wire injury, suggesting a regulatory role for inflammatory and/or immune cells in wound repair and VSMC proliferation. Similarly, Grunewald et al. (5) reported that tissue-specific hepatic and cardiac VEGF overexpression stimu-
lated SDF-1 expression in perivascular “fibroblastic or smooth muscle” cells, which was sufficient to recruit and retain circulating CD45+CD11b+CXCR4+ myeloid cells. These investigators also demonstrated that the induction of perivascular SDF-1 expression is an integral aspect of angiogenesis and that the administration of a CXCR4 antagonist significantly inhibits this process (5). Recently, SDF-1 was also shown to be an important downstream mediator of HIF-1α, both of which were found to be upregulated during the hyperproliferative response to vascular wire injury in ApoE−/− mice (64). Our results corroborate these publications, and we similarly found that SDF-1/CXCR4 signaling is of importance in both p21−/− and p21+/− mice. However, we further expand these intricate regulatory networks and suggest that p21Cip1 is an important mediator of SDF-1 transcription.

p21Cip1 is known to be a direct modulator of several transcription factors including STAT3 (34, 35, 37). We investigated this relationship in the context of SDF-1 transcription and were able to identify a putative STAT3-binding site within the murine SDF-1 promoter region. We also demonstrated that in VSMCs, activation of STAT3 signaling increases SDF-1 expression and, conversely, that inhibition of STAT3 signaling decreases SDF-1. In addition, we performed ChIP experiments on VSMCs and found that STAT3 occupancy could be detected on the SDF-1 promoter. Although SDF-1 was described as signaling through the JAK/STAT pathway almost a decade ago (9), we are unaware of any previous reports describing this reciprocal signaling arrangement, whereby the JAK/STAT pathway regulates SDF-1 transcription.

Integrating our findings, we suggest that in VSMCs and during vascular wound repair, p21Cip1 is able to modulate vascular SDF-1 levels by inhibiting the binding of STAT3 to the SDF-1 promoter. Adding complexity, it has also recently been shown that p21Cip1 is a direct STAT3 target, and furthermore, that STAT3 functions as a transcriptional repressor of p53, an important transcriptional activator of p21Cip1 (65, 66). However, it is also known that STAT3 and HIF-1α form a complex that regulates VEGF transcription (52), thus the possibility exists that HIF-1α also participates in the regulation of SDF-1 via this higher order transcriptional complex. Therefore, considering all available data and reports, we consider the following possible in the context of vascular wound repair and remodeling: (a) A positive feedback loop for SDF-1 operates via JAK/STAT signaling in the early phase of this process. (b) A negative feedback loop controls STAT3 transcriptional activity through p21Cip1, connecting the p53-mediated cellular stress response to the inflammatory response and restraining the latter in a p21Cip1-dependent manner. (c) Medial VSMCs modulate the immune response to vascular injury by the synthesis and secretion of SDF-1.

Importantly, numerous aspects of this study are of clinical relevance. However, none is perhaps more obvious than the potential application of our findings to vascular stent technologies. Thus, we speculate that several of the important regulators of the vascular remodeling process that were identified in this study (or their inhibitors) may have potential clinical utility if appropriately loaded onto “drug-eluting” stents. Conceivably, this could be either as adjuncts to, or replacements for, either sirolimus or paclitaxel, the agents commonly used for this purpose at the current time.

In conclusion, our findings indicate that p21Cip1 plays an important role in the modulation of vascular remodeling after arterial injury. In local vascular cells, p21Cip1 restrains proliferation and the inflammatory response during vascular wound repair. Under the influence of p21Cip1, VSMC-derived SDF-1 plays a pivotal role in recruiting inflammatory cells to the injured area, and we propose that p21Cip1 regulates SDF-1 via the JAK/STAT signaling pathway. Importantly, this provides a possible link between the p53/p21Cip1-mediated cellular stress signaling program and the inflammatory response during vascular remodeling. Collectively, these findings suggest a central role for SDF-1 in vascular injury repair. We are cautiously optimistic that the targeted manipulation of key biological regulators of the vascular repair process, in particular p21Cip1 and the SDF-1/CXCR4 axis, may be useful clinical adjuncts in the treatment or primary prevention of atherosclerotic vascular disease.

**Methods**

*Generation of homozygous mice.* Heterozygous 129/B6 p21−/− mice were obtained from Tyler Jacks (David H. Koch Institute for Integrative Cancer Research at MIT, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA). All animal experiments were approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee. We back bred the mice for 12 generations against a C57BL/6 background and studied male and female mice at 10 weeks of age. p21−/− littermates were used as controls. Genotyping was performed by PCR amplification of mouse tail DNA using allele-specific probes. Each experimental group contained a minimum of 5 mice.

*Primary VSMCs, cell proliferation, and cellular stress response.* VSMCs were isolated by enzymatic and outgrowth methods (40), with careful attention given to the complete removal of the adventitial layer. Dissected pieces of aortic media were minced and incubated with collagenase (1 mg/ml) and elastase type III (0.125 mg/ml) in DMEM for 30 min. Tissues were plated with DMEM containing 10% FBS. Cell numbers were determined 24 to 96 hours later. Cellular stress was induced by incubating VSMCs with H2O2 at 375 μM and 750 μM for 1 hour. The apoptotic rate was determined 6 hours after incubation with H2O2. Apoptosis was induced with 10 and 50 ng/ml recombinant FasL (R&D Systems) for 12 hours in the presence of 10 μg/ml of a cross-linking antibody (mouse anti-6X histidine; R&D Systems). Annexin V staining was performed according to the manufacturer’s protocol (BD Biosciences — Pharmingen). STAT3 activation and inhibition was performed in low-serum cultured (0.5% FBS) VSMCs. Cells were incubated with 40 ng/ml LIF (Sigma-Aldrich), and SDF-1 mRNA expression was analyzed at the indicated time points. LIF-stimulated VSMCs were incubated with the STAT3 activation inhibitor Pyr1LKT-mts (Calbiochem) at the indicated concentrations and SDF-1 mRNA expression analyzed 4 hours later.

*Quantitative RT-PCR.* RNA was extracted from blood vessels using the Qagen RNeasy kit. A total of 2 μg total RNA was annealed to 1 μg of random primers at 70°C, and first-strand cDNA synthesis was performed in a 25-μl reaction volume following the Promega protocol for M-MLV reverse transcriptase. p21Cip1, p53, and SDF-1 expression levels were analyzed by real-time PCR using the DyNaMo HS SYBR Green qPCR kit (New England Biolabs Inc.), with 0.2 μl from each cDNA synthesis (or the equivalent cDNA from 15 ng of RNA) and a final primer concentration of 0.3 μM in each 20-μl PCR reaction. Real-time PCR was performed with an initial 10-min cycle at 95°C (denaturation), followed by 40 amplification cycles as follows: 10 s at 95°C (denaturation), 20 s at 60°C (annealing), and 30 s at 72°C (extension). Primer sequences are available in Supplemental Methods.

*Wire injury in mice.* p21−/− and p21+/− mice were investigated using an established model of femoral artery wire injury, which results in complete endothelial denudation (66). This procedure was performed by a surgeon who was blinded to the mouse genotype. BrdU (25 mg/kg) was injected at 24 hours and 1 hour prior to tissue harvest. The percent of BrdU-positive cells in the intima and media area ratio were analyzed by computer-assisted morphometry (n = 5 mice, 10 arteries/group). For each mouse, 4 sections were analyzed per artery.
BM transplantation. BM was obtained from 8- to 12-week-old p21−/− and p21+/− mice after euthanasia with CO2. BM cell suspensions were flushed from femurs and tibias, filtered, and stored on ice until use. Recipient mice were lethally irradiated with 900 rads of whole-body irradiation and then received 5 x 106 unfractionated BM cells by intravenous tail vein injection. Successful engraftment was confirmed 12 weeks later by quantitative PCR for the presence or absence of p21Cip1 or Sry to distinguish female and male BM cells (n = 5 mice, 10/aries/group).

Immunohistochemistry, western blot, and immunoprecipitation analysis and arterial cytokine concentrations. Immunohistochemistry was performed on paraffin-embedded tissues using an ABC immunoperoxidase protocol including antigen retrieval. The following primary antibodies were used: anti–smooth muscle α-actin antibody (1:1000, 1A4; Roche), F4/80 antibody against macrophages (1:100, A3-1; Serotec), 7/4 antibody against neutrophils (1:10, 7/4; Cedarlane), CD3 antibody against T lymphocytes (1:100; DAKO Cytomation), and alkaline phosphatase-conjugated mouse monoclonal antibody against BrdU (1 U/ml; Roche). Immunofluorescence staining was performed on fresh frozen tissue sections without antigen retrieval. Methanol-acetone-fixed sections were stained with primary antibodies against SDF-1 (1:100; eBioscience), CD31 (1:100, MECA 137; BD Biosciences — Pharmingen), CD45 (1:50, 30-F11; eBioscience), F4/80 (1:100; Invitrogen), p21Cip1 (1:50, ab2961; Abcam Inc.), p53 (1:50, sc-6243; Santa Cruz Biotechnology Inc.), and smooth muscle α-actin (FITC-conjugated) (1:100, 1A4; Sigma-Aldrich). Where appropriate, these were incubated with Alexa Fluor 488– or Alexa Fluor 594–conjugated secondary antibodies (1:200 dilution; Invitrogen). Apoptotic vascular cells were identified by TUNEL assay. Images were acquired using either a Zeiss Axioskop plus light microscope with AxiosVision V4.3 software or, for confocal microscopy, a Zeiss LSM 510 UV laser scanning confocal microscope system (Carl Zeiss GmbH). Cell counting (minimum 100 cells/section) was performed by a blinded member of our laboratory with extensive experience in both cell morphology and the techniques described above. Western blot analysis was performed on homogenized arterial and VSMC samples in RIPA lysis buffer using antibodies against mouse p21Cip1 (sc397, 1:1000; Santa Cruz Biotechnology Inc.), STAT3 (1:1000; Cell Signaling Technology), and p-STAT3 (1:1000; Cell Signaling Technology). Each lane was loaded with 50 μg protein (Bradford assay). For immunoprecipitation, cells were lysed in NP-40 buffer (75 mM NaCl, 1.0% NP-40, 50 mM Tris, pH 8.0, and protease inhibitors). Cell lysate (200 μg) was incubated with the antibodies indicated in Figure 2A and Figure 8D and/or with isotype control IgG and analyzed by western blot. Arterial cytokine concentrations were measured using a mouse SearchLight Proteome Array (Pierce Biotechnology).

CXCR4 in vivo inhibition. Commencing 24 hours prior to vascular injury, a continuous 20-ng/ml dose of AMD3100 (Sigma-Aldrich) was administered to mice via osmotic minipump (Alzet) at a rate of 2 mg/wk for 2 weeks. CXCR4-blocking antibody (clone 2B11; BD Biosciences) was administered intravenously commencing 24 hours prior to vascular injury at 3-day intervals for 1 or 2 weeks at a dose of 20 μg. IgG2b rat isotype antibody was used as a control for this experiment (clone A95-1; BD Biosciences) (48).

Immunophenotyping. Peripheral blood was isolated from mice by cardiac puncture before and 7 days after injury. Complete blood cell counts were performed using a standard automated cell counting hematology analyzer puncture before and 7 days after injury. Complete blood cell counts were performed using a standard automated cell counting hematology analyzer (Cell-dyne 3500; Abbott). Automated image identification for cell count per 1 or 2 weeks at a dose of 20 ng/wk for 2 weeks. CXCR4-blocking antibody (clone 2B11; BD Biosciences) was administered intravenously commencing 24 hours prior to vascular injury at 3-day intervals for 1 or 2 weeks at a dose of 20 μg. IgG2b rat isotype antibody was used as a control for this experiment (clone A95-1; BD Biosciences) (48).

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