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General Discussion

During embryonic development, tissues obtain their specific shapes and functions through dynamic morphogenetic processes. The functional and highly conserved patterns of skeletal tissues, such as the alternating pattern of bone and cartilage of the vertebral column, are determined during early skeletal development and derived from a common cell source. This involves a tightly orchestrated process of pattern formation and skeletal progenitor cell differentiation, which take place in a mechanically dynamic environment, because cellular traction forces and tissue growth in combination with mechanical boundary conditions cause tissue-level stresses and strains. The role of these mechanical cues in early skeletal development is poorly understood, partly due to a lack in interdisciplinary model systems. The overall aim of this thesis is to investigate the role of cell-mediated contraction, growth-mimicking strain, and geometric boundary conditions on early skeletal development *in vitro*. This was done by developing novel cross-disciplinary model systems combining tools from developmental biology and engineering.

In the following, the results of the studies presented in this thesis are discussed in a broader context. Aspects addressed include the role of cellular traction forces in morphogenetic processes, the translation of mechano-biological studies to the challenges of tissue engineering, and the effect of growth and geometry on the patterning of condensations. Furthermore, a range of models and techniques are described that could be employed to improve our understanding of the mechanism of mesenchymal condensation and provide insight into the interdisciplinary character of skeletal development.

Cellular traction forces

Cells use their contractile machinery to exert forces - cellular traction forces - on the extracellular matrix and neighboring cells that they are adhered to. Cellular traction forces play an active, multi-faceted role in morphogenesis (1). On the tissue level, the collective contraction by a group of cells can lead to considerable stresses and deformation of the tissue. For example, tension generated by contractile forces in the lateral epidermis and amnioserosa in the Drosophila embryo is shown to be essential for dorsal closure (2, 3). As a result of these stresses and strains, the mechanical properties of the extracellular matrix can also be altered, which directly feeds back to the cell and its ability to contract the matrix.

In **chapter 2**, a three dimensional gel-cell construct was used to study the effect of cell-mediated contraction under inhomogeneous boundary conditions on mesenchymal stem cell differentiation and the patterning thereof. The experimental model was compared with a computational model to predict the stresses generated in the construct while it was contracted by the encapsulated MSCs. It was observed that contraction under inhomogeneous boundary conditions led to a significant shape change of the overall construct as well as time-varying inhomogeneous stress distributions. These results correspond to the notion that cell-generated forces can cause tissue-level strains and stresses, and that their net effects are influenced by the mechanical boundary conditions of the system. The inhomogeneous contraction was subsequently shown to lead to patterned osteogenic differentiation. Regions of eventual osteogenic differentiation were shown to correlate with regions predicted

to have experienced high stress for a limited amount of time during different phases of culture. This indicates that a few days in a high-stress environment might be sufficient to induce lineage commitment. This underscores the dynamic character of cell-mediated contraction and boundary conditions, causing the mechanical microenvironment to change in time and space, resulting in patterned differentiation.

Insight into the molecular mechanisms of cellular traction would enable to better understand the macroscopic effects of cell-generated forces. Reconstituted contractile networks have been used to study the molecular regulation of contraction in a highly quantitative manner. These studies have for example implicated that network contractility is regulated by local motor activity and cross-linker density (4). If one could extend such investigations to live cell cultures, it would be interesting to study how the expression pattern of such motor and cross-linker proteins is affected by mechanical cues like matrix stiffness and tissue-level stresses. This could provide insight into how contractility is regulated in time and space.

The direct cellular microenvironment is also known to affect the contractile behavior of a cell, and the cell in turn uses traction forces to probe and respond to the mechanical properties of the matrix, a process known as mechanotransduction (5, 6). For example, it was shown that traction-dependent integrin binding and adhesion ligand clustering regulates mesenchymal stem cell fate in 3D cell cultures (7). In line with our findings in chapter 2, which point out the dynamic character of the mechanical environment, it was recently shown that mesenchymal stem cells possess 'mechanical memory' (8). By storing information from past physical environments, they are sensitive to the amount of time they have been exposed to mechanical cues such as substrate rigidity (8, 9). This mechanical memory was found to affect stem cell fate through the YAP/TAZ transcription pathway (8, 10). Since these experiments have only been performed with adult mesenchymal cells, it would be insightful to investigate whether the same mechanotransductive mechanisms also apply to skeletal precursor cells in the embryo.

Collectively, cellular traction forces have the ability to alter the properties of the extracellular matrix. For example, it was quantitatively shown that fibroblasts embedded in a fibrin construct stiffen the fibrin matrix by a factor of 3 by generating contractile prestress (11). A recently described model that facilitates the study of active matrix remodeling by cellular traction forces constitutes arrays of mircotissues, consisting of collagen gels with embedded cells that contract around microcantilevers (12, 13). With this model, overall morphological changes, force generation, and cellular level matrix remodeling can be quantified simultaneously. This model could be further explored to generate a better understanding of how traction forces actively adapt the cellular microenvironment.

Translation to tissue engineering

Insight into the cues that regulate skeletal progenitor cell differentiation and patterning could be valuable for skeletal tissue engineering strategies, which aim to regenerate injured or degenerated bone and cartilage tissues in the adult body. For instance, as mentioned above, it was shown that cell-mediated contraction under specific

boundary conditions could guide patterned differentiation through the creation of dynamic stress and strain patterns (chapter 2). If we could quantify such effects, these insights could be used to guide the design of tissue engineering constructs and their boundary conditions to elicit controlled and desired differentiation and patterning.

In **chapter 3**, it was specifically addressed what the tissue engineering field can learn from a model that recapitulates early skeletal development *in vitro* using embryonic skeletal precursor cells: the micromass model. This assay can be performed with skeletal progenitor cells from different anatomic locations and different developmental stages. Hence, it could be used to investigate whether intrinsic cellular differences or rather environmental cues determine their commitment to bone versus cartilage and the distinctive skeletal patterns. The assay can also be performed with cells from transgenic embryos, which could shed light on the signaling pathways that regulate chondrocyte hypertrophy and other developmental processes. Viral gene transduction and molecular inhibitors can further enhance our insights by silencing specific genes during the morphogenetic processes. Results of such studies could guide the controlled prevention of undesired hypertrophy and mineralization in cartilage tissue engineering constructs. 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 and described in chapter 4 and 5 of this thesis.

When translating the results from developmental biology studies to skeletal tissue engineering applications, one has to take into consideration two important differences between developmental and regenerative skeletogenesis: cell type and scale. For example, adult mesenchymal stem cells do not spontaneously form mesenchymal condensations, while this is a key process in early skeletal development. Several engineering approaches, such as 3D cell-gel printing (14) or cadherin mimetic peptides (15), could be used to ensure the desired cell-cell signaling that is typically brought about by the high cell density in condensations. Also, tissue-engineering constructs are generally much larger than the developing embryonic tissue. This has consequences for the diffusion of oxygen and other molecules, and also patterning cues such as gradients of morphogens and substrate properties should be adjusted and optimized to account for the larger sized constructs.

The effect of growth

The developing embryo is growing rapidly. When two adjacent tissues grow at different rates, this results in growth-generated strains and/or stresses, which are thought to play a role in morphogenetic processes. For example, it is hypothesized that the segmentation of the paraxial mesoderm, which gives rise to the specific pattern of somites, is a result of differential strains between the segmenting mesoderm and the surrounding tissues (16). To address the role of growth-generated strain in 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 strain. This loading is applied for 20 hrs at three different time points during culture: at the beginning, the middle, or the end of the 60 hr

culture. Proliferation, condensation morphology, and chondrogenic differentiation were not affected by this loading regime. The number of condensations per unit area was similar for the non-stretched condition and the 0-20 hr stretch, but significantly lower for the 20-40 hr and 40-60 hr stretch condition. This observed decrease is likely due to the expansion of the area on which condensations had already formed, since the fold change (~1.25) corresponds to the percentage strain applied (25%). When the number of condensations is corrected for the applied strain, it becomes clear that the number of condensations per unit original surface area is increased only when stretch is applied at the first 20 hr of culture. This might suggest that the initiation of condensation is sensitive to growth-mimicking strain, similar to for example the continuous rearrangement of skin patterns of marine anglefish during growth (17). Alternatively, the eventually similar number of condensations per unit area for the non-stretched and the 0-20 hr stretch conditions might indicate that condensations start to form only at the end of the first 20 hrs of culture, when the total stretch was already applied. Further studies, for example by the application of additional stretch regimes, should provide better insight into the exact role of growth-mimicking stretch on the spacing and patterning of condensations. As the pattern of condensations is key in skeletal patterning *in vivo*, and the spacing *in vitro* is shown to be affected by the timing of stretch, these results suggest that dynamic tissue deformation can affect skeletal patterning.

Under these conditions, proliferation and chondrogenic differentiation are not directly affected by growth-mimicking strain. Skeletal progenitor cells *in vitro* are known to deposit abundant extracellular matrix, so the question rises whether the strain applied to the underlying substrate is actually sensed by the cells, and how this affects their response. Also, different developing skeletal structures *in vivo* might be subjected to different growth-generated stress/strain fields. For example, the skeletal elements of the vertebral column develop along the relatively stiff notochord, while surrounded by various other developing tissues, whereas the skeletal elements of the limbs develop away from the bulk embryo, closely surrounded by an ectodermal layer. The improved design of future model systems would benefit from a more detailed mechanical characterization of developing tissues. Tissue-level strains may also have a profound effect on extracellular matrix architecture and the remodeling thereof. Such strain-induced ECM remodeling has for example been characterized in *in vitro* fibrin-based tissue constructs (18), and can be further explored in models of early skeletal development.

The effect of geometry

The net effect of cellular traction forces and differential growth rates is also affected by the mechanical boundary conditions. For example, the development of a tissue is often spatially restricted within the embryo, as it is closely surrounded by neighboring structures. Geometric boundary conditions have the ability to induce patterned differentiation of mesenchymal stem cells in culture (19), and tissue geometry can elicit patterns of mechanical stress that regulate branching morphogenesis of mammary epithelium (20). In **chapter 5**, it was shown that geometric boundary conditions modulate linear patterning of mesenchymal condensations in micromass culture. Embryonic skeletal progenitor cells were cultured on long, narrow adhesive

islands using a microchannel patterning technique to mimic the geometric boundary condition set by neighboring tissues at the site of the developing vertebral column. With increasing island width, the inter-condensation distance, as well as the number of condensations per unit area, was found to decrease significantly. It was confirmed that these effects were not the result of differences in initial cell density or proliferation, as both parameters were similar across all island widths. These findings suggest a potential role of geometric constraints in regulating skeletal patterning in a process of self-organization.

Microcontact printing and microchannel patterning are widely used techniques to pattern single or groups of cells on 2D substrates (21). However, skeletal development is a three-dimensional process with three-dimensional geometric boundaries. Therefore, it would be insightful to study the process of early skeletal development in a more physiological 3D model system. In order to achieve such 3D boundary conditions, 3D printing techniques could be used to create small well-defined micro-tissues that recapitulate the essential geometry and size of embryonic tissues (14, 22).

In **chapter 5**, the linear correlation that was found *in vitro* between inter-condensation distance and geometric constraints was compared with the pattern of condensations *in vivo* at the site of the developing vertebral column in chicken embryos. The *in vivo* pattern along with its geometric constraints was found to match the correlation found *in vitro* quite well. To further evaluate this correlation, it would be interesting to study the linear pattern of condensations of the vertebral column in additional species with different embryonic dimensions in which the cell population is thus subjected to different geometric constraints.

Mesenchymal condensation: understanding the mechanism

In vivo, mesenchymal condensations determine the local pattern of skeletal elements and induce chondrogenic differentiation. It is thus key in skeletal morphogenesis. In chapter 4 and 5 of this thesis it was shown that mechanical cues such as growth-mimicking strain and geometric boundary conditions affect the pattern of condensations *in vitro*. However, it is currently poorly understood how exactly condensations are initiated and formed.

On the molecular level, various signaling pathways have been identified that are involved in the condensation process (23). For example, TGF-β has been shown to play an important role by stimulating FN production as well as its receptor (24), which in turn regulates the cell-adhesion molecule NCAM, which is associated with condensation (25). FN would also cause an increase in cAMP in cells within the condensation (26), which in turn would trigger cartilage-specific gene expression (23). Syndecan and tenascin are found to be associated with the determination of condensation size and boundaries (27). Surprisingly, knock-out of specific genes that are thought to be essential, such as NCAM (28) and tenascin (29), turned out to have little effect on condensation and skeletal development, potentially due to functional redundancy. This makes it difficult to interpret the exact roles of these pathways. Also, it is unclear what

triggers the initial onset of the signaling pathways involved in condensation at their specific locations.

On the cellular level, single cell tracking experiments performed *in vitro* have indicated that the increased cell density in condensations is not the result of active cell migration toward the condensation-center or increased localized proliferation (30). Instead it is thought that condensation occurs through passive cell movements and cellular rearrangements (31-33). However it is poorly understood what drives these motions. It has been hypothesized through mathematical modeling that the local increase in cell density could be a result of a positive feedback loop between traction forces applied at cell-matrix interactions, extracellular matrix concentration and associated ligand availability (34, 35), triggered by a mechanical instability (36). In a different model, it was hypothesized that localized degradation of hyaluronic acid, which is a hydrophilic polymer that caused osmotic swelling, by hyaluronidase enzymes brings the skeletal progenitor cells closer together, to the extent that intercellular traction forces come into play and create dense cellular aggregates (37). Experimental models are required to test the proposed hypotheses.

Further experimentation is needed to provide insight into the exact mechanisms of the formation of mesenchymal condensations and the patterning thereof, and in particular the role of mechanical stress. The role of cellular traction forces could be addressed in micromass culture by the use of molecular inhibitors against key components of the contractile machinery, such as blebbistatin which inhibits non-muscle myosin II or Y27632 which inhibits Rho kinase. However, as these drugs might also affect other cell behavior such as proliferation and migration, such experiments might be too disruptive. Instead, it could be insightful to map the cell-matrix and cell-cell traction forces by traction force microscopy (38-40), in combination with direct single cell tracking. It could then be investigated how these force maps change when critical cell-cell or cell-matrix adhesion molecules are blocked or overexpressed, when growth factors or enzymes are added to the medium, or when mechanical stimuli are applied. Such novel approaches that combine bioengineering tools with *in vitro* cultures of embryonic skeletal precursor cells will provide a better understanding of the mechanisms underlying mesenchymal condensation and the patterning thereof.

Towards a cross-disciplinary understanding: useful techniques

In vitro model systems aim to recapitulate a biological process, thereby mimicking key aspects of that process as well as possible. Consequently, if one is to investigate the role of mechanical cues in early skeletal development *in vitro*, such as the stress/strain fields caused by cell-generated forces and growth, it is important to know their magnitude as they exist in the developing embryo *in vivo*. However, direct measurements of such characteristics have been challenging due to the embryo's fragility, small size, difficult accessibility, and the rapidly changing structures (41). Accurate techniques have been developed to measure the viscoelastic properties of explanted embryonic tissues (41), but performing such measurements in the intact embryo remains challenging. Measuring stress and force production in the embryo has been mostly approached by laser ablation techniques (1, 2). A tightly focused laser beam is employed to destroy a single cell or cell-cell junction, and live imaging

is used to follow the cell and tissue movements in response to the ablation, the recoil-response, which can be used as an indication of the magnitude and principle direction of stresses in the original tissue. Apart from the notion that this technique is only semi-quantitative, an important limitation is that the recoil-response is determined by both actively generated forces and the mechanical resistance of the tissue. Additional measurements are thus required to determine the contribution of each of those effects. Particle tracking methods have been developed that circumvent the need of direct contact between a probe and the tissue. In particle tracking microrheology for example, the movement of microinjected nanoparticles is monitored to determine the viscoelastic properties of the embryonic tissue (42). Also, a micromanipulation technique has been described in Drosophila embryos, where ferrofluid was injected in specific locations of the embryo and magnetic tweezers were used to apply controlled tissue deformations (43). Recently, a promising method was developed to quantify local mechanical stresses in living embryonic tissues by injecting fluorescent oil microdroplets, whose shape deformation can be used to calculate the stresses that act upon it (44).

In addition to the characterization of the mechanical aspects of the developing skeletal tissues, the design of accurate model systems would also benefit from a detailed understanding of the anatomy at different stages of development. Apart from the classic sectioning and histological techniques, new techniques have been developed that allow for whole embryo imaging and 3D rendering, such as Optical Projection Tomography (OPT), which provides high resolution three dimensional anatomical data (45). As this technique can currently only be used on fixed embryos, it does not capture the real-time dynamics of the developing tissues. For this purpose other, increasingly quantitative, methods are being developed, such as the use of genetically encoded fluorescent probes or engineered nanoprobes in combination with recent innovations in for instance multiphoton and light-sheet microscopy (46).

Together these techniques could be exploited to gain a quantitative understanding of the mechanical properties, the overall growth and the specific tissue deformations of the developing skeletal tissues. This knowledge could then be used for the improved design of interdisciplinary model systems that aim to investigate the role of cell- and growth-generated stresses and boundary conditions on skeletal progenitor cell differentiation and patterning.

By fostering cross-disciplinary approaches and collaborations, this thesis and further investigations will provide a more profound understanding of the role of mechanical cues in early skeletal development and may guide the design of skeletal tissue engineering constructs.

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