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

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General Introduction

During embryonic development, morphogenetic processes give rise to a wide variety of shapes and patterns, creating the complex tissue structures and functionalities that enable life. Morphogenesis involves an intricate orchestration of molecular and cellular processes that interrelate to create progressive levels of organizational complexity. Understanding the creation of form and function in the developing embryo is a major and ongoing quest in biology, and despite major advancements in our knowledge, many aspects are still poorly understood. One of those aspects is the role of mechanics, in particular the role of stresses and deformations due to cellular traction forces, growth, and mechanical boundary conditions during early stages of development. In the early 20th century, D'Arcy Thompson was a major advocate of using the rules of mathematics and physics to describe the creation of form in the developing embryo in his book 'On Growth and Form' (1). However, after the discovery of the structure of DNA (2) and the subsequent emergence of genetic and molecular techniques, focus shifted to signaling pathways and transcriptional networks. Recently, the role of mechanics in morphogenetic processes has regained appreciation and stimulated insightful cross-disciplinary studies (3-5). For example, it was found that the forces resulting from differential growth rates of the gut tube and the anchoring dorsal mesenteric sheet lead to the reproducible looping pattern of the gut (6). Further, mechanical compression of mesenchymal cells due to antagonistic migratory factors from the adjacent epithelium results in cell-shape changes, which are shown to guide embryonic tooth formation in mice (7). The interdisciplinary nature of morphogenesis is becoming increasingly clear and investigations will greatly benefit from cross-disciplinary experimental approaches.

Cellular differentiation and pattern formation are two major components of morphogenesis, as they determine functionality and form. Take for example the vertebral column, with its characteristic alternating pattern of bone and cartilage. This robust pattern provides stability as well as flexibility to the vertebrate body and is highly conserved across species. During embryonic development, bone and cartilage are both derived from a common cell source through closely related differentiation pathways (8). Disruption of the normal process of differentiation and patterning can lead to severe skeletal malformations and concomitant health issues, as illustrated by various skeletal congenital conditions. For example, failure of correct patterning of the spine during early development can lead to the development of hemivertebrae, where the development of half a vertebral body results in lateral asymmetry (9). This leads to asymmetric growth and can result in severe scoliosis and associated neurological problems (10). Also, a rare mutation of the brachyury gene, which is a key player in axial differentiation and patterning, causes abnormal ossification of the vertebral bodies due to delayed regression of the notochordal canal in the developing spine (11, 12), leading to overall malformation of the spine. Although the investigation of genetic determinants and signaling pathways in skeletal development is rapidly advancing, the role of mechanical cues in skeletal tissue differentiation and patterning is still poorly understood, partly due to a lack of accurate model systems.

In this thesis, the role of specific mechanical cues in guiding cellular differentiation and pattern formation in the early stages of skeletal development will be explored by developing novel cross-disciplinary cell culture models. This chapter proceeds

with background on the process of skeletal development, the current knowledge regarding the role of mechanics in this process, and the models that are typically used to investigate skeletal differentiation and patterning *in vitro*. The specific aim of this thesis will then be described followed by an overview of the studies presented in the following chapters.

Skeletal development

During embryonic development, a range of skeletal tissues varying widely with regard to structure, composition and mechanical properties, is derived from the skeletal progenitor cells in the mesoderm. The majority of bone structures develop through endochondral ossification, where bone is created via a cartilage intermediate. As a first stage, skeletal progenitor cells form mesenchymal condensations, characterized by a local physical clustering of mesenchymal cells. This is an essential step in skeletal development, as this determines the location and pattern of future skeletal elements (13-15). The increased cell density in the condensations is accompanied by an increase in cell-cell contacts mediated through NCAM and N-cadherin, which is implied to induce the onset of chondrogenic differentiation (16-18). The induced chondrocytes deposit glycosaminoglycans and collagen type II matrix, and eventually become hypertrophic with the concomitant expression of collagen type X (19). The cartilage then starts to calcify and subsequently becomes vascularized, which allows osteoblasts to enter and replace the cartilage anlage by mineralized bone (8, 20). Stable cartilage, which exists in varying forms with distinctive structural organizations (21), is created through a similar process. However, instead of progressing towards the end-stage of endochondral ossification, the chondrocytes are prevented from proceeding into the hypertrophic fate, resulting in mature, stable cartilage.

Mechanics in skeletal development

The maintenance of mature skeletal tissue is highly dependent on mechanical loading, as bone continuously remodels in response to the magnitude and direction of imposed forces (22). Osteocytes within the bone tissue are mechanosensitive: in response to mechanical loading, they orchestrate the activity of bone-forming osteoblasts and bone-resorbing osteoclasts (23, 24). Furthermore, healthy homeostasis of articular cartilage as well the intervertebral disc is known to benefit from moderate dynamic loading (23-27). Mechanical cues have also been shown to affect embryonic development of the skeletal elements. In late stages of development, repetitive skeletal muscle contractions subject the developing bones to direct mechanical loading. The absence of such loading, through ablation of muscle contraction in developing chicken embryos, or specific neuromuscular disorders in human embryos, has been shown to severely affect normal bone development (28-32), indicating the importance of mechanical loading in that stage of skeletal development. Also, in developing zebrafish it was shown that increased swimming activity, which likely increases mechanical loading on the developing skeleton via increased muscle activity, results in an earlier onset of chondrogenesis and osteogenesis (33).

Early skeletal differentiation and patterning takes place in the absence of muscle contractions, where mechanical forces are generated through different mechanisms. First of all, individual cells may use their contractile machinery to exert traction forces

on the extracellular matrix and neighboring cells with which they are in direct contact (34) (Fig. 1A). Apart from probing their environment, which directly feeds back to their behavior, collectively these forces can generate tissue-level stresses (cell-generated stresses) and tissue deformation (cell-generated strains) (35-37) (Fig. 1B). A single cell's ability to exert traction forces was first observed when single fibroblasts were cultured on a soft deformable sheet (38), and their collective potential was shown by their ability to contract soft 3D matrices and control patterning on 2D substrates and in 3D hydrogels (39-42). Differential growth rates of distinctive tissues can also lead to tissue-level stresses and strains (Fig. 1). Such growth-generated stresses have been hypothesized for example to control the periodicity along the embryonic axis that leads to somitogenesis (43), and the physical cracking of the developing skin on a crocodile's head (44). Eventually, the net effect of cellular traction forces and differential growth is determined by a set of mechanical boundary conditions, such as the stiffness and cohesion of the tissue and the proximity or adhesion to other tissues. Together, these mechanical cues are hypothesized to play a role in early skeletal development (36, 37, 45) but well-controlled experiments are required to provide insight into their specific role in guiding skeletal progenitor cell differentiation and patterning.

Insight into the cues that guide skeletal development could greatly facilitate skeletal tissue engineering strategies (46, 47). There is a great need for such strategies that aim to regenerate functional bone and cartilage in the adult body, as damage, loss and degeneration of skeletal structures cause a significant disease burden, which is rapidly increasing due to our progressively aging population (48).

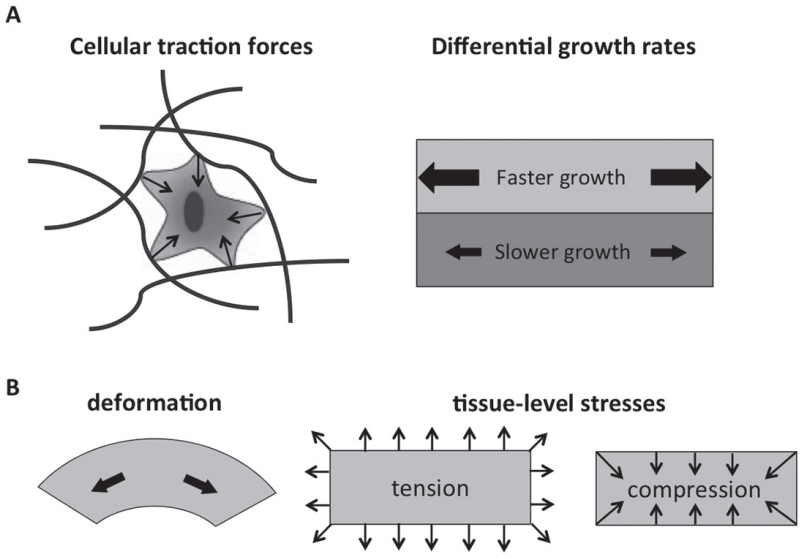

Fig. 1. Cellular traction forces exerted on the extracellular matrix, and differential growth rates of embryonic tissues (**A**) result in tissue-level deformations and tensional and compressive stresses (**B**).

Model systems

Over the years, a range of *in vitro* models has been developed that have enabled the study of skeletal progenitor cell differentiation and patterning, and the cues that guide or potentially disrupt these processes. Models focusing on mesenchymal stem cell (MSC) differentiation and patterning have become progressively more complex. The earliest investigations were performed on classic 2D tissue culture plastic and identified soluble factors that guide MSC lineage commitment (49). 2D hydrogel and rubber substrates enabled the study of the independent and interdependent effects of substrate rigidity, extracellular matrix composition, and ligand density, identifying important effects on MSC differentiation (50-55). Cell-patterning techniques allowed for the tight control of the shape and size of single cells and groups of cells, and such studies showed that MSC differentiation is modulated by cell shape and associated cytoskeletal tension (56-58). Moreover, the geometry of multicellular patterns was found to affect spatial patterns of proliferation and differentiation (59-61).

Mechanical loading devices have been used to investigate the role of explicit loading on cellular differentiation and patterning, showing for example enhanced chondrogenic differentiation under dynamic compressive loading (62-64). Three-dimensional hydrogel and scaffold systems were developed to study stem cell differentiation in a more physiologically relevant 3D environment, and include the ability to change the matrix elastic and viscoelastic properties, matrix composition, ligand presentation, pore size, and even the spatiotemporal presentation of morphogens and mechanical cues (65-70). All these *in vitro* studies have greatly enhanced our understanding of the cues that guide mesenchymal stem cell differentiation and patterning, however these investigations are mostly performed with adult mesenchymal stem cells. The role of mechanical cues in embryonic skeletal precursor cell differentiation and patterning is less well understood. This is partly because embryonic cells are more difficult to obtain for cell culture, and because of a lack of interdisciplinary approaches. The role of mechanical cues in embryonic skeletal progenitor differentiation and patterning could be addressed by developing new cross-disciplinary model systems that combine tools from developmental biology and engineering.

Aim of this thesis

During embryonic development cellular traction forces and growth cause considerable tissue-level stresses and macroscopic deformations. The role of cell- and growth-generated stresses and strains, as well as the mechanical boundary conditions, in guiding the onset of skeletal development is still poorly understood. The **specific aim** of this thesis is to investigate the role of cell-mediated contraction, growth-induced strain and geometric boundary conditions in skeletal progenitor cell differentiation and patterning *in vitro*, by employing novel cell culture models that combine tools from developmental biology and bioengineering.

In **chapter 2**, the effects of cell-mediated contraction on mesenchymal stem cell differentiation and patterning thereof are investigated. A three dimensional model is used consisting of a collagen matrix with encapsulated adult immortalized mesenchymal stem cells, which exert traction forces on their substrate under time-varying mechanical boundary conditions, leading to overall contraction of the construct. This experimental model is combined with a computational model

to predict the stresses generated in the construct, and these stress patterns are compared with the eventual spatial pattern of osteogenic differentiation.

Understanding the cues that guide skeletogenesis during embryonic development could be valuable for skeletal tissue engineering strategies. In order to study developmental skeletogenesis in a controlled manner *in vitro*, developmental biologist have established a particularly useful model that recapitulates early skeletal development using embryonic precursor cells: the micromass model (71-73). Typically, skeletal precursor cells are freshly isolated from mouse or chicken embryonic limb buds and cultured in a high-density drop. In culture, the cells undergo abundant proliferation, mesenchymal condensation and subsequent chondrogenic differentiation (72). In **chapter 3** the micromass model is described and it is investigated what lessons the tissue engineering field can learn from studying skeletal development using this model. This chapter focuses on two major differences between developmental and regenerative skeletogenesis, cell type and tissue size, and how those differences may limit the lessons that can be learned from studying skeletal development *in vitro*.

After establishing the potential of the micromass assay to study early skeletal development and the cues that guide the process, this assay is combined with bioengineering tools to investigate the effect of two specific cues on embryonic skeletal precursor cell differentiation and patterning: growth and geometry. In **chapter 4**, growth-induced strain is mimicked by applying continuous stretch to chicken embryonic limb bud cells in micromass. The effect of this growth-mimicking strain on proliferation, the morphology and number of mesenchymal condensations, and chondrogenic differentiation is investigated. In **chapter 5**, a microchannel patterning technique is employed to allow micromass culture of skeletal progenitor cells under strictly controlled geometric boundary conditions. This approach is used to study the patterning of mesenchymal condensations and subsequent chondrogenic differentiation under varying geometric constraints, mimicking the geometric boundaries set by neighboring tissues. Ultimately, the results from the *in vitro* model are compared with the *in vivo* pattern of condensations at the developing vertebral column in chicken embryos.

In **chapter 6**, the main findings of this thesis as well as their implications and limitations are summarized and integrated into a general discussion and a framework for future work.

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