In vitro studies of the role of mechanical cues in skeletal patterning and differentiation

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2014

document version
Publisher's PDF, also known as Version of record

Summary

During embryonic development, tissues obtain their specific shapes and patterns through dynamic and complex morphogenetic processes. The skeletal system consists of rigid bony elements and flexible cartilaginous structures. Their patterns are highly functional, such as the alternating pattern of bone and cartilage of the spine, which provides stability and flexibility to the vertebrate body. These skeletal patterns are determined during early development. Both bone and cartilage are derived from a common stem cell source, and their development involves a tightly orchestrated process of pattern formation and stem cell differentiation. The first step in this process is mesenchymal condensation. Condensations are characterized by a local physical clustering of mesenchymal cells, and are believed to induce differentiation. It is an essential step in skeletal development, as this determines the location and pattern of future skeletal elements. Despite growing insight into the role of molecular signaling pathways in regulating the process of early skeletal development, the impact of mechanical cues is largely unknown.

Skeletal development takes place in a mechanically dynamic environment. Individual cells exert forces on the surrounding matrix and/or neighboring cells, and distinctive tissues grow at different rates. Collectively, such cell-generated forces and differential growth rates can generate large tissue deformations and stresses. Their net effect is influenced by a set of mechanical boundary conditions, such as the proximity or adherence to neighboring tissues. The role of these mechanical cues in early skeletal patterning and differentiation is poorly understood, partly due to a lack of interdisciplinary model systems.

The general aim of this thesis is to investigate the role of cell-mediated contraction, growth-induced stretch, and geometric boundary conditions on skeletal stem cell differentiation and patterning *in vitro*, by developing novel cross-disciplinary model systems combining tools from developmental biology and engineering.

In **chapter 2**, a three-dimensional cell-gel model was used to investigate the effect of cell-generated forces on skeletal progenitor cell differentiation and patterning thereof. Mesenchymal stem cells were embedded in a collagen matrix, which they collectively contracted while subjected to inhomogeneous boundary conditions. The experimental model was compared with a computational model to predict the stresses generated in the construct during contraction. It was found that contraction under these conditions led to a significant shape change of the construct as well as an inhomogeneous stress distribution that changed over time. This was shown to result in patterned osteogenic differentiation, independent of osteogenic factors in the culture medium. The regions of distinct osteogenesis correlated with regions that were predicted to have experienced relatively high shear stress at any time during contraction. Together, this indicates that the interplay of cell-mediated contraction and mechanical boundary conditions alone can result in patterned differentiation. Also, the results suggest that a transient exposure to a high-stress environment might be sufficient to induce lineage commitment. This emphasizes the importance of the role of cell-mediated contraction and boundary conditions in creating a microenvironment that changes in time and space, which can lead to patterning differentiation. Such insights into the cues that regulate skeletal progenitor cell

differentiation and patterning could be valuable for skeletal tissue engineering strategies.

Disease, trauma, and aging can lead to damage and degeneration of the skeletal elements, resulting in a significant socio-economic burden. Skeletal tissue engineering strategies aim to regenerate functional bone and cartilage in the adult body. In order to improve tissue-engineering strategies, it could be useful to look at the robust morphogenetic process of skeletal development. Aspects of this process can be modeled *in vitro* by the micromass assay, where embryonic skeletal progenitor cells are cultured in a high-density drop. In **chapter 3**, we specifically address what lessons the skeletal tissue-engineering field can learn from this model system. Embryonic skeletal progenitor cells from different anatomic locations as well as from different developmental stages could be compared in culture to assess intrinsic cellular differences and environmental cues that determine their commitment. As undesired chondrocyte hypertrophy and mineralization is a major issue in cartilage tissue engineering, genetic and molecular tools can be used in combination with the micromass to shed light on the signaling pathways that regulate chondrocyte hypertrophy. Also, the cues that guide patterning and boundary formation can be studied by combining the micromass with various engineering tools. Examples of such studies are performed in chapter 4 and 5 described below. The lessons that can be drawn from the micromass assay are limited by two major differences between developmental and regenerative skeletogenesis: cell type and scale. For example, adult progenitors do not spontaneously form mesenchymal condensations, which is key in skeletal development. Also, the mechanisms of tissue patterning need to be adjusted to the larger tissue engineering constructs. Altogether, fundamental insights gained from the micromass model could lead to a better mechanistic understanding of skeletal tissue (re)generation and can guide the improved design of tissue-engineering constructs.

Rapid growth of distinctive tissues in the developing embryo leads to growth-generated stresses and deformations. To address the effect of growth on early skeletal development, in **chapter 4** a novel culture model was developed in which embryonic skeletal progenitor cells in micromass culture are subjected to slow, growth-mimicking stretch. A total of 25% strain was applied during three different phases of culture, at the beginning, the middle or the end, to specifically address the effect of mechanical loading on the sequential stages of early skeletal development: proliferation, condensation and differentiation. It was found that growth-mimicking stretch at either time point did not affect proliferation and chondrogenic differentiation under the tested conditions. However, the number of condensations per unit area was affected by the timing of stretch, showing a decreased number per unit area when stretch was applied at later time points. When these results were corrected for the applied strain, it became clear that the number of condensations per unit original surface area increased only when stretched during the first 20 hrs, possibly indicating a sensitivity to stretch during the initiation phase of the condensation process. This suggests a role for the mechanically dynamic environment in skeletal patterning deformation.

Developing tissues are also subjected to a set of mechanical boundary conditions, such as attachment to specific structures or the geometric constraints set by neighboring tissues. The cell population that gives rise to the vertebral column for example is restricted to a long narrow space in the embryo closely surrounded by adjacent structures. In **chapter 5**, the effect of such geometric boundary conditions on the linear patterning of mesenchymal condensations was investigated. The micromass model was combined with a microchannel patterning technique that allowed for the culture of skeletal progenitor cells on long, narrow adhesive islands of varying width. It was found that the spacing between condensations increased on narrower islands, a phenomenon that could not be explained by cell availability alone. Also, the alignment of condensations and the overall chondrogenic differentiation was increased when subjected to tighter geometric constraints. When the pattern of condensations *in vivo* at the site of the developing vertebral column in chicken embryos was investigated, it was found to closely fit the linear correlation between inter-condensation distance and geometric constraints that was found *in vitro*. Together, these findings indicate that geometric boundary condition might play a role in skeletal patterning through a process of self-organization.

Taken together, this thesis addresses the role of mechanical cues in early skeletal development by employing novel cross-disciplinary cell culture models. Mesenchymal condensation is a key process in skeletal development *in vivo* and it was observed that mechanical cues such as growth-mimicking stretch and geometric constraints modulate their patterning *in vitro*. To better understand how mechanical cues affect condensation and subsequent differentiation, more insight into the molecular, cellular and physical mechanisms underlying the initiation and maturation of condensations is required. Insights into the cues that guide skeletal progenitor cell differentiation and patterning could greatly benefit the design of skeletal tissue engineering constructs. In future studies, a more detailed quantitative characterization of the dynamic stress/strain fields in the developing embryo would be required to improve current models. Furthermore, advanced techniques such as 3D printing and traction force microscopy could be useful to further exploit the cross-disciplinary models described in this thesis. Such approaches will provide a more profound understanding of the role of mechanical cues in skeletal development.