

Housekeeping Gene Selection in Mesenchymal Stem Cells: Challenges, Variability, and Standardization Strategies

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ABSTRACT

Housekeeping genes (HKGs) serve as essential internal controls for transcript normalization in quantitative gene expression analyses, predominantly reverse transcription quantitative real-time PCR (RT-qPCR) and RNA sequencing (RNA-seq). In mesenchymal stem/stromal cell (MSC) research, conventional HKGs—such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-actin (*ACTB*), 18S ribosomal RNA (18S rRNA), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*)—have historically been presumed to be constitutively and stably expressed across diverse experimental conditions. However, accumulating empirical evidence reveals that their transcript abundance fluctuates markedly according to tissue origin, donor demographics, in vitro passage number, culture confluency, differentiation status, and external microenvironmental stimuli. Such uncharacterized expression instability invalidates numerical normalization, distorts relative target quantification, and introduces substantial reproducibility challenges into both fundamental cell biology and translational MSC investigations. This comprehensive review synthesizes current insights into the biological mechanisms driving HKG transcriptional instability in MSCs across diverse experimental settings, including osteogenic, adipogenic, and chondrogenic differentiation, hypoxic culture, inflammatory priming, and mechanical stimulation. We critically compare standard algorithmic frameworks for reference gene selection—including geNorm, NormFinder, BestKeeper, the comparative ΔC_t method, and RefFinder—and highlight candidate reference genes displaying superior empirical stability (e.g., *TBP*, *RPL13A*, *YWHAZ*, and *PPIA*). Furthermore, we examine emerging paradigms, including the use of small non-coding RNAs for extracellular vesicle characterization, machine learning-based predictive modeling, and the formulation of standardized reference panels for clinical-grade MSC biomanufacturing under Good Manufacturing Practice (GMP) compliance. Finally, we formulate practical, evidence-based recommendations aligned with the updated Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE 2.0) guidelines to guide rigorous reference gene validation, ensuring the validity, reproducibility, and therapeutic translatability of MSC gene expression data.

Key words: Housekeeping genes, Reference gene selection, Gene expression normalization, Mesenchymal stem cells (MSCs), RT-qPCR, RNA-seq, Gene expression stability, Standardization strategies, MIQE 2.0

INTRODUCTION

Housekeeping genes (HKGs) serve as foundational internal standards for normalizing gene expression profiles in quantitative molecular profiling assays, most notably reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and RNA sequencing (RNA-seq)^{1,2}. Accurate transcript normalization remains indispensable for mitigating confounding technical variability introduced by fluctuations in initial RNA yield, RNA integrity, enzymatic reverse transcription efficiency, and exponential PCR amplification kinetics^{2,3}. Historically, classical reference transcripts—such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-actin (*ACTB*), 18S ribosomal

RNA (18S rRNA), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*)—were widely assumed to be constitutively and uniformly expressed across distinct cell lineages, leading to their routine application without prior experimental validation^{1,4}. Mesenchymal stem/stromal cells (MSCs) have emerged as an essential platform in regenerative medicine, immunomodulatory therapy, and tissue engineering, underpinning extensive preclinical investigations and clinical trials⁵. MSCs can be isolated from a diverse spectrum of anatomical sources, including bone marrow, adipose tissue, umbilical cord matrix, placenta, and other perinatal tissues, with each compartment imparting distinct biological, phenotypic, and transcriptomic properties⁵⁻⁹. This pronounced innate heterogeneity,

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compounded by the extensive in vitro ex vivo expansion required to generate clinically meaningful cell dosages, poses substantial hurdles for standard transcript normalization strategies^{10,11}.

A burgeoning body of empirical evidence underscores that the transcript abundance of conventional HKGs in MSCs is exquisitely sensitive to culture context. Factors including anatomical tissue derivation, inter-donor variability, cumulative passage number, cellular seeding density, multilineage differentiation commitment, ambient hypoxia, inflammatory activation, and mechanical loading have all been shown to disrupt HKG expression stability^{12,13}. Crucially, classical reference genes such as *GAPDH* and *ACTB* actively participate in core glycolytic and cytoskeletal networks, rendering their transcription highly vulnerable to common experimental perturbations¹⁴. Consequently, the uncritical adoption of single unvalidated HKGs in MSC research has frequently precipitated flawed normalization, compromised data reproducibility, and yielded contradictory biological interpretations¹⁵ (Figure 1).

The ramifications of reference gene instability are particularly acute in translational and clinical manufacturing settings. Clinical MSC therapies demand extensive ex vivo expansion to attain targeted therapeutic doses, an intervention inextricably linked to replicative senescence, profound cytoskeletal remodeling, and genome-wide transcriptomic rewiring¹⁶. Furthermore, the regulatory transition from fetal bovine serum (FBS)-supplemented culture media toward chemically defined serum-free (SFM) or xeno-free (XFM) formulations—mandated by Good Manufacturing Practice (GMP) compliance—imposes additional transcriptional shifts that profoundly alter baseline HKG stability¹³. In these bioprocessing environments, selecting an inappropriate reference gene can introduce false readouts in quantitative potency assays, distorting the perceived efficacy and release criteria of critical immunomodulatory and differentiation pathways^{13,16}.

To address these limitations, a variety of statistical and computational validation frameworks have been established, including geNorm, NormFinder, and BestKeeper, alongside integrated consensus tools such as RefFinder^{17,18}. Because reference gene stability is inherently context-dependent, no single universal HKG can perform reliably across all MSC experimental conditions¹³. Instead, genes participating in basal transcription initiation or proteostatic turnover—such as TATA-box binding pro-

tein (*TBP*), ribosomal protein L13a (*RPL13A*), peptidylprolyl isomerase A (*PPIA*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*)—frequently demonstrate superior stability within tailored biological contexts^{19–21}. In recent years, these accumulated insights have catalyzed a fundamental paradigm shift toward multi-gene normalization strategies and rigorous pre-validation, principles formally codified in the updated Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE 2.0) guidelines²².

This narrative review synthesizes contemporary experimental and computational findings examining HKG expression variability in MSCs across diverse biological and technical landscapes, including multilineage differentiation, hypoxia, inflammatory priming, mechanical stimulation, and GMP-compliant biomanufacturing. We critically evaluate established validation algorithms, explore emerging frontiers—such as RNA-seq-assisted candidate pre-selection, small non-coding RNA normalization, and machine learning-driven discovery models¹⁵—and provide a structured series of practical recommendations to maximize experimental validity, methodological reproducibility, and translational reliability in MSC gene expression profiling.

This narrative review was informed by a systematic literature search conducted across PubMed, Web of Science, and Google Scholar, capturing peer-reviewed studies published up to 2025. Search strings incorporated combinations of the following terms: "mesenchymal stem cells", "mesenchymal stromal cells", "housekeeping genes", "reference genes", "gene expression normalization", "RT-qPCR", "RNA-seq", "hypoxia", "multilineage differentiation", "inflammatory priming", "serum-free media", "xeno-free culture", and "GMP biomanufacturing". Articles evaluating reference gene stability in MSCs under defined biological or physical perturbations were critically appraised. Given the broad methodological diversity across MSC sources and experimental systems, findings were qualitatively synthesized and structured thematically by experimental context. Representative empirical studies were prioritized to illustrate recurrent patterns of transcript stability and instability rather than to provide an exhaustive enumeration.

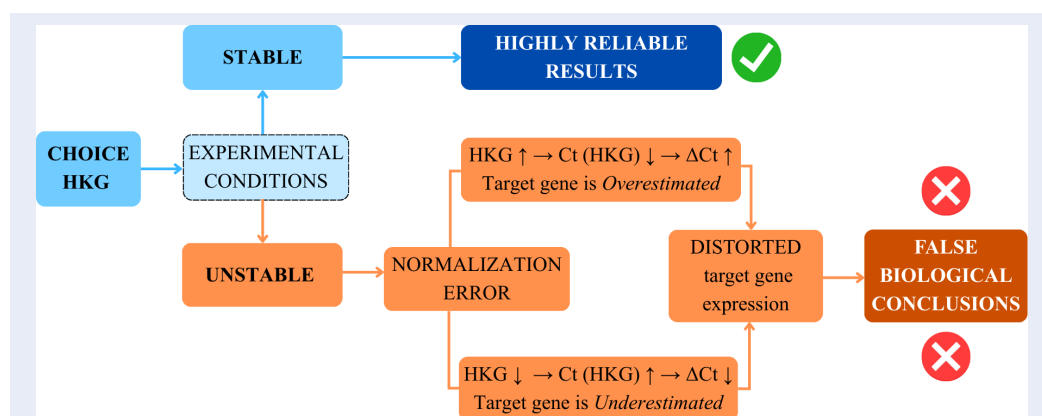


Figure 1: Multifactorial determinants of reference gene stability in mesenchymal stem/stromal cells (MSCs). This diagram illustrates the extrinsic and intrinsic variables that undermine the transcriptional stability of traditional housekeeping genes (HKGs) in mesenchymal stem/stromal cells (MSCs). The central core highlights the hallmark properties of MSCs—namely, their high cellular plasticity and context-responsive transcriptome—which inherently predispose candidate HKGs to transcriptional fluctuations and demonstrate that no universal reference gene exists. These perturbations are categorized into four major interconnected quadrants: **Biological Heterogeneity:** Variations driven by diverse anatomical tissue sources (e.g., BM-MSC, AD-MSC, UC-MSC), donor demographics (e.g., chronological age, biological sex, underlying clinical pathologies), and cellular expansion quality (e.g., cumulative in vitro passage number, seeding density, replicative senescence). **Functional Reprogramming:** Dynamic transcriptional shifts induced during trilineage commitment (osteogenic, adipogenic, and chondrogenic differentiation), inflammatory priming (e.g., licensing with IFN- γ , TNF- α , or IL-1 β), and mechanical cues (e.g., tensile stretching, shear stress, substrate stiffness, and 3D architectural constraints). **Experimental & Culture Conditions:** Microenvironmental influences originating from basal media formulations (e.g., fetal bovine serum [FBS] vs. serum-free [SFM], xeno-free [XFM], or human platelet lysate [hPL] supplementation) and specific physicochemical stress factors (e.g., oxidative stress, pericellular hypoxia [$<1\%$ O $_2$], and nutrient deprivation). **Analytical & Technical Factors:** Methodological and pre-analytical confounders that introduce technical variance, including RNA extraction purity, RNA integrity number (RIN), reverse transcription enzymatic efficiency, real-time PCR amplification efficiency, inter-instrument detection variance, and quantitative data analysis algorithms. **Abbreviations:** AD-MSC: adipose-derived mesenchymal stem cell; BM-MSC: bone marrow-derived mesenchymal stem cell; FBS: fetal bovine serum; HKG: housekeeping gene; hPL: human platelet lysate; IFN- γ : interferon gamma; PCR: polymerase chain reaction; SFM: serum-free medium; TNF- α : tumor necrosis factor alpha; UC-MSC: umbilical cord-derived mesenchymal stem cell; XFM: xeno-free medium.

CONCEPT OF HOUSEKEEPING GENES IN THE CONTEXT OF MESENCHYMAL STEM CELL BIOLOGY

Classical molecular biology traditionally defines housekeeping genes as an essential cohort of constitutively expressed genes required for basal cellular maintenance, encompassing central metabolic processing, cytoskeletal integrity, ribosome biogenesis, and basic transcriptional upkeep²³. Historically, these genes were assumed to exhibit uniform transcription across diverse cell lineages and physiological states, independent of external conditions²³. However, modern high-throughput transcriptomic profiling and rigorous empirical validation have revealed this paradigm to be fundamentally oversimplified. Even under baseline homeostatic conditions, conventional candidate HKGs dis-

play notable transcriptional fluctuations across distinct cell types, developmental stages, and microenvironmental niches, undermining their validity as invariant internal standards²³. Consequently, candidate reference genes must no longer be viewed as absolute constants, but rather as empirical candidates whose expression stability must be experimentally verified in each specific biological system¹⁰. This conceptual limitation is uniquely pronounced in MSCs. Beyond their characteristic trilineage differentiation capacity, MSCs possess exceptional phenotypic plasticity and environmental sensitivity. During osteogenic, adipogenic, and chondrogenic differentiation, MSCs undergo sweeping transcriptional reprogramming that reconfigures wide functional networks rather than merely altering isolated lineage markers^{24,25}. These lineage specifications are intrinsically coupled to extensive metabolic transitions, such as switching from aerobic gly-

colysis to mitochondrial oxidative phosphorylation (or vice versa), which causally drives the upregulation or downregulation of metabolic genes routinely employed as reference standards^{24,25}. Furthermore, culturing MSCs under physiological or pathophysiological hypoxia—frequently utilized to sustain stemness, promote expansion, or mimic the native in vivo niche—stabilizes hypoxia-inducible factor 1- α (HIF-1 α) and transactivates glycolytic target genes, triggering substantial expression shifts in metabolic HKGs such as *GAPDH* and *PGK1*²⁶.

In addition to metabolic rewiring, MSCs mounting immunomodulatory responses exhibit pronounced transcriptional plasticity. Exposure to proinflammatory cytokines, such as interferon- γ (IFN- γ) or tumor necrosis factor- α (TNF- α), initiates broad stress-responsive and immune-regulatory cascades^{27,28}. Concurrently, biophysical cues—such as substrate stiffness, cyclic tensile stretch, and three-dimensional (3D) scaffold architecture—activate cytoplasmic mechanotransduction pathways and induce extensive cytoskeletal remodeling^{28,29}. Because structural genes such as *ACTB* and *TUBA1B* are central components of the actin and microtubule machinery, their transcription fluctuates dynamically during physical perturbation. Moreover, baseline transcriptomic heterogeneity stemming from donor demographics, tissue origin, and isolation procedures introduces substantial inter-donor variability even prior to experimental stimulation²⁷.

Collectively, these unique attributes position MSCs as a biologically sensitive system in which subtle experimental perturbations elicit pronounced transcriptional responses. Secondary variables that exert minimal impact on immortalized cell lines—such as subculture passage number, cell confluency, pericellular oxygen tension, and basal medium formulation—profoundly modulate the MSC transcriptome^{10,27}. From a conceptual standpoint, reference gene stability in MSC models cannot be assumed a priori but must be rigorously demonstrated within each designated experimental framework.

Mechanistic insight into reference gene instability can be obtained by examining the functional roles of candidate genes within MSC biology. Instability is rarely a stochastic phenomenon; rather, it directly mirrors the functional involvement of candidate genes in the cellular pathways undergoing experimental remodeling^{26,30}. Cytoskeletal genes (e.g., *ACTB*, *TUBA1B*) fluctuate extensively because actin dynamics and cell morphology are

tightly linked to lineage commitment and mechanotransduction^{28,29}. Ribosomal protein genes (e.g., *RPL13A*, *RPLP0*) frequently exhibit robust stability under moderate conditions but can drift significantly in response to altered proliferation rates, fluctuating translational demand, or metabolic stress³¹. Transcripts involved in basal transcriptional initiation (e.g., *TBP*, *POLR2A*) generally display superior stability because they regulate global RNA polymerase II assembly rather than stimulus-specific responses^{4,30}. Similarly, molecular chaperones and proteostatic regulators (e.g., *PPIA*, *HSP90AA1*, *UBC*) often maintain stable expression during standard expansion and mild physical stimulation, although they may fluctuate under severe cellular stress, proteasomal saturation, or replicative senescence^{32,33}.

This biological interconnectedness introduces a major hazard in reference gene selection: functional co-regulation. Evaluating multiple candidate genes belonging to identical metabolic or structural pathways can yield artificially low pairwise variation scores, generating misleadingly stable normalization factors that merely mirror shared biological regulation. As detailed in Table 1, candidate panels must deliberately integrate genes from disparate, non-overlapping functional categories. Given the intrinsic plasticity of MSCs, an ideal reference gene represents an operational definition rather than a universal transcript. Endogenous reference controls should possess low intra- and inter-group transcriptional variance, moderate baseline expression (avoiding extreme saturation or low-abundance dropout), consistent amplification efficiency (90–110%), and no mechanistic involvement in the experimental conditions under study. Because no single gene completely fulfills these criteria across all MSC platforms, contemporary consensus strongly advocates a multi-gene normalization paradigm: screening a diverse set of candidate genes and applying the geometric mean of two or three validated, stably expressed transcripts (e.g., *TBP*, *RPL13A*, *PPIA*, *YWHAZ*) (Table 1).

FACTORS INFLUENCING THE VARIABILITY OF HOUSEKEEPING GENES IN MSCS

Culture Environment and Bioprocessing Conditions

Extrinsic culture parameters exert a profound influence on MSC gene expression profiles⁷. Because MSCs dynamically adapt to their in vitro niche,

Table 1: Functional classification, biological roles, and context-dependent stability of candidate reference genes in mesenchymal stem cells.

Functional Category	Candidate Gene Symbols	Biological Roles, Stability Factors, and Contextual Risk Considerations
Cytoskeletal	<i>ACTB</i> , <i>TUBA1B</i>	Encodes core structural microfilaments and microtubules. Heavily altered during osteogenic/adipogenic differentiation, mechanical loading, cell passaging, and donor variation. Frequently identified as the least stable reference gene in MSCs (poor geNorm ranking; highly unstable under extensive expansion or inflammatory priming) ¹² .
Metabolic	<i>GAPDH</i> , <i>PGK1</i>	Directly involved in the glycolytic energy cascade. Strongly influenced by pericellular hypoxia, glucose fluctuations, inflammatory activation, and nutrient starvation. Markedly unstable in adult MSCs (BMSCs and ASCs), although relatively more stable in neonatal hAMSCs; displays significant expression shifts during early tenogenic commitment ³⁰ .
Ribosomal	<i>RPL13A</i> , <i>RPLP0</i>	Structural constituents of the 60S ribosomal subunit mediating protein synthesis. Generally stable across multiple standard culture conditions; achieves optimal stability when combined with <i>YWHAZ</i> under tenogenic stimulation (e.g., BMP-12 induction in BMSCs/ASCs). However, shows increased variance in specific cross-source comparisons involving hAMSCs or ASCs ³¹ .
Regulatory / Signaling	<i>TBP</i> , <i>YWHAZ</i>	Participates in basal transcriptional assembly (<i>TBP</i>) and signal transduction/apoptosis regulation (<i>YWHAZ</i>). Exhibits excellent multi-condition stability; frequently top-ranked (lowest geNorm <i>M</i> -values) across diverse adult and perinatal MSC platforms; minimally affected by tissue origin or microenvironmental stress, though empirical validation remains necessary in diseased donor contexts ³⁰ .
RNA Polymerase / Basal Transcription	<i>POLR2A</i> (<i>RPB1</i>), <i>TBP</i>	Encodes the largest catalytic subunit of RNA polymerase II (<i>POLR2A</i>) and the TATA-box binding subunit of transcription factor TFIID (<i>TBP</i>). Generally stable due to essential roles in basal transcriptional machinery; ranks among the most stable reference genes across numerous MSC models (geNorm/NormFinder); minimal risk of fluctuation except under severe global transcriptional arrest ⁴ .
Protein Folding / Molecular Chaperones	<i>HSP90AA1</i> , <i>PPIA</i> (Cy- clophilin A)	Catalyzes cis-trans isomerization of proline peptide bonds (<i>PPIA</i>) and mediates stress-responsive protein folding (<i>HSP90AA1</i>). <i>PPIA</i> exhibits exceptional stability during long-term ex vivo expansion and pulsed electrical stimulation of MSCs (top-ranked by geNorm and NormFinder); <i>HSP90AA1</i> remains stable under mild thermal/osmotic stress but may be transactivated under severe proteotoxic shock ³² .
Ubiquitin-Proteasome Pathway	<i>UBC</i> , <i>UBB</i>	Encodes polyubiquitin precursors essential for proteasome-mediated protein degradation and cellular homeostasis. <i>UBC</i> frequently demonstrates high stability under defined stress conditions; however, its expression is systematically downregulated during replicative senescence and proteasomal exhaustion in aging MSCs ³³ .

This table provides a comprehensive classification of candidate reference genes commonly evaluated in mesenchymal stem/stromal cell (MSC) gene expression studies. Transcripts are grouped according to their primary cellular, metabolic, or structural functions. The table summarizes specific biological perturbations (e.g., lineage differentiation, mechanical strain, hypoxia, inflammatory licensing, or replicative senescence) that destabilize each gene family, highlights reported empirical rankings, and provides supporting literature citations. Evaluating candidate genes across distinct functional classes is essential to prevent false stability rankings arising from pathway co-regulation. **Abbreviations and Gene Symbols:** *ACTB*: beta-actin; AD-MSCs / ASCs: adipose-derived mesenchymal stem/stromal cells; *B2M*: beta-2-microglobulin; BMP-12: bone morphogenetic protein 12; BM-MSCs / BMSCs: bone marrow-derived mesenchymal stem/stromal cells; *EF1A*: eukaryotic translation elongation factor 1 alpha 1; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; hAMSCs: human amniotic membrane-derived mesenchymal stromal cells; *HPRT1*: hypoxanthine phosphoribosyltransferase 1; *HSP90AA1*: heat shock protein 90 alpha family class A member 1; *PGK1*: phosphoglycerate kinase 1; *POLR2A* (*RPB1*): RNA polymerase II subunit A; *PPIA*: peptidylprolyl isomerase A (cyclophilin A); *RPL13A*: ribosomal protein L13a; *RPLP0*: ribosomal protein lateral stalk subunit P0; *TBP*: TATA-box binding protein; *TUBA1B*: tubulin alpha 1b; *UBB*: ubiquitin B; *UBC*: ubiquitin C; *YWHAZ*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

candidate reference genes must maintain transcriptional stability across diverse tissue sources (bone marrow, adipose tissue, umbilical cord matrix, placenta), long-term passaging (passages 2 to 10+), multilineage differentiation, physiological hypoxia, proinflammatory challenge, 3D scaffold encapsulation, and cellular senescence^{8,10,34}.

The metabolic sensitivity of MSCs represents a primary driver of HKG variability. MSCs toggle between glycolytic flux and oxidative phosphorylation in response to pericellular oxygen levels and nutrient availability. Hypoxic culture—widely employed to maintain naive stemness or replicate physiological stem cell niches—stabilizes HIF-1 α , which directly transactivates glycolytic enzymes, including GAPDH²⁶. Consequently, *GAPDH* transcript abundance rises substantially under hypoxia, invalidating its utility as a reference gene in low-oxygen studies. Similarly, exposure to inflammatory mediators (e.g., IFN- γ , TNF- α , IL-1 β) and physical stimuli (e.g., fluid shear stress, scaffold stiffness, 3D encapsulation) activates broad immunomodulatory and mechanosensitive cascades that profoundly dysregulate structural genes such as *ACTB*²⁸.

Biomanufacturing parameters—including media supplementation, cell seeding density, passage number, and oxidative stress—introduce additional layers of variability. A seminal study by Ragni et al. (2024) demonstrated pronounced differences in HKG stability when adipose-derived stem cells (AD-MSCs) were transitioned from standard FBS-containing media to clinical-grade SFM or XFM formulations¹³. Similarly, Fitzgerald et al. (2023) highlighted the decisive impact of medium composition on MSC phenotypic stability, observing that XFM systems reduced inter-donor and batch-to-batch transcriptomic variability compared to undefined FBS-supplemented cultures³⁵. Chemically defined and serum-free platforms eliminate the uncharacterized growth factors and microvesicles inherent to FBS, thereby providing a more consistent transcriptional baseline³⁶. Aussel et al. (2022) corroborated this finding, demonstrating reduced HKG variability in FBS-free expansion protocols designed for clinical-grade MSC manufacturing³⁷.

Crucially, media formulations alter the relative stability hierarchy of individual HKGs. For example, in AD-MSCs, *EF1A* exhibited high stability in FBS-supplemented cultures, whereas *GAPDH* demonstrated superior stability in specific xeno-free media, and *TBP* remained consistently robust across all tested formulations¹³. These findings indicate

that distinct media compositions imprint unique transcriptomic signatures on MSCs. Consequently, early and integrated reference gene validation must be incorporated into standard quality control (QC) protocols and institutional standard operating procedures (SOPs) for GMP-compliant cell therapy biomanufacturing.

In addition to biochemical factors, mechanical loading forces play a decisive role in regulating MSC fate. Dynamic compressive, tensile, or shear stresses trigger mechanotransductive signaling through integrins and focal adhesion complexes, inducing major cytoskeletal rearrangements and destabilizing cytoskeleton-associated reference transcripts²⁹.

Biological Heterogeneity and Tissue-Source Specificity

MSCs exhibit profound phenotypic plasticity, undergoing extensive transcriptomic reprogramming during osteogenic, adipogenic, and chondrogenic differentiation^{24,25}. These lineage commitment processes systematically alter basal cellular metabolism and structural architecture, directly impacting candidate reference genes.

MSCs isolated from distinct anatomical niches (e.g., bone marrow, adipose tissue, umbilical cord, dental pulp) display distinct baseline transcriptional signatures, contributing to significant variability in HKG performance⁹. Calcat-i-Cervera et al. (2023) demonstrated that under harmonized culture conditions, bone marrow MSCs (BM-MSCs) exhibited substantial inter-donor variability, adipose-derived MSCs displayed the greatest consistency, and umbilical cord MSCs (UC-MSCs) showed attenuated differentiation responsiveness³⁸. Furthermore, donor demographics—such as age, sex, and health status—strongly dictate reference gene stability; adult MSCs exhibit divergent HKG expression compared to fetal or neonatal counterparts¹⁰. Notably, the disruptive effect of HKG variability is more pronounced when analyzing individual donors than when evaluating pooled or averaged datasets¹⁰.

In accordance with International Society for Cell & Gene Therapy (ISCT) criteria, MSC identity requires demonstrable multipotent trilineage differentiation³⁹. However, lineage specification triggers functional reprogramming that directly perturbs candidate reference transcripts. Osteogenic and adipogenic inductions induce a metabolic shift from glycolysis toward mitochondrial oxidative phosphorylation⁴⁰. Luque-Campos et al. (2021) demonstrated that this metabolic reprogramming not

only governs MSC differentiation and macrophage-polarizing capability but also causes significant fluctuations in glycolytic transcripts such as *GAPDH*⁴¹. Simultaneously, morphological reshaping during osteogenesis alters actin microfilament dynamics, directly driving the downregulation or instability of *ACTB*³⁴. Thus, both metabolic and structural remodeling during differentiation actively destabilize classical HKGs.

Temporal Dynamics and Microenvironmental Perturbations

Beyond culture media and tissue origins, subculture passage number and cell density significantly dictate reference gene stability. Greer et al. (2010) established that cell seeding density markedly modulates HKG transcript abundance⁴². Extended in vitro passaging drives MSCs into replicative senescence, characterized by permanent cell-cycle arrest, enlarged flattened morphology, cytoskeletal reorganization, and widespread transcriptional senescence-associated secretory phenotype (SASP) expression. Low-density cultures favor rapid self-renewal and stemness marker expression, whereas high confluence triggers contact inhibition, cell-cycle withdrawal, and altered HKG expression. These temporal parameters must be rigorously standardized during experimental setup.

Under specific stress conditions, reference gene instability becomes acute. Ong et al. (2023) demonstrated that in human AD-MSCs subjected to hypoxia, *GAPDH* and *PGK1* are entirely unsuitable for normalization due to the presence of functional hypoxia response elements (HREs) in their promoter regions; conversely, 18S rRNA and *RRP1* maintained remarkable stability⁴³. Rahimi et al. (2021) further documented that oxidative stress generated during MSC isolation, passaging, or cryopreservation alters secretome dynamics and induces severe fluctuations in HKG expression⁴⁴.

To simulate in vivo pathophysiological niches, researchers frequently treat MSCs with inflammatory cytokines, growth factors, or biophysical forces (Table 1). Wiese et al. (2022) revealed that stimulation with TNF- α , IL-1 β , or IFN- γ markedly alters MSC global gene expression relative to unprimed controls⁴⁵. In contrast, Valencia et al. (2025) reported that short-term proinflammatory cytokine priming enhances immunomodulatory capacity (e.g., suppressing T-cell and NK-cell proliferation, attenuating dendritic cell activation, and promoting M2-like macrophage differentiation) without compromising

core immunophenotypes or trilineage differentiation⁴⁶. The divergent transcriptional impacts observed between these studies stem from differences in cytokine concentrations, exposure kinetics, and basal culture status, highlighting the decisive role of experimental design. Furthermore, mechanical cues such as cyclic tensile stretch, fluid shear, and matrix stiffness trigger RhoA/ROCK signaling and actin filament bundling, leading to marked shifts in *ACTB* transcription. Consequently, *ACTB* should be strictly avoided in mechanobiology studies involving bioreactors or stiffness-tuned scaffolds.

These cumulative observations emphasize that candidate HKGs must be experimentally screened and validated for each specific experimental model rather than adopted by convention (Table 2). In MSC systems, *GAPDH* and *ACTB* frequently rank among the most unstable transcripts (Table 1), reflecting their central positions in glycolysis and cytoskeletal dynamics. Crucially, Ragni et al. (2024) observed that even under standard FBS-supplemented culture, *GAPDH* displayed severe expression instability⁴⁷, debunking the assumption of its universal utility. While reference gene stability remains donor- and context-dependent, avoiding *GAPDH* and *ACTB* as default normalization controls—particularly in adult MSCs subjected to differentiation, hypoxia, or cytokine challenge—represents a necessary methodological improvement.

Quantitative benchmarks for validating candidate HKGs in MSC models include: low statistical variance (coefficient of variation [CV] < 20%, geNorm stability measure $M \leq 0.5$, NormFinder stability value ≤ 0.15), moderate transcript abundance (quantification cycle [C_q] values between 18 and 28), robust amplification efficiency (90–110%), and verified absence of functional participation in the pathways under investigation^{7,52}. Because MSCs exhibit pronounced donor and tissue heterogeneity and undergo replicative senescence during prolonged culture, no universal reference gene exists across all MSC paradigms^{7,8,10,34}. Current international guidelines therefore recommend screening 8–12 candidate genes (e.g., *RPL13A*, *PPIA*, *TBP*, *HPRT1*, *YWHAZ*) across representative experimental samples and utilizing the geometric mean of the top 2–3 validated genes for accurate normalization (Table 3)^{1,31,53}.

Table 2: Summary of representative empirical studies evaluating reference gene variability in mesenchymal stem cells across defined experimental conditions.

Cell Type & Condition	Experimental Model & Mechanism	Biological & Transcriptomic Impact	Unstable HKGs	Stable Alternatives	Reference
Gingival MSCs Oxidative stress	Exposure to high ambient glucose concentrations (25–30 mM)	Alters glycolysis, elevates intracellular ROS, and modulates apoptotic pathways	<i>GAPDH</i> , <i>ACTB</i>	18S rRNA	Junaid et al., 2021 ⁴⁸
AD-MSCs Serum starvation	Cell culture in basal DMEM without FBS supplementation	Suppresses cell cycle progression, induces quiescence, and initiates cytoskeletal remodeling	<i>ACTB</i>	<i>B2M</i>	Ragni et al., 2024 ⁴⁷
AD-MSCs Serum-free / Xeno-free media	Culture in chemically defined media without animal serum or xenogeneic additives	Modulates integrin-mediated adhesion, membrane trafficking, and translation rate	<i>RPLP0</i>	<i>GAPDH</i> , <i>EF1A</i> , <i>TBP</i>	Ragni et al., 2024 ¹³
AD-MSCs Hypoxic culture	Incubation under normoxia (21% O ₂) vs. physiological/severe hypoxia (<1% O ₂)	Stabilizes HIF-1α signaling, upregulating glycolytic target genes containing HREs	<i>GAPDH</i> , <i>PGK1</i>	18S rRNA, <i>RRP1</i>	Ong et al., 2023 ⁴³
AD-MSCs Inflammatory stimulation	Basal medium supplemented with IL-1β or TNF-α	Activates NF-κB signaling and induces inflammatory and cytoskeletal restructuring	<i>ACTB</i>	<i>GAPDH</i>	Ragni et al., 2024 ⁴⁷
MSCs Electrical stimulation	Application of a direct-current electric field (100 mV/mm)	Alters cell polarization, cytoskeletal alignment, and intracellular calcium mobilization	<i>GAPDH</i> , <i>ACTB</i>	<i>PPIA</i> , <i>YWHAZ</i>	Steel et al., 2022 ³²
hBM-MSCs Osteogenic differentiation	Osteogenic cocktail (0.2 mM ascorbic acid, 10 mM β-glycerophosphate, 100 nM dexamethasone)	Dynamic transcriptional lineage commitment, mineralization, and cell morphology changes	<i>ACTB</i>	<i>RPL13A</i> , <i>GAPDH</i>	Quiroz et al., 2010 ³⁴
BMSCs / ASCs Tenogenic differentiation	Medium supplemented with BMP-12, ascorbic acid, and basic fibroblast growth factor	Stimulates tenogenic transcription factors (<i>SCX</i>), collagen synthesis, and ECM remodeling	<i>GAPDH</i>	<i>YWHAZ</i> , <i>RPL13A</i>	Viganò et al., 2018 ³¹
Cross-source baseline (AD-, BM-, UC-MSCs)	Unstimulated baseline conditions across distinct anatomical tissue derivations	Inherent epigenetic and metabolic divergence governed by tissue ontogeny	<i>GAPDH</i> , <i>ACTB</i>	<i>B2M</i> , <i>PPIA</i>	Li et al., 2015 ¹²
Adult MSCs (ADSC, BM-MSC, hAMSC)	Comparison of adult donor cohorts across increasing age ranges	Age-related senescence shifts, reduced proliferative capacity, and metabolic decline	<i>GAPDH</i> , <i>ACTB</i>	<i>TBP</i> , <i>EF1A</i>	Ragni et al., 2024 ¹⁰
Fetal vs. Neonatal MSCs	Ontogenetic comparison of developmental stages (placental/umbilical vs. adult)	Disparate baseline cell division rates and global transcriptional activity	<i>ACTB</i>	<i>TBP</i> , <i>GAPDH</i>	Ragni et al., 2024 ¹⁰
AD-MSCs Proliferation & differentiation	Logarithmic growth phase vs. multidirectional lineage specification	Dynamic reconfiguration of metabolic, ribosomal, and structural gene networks	<i>GAPDH</i>	<i>EF1A</i> , <i>RPLP0</i> , <i>RPL13A</i>	Ayanoğlu et al., 2020 ⁴⁹

AD-MSCs FBS- containing medium	Standard expansion in 10% FBS-supplemented basal medium	Heterogeneous serum growth factors stimulate cell motility and basal glycolysis	ACTB, GAPDH	EF1A, TBP, HPRT1	Ragni et al., 2024 ⁴⁷
BM-MSCs culture + inflammation	Hydrogel/scaffold 3D encapsulation combined with TNF-α or IFN-γ priming	Synergistic biophysical confinement and cytokine-induced cytoskeletal tension	ACTB, UBC	RPS13, RPL30	Gonzalez- Pujana et al., 2020 ⁵⁰
UC-MSCs Lentiviral transduction + diff.	Lentiviral gene transfer followed by trilineage differentiation induction	Cellular defense response to vector integration coupled with lineage remodeling	ACTB, GAPDH	RPS17, 18S rRNA	Borkowska et al., 2020 ⁵¹

This table compiles representative published studies assessing reference gene stability in diverse human mesenchymal stem/stromal cell (MSC) models. For each experimental paradigm, the table details the tissue origin, specific biological or physical stimulus, cellular impact, unstable reference transcripts, identified stable alternatives, and primary literature citations. The summarized data highlight the recurrent instability of classical housekeeping genes (such as *GAPDH* and *ACTB*) under differentiation, hypoxia, and inflammatory conditions, reinforcing the necessity of tailored multi-gene validation. **Abbreviations:** 18S rRNA: 18S ribosomal RNA; *ACTB*: beta-actin; AD-MSCs / ADSCs: adipose-derived mesenchymal stem cells; *B2M*: beta-2-microglobulin; BM-MSCs / BMSCs: bone marrow-derived mesenchymal stem cells; DMEM: Dulbecco’s modified Eagle’s medium; *EF1A*: eukaryotic translation elongation factor 1 alpha 1; FBS: fetal bovine serum; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; hAMSC: human amniotic membrane-derived mesenchymal stromal cell; hBM-MSC: human bone marrow-derived mesenchymal stem cell; HIF: hypoxia-inducible factor; HKG: housekeeping gene; *HPRT1*: hypoxanthine phosphoribosyltransferase 1; *PGK1*: phosphoglycerate kinase 1; *PPIA*: peptidylprolyl isomerase A; ROS: reactive oxygen species; *RPL13A*: ribosomal protein L13a; *RPL30*: ribosomal protein L30; *RPLP0*: ribosomal protein lateral stalk subunit P0; *RPS13*: ribosomal protein S13; *RPS17*: ribosomal protein S17; *RRP1*: ribosomal RNA processing 1; RT-qPCR: reverse transcription quantitative real-time PCR; SFM: serum-free medium; *TBP*: TATA-box binding protein; *UBC*: ubiquitin C; UC-MSCs: umbilical cord-derived mesenchymal stem cells; XFM: xeno-free medium; *YWHAZ*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

METHODOLOGICAL
FRAMEWORKS AND
ALGORITHMS FOR EVALUATING
REFERENCE GENE STABILITY

To quantify reference gene stability rigorously, researchers employ an array of statistical algorithms alongside high-throughput transcriptomic profiling tools (RT-qPCR, RNA-seq, and microarrays). For instance, Ong et al. (2023) established an RNA-seq-guided selection pipeline in human AD-MSCs: candidate genes from literature databases were cross-referenced against whole-transcriptome RNA-seq profiles of normoxic and hypoxic cells. Candidates exhibiting minimal biological variance (low CV and log₂ fold-change near zero) were shortlisted, validated by RT-qPCR, and evaluated using geNorm, NormFinder, and BestKeeper to pinpoint the most resilient reference set⁴³. Algorithmic platforms such as geNorm, NormFinder, and BestKeeper assess relative stability by evaluating candidate gene expression across experimental cohorts (Table 2). To streamline multi-algorithm comparisons, RefFinder integrates geNorm, NormFinder, BestKeeper, and the com-

parative ΔC_t method into a unified web-accessible platform¹⁷. RefFinder assigns an overall stability ranking based on the geometric mean of the individual algorithm ranks, thereby minimizing single-method bias. Nevertheless, RefFinder possesses intrinsic constraints: it lacks customizable weighting parameters, cannot perform advanced multivariate modeling, handles large-scale genomic datasets slowly, and its consensus metric may obscure nuanced, context-dependent variances. An enhanced geNorm module has been incorporated into Biogazelle’s qbase+ analytical software suite⁵⁴. Compared to the original macro-based geNorm program, qbase+ offers accelerated processing speeds, automated QC reporting, robust handling of missing values, identification of the single most stable reference transcript (rather than only the final stable gene pair), and cross-platform compatibility across Windows, macOS, and Linux systems. Despite their widespread adoption, geNorm and BestKeeper rely predominantly on pairwise correlation and similarity metrics. As a consequence, functionally co-regulated genes may be falsely categorized as highly stable if they exhibit parallel

Table 3: Comparative evaluation of mathematical algorithms and software tools for reference gene stability analysis.

Evaluation Dimension	geNorm	NormFinder	BestKeeper	Comparative ΔC_t Method	RefFinder
Mathematical Principle	Calculates the pairwise variation of each candidate gene against all others, defining stability measure M as the mean standard deviation. Iteratively eliminates the least stable gene. Computes pairwise variation ($V_{n/n+1}$) to determine the optimal number of reference genes ⁵⁴ .	Employs an ANOVA-based model to evaluate expression variance, partitioning variance into intra-group (within a condition) and inter-group (across conditions) components to yield a direct stability value (SV) ⁵⁵ .	Evaluates candidate gene stability using raw C_t values through descriptive statistics (SD, CV) and pairwise Pearson correlation coefficients (r) against an internal BestKeeper index ⁵⁶ .	Performs pairwise comparisons of relative cycle threshold differences (ΔC_t) between all candidate gene pairs within each sample; ranks stability based on mean ΔC_t standard deviations across samples.	Integrative web-based algorithm that calculates the geometric mean of candidate gene ranking positions generated across geNorm, NormFinder, BestKeeper, and the comparative ΔC_t method.
Input Data Format	Relative linear quantities ($Q = E^{-\Delta C_t}$), corrected for PCR amplification efficiency (E).	Linear relative quantities (same format as geNorm), with optional sample group annotations.	Raw, un-transformed cycle threshold (C_t/C_q) values and assay-specific amplification efficiencies.	Raw or normalized cycle threshold (C_t/C_q) values.	Raw C_t values or individual stability ranks generated by the four primary algorithms.
Stability Metric & Interpretive Threshold	$M \leq 0.5$: highly stable expression; lower M denotes greater stability. $V_{n/n+1} < 0.15$: indicates that n reference genes are sufficient; additional genes are unnecessary ⁵⁷ .	Stability value (SV) ≤ 0.15 indicates acceptable stability for reliable normalization; lowest SV indicates the single most stable gene ⁵⁸ .	$SD > 1.0$: unstable gene (unacceptable for normalization). r approaching 1.0: high stability. CV $< 2-5\%$: acceptable variance ⁵⁸ .	Lowest mean standard deviation (SD) of ΔC_t values across samples indicates the most stable candidate reference gene.	Comprehensive ranking based on the geometric mean of individual algorithm ranks; convergence across algorithms indicates high confidence.
Ease of Implementation	++	+	++	++	++
Quantitative Stability Ranking	++	++	+	+	++
Statistical Robustness	+	++	+	—	+

Resistance to Co-Regulation Bias	– (high vulnerability due to pairwise correlation)	++ (robust against co-regulation via ANOVA modeling)	– (vulnerable to correlated expression shifts)	– (vulnerable to pairwise co-regulation)	+ (partially buffered by multi-method aggregation)
Performance in Heterogeneous Cohorts	– (assumes constant expression ratios)	++ (models inter-group and intra-group variance separately)	– (confounded by biological subgroup shifts)	– (sensitive to subgroup sample variance)	+ (reflects composite performance across tools)

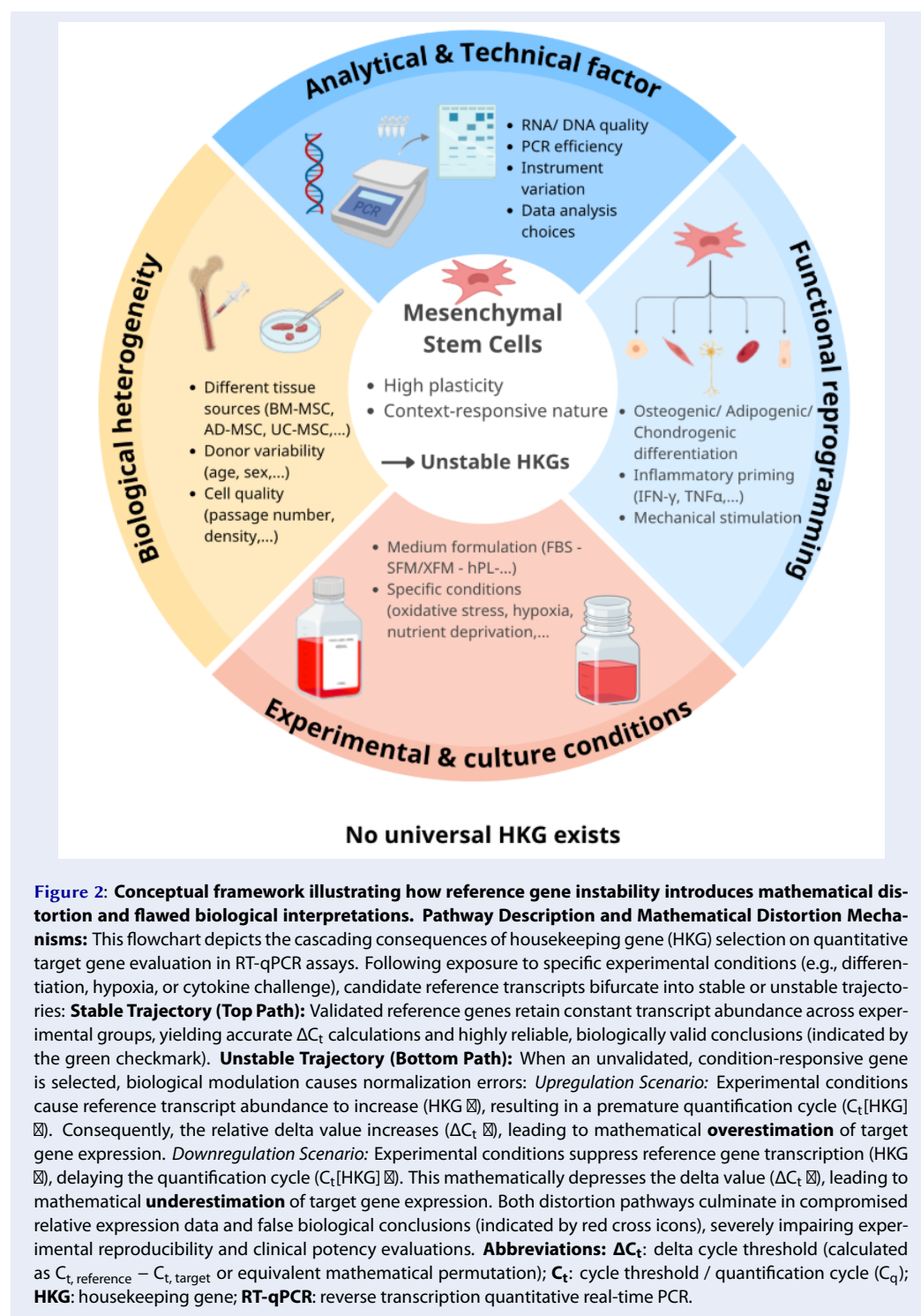
This table systematically compares the five most widely utilized mathematical algorithms and software tools for reference gene validation in mesenchymal stem cell (MSC) gene expression studies: geNorm, NormFinder, BestKeeper, the comparative ΔC_t method, and RefFinder. Each tool is characterized according to its underlying mathematical rationale, required input data format, primary stability metrics and interpretive thresholds, and operational performance across key experimental criteria. A nuanced understanding of these distinct mathematical assumptions is essential for executing robust multi-algorithmic consensus evaluations and avoiding co-regulation artifacts. **Symbols and Abbreviations:** ++: strong / high performance; +: moderate / acceptable performance; –: absent / poor performance; ANOVA: analysis of variance; C_t / C_q : cycle threshold / quantification cycle; CV: coefficient of variation; ΔC_t : delta cycle threshold; E : PCR amplification efficiency; M value: geNorm average expression stability measure; Q : relative expression quantity; r : Pearson correlation coefficient; RT-qPCR: reverse transcription quantitative real-time PCR; SD : standard deviation; SV : NormFinder stability value; V value: geNorm pairwise variation value.

expression shifts in response to a common perturbation. For example, transcripts involved in shared metabolic pathways may display strong mutual correlation despite both being modulated by experimental treatments. NormFinder partially circumvents this limitation through an ANOVA-based model that separates overall expression variance into intra-group and inter-group components. Nonetheless, if a gene displays uniform, low-variance changes across all experimental groups, NormFinder may still classify it as stable despite biologically meaningful modulation. Therefore, mathematical stability rankings must always be cross-referenced against biological plausibility, ensuring that candidate panels are populated by genes originating from distinct, non-overlapping functional categories to preclude co-regulation artifacts.

IMPLICATIONS OF REFERENCE GENE INSTABILITY FOR FUNDAMENTAL RESEARCH AND CLINICAL TRANSLATION

Reference gene stability directly dictates the precision, validity, and biological interpretability of gene expression profiling. Normalizing datasets against unverified, unstable internal controls introduces severe mathematical bias and systematic error (Figure 2)⁵⁹. The updated MIQE 2.0 guidelines explicitly assert that qPCR datasets normalized against unvalidated reference genes cannot be considered analytically valid²². Across diverse MSC sources (adipose tissue, bone marrow, umbilical cord matrix), conventional HKGs

such as *GAPDH*, *ACTB*, and *PGK1* frequently display pronounced expression instability, whereas non-traditional candidates such as *PPIA*, *YWHAZ*, *TBP*, *RPL13A*, *RPLP0*, 18S rRNA, or *RRP1* provide superior stability in specific biological contexts (Table 1). External factors—including tissue harvest site¹², donor characteristics¹⁰, pericellular hypoxia⁴³, culture media shifts (FBS to SFM/XFM)¹³, inflammatory activation, serum starvation⁴⁷, and 3D scaffold encapsulation⁵⁰—profoundly alter basal HKG transcription. Choosing an inappropriate reference gene can artificially amplify or dampen apparent expression differences in target genes. In MSC differentiation assays, sub-optimal reference gene selection can entirely mask lineage-specific induction markers or, conversely, create artifactual differences in differentiation efficiency⁶⁰. In severe instances, improper normalization can invert biological conclusions. For example, Casas et al. (2022) demonstrated that standardizing stroke-related *NOX4* expression against different HKGs generated contradictory conclusions regarding whether the target transcript was significantly upregulated or downregulated⁵⁹. In cytokine-primed MSCs, normalizing against *ACTB* completely obscured the upregulation of the key immunosuppressive enzyme indoleamine 2,3-dioxygenase (*IDO*), whereas stable reference genes revealed robust induction. Such discrepancies are especially detrimental in quantitative potency assays, where misquantifying immunomodulatory transcripts can compromise therapeutic efficacy and product characterization.



At the bioprocessing and translational scale, reference gene instability increases experimental variance, generating false-positive or false-negative readouts that directly impact cell therapy release criteria¹³. Ragni et al. (2024) demonstrated that

when comparing AD-MSCs expanded in SFM versus FBS, unstable *ACTB* expression falsely indicated an upregulation of the proinflammatory chemokine *CCL5* in SFM products; conversely, normalization against the stable reference gene *TBP* demonstrated

that *CCL5* transcription was unchanged or slightly downregulated¹³. Similarly, during immunological evaluations of MSCs encapsulated in 3D biomaterials or subjected to inflammatory challenge, *ACTB* normalization severely skewed perceived immunomodulating potency^{50,61}.

In commercial cell manufacturing and clinical translation, MSC products must adhere strictly to GMP regulations. Regulatory agencies, including the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), mandate validated quantitative potency testing prior to lot release. Within these release assays, reference genes represent an indispensable component of the analytical validation framework, ensuring assay accuracy, linearity, and precision⁶². Normalizing against unvalidated reference genes can cause release metrics to fall artificially outside predefined acceptance ranges. This introduces substantial operational risks: high-quality cell batches may be erroneously rejected due to normalization artifacts—resulting in substantial economic loss—or compromised cell batches may be mistakenly released, posing direct clinical risks to patients.

RECOMMENDATIONS AND EMERGING STANDARDIZATION STRATEGIES

Methodological Recommendations Aligned with MIQE 2.0

Inter-laboratory variability in MSC research remains substantial due to divergent tissue isolation techniques, expansion protocols, cryopreservation methods, oxygen concentrations, and culture media lots, all of which alter baseline gene expression⁶³. For instance, transitioning from FBS-based media to SFM/XFM bioprocesses directly shifts reference gene stability hierarchies¹³. Although regulatory authorities mandate potency testing for MSC product release⁶⁴, specific standardized panels for reference gene selection are not formally codified in GMP or FDA guidance documents. Consequently, reference gene validation must be treated as a facility- and protocol-specific process, precluding the direct extrapolation of HKG sets across different laboratories or distinct biomanufacturing runs. Each facility must empirically validate candidate reference genes upon establishing or modifying production parameters, locking validated panels into formal SOPs to preserve batch-to-batch consistency.

Assay reproducibility represents the cornerstone of reliable quantitative molecular testing. In this regard, inter-laboratory standardization is not merely

recommended—it is essential. The original MIQE guidelines served as the technical foundation for the international standard ISO 20395:2019⁶⁵, demonstrating the universal relevance of these validation frameworks. The updated MIQE 2.0 consensus statement (published in 2025) introduces several critical enhancements³:

- Provides comprehensive specifications for inter-laboratory comparability, cross-platform instrument calibration, and transparent metric reporting.
- Mandates empirical validation and confidence intervals rather than reliance on nominal C_q values.
- Enforces the routine inclusion of rigorous negative controls (no-template controls [NTC] and no-reverse-transcription controls [NRTC]) and strongly recommends exogenous RNA spike-in controls to monitor RNA recovery, reverse transcription inhibition, and PCR amplification efficiency.
- Explicitly requires the geometric mean of multiple experimentally validated reference genes for normalization, strictly forbidding the use of single unvalidated housekeeping genes.

To facilitate preselection, public expression databases such as Genevestigator—which curates thousands of high-quality transcriptomic datasets—provide empirical confirmation that no mammalian transcript is universally stable across all physiological settings⁶⁶. The "Reference Gene Selection" tool within Genevestigator enables researchers to query specific biological contexts and shortlist stable candidate genes⁶⁷. In practice, researchers should design a candidate panel comprising 8–12 genes representing diverse, non-overlapping functional pathways to prevent co-regulation bias. Initial selection can be informed by whole-transcriptome RNA-seq datasets (prioritizing genes with low standard deviation and minimal CV across replicates) or prior validation studies in comparable cell types⁴³. Following candidate selection, MSCs should be cultured under representative experimental conditions, and total RNA must be extracted while ensuring high purity ($A_{260}/A_{280} > 2.0$; $A_{260}/A_{230} > 1.8$) and structural integrity (RNA Integrity Number [RIN] ≥ 7.0), as partially degraded RNA introduces substantial normalization artifacts. Furthermore, primer amplification efficiency must be rigorously determined using multi-point serial cDNA dilutions (optimal slope -3.32 , efficiency 90–110%, $R^2 > 0.99$),

primer specificity must be confirmed via single-peak melting curve analysis or gel electrophoresis, and all reactions should be performed in technical and biological triplicate^{12,68,69}. Finally, stability should be evaluated using a multi-algorithmic consensus strategy (geNorm, NormFinder, BestKeeper, comparative ΔC_t , and RefFinder) to identify the optimal number of reference genes (typically 2 to 3) for geometric mean normalization (Figure 3, Table 3)⁶⁶.

Alongside gene validation, investigators must comprehensively document experimental parameters in accordance with the MIQE 2.0 reporting checklist—including primer sequences, amplification efficiencies, C_q distributions, sample cohort sizes, biological replicates, and algorithmic stability values—thereby providing full transparency for peer reviewers and academic readers³.

Emerging Approaches: Small RNA Reference Panels and Machine Learning

The establishment of robust reference standards is gaining rapid traction as MSC applications expand from whole-cell therapy toward cell-free biologics, particularly microRNAs (miRNAs) packaged within extracellular vesicles (EVs). Because traditional mRNA-based HKGs are largely absent or highly fragmented within EV cargo, endogenous miRNAs have emerged as candidate reference controls for normalizing EV molecular content. However, widely used small RNA controls (e.g., U6 snRNA) are susceptible to processing variability and do not accurately reflect EV packaging dynamics. Profiling of adipose-derived MSC-EVs revealed that miR-16-5p and miR-23a-3p were stably and consistently detected, whereas let-7a-5p, miR-425-5p, and U6 snRNA exhibited substantial variability⁷⁰. Similarly, in amniotic fluid MSC-EVs, miR-101-3p and miR-22-5p demonstrated high stability, while miR-423-5p and U6 snRNA showed severe expression fluctuations⁷¹. These collective findings emphasize that U6 snRNA should be avoided in quantitative EV-miRNA studies due to its high vulnerability to bioprocessing artifacts. In specialized differentiation systems, multi-miRNA panels are increasingly necessary: for example, the combination of miR-191-5p and let-7a-5p provides optimal normalization during BM-MSC chondrogenesis⁷², while miR-103a-3p and miR-22-5p display cross-source stability across bone marrow, adipose, and chondrocyte-derived EVs⁷³.

Concurrently, *in silico* bioinformatic pipelines and artificial intelligence (AI) methodologies are reshaping reference gene selection. Algorithmic tools such as HouseKeepR automate the retrieval and meta-analysis of public transcriptomic repositories, ranking candidate genes in an unbiased, context-specific manner⁵⁹. This data-driven approach overcomes the inherent biases of literature convention. Furthermore, advanced machine learning architectures—including Random Forest, Support Vector Machines (SVM), and deep neural networks—are increasingly deployed to parse high-throughput multi-omics data (transcriptomics, epigenomics, and proteomics) to identify stable, context-tailored reference networks⁷⁴. These algorithms evaluate not only mean expression and variance but also gene co-expression networks, topological connectivity, tissue specificity, and experimental covariates¹. As curated biomedical repositories expand and AI-driven feature selection matures^{75,76}, machine learning-assisted reference gene selection represents a transformative strategy for standardizing MSC research and large-scale clinical biomanufacturing.

CONCLUSION

The selection of housekeeping genes in mesenchymal stem cell research cannot be addressed through simple, one-size-fits-all assumptions. MSCs display remarkable biological plasticity, undergoing profound transcriptomic rewiring during multilineage differentiation, metabolic reprogramming, hypoxic adaptation, inflammatory licensing, mechanical stimulation, and extended *ex vivo* expansion. This intrinsic responsiveness systematically disrupts the transcriptional stability of traditional reference genes such as *GAPDH* and *ACTB*. Empirical evidence firmly establishes that no universal reference gene exists across all MSC experimental conditions. Accurate, reproducible normalization requires a context-specific validation strategy that merges rigorous mathematical algorithms with sound biological rationale. Failing to validate reference genes risks generating distorted expression metrics, false-positive or false-negative findings, and contradictory conclusions in potency and translational assays. Integrating multi-gene normalization panels, adhering strictly to MIQE 2.0 reporting standards, and leveraging RNA-seq preselection and machine learning models provide a robust methodological foundation to ensure experimental rigor, reproducibility, and therapeutic translatability in MSC biology.

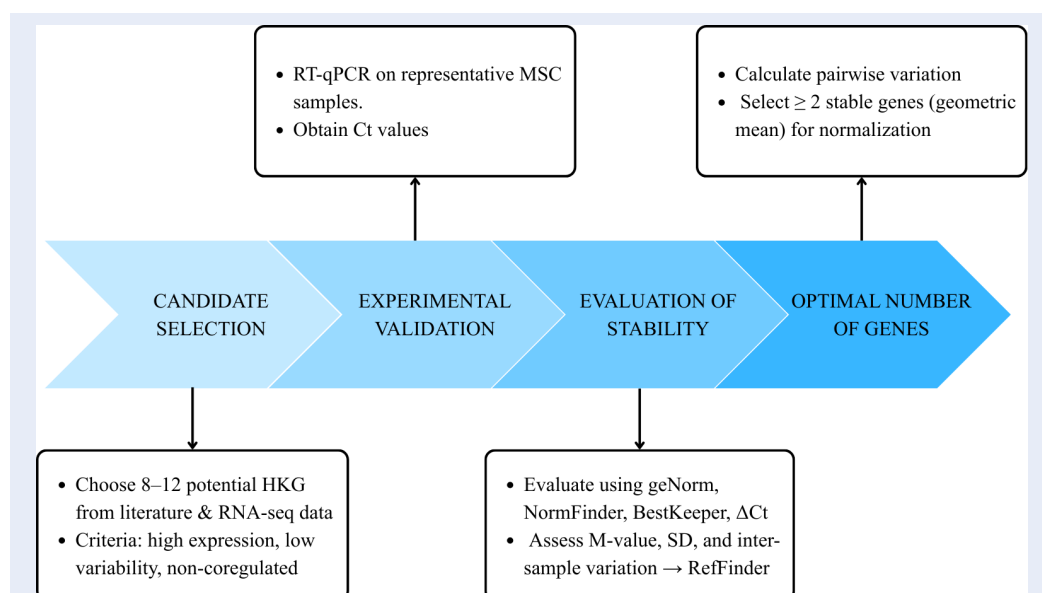


Figure 3: Standardized four-phase workflow for reference gene selection and experimental validation in MSC research. Methodological Workflow and Decision Architecture: This multi-stage schematic outlines a systematic, guideline-compliant protocol (conforming to MIQE 2.0 recommendations) for identifying and validating stable internal reference controls for gene expression profiling in mesenchymal stem cells (MSCs): **Phase 1: Candidate Selection:** Pre-screen 8–12 candidate reference genes from curated literature databases and public RNA-seq datasets (e.g., Genevestigator). Selection criteria mandate moderate-to-high transcript abundance (C_t 18–28), low baseline coefficient of variation ($CV < 20\%$), and deliberate distribution across non-overlapping functional and metabolic pathways to eliminate co-regulation artifacts. **Phase 2: Experimental Validation:** Culture MSCs under experimental conditions precisely mirroring the study design. Extract high-purity, intact total RNA ($RIN \geq 7.0$), verify primer specificity (single melt curve peak), assess PCR amplification efficiency via standard curves (90–110%), and execute RT-qPCR across biological and technical triplicates to generate raw quantification cycle (C_t/C_q) values. **Phase 3: Evaluation of Stability:** Perform statistical evaluation across candidate genes using established mathematical algorithms—including geNorm (stability measure $M \leq 0.5$), NormFinder (stability value ≤ 0.15), BestKeeper (standard deviation $SD < 1.0$), and the comparative ΔC_t method. Synthesize individual algorithm rankings using RefFinder to derive a comprehensive stability consensus. **Phase 4: Determination of Optimal Number of Genes:** Calculate geNorm pairwise variation ($V_{n/n+1} < 0.15$) to identify the minimum number of reference transcripts required for robust normalization. Incorporate the geometric mean of ≥ 2 top-ranked, validated reference genes into downstream target quantification. **Abbreviations:** ΔC_t : delta cycle threshold; C_t : cycle threshold; CV : coefficient of variation; HKG: housekeeping gene; MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments; RIN : RNA integrity number; RNA-seq: RNA sequencing; RT-qPCR: reverse transcription quantitative real-time PCR; SD : standard deviation; V value: geNorm pairwise variation value.

ABBREVIATIONS

18S rRNA: 18S ribosomal RNA; **ACTB:** Beta-actin; **AD-MSCs / ASCs:** Adipose-derived mesenchymal stem/stromal cells; **AI:** Artificial intelligence; **B2M:** Beta-2-microglobulin; **BM-MSCs / BMSCs:** Bone marrow-derived mesenchymal stem/stromal cells; **C_q / C_t :** Quantification cycle / Cycle threshold; **CV :** Coefficient of variation; **ΔC_t :** Delta cycle threshold; **ECM:** Extracellular matrix; **EF1A:** Eukaryotic translation elongation factor 1 alpha 1; **EMA:** European Medicines Agency; **EVs:** Extracellular vesicles; **FBS:** Fetal bovine serum; **FDA:** Food and Drug Administration; **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase; **GMP:** Good Manu-

facturing Practice; **hAM-MSCs / hAMSCs:** Human amniotic membrane-derived mesenchymal stem/stromal cells; **HIF-1 α :** Hypoxia-inducible factor 1 alpha; **HKGs:** Housekeeping genes; **hPL:** Human platelet lysate; **HPRT1:** Hypoxanthine phosphoribosyltransferase 1; **HRE:** Hypoxia response element; **HSP90AA1:** Heat shock protein 90 alpha family class A member 1; **IDO:** Indoleamine 2,3-dioxygenase; **IFN- γ :** Interferon gamma; **IL-1 β :** Interleukin 1 beta; **ISCT:** International Society for Cell & Gene Therapy; **miRNA:** MicroRNA; **MIQE:** Minimum Information for Publication of Quantitative Real-Time PCR Experiments; **ML:** Machine learning; **MSCs:** Mesenchymal stem/stromal cells;

NRTC: No-reverse-transcription control; **NTC:** No-template control; **PGK1:** Phosphoglycerate kinase 1; **POLR2A:** RNA polymerase II subunit A; **PPIA:** Peptidylprolyl isomerase A (Cyclophilin A); **QC:** Quality control; **RIN:** RNA integrity number; **RNA-seq:** RNA sequencing; **RPL13A:** Ribosomal protein L13a; **RPLP0:** Ribosomal protein lateral stalk subunit P0; **RPS13:** Ribosomal protein S13; **RPS17:** Ribosomal protein S17; **RRP1:** Ribosomal RNA processing 1; **RT-qPCR:** Reverse transcription quantitative real-time polymerase chain reaction; **SASPs:** Senescence-associated secretory phenotypes; **SD:** Standard deviation; **SFM:** Serum-free medium; **SOP:** Standard operating procedure; **SV:** Stability value; **TBP:** TATA-box binding protein; **TNF- α :** Tumor necrosis factor alpha; **TUBA1B:** Tubulin alpha 1b; **UBB:** Ubiquitin B; **UBC:** Ubiquitin C; **UC-MSCs:** Umbilical cord-derived mesenchymal stem/stromal cells; **XFM:** Xeno-free medium; **YWHAZ:** Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

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AUTHOR'S CONTRIBUTIONS

T.K.N. Nguyen, N.T.T. Nguyen, and A.M. Nguyen conducted the literature investigation, performed data curation, and prepared the original manuscript draft. **K.V.T. Nguyen and K.D. Bui** supported formal comparative analyses and contributed to critical manuscript editing. **P.D. Huynh** conceptualized and supervised the study, oversaw methodology and project administration, and critically revised and finalized the manuscript. All authors read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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