Comparative Analysis of Thrombopoietin (TPO), a Novel Agent to Heal Segmental Bone Defects, with Bone Morphogenetic Protein-2 (BMP-2): A Hypothesis-Generating Transcriptomic Study

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Background/Purpose: Segmental bone defects (SBDs), typically resulting from high-energy open fractures or infections, frequently have poor regional soft tissue and vascularity. Successful treatment typically requires composite interventions that restore soft tissue, reconstitute regional vascularity, and augment SBDs with autograft or synthetic agents. BMP-2, a mesenchymal cell stimulant, has emerged as the primary synthetic compound for SBD healing. We have developed an alternative compound, TPO, to heal SBDs. In contrast to BMP-2-mediated mesenchymal stimulation, TPO stimulates hematopoietic stem cells. Our preliminary studies have shown that TPO-mediated hematopoietic stimulation effectively heals bone, but also stimulates muscle healing and angiogenesis. Furthermore, phenotypic features of SBD healing stimulated by TPO demonstrate robust thickened cortical mature bone in the defect (Figure 1a), in contrast to secondary woven bone typically seen with BMP-2. These observations indicate that TPO-stimulated angiogenesis plays a central role in its bone formation. Collectively, TPO's expanded therapeutic footprint makes it an ideal agent for SBDs with compromised vascularity. The purpose of this study was to compare basic biologic signaling effects of TPO and BMP-2 on marrow cells using a computational biologic approach. We hypothesized that TPO and BMP-2 augmented transcriptomes would demonstrate fundamentally different signatures. We anticipated that both TPO and BMP-2 would augment networks of transcripts directly involved in bone healing, but increased concentrations of transcripts that code for muscle healing and angiogenesis would be measured in TPO-stimulated cells.

Methods: Femoral bone marrow cells were collected from 12 C57/BL6j mice and cultured in presence of BMP-2 (200 ng/mL), TPO (10 ng/mL), or saline as a negative control for 3 days (n = 4/group). Post treatment, cells were retrieved in Trizol, and mRNA was extracted and converted to cDNA. Gene expression analysis was done with high throughput dual-dye cDNA microarrays (Agilent). Pairwise t test with $P < 0.005$ found 756, 1033, and 2488 transcripts differentially expressed between the BMP-2 vs. saline, TPO vs. saline, and BMP-2 vs. TPO treatment groups, respectively. The genes were functionally annotated using a host of Systems Biology tools, including Ingenuity Pathway Analysis (IPA) and DAVID. Expression levels of select genes relevant to this study were validated using quantitative PCR (polymerase chain reaction) method.

Results: Principal component analyses revealed that 98% of variation between TPO and BMP-2 were accounted for within the first two principal components, confirming that treat-

ment differences were captured by our analyses. TPO-stimulated cells showed an expanded signature of transcripts for a variety of growth factors that augment muscle healing, angiogenesis, and wound healing compared to BMP-2 treated cells (Figure 1b). In addition, TPO preferentially augmented transcription for networks of proteins located in the cell membrane. TPO-stimulated cells had particularly enriched genomic clusters that code for tubulin and actin, both cytoskeleton constituents, and plasma-membrane bound integrins suggesting that TPO plays an undiscovered role in orchestrating cellular mechanotransduction through the cell membrane into the cytoplasm.

Conclusion: TPO is a novel bone-healing agent with ubiquitous healing effects. Our initial studies have shown that TPO is effective in healing SBDs, and subsequently we have demonstrated that TPO also has potent effects on muscle healing and angiogenesis. The results from this study indicate that TPO may be an ideal agent to treat SBDs with poor adjacent soft tissue and compromised vascularity. Physiologically, TPO primarily stimulates hematopoietic tissue, which is responsible for initiating and orchestrating wound-healing in all injured tissues. Our study demonstrated that stem cells stimulated by TPO produced an expanded signature of growth factors and healing factors. These results, in concert with our foundational experiments, support trials to explore TPO efficacy in healing SBDs with poor soft tissue and compromised vascularity (ie, infection, open fractures).

See pages 47 - 108 for financial disclosure information.

The FDA has stated that it is the responsibility of the physician to determine the FDA clearance status of each drug or medical device he or she wishes to use in clinical practice.

Figure 1a

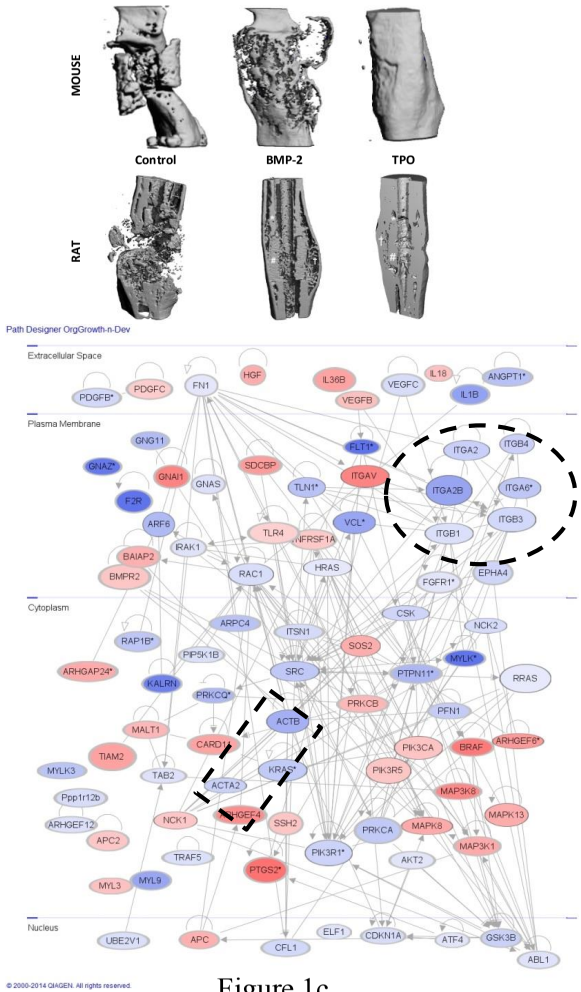

Figure 1c

Figure 1b

Biologic Function	Transcripts Upregulated by BMP	Transcripts Upregulated by TPO
Cytokines	- IL-4, IL-10, IL-22 signaling pathways; TGF-beta (primarily anti-inflammatory)	- IL-1, IL-3, IL-8, IL-16 and IL-17 signaling pathways (primarily pro-inflammatory)
Immune Response	- PKC8 signaling in T lymphocytes - CD28 signaling in T helper cells (mainly Bcell and Tcell receptor signaling)	- Leukocyte extravagation - Granulocyte adhesion (inflammatory cell peripheral adhesion)
Growth Factors	- EGF; ErbB; TGF-beta	- VEGF ; IGF-1; FGF; GM-CSF - Angiopoietin; Erythropoietin; (highly angiogenic and wound healing)
Cell Growth and Development	- Wnt/beta-catenin signaling - Mouse embryonic stem cell pluripotency	- Integrin signaling - Gap junction signaling - Epithelial adhesion junction
Skeleton and Muscle Development and Function	- Osteoblast differentiation (bone formation)	- Bone morphology - Muscle formation (balanced muscle and bone formation)

Figure 1 a – c: Rat and mouse SBDs treated with TPO invariably showed thickened mature cortices compared to abundant woven bone in BMP-2 treated specimens (Figure 1a) TPO stimulated cells demonstrated an expanded envelope of growth factors that affected angiogenesis and wound healing (Figure 1b red dashed circle). Fundamental differences in inflammatory cytokines and immune response were also detected. Representative pathway map (Figure 1c) of growth factor signaling demonstrates that TPO-enriched pathways (blue shaded) preferentially coded for proteins that are located in the plasma membrane. Note transcripts for proteins associated with mechanotranduction in the plasma membrane (integrins; dashed black circle) and corresponding cytoplasmic cytoskeletal components (actin; dashed black box).