STATE-OF-THE-ART REVIEW

Clonal Expansion in Cardiovascular Pathology

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HIGHLIGHTS

- Smooth muscle cells, endothelial cells, and sometimes macrophages undergo clonal expansion in a variety of cardiovascular diseases regardless of the different pathological milieus.
- Many of these clones further undergo phenotypic modulation to adopt beneficial and/or detrimental phenotypes which appears to be disease-dependent.
- Although the sources of these clones are debated, functionally, a progenitor-like population within the vasculature might be responsible.
- The underlying mechanisms responsible for clonal expansion are still mostly unknown.
- Specific targeting of clones offers promise for future therapeutic options for cardiovascular disease treatment.

SUMMARY

Clonal expansion refers to the proliferation and selection of advantageous "clones" that are better suited for survival in a Darwinian manner. In recent years, we have greatly enhanced our understanding of cell clonality in the cardiovascular context. However, our knowledge of the underlying mechanisms behind this clonal selection is still severely limited. There is a transpiring pattern of clonal expansion of smooth muscle cells and endothelial cells—and, in some cases, macrophages—in numerous cardiovascular diseases irrespective of their differing microenvironments. These findings indirectly suggest the possible existence of stem-like vascular cells which are primed to respond during disease. Subsequent clones may undergo further phenotypic changes to adopt either protective or detrimental roles. By investigating these clone-forming vascular cells, we may be able to harness this inherent clonal nature for future therapeutic intervention. This review comprehensively discusses what is currently known about clonal expansion across the cardiovascular field. Comparisons of the clonal nature of vascular cells in atherosclerosis (including clonal hematopoiesis of indeterminate potential), pulmonary hypertension, aneurysm, blood vessel injury, ischemia- and tumor-induced angiogenesis, and cerebral cavernous malformations are evaluated. Finally, we discuss the potential clinical implications of these findings and propose that proper understanding and specific targeting of these clonal cells may provide unique therapeutic options for the treatment of these cardiovascular conditions. (J Am Coll Cardiol Basic Trans Science 2024;9:120–144) © 2024 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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The concept of "survival of the fittest" has underpinned our ideas about evolutionary biology since it was first presented in the mid-1800s.[1] This describes the biological imperative of all organisms to pass on their favorable traits to their progeny and thus continue the existence of the species. Although first considered on an organism level, it is becoming increasingly apparent that this idea should be applied at a cellular level as well. A prominent example of this is in cancer, whereby favorable genetic mutations—at least from the cancer cell's perspective—in even a single cell can lead to rapid proliferation and migration, and thus the development of cancerous tissue.[2,3] This mitotic replication of single cells into identical progeny is known as clonality, albeit the daughter cells may not necessarily remain identical over time.

In the field of vascular biology, cells of the vascular system (smooth muscle cells [SMCs], endothelial cells [ECs], and macrophages) have been discovered to show degrees of clonal expansion during injury and disease (**Central Illustration**, **Table 1**). Although the clonal architecture of some of these cell populations has been suspected for decades, recent developments in Cre-*Lox* recombination technologies with multicolor fluorescent reporter mice (**Table 2**) have allowed the marking of specific cells, and thus the definitive determination of a clonal origin (**Figure 1**).[4,5] This could arise from the positive selection of cells that have acquired an advantageous mutation, the negative selection of less-able cells, or the proliferation of progenitor-like cells present in a heterogenous vascular population (**Figure 2**). In either case, vascular cell clonal expansion could be described as the selection of cells that are "fitter" to respond to abnormal conditions. An important caveat that must be highlighted is that these "gold-standard" genetic recombination techniques are limited to animal models. Although there is evidence for clonal expansion in human pathology, the inability to mark specific cells hinders the same level of conclusiveness. In this review, we discuss the transpiring commonality of clonality of vascular cells in a variety of cardiovascular diseases (CVDs) which has not been comprehensively reviewed to date (**Tables 3 and 4**). The CVDs that we discuss include atherosclerosis, clonal hematopoiesis of indeterminate potential (CHIP), pulmonary artery hypertension (PAH), aortic aneurysm, vessel injury, ischemia- and tumor-induced angiogenesis, and cerebral cavernous malformations (CCMs).

Although the clonal nature of vascular cells in injury and disease is undoubtedly fascinating, the question of whether this phenomenon has clinical relevance remains. Numerous groups are researching the plasticity of these vascular cells during disease and the underlying mechanisms involved. However, before this can occur, these cells must be first selected and undergo clonal expansion. Therefore, it is crucial that we understand the driving forces and mechanisms responsible for clonality that is often an inherent precursor to phenotypic modulation. If we know that a particular cell is responsible for responding to disease or injury—or even causes disease—perhaps we can target this cell for therapeutic benefit. However, identifying the clonal cell before it has clonally expanded may be difficult, especially if we have yet to determine the mutation or genes responsible for its clonal nature.

CLONALITY OF VASCULAR CELLS IN VASCULOPATHIES

ATHEROSCLEROSIS. One of the most well-known cases of cell clonality in vascular diseases is atherosclerosis, a condition characterized by the formation of a lipid-rich plaque within the arteries. Plaque development is the culmination of atherogenic risk factors (such as smoking, diet, and lipid levels) that lead to a complicated network of cellular interactions involving SMCs, ECs, and macrophages.[69-72] Low-density lipoprotein (LDL) build-up leads to chemokine and adhesion factor secretion from ECs, all resulting in the recruitment of monocytes from the bloodstream. Monocytes differentiate into macrophages, migrate through the intimal EC layer, and engulf these LDLs, forming foam cells within the plaque.[70-72] Macrophages and foam cells contribute to the prothrombotic necrotic core whereas SMCs line the atheroprotective fibrous cap.[70-73] However, the heterogeneity and different developmental origins of cells within blood vessels may affect the propensity of different vessels to plaque formation.[74,75]

CENTRAL ILLUSTRATION Vascular Cell Clonal Expansion in Vasculopathies

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Smooth muscle cells, endothelial cells and macrophages all exhibit degrees of clonal expansion in a variety of cardiovascular diseases.

SMC clonality in human atherosclerotic lesions. Early studies of clonal expansion in humans were based upon X-inactivation. During female embryonic development, the random inactivation of an X chromosome, termed lyonization, results in different cells expressing different alleles of X-linked genes.[76] The phenomenon of clonal plaque development was first suggested by Benditt and Benditt[62], a report that received criticism at the time. X-inactivation of glucose-6-phosphate dehydrogenase isoenzymes suggested that human plaques were largely monoclonal (expressing one isoenzyme). They presumed that this was due to the clonal expansion of resident SMCs but were unable to validate this conclusively. Although they noted that not all of the zymograms

contained only 1 isoenzyme, they attributed this to macrophage contamination, which only highlights the shortcomings of their methodology.[62] These observations were corroborated by some but argued against by others.[63,64] Thomas et al[64] split large plaques into layers and only found significant numbers of single isoenzymes in the top layer (luminal side).

Further insight into atherosclerotic plaque clonality was provided by using immunostaining for an SMC marker (ACTA2$^+$) which allowed for more precise microdissection of specific cells. Analysis of the X-linked androgen receptor gene CAG repeat patterns revealed that SMCs were the monoclonal population.[65,66] It can be assumed that the observation of some polyclonal "SMC" populations could be a result

TABLE 1 Murine Models for Cardiovascular Diseases Used in Clonality Studies

Atherosclerosis

Mice harboring the $Apoe^{-/-}$ or $Ldlr^{-/-}$ mutations, or induced via PCSK9, are predisposed to increased cholesterol and lipid levels in the bloodstream. This leads to susceptibility to Atherosclerotic plaque formation when fed a high-fat diet.[6-8]

Clonal hematopoiesis of indeterminate potential (CHIP)

Bone marrow transplant from $Tet2^{-/-}$ or $Tet2^{+/-}$ mice into $Tet2^{+/+}$ irradiated mice mimic the endpoint clonal expansion of these mutated cells, whereas injection of a mixture of $Tet2^{-/-}$ and $Tet2^{+/+}$ bone marrow cells into lethally irradiated mice or partial transplant of $Tet2^{-/-}$ into nonirradiated mice better recapitulate the competitive nature of clonal expansion during aging. Similar models have been used with $Jak2$ and $Trp53$ mutations.[9-14] Bone marrow reconstitutions have also been performed in atherosclerosis mouse models to investigate the links between these diseases.[9,10,13,14]

Pulmonary artery hypertension (PAH)

Mice exposed to chronic hypoxic conditions have PAH with distal artery muscularization.[15] However, chronic exposure to house dust mites has been suggested to be a better model as it produces neointimal lesions, better mimicking human pathology.[16]

Aneurysm

Mice develop aortic aneurysms after infusion with angiotensin II.[17] This is sometimes combined with $Apoe^{-/-}$ or $Ldlr^{-/-}$ mutations.[17] Alternatively, $Tgf\beta r2$ knockout at 6 weeks in vascular smooth muscle cells (SMCs) of $Apoe^{-/-}$ or $Ldlr^{-/-}$ mice fed a high-fat, high-cholesterol diet develop aortic aneurysms that well resemble human pathology.[18]

Vessel injury

There are many different mouse models used for vessel injury. Ligation of the left carotid artery causes rapid proliferation of SMCs into the lumen, leading to neointima formation.[6] Surgical clamping of the abdominal aorta for 1 minute caused removal of the endothelial layer at the clamping site.[19] Laser ablation, streptozotocin-injection, and NG-nitro-L-arginine methyl ester treatment have been used to mimic endothelial cell (EC) injury in specific locations, diabetes, and hypertension, respectively.[20]

Ischemia-induced angiogenesis

The method of inducing ischemia depends on the ischemic tissue that must be studied. Permanent ligation of the left anterior descending coronary artery promotes the development of myocardial infarction and subsequent neovascularization.[21,22] Exposure to 75% oxygen for 5 days during postnatal development produces retinopathy of prematurity.[22] Permanent ligation of the femoral artery and vein leads to hindlimb ischemia.[22]

Tumor angiogenesis

Subcutaneous injection of a melanoma cell line into healthy mice led to the development of tumorous tissue. This type of tumor produces an abundant network of new blood vessels in 4 weeks.[23]

Cerebral cavernous malformations (CCMs)

$Ccm3$ knockout at postnatal days 1-5 specifically within ECs leads to lesion formation. Size and distribution of the lesions depends on tamoxifen dose, and thus the number of ECs undergoing recombination.[24,25]

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of cell plasticity, which will be briefly discussed in subsequent sections. The investigators found that some medial and diffuse intimal thickening samples were also skewed towards a single allele.[65,66] However, these zymogram and polymerase chain reaction-based techniques are inferior to fluorescent labeling that has been developed in mice. This is because these studies do not rule out the presence of more than 1 X-inactivated progenitor cell in a single patch.[66] Moreover, up to 31% of females may have a skewed pattern of X-inactivation, which is further complicated as skewing patterns may be tissue dependent.[77,78] Ultimately, altered skewing patterns may mimic clonal expansion and renders these techniques unreliable. Nevertheless, although results of SMC clonality in the atherosclerotic plaque have largely been corroborated in mice, the differences in clonal analysis techniques and intrinsic interspecies differences may be the cause of some discrepancies. Thus, better clonal analysis techniques in humans are direly needed. Mitochondrial DNA mutations amass with age and offer promise as a natural cellular barcode that can be used for lineage tracing[79]; however, this remains unexplored in the context of cardiovascular disease clonality.

SMC clonality in atherosclerotic murine models. Building upon the early suggestions of atherosclerotic plaque clonality, it has now been well established using advanced lineage tracing mice that SMCs are the cells of interest that undergo clonal expansion.[6-8] It has become apparent that a select few SMCs from the polyclonal vascular medial wall undergo extensive proliferation and migration to form the monoclonal/oligoclonal fibrous cap. From here, some cells migrate to the necrotic core where they can undergo further phenotypic changes, which is referred to as SMC plasticity.[8,80] The Yamanaka transcription factors KLF4 and OCT4 are suggested to play critical roles in SMC plasticity, which is perhaps not surprising given their roles in induced pluripotent stem cell reprogramming.[80-82] The various smooth muscle (SM)-derived phenotypes are not a focus of this review and have been reviewed extensively by others.[83-85]

Given that SM-derived cells in the fibrous cap and necrotic core are all derived from 1 or 2 cells, an interesting question that arises is whether all SMCs have the capacity to undergo phenotypic modulation, or whether this is a property limited to the clonally expanding SMCs. More fundamentally, are all SMCs

TABLE 2   Common Cre-lox Systems for Clonal Analysis

Myh11-promoter

Currently considered to be the gold standard for SMC lineage tracing due to its specificity for SMCs.[6-8,16-18,26,27] However, there are recent reports that it may mark some pericyte populations as well.[28] The main limitation with this Cre-driver is that the transgene is located on the Y-chromosome, limiting lineage tracing analysis to males only.[27] Recent developments have overcome these issues, but their use is not yet widespread.[29,30]

Sm22α-promoter

Although this promoter is used for SMC lineage tracing, its major drawback is that it is has a lower specificity than the *Myh11*-promoter.[27,31,32] SM22α is known to be expressed in developing cardiomyocytes, megakaryocytes, and perivascular adipocytes.[33-35]

Acta2-promoter

This is also a less-used Cre-driver for SMC lineage tracing because of its lack of specificity.[8,15,27,36] ACTA2 is known to be expressed in fibroblasts, myofibroblasts, and myoepithelial cells.[37-39]

Cdh5-promoter

A well-known Cre driver for EC lineage tracing.[19,20,22,23,25,40,41] The only known drawback is its nonspecific expression in hematopoietic cells if marked early during development due to the common hematopoietic and endothelial progenitor.[42]

Pdgfb-promoter

A less-used Cre driver for EC lineage tracing because of its lack of specificity and sensitivity.[21,24,41,43] This promoter does not mark the endocardium, and expression of PDGFB can be observed in SMCs and megakaryocytes.[44-46]

Csf1r-promoter

This promoter is used for macrophage lineage tracing, but care must be taken as it has a low specificity, with expression also in dendritic cells, T lymphocytes, and granulocytes.[8,47-50]

Cx3cr1-promoter

Another promoter used for macrophage lineage tracing but is also expressed in some other immune cell types, such as dendritic cells and mast cells.[47,49,51,52] Furthermore, its efficiency may depend on the macrophage population of interest.[52]

Rainbow-reporter

A multicolor reporter that allows analysis of cell clonality, based on the *Brainbow*-1.1 construct.[4,53] In this construct, fluorescent reporter genes are flanked by incompatible *lox* variants. These *lox* mutations force mutually exclusive excision events upon Cre recombination. The construct also transiently expresses one of the colors, acting as an internal control.[53]

Confetti-reporter

Another type of multicolor reporter for clonal analysis, based on the *Brainbow*-2.1 construct.[4,54] This construct contains inverted *loxP* sites surrounding fluorescent reporter genes in different orientations. Cells do not express any fluorescent reporter before recombination, but Cre-induced excision and/or inversion generates 1 of 4 different colors. Importantly, transient Cre expression can continue to invert the construct and cause color changes, which is a major drawback for clonality studies.[54,55] The confetti reporter has been shown to attenuate PCSK9-induced atherosclerotic plaque burden.[7]

Corollary

The above Cre-lox promoters and reporters are the most widely used systems for clonal analysis in the cardiovascular disease context. The Rainbow and Confetti reporters are variants of the original *Brainbow* constructs, and multiple other variations exist.[4,53,54,56-61] Increased numbers of distinctive fluorescent protein colors reduce the chance of low-probability recruitment of same color cells and overcomes the need for low-dose tamoxifen to reduce labelling efficiency. This has been achieved via increased number of transgene copies and using different subcellular localizations of the fluorescent proteins.[4,56-61]

Abbreviations as in **Table 1**.

equally likely to undergo proliferation and migration to form the atherosclerotic plaque, or are some select SMCs "primed" or acquire selectional advantages that allow them to clonally expand? In the context of atherogenesis, stem cell antigen-1 (SCA1/LY6A) may be a marker for these hyperproliferative SMCs that are responsible for cap formation. However, there have been mixed results as to whether SCA1 marks SMCs that are predisposed for rapid proliferation, or whether it is upregulated in select cells during disease progression. Dobnikar et al[86] suggest that approximately 1% of medial MYH11+ cells are SCA1+, identified using antibody staining and constitutively active *Sca1-GFP* mice. In comparison, use of new *Sca1*-Cre-driven mice have not corroborated these findings.[87,88] To explain these apparent contradictions, it is possible that this *Sca1*-Cre driver does not have a high enough recombination efficiency to consistently mark this rare population of cells.

Alternatively, SCA1 expression is induced by pro-atherogenic conditions, as reported by Tang et al[88] who investigated SCA1 expression in healthy adult mice, and Wang et al[87] who used atherogenic mice but induced recombination before feeding the mice the specialized high-fat diet. In cultured SMCs, cholesterol loading has been shown to increase *Sca1* mRNA expression.[89] This proposal is further supported by the observations that the necrotic core is SCA1+ whereas the fibrous cap is SCA1-, despite their mono-/oligoclonal origins, pointing in favor of *Sca1* expression modulation during atherogenesis.[86]

Regardless of whether SCA1 can mark predisposed SMCs in the medial wall, there is no doubt that a population of plaque SMCs are SCA1+ during disease progression. These SCA1+ SMCs exhibit hyperproliferative capacity and upregulate complement C3 secretion.[90,91] Wang et al[91] suggest that because C3 marks cells for removal via phagocytosis, the

FIGURE 1 Historic Milestones for the Study of Clonality in Vasculopathies

Studies of clonal expansion in humans have been based on X-inactivation, starting with zymogram-based approaches, followed by polymerase chain reaction methods. Recent developments into fluorescent lineage tracing with multicolor reporters has revolutionized clonal analysis in murine models. The first studies of clonal expansion in each cardiovascular disease are shown.[6,15,17,19,22-24,62,63] EC = endothelial cell; SMC = smooth muscle cell.

clonality of these specialized SMCs is due to a combination of hyperproliferation and defective efferocytosis, rather than an inherent capacity to avoid being phagocytosed. Polyclonality of SMCs in the lesion was observed when treating mice with an anti-CD47 antibody.[91] Although we still do not know how blocking CD47 inherently causes polyclonal expansion, it again raises the question of whether all SMCs have the capacity for clonal expansion and, if so, do they exhibit the same propensity for plaque stabilization and subsequent phenotypic modulation? It could be interesting to verify that even in the context of polyclonality these newly recruited SMCs also gain SCA1 expression. Given that the investigators found no incidence of plaque destabilization, it may be easy to assume that all SMCs do have the same

atheroprotective capacity, but care must be taken especially given the newly suggested role of other cell sources in plaque stability that compensate when SMC investment is altered.[91,92]

Polyclonality of SMCs has also been shown to be induced by the global knockout of integrin β3 (*Itgb3*).[8] It has been previously established that *Itgb3* deficiency accelerates disease pathogenesis, which is thought to be due to increased inflammation and an increase in SMC transdifferentiation to a macrophage-like phenotype.[8,93,94] Bone marrow transplanted from *Itgb3* null to wild-type atheroprone mice has shown polyclonality as well, suggesting that paracrine effects of myeloid cells play a role in SMC recruitment to plaque development.[8] It is extremely fascinating to note that both methods to alter SMC clonality within

FIGURE 2   Possible Mechanisms Underlying the Clonal Expansion of Cells

One possibility is that cell populations are inherently heterogenous and some of these cells remain in a more progenitor-like state which are more proliferative and responsive to disease. In cancer, clonal expansion is observed because of the acquisition of mutations that allow a selective advantage over regular cells (eg, more proliferation, apoptosis resistant, or increased self-renewal capability). Another possibility is that the heterogenous cell population contains a subset which is more resistant to apoptosis. Removal of non-apoptotic cells results in a clonal population. These mechanisms are not necessarily distinct.

the plaque have roles in inflammation. Anti-CD47 antibodies have been shown to increase macrophage phagocytic potential and *Itgb3* knockout induces more inflamed macrophages.[8,91,94] This raises the distinct possibility that inflammatory macrophages are key regulators of clonal expansion, and conditions that affect this inflammatory state may thus influence SMC recruitment.

What drives the phenotypic modulation of these clonal SMCs? If the clonally expanded SMCs do not gain other mutations that control their plasticity (ie,

all the fibrous cap SMCs are the same), why do SMCs adopt various phenotypes such as macrophage-like and osteoblast-like properties? Although it has been suggested that all the different phenotypes are due to a linear transition to a singular phenotype, it is more likely that a "stem-like" phenotype is reached, followed by subsequent phenotypic branching.[82,89,95,96] Despite numerous studies that have investigated the genes involved in these phenotypic transitions, there is a lack of understanding of the fundamental driving forces that initiate many of these transitions. It is

TABLE 3 Evidence for Clonal Expansion of Vascular Cells in Human Cardiovascular Pathology

Cardiovascular Pathology	Disease Model	Vascular Cell Clonality	X-linked Gene Analyzed	Analysis Technique	First Author (Year)
Atherosclerosis					
	Abdominal aorta and common iliac arteries	Monoclonal SMCs (bulk plaques)	Glucose-6-phosphate dehydrogenase isoenzymes	Zymogram	Benditt and Benditt[62] (1973)
	Abdominal aorta	Monoclonal SMCs (bulk plaques)	Glucose-6-phosphate dehydrogenase isoenzymes	Zymogram	Pearson et al[63] (1975)
	Aorta	Polyclonal SMCs (bulk plaques split into layers)	Glucose-6-phosphate dehydrogenase isoenzymes	Zymogram	Thomas et al[64] (1979)
	Aorta and coronary arteries	Monoclonal SMCs (plaques split into regions and microdissected based on ACTA2 expression)	Androgen receptor CAG tandem repeats	Polymerase chain reaction	Murray et al[65] (1997)
	Abdominal aorta	Monoclonal SMCs (plaques split into regions and microdissected based on ACTA2 expression)	Androgen receptor CAG tandem repeats	Polymerase chain reaction	Chung et al[66] (1998)
PAH					
	Primary PAH	Monoclonal ECs (microdissection)	Androgen receptor CAG tandem repeats	Polymerase chain reaction	Lee et al[67] (1998)
	Secondary PAH (congenital heart disease, CREST syndrome)	Polyclonal ECs (microdissection)	Androgen receptor CAG tandem repeats	Polymerase chain reaction	Lee et al[67] (1998)
	Anorexigen-induced primary PAH	Monoclonal ECs (microdissection)	Androgen receptor CAG tandem repeats	Polymerase chain reaction	Tuder et al[68] (1998)

CREST = calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia; other abbreviations as in **Table 1**.

likely that small variations in the plaque microenvironment or paracrine effects from other cells are responsible. Nevertheless, SMCs play a crucial role in atherosclerotic plaque stability and determining the mechanisms by which they are recruited is vital for the development of therapeutics that are better able to influence atherosclerosis on a cellular level.

Macrophage clonality in atherosclerosis. In comparison to SMCs, macrophages appear to exhibit polyclonality within the atherosclerotic lesion.[8] However, analysis of macrophage clonality occurred from 6 to 9 weeks after initiation of the atherogenic high-fat diet. Recently, Williams et al[47] have elegantly used lineage tracing mice to conclude that a population of aortic intimal resident macrophages (dubbed Mac[AIR]) are the first responders to form the foamy macrophage population within the necrotic core. These have only a limited capacity for proliferation and are slowly replaced by bone marrow (BM)-derived macrophages over approximately 12 weeks of high-fat diet.[47] Given that clonal analysis occurred during this "replacement period," it is unsurprising that the investigators observed a polyclonal macrophage contribution.[8] A recent study of BM-derived macrophages at 16 weeks of high-fat diet suggests that even at this later timepoint macrophages exhibit polyclonality.[13] That being said, macrophage clonality was not a major focus of this study and further

analysis is required at different timepoints. This would be interesting to investigate given that the myeloid-derived macrophage population within established plaques maintains itself in part via local proliferation, which may be hypothesized to be due to the proliferation of a "progenitor-like" subpopulation, with an ongoing contribution from newly recruited circulating monocytes.[97,98] This may have clinical relevance, given that preventing this self-renewal of the myeloid-derived macrophages may play a key role in plaque regression.[99]

Clonal hematopoiesis of indeterminate potential. Clonal hematopoiesis of indeterminate potential (CHIP) is a condition associated with the clonal expansion of hematopoietic stem and progenitor cells (HSPCs) due to the accumulation of somatic mutations that can lead to an altered immune response. Recently, CHIP was found to be a risk factor of atherosclerosis, stroke, and myocardial infarction.[9,10,13,100] Using whole-exome sequencing, *DNMT3A, TET2, ASXL1,* and *JAK2* mutations were identified and associated with increased risk of CVD.[9,100] Exact mechanisms whereby these mutations induce the clonal expansion of HSPCs remains unknown. However, different genetic variants affect clonal hematopoiesis via different mechanisms and thus confer differing risks of CVD, suggesting that future therapies should be mutation specific.[101,102] Nevertheless, investigators

TABLE 4 Clonal Analysis of Vascular Cells in Cardiovascular Diseases Using Lineage Tracing Murine Models

Cardiovascular Pathology	Experimental Model	Vascular Cell Clonality	Clonal Analysis Technique	First Author (Year)
Atherosclerosis	$Apoe^{-/-}$ mice fed a normal diet for 52 weeks or high fat diet for 16 weeks	Monoclonal SMCs	$Sm22\alpha$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in aortic plaque	Feil et al[31] (2014)
	$Apoe^{-/-}$ mice fed a high fat diet for 16-19 weeks	Oligoclonal SMCs	$Myh11$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in aortic arch, carotid arteries and descending aortic plaques	Chappell et al[6] (2016)
	Wild-type mice injected with PCSK9 and fed a high-fat diet for 12, 24 and 36 weeks	Oligoclonal SMCs	$Myh11$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in aortic root and aortic arch plaques	Jacobsen et al[7] (2017)
	$Apoe^{-/-}$ mice fed a high-fat diet for 6, 12 or 16 weeks	Monoclonal SMCs	$Myh11$-CreER[T2] or $Acta2$-CreER[T2], $Rosa26$-$Rainbow$ lineage tracing in aortic root and BCA plaques	Misra et al[8] (2018)
	$Apoe^{-/-}$ mice fed a high-fat diet for 6-9 weeks	Polyclonal macrophages	$Csf1r$-Mer-iCre-Mer, $Rosa26$-$Rainbow$ lineage tracing in aortic root plaques	Misra et al[8] (2018)
	$Ldlr^{-/-}$ mice fed a high-fat diet for 16 weeks	Polyclonal macrophages	Lethal irradiation and bone marrow transplant from $Mx1$-Cre, $Rosa26$-$Confetti$ mice. Lineage tracing in aortic root plaques.	Fidler et al[13] (2021)
Atherosclerosis (CHIP)	$Ldlr^{-/-}$ mice fed a high-fat diet for 16 weeks	Oligoclonal $Jak2^{V617F}$ macrophages	Lethal irradiation and bone marrow transplant from $Mx1$-Cre $Jak2^{V617F}$, $Rosa26$-$Confetti$ mice. Lineage tracing in aortic root plaques.	Fidler et al[13] (2021)
PAH	Chronic hypoxia (10%) for 7 or 21 days	Monoclonal SMCs	$Acta2$-CreER[T2], $Rosa26$-$Rainbow$ lineage tracing in distal arteriole muscularization	Sheikh et al[15] (2015)
	House dust mite exposure for 6 weeks	Oligoclonal SMCs	$Myh11$-CreER[T2], $Rosa26$-$Rainbow$ lineage tracing in neointimal lesions	Steffes et al[16] (2020)
Aneurysm	$Apoe^{-/-}$ or wild-type mice infused with angiotensin II	Oligoclonal SMCs	$Myh11$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in the aortic adventitia	Clément et al[17] (2020)
	$Tgfbr2^{i\Delta SMC}$ (knockout at 6 weeks) in $Apoe^{-/-}$ mice fed a high-fat high cholesterol diet for 16 weeks	Monoclonal SMCs	$Myh11$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in the medial bone nodule areas of the ascending aorta	Chen et al[18] (2020)
Vessel injury	Ligation of the left carotid artery for 28 days	Oligoclonal SMCs	$Myh11$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in the neointima	Chappell et al[6] (2016)
	Clamping of the abdominal aorta for 60 seconds	Oligoclonal ECs	$Cdh5$-CreER[T2], $Rosa26$-$Rainbow$ lineage tracing in the injury region	McDonald et al[19] (2018)
	Laser ablation of glomerular vascular pole	Oligoclonal ECs	$Cdh5$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in glomeruli	Desposito et al[20] (2021)
	Streptozotocin injection	Oligoclonal ECs	$Cdh5$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in glomeruli	Desposito et al[20] (2021)
	NG-nitro-L-arginine methyl ester delivered via drinking water for 10-60 days	Oligoclonal ECs	$Cdh5$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in glomeruli and other organs (heart, lung, kidney, brain, pancreas, liver, spleen)	Desposito et al[20] (2021)
Ischemia-induced angiogenesis	75% oxygen exposure for 5 days at postnatal day 7	Oligoclonal ECs	$Cdh5$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in ischemic retina	Manavski et al[22] (2018)
	Ligation of the left anterior coronary artery for 7 or 14 days	Oligoclonal ECs	$Cdh5$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in ischemic heart	Manavski et al[22] (2018)
	Ligation of the left anterior coronary artery for 7 days	Monoclonal ECs	$Pdgfb$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in ischemic heart	Li et al[21] (2019)
	Ligation of the femoral artery and vein for 14 days	Oligoclonal ECs	$Cdh5$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in ischemic calf	Manavski et al[22] (2018)
Tumor angiogenesis	Melanoma cell line injection for 7-40 days	Oligoclonal ECs	$Cdh5$-CreER[T2], $Rosa26$-$Rainbow$ lineage tracing in tumor	Corey et al[23] (2016)
	Melanoma cell line injection for 7-40 days	Polyclonal stromal cells	$Acta2$-CreER[T2], $Rosa26$-$Rainbow$ lineage tracing in tumor	Corey et al[23] (2016)
Cerebral cavernous malformations	$Ccm3^{i\Delta EC}$ (knockout at 3-5 days)	Monoclonal ECs	$Pdgfb$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in lesion	Detter et al[24] (2018)
	$Ccm3^{i\Delta EC}$ (knockout at 1 or 2 days)	Monoclonal ECs	$Cdh5$-CreER[T2], $Rosa26$-$Confetti$ lineage tracing in lesion	Malinverno et al[25] (2019)

BCA = brachiocephalic artery; other abbreviations as in Table 1.

found that in mouse models, *Tet2* mutant hematopoietic cells have a competitive advantage over their wild-type counterparts, which could be due to the ability of TET2 to inhibit the self-renewal of HSPCs.[10] In atherosclerosis-prone, LDL receptor–deficient ($Ldlr^{-/-}$) mice, these *Tet2*-mutant hematopoietic cells clonally expanded and led to a NLRP3 inflammasome–mediated marked increase in atherosclerotic lesion size.[10] Tet2-deficient mice have increased secretion of interleukin 1β (IL-1β) and IL-6 when induced with lipopolysaccharide and interferon gamma.[10] These findings suggest a strong link between clonal hematopoiesis with proinflammatory pathways involving IL-1β and NLRP3 inflammasome activation in CVD.[10,103]

More recently, Fidler et al[13] investigated the relationship between atherosclerosis and CHIP using BM transplants of the HSPC or macrophage-specific inducible gain of function mutation $Jak2^{V617F}$. In accordance with previous reports, this increased plaque size as well as macrophage proliferation and macrophage inflammation (increased NLRP3 and AIM2 inflammasome activation, and IL-1β).[13] The investigators note that in a BM mixture competition model (20% mutant and 80% nonmutant HSPCs), the $Jak2^{V617F}$ macrophages were much higher in abundance within the lesion (60% of macrophages) compared with in the bloodstream (30% of monocytes), which was thought to be a result of increased adhesion to the endothelium and increased local proliferation.[13] Using a mutant BM transplant from a confetti mouse, the investigators show oligoclonal expansion of BM-derived cells within the plaque, corresponding with increased rates of proliferation from these recruited mutant macrophages.[13] These findings of increased proliferation and plaque burden have been mirrored using a 20% $Trp53^{-/-}$ competitive bone marrow transplant model.[14] Furthermore, it will also be fascinating to investigate the effects of other inflammation-associated CVD risk factors on the clonal architecture of plaque cells.

The findings from these mouse studies on clonal hematopoiesis may have strong significance to the outcomes of the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study).[104] The overall benefit of canakinumab (IL-1β blocking antibody) was modest, with no change in overall mortality rate, in part because canakinumab was associated with the increased risk of fatal infection.[104] However, the patients who achieved high-sensitivity C-reactive protein reduction to <2 mg/L had significantly reduced cardiovascular mortality and all-cause mortality compared with the patients who did not achieve significant reductions in high-sensitivity C-reactive protein after treatment of canakinumab.[104] Given that CHIP patients have a heightened inflammatory milieu, this raises a strong possibility that patients harboring the *TET2* mutation (or equivalent) and having IL-1β producing clones of hematopoietic cells may be responding better to canakinumab.[105-109] Taken together, these results are consistent with the notion that clonal hematopoiesis contributes to CVD and CHIP should be considered to be a CVD risk factor.

Finally, the term "risk factor" implies that CHIP develops before atherosclerosis, which may not always be true. In humans and mouse models, atherosclerosis leads to a marked increase in HSPC proliferation.[110] Using mathematical modelling, the investigators estimate that atherosclerosis could accelerate CHIP emergence from approximately 85 to 65 years of age (depending on extent of atherosclerotic CVD).[110] Given that the hyperlipidemic atherogenic diet was insufficient to induce clonal expansion of $Tet2^{-/-}$ clones in healthy murine models, this would indicate that the inflammatory nature of atherosclerosis has systemic consequences and is responsible for accelerating CHIP.[110] Taken together, CHIP and atherosclerosis appear to exacerbate each other in an inflammatory cascade. These findings may provide the link as to why patients suffering from many other diseases with heightened inflammation experience increases in adverse cardiovascular events, and highlights the importance of targeting pathological inflammation for the treatment of CVD.

PULMONARY ARTERY HYPERTENSION. PAH is the culmination of a complex series of cellular events involving all 3 layers of the vessel wall, collectively termed vascular remodelling.[111-113] It primarily entails medial thickening due to SMC hypertrophy, along with increased production of cytokines and extracellular matrix proteins.[114-117] Studies in rodent and large animal models show considerable spatial heterogeneity of pulmonary artery (PA) SMCs, which correspond to distinct responses to pathological stimuli.[118-120] The media of the proximal region of bovine PAs consists of an oligoclonal mosaic of differentiated SMCs, whereas the distal part is more "muscular," consisting of a uniform population of SMCs expressing myosin heavy chain proteins.[121] Lineage tracing studies in murine models have revealed diverse mesenchymal progenitors in the PA that differ in their proliferative and migration patterns.[122] These likely give rise to phenotypically distinct SMCs that are pathologically relevant.[123] In addition to the lineage-specific progenitors, SM-like

cells can also be derived from ECs undergoing transition to the mesenchymal lineage, and from fibrotic cells from the adventitia.[124,125]

SMC clonality in PAH. In a hypoxia-induced murine model of PAH, PDGFR-β^+ SMCs in the proximal and middle arterioles dedifferentiate, migrate distally, clonally expand, and re-differentiate into the distal arterioles.[15] Only a specific few SMCs near the muscular-unmuscular border express the undifferentiated mesenchymal marker platelet-derived growth factor receptor-β (PDGFR-β) under normoxic conditions, suggesting the existence of a subset of primed SMCs that are responsible for clonal expansion.[126] It has been shown that a pool of PDGFR-α^+ progenitors in the perivasculature differentiate into SMCs and contribute during PAH-induced muscularization, which raises the question whether these PDGFR-α^+ progenitors maintain the pool of rare PDGFR-β^+ cells at the unmuscularized border.[127] Regardless, initiation of hypoxia induces the expression of the hypoxia inducible factor HIF1-α and the pluripotent factor KLF4 in these primed SMCs, which clonally expand to muscularize the distal arterioles.[15,128] KLF4 and HIF1-α appear to be critical for the initial migration and subsequent clonal expansion, respectively, and without either, PAH is limited.[15,128] In addition, HIF1-α in ECs and macrophages is responsible for PDGF-B secretion, which increases SMC proliferation and migration in a paracrine manner.[15,128,129] Future studies should also investigate the contribution of PDGF-B secretion by platelets and the relative contribution of each cell type to PDGF-B secretion, along with PDGF-D secretion, another activator of PDGFR-β. Limiting SMC clonal expansion by nanoparticle administration of *Pdgfb* small interfering RNA to lung macrophages or by inducing *Pdgfb*$^{+/-}$ genetically appears to provide some protection against hypoxia-induced PAH.[15,129] Not all of the ACTA2$^+$ cells within the newly muscularized distal arteriole are SM-derived, and macrophages may be a contributing source.[128] These observations highlight the importance of SMC progenitors and the potential involvement of KLF4 and PDGF signaling in the pathogenesis of PAH and perhaps other vascular disorders. Although these murine studies appear promising, further research and care must be taken when considering this pathway for clinical translation. The IMPRES (Imatinib [QT1571] in Pulmonary Arterial Hypertension) clinical trial showed some pulmonary benefits of the tyrosine kinase receptor inhibitor imatinib, but this was overshadowed by the high rate of serious adverse events including multiple mortalities.[130]

However, the hypoxia-driven model of PAH has been criticized because of the lack of neointimal lesions which are present in human pathogenesis.[16] Using an inflammatory model induced by house dust mite exposure, it has also been proposed that a "primed" subset of medial SMCs are responsible for neointima formation.[16] Steffes et al[16] identified this subset as NOTCH3^{high}, and elegantly show that these cells are responsible for the clonally derived neointimal lesions. Furthermore, inhibition of Notch signaling using dibenzazepine prevented neointima formation, suggesting that the SMC dedifferentiation and migration are Notch-dependent. This is perhaps unsurprising given the role of Notch signaling during vascular development.[131] Medial wall thickening did not appear to be regulated by Notch.[16] Care must be taken when interpreting these results because Notch inhibition was not cell specific and may possibly cause other paracrine effects from other cell types. Future studies should include inducible Notch knockouts in SMCs specifically. Although this "primed" NOTCH3^{high} subpopulation is likely to be also PDGFR-β^+ and later KLF4$^+$, this remains to be conclusively proven. These findings raise additional important questions: when do primed cells appear in the blood vessels of developing lungs, is it in very early development, and what is their role in lung morphogenesis?

These studies revealed seminal insights into cellular processes of PAH progression; however, from a clinical perspective, it is essential to delineate cellular and molecular mechanisms of reverse distal arteriole muscularization to mimic advanced muscularized distal arterioles when patients seek clinical intervention. Hypoxia-induced PAH reverts upon reexposure to normoxia in murine models. However, in reversal, the fate of primed cells is unknown and whether primed cells undergo apoptosis and disappear from vasculature or whether primed cells maintain plasticity and undergo further differentiation into any other lineage to maintain normal lung functioning is currently unexplored. Thus, further investigations are needed to uncover the role of primed cells and the underlying mechanisms to maintain them in the lung vasculature. This may provide a pathway for novel therapeutics to improve clinical outcomes. Taken together, the results from clonal expansion of SMCs in atherosclerosis and PAH, it is intriguing to consider the existence of specialized cells in the blood vessel wall that may clonally expand in CVD pathology.[132]

EC clonality in PAH. In a similar manner to the early polymerase chain reaction-based atherosclerosis clonality studies, Lee et al[67] used the X-linked CAG

repeats of the androgen receptor gene to investigate the clonality of vascular cells in PAH.[65,66] Plexiform lesions are intraluminal vascular tumors that arise from the abnormal proliferation of ECs from the pulmonary arteries and are key features of severe PAH.[133,134] It was found that the plexiform lesions in primary PAH, but not secondary PAH, were formed from monoclonal ECs. However, using microdissection, the investigators had no way of ensuring these were solely ECs, and they suggest that SMC or inflammatory cell contamination could explain the 2 alleles found in some samples.[67] Similar findings were observed in the analysis of anorexigen-induced PAH patients.[68]

To uncover the molecular mechanisms behind this clonal expansion, Yeager et al[133] looked for evidence of somatic mutations that confer to these cells a selectional advantage. The investigators found various microsatellite mutations in TGF-βR2, BAX, and MSH2. These mutations may explain the aberrant growth of ECs during lesion formation, as TGF-βR2 is involved in limiting EC proliferation, BAX is a well-known pro-apoptotic protein, and MSH2 helps prevent genomic instability and cancer formation.[135-137] These findings are supported by observations that plexiform lesions do not have many cells undergoing apoptosis.[138] However, all these studies have been performed on human samples with well-established PAH. Given that clonal expansion could be a result of apoptosis of "less capable" ECs, clonal analysis during lesion formation should be performed to establish the temporal nature of clonality.

Alternatively, or perhaps in addition, there may be a subpopulation of primed ECs responsible for plexiform lesion monoclonality during PAH. Lesional ECs have been shown to express CD117 and CD133, which have been considered as progenitor markers, and may suggest the clonal expansion of a "progenitor" population.[139] To investigate the properties of this primed population, Bhagwani et al[140] isolated CD117+ ECs (CD31+) from the lungs of constitutively expressed GFP+ rat lungs and transplanted them into GFP- chronic hypoxia rats. This increased pulmonary artery vaso-occlusion. They also show that these primed ECs are hyperproliferative and underwent endothelial to mesenchymal transition (EndoMT, the conversion of ECs to a mesenchymal phenotype) to SM-like and osteoblast-like phenotypes.[140] Whether these CD117+ ECs have a greater propensity to undergo phenotypic modulation compared with more mature ECs remains unknown, but would be interesting to investigate, especially in the context of the newfound role of EndoMT in atherosclerotic plaque stabilization.[92] However, the lack of EC lineage

tracing in PAH models to date greatly hinders the argument of clonal expansion.

ANEURYSM. Abdominal aortic aneurysm is the most common type of aneurysm, accounting for approximately 80% of all aneurysms.[141,142] SMCs and ECs are also involved in aneurysm formation, a degenerative disease of the vessel wall characterized by focal dilation of blood vessels. These pathological changes are the result of EC remodeling and SMC apoptosis, leading to degradation of elastin and increased collagen I/III ratios throughout the adventitia.[143] In addition to genetic factors, atherosclerosis, hypertension, and chronic obstructive pulmonary disease are implicated in aneurysm formation.[144]

SMC clonality in aneurysm. In murine models of angiotensin II-induced dissecting aortic aneurysm (AA), Clement et al[17] found that SMCs clonally expanded in dissecting AA disease. Moreover, they reported that these SMC clones de-differentiate, lose SMC markers, and contribute to autophagy, phagocytosis, and endoplasmic reticulum-mediated stress response. SMC-specific deletion of the gene *Atg5* (autophagy-related 5) enhanced aneurysm-related mortality due to increased inflammation, endoplasmic reticulum stress, and SMC apoptosis.[17] ATG5 is a key player in regulating both canonical and noncanonical autophagy pathways and recent studies have shown that it plays a crucial role in linking autophagy with immune response by regulating lymphocyte proliferation and differentiation.[145] In human abdominal aortic aneurysm, autophagy-related pathways are reported to be dysregulated suggesting that lineage tracing results from murine models of AA may have translational relevance.[17,146] It is yet to be discovered if signaling pathways give survival benefits to the clonal cells to induce a stem-cell like proliferative advantage over other medial cells, or whether these rare medial cells have an intrinsic ability to suppress neighboring cells to undergo clonal expansion.

Transforming growth factor (TGF)-β signaling plays an important role in AA and dysregulating TGF-β signaling in aortic wall leads to aneurysm.[147-149] The pathological role of TGF-β is supported due to detection of low levels of TGF-β in human and murine models of abdominal aortic aneurysm.[150] Loss of TGF-β signaling in SMCs has been implicated in AA formation, aortic dilation, and arterial inflammation.[147] However, in an angiotensin II-induced dissecting AA model, the clonal expansion of SMCs was independent of TGF-β signaling[17] indicating that other pathways such as mitogen-activated protein kinases, AKT, and mammalian target of rapamycin may play a role in

this process.[151-153] Recently, Chen et al[18] showed that SMC-specific deletion of TGF-β signaling in *Apoe*^{-/-} mice fed a high-cholesterol, high-fat diet developed a phenotype of AAs that closely resembles non-Marfan/Loeys-Dietz types of human disease. Interestingly, single cell RNA sequencing and clonal analysis on confetti mice revealed that SMCs reprogrammed and clonally expanded into several mesenchymal cell-like fates including osteoblasts, macrophages, adipocytes, and chondrocytes during aneurysm progression. It was suggested that dysregulated TGF-β signaling plays a role in the conversion of SMCs to detrimental phenotypes, as downstream TGF-β proteins are responsible for suppressing the plasticity marker KLF4.

These findings warrant additional studies on the role of autophagy and TGF-β signaling on SMC clonality and phenotypic switching in AA. Overall, these findings raise an interesting possibility that, although SMC undergo clonal expansion in multiple vascular disorders, the signaling pathways regulating these processes may be dependent on the local environment and disease specific cytokines and chemokines secretion.

BLOOD VESSEL INJURY. There are many diverse causes of blood vessel injury, and yet the cellular responses are often similar. This includes regeneration of the endothelial lining, and proliferation of SMCs into the neointima.[6,154,155] The extent to which SMCs proliferate and invade the lumen appears to be dependent on the degree of injury, as well as molecular signals such as PDGF signaling.[154,156] Although there has been some debate as to the source of cells in the regenerating endothelium, it is now generally accepted that local ECs are responsible for local tissue repair.[19,157-162]

SMC clonality in blood vessel injury. In addition to examining the clonality of atherosclerotic plaques, Chappell et al[6] also looked at SMC clonal expansion in a ligation injury model. This was performed to determine whether the hyperlipidemic environment was the underlying cause of clonality. They observed oligoclonal patches within the neointima, suggesting that there is a subpopulation of hyperproliferative or "fitter" SMCs that respond to pathological conditions regardless of the disease. In contrast to the disease-independent phenomenon of SMC clonal expansion, there appeared to be less phenotypic modulation of SMCs in the vessel injury model compared to within the atherosclerotic plaque. The neointimal SMCs expressed the mature SMC marker SMMHC, suggesting that SMCs do not inherently undergo plasticity changes, and that the conversion to detrimental phenotypes is dependent on the differing cues present in different vascular diseases.[6] This observation is promising in the context of atherosclerosis, as the initial clonal expansion is considered protective whereas subsequent phenotypes are mostly considered detrimental. In the future it may therefore be possible to target these clones and keep them in a protective myofibroblast-like state before they convert phenotype.

EC clonality in blood vessel injury. Vascular ECs play an essential role in regulating blood flow by secreting vasoactive substances that act across the vascular beds in a paracrine manner.[163] Schwartz and Benditt[164] were the first to notice that under homeostatic conditions, ECs replicate in a non-uniform manner. They observed focal regions of higher replication, which could indicate a proliferative subpopulation.[164] However, without lineage tracing techniques at their disposal, their labelling method only identified cells which have proliferated, without any information regarding their original source.

Although ECs are normally in a quiescent state, they are activated in response to pathological stimuli such as inflammation or hydrodynamic stress to induce a prothrombotic state.[165-167] In response to injury or pathological conditions during development, some quiescent ECs have the capacity to differentiate into other cell types, including mesenchymal cells, stem cell progenitors, and hematopoietic stem cells.[168,169] This again raises the fundamental question: apart from producing progenitor stem cells, do ECs have definable stem cell potential and retain phenotypic flexibility? Recently, McDonald et al[19] and Wakabayashi et al[158] suggested that ECs respond to injury and regenerate the entire vascular surface, including vein and sinusoids in the transplanted liver. To identify if there is a unique set of ECs with regenerative potential, lineage tracing using the multicolor rainbow reporter was performed in a murine aortic injury model. The investigators found that 2 distinct populations of ECs with different proliferative potential participated in blood vessel regeneration.[19] Injury-adjacent ECs initiate the repair response, whereas a hyperproliferative subpopulation dominates over time. Similar findings of clonal proliferation were shown in renal glomeruli after laser ablation, diabetes-induced injury, and hypertensive injury.[20] These observations raise additional questions: Do quiescent ECs residing in specific microenvironments get activated by particular external stimuli? Do endothelial stem cells have definable lineage progression from progenitor to mature ECs? Collectively, these studies support the idea of

endothelial stem/progenitor cells in the vessel wall and provide seminal insights into cellular mechanisms of vessel injury.

To investigate the source of endothelium repair, Tang et al[170] recently used *Sca1*-CreER mice to show that SCA1+ ECs preferentially proliferate after injury. Furthermore, single cell RNA sequencing analysis revealed that these cells have increased angiogenic and proliferation potential. Multicolor lineage tracing is thus needed to confirm that these SCA1+ cells play a role in clonal regeneration. Although it was not investigated, further study into the angiogenic properties of these SCA1+ cells in murine models is necessary given that ECs clonally expand during angiogenesis after ischemia as well.

NEOVASCULARIZATION. Neovascularization describes the overall process of blood vessel formation.[171] Angiogenesis is a subset of this, whereby new blood vessels grow from the existing vasculature.[172] During this process, the existing vascular network is destabilized, which allows for the proliferation and migration of pioneer ECs. Signaling molecules such as vascular endothelial growth factor (VEGF) play crucial roles in regulating angiogenesis.[172] Mural cells (SMCs and pericytes) are also recruited to stabilize the newly formed blood vessels, influencing EC growth in a paracrine manner.[172,173]

EC clonality in ischemia induced neovascularization. Ischemia is a well-known complication of atherosclerosis, and angiogenesis plays an essential role in the restoration of blood flow to these affected tissues. Hypoxia initiates the secretion of pro-angiogenic growth factors via the activation of the hypoxia inducible factors (HIF-1).[174,175] Manavski et al[22] provided novel insights into the clonal expansion of ECs by comparing developmental vascularization with pathological vascularization during ischemia. The investigators studied angiogenesis in multiple ischemic diseases, including retinopathy of prematurity, myocardial infarction, and hindlimb ischemia. Although polyclonal expansion of ECs was observed in healthy tissues, all ischemic vascular beds manifested oligoclonal EC expansion.[22] Anti-vascular endothelial growth factor receptor 2 (anti-VEGFR2) antibodies were injected into the hindlimb ischemia model to investigate the possible mechanisms underlying this hypoxia-driven clonal expansion. This reduced the clonal proliferation of a single progenitor.[22] However, care must be taken when considering VEGF signaling as a clonal determinant because VEGF is crucial for

angiogenesis and the observation of reduced clonal expansion is likely secondary to the change in angiogenic architecture.

Furthermore, transcriptome analysis of clonal ECs within the heart post-myocardial infarction identified the upregulation of genes involved in EndoMT, which corresponds with previous reports that hypoxia drives EndoMT.[22,176] Although another recent study has corroborated these observations regarding the clonal expansion of ECs during ischemia, they do not provide evidence for a transition of ECs to mesenchymal lineages.[21,177] Tombor et al[177] recently suggested that ECs only transiently acquire a mesenchymal state. Consistent with Li et al[21], mesenchymal marker gene expression in ECs after myocardial infarction was only modestly elevated and was lower compared to the expression observed in mesenchymal cells, suggesting that ECs may not fully transition to mesenchymal lineages. Although the functional consequences of this transient and incomplete mesenchymal transition are still unclear, inhibition of TGFβ signaling (a known driver of EndoMT) reduced clonal expansion, suggesting that partial mesenchymal transitioning may contribute to clonal expansion.[177] This may be in line with the need of ECs to reduce the tight EC-EC connection, allowing them to migrate into the tissue to clonally expand. But this still raises the question of whether all ECs have the same capacity to undergo EndoMT, or whether this is restricted to clonal ECs. Overall, these results only serve to highlight how little we still know regarding EndoMT.[178]

Taken together, these results suggest that the clonal expansion of a pre-existing progenitor-like population of ECs may be responsible for the formation of new blood vessels during ischemia. Although these clonality studies did not try to characterize the progenitor-like EC population, there are reports that CD117+ ECs may be responsible for angiogenesis in adults.[179] In the context of myocardial infarction, the importance of these resident ECs in overall tissue repair may be a contributing factor to explain why BM stem cell therapy has not been as promising as first envisioned in clinical trials.[180] Furthermore, these findings indicate that future therapies should focus on these special clone-forming ECs to enhance angiogenesis for the treatment of ischemia. Although multiple clinical trials have investigated VEGF treatment for revascularization, current results are far from promising, highlighting a need for further understanding of this clonally driven process.[181] Given that these progenitor ECs may adopt the properties of other cell types (EndoMT and perhaps other unknown

phenotypic changes), further study is also needed into these various phenotypes and their underlying driving factors.

EC clonality in tumor-induced neovascularization. Hypoxia-driven angiogenesis not only takes place after ischemia, but also in tumor development. The hypoxic microenvironment stimulates angiogenesis to provide malignant cells with the nutrients they need for uncontrolled growth.[182] Corey et al[23] induced melanoma formation in rainbow lineage tracing mice, which readily formed new blood vessels. Twenty-eight days post tumor cell inoculation, they observed large regions of mono/oligoclonal ECs in the new vasculature. Polyclonality was observed at 14 days, which corresponds with findings of clone size expansion over time in the ischemic heart.[22,23] These results suggest that although many ECs may respond to angiogenic cues, a subset of these have properties that allow them to dominate. Transcriptomic profiling of the initial polyclonal ECs and the late mono-/oligoclonal ECs showed that the "late-stage" ECs have lower expression of numerous adhesion molecules and lymphocyte chemokines.[23] Furthermore, there was an upregulation of angiogenic ligands and receptors, which may explain their preferred selection. Understanding the complex cellular behavior involved in tumor vascularization is extremely important given that angiogenesis inhibitors have clinical relevance.[183]

EC clonality in cerebral cavernous malformations. CCMs are characterized by the growth and aggregation of abnormal blood vessels within the central nervous system. In these cavernoma lesions, mural cells are often absent, which can result in hemorrhaging and seizures.[184,185] Loss of function mutations in *Ccm1*, *Ccm2,* and *Ccm3* have been implicated in disease pathogenesis.[186-190] CCM1 appears to play a role in EC junction stability, as well as regulate EC proliferation and angiogenesis via the Notch signaling pathway.[191-194] CCM2 can bind with CCM1 and the kinase MEKK3 to regulate KLF2 and KLF4 expression, which will be briefly discussed shortly.[195] CCM3 may be able to also bind with CCM2 to prevent degradation of both proteins, and is thought to be crucial for angiogenesis.[196]

Two recent studies using EC-specific inducible *Ccm3* knockout confetti reporter mice have shown the clonal expansion of ECs during lesion formation.[24,25] Although smaller lesions exhibited monoclonality, larger lesions were polyclonal. Using a low dose of tamoxifen, the investigators note that large lesions also contained unmarked (confetti⁻) ECs (CCM3⁺).[25] Malinverno et al[25] elegantly show that mutations within a protein C receptor⁺ (Procr⁺) endothelial progenitor population are responsible for clonal expansion during the early stages of lesion formation, and that these Procr⁺ CCM3⁻ ECs recruit nonmutant ECs during later lesion growth. These observations further highlight the importance of clonal analysis at different timepoints during disease progression. In this context, if we know that mutant and wild-type ECs contribute to lesion development, this time-dependent clonal expansion suggests that we should focus on targeting mutant progenitors before they have time to recruit nonprimed cells. Although Procr has been used to mark a subset of ECs that exhibit more stem-like properties, Procr and CD117 (mentioned previously) marked distinct subpopulations of ECs.[197] This raises further questions regarding the heterogeneity of ECs (and perhaps other cell types) within the adult vasculature. Do multiple progenitor-like populations exist within each cell type? And, if so, do each of these subpopulations have properties that allow them to better respond to different conditions?

The initially selected CCM3⁻ ECs lining these lesions underwent EndoMT and also induced EndoMT in later recruited CCM3⁺ ECs in a paracrine manner.[25] The large number of phenotypically plastic ECs during lesion formation correspond with previous reports that KLF4 is crucial for pathogenesis.[198] In the context of CCMs, EndoMT has been considered detrimental for disease progression.[199] These observations pose further questions regarding the plasticity of ECs and their relationship with clonal expansion. Given that it appears that both clonal and nonclonal ECs can undergo EndoMT, do both subpopulations undergo EndoMT to the same extent? This can be considered, given that CCM1⁻ ECs respond more to TGF-β stimulation than wild-type ECs.[199] If some ECs are more progenitor-like, then it may be hypothesized that these cells are more plastic and may express higher levels of EndoMT markers. This distinction is important and should be further investigated given the apparent duality of the effects of EndoMT in different vascular diseases.

On a slightly separate note, the ability for primed ECs to induce the recruitment of wild-type ECs has interesting consequences for the observation that polyclonal expansion of SMCs can be induced in atherosclerosis.[8,25,91] It has been shown that paracrine signaling from inflammatory macrophages may be recruiting multiple SMCs from the medial wall.[8] However, if we consider recent results in CCMs, this raises another possibility that primed SMCs are the first to respond, and in the anti-CD47 antibody/*Itgb3*⁻/⁻ environment recruit regular SMCs which

would lead to the polyclonal observations. Timepoint analysis of these polyclonal plaques is therefore required to investigate this interesting possibility. This line of thought highlights the idea that polyclonal expansion does not necessarily preclude the existence of a progenitor-like population.

CLONAL EXPANSION IS AN EMERGING TREND

When reconciling the results of both human and animal studies across all these numerous CVDs, it becomes apparent that the clonal nature of these vascular cells perhaps should not be considered as a disease-dependent phenomenon. SMC clonal expansion has been observed in atherosclerosis, PAH, aneurysm, and blood vessel injury. ECs have also shown degrees of clonal expansion during angiogenesis, blood vessel injury, PAH, and CCMs. This raises the question of how this clonal phenomenon occurs, as these vascular diseases will have a variety of complex microenvironments and signaling pathways. In the context of SMC clonality, the atherosclerotic plaque microenvironment is primarily driven by lipid accumulation and chronic inflammation.[71,200] Although inflammation will inherently be a factor in all cardiovascular diseases, hyperlipidemia does not play a role in pulmonary hypertension, which can be driven primarily by hypoxia.[15] Likewise, in the context of EC clonality, hypoxia drives angiogenesis in ischemic and tumorous tissue, but is not a major focus in endothelium regeneration.[19,21-23] Taken together, this suggests that clonal expansion may be regulated by some signaling pathways that are conserved across each diseased state, and/or by fundamental properties of the cells themselves.

These clonality studies suggest that there may be subsets of SMCs and ECs within the vascular population that are more progenitor-like or are more primed to respond during injury or disease (**Figure 3**). Whether this is a single progenitor-like population or there are multiple subpopulations that respond to different conditions remains unknown. If these cells exist, a major hurdle that still must be addressed is the identification of these responsive populations because there is no clear consensus of markers that should be used. Identifying these cells may be vital in targeting clonal cells for therapeutic intervention in the future.

Although clonal expansion seems to be a common theme among vascular disorders, many of these clones undergo further phenotypic modulation. Although clonal expansion may not be determined by the microenvironment of any disease, the subsequent cellular plasticity is very likely affected by the

differing microenvironments. For example, SMCs convert into a variety of lineages during atherosclerosis and AA, but they remain predominantly in a contractile state after vessel injury.[6,17,18,82,84,89,95] Likewise, ECs have been shown to convert into a variety of mesenchymal lineages.[25,140,199] Further investigation into these different phenotypic conversions and the driving forces behind these transitions is needed, especially because these transitions may be beneficial or detrimental to disease progression.

THERAPEUTIC APPLICATIONS

CVDs are the leading cause of death around the world and remain so despite rapid advances in science and medicine. Current approaches to treating these diseases are predominantly reactive, whereby therapeutic intervention is provided after the disease has significantly progressed. The future of medicine relies on a better understanding of disease pathology at a cellular level so that we can predict disease progression and intervene at appropriate times. We have discussed that SMCs, ECs, and macrophages display degrees of clonal expansion in numerous CVDs. After expansion, these clones often undergo phenotypic changes to a variety of beneficial and/or detrimental phenotypes. Thus, by identifying and targeting these clonal cells, we may be able to prevent these detrimental lineage conversions or control cellular plasticity towards helpful phenotypes. In some cases, it may be beneficial to suppress clonal expansion entirely, such as inhibiting proliferation of angiogenic clones in tumor vascularization. In other cases, we may want to promote clonal expansion, such as the beneficial angiogenesis that occurs in ischemic tissue. However, care must be taken because suppressing clonal expansion of one cell type may induce the recruitment of other cells in a compensatory form, as recently shown in atherogenesis.[92] Although our knowledge of clonal expansion in these vasculopathies is still in its infancy, better understanding the underlying mechanisms and how these cells interact with the diseased microenvironment and other nearby cells is needed.

There is newfound evidence showing that vascular cell clonality is influenced by some cardiovascular risk factors, which may suggest that there is a link between clonal composition and patient outcome. Mimicking the inflammatory milieu during aging and CHIP by transplanting aged and *Tet2*^{-/-} bone marrow into young atherogenic mice induced the polyclonal expansion of SMCs[201]. Lesional macrophages have shown an opposite trend of oligoclonal

FIGURE 3 SMCs and ECs Clonally Expand During Cardiovascular Disease
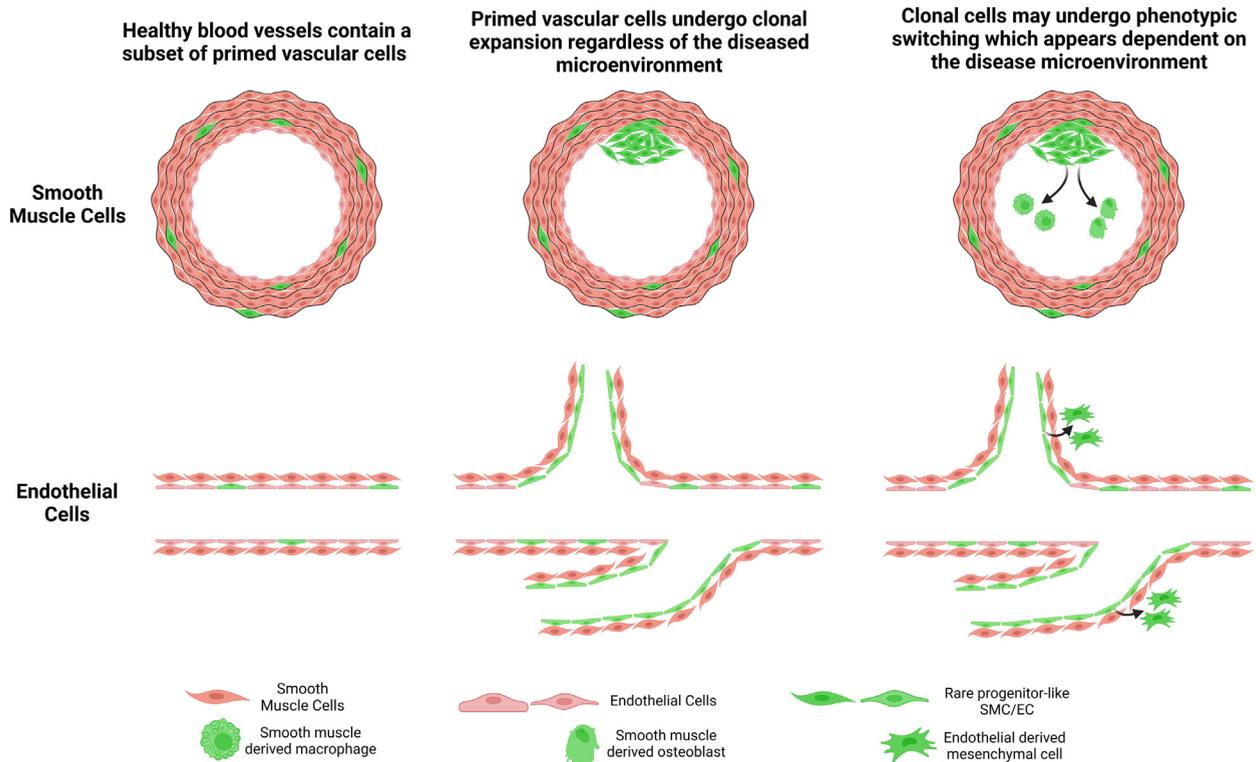
The healthy vasculature is composed of heterogenous populations of SMCs and ECs, some of which are primed to respond during injury or disease. These rare vascular cells clonally expand, which appears independent of the diseased microenvironment. In many diseases, these clones undergo phenotypic switching to disease-prone or disease-protective phenotypes. This subsequent phenotypic switching appears disease dependent. Abbreviations as in **Figure 1**.

proliferation.[13,201] It has been suggested that aging and CHIP reduce ITGB3 expression in macrophages, leading to elevated secretion of the proinflammatory cytokine tumor necrosis factor alpha (TNFα).[201] Elevated TNFα levels are observed in high-risk CVD patients and are thought to exacerbate atherosclerosis.[202,203] Anti-TNFα antibody treatment has been suggested to reduce the incidence of adverse cardiovascular events, favorably influence plaque morphology, and increase the preferential expansion of a single SMC.[201,204,205] However, key questions relating these observations of clonal expansion to patient outcome remain unanswered. Does the recruitment of multiple clones affect plaque stability, or is this relationship merely correlative? If there is a causative connection, do differing clones have differing capacities for phenotypic switching which may explain the changes in plaque vulnerability? Do other plaque-burdening inflammatory risk factors (eg, sleep and stress) also influence intraplaque vascular

cell clonal architecture?[206,207] Is TNFα the only clonality determining cytokine? It would be interesting to investigate the effect of IL-1β inhibition on SMC clonality, given that anti–IL-1β antibodies affect SMC plaque investment and have shown patient benefit in the CANTOS trial.[104,208]

To date, statins remain unsurpassed in reducing atherosclerotic CVD. It has been recently shown that although statins are most well known for their lipid-lowering effects, they also suppress CD47, a key regulator of efferocytosis.[209] Defective efferocytosis is a driver of plaque burden, and multiple preclinical models have shown plaque attenuation via CD47 inhibition[210-212] In a small clinical trial, magrolimab (anti-CD47 antibody) suppressed vascular inflammation, as assessed by ^{18}F-fluorodeoxyglucose uptake in the carotid arteries.[213] However, ^{18}F-fluorodeoxyglucose is a glucose analog and indicates metabolic activity rather than inflammation exclusively. Furthermore, how this influences atherosclerotic

FIGURE 4   Personalized Medicine May Rely on Predictive Exploitation of Vascular Cell Clonal Behavior

Clonal expansion and transdifferentiation of vascular cells contribute to disease pathogenesis, either playing protective or detrimental roles. Future therapies must beneficially alter clone behavior, but this remains mostly unexplored to date. Abbreviation as in **Figure 1**.

plaque behavior and adverse cardiovascular events in the long term remain unknown. As previously mentioned, anti-CD47 antibodies induce polyclonal SMC expansion without any change in plaque stabilization.[91] CD47 inhibition and TNFα inhibition have opposite effects on macrophage activation, which explains the clonal SMC observations, despite both showing benefit on plaque behavior. Thus, inflammatory status may also be a key regulator of clonal expansion, in addition to progenitor-predisposition. Do these clonal determinants influence vascular cell clonality in other CVDs? Are clonal determinants cell-type specific? This would be interesting to consider, given that it is well known that different vascular cell types respond differently to the same molecular pathways. For example, KLF4 deficiency in SMCs reduces adverse phenotypic switching and promotes plaque stability[80,82], whereas KLF4 knockdown in

myeloid cells promotes inflammation and plaque burden.[214] Ultimately little is known regarding clonal determinants, and further study is needed to determine how clonal expansion influences patient outcome.

Additionally, fundamental limitations with these analysis techniques and interspecies differences have hindered investigations into clonality and subsequent plasticity in humans. For example, some groups have proposed that SCA1 may mark these progenitor populations in murine models, and yet *Sca1* lacks a human homolog.[86,170] Albeit, rare responsive SMCs in the healthy vasculature are likely to exist in humans too, suggesting that the overall trends remain.[90] Better technology is needed to show and confirm the translatability of these mouse findings for therapeutic use in humans. Predictive exploitation of the clonal nature of vascular cells may

allow us to take a more proactive approach to cardiovascular disease management in the future (Figure 4).

CONCLUSIONS

Studying the clonal expansion of tumor cells has revealed seminal insights into cancer progression. Recent studies have brought to light the phenomenon of clonal expansion of several cell types and their impact on disease progression in several cardiovascular conditions. These findings reinforce this transpiring concept that vascular cells (eg, SMCs and ECs) clonally expand and may adopt various phenotypes across a broad spectrum of vascular diseases. In most of these disorders, the evidence suggests that there may be a subpopulation of cells that are more progenitor-like, which contribute to the lesion and have the capacity to persevere in the pathological milieu.

Although vascular cells follow a clonal pattern in most vascular diseases, the underlying cellular and molecular mechanisms of such clonal expansion in selected progenitors may vary depending on the disease and between cell types. Understanding the intricate unique mechanism in each disease and how to manipulate clonal expansion in each disease may enable better monitoring and/or treatment of disease progression. However, whether such inhibition promotes the proliferation of other less potential cells is important to consider. This line of thought arises if we consider that the clonally expanding progenitors not only have a proliferative advantage but may also have the capacity to inhibit expansion from other nearby cells. Ultimately, the key question that arises is whether clonal expansion plays protective or harmful roles in these cardiovascular diseases. Given that these clones adopt both atheroprotective and atheroprone phenotypes during atherogenesis, it is likely that future therapies should focus on intervention at appropriate times rather than preventing clonal expansion altogether.

Currently, we still only have a limited knowledge of the role of clonal expansion in cardiovascular disorders. We have yet to uncover the underlying signaling mechanisms involved and the fundamental driving forces behind many of the subsequent phenotypic switching that occurs. Increasing our understanding of vascular cell clonality will embolden its potential in therapeutics targeting vascular diseases.

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KEY WORDS cardiovascular diseases, clonal expansion, endothelial cells, macrophages, vascular smooth muscle cells

APPENDIX For a list of supplemental references for Figure 1, please see the online version of the article.