6.1 Thesis summary

During somitogenesis, the paraxial mesoderm of the early vertebrate embryo segments from head to tail into blocks of cells, called somites. Somites are the building blocks of the segmented vertebrate body plan and their formation is used as a research model for morphogenesis, body plan patterning and mesenchymal-to-epithelial transitions (MET). Therefore, studying the differentiation and maturation of the embryonic tissues during somitogenesis gives us insights into the origin of congenital diseases, the evolution of vertebrate species, regulating cellular behavior during cancer progression, and helps to improve tissue engineering and cell therapy, aiming at the regeneration of damaged or lost tissues after disease or injury.

The most prevalent model of somitogenesis, the clock-and-wavefront model, describes the mesoderm segmenting by cyclic gene expression and signaling gradients. Next to chemical signals, mechanics is also essential to tissue formation and biomechanical cues can guide cell behavior and differentiation. However, most studies on somitogenesis do not consider mechanical factors and cannot yet explain the translation from the genetic segmentation in the unsegmented tail to the physical somite formation. The general aim of this thesis was to establish a possible role of mechanics in the segmentation of the vertebrate embryo.

For this, I used the chicken embryo as a vertebrate model, because it provides a practical, cheap and ethical way to study early vertebrate embryogenesis. Within this project, four studies were performed, with the following specific four objectives:

1. Define the potential for mechanobiology in somite formation and take a mechanical perspective for future experiments to further investigate this concept.
2. To develop an ex ovo culture method for chicken embryos that allows access for manipulation experiments and long-term high-resolution time-lapse imaging.
3. To explore the mechanical properties of early chicken embryos, to better understand the physics of tissue development during somitogenesis.
4. To assess the potential for mechanical cues to guide somitogenesis in the chicken embryo.
Objective 1: To define the potential for mechanobiology in somite formation and take a mechanical perspective for future experiments to further investigate this concept. It is still unclear how the genetic patterning in the presomitic mesoderm (PSM) is translated into the physical somite formation. In Chapter 2, we performed a literature review, in which we listed open questions in the clock-and-wavefront model and the mechanical changes before somite formation. Essentially, the genetic patterning and somite formation are two overlapping mechanisms, in which the patterning is a determined process and the formation seems to be a local, cellular self-organization. We proposed that a positive feedback loop might arise from the interplay between chemical and mechanical aspects of the rostral PSM that leads to increase in tissue tension, preceding somite formation. This feedback loop could lead to a physical phenotype of the PSM that is ready to undergo somite formation. Therefore, we suggested more studies to be focused on the quantitative aspects of somitogenesis: cell behavior, matrix buildup, mechanical behavior, stress patterns and geometry in the PSM, as they can determine how the cells cluster into somites. All these data can be evaluated for possible mechanical effects by mathematical modeling. Studies of in vitro engineered PSMs could be a fruitful route to unravel the mechanobiology of somite formation in the upcoming years. In these, we can combine new experimental techniques, such as PSM-derived cell lines, CRISPR-Cas9-mediated gene modifications, imposing mechanical stimuli, while monitoring the cells and matrix over time with quantitative imaging techniques, so that the mechanical environment of such PSM-cultures can be tested as an instructive component for segmentation.

Objective 2: To develop an ex ovo culture method for chicken embryos that allows access for manipulation experiments and long-term high-resolution time-lapse imaging. In Chapter 3, we developed an in vitro protocol that provided a stable environment to study early chicken morphogenetic processes, with long-term, high-resolution visualization in a widefield view. This was accomplished by adapting an existing filter paper carrier technique: we sandwiched chicken embryos between two filter paper carriers, cultured fully submerged in a simple culture medium (buffer and thin albumen), and covered with light mineral oil in a temperature-controlled container. We call our new in vitro culture method the 'submerged filter paper sandwich culture'. This culture technique is cheap and easy to set up as it bypasses
the need for a climate chamber with a heated lid. Also, it allows for long time windows to image high-resolution time-lapses of morphogenetic processes in chicken embryos, both ventral and dorsal, starting from the primitive streak stage (HH5) up to at least the 28-somite stage (HH16). The submerged filter paper sandwich provides a simple and stable environment for microsurgery, bead implantation, microinjection, gene silencing, and electroporation studies. Its imaging potential can be pushed much further by using laser-based imaging, including light-sheet microscopy and immersion objectives.

Objective 3: To explore the mechanical properties of early chicken embryos, to better understand the physics of tissue development during somitogenesis. Since cells are mechanosensitive, their migration and differentiation partially depend on their mechanical environment, such as contractile neighbor cells and the stiffness of the extracellular matrix. The stiffness of tissues could thus be a morphogenetic factor and is valuable information for understanding embryonic development. The early vertebrate embryo has a morphological age difference over its rostrocaudal axis this suggests differences in mechanical properties, but it is practically undocumented. In Chapter 4, we developed a novel combination of techniques that allows measuring the viscoelastic properties of embryos and set out to explore the potential stiffness inhomogeneities along the segmenting chicken mesoderm from caudal to rostral in embryos stage HH10-11 in vivo. For this, the filter paper sandwich culture was combined with a cantilever-based indentation method, in a self-built setup, monitored with optical coherence tomography. We demonstrated that this new protocol makes it possible to investigate the relationship between microscopic embryo structures and their dynamic mechanical properties. Also, we showed the first stiffness measurements on chicken mesoderm during somite formation, with high spatial resolution from the mesenchymal tail bud up to the epithelialized somites. The stiffness of the mesoderm ranges from 200 Pa in the tail to 700 Pa in the somites. The midline in the caudal PSM is the stiffest structure with 900 Pa, thereby mechanically supporting the surrounding tissues. The difference in stiffness between midline and presomitic mesoderm decreases when the mesoderm forms somites, but it still takes somite S0 to SIV for the somitic mesoderm to develop a higher viscoelastic response. The results show that major changes in stiffness are related to epithelialization. While there is variation in the absolute stiffness between individual embryos, the trends along
the anatomical positions appear similar. **Our method can reliably measure stiffness of 200 Pa and higher in embryonic tissues and allows investigating the correlations between local mechanical properties and tissue morphology in situ, with more precision than previous studies.**

**Objective 4: To assess the potential for mechanical cues to guide somitogenesis in the chicken embryo.** In Chapter 5, we assessed the potential for mechanical cues to guide somitogenesis in the chicken embryo. We hypothesized that if somitic mesoderm self-organizes under influence of biomechanical cues, as discussed in literature, mechanical stretching should then suffice to induce morphological changes in the segmented vertebrate body plan. To test this hypothesis, we developed a novel experimental setup to apply controlled strains to live chick embryos. This system combined the filter paper sandwich culture with the possibility to stretch developing chicken embryos in vivo at micrometer speeds, while visualizing changes in somite development by high-resolution widefield time-lapse imaging. **Our experiments showed that a physical cue, mechanical stretching, can induce the formation of additional somites in developing chicken embryos and thereby modify the early vertebral patterning.** Stretching of live chicken embryos deforms the somites, resulting in a slow cellular reorganization to form more well-shaped and stable daughter somites. **We concluded that the somitic mesoderm has self-organizing properties, and in this way, generates phenotypic plasticity under variations in the mechanical environment.** This phenomenon may provide a different mechanism towards the evolution of the large variation of vertebral numbers between vertebrate species, next to earlier proposed mechanisms involving mutations leading to changes in the segmentation clock period or axial growth rate. Additionally, these somite qualities can be selectively advantageous by preventing the formation of transitional vertebrae. Somites’ self-organizational properties provide a promising basis for further exploration into the physical component of somite formation and the possible role of mechanics in body-plan evolution.

Understanding the principles that underlie physical somite formation will contribute to a more detailed understanding of tissue development and stem cell differentiation, which is key to identifying defects underlying diseases and setting up tissue engineering therapies.