Spatio-temporal expression of ChM-1 and its role in steering cartilage formation and angiogenesis in bone healing

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INTRODUCTION: The mechanisms driving cartilage development from mesenchymal cells to hypertrophic chondrocytes during skeletogenesis have been under intense research [1,2]. In skeletal development, the differentiation of chondrocytes into hypertrophic cells is followed by the invasion of blood vessels, promoting cartilage to bone remodeling known as endochondral ossification. This process is also assumed to occur during fracture repair. In development, it has been observed that blood vessels within the mesenchyme undergo regression, creating a hypoxic environment. This hypoxia, following vascular regression, upregulates HIF1alpha, a factor known to promote the expression of Sox9 and chondrocyte differentiation [3]. In previous work, we demonstrated that similar to developmental processes, in bone healing blood vessels in a fully vascularized gap undergo regression in regions concurrently developing cartilage. However, the specific factors that drive this regression remain elusive. Chondromodulin-1 (ChM-1) is a glycoprotein known for its dual properties of promoting cartilage formation while also being anti-angiogenic. ChM-1 exerts its inhibitory effect on angiogenesis by inhibiting migration and tube formation of endothelial cells (ECs). We hypothesized that ChM-1 is key in steering cartilage formation and angiogenesis during bone healing. Here, we validated the spatio-temporal expression of ChM-1 during both the early and later phases of fracture healing and its spatial correlation to vascular structures.
METHODS: In this study, we employed a mouse osteotomy model where we used two different fixators: a rigid one that led to successful healing within 21 days, and a semi-rigid fixator that caused a substantial delay in healing within the same timeframe. The femur osteotomies were conducted as previously described [4,5] and bone samples were collected either at 7 or 14 days after the osteotomy. We employed two mechanically distinct fixation systems (RISystem) with either a rigid, fast healing or a semi-rigid, delayed healing fracture fixation. All animal procedures were conducted in accordance with the approval of LaGeSo, Berlin, Germany (#G0322/18). Histological and immune-histological analyses were conducted to investigate the progression of vascular development, the organization of collagen networks, and cartilage development and maturation at both time points post-osteotomy. Progression of vascular development was analyzed using the EC markers, Emcn and CD31; fibrillar collagen by using second harmonic generation (SHG) imaging with confocal microscopy. Finally, the mouse monoclonal antibody ChM-1 (#sc-365693) was employed to investigate the temporal and spatial expression patterns of ChM-1 during the early and advanced phases of bone healing. All images where quantified using histomorphometric measures of proximal bone marrow region separated from the osteotomy gap region.
RESULTS: At seven days post-osteotomy, with the use of rigid fixation, we observed complete vascularization of the gap. The proximal half of the gap was filled with a collagen matrix, with the distal half of the gap still being filled with a hematoma (Fig. 1A). At 14 days under rigid fixation, we observed the development of cartilaginous tissue at the distal side of the gap, flanking the cortical regions (Fig. 1B). This marked the onset of endochondral ossification and the beginning of a second phase of angiogenesis. This region had become avascular, indicating a regression of the vascular structures within it. We hypothesized that the presence of ChM-1 may be linked to this observed vascular regression. To investigate ChM-1 expression within the osteotomy gap and surrounding soft tissue, we utilized osteotomy gaps stabilized with a semi-rigid fixator, as this fixation method provokes even more cartilage development. At the 7 day time-point, we noted a co-occurrence of ChM-1 expression with the vascular structures in the proximal bone marrow cavity (Fig. 2A,a). However, no ChM-1 expression was found in the osteotomy gap (Fig. 2A,b) or adjacent soft tissue from which vessels invade the gap (Fig. 2A,c). In contrast, at 14 days, ChM-1 expression was absent in vascularized areas at the proximal bone marrow side (Fig. 2B,a) but became notably prominent in developing avascular cartilage islands in both, the gap (Fig. 2B,b) and surrounding callus region (Fig. 2B,c).
DISCUSSION: Our study revealed the expression of ChM-1 at sites of angiogenesis during early bone healing. Notably, ChM-1 expression was largely absent in the osteotomy gap, which primarily consisted of granulation tissue at this point. In contrast, as the healing process progressed to later stages, ChM-1 expression was predominantly in regions associated with cartilage development, while being absent in areas with an established vascular network. These results suggest that expression of ChM-1 in vascular regions prior to the onset of cartilage development may play a critical role in the regression of blood vessels. This may occur by inhibiting EC migration and disrupting tube formation, which in turn paves the way for cartilage formation in these regions.
SIGNIFICANCE/CLINICAL RELEVANCE: The dynamic role of ChM-I throughout the healing process holds promise for therapeutic applications in steering angiogenesis and cartilage development to optimize the healing outcome. It presents implications for orthopedics, regenerative medicine, but also for the treatment of angiogenesis-related disorders.
REFERENCES: [1] Kozhemyakina and Zelzer et al., *Development* 2015; [2] Stricker and Mundlos et al., *Developmental Biology* 2002; [3] Amarilio and Zelzer et al., *Development* 2007; [4] Kruck and Willie et al., *J Bone Miner Res* 2018; [5] Hoerth and Wagermaier et al., *J Mech Behav Biomed Mater* 2018
IMAGES AND TABLES:

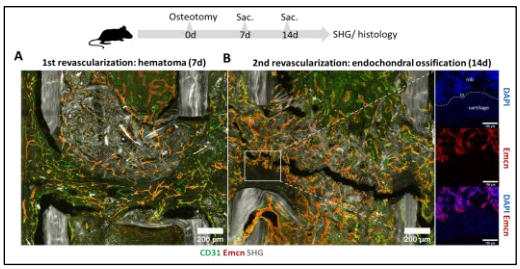

Figure 1: A. Representative image of an osteotomy gap 7 days post-osteotomy stabilized with a rigid fixator. Blood vessel visualization was conducted using Emcn and CD31 staining, combined with second harmonic generation (SHG) imaging to visualize fibrillar collagen. The gap was fully vascularized with a collagen matrix, predominantly on the proximal half of the gap. **B.** At 14 days post-osteotomy, a zoom-in view of the region where endochondral ossification is initiated was captured. Notably, vessels at the site of cartilage development vanished.

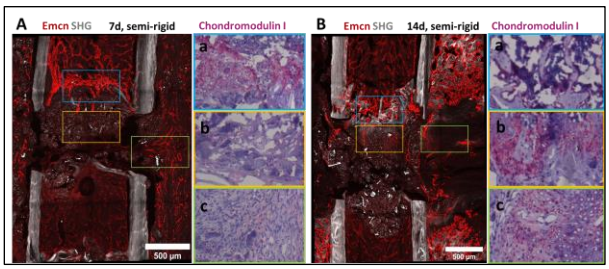

Figure 2: Representative image of osteotomy gaps 7 (**A**) and 14 (**B**) days post-osteotomy, respectively, stabilized with a semi-rigid fixator. Blood vessel visualization using Emcn staining was conducted, combined with second harmonic generation (SHG) imaging to visualize fibrillar collagen. Zoom-in images show ChM-1 staining (magenta) in distinct areas. **A**. At 7 days post-osteotomy, ChM-1 is expressed at sites of angiogenesis (A,a), while being absent in the osteotomy gap (A,b) and surrounding tissue (A,c). **B.** At 14 days post-osteotomy, ChM-1 is absent at sites of angiogenesis (B,a), but prominently expressed in the avascular regions (B,b and B,c). This suggests its regulatory role in preventing blood vessel growth, creating an avascular zone conducive to cartilage development.