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Editorial

# Beyond Protein Quantification: Functional Bioassays as the Next Step in Demineralized Bone Matrix Quality Assessment

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Demineralized bone matrix (DBM) remains one of the most widely used biological bone graft substitutes in orthopedic, spinal, craniofacial, and dental surgery. Yet, despite decades of clinical use, a fundamental challenge persists: how should biological activity be assessed before implantation? While conventional characterization approaches focus primarily on protein content and composition, increasing evidence suggests that the presence of biologically relevant molecules does not necessarily predict their functional activity. DBM products exhibit substantial variability arising from donor characteristics, tissue source, processing methods, sterilization procedures, and carrier formulations, highlighting the need for more biologically informative quality-control strategies [1,2].

In a recent article in the Journal of Functional Biomaterials, Lendvai and colleagues address this important challenge by evaluating a functional TGF-β/Smad reporter assay for assessing of DBM-derived allograft bioactivity [2]. Rather than quantifying proteins alone, the authors investigated whether processed DBM materials could activate a biologically relevant signaling pathway in living cells. Specifically, they employed HEK-Blue™ TGF-β reporter cells from InvivoGen, which produce secreted embryonic alkaline phosphatase (SEAP) as a readout of Smad-dependent pathway activation. Similar cellular assay system were already introduced over three decades ago, including system in which, for example, mink lung epithelial cells (MLCs) were stably transfected with an expression construct containing a TGF-β-sensitive truncated plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase reporter gene [3]. Such systems are significantly more sensitive than ELISAs in detecting active TGF-β in serum and both active and total TGF-β in solid organ tissues [4]. Furthermore, they require minimal sample volumes, do not require prior extraction, and allow discrimination between latent and mature TGF-β [5]. Because firefly luciferase is expressed intracellularly, the reporter cells must be lysed to release the enzyme and enable substrate catalysis. Therefore, other systems that rely on enhanced green fluorescent protein gene (EGFP) have been established. In one of these systems, a Smad-binding element (SBE), i.e. the so-called (CAGA)12 box, was fused to the EGFP gene and transfected into Expi293FTM cells (RRID:CVCL_D615), which can be cultured in suspension or as adherent cells using chemically defined medium without fetal calf serum, which may contain (traces of) TGF-β [6].

Against this background, the study by Lendvai and co-workers shifts the focus from measuring molecular abundance to evaluating functional cellular responses. The significance of this work extends beyond the specific assay examined. Regenerative biomaterials are increasingly being evaluated not only by their composition but also by their capacity to engage cellular signaling networks that drive tissue repair. Functional bioassays that directly measure pathway activation are therefore emerging as valuable complements to traditional biochemical analyses. In skeletal biology, TGF-β signaling plays a central role in osteoblast differentiation, extracellular matrix remodeling, bone homeostasis, and regenerative responses, making it a particularly relevant target for functional assessment [7,8].

This conceptual shift is summarized in figure 1. Conventional DBM quality assessment largely relies on compositional analyses, including protein extraction and quantification using assays such as the enzyme-linked immunosorbent assay (ELISA) or the bicinchoninic acid (BCA) assay, which provide information on molecular abundance but do not directly establish biological activity. Manufacturing-related variables, including donor-to-donor variability, demineralization, thermal processing, putty formulation, and γ-sterilization, may further uncouple detectable protein content from functional signaling capacity. Functional reporter assays address this limitation by measuring whether DBM-derived factors can activate defined cellular pathways, such as TGF-β/Smad-dependent SEAP expression in reporter cells. Consequently, pathway-based bioassays can complement conventional protein-based characterization and provide a more biologically meaningful assessment of DBM-derived graft materials.

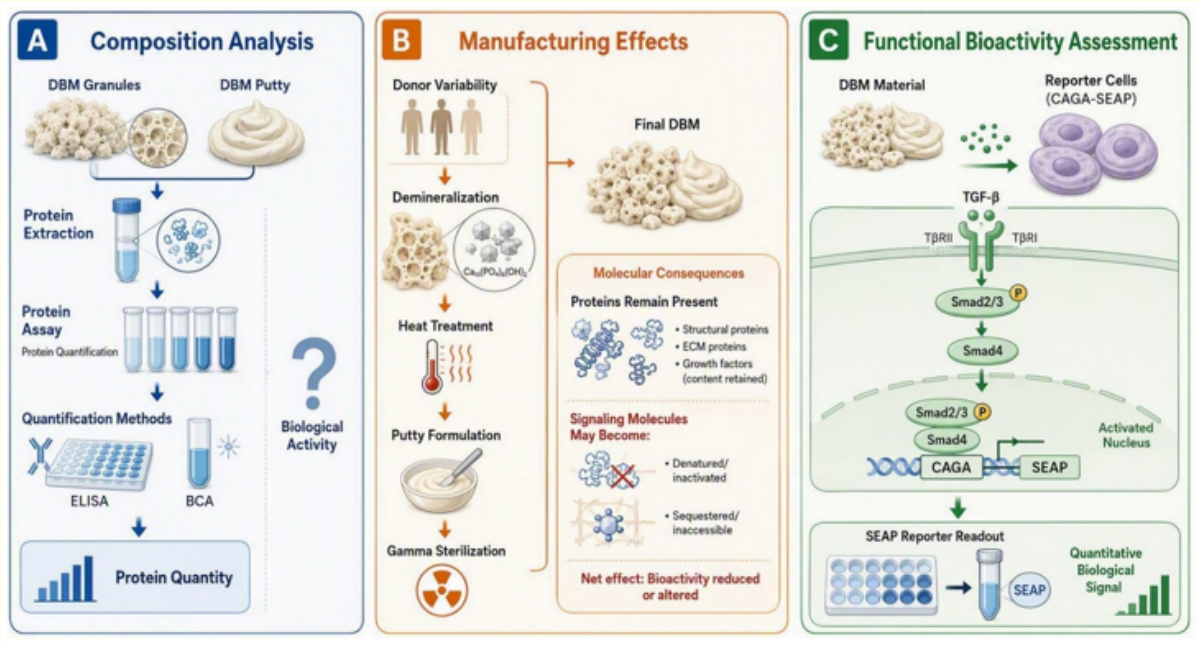


***Figure 1:*** *Protein composition versus functional bioactivity of demineralized bone matrix (DBM). (A) Conventional characterization of DBM products, including granular and putty formulations, typically involves protein extraction followed by protein quantification using biochemical assays such as enzyme-linked immunosorbent assay (ELISA) and bicinchoninic acid (BCA) assays. These approaches provide information on total protein content but do not directly assess the biological activity of the detected proteins and growth factors. (B) Multiple manufacturing variables, including donor-to-donor variability, demineralization, thermal processing, putty formulation, and γ-sterilization, can influence the biological properties of DBM. Although proteins and growth factors may remain detectable after processing, biologically relevant signaling molecules can become denatured, inactivated, sequestered, or otherwise inaccessible, resulting in reduced or altered bioactivity despite preserved protein content. (C) Functional reporter-cell assays provide a direct measure of biologically active signaling molecules released from DBM. In the example shown, DBM-derived transforming growth factor-β (TGF-β) activates the canonical TGF-β/Smad signaling pathway in CAGA-SEAP reporter cells, resulting in expression of secreted alkaline phosphatase (SEAP) as a quantitative readout of pathway activation. Such functional assays complement conventional compositional analyses by assessing the biological potency of DBM products rather than the presence of proteins alone. Adapted from concepts presented by Lendvai., et al. [2].*

One of the most striking observations reported by Lendvai., *et al*. is the apparent disconnect between protein recovery and biological activity. Urea extraction yielded higher apparent protein concentrations than guanidine hydrochloride (GuHCl) extraction. However, although the GuHCl workflow recovered less apparent protein, the GuHCl-derived extracts induced substantially greater TGF-β reporter activation [2]. This finding highlights a critical limitation of conventional compositional analyses: protein abundance alone may not reflect biological functionality. Similar concerns about the predictive value of protein measurements for osteoinductive potential have been raised previously [9]. The results reinforce the concept that growth factors and matrix-associated signaling molecules must be evaluated not only for their presence but also for their ability to activate cellular signaling pathways.

The study further demonstrated that clinically relevant DBM products retained measurable reporter activity after processing and manufacturing. Cortical demineralized bone granules, wet heat-treated putty formulations, and γ-irradiated putty products all induced reporter activation above the negative-control levels. These findings are particularly noteworthy because extensive processing steps are often assumed to diminish osteoinductive potential. Although processing can alter protein integrity, matrix architecture, and factor availability, appropriately manufactured DBM products may retain biologically meaningful signaling activity [1,2].

Importantly, the authors avoid overstating the implications of their findings. The assay is not presented as a comprehensive measure of osteoinductivity. Bone regeneration is inherently multifactorial and depends on coordinated interactions among TGF-β, BMP, and Wnt signaling pathways. Moreover, it is influenced by angiogenic and inflammatory processes, and extracellular matrix remodeling. Recent studies have highlighted the extensive crosstalk among these regulatory networks and their collective contribution to skeletal repair and homeostasis [7,8]. Consequently, no single pathway can fully represent the regenerative potential of a bone graft material.

Several limitations deserve consideration. First, receptor-level confirmation using TGF-β neutralization or pathway inhibition was not included, which prevents definitive attribution of the observed responses to specific receptor-mediated signaling events. Second, the direct-contact material experiments were not normalized for cell number, dry DBM content, hydration state, or accessible matrix surface area. Such normalization will be required before quantitative comparisons among products, batches, or manufacturing workflows can be performed. Moreover, donor-specific analyses were limited, leaving unanswered questions regarding inter-donor variability, an issue that remains a major challenge in DBM research [2,10]. Finally, the assay shares a specificity limitation with related reporter systems. It quantifies Smad3-dependent signaling, which can be triggered by all three TGF-β isoforms and potentially by other TGF-β superfamily members interconnected with the Smad3 pathway.

Nevertheless, the broader impact of this work should not be underestimated. Historically, the evaluation of osteoinductive potential has relied heavily on ectopic implantation models and other animal-based approaches. Although these models remain valuable, they are costly, time-consuming, and difficult to standardize. Functional cell-based assays offer an attractive complementary strategy that may enable rapid screening of material bioactivity during product development, manufacturing optimization, and batch-release testing [2,11]. Such assays have the potential to bridge the gap between simple compositional measurements and complex *in vivo* studies.

Looking ahead, the greatest value of pathway-specific reporter assays may emerge from their integration into multidimensional testing platforms. Rather than relying solely on protein quantification or a single biological pathway, future quality-control frameworks could combine measurements of TGF-β activity with assays addressing BMP signaling, angiogenesis, immune modulation, osteogenic differentiation, and matrix remodeling. Such functional "bioactivity fingerprints" could provide more comprehensive and clinically meaningful assessments of regenerative biomaterials than compositional analyses alone. Emerging insights into the integrated regulatory roles of TGF-β/BMP signaling in skeletal regeneration strongly support the feasibility of such approaches [7,8].

In summary, Lendvai and colleagues provide compelling evidence that functional pathway activation can reveal properties of DBM-derived materials that remain invisible to conventional protein quantification. Their work reflects a broader shift occurring across

regenerative medicine, from asking what molecules are present to asking whether those molecules remain biologically functional. The HEK-Blue™ TGF-β reporter assay may therefore represent an important step toward more biologically informed and mechanism-based assessment of bone

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Not applicable.

## Consent for Publication

Not applicable.

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Not applicable.

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