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COMMENTARY

Two birds with one bone?

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Guan *et al.*[1] have proposed a novel solution to two key problems in mesenchymal stem cell (MSC) biology and bone disorders. First, the problem of how to engraft MSCs into the bone marrow, and second, the problem of how to drive osteogenesis in aged bone. It has become clear that age-associated bone loss is in large part the result of a loss of osteoblast precursor MSCs with age. Although not well understood, this bone marrow MSC loss may be due to increased cell death, reduced proliferation, mobilization from the bone marrow—possibly with interference of MSC recirculation to the bone marrow niche, or a change in the rate of differentiation into an adipocyte cell fate rather than an osteogenic fate.[2–7] MSC bone marrow transplantation to replace these cells has been problematic. Attempts to transplant MSCs from whole bone marrow, or from MSC-enriched peripheral blood (for example, from G-CSF-mobilized donor bone marrow) or highly purified and cultured MSCs, even low passage MSCs, almost universally fail to significantly engraft within the bone marrow when infused into the peripheral circulation of humans and animal transplantation models.[8–10] This seems owing to both the 'pulmonary first-pass effect,' where more than 96% of cells are entrapped in the lung microvasculature together with poor long-term engraftment (for example, beyond 1–2 months), within the bone marrow.[11,12]

Guan *et al.*[1] have in part bypassed the problem of engrafting the MSCs into their endogenous niche by guiding the infused cells directly to the bone-forming surfaces and having the cells engage in *de novo* bone formation (**Figure 1**). This has been achieved by creating a unique bi-functional molecule (LLP2a–Ale) that binds to the MSC's $\alpha4\beta1$ integrins and to bone. A novel combinatorial library was screened for peptidomimetric ligands able to bind to 'active' $\alpha4\beta1$ integrins, and LLP2A was identified as a high-affinity and specific ligand. LLP2A was then conjugated with the bisphosphonate alendronate (Ale), allowing for direct attachment to bone. As they show, LLP2A alone may help drive endogenous bone formation; however, when coupled with alendronate it significantly increases bone formation (**Figure 2**). Importantly, this innovative approach avoids the significant problems now recognized to be associated with blocking bone resorption with bisphosphonates. The issues with bisphosphonates include an inability to remodel in response to changing biomechanical needs, and the rare, but extremely serious, outcomes of bone necrosis and increased fracture risk.[13]

The integrin pair $\alpha4\beta1$ was selected as a target, as earlier work suggested overexpression of $\alpha4$ leads to increased MSC homing to bone, and $\beta1$ is associated with integrins in MSCs and osteoblasts.[14] Although not described here, it is presumed that LLP2A induces $\alpha4\beta1$ signal transduction. As such, there is still the question of what happens to transplanted, or endogenous, MSCs when LLP2A binds to the target $\alpha4\beta1$ integrins. Does this increase the surface expression, or activation, of other integrins and extracellular matrix (ECM) receptors (for example, CD44) that may drive interactions with ECM target antigens? This signaling may enhance MSC proliferation, osteogenic differentiation, binding to bone surface molecules or expression of ECM molecules needed for bone matrix formation *in vivo*. Some of these possibilities are suggested by their outcome data that LLP2A alone leads to an increased osteoblast surface (Ob/BS) and possibly an increased bone formation rate (BFR/BS) (**Figure 2**).

Human and murine MSCs transplanted with LLP2A–Ale into immunocompromised mice were demonstrated to accumulate at endosteal surfaces on both cortical and trabecular bone, as well as on periosteal bone surfaces within 24 h. This suggests that the MSCs were able to exit blood vessels near both interior and exterior bone-forming surfaces and quickly move, and bind, to those surfaces. Over the course of 3 weeks, the MSCs were incorporated as osteocytes into newly formed bone adjacent to the bone-forming surfaces. Additionally, markers of bone formation were increased, including serum osteocalcin levels and the morphometric measures for mineralizing surface (MS/BS) and BFR/BS (**Figure 1**). Excitingly, this presents a new therapeutic approach that could potentially be used for numerous bone defect scenarios, and especially for osteoporosis.

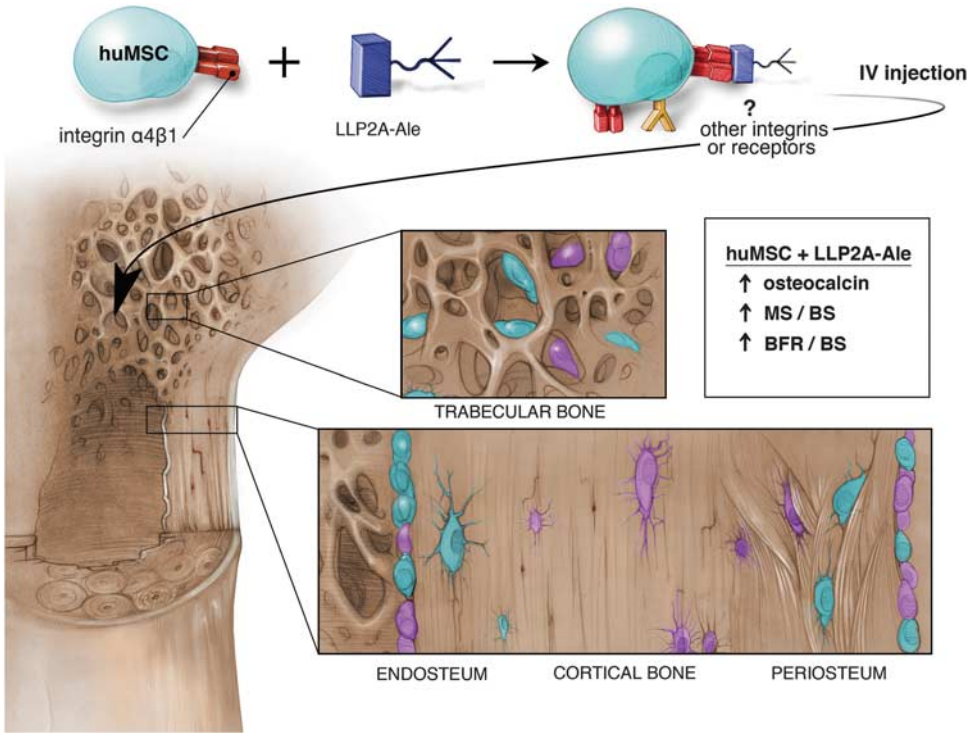

Figure 1 Exogenous human, or murine, mesenchymal stem cells (MSCs), when co-transplanted with LLP2A–Ale were directed to bone-forming surfaces on trabecular and cortical bone; both to endosteal and periosteal surfaces. The combined treatments demonstrated homing and engraftment of the MSCs, and their integration into newly formed bone as osteocytes, through at least 3 weeks post transplantation. In addition, serum osteocalcin and morphometeric measures showed increased bone formation compared with controls lacking LLP2A–Ale. Without LLP2A–Ale, the intravenously (IV) injected exogenous MSCs were not detectable in the bone marrow or bone, suggesting a lack of engraftment.

However, a number of questions remain to be answered related to MSC transplantation with these novel ligands. Do MSCs co-transplanted with LLP2A–Ale mainly home to the bone, or are the majority of cells still entrapped in the microcapillary beds of the lungs, or other tissues, as typically seen with MSCs? Do the newly transplanted MSCs remain engrafted beyond the 2 months that have been achieved with other transplantation methods? If the MSCs do not also engraft in the stem cell niche, will it require multiple transplantations to maintain bone growth? Would MSCs treated with LLP2A, without alendronate, target bone marrow? As all of the transplantation work was performed in young mice, will this approach also work in older mice with a different bone marrow microenvironment?

Importantly Guan *et al.*[1] also tested simple injection of LLP2A or LLP2A–Ale in three types of immunocompetent mice. The effect on bone formation in 2-month-old 129SVJ female mice with LLP2A–Ale was similar to that seen when MSCs were injected together with LLP2A–Ale (**Figures 1 and 2**). Both serum (osteocalcin) and morphometric measures (microtomography for trabecular bone volume, Ob/BS, MS/BS and endosteal (ec) BFR/BS), as well as biomechanical testing, suggested that the injection of LLP2A–Ale significantly increased bone formation and bone 'quality', at least on the interior endosteal surfaces. Significant changes to the periosteal bone were not in evidence with the ligand-only treatment. Whether this was due to differences in periosteal MSC or osteoblast responses to the ligands, or difficulty in tracking endogenous mMSCs is not known. Two of the mouse models used were to determine if LLP2A or LLP2A–Ale could prevent, or reduce, bone loss seen with ageing. These included C57BL/6 male mice between 2 and 4 months of age, where bone mass and formation are expected to decline 50%, and 2.5-month-old ovarectomized C57BL/6 female mice followed over the course of 6 weeks. A single injection of LLP2A–Ale appeared to stave off bone loss in both 'ageing' models. Because other research groups use 3- to 6-month-old C57BL/6 mice as 'young adults' and see measures of bone formation still increasing until at least 12 months, it would be useful to see these experiments repeated in older mice.[15,16] Irrespectively, these studies strongly support that LLP2A–Ale can direct endogenous MSCs to the bone surface and drive new bone formation. Possibly, the injected LLP2A–Ale is engaging MSCs within the stem cell niche and inducing them to proliferate, differentiate and migrate to the bone surface *in vivo* in a manner similar to that suggested by the *in vitro* studies. *In vitro* LLP2A–Ale increased Runx2 and Bglap expression, CFU-Ob counts, Alizarin red-positive mineralization and transwell migration of MSCs.

The authors suggest that because there were no frank bone architectural changes with LLP2A, it is possibly having its effect on osteocalcin and Ob/BS mainly through osteoblasts, rather than MSCs. However, it is not obvious why LLP2A would not have the same effect on MSC $\alpha 4\beta 1$ signaling, and any subsequent action on MSC proliferation, differentiation or migration as LLP2A–Ale, unless localization to the bone surface ECM microenvironment is critical for other signaling pathways. Possibly additional treatments and time are needed to see an unambiguous effect on bone formation for LLP2A, as it would not be predicted to directly link the MSCs or osteoblasts to bone.

Again, excitingly, the work of Guan *et al*[1] suggests that treatment with the ligands LLP2A or LLP2A–Ale might drive

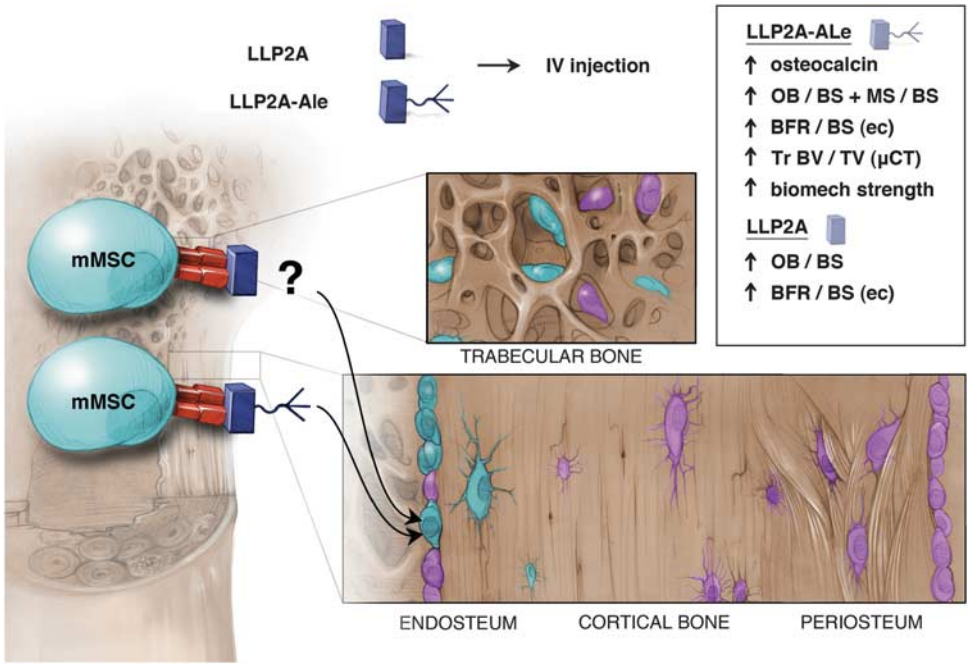

Figure 2 Serum, morphometric measures and biomechanical testing, demonstrated that the intravenous (IV) injection of LLP2A–Ale significantly increased bone formation and bone 'quality' on the endosteal (ec) surfaces. This is thought to be due to LLP2A–Ale binding to the $\alpha 4\beta 1$ integrin receptors on endogenous murine mesenchymal stem cells (mMSCs), as well as linking them to the bone-forming surfaces. However, it did not appear that there was bone growth at the periosteal bone surface. LLP2A, which lacks the conjugated bisphosphonate alendronate showed some limited increase in bone formation, but not to the extent seen with LLP2A–Ale. Abbreviations: Alendronate (Ale); biomechanical stress and load (biomech strength); bone-forming rate per bone surface (BFR/BS); human mesenchymal stem cells (huMSCs) labeled blue; mineralizing surface per bone surface (MS/BS); microtomography for trabecular bone volume (Tr BV/TV); osteoblast surface per bone surface (Ob/BS).

endogenous MSCs and osteoblasts to increase bone formation. It is clear that their $\alpha 4\beta 1$ ligand treatment can strongly induce acute bone formation; although it still has to be shown if this can be sustained over a longer period of time in chronic disease states. Critical questions remain, such as whether LLP2A–Ale treatment ultimately might reduce long-term osteogenesis due to depletion of the MSC niche population as a consequence of driving MSC differentiation and movement to bone without increased MSC proliferation in the stem cell niche. Alternatively, this treatment might supplement the MSC population by enhancing MSC proliferation prior to differentiation. Regardless, both the potential of ligand-enhanced MSC transplantation, or endogenous MSC/osteoblast-driven osteogenic induction is of great import. They appear to be superior approaches compared with current pre-clinical models of genetic, or cytokine manipulation of MSCs based on near-term feasibility and costs. Because LLP2A–Ale promotes remodeling, and increases biomechanical bone quality, it could obviate the need for the clinical use of anti-resorptive therapies for osteoporosis with their negative side effects. Or it could be integrated into a bisphosphonate therapy to reduce the risk of those side effects. Further, targeting ECM receptors with mimetic ligands has great potential not only for $\alpha 4\beta 1$ integrins but also for other ECM receptors as well. If this initial work holds up in additional models of ageing, for both males and post-menopausal females, it opens a new avenue for enhancing bone formation not only in osteoporosis but also in numerous bone defect models.

Conflict of Interest

The authors declare no conflict of interest.

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