Review

Engineering the Vero Cell Lineage: Integrating Membrane, Cytoplasm, and Nucleus for a Programmable Vaccine Manufacturing Platform

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Article

Engineering the Vero Cell Lineage: Integrating Membrane, Cytoplasm, and Nucleus for a Programmable Vaccine Manufacturing Platform

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Abstract

Vero cells are an validated continuous substrate for human viral vaccine manufacturing. Decades of performance improvements have relied on extrinsic process optimization; however, intrinsic genomic instability, including segmental aneuploidy and dynamic chromatin rearrangements, are currently limiting the durability of engineered phenotypes under sustained viral burden and bioreactor stress. In this Perspective, we examine how a conceptual transition from empirical permissiveness toward genome-informed architectural redesign could substantially expand the manufacturing capabilities of the Vero platform. We organize the emerging landscape of Vero cell engineering across three interdependent functional layers: the membrane interface, where receptor redesign and suspension adaptation extend viral entry range and culture scalability; the cytoplasmic foundry, where metabolic flux management, ER stress buffering, and temporally controlled apoptosis modulation address production bottlenecks; and the nuclear blueprint, where epigenetic insulation and genomic precision engineering determine the long-term durability of all upstream gains. We further discuss how infection-responsive dynamic logic circuits and the systematic identification of Vero-specific genomic safe harbors could shift the paradigm from static trait installation toward a continuously programmable, conditionally responsive manufacturing architecture. Collectively, these advances suggest a pathway for transitioning the Vero lineage from a passive biological substrate into a programmable platform capable of meeting the accelerated timelines and distributed manufacturing imperatives of modern global health preparedness.

Keywords: vaccine; vero cells; cellular engineering; gene editing

1. Introduction

Established in 1962, Vero cells became a major continuous substrate for viral vaccine manufacturing as production moved away from variable primary monkey kidney cultures toward more standardized cell-based systems [1–4]. A defining biological feature of the lineage is a homozygous deletion affecting the type I interferon locus, which contributes to broad susceptibility to diverse viruses and has long supported its utility in virology and vaccine production [5,6]. At the same time, Vero cells have retained an unusually important regulatory position among continuous substrates, with an established safety record within defined passage limits and extensive use in the manufacture of vaccines such as inactivated poliovirus, rabies, and rotavirus vaccines [7–11].

However, the historical success of Vero cells has depended more on empirical permissiveness and process adaptation than on systematic redesign of the host cell itself [12–15]. Such distinction has become increasingly important, as contemporary vaccine and viral-vector manufacturing demands not only permissiveness, but also stable performance under high cell density, infection-associated metabolic burden, and industrial scale-up conditions [16–20]. Currently, the most accessible engineering layer is the membrane interface, where receptor availability and anchorage dependence directly influence host range, infection efficiency, and culture format [19,21–24]; yet improvements at

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this boundary propagate inward by reshaping intracellular stress and, ultimately, the durability of engineered phenotypes [25–28]. Meanwhile, Vero cells display genomic plasticity, including karyotypic variation and stress-associated chromatin remodeling, which may limit the long-term stability of inserted traits or regulatory circuits [29,30]. Therefore, we frame this Perspective around Vero cell engineering across the membrane interface, cytoplasmic foundry and the nuclear blueprint **(Figure 1**), hence discuss how dynamic control logic and genome-context-aware integration may help convert a historically permissive substrate into a more programmable manufacturing platform.

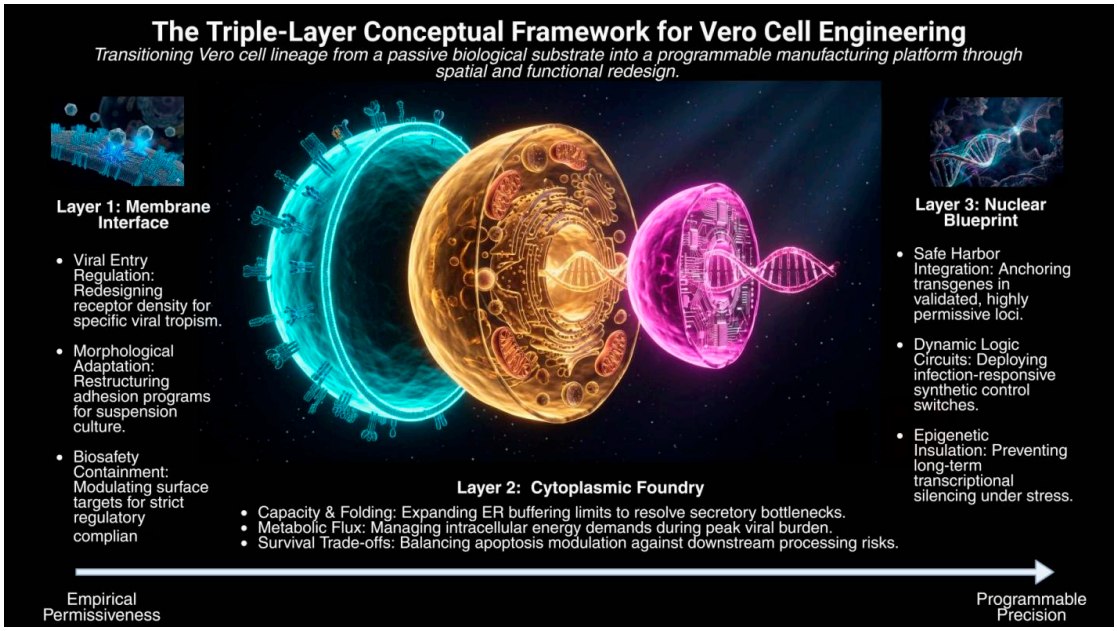

Figure 1. The Three-Layer Conceptual Framework for Vero Cell Engineering. The framework organizes engineering strategies across three interdependent functional layers. Left: The Membrane Interface governs viral entry efficiency and culture format scalability through receptor reprogramming (e.g., SLAM, SCARB2, ACE2/TMPRSS2) and suspension adaptation (e.g., anoikis resistance via CDH18/PTEN modulation). Middle: The Cytoplasmic Foundry addresses intracellular production bottlenecks by managing metabolic flux, buffering endoplasmic reticulum (ER) stress (e.g., XBP1s overexpression), and modulating apoptosis timing (e.g., BCL-XL, ISG15 deletion). Right: The Nuclear Blueprint determines the long-term durability of engineered phenotypes through epigenetic insulation (e.g., UCOE elements), precision editing (e.g., CRISPR/Cas9), and integration into validated genomic safe harbors (GSHs). Arrows indicate the propagation of stress and regulatory signals between layers, emphasizing that durable manufacturing robustness requires coordinated, context-aware engineering rather than isolated trait optimization.

2. Current Landscape and Methods of Genetic Engineering Vero Cells

2.1. Engineering the Membrane Interface: From Viral Entry to Suspension Adaptation

Currently, the cellular membrane is regarded as a programmable, intelligent interface through which extracellular materials, signaling cues, and viral particles are selectively negotiated. In wild-type Vero cells, receptor composition reflects species-specific evolutionary history rather than rational optimization for industrial vaccine manufacturing. A conceptual shift from passive permissiveness to active reprogramming has therefore emerged as the guiding principle of membrane interface engineering. Underneath such shift, membrane receptors are hence treated as modular components whose composition and density can be redesigned to align viral entry efficiency with defined bioprocessing constraints. To reflect in a more direct way, we created **Table 1** for comparing and contrasting.

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Membrane-located receptor transplantation has proven to be a direct and effective strategy for overcoming host-range limitations. Introduction of SLAM (CD150) enabled productive infection of measles virus [21] and canine distemper virus [31], while expression of KREMEN1 facilitated Coxsackievirus A6 entry [32].These cases demonstrate that supplementation of missing entry receptors achieves 10^6 - 10^8 $TCID_{50}$ / mL titers in specific Vero systems at proof-of-concept scale, though systematic industrial-scale validation remains needed, however such demonstration still avoided regulatory complexities associated with the application of more infectious, but tumor-derived cellines such as RD cells. For viruses relying predominantly on endocytic pathways, including EV71, kinetic bottlenecks have been relieved through increased membrane anchor density, as demonstrated by SCARB2 overexpression producing order-of-magnitude yield improvements [22]. Comparable gains in viral attachment and internalization have been observed following AXL [33] or TIM-1 [34] expression for filoviruses and ICAM1 augmentation for rhinovirus replication [35]. Receptor density amplification therefore represents a highly efficient lever for enhancing volumetric productivity. However, diminishing returns have been documented when single-receptor augmentation is applied to viruses with multi-factorial entry requirements or extremely low infectious inputs. In the case of SARS-CoV-2, efficient entry required coordinated expression of ACE2 and the priming protease TMPRSS2 rather than receptor amplification singly, resulting in a 1.6-fold overall improvement in viral isolation efficiency and a 2.6-fold increase in ultra-low-load clinical specimens [36]. Meanwhile, membrane programmability has been extended toward intrinsic biosafety. Disruption of CD155 rendered Vero cells non-permissive to poliovirus, thereby establishing a genetic barrier compatible with WHO GAPIII containment standards [37].

In parallel, morphological engineering has addressed the anchorage dependence intrinsic to Vero cells. Traditional suspension adaptation relied on prolonged environmental selection, including gradual suppression of cadherin-mediated aggregation [19]. Although empirically successful, such approaches provide limited control over phenotypic stability and genomic robustness. A transition toward genotype-driven suspension engineering has therefore been pursued. Anchorage-independent survival requires coordinated activation of anoikis-resistance pathways. IGF-1 has been shown to sustain viability through persistent activation of the PI3K/Akt axis [38]. Targeted downregulation of CDH18 weakens intercellular adhesion [39], while PTEN knockdown markedly reduces matrix attachment strength [40]. Through such interventions, homogeneous suspension cultures exceeding 10^7 cells per milliliter have been established [23]. Nevertheless, transcriptomic and metabolic analyses reveal an intrinsic vulnerability underlying suspension phenotypes. Adaptation to anchorage independence is accompanied by broad suppression of adhesion and extracellular matrix programs, including cadherins, tight junction components, and versican. Loss of physical anchoring cues imposes chronic cellular stress, necessitating compensatory activation of unfolded protein response pathways. Persistent upregulation of stress markers such as DDIT3 (CHOP) and CHAC1 indicates sustained endoplasmic reticulum strain [41], while downregulation of signaling receptors, including FGFR2, requires exogenous FGF2 supplementation to partially restore proliferative capacity [24]. Paradoxically, the genetic modifications enabling scalable suspension entrench the cells within a persistent stress-adaptation loop.

Collectively, membrane interface engineering, encompassing both receptor repertoire expansion and morphological reconfiguration, has yielded substantial gains in viral permissiveness and scalable culture formats. However, these interventions remain fundamentally static. While surface receptor overexpression alleviates entry barriers, it theoretically imposes an additional burden on limited ER folding capacity shared with viral glycoproteins [25], potentially precipitating premature secretory stress [26,42,43]. Suspension adaptation alleviates aggregation constraints yet might activates chronic stress-response pathways [44]. Therefore, principally, any membrane-level enhancement must be paired with cytoplasmic-nuclear safeguards to prevent stress-driven epigenetic silencing of engineered traits [27,28]. Long-term industrial robustness therefore depends on resolving the intracellular and genomic cascades inevitably triggered by boundary-level reprogramming [45].

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2.2. The Cytoplasmic Foundry: Capacity, Stress Handling, and Survival Trade-Offs

Post-fusion viral replication imposes coordinated demands on intracellular trafficking, metabolic flux [46], protein folding [13], and survival control [44], suggesting the cytoplasm often emerges as a central bottleneck in Vero manufacturing contexts. Under acute infection, Vero cells exhibit increased ATP consumption [47], substantial lactate accumulation [48], and pronounced ER stress [42], indicating that intracellular capacity frequently limits volumetric output. Expanding ER capacity via XBP1s overexpression enhances glycoprotein secretion under high-load conditions [49]. Anti-apoptotic reinforcement via BCL-XL extends productive lifespan [50], validated in Vero systems where CRISPR-mediated ISG15 deletion increased influenza and rVSV yields 70- to 87-fold [51]. Meanwhile, dual IFNG/IFNGR1 knockout enhanced replication of interferon-sensitive viruses by up to 3-fold [52], as well as UPR axis modulation represents a rational strategy to resolve folding bottlenecks under industrial MOI [24].

On the other hand, constitutive cytoplasmic manipulation appears to yield conditional gains. While BCL-XL overexpression extends replication windows, unresolved ER stress could potentially compromise assembly fidelity. Under sustained burden, apoptosis-resistant but metabolically strained cells have been observed in other systems to accumulate defective interfering particles and incomplete virions [53,54]. In parallel, apoptosis blockade is known to occasionally redirect death toward necrotic pathways [55], which could in theory release host cell proteins and genomic DNA directly into the bioreactor [56] and thereby increase downstream processing burden. Although these observations rely largely on cross-system extrapolation rather than direct industrial Vero validation, they suggest that apoptosis suppression without upstream stress mitigation carries the risk of displacing the bottleneck from replication kinetics to downstream processing.

Therefore, we propose that relying solely on cytoplasmic reinforcement may represent a capacity-limited strategy rather than a comprehensive structural solution. Static survival edits can transiently elevate output, but they do not inherently reconcile productivity with biosafety or eliminate cumulative transcriptional drift. At the same time, durable apoptosis resistance inevitably raises tumorigenicity concerns within the strict regulatory framework of vaccine substrates. Industrial robustness is therefore likely to depend upon temporally controlled, genomically anchored interventions that can coordinate survival, folding capacity, and replication fidelity within a stable nuclear architecture.

2.3. The Nuclear Blueprint: Genomic Stability and Precision Engineering

As Vero cell engineering advances from permissiveness optimization toward durable manufacturability [57], the nucleus becomes the decisive determinant of phenotypic persistence [58], population uniformity [59], and long-term passaging stability [60]. Nuclear engineering extends beyond simple gene disruption; it encompasses rational optimization of promoters, enhancers, codon architecture, chromatin-opening elements, and epigenetic insulators that collectively define transcriptional topology under stress. Whereas membrane and cytoplasmic interventions function as execution layers modulating entry, folding, and survival kinetics, while nuclear modifications restructure the transcriptional command architecture [61,62]. These changes govern how engineered traits are inherited, buffered, or progressively attenuated under sustained viral burden and bioreactor stress [51]. Consequently, genomic location, chromatin accessibility, and epigenetic landscape delimit the permissible boundaries of phenotypic stability, making long-term robustness therefore inseparable from genomic context.

Targeted nuclear perturbations in Vero cells illustrate how redesign at the transcriptional command layer can measurably reshape viral productivity. CRISPR/Cas-mediated knockout of nuclear regulators such as EMX2 has been reported to markedly enhance rotavirus replication and vaccine antigen yield, with homozygous deletions exhibiting superior performance relative to heterozygous counterparts [9]. Parallel strategies address transcriptional durability rather than pathway removal. Incorporation of chromatin-opening elements such as miniUCOE upstream of EF1α or CMV promoters has been shown in Vero-like production systems to reduce CpG

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methylation-associated silencing and sustain transgene expression across serial passages despite progressive heterochromatinization [63]. Similar durability-oriented designs, including modulation of p53/ATM/ATR signaling axes and editing of intrinsic restriction factors [64–66], demonstrate that nuclear tools, such as CRISPR [9], RNAi-derived persistent knockdowns [67], or recombinase-based targeted insertions [68], can install phenotypes that are initially robust at the DNA level and functionally advantageous during early production phases.

However, early-passage success frequently conceals a deeper instability. In Vero cells, stable sequence integration does not ensure sustained transcriptional output. Over extended passaging, engineered lines commonly exhibit expression drift , mosaicism, and clone-to-clone heterogeneity [13,29,51], phenomena rarely attributable to physical loss of edited loci. Translation of localized nuclear edits into durable industrial phenotypes is further constrained by the intrinsic genomic plasticity of Vero cells. Extended passaging repeatedly reveals expression drift and phenotypic heterogeneity, conclusions largely extrapolated from limited-scale or non-Vero model systems, phenomena that are rarely attributable to physical loss of the engineered sequence itself [69,70]. Instead, sustained viral amplification and bioreactor-associated stress may induce global chromatin remodeling [66,71], redistribution of histone modifications [66], and widespread transcriptional repression [65]. DNA methylation, heterochromatin spreading, and promoter-independent silencing repeatedly emerge as dominant mechanisms underlying long-term transgene attenuation, demonstrating that reliance on strong constitutive promoters is insufficient to secure sustained output [69,70,72]. Collectively, these findings indicate that collaboration with epigenetic context, rather than promoter strength in isolation, ultimately dictates expression longevity.

Superimposed upon these epigenetic constraints, higher-order genome organization exerts a decisive influence on engineering outcomes. Vero cells exhibit karyotypic instability, segmental aneuploidy, and evolving nuclear topology during serial passage, continuously reshaping regulatory neighborhoods [13,29]. Randomly integrated loci may become repositioned relative to Topologically Associating Domains (TADs), decoupled from enhancer networks, or exposed to repressive compartments as nuclear architecture remodels [30,73,74]. Such structural plasticity, while biologically adaptive, directly undermines batch-to-batch reproducibility and amplifies regulatory uncertainty in large-scale manufacturing.

Together, these dynamics suggest that Vero engineering might no longer proceed as isolated trait optimization. Membrane and cytoplasmic interventions resolve immediate constraints, yet the nucleus functions as the integrative command layer determining whether engineered gains persist under multidimensional stress. Advancement toward a programmable Vero platform requires coordinated strategies stabilizing genomic context, enforcing spatial insulation, and enabling dynamic control.

3. Toward a Fully Programmable and Scalable Vero Cell Platform

3.1. Engineering Robustness Under Multidimensional Stress and Regulatory Constraints

In industrial biomanufacturing, cellular robustness is not ordinarily treated as an intrinsic property, but is more often established through deliberate engineering. Chinese Hamster Ovary cells provide the clearest precedent. In that system, metabolic overflow has been reduced, apoptosis has been restrained, and culture longevity has been extended, with corresponding gains in viable cell density, process duration, and product output under fed-batch and perfusion conditions [75,76]. Such gains were not obtained merely by accelerating biosynthesis, but by preserving functional stability under prolonged metabolic and oxidative stress, as a result of which the cells were progressively converted into robust biological factories [77].

Even in the CHO, however, robustness engineering has not been unconstrained [78]. Anti-apoptotic interventions can improve manufacturing performance, yet they also alter growth control, death commitment, and long-term population behavior [79,80]. For such reasons, current strategies are generally interpreted within the comparatively permissive framework of recombinant protein

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production rather than within the tighter safety logic that governs vaccine substrates. The CHO literature therefore illustrates not only that stress can be engineered away, but also that the acceptability of doing so depends strongly on the product class and the regulatory context [80,81]. Vero cells, however, occupy a markedly different position. Their industrial value was established primarily through viral permissiveness, broad regulatory familiarity, and suitability for human vaccine manufacture, rather than through sustained platform optimization for stress tolerance, clonal uniformity, or scalable robustness [3,82,83]. Accordingly, the crucial challenge in Vero engineering has not been whether more survival, more biomass, and more persistence would be useful, but whether such traits can be introduced without materially altering the safety identity of the substrate.

Some progress has certainly been made, although mostly at the level of process adaptation rather than deep cellular redesign [16]. Microcarrier systems [84], fixed-bed bioreactors [8], serum-free media [85], perfusion strategies [86], and more recently suspension-adapted Vero platforms [17] have each been used to reduce scale-up burden, increase cell density, or improve volumetric productivity. Suspension Vero culture is particularly attractive because it simplifies manufacturing and can improve productivity when infection is performed at higher cell density, especially under perfusion-supported conditions [18,87]. Indeed, recent comprehensive characterization of a suspension Vero line demonstrated viabilities and densities compatible with bioreactor scaling, yet transcriptomic profiling revealed that this morphological adaptation requires broad suppression of adhesion programs alongside a persistent, compensatory upregulation of ER stress markers such as DDIT3/CHOP, locking the cells into a chronic stress-adaptation loop [88]. Yet these advances remain partially analogous to what has been achieved in CHO, because the environment surrounding the cell is more often improved than the cell itself, as shear can be mitigated, oxygen transfer can be managed, and inhibitory metabolites can be diluted or removed, while the underlying liabilities of the host remain largely intact [16]. In agitated systems, Vero cells are still vulnerable to hydrodynamic stress associated with stirring and sparging, whereas in high-density cultures oxygen limitation, nutrient heterogeneity, lactate accumulation, and ammonia buildup continue to narrow the physiological margin for productive infection [20,89]. Meanwhile, during the replication phase, viral production appropriates host biosynthetic capacity and progressively depletes essential cellular resources, after which cell death becomes structurally embedded in the process itself [90]. Recent multi-omics evidence further confirms that such viral burden does not merely deplete metabolic reserves, but induces large-scale chromatin remodeling and persistent transcriptional reprogramming that fundamentally reshape the regulatory landscape of the host genome [30].

A similar limitation has been observed when more direct engineering has been attempted. Productivity-enhancing knockdown or knockout strategies have been reported in small-scale studies, suggesting that Vero may in principle be amenable to rational host engineering [12,91]. This approach has recently advanced to genome-wide CRISPR/Cas9 loss-of-function screens that systemically identify novel pro- and anti-viral host factors (e.g., SERPINB1, TMEM236) [92], alongside targeted interventions such as fibronectin knockout that directly alleviate host-cell protein burden during downstream purification [93]. However, gains have proved difficult to stabilize across Vero sublines [67], and stress tolerance appears limited by a broader mismatch among genome annotation, lineage heterogeneity, and long-term stability [16]. Regulatory concerns become sharper when CHO-like solutions are considered more seriously, since permanent attenuation of apoptosis or constitutive enhancement of survival signaling in a vaccine substrate is more likely to be evaluated through the lens of tumorigenicity, genomic instability, and cell-substrate qualification [81,83]. The presence of endogenous simian retroviral sequences in Vero genomes further reinforces that caution [94], , while high-throughput sequencing has been advanced as an important tool for adventitious virus detection in biological products [95]. Consequently, any modified Vero line would require comprehensive adventitious-agent screening, substantially increasing the Chemistry, Manufacturing, and Controls burden associated with substrate qualification. The principal barrier is therefore not merely that multiple liabilities coexist, but that the most intuitive routes to robustness are either operational

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rather than intrinsic, or biologically plausible yet regulatorily difficult to defend, thereby shifting the center of gravity toward programmable intracellular control.

3.2. From Static Optimization to Dynamic Control: Engineering Conditional Logic

(Note: The engineering strategies proposed below represent testable hypotheses drawn from broader synthetic biology precedents, providing a conceptual framework for future validation in Vero cells.)

The stringent regulatory barriers and multidimensional stresses outlined previously suggest that future Vero cell engineering will likely need to move beyond purely static genomic interventions. In conventional recombinant protein platforms, constitutive overexpression, inducible-expression platforms, and durable attenuation of cell-death pathways have all been actively developed to sustain output and process stability, including cumate- and Tet-based control systems in CHO cells and explicit anti-apoptosis engineering strategies for improved perfusion performance [77,96,97]. However, in continuous cell substrates for vaccine manufacturing, the same logic becomes more difficult to defend, because permanent enhancement of survival or impaired apoptotic commitment is more readily scrutinized through the lenses of tumorigenicity, genomic stability, and cell-substrate qualification [81,98]. Under such constraints, the solution may lie in the temporal dimension: not perpetual survival, but transient support delivered only when viral burden and ER stress become limiting. On the other hand, if such regulation is to remain practical at manufacturing scale, the trigger would ideally be endogenous and directly coupled to the viral life cycle rather than imposed by exogenous chemical induction [99].

Instead of simply relying on constitutive promoters, such strategies would need to leverage synthetic biology to create infection-responsive logic gates, especially because Vero cells carry a large homozygous deletion encompassing the type I interferon gene cluster, which already places antiviral signaling in an unusual genomic context [100,101]. A more programmable route would be to exploit protease-dependent synthetic switches, allowing virus-associated protease activity to cleave and release membrane-anchored synthetic transcription factors. Such feasibility is supported by engineered receptor platforms in mammalian cells, including SNIPR-related and other modular receptor systems, in which membrane-tethered modules are conditionally liberated and converted into transcriptional outputs once activating thresholds are reached [102,103]. Translated into Vero cells, similar design logic suggests a testable hypothesis:replication-associated protease activity might serve as the endogenous trigger that initiates nuclear circuit activation at the point of maximal production stress..

Upon viral infection reaching such a threshold, the liberated synthetic effectors could be designed not merely as simple on/off switches for transient anti-apoptotic buffering, but as programmable interfaces with the epigenetic and transcriptional architecture discussed previously. By coupling protease-sensitive modules to d*Cas*9-based transcriptional effectors such as VPR or KRAB, the host nucleus could in principle be reprogrammed in real time, extending the programmable activation framework already established in mammalian cells [104]. In such a cascade, peak viral replication could theoretically be leveraged to open selected chromatin regions at metabolic or UPR-buffering loci, including XBP1-linked stress-response nodes, while temporarily repressing competing host-defense programs [105]. Meanwhile, the broader design logic closely parallels recently described feedback-responsive XBP1s circuits that dynamically modulate the unfolded protein response without requiring constitutive activation [49]. At the same time, several implementation barriers remain substantial: protease specificity differs across viral families, large dCas9 cargos complicate delivery architecture, threshold calibration would likely need to be optimized for each virus-cell combination, and circuit performance would still need to remain stable through banking and passaging [106–109].

However, even low-level leakage during uninfected seed-train culture could theoretically alter baseline epigenetic state, making resting-state repression as important as induced-state performance for vaccine substrates. UCOE-based insulation may help stabilize expression, but it does not by itself eliminate basal activity, so conditional circuits will likely require particularly stringent off-state

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control to preserve seed-line purity [98,108,110–112]. Accordingly, the bottleneck shifts from circuit design to genomic placement, and a practical next step would be the identification of Vero-specific genomic safe harbors combined with insulated expression cassettes, much as safe-harbor mapping has already been pursued in CHO to identify chromatin regions with enhanced genetic and epigenetic stability [113,114].

3.3. Genomic Precision as the Integrative Bottleneck

The concept of Genomic Safe Harbor originally belonged to the language of gene therapy, where it denoted a locus able to support durable transgene expression without materially disturbing endogenous regulation, genome integrity, or cell phenotype [115]. Classic examples, including AAVS1 and ROSA26, became influential precisely because they combined expression competence with relative functional neutrality across repeated engineering studies [116]. However, as the concept expanded into industrial cell engineering, particularly within Chinese Hamster Ovary systems, the defining criteria has slightly shifted: a useful harbor is increasingly evaluated by whether it remains transcriptionally permissive, copy-number stable, and industrially productive under high-yield manufacturing conditions [113,117]. Hilliard and Lee [113] made such a conceptual transition unusually visible by defining CHO safe harbors through active and stable three-dimensional chromatin features, effectively reducing the practical search space to a fraction of the genome, while subsequent studies extended the same logic toward stable hotspot compendia and flexible landing-pad platforms for evaluating expression cassettes across multiple loci [118]. Consequently, safe-harbor discovery has begun to acquire cell-line-specific styles: human and stem-cell contexts still foreground insertional neutrality and oncogenic distance [115], whereas CHO increasingly foregrounds robust expression from loci that remain stable enough for process development [117].

On the other hand, Vero engineering appeared not to be following similar trajectory. The reason appears unlikely to be a simple delay; rather, a continuous vaccine substrate is judged under a much sharper regulatory lens than a purified-protein host, with explicit concern for tumorigenicity, genomic stability, and rigorous substrate qualification [98]. Furthermore, the Vero genome itself already exhibits pronounced karyotypic instability, subline divergence, aneuploidy, and structural rearrangement [101]. or the Vero platform, the central question is not merely where a construct can be expressed, but how to define a harbor safe enough to satisfy vaccine-substrate scrutiny and transcriptionally active enough to remain useful. Such a tension makes the direct transplantation of CHO hotspot logic inherently difficult, arguably explaining why the Vero lineage still lacks a mature safe-harbor paradigm of its own [117].

Despite the obvious advantages of targeted integration, several historical and biological constraints might elucidate why systematic safe-harbor discovery did not emerge earlier in Vero cells. Historically, the lineage was developed and optimized not as a platform for durable recombinant expression, but as a continuous vaccine cell substrate operated largely in adherent or microcarrier-based formats; accordingly, the dominant engineering objectives were viral permissiveness, cultivation scalability, and manufacturing robustness rather than the identification of chromosomal loci for predictable transgene deployment [15,16,24]. Such an architectural focus matters at a fundamental level. Systematic safe-harbor discovery becomes strategically central only when the long-term behavior of an inserted transgene is a primary product attribute. By contrast, the Vero lineage has traditionally been treated as a qualified biological substrate, where acceptability is judged primarily through empirical safety characterization and process control [98]. The underlying challenge is further complicated by the fact that Vero is not a genomically uniform entity: whole-genome and subline-resolved analyses have revealed copy-number variation and structural divergence across the lineage, weakening any simple assumption that a highly active locus identified in one background would be automatically portable across others [14,101]. From a contemporary perspective, an apparent paradox emerges: a cell line utilized for more than six decades has only relatively recently acquired foundational genomic resources at higher resolution, while much of the

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expanding omics literature remains organized around infection biology or subline characterization rather than around a unified engineering atlas [119,120].

By contrast, CHO has already moved toward epigenome-guided hotspot frameworks in which locus selection is treated as a formal engineering problem [117]. However, such an asymmetry between the two systems might now become less prohibitive. Not only have high-quality multi-omics determinations of Vero cells been frequently conducted in recent years [121], but genome sequence-based, prediction-guided artificial intelligence models, such as AlphaGenome, are beginning to make computational prioritization of candidate loci conceptually possible, although sequence-only inference remains limited when Vero subline chromatin topology is unstable [122]. Accordingly, topologically aware frameworks, including Enformer [123], Borzoi [124], and Orca [125], may be more informative for integrating Hi-C or ATAC-seq feedback into Vero-specific locus prioritization, particularly where long-range cis-regulatory interactions are likely to matter.

Yet the fact that Vero-specific safe-harbor discovery has become newly conceivable does not imply that it will be straightforward in practice. A first foreseeable translational challenge is that current sequence-to-function frameworks, including AlphaGenome and related methodological predecessors, have been trained primarily on human and murine regulatory data [122–124]. When such models are transferred to Vero cells, the problem is not merely one of species difference in the abstract, but of domain shift into a non-human primate cell system with documented subline-level genomic divergence [101,126]. Existing cross-species regulatory modeling work already suggests that while transferability can be substantial without being complete, genome-specific activity remains a real source of residual error [126]. It is therefore likely that useful Vero-specific prediction will depend less on direct transplantation of pre-trained models than on iterative refinement through experimentally grounded feedback. A second profound challenge is that safe-harbor suitability cannot be judged solely by the pre-integration state of an apparently favorable locus, because the insertion of a large regulatory cassette may itself reshape the local spatial environment. Therefore, early progress will probably require a calibration strategy in which prediction is repeatedly confronted with empirical manufacturing outcomes. To convert theoretical predictions into an executable engineering roadmap, we propose that Vero-specific safe-harbor identification should integrate predictive modeling with empirical validation across four fundamental design dimensions (**Figure 2**).

Ultimately, variables including boundary distance, local interaction topology, and chromatin openness are unlikely to function as fixed computational filters at the beginning. Rather, they will more plausibly emerge through repeated cycles of prediction, biological correction, and re-parameterization.

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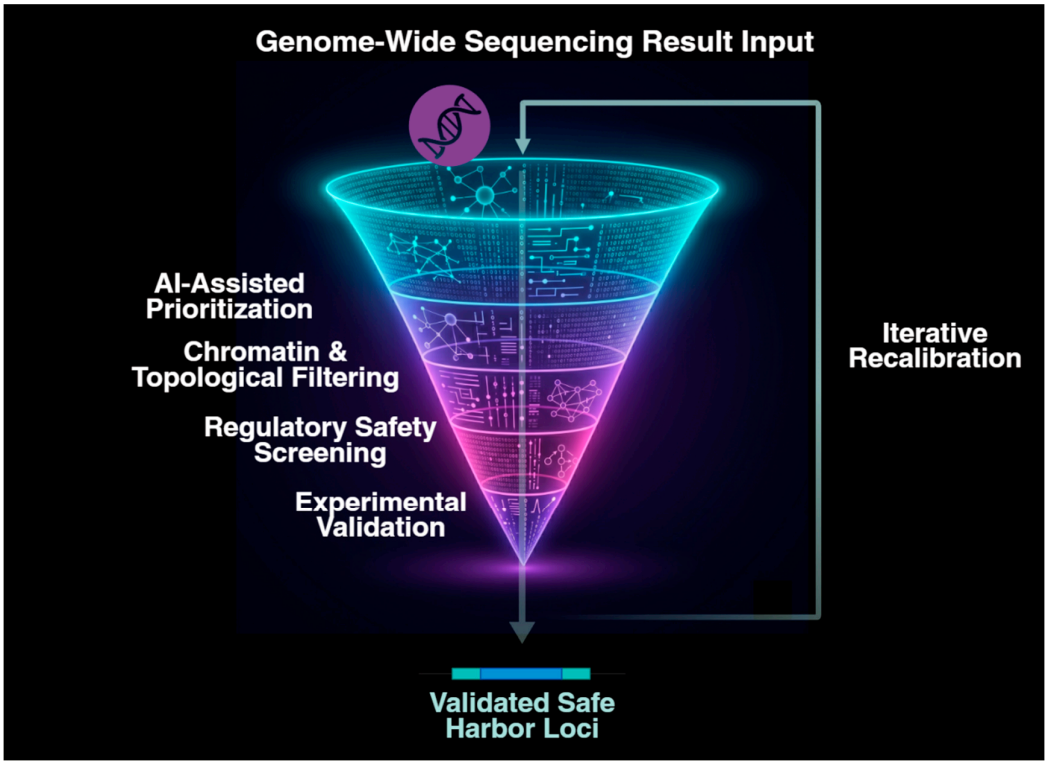

Figure 2. Proposed Genome-Wide, AI-assisted, quad-layered GSH Filter. Candidate loci derived from genome-wide sequencing are refined through AI-assisted prioritization, chromatin and topological filtering, regulatory safety screening, and experimental validation. The iterative structure reflects the need for feedback-guided recalibration when translating computational predictions into experimentally validated safe harbor loci.

3.4. From Cell Lines to Manufacturing Platforms: The Public Health Imperative

Integration of stress-tolerance engineering, conditional regulatory logic, and validated genomic safe harbors reframes Vero cells from permissive substrates into programmable manufacturing infrastructure. In mammalian cell-line development, random genomic integration is associated with positional effects, copy-number heterogeneity, and substantial clone-to-clone expression variability, all of which enlarge Chemistry, Manufacturing, and Controls (CMC) uncertainty [127,128]. For continuous cell substrates, such uncertainty is not merely technical, because Master Cell Bank qualification requires extensive characterization related to tumorigenicity, adventitious agents, and stability across passage history [10,129]. Regulatory burden therefore scales, at least in part, with the indeterminacy of the genomic backbone being advanced into manufacturing.

A standardized Vero chassis containing a validated genomic safe harbor linked to an RMCE-compatible landing pad would address that problem at its source. In CHO and other mammalian systems, site-specific integration and recombinase-mediated cassette exchange have been used to reduce clonal heterogeneity, preserve genomic context, and enable repeated cassette replacement without re-randomizing the insertion environment [127]. Under such a framework, regulatory uncertainty would not disappear, but it could shift away from repeated substrate re-establishment and toward payload-specific evaluation within a pre-characterized cellular backbone [128]. CMC development would thus become more platform-iterative and less substrate-reconstructive.

Such translational relevance is perhaps most tangible in two specific scenarios. First, highly pathogenic H5-subtype avian influenza strains, which impose compressed timelines and often attenuated replication kinetics in conventional substrates, may benefit from genomic safe harbor-anchored chassis whose transcriptional stability under accelerated passaging could reduce the cumulative genetic drift risk that currently complicates seed-strain qualification at pandemic scale

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[130]. Second, structurally complex antigen formats such as HCMV VLP candidates, whose assembly depends on coordinated multi-glycoprotein folding and stoichiometric co-expression, impose ER burdens that infection-responsive XBP1s-linked cytoplasmic buffering could theoretically help mitigate without constitutively altering baseline cellular homeostasis [131–133]. Meanwhile, the significance of such a conceptual transition extends beyond single-process optimization; it bears directly on manufacturing readiness during outbreak responses. Global initiatives, including frameworks analogous to CEPI's 100 Days Mission, increasingly emphasize the importance of scalable and distributed manufacturing capacity [134]. Such an objective becomes inherently difficult to realize when each production site must independently derive and revalidate product-specific producer clones, thereby multiplying Chemistry, Manufacturing, and Controls (CMC) uncertainties. A regulator-familiar, genomically standardized Vero platform could theoretically support a more decentralized manufacturing model. Under such a framework, distinct facilities would operate from fixed genomic coordinates and a shared safety profile, exchanging pathogen-specific payloads as needed. Consequently, the practical value of Vero safe harbors lies in converting a historically empirical substrate into a modular platform, yielding biological behavior that is highly portable, reproducible, and rapidly deployable for continuous vaccine production.

Table 1. Representative Genetic Engineering Strategies in Vero Cells and Their Associated Manufacturing Trade-offs. Interventions at the membrane, cytoplasmic, and nuclear layers frequently enhance specific viral permissiveness, production capacity, or transient transcriptional output. However, such static modifications often introduce secondary bioprocessing constraints, structural instabilities, or regulatory complexities.

Engineering Layer & Component	Biological Intervention	Primary Manufacturing Benefit	Inherent Trade-off / Implementation Barrier	Reference
Membrane Interface	SLAM (CD150) / KREMEN1 knock-in	Circumvents host-range limitations (e.g., Measles, Coxsackievirus) to enable productive infection without relying on tumor-derived cell lines.	Receptor amplification potentially imposes additional burden on limited endoplasmic reticulum (ER) folding capacity, precipitating premature secretory stress.	[21,32]
Membrane Interface	SCARB2 / AXL / TIM-1 overexpression	Relieves entry kinetic bottlenecks (e.g., for EV71, filoviruses), producing order-of-magnitude yield improvements.	Yields diminishing returns for viruses with multi-factorial entry requirements or extremely low infectious inputs (e.g., SARS-CoV-2 requiring coordinated ACE2/TMPRSS2).	[22,33-36]
Membrane Interface	CD155 disruption	Renders cells non-permissive to poliovirus, establishing intrinsic biosafety barriers compatible with WHO GAPIII containment standards.	Narrows the broad-spectrum permissiveness characteristic of the platform, requiring separate maintenance of distinct engineered sublines.	[37]

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Membrane Interface	CDH18 / PTEN downregulation & IGF-1 activation	Weakens intercellular adhesion and activates anoikis resistance, enabling high-density suspension cultivation.	Broad suppression of anchoring cues triggers compensatory, chronic ER stress-adaptation loops (e.g., DDIT3/CHOP upregulation), entrenching cells in persistent stress.	[38-41]
Cytoplasmic Foundry	XBP1s overexpression	Expands ER buffering capacity, significantly enhancing viral glycoprotein secretion under high-load conditions.	Constitutive stress-response activation globally alters host metabolic homeostasis, potentially disrupting baseline cellular functions prior to viral infection.	[49]
Cytoplasmic Foundry	BCL-XL overexpression	Delays apoptotic commitment, significantly extending the productive viral replication window.	Prolonged strain risks the accumulation of defective interfering particles and incomplete virions, while exacerbating tumorigenicity concerns for vaccine substrates.	[50]
Cytoplasmic Foundry	ISG15 deletion / IFNG–IFNGR1 knockout	Dramatically amplifies volumetric yields for highly interferon-sensitive viruses (e.g., influenza, rVSV) by removing residual host defense.	Apoptosis blockade without upstream stress mitigation may redirect death toward necrotic pathways, releasing host cell proteins/DNA and inflating downstream processing burdens.	[51]
Nuclear Blueprint	EMX2 knockout / Restriction factor editing	Remodels the transcriptional command layer to measurably reshape early-passage viral productivity.	Fails to ensure sustained transcriptional output; relies on physical locus disruption rather than enduring epigenetic collaboration.	[52]
Nuclear Blueprint	miniUCOE incorporation (Upstream of EF1α/CMV)	Reduces CpG methylation-associated silencing, sustaining transgene expression across serial passages.	Requires integration into randomly distributed loci, leaving circuits vulnerable to position effects, subline heterogeneity, and broader chromatin neighborhood remodeling.	[63]

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Nuclear Blueprint	Static Recombinase / RMCE integration	Enables targeted cassette exchange to reduce clone-to-clone heterogeneity and preserve genomic context.	Still limited by the intrinsic karyotypic instability of Vero cells if not anchored within explicitly validated, stress-resilient Genomic Safe Harbors (GSHs).	[127]

4. Discussion & Conclusions

Over more than six decades of use, Vero cells have been established as indispensable vaccine substrates largely through empirical permissiveness rather than rational architectural design. Yet permissiveness is not equivalent to industrial robustness: under extended passaging, high viral burden, oxidative stress, and bioreactor-associated shear, the durability of engineered phenotypes becomes increasingly constrained by epigenetic silencing, copy-number fluctuation, and structural genome plasticity. In this context, receptor optimization and cytoplasmic reinforcement can improve performance, but they remain execution-layer interventions whose long-term value is ultimately conditioned by nuclear integration context. The central implication of this review is therefore that durable Vero engineering cannot be achieved through isolated trait repair alone; it requires an integrative genomic framework in which conditional regulatory circuits and productivity-enhancing modules are anchored within validated Vero-specific genomic safe harbors capable of preserving transcriptional activity and regulatory insulation across manufacturing stress states.

The major challenge, however, is translational rather than merely conceptual. Although gene-therapy and CHO-based manufacturing studies have shown that safe harbors can be defined through exclusion criteria, spatial rules, and longitudinal transcriptional validation, those principles cannot be transferred directly into Vero cells without accounting for lineage-specific aneuploidy, structural divergence, and the stricter safety logic applied to continuous vaccine substrates. Accordingly, future progress will depend on combining Vero-centered multi-omics, iterative computational prediction, and targeted empirical validation to identify loci that are not only transcriptionally permissive, but also regulatorily defensible over industrially relevant passage histories. Such transition beyond cell-line engineering singly, as pandemic-response initiatives such as CEPI's 100 Days Mission depend on manufacturing platforms that are more standardized, comparable, and rapidly deployable than empirically derived clone-by-clone workflows can readily support. In that sense, the evolution of Vero cells from historically permissive hosts into programmable manufacturing platforms should be understood not as incremental optimization, but as a necessary modernization of the cellular infrastructure underlying future vaccine preparedness.

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