-Title page

The mechanical force with the physiological movement works as a biological cue in tendon development during the postnatal phase in mice.

running title: (**running title of 32 characters or less**) Role of force in tendon development

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2. Summary statement

Biological and morphological events of tendon development in the embryo to postnatal phase are related to external mechanical forces depending on the physical environment and limb movement patterns.

3. Abstract

The musculoskeletal system provides structural stability and coordinates to enable movement. The tendon has the important role of transmitting the muscle contraction force to bone and resisting an external force. It is well known that muscle contraction is required to maintain tendon differentiation. Although tendon always bears mechanical force from muscle and external, it is hard to evaluate them *in vivo*. In this study, we tried to define the mechanical force from an original point of view as a limb physiological environment. Here we showed that late-stage embryos have not enough space in the amniotic membrane and disturbed limb movement. In the neonatal phase, to get the physical ability of righting reflex and locomotion increases mechanical force for their limbs. We also clarified this relationship by detecting the expression of tendon developmental factors during the embryo to postnatal phase. We found that the upregulation of Scx and Tnmd is accompanied by the development of physiological movement with increasing mechanical force. Our results reveal the part of the role of mechanical force, including internal and external factors, in the tendon development process.

4. Introduction

The musculoskeletal tissues are generated to enable us to provide structural stability and coordinate joint movement. During the developmental process, the cells at the joint area were gradually organized in the embryo phase, regulated by the redundant biological process. Mechanical force is an essential factor as a switching cue of the biological phase translations. This mechanobiological mechanism is a recent remarkable topic in musculoskeletal development. Therefore, recent studies focused on revealing the role of mechanical force and capitalized on some kinds of transgenic mice in the great field of science. Especially several types of mice-modulated muscle functions were used in musculoskeletal development. The muscle is the one and only organ *in vivo* to generate the mechanical force on itself.

The tendons connect muscle and bone, so the tendon transmits the muscle contraction force to bone and drives joint movement. These functions also enable us to resist an external force. This means the tendon was always exposed to mechanical forces inevitably and had a specific developmental process during the embryo to postnatal phase. In tendon developmental research, some kind of mechano-biological key factors have been found, like a Scleraxis (Scx) (R Schweitzer et al., 2001), Transforming growth factor-beta (TGF-β) (Havis et al., 2016; Pryce et al., 2009) and Tenomodulin (Tnmd) (Docheva et al., 2005). These studies revealed that the tendon development process has three major processes; induction, differentiation, and maturation (V. Arvind and A. H. Huang, 2017), and led to paying attention to the relationship between the expression of biological factors and mechanical force. The initiating phase of tendon development is independent of muscle, however, in the late embryonic stage tendon requires mechanical force generated by muscle contraction (Brent et al., 2005; Eloy-Trinquet et al., 2009; Huang et al., 2015; R Schweitzer et al., 2001). Previous studies showed some evidence that muscle contraction is an essential factor in maintaining to regulate the expression of tendon-related factors. TGFβ in chick limbs during the differentiation phase was highly expressed (Havis et al., 2016). In the mice model, Pax3$^{spd/spd}$ mutants embryos that are muscle-less mice formed autopod tendon segments but failed to form zeugopod tendons. However, in muscular dysgenesis (mdg) mice embryos, the loss of muscle contraction to induce paralyzed succeeded zeugopod tendon formation (Huang et al., 2015). These results suggested that the muscle is necessary for tendon formation in the embryo and postnatal phases (Felsenthal and Zelzer, 2017; V. Arvind and A. H. Huang, 2017) because of the connecting tissue between the tendon to the bone. However, whether muscle contraction is an essential factor in tendon formation or not has remained.

The developmental process involves multi-biological organizations from the embryonic period to the early postnatal period. During this process, musculoskeletal organs are exposed to several types of mechanical forces. Among these, both muscle contraction and skeleton elongation were well investigated and led to activating the expression of several growth factors for tendon maturation (Havis et al., 2016). However, any kind of external mechanical forces also are applied to the limb musculoskeletal tissues. Between the embryonic and neonatal phases, the mechanical environment of neonatal mice is dramatically changed. In addition, the rapid development of behavioral abilities and reflexes begins after P0. In detail, at first, growth of muscle function occurred, then the postural orientation was changed and gradually began to move by itself based on the central nervous system maturation leading to improvement of motor function. Initial complex motor skills such as forelimb

grasping, placing of hindlimbs, standing, and self-grooming develop continuously over the first six days of leftfield (Brust et al., 2015). These dynamic changes in physical movement modulate the molecular response as a mechanical cue. Some previous studies in the tendon development field showed that muscle contraction was needed (Havis et al., 2016; Huang et al., 2015). However, little is known about whether the external mechanical force depends on postural changes and weight bearing in physical movement affects to tendon development likely to muscle contraction. Therefore, we hypothesize that the applied force, both muscle contraction and an external passive force that was generated by limb contact with the ground and weight-bearing, have an equal effect on the tendon developmental phase. However, the mechanical forces of the limbs are difficult to evaluate the qualitative properties and quantitative amounts for limbs *in vivo*. In this context, we focused on the relationship between mechanical force and physical movement. Recent studies focused on the motor function and behavior of the animals to assess the changes of mechanical force for limbs (S. K. Theodossiou et al., 2019; S. K.Theodossiou et al., 2021).

Moreover, even though muscle activity is inhibited, joint movement remains generated with limb contact and weight-bearing. It is impossible to exclude this external mechanical force applied to limbs in vivo study. This is important to redefine the role of mechanical force for limb development because many of the biological changes were reported only using the muscle dysfunction model. These models have been treated as a model for reducing mechanical force depending on muscle contractions, but the limbs were affected by joint movement by externally applied to the load. This is the "pitfall" to insights into the understanding of the mechanotransduction mechanism in tendon development. To address this hypothesis, we measured the righting reflex commonly used as the motor ability outcome for mice pups to flip onto their feet from a supine position. In this position, limbs weigh their own weight as a mechanical force. We used this test to determine when the pup's limbs were exposed to mechanical force by their weight. Before attaining the ability to right reflex, all limbs of pups moved only generated by the muscle contraction force. In rehabilitation medicine, these movements are called the Open Kinetic Chain movement (OKC) (Steindler, 1955) in the kinematic research field. Every mammalian showed this kind of movement at first. Then after attaining the righting reflex, every limb is exposed to mechanical force by their weight-bearing. In this phase, the limbs must generate the force to resist gravity and weight-bearing. These movements are defined as the Closed Kinetic Chain movement (CKC) (Steindler, 1955). Finally, during the initial locomotion phase, limbs expose both internal and external forces.

In this context, we hypothesize that both the mechanical force generated by muscle contraction and external force affects tendon development leading to a mechanobiological response. To address this hypothesis, we investigate the changes in mechanical force applied to limb development during the embryonic to the early postnatal period. Additionally, we also explore the relationship between the changing mechanical force and the expression of existing tendon-related factors and tendon morphology. The goal of this study was to reveal the role of mechanical force, including internal and external factors, in the tendon development process.

5. Results

5.1. Embryonic limbs don't move in the womb.

We observed embryonic limb movement by using ultrasound imaging. Embryos from E15.5 to E19.5 showed no spontaneous limb movement independent of their heartbeat (Fig.1, Supplementary Video1). The space in each amnion decreased with age since their body size increased, which was one of the limitations of limb movement depending on the womb environment. As a result, the embryos are forced under space limitations. (Fig.1D).

5.2. Limbs' movement pattern and gait pattern change with age.

Bodyweight gradually increased with age (Fig.2A). P0 mice were only in a supine position. However, the P0 mouse's limb motion is very few. After P1, they irregularly move their arms and legs in a spine position. This movement is OKC. Then they gradually gained the righting reflex, the motor ability to flip onto their feet from a supine position from P1 to P5. P5 mice became to success it three times. All mice were turned over on P7 (Fig.2B). After they got righting reflex, they could touch their limbs on load, which means their limbs moved in CKC. However, from P1 to P7, mice could not move hindlimbs joints under load and locomote in the prone position. Improvement in walking ability was recognized from P8 onwards(Fig.2C). The gait of P8 showed crawling with asymmetric limb movement. With increasing gait scores, they obtained a symmetric gait pattern with age. They could be up tail, head, and heal during ambition(Fig.2D).

5.3. ECM organization increases with age.

In Alcian Blue/HE stains, the length of the tendon became longer with time course, and the line of the muscle-tendon junction became remarkable. At E15, the cohesion of cells was observed at the site where tendon tissue will be formed, but no extracellular matrix (ECM) stained with eosin was observed at this time. At E17, the cell density increased, and the border between the tendon tissue and the surrounding tissue became clear. The tendon tissue didn't have a precise fibril arrangement in the embryonic period. It was a simple cell aggregation image, but in the tissue after P0, the tendon cells became elliptic and aligned in the long-axis direction (Fig. 3A). We detect collagen type 1 (col1) that the most abundant form of collagen in tendons in immunofluorescence staining. Col1 didn't show at E17.5. But we can see collagen type 1 after P1 and increased for P14(Fig. 3B). At P14, fibril formation progressed with the increased ECM in the long-axis direction, and the decrease of nuclei was confirmed. Like tendons, the muscle tissue had cell aggregation during the embryonic period, but at P3, the muscle-tendon junction became apparent, and the muscle tissue fiber arrangement was formed (Fig. 3A).

5.4. Gene expression tends to change in the developmental phase.

We performed qPCR to understand tendon-related gene expression with changing mechanical force associated with the physical environment or locomotion. We focused on tendon-related factors crucial for tenocyte differentiation(Scx), associated with Scx expression and induced by mechanical force(TGFβ2) and known as tendon maturation makers and matrix production(Tnmd).

The tissue at E15 was excluded from analysis because it was too immature to collect muscle and tendon tissue under the stereoscopic microscope. The expression levels of Scx gradually increased for P12, and the expression level of Scx displayed a higher level before P5 compere to E17.5 to P3. This tendency also showed in Tnmd. The highest expression of TGFβ2 was P5. But values of all time points were stable at the standard value of E17.5(Fig. 4).

6. Discussion

In this study, we identified the relationship between increasing the mechanical force for limbs with motor behavior development and tendon development in the postnatal phase. Although tendon always bears mechanical force, it is hard to evaluate them *in vivo*. For this problem, we defined mechanical force from an original point of view using a limb weight-bearing and movement. We clarified this relationship by detecting the expression change of some developmental factors during the early to mid-postnatal phase chronologically. Because the mice in this phase showed a dramatic change in their biomechanical environment for the limb, we found that the upregulated tendon-related factor while limb movement was gradually changed. The results of this study supported the hypothesis that mechanical force has a critical role in tendon development in the early postnatal phase. Especially external mechanical stress also induces the tendon maturation process is a novel point of view for mechanotransduction in tendon development. Understanding the true mechanotransduction mechanism in tendon maturation provides insights into tendon degeneration and the treatment after tendon injury.

6.1. Limb Movement and Motor Behavior Development in the Embryo to Early Postnatal Phase

We observed embryonic movement in the womb using ultrasound imaging. Previous studies have shown that muscle contraction is needed for tendon elongation during the embryo phase (Huang et al., 2015). However, our results showed that limbs didn’t move spontaneously during E15.5 to 19.5.

Previous works showed that the first innervation of the mouse limb appeared after E12.5 (Martin, 1990). Mouse embryos at the early developmental stage of E12.5 can generate organized movements in vitro transplacental perfusion method (Suzue, 1996). Lipp et al. exposed freshly explanted limbs to acetylcholine to determine when muscle contraction begins. They reported muscle contraction in WT limbs was observed at E13.5 and E14.5, but not at E12.5 (Lipp et al., 2023). Suzue et al. detected mouse hindlimb movement in an area of 300µm square, which is insensible movement. We thought that this was one of the reasons for the difference in our result that didn’t detect their limb movement in ultrasound images. We thought the reason why limbs couldn’t move in the womb was a circumambient environment, especially the body surrounding space, too. The embryo lives in the space of an amniotic membrane, so the body was arched, and limbs were folded in front of the trunk. Physiological development led to the maturation of muscle but also gradually reduced space in the amniotic membrane and disturbed embryonic limb movement. In fact, the spontaneous leg muscle activity of the chick embryo is affected by atypical postural constraint and modified by the stretch of leg muscles (Bradley et al., 2014). This means that the muscle-tendon formation in the embryonic phase may not be necessary for the potential mechanical cue. In other words, the required mechanical force for embryonic tendon development is quite slight compared to postnatal visible movement. It is like a passive movement, such as a shaky vibration from the mother's movement can compensate for tension from muscle contraction even if they have muscle dysfunction.

Then after birth, the limb is released from space limitation and begins dynamic movement in the spine position, which is OKC joint movement. We expect a change in the mechanical environment of limbs to arrow the free movement; this leads to an increase in muscle activity. However, P0 mice immediately after birth had not matured enough muscle function to move their limbs. As a result, the

212 limbs had only limited movement because gravity was applied to the limbs even if they didn't bear
213 weight loading.
214 To confirm the biological tendon response to the gradually induced mechanical stress, we
215 analyzed the expression of tendon development-related genes. Scx is known for specific tendons
216 maturation markers from the early progenitor stage to the formation of mature tendons. Previous
217 studies showed that Scx expressions are sensitive to mechanical signals in mouse (Maeda et al., 2011)
218 and chick limbs (Eloy-Trinquet et al., 2009; Havis et al., 2016). Moreover, mechanical forces maintain
219 Scx expression through the TGFb signaling pathway (Maeda et al., 2011). TGFb signaling has a role in
220 adapting to tension released in response to force. TGFb acts on tenocytes to alter their morphology and
221 ECM production, revealing a feedback mechanism (A. Subramanian et al., 2018; Maeda et al., 2011).
222 Tnmd is also required for proper tendon tissue adaptation (Dex et al., 2017) and to induce tendon
223 maturation (Docheva et al., 2005). Scx is necessary for the induction of Tnmd expression in mature
224 tenocytes (Shukunami et al., 2018). Therefore, we analyzed the expression of these factors to
225 understand whether mechanical force influences tendon maturation or not. Consequently, Scx, TGFb2,
226 and Tnmd have not shown dynamically changed between E17.5 to P1. The embryo phase showed no
227 specific limb movement in the amniotic membrane, and early postnatal could not move immediately
228 their limb on the gravity environment. The neonatal mice showed little movement of their limbs during
229 P0 to P1. Results of qPCR showed the expression of tendon development-specific factors; Scx and Tnmd
230 showed low-level expression during E17.5 to P1. The expression of Scx showed increasing between 0.8
231 to 1.2 times compared to E17.5. Tnmd expression levels exhibited 0.8 to 1.8 times, and TGFβ2 level
232 showed little change from 0.7 to 1 during E17.5 to P1(Fig.4). These results demonstrate a part of our
233 hypothesis that the functional relationship between tendon development and mechanical force.
234 Additionally, other factors also may have affected tendon development as a mechanical force. In
235 bone development, Nowlan et al. reported movements of the mother or littermates to compensate for
236 passive stimuli during embryonic skeletogenesis, using a lack of skeletal muscle model (Nowlan et al.,
237 2012). The previous study in neurological research reported that late pregnant mother is active
238 throughout late gestation like those of nonpregnant females. Locomotor behaviors in pregnant mothers
239 are rearing, head and body grooming, and stirring. That provides a sensory experience for rodent pups
240 in utero (Ronca et al., 1993). In mammals, these mother's movements have the potential to provide
241 mechanical force for the embryo to make joint movements by touching limbs to the amniotic
242 membrane. However, in this study, the embryo did not show limb movement confirmed ultrasound
243 imaging and low-level expression of tendon development factors. The divergence between previous
244 studies and our results may relate to the body size. Previous studies suggested the possibility of the
245 effect of passive stimuli and used transgenic mice. In general, transgenic musculoskeletal gene mice
246 showed small and thin limbs rather than normal mice. Bone cells can receive passive stimuli, and
247 compression and share stress, directly. But tendon cells are quite different from bone cells and need
248 joint movements to get the stretch stress depending on the muscle contraction or passive stimuli.
249 However, the space in the amniotic membrane is limited. This difference is quite important to consider
250 in the role of mechanical force in musculoskeletal developmental studies. Our results suggest that
251 passive and external mechanical stress may be a piece of an essential factor in tendon development in
252 the early postnatal phase. Additionally, our results give another point of view to consider the results of

transgenic mice studies because previous studies do not refer to this point (Usami Y. et al., 2022). Further study is needed to reveal the role of these external mechanical stimuli on tendon development.

6.2. Tendon development in the Mid to Late Postnatal Phase

Next, we analyzed the righting reflex to define the increase of mechanical force on the postnatal limb, depending on behavior patterns. Before getting the ability to righting reflex, limbs move not on loading conditions, which is called the OKC (Steindler, 1955). Then, they get the righting reflex, limbs touch the ground, and limbs are exposed to mechanical force by their own weight, which is called CKC movement (Steindler, 1955). P5 mice increased the time of a prone position that makes their limbs on load; CKC. To get the righting reflex, limbs received increasing mechanical force compere to OKC, depending on the weight-bearing load. In this phase, three genes showed tend to increase even though it was not significant. Regarding the Scx expression, it tends to increase to P12. It may give mechanical force to Achilles tendons through growing gait function especially raising the heel. During the crawling phase, they cannot raise their heel during locomotion. It showed that their limbs did not get enough muscle function to bear the weight. After the transition from crawling to walking, they can raise the heel, which means maturing the muscle function (Fig.5).

In the previous study, the detail of postnatal tendon formation was few reported compared with embryonic formation. Only two reports mentioned the relationship between mechanical force and tendon development. One is the mechanical development may proceed differently in weight-bearing Achilles tendons and non-weight-bearing tail tendons (S. K. Theodossiou et al., 2019). The same group then reported that elastic modulus and cross-sectional area of the Achilles tendon were altered with the spinal cord transection (S. K.Theodossiou et al., 2021). They used a spinal cord transection rat model to prepend weight bearing but didn't mention which affects the decreased muscle contraction or weight bearing. Finally, they suggested that the weight-bearing movement in the postnatal may provide the mechanical loading needed to direct postnatal tendon mechanical properties. We agree with their perspective on the importance of weight-bearing; moreover, we subdivided weight-bearing into CKC and gait patterns. The other study that only focused on natural development reported linear region stiffness and elastic modulus of Achilles tendons increased with age from P4 to P28 (Ansorge et al., 2011). Fibril diameters and the distribution of fibril diameter sizes increased with age (Ansorge et al., 2011). There is significant proliferation even as the tendon cells are reduced in density from P0 to P28 Achilles tendon (Grinstein et al., 2019). This result indicates that the postnatal tendon cell has cellular activities dynamically, or the transition of dynamic physical motion activates the tendon cells. To understand how much affected mechanical force by the postnatal activity on tendon cells, it is important to consider not only tendon growth but also tendon homeostasis and repair from injury. We suggest which is all condition bearing mechanical force. Because tendons always respond to mechanical force to maintain a functioning organism. Our work is the first report to suggest the relationship between external mechanical force and tendon development-specific gene expression in normal mice development.

Our study has some limitations. We used behavior tests to evaluate mechanical force, but we could not determine directly how much-increased muscle tension and changing muscle-tendon stretching occurred during postural change. This question should be addressed in future studies by

evaluating muscle tensile strength in the triceps surae. We only used wild-type mice and did not investigate specific gene functions use of transgenic mice such as Tgfb or Scx knockout mice. However, previous studies represented that these mice showed significantly different phenotypes for their limbs. To reconsider the meaning of the reported results, our result presented the baseline data and enable us to understand the mechanotransduction mechanism more clearly for the future study to use some of the transgenic mice. Finally, we detected the changing timeline in mechanical force for limbs using their physical movement ability and patterns but could not test their frequency or cage activity. We cannot yet exclude why mouse embryos need muscle contraction though they have no movement. In the future, it is necessary to evaluate the extent to which their abilities are reflected in actual activities.

In summary, our findings underscore that tendon development needs mechanical force from changing limb movement depending on muscle contraction and external mechanical force. Future studies need to focus on not only muscle contraction as an internal factor but also an external factor, such as environment and gravity between the embryo to postnatal contribute the tendon development.

7. Materials and methods

Mouse lines

All animal procedures were approved by the Animal Research Committee and were consistent with animal care guidelines (Approval Number: 2019-10). The mouse lines were used wild-type C57BL/6J (Tokyo Laboratory Animals Science Co., Ltd) backgrounds were bred in-house. For embryo harvest, timed mating was set up in the afternoon, and identification of a mucosal plug the next morning was considered 0.5 days of gestation (E0.5). Animal subjects were housed in a controlled environment with a 12:12-h light-dark cycle with ad libitum access to water and food. The experimental design is illustrated in Fig. 6.

Ultrasound imaging

The embryonic movement was observed using Real-time ultrasound imaging Vevo3100 (FUJIFILM VisualSonics, Inc., Toronto, Canada). This data showed how moves embryonic limb. Pregnant mice were transferred to the imaging center. Ultrasound imaging was performed under general anesthesia, induced with 5% isoflurane, and maintained with isoflurane 2%. During the scan, physiological changes were monitored with in-built ECG electrodes, and body temperature was maintained at 37 °C. Their abdomen's fur was removed using a chemical hair remover, and the pre-warmed gel was applied to their abdominal region.

Neonatal Motor Tests

Mouse pups were tested for motor behavioral development according to the previous study50). Pups were tested 6hr (±1.5hr) after the light cycle started to eliminate the time-of-day difference in behavior. Pups were removed from the dam for no more than 15 minutes to avoid losing their body heat, hunger, and separation issues.

The righting reflex is commonly used as the motor ability for mice pups to be able to flip onto their feet from a supine position. In accordance with a previous study 50), mice aged P0 to P7 tested the righting reflex. Ambulation was assessed using the following scale: 0 = no movement, 1= Crawling with asymmetric limb movement, 2 = slow crawling but symmetric limb movement, and 3 = fast crawling/walking. In detail, the head and tail of a crawling mouse were low to the ground, and the head began to rise during the transition from crawling to walking. The transition was complete when both the head and tail were elevated, and only the front of the hind paw touched the ground. The original test arrowed mice to score ambulation arrowed be repeated many times, but this research arrowed them for 1 min three times to ambulation and gave rest for 1 min considering the effect of fatigue. The score of each mouse was adopted at the highest score three times based on mentioned ambulation, was no potential for learning during the test.

RNA isolation, reverse transcription, and qRT-PCR

Total RNAs were extracted from E17 to P14 Achilles tendon using ISOGEN(Nippon gene, Tokyo, Japan). The reverse transcription was TaqMan™ Gene Expression Master Mix. Primer sequences used are Scx, Transcription growth factor-β2, and Tnmd. For mRNA level analyses, gene expression changes were quantified using the -2ΔΔCt method. Housekeeping was glyceraldehyde-3-phosphate dehydrogenase (Gapdh).

Histology

After sacrifice, forelimbs were skinned (E17.5~) and fixed in 4% paraformaldehyde for 24hr at 4°C. Postnatal tissues were decalcified in 50 mM EDTA for one day to 2 weeks at 4°C, then incubated in 5% sucrose (4hr) and 30% sucrose (overnight) at 4°C. Limbs were then embedded in OCT compound and longitudinal cryosections 12 μm were collected using Cryostar (Thermo Fisher). Sections were then stained with Alcian blue, Hematoxylin, and Eosin. For immunostaining, sectioned slides were rinsed in PBS-T and blocked in Antibody Diluent with Background Reducing Components (Dako) for 1h at room temperature and using M.O.M. kit (vector). Then sections were incubated in primary antibodies Collagen type Ⅰ (Col1)(Abcam) overnight at 4℃ and secondary antibodies for 4h each at room temperature and My32 (Sigma) was used to detect muscle-specific type II myosin heavy chain (MHC) for 30min at room temperature with secondary antibody Dylight588 (Abcam) and Cy3 secondary detection (Jackson ImmunoResearch) and remove unwanted fluorescence (Vector TrueVIEW Autofluorescence Quenching Kit with DAPI, Vector). DAPI counterstaining to visualize cell nuclei. Sections were observed under a BZ-X710 microscope (Keyence, Osaka, Japan)

Statistical analysis

GraphPad Prism (version 9.4.1) was used for statistical analysis and to generate all graphs. Non-statistically significant comparisons were not shown. A one-way ANOVA with Tukey-Kramer posthoc test was performed across ages for each measured parameter.

8. Acknowledgements

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9. Competing interests

The authors declare that no conflict of interest exists.

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11. Data availability

The Data that supports the findings of this study are available in the supplementary material of this article.

12. References

A. Subramanian, Kanzaki, L. F., Galloway, J. L. and Schilling, T. F. (2018). Mechanical force regulates tendon extracellular matrix organization and tenocyte morphogenesis through TGFbeta signaling. *Elife* **7**.

Ansorge, H. L., Adams, S., Birk, D. E. and Soslowsky, L. J. (2011). Mechanical, compositional, and structural properties of the post-natal mouse Achilles tendon. *Ann Biomed Eng* **39**, 1904-1913.

Bradley, N. S., Ryu, Y. U. and Yeseta, M. C. (2014). Spontaneous locomotor activity in late-stage chicken embryos is modified by stretch of leg muscles. *J Exp Biol* **217**, 896-907.

Brent, A. E., Braun, T. and Tabin, C. J. (2005). Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development. *Development* **132**, 515-528.

Brust, V., Schindler, P. M. and Lewejohann, L. (2015). Lifetime development of behavioural phenotype in the house mouse (Mus musculus). *Frontiers in Zoology* **12**, S17.

Dex, S., Alberton, P., Willkomm, L., Sollradl, T., Bago, S., Milz, S., Shakibaei, M., Ignatius, A., Bloch, W., Clausen-Schaumann, H., et al. (2017). Tenomodulin is Required for Tendon Endurance Running and Collagen I Fibril Adaptation to Mechanical Load. *EBioMedicine* **20**, 240-254.

Docheva, D., Hunziker, E. B., Fassler, R. and Brandau, O. (2005). Tenomodulin is necessary for tenocyte proliferation and tendon maturation. *Mol Cell Biol* **25**, 699-705.

Eloy-Trinquet, S., Wang, H., Edom-Vovard, F. and Duprez, D. (2009). Fgf signaling components are associated with muscles and tendons during limb development. *Dev Dyn* **238**, 1195-1206.

Feather-Schussler, D. N. and Ferguson, T. S. (2016). A Battery of Motor Tests in a Neonatal Mouse Model of Cerebral Palsy. *J Vis Exp*.

Felsenthal, N. and Zelzer, E. (2017). Mechanical regulation of musculoskeletal system development. *Development* **144**, 4271-4283.

Grinstein, M., Dingwall, H. L., O'Connor, L. D., Zou, K., Capellini, T. D. and Galloway, J. L. (2019). A distinct transition from cell growth to physiological homeostasis in the tendon. *Elife* **8**.

Havis, E., Bonnin, M. A., Esteves de Lima, J., Charvet, B., Milet, C. and Duprez, D. (2016). TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* **143**, 3839-3851.

Huang, A. H., Riordan, T. J., Pryce, B., Weibel, J. L., Watson, S. S., Long, F., Lefebvre, V., Harfe, B. D., Stadler, H. S., Akiyama, H., et al. (2015). Musculoskeletal integration at the wrist underlies the modular development of limb tendons. *Development* **142**, 2431-2441.

Lipp, S. N., Jacobson, K. R., Colling, H. A., Tuttle, T. G., Miles, D. T., McCreery, K. P. and Calve, S. (2023). Mechanical loading is required for initiation of extracellular matrix deposition at the developing murine myotendinous junction. *Matrix Biol* **116**, 28-48.

Maeda, T., Sakabe, T., Sunaga, A., Sakai, K., Rivera, A. L., Keene, D. R., Sasaki, T., Stavnezer, E., Iannotti, J., Schweitzer, R., et al. (2011). Conversion of mechanical force into TGF-beta-mediated biochemical signals. *Curr Biol* **21**, 933-941.

Martin, P. (1990). Tissue patterning in the developing mouse limb. *Int J Dev Biol* **34**, 323-336.

Nowlan, N. C., Dumas, G., Tajbakhsh, S., Prendergast, P. J. and Murphy, P. (2012). Biophysical stimuli induced by passive movements compensate for lack of skeletal muscle during embryonic skeletogenesis. *Biomech Model Mechanobiol* **11**, 207-219.

Pryce, B. A., Watson, S. S., Murchison, N. D., Staverosky, J. A., Dunker, N. and Schweitzer, R. (2009). Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development* **136**, 1351-1361.

R Schweitzer, Jay H. Chyung, Lewis C. Murtaugh, Ava E. Brent, Vicki Rosen, Eric N. Olson, Lassar, A. and Tabin, a. C. J. (2001). Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* **128**, 3855-3866.

Ronca, A. E., Lamkin, C. A. and Alberts, J. R. (1993). Maternal contributions to sensory experience in the fetal and newborn rat (Rattus norvegicus). *J Comp Psychol* **107**, 61-74.

S. K. Theodossiou, Bozeman, A. L., Burgett, N., Brumley, M. R., Swann, H. E., Raveling, A. R., Becker, J. J. and Schiele, N. R. (2019). Onset of neonatal locomotor behavior and the mechanical development of Achilles and tail tendons. *J Biomech* **96**, 109354.

S. K.Theodossiou, Pancheri, N. M., Martes, A. C., Bozeman, A. L., Brumley, M. R., Raveling, A. R., Courtright, J. M. and Schiele, N. R. (2021). Neonatal Spinal Cord Transection Decreases Hindlimb Weight-Bearing and Affects Formation of Achilles and Tail Tendons. *J Biomech Eng* **143**.

Shukunami, C., Takimoto, A., Nishizaki, Y., Yoshimoto, Y., Tanaka, S., Miura, S., Watanabe, H., Sakuma, T., Yamamoto, T., Kondoh, G., et al. (2018). Scleraxis is a transcriptional activator that regulates the expression of Tenomodulin, a marker of mature tenocytes and ligamentocytes. *Sci Rep* **8**, 3155.

Steindler, A. (1955). *Kinesiology of the human body under normal and pathological conditions*: Charles C Thomas.

Suzue, T. (1996). Movements of mouse fetuses in early stages of neural development studied in vitro. *Neuroscience Letters* **218**, 131-134.

Usami Y., Iijima H., Kokubun T., et al. (2022). Exploring the Role of Mechanical Force on Tendon Development in vivo Model: a Scoping Review, 23 May 2022, PREPRINT (Version 1) available at Research Square [https://doi.org/10.21203/rs.3.rs-1681832/v1]

V. Arvind, V. and A. H. Huang (2017). Mechanobiology of limb musculoskeletal development. *Ann N Y Acad Sci* **1409**, 18-32.

13. Figure legends

Fig. 1. Ultrasound imaging showed the embryo limbs had no spontaneous movement.

The axial shows limbs of (A)E15.5 and (C)E17.5 and sagittal imaging of (B)E17.5 and (D)E19.5. E19.5 had no space compere to E15.5 and E17.5. White arrowheads depicting hindlimb and black arrows mark the room in each embryo. The trunk and the head were marked with asterisks.

Fig. 2. Neonatal motor test detected increased mechanical force for limbs (n = 6/time points).

(A) Bodyweight increased with age. The Dods mean the weight of each mouse. (B) The number of successes in righting reflex test 3 times. [P0 vs. P5 P=0.0367, P0 vs. P7 P<0.001, P1 vs. P7 P=0.0019, P3 vs. P7 P=0.0019](C) The ambulation score (0 = no movement, 1= Crawling with asymmetric limb movement, 2 = slow crawling but symmetric limb movement, and 3 = fast crawling/walking) was adopted at the highest score three times[P9 vs. P14 P=0.0012]. Error bars represent SDs. (D) The heel (white arrowhead) begins to rise during the transition from crawling to walking, as agreed with the previous study (Feather-Schussler and Ferguson, 2016).

Fig. 3. Alcian Blue and HE stains and Immunofluorescence of hindlimbs showed increased Achilles tendon ECM.

(A) Sagittal sections of the ankle were shown. The tendon matured gradually with time course. Scale bars, 200μm. Arrowhead shows the Achilles tendon (E15.5). (B) Double immunostaining of Col1 (green) and muscle (MHC) was performed on frozen sections.

Fig. 4. mRNA expression tended to change with age.

qPCR analysis of Scx, TGFβ2, and Tnmd of Achilles tendon compere to E17.5. All factors have no significant deference as evaluated by a one-way ANOVA (time points). Mean values were shown, n= 6/group.

Fig. 5. Summerly of limb movement and motor behavior in the postnatal phase

(A) P0 mouse limbs move with no loading conditions; OKC. P5 mouse got loading; CKC. (B) The schema of kinetics chain for the limbs. (C)Translation of gait pattern with age. The red arrow is shown the heel raise.

Fig. 6. Experimental scheme of this study.

Research Design Overview

Supplement Video 1.

The Ultrasound imaging showed the embryo limbs had no spontaneous movement.

(A) At E15.5 embryo (cronal) and (B) at E17.5 embryo (sagittal) ultrasounds visualizing. These videos showed the embryo had no spontaneous limb movement.

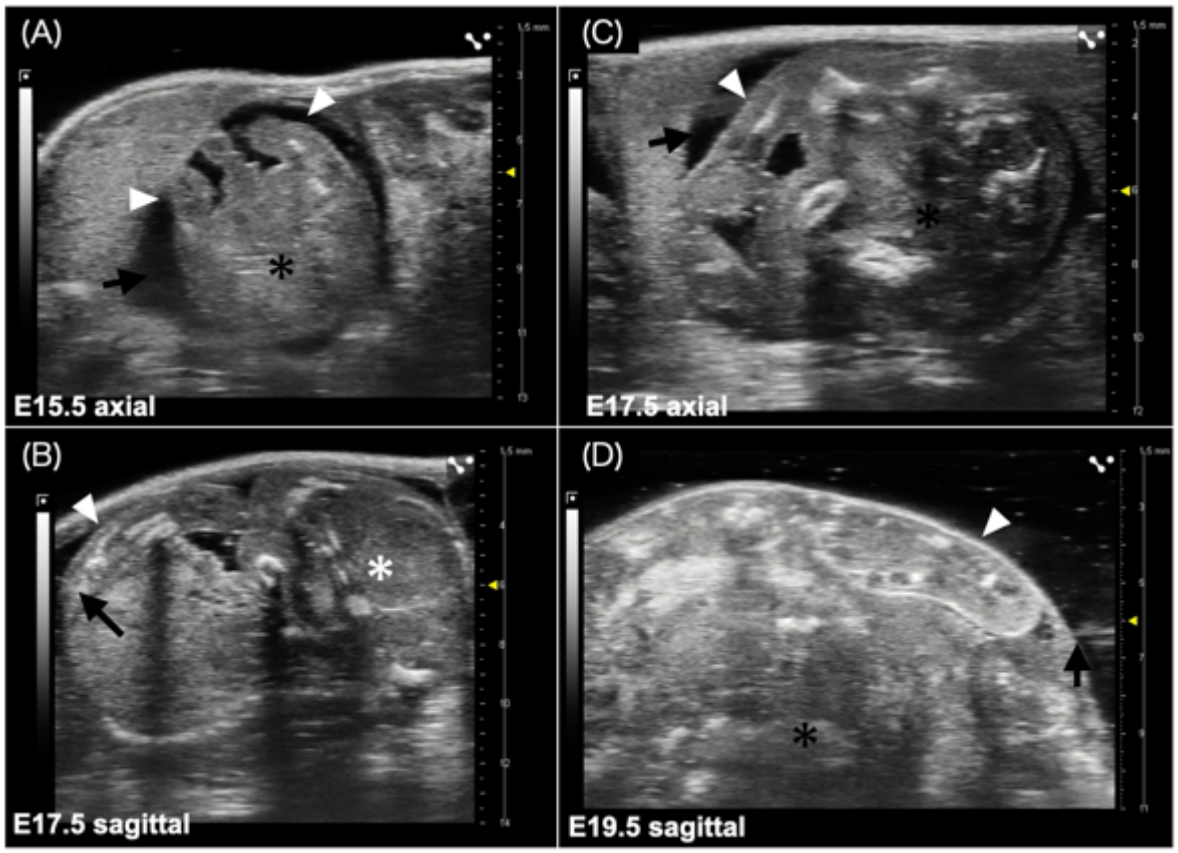

Fig. 1. Ultrasound imaging for the embryo in the womb.

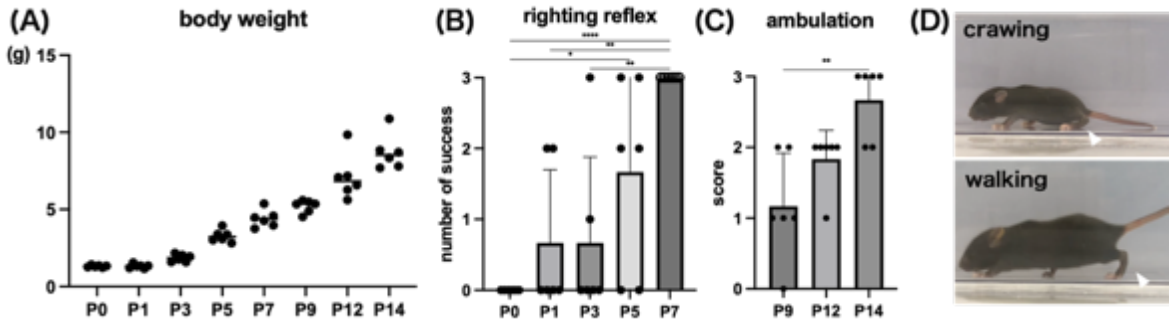

Fig. 2. Results of Neonatal motor test (n = 6/time points).

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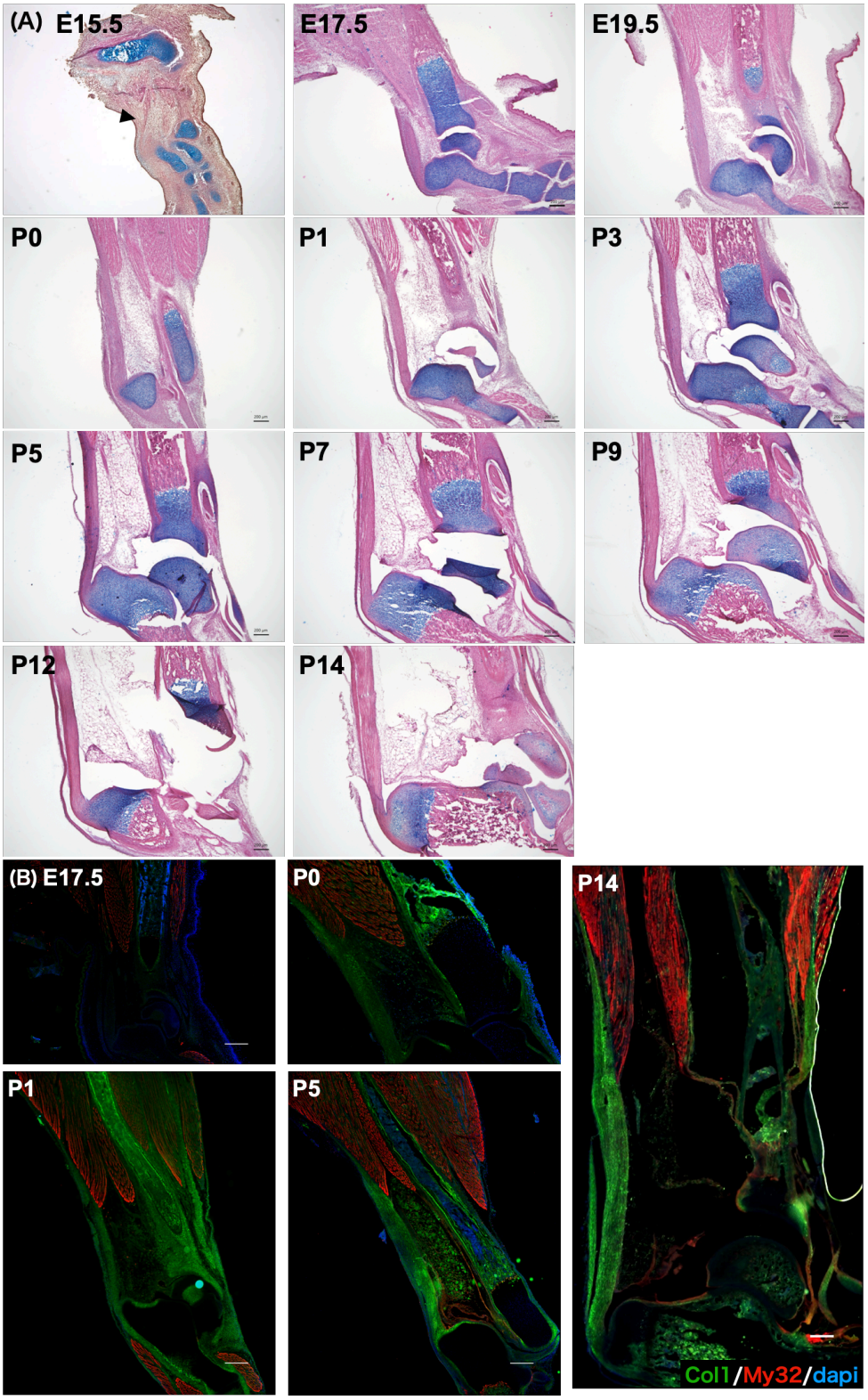

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511 Fig. 3. Alcian blue and HE and immunofluorescence staining of hindlimbs.

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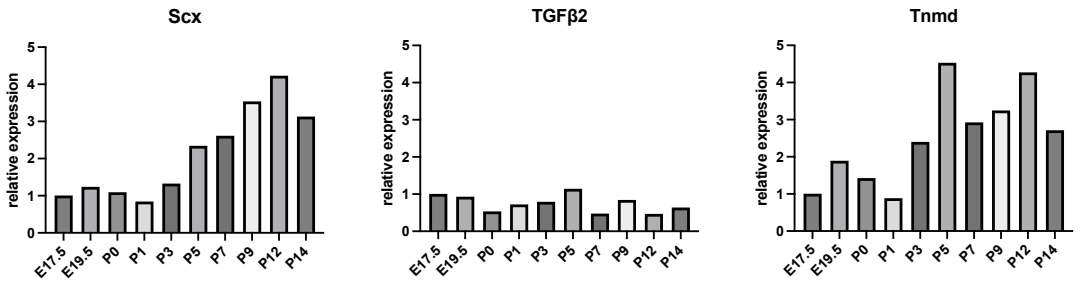

Fig. 4. Results of mRNA expression.

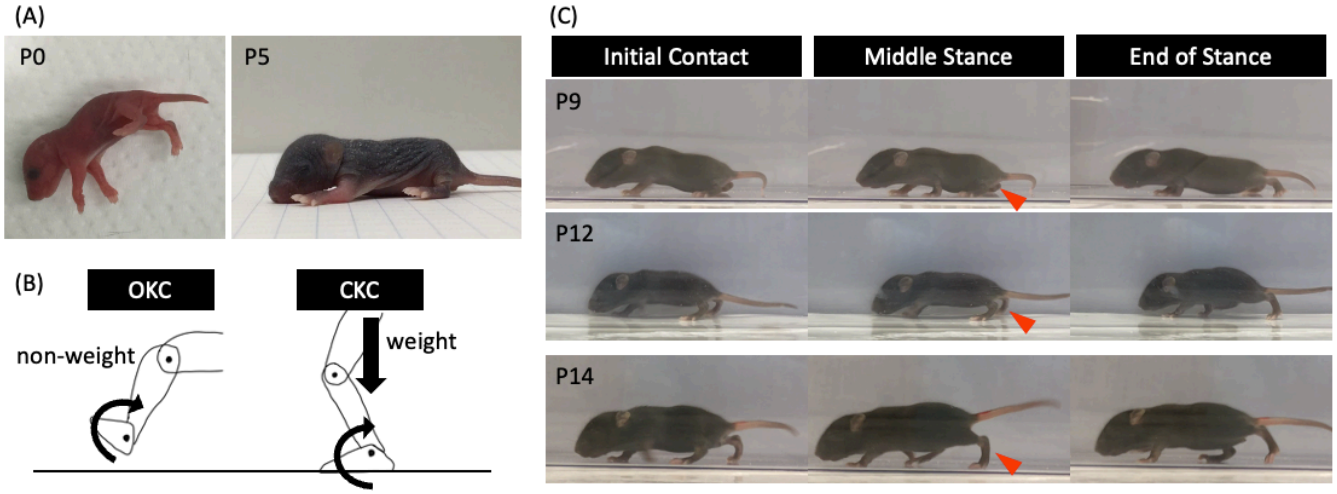

Fig. 5. Results of mRNA expression.

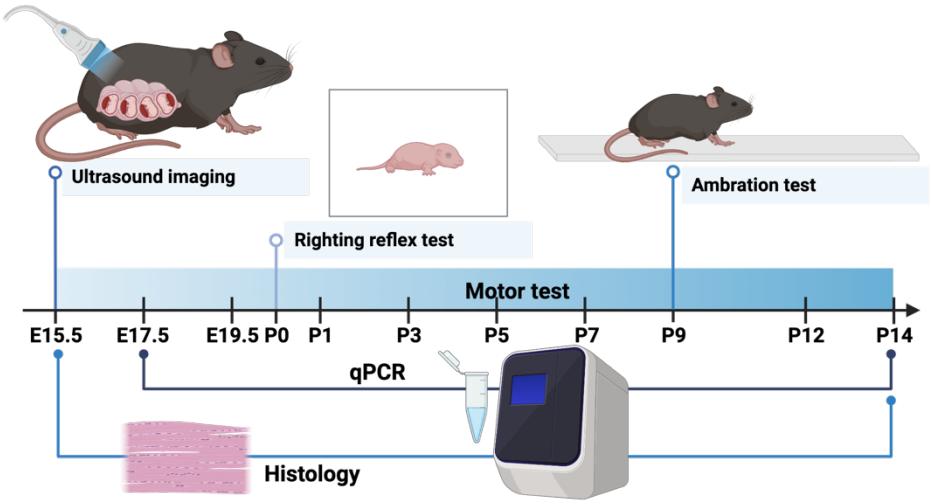

Fig. 6. Study design.