Skeletal Development in Embryonic Stem Cell 3D Aggregates
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Introduction
Skeletal development is a complex process governed by a sequence of temporal and spatial cues, thus a deeper understanding of the underlying differentiation events can provide valuable guidance for skeletal tissue regeneration. Due to their pluripotent differentiation ability, embryonic stem cells (ESCs) are a useful tool to investigate various morphogenic processes including bone and cartilage. Stem cells (ESCs) are a useful tool to investigate various morphogenic processes including bone and cartilage formation in vitro. The objective of this study was to establish a model of osteochondral development using 3D ESC aggregates in combination with biochemical cues and mineral particles and apply this model to examine the temporal morphogenetic process during osteo-chondrogenic differentiation.

Materials and Methods
Uniform 3D ESC aggregates were formed by forced aggregation of mouse D3 ESCs in 400x400 μm AggreWell™ microwells. Hydroxyapatite microparticles (MPs) coated in a modified simulated body fluid solution were simultaneously incorporated into some ESC aggregates at the time of formation. After 24 hours, ESC aggregates (~1000 cells each) with or without MPs were cultured on rotary orbital shakers (45 rpm) in serum-free (SF) media (+N2/B27) or serum containing (SC) media (+15% FBS) or for 5 days of pre-mesoderm culture (Fig. 1A). An additional 28 days were assessed under different culture conditions after 33 days of culture.

Results
A temporal gene expression of growth factors by ESC aggregates in response to βGP+VitC+Dex treatment was observed during the course of culture (Fig. 1A). The relative early expression of pro-chondrogenic growth factors (tgfb1s) and later expression of pro-osteogenic growth factors (bmps) were in accordance with the expression pattern of chondrogenic markers (col2) and osteogenic markers (col1), respectively. βGP treatment led to significant increases in the expression of osteogenic genes (P<0.05) as well as the mineral deposition as indicated by alizarin red staining compared to basal culture (Fig. 1B). Additional VitC and Dex did not affect βGP-induced osteogenic differentiation, but promoted chondrogenic differentiation as evidenced by the formation of positive safranin o stained cartilage-like structures (Fig. 1B). Compression testing also revealed a time-dependent increase in the bulk stiffness of ESC aggregates with the highest value found in the βGP-treated group (P<0.05). Interestingly, a significant decrease of mineralization but increase in chondrogenesis was observed when these chemical cues were supplemented to SF media compared to SC media (Fig. 1C). Furthermore, MP incorporation alone increased the expression of osteogenic and chondrogenic markers as well as the mineralization of ESC aggregates (Fig. 1D) to the extent similar to those induced by chemical cues in SC culture (Fig. 1B). In combination with chemical cues, MPs induced a further increase of osteo-chondrogenic differentiation compared to MP alone in SC culture.

Discussion and Conclusions
This study indicates that 3D ESC aggregates can recapitulate various aspects of skeletal development in terms of temporal expression of osteogenic and chondrogenic markers and morphogens as well as the progression of structural remodeling. Our results also reveal the distinct roles of different chemical and material cues in the osteogenic and chondrogenic differentiation process. The unique features of pluripotent 3D ESC aggregates make it a desirable model to explore the skeletal development as well as examine tissue engineering strategies for skeletal regeneration.