Study Reports of Research Stay

—The Chihiro and Kiyoko Yokochi Fund—

2017–2020

The Ichiro Kanehara Foundation/Tokyo

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Preface to the Study Reports (2017–2020)

The Chihiro and Kiyoko Yokochi Fund was established in January 1999 through a donation by Chihiro Yokochi, M. D., Emeritus Professor of Anatomy of the Kanagawa Dental College, Yokosuka, Japan, in memory of his beloved wife, the late Kiyoko Yokochi, who died in September 1995. It was her wish to organize a scholarship program for German medical researchers. The roots of this intimate relationship trace back to the 1970s, when an extraordinarily fruitful and successful co-project between Prof. Chihiro Yokochi and Prof. Johannes W. Rohen at the University of Erlangen, Germany, was initiated. It finally resulted in the famous "Color Atlas of Anatomy: A Photographic Study of the Human Body", published by Igaku-Shoin (Tokyo) and F. K. Schattauer Verlag (Stuttgart), which up to now provides unsurpassed insights into the delicate complexity of the human body to medical students all over the world. In the course of this project, Chihiro and Kiyoko Yokochi visited Erlangen several times, and a mutual admiration of German and Japanese cultures developed.

The aim of the fund is to promote a special program to financially support young German researchers who wish to pursue basic medical research in medical schools, dental schools or institutes in Japan. This generous offer was well appreciated, and the turn of the millennium marked the beginning of what turned out to develop as an extraordinary scientific and cultural scholarly program. Between 2000 and 2004, ten recipients of a scholarship visited basic research institutes and clinical departments of medical schools in Japan to conduct collaborative projects and deepen cultural interrelationship. The scientific reports of these initial ten scholars have been published in a first volume in 2007. In the upcoming next eleven years, grants have been awarded to another eleven German researchers, selected on the basis of the scientific quality of their proposed project to be conducted with their Japanese host. Their projects covered indeed the full spectrum of medical research, spanning from basic questions in anatomy and physiology to the immediate needs of patients in developing artificial organs, summarized in a second volume of the study reports. To allow for competent review and judgement of such a broad spectrum of proposals, the Scientific Evaluation Committee consisting of both Japanese and German scientist had been expanded in this period by including another Chair, Prof. Stefan Bauer from the Institute of Immunology at the Philipps University Marburg, Germany.

The present third study report volume documents the achievements of the last 4 German scholars visiting host institutions in Japan in the years 2017 to 2020, which altogether resulted in 7 peer-reviewed joint scientific publications of the scholarship holders together with their hosts. They addressed basic aspects of areas of utmost clinical relevance such as stem cell therapy after stroke, underlying mechanisms of dry eye disease, and functional understanding of cartilaginous and connective tissue elements of

the pelvic ring that contribute so much to the hard-to-treat syndromes of pelvic girdle pain.

Thus, over a period of 21 years, altogether 25 young German scientists enjoyed an outstanding opportunity. A distinctive feature of this program from most others is that fostering of new cultural bonds between the participating countries, Germany and Japan, is as much intended and indeed achieved as the rationale aspect of research. This is supported by the unusual approach to bring together people who share interests but not have worked together extensively in the past, rather than just boosting pre-existing collaborations. The reports published in this volume document not only the scientific success but also the cultural exchange that developed, very much in the spirit of the sponsors. I wish to express my gratitude to the generous sponsors, the Ichiro Kanehara Foundation for the very effective handling and support of the program, and of course all those in both countries, scholars and hosts, who filled it with the spirit in which it was established.

Wolfgang Kummer, M. D.
Professor of Anatomy and Cell Biology
Justus Liebig-University, Giessen
Chair of the Evaluation Committee

History of The Chihiro and Kiyoko Yokochi Fund

The Chihiro and Kiyoko Yokochi Fund was established in January 1999 through a donation by Chihiro Yokochi, M.D., Emeritus Professor of Anatomy of the Kanagawa Dental College, Yokosuka, Japan in memory of his beloved wife, the late Kiyoko Yokochi, who died in September 1995. It was her wish to organize a scholarship program for German medical students. The aim of the Fund is to promote a special program to financially support young German researchers who wish to study basic medical or dental science in schools or research institutes in Japan.

Prof. Chihiro Yokochi was born in Morioka (Iwate Pref.) on October 25, 1918 and graduated from Nippon Medical School (Tokyo) in 1942. After World War II he obtained his M.D. degree from Keio University School of Medicine (Tokyo) and began his studies of anatomy at the Gunma University School of Medicine in Maebashi (Gunma Pref.). He then moved to Yokohama (Kanagawa Pref.) in 1953 to accept a position as Associate Professor of Anatomy at the Yokohama City University School of Medicine. In 1965 he was promoted to Full Professor and Chairman of Anatomy at Kanagawa Dental College in Yokosuka (Kanagawa Pref.). He dedicated his efforts mostly to the study of macroscopic anatomy and student education in courses of anatomy in medical and dental schools.

Prof. Yokochi wrote and edited four anatomy atlases for medical, dental and allied health students. He published his first photographic atlas “Photographic Anatomy of the Human Body” in Japanese (Igaku−Shoin,1962). The first English edition was published by Igaku−Shoin in 1969, and its subsequent editions were published in 1978 (second) and 1989 (third). This “Small Atlas” was the milestone for a more extensive anatomy atlas for medical students, which was to be co−authored later by Prof. Johannes Rohen (Marburg, Erlangen).

In the mid 1970’s a co−publication project with Chihiro Yokochi was proposed by Professor Rohen with the publishers Igaku−Shoin (Tokyo) and F. K. Schattauer Verlag (Stuttgart). During the preparation of this manuscript, Prof. Yokochi visited Erlangen several times for discussion of editorial matters and to dissect new specimens, with his wife, Kiyoko, who accompanied him frequently. During these visits she developed an admiration for German culture and a mutual admiration arose between herself and the German people. The first edition of the atlas appeared in 1983 under the title “Rohen/ Yokochi: Color Atlas of Anatomy; A Photographic Study of Human Body” (Igaku−Shoin/F. K. Schattauer Verlag) and it has been revised periodically since then, the ninth edition was published by Wolters Kluwer in 2021.

In 1997, during the preparation of its Fourth edition the Igaku-Shoin's share in the title was sold to Wavery Press [Lippincott, Williams and Wilkins (Baltimore, USA)] and the new Fourth edition was published in 1998, inviting Prof. Elke Lütjen-Drecoll (Erlangen) as the third author.

Even after his retirement in 1986, Prof. Yokochi continued to make new dissections for the revised editions of "Color Atlas of Anatomy". In 1997 he published in Japanese a small book called "Stereoscopic Atlas of Anatomy" (Igaku-Shoin) and its second revised edition with 3D viewer in 2017.

A large portion of the donation by Prof. and Mrs. Yokochi includes royalties from his four books. Prof. Yokochi truly believes that this work "Color Atlas of Anatomy" is literally a co-work between two academic institutes in two different countries, or a masterpiece produced by the magnificent collaboration of German and Japanese cultures (From the eighth edition published in 2016 the title has been changed to "Anatomy - A Photographic Atlas"). This scholarship program was initially proposed by Mrs. Yokochi and was verified in her last testament. In the 20 years since 2000, the Chihiro and Kiyoko Yokochi Fund granted a total of 80,762,979 yen in subsidies to 25 researchers. We are sure that the Chihiro and Kiyoko Yokochi Scholarship has been a very useful program for those who have studied in Japan from research institutes in Germany, and we are convinced that the long-nurtured hope of Prof. and Mrs. Yokochi was fulfilled in the creation of the Yokochi Fund and its successful operation over many years.

In accordance with the intention expressed by Prof. Yokochi, the founder, applications for the Chihiro and Kiyoko Yokochi Scholarship program was closed as of the 31st day of March 2019. It has been 20 years after the establishment of the program. The Ichiro Kanehara Foundation would like to express our deepest gratitude to everyone concerned for their efforts and cooperation over the years.

Contents

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1. Transient receptor potential channel expression in lacrimal gland and association with the aqueous deficient form of Dry Eye Disease

Anna Kanewska

Scientific Background

The Dry Eye Disease (DED) as a disease which has a higher prevalence in the elderly population [1], has been gaining importance due to the demographic changes. There are several attempts on identifying the causes, which concentrate on different parts of the lacrimal functional unit. In my studies, I focused on the lacrimal gland.

Aside, there had been studies in the role of transient receptor potential channels (TRP−channels) in the ocular surface for the development of DED [2]. TRP−channels are ion channels somewhat permeable for Ca^{2+} and mediate sensory sensation activated by e. g. changes in the osmolarity [3].

Our group has already shown the expression of TRP channels in human corneal [4] and conjunctival epithelial cells [5], as well as human corneal endothelial cells [6]. However, the expression of TRP−channels in the lacrimal gland has not been explored yet. Since the lacrimal gland plays an essential role in the tear production and, therefore, might be affected in case of DED, the goal of the studies was to characterize the TRP−channel expression and relevance in the lacrimal gland.

Results

Gene expression results

The aim was to provide an overview of TRP−channel expression in the murine lacrimal gland (LG), submandibular gland (SMG) and Harderian gland (HG). For this, we chose representatives of the TRPM−family based on preliminary microarray results of Prof. Dr. Ito's laboratory at the Department of Developmental Anatomy and Regenerative Biology, National Defense Medical College, as well as TRPV1 based on own preliminary work.

【Keywords】 lacrimal gland; developmental anatomy; ocular diseases; TRPM3; TRP channels

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Period of Stay: From April 1, 2017 to January 3, 2018
Submission Date of Manuscript: June 22, 2018

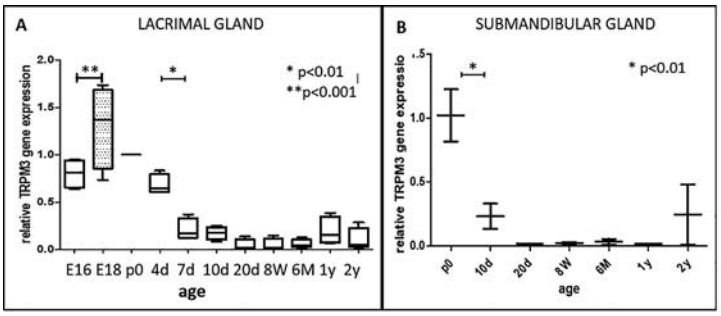

Figure 1. Relative TRPM3-gene expression in murine lacrimal gland at different stages of development. (A) shows the expression of TRPM3 in the murine lacrimal gland (LD) at different stages (E=embryonic, p=postnatal). For each stage, four mice (n=4) were analyzed. There is a significant ($p < 0.001$) 1.6-fold increase in TRPM3 gene expression from E16 (0.80 ± 0.13) to E18 (1.31 ± 0.37) and a significant ($p < 0.01$) reduction by about two thirds (69%) from 4d (0.68 ± 0.10) to 7d (0.21 ± 0.10). (B) shows a significant (N=3, $P < 0.01$) reduction by 77% in TRPM3 expression in the murine submandibular gland (SMG) from day 0 (1.02 ± 0.20) to day 10 (0.27 ± 0.10). For statistical analyses, Bonferroni multiple-t-test was used.

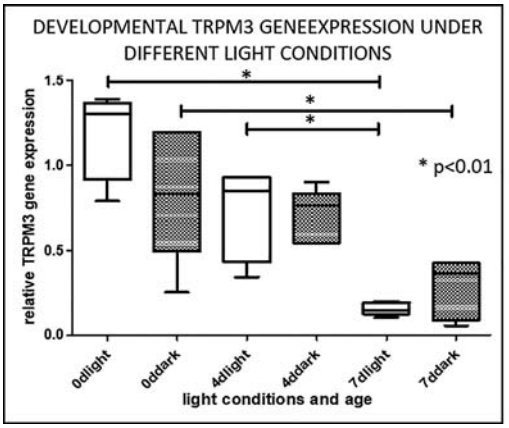

Figure 2. Relative TRPM3 gene expression in p0d, p4d and p7d old mice kept under different light conditions. The statistical analyses with Bonferroni multiple-t-test (N=4) reveals a significant ($p < 0.01$) 87% decrease from p0 (1.19 ± 0.27) to 7d (0.15 ± 0.04) and a 78% decrease from 4d (0.71 ± 0.27) to 7d in normal mice and a decrease by two thirds (67%) from p0 (0.84 ± 0.39) to 7d (0.28 ± 0.18) in light deprived mice.

The TRPM2-, TRPM4-, TRPM6- and TRPV1-gene expression in LG, SMG and HG did not reveal any conspicuous pattern. TRPM3-gene expression, however, showed a significant and continuous decrease after the stage E18. This developmental pattern was similar for the submandibular gland (Figure 1).

The decrease might be unrelated to light conditions: Since the decrease of TRPM3-gene expression occurred after birth and seemed to continue significantly until about the stage

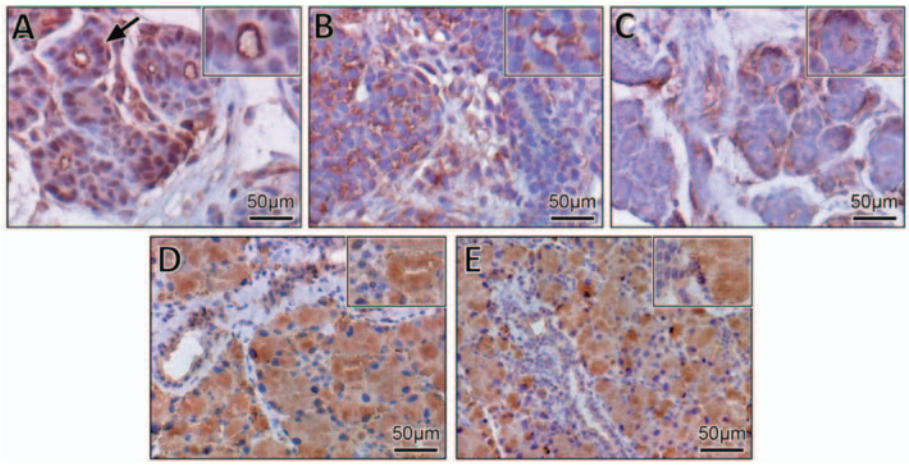

Figure 3: TRPM3 expression pattern in murine LG in different age stages. Immunohistochemical analyses with paraffin embedded sections of murine lacrimal gland. Brown signal shows reactivity with αTRPM3 antibody. In mice of embryonic age 18 (A), newborn (B) and 7d postnatal (C) there seems to be a positive signal in the apical membranes of ducts and acini (black arrow in A). In older stages, 8W (D) and 6M (E), the signal is either membranous in bigger ducts or diffusive cytoplasmic. Nuclei are counterstained with Hematoxylin.

of eye opening (10d), light-presence was a hypothetical influence factor for TRPM3 expression. Therefore, an animal model of light deprived mice was established and analyzed. The gene expression in LG of model mice, which were born and grew up in complete darkness, was compared with the expression in LG of age-matched mice, which were held under normal light conditions. To make sure whether eye opening itself had an impact, only age stages before eye opening, p0, 4d and 7d were used. Despite of the significant decrease from p0 to the other stages in both, model and normal mice, light exposure was not identified as a relevant factor for TRPM3-gene expression (Figure 2).

Histology

Membranous expression of TRPM3 in younger stages: Due to its character as an ion channel, TRPM3 cellular localization was expected to be in the cell membrane of the lacrimal gland cells. This could be observed for the younger stages (Figure 3). For the older stages, where qPCR-results indicate a low expression of TRPM3, this was not the case. These stages contained weak, diffuse cytoplasmic signals for TRPM3 channel in the LG.

Conclusions and Future Directions

TRPV1 and Dry Eye Disease

TRPV1 is a thermo-TRP-channel involved in inflammational processes, either as a nociceptor in peripheral nerve endings or a contributor to the IL-6 release [7]. It has been shown that IL-6 specifically is involved with ocular autoimmune diseases, as in Sjögren

syndrome [8].

As the expression of TRPV1 in the murine lacrimal gland showed to be almost below the threshold for qPCR analyses (results not shown) despite of positive signals in prior RT−PCR, we would like to focus more on the neuroimmunological role of TRPV1 in the lacrimal gland, which may be not necessarily associated within the LGEC itself.

Role of TRPM3 in the ocular surface

Our studies indicate a peak of TRPM3 expression in the murine lacrimal gland at E18. At this point, there also is a crucial term for the development of the LG [9]. Since there are indications for the co−relation of TRPM3 and Pax−6, an essential transcription factor for development of ocular structures [9], it is possible that TRPM3 plays an important role in the development of the lacrimal gland.

References

[1] Craig JP, Nichols KK, Akpek EK, Caffery B, Dua HS, Joo C−K, et al. TFOS DEWS II Definition and classification report. *The Ocular Surface.* 2017;15(3):276−283. doi:10.1016/j.jtos.2017.05.008.

[2] Almaraz L, Manenschijn J−A, de La Pena E, Viana F. TRPM8. *Handbook of Experimental Pharmacology.* 2014;222:547−579. doi:10.1007/978−3−642−54215−2_22.

[3] Ramsey IS, Delling M, Clapham DE. An introduction to TRP channels. *Annu Rev Physiol*. 2006;68:619−647. doi:10.1146/annurev.physiol.68.040204.100431.

[4] Mergler S, Garreis F, Sahlmüller M, Reinach PS, Paulsen F, Pleyer U. Thermosensitive transient receptor potential channels in human corneal epithelial cells. *J Cell Physiol.* 2011;226(7):1828−1842. doi:10.1002/jcp.22514.

[5] Mergler S, Garreis F, Sahlmüller M, Lyras E−M, Reinach PS, Dwarakanath A, et al. Calcium regulation by thermo− and osmosensing transient receptor potential vanilloid channels (TRPVs) in human conjunctival epithelial cells. *Histochem Cell Biol.* 2012;137(6):743−761. doi:10.1007/s00418−012−0924−5.

[6] Mergler S, Mertens C, Valtink M, Reinach PS, Székely VC, Slavi N, et al. Functional significance of thermosensitive transient receptor potential melastatin channel 8 (TRPM8) expression in immortalized human corneal endothelial cells. *Exp Eye Res.* 2013;116:337−349. doi:10.1016/j.exer.2013.10.003.

[7] Seki N, Shirasaki Hideaki, Kikuchi M, Himi T. Capsaicin induces the production of IL−6 in human upper respiratory epithelial cells. *Life Sci.* 2007;80(17):1592−1597. doi:10.1016/j.lfs.2007.01.037.

[8] Zahir−Jouzdani F, Atyabi F, Mojtabavi N. Interleukin−6 participation in pathology of ocular diseases. *Pathophysiology: the official journal of the International Society for Pathophysiology.* 2017;24(3):123−131. doi:10.1016/j.pathophys.2017.05.005.

[9] Makarenkova HP, Ito M, Govindarajan V, Faber SC, Sun L, McMahon G, et al. FGF10 is an inducer and Pax6 a competence factor for lacrimal gland development. *Development (Cambridge, England).* 2000;127(12): 2563−2572.

Publication of Project Results

- Kanewska A, Ito M, Karasawa Y, Inada M, Garreis F, Paulsen F, Takeuchi M. Developmental change in the gene-expression of transient receptor potential melastatin channel 3 (TRPM3) in murine lacrimal gland. *Ann Anat.* 2020;231:151551.

Project-related Presentations

- Kanewska, Anna (2017): Transient receptor potential channel expression in lacrimal gland and association with the aqueous deficient form of Dry Eye Disease, 27th Tokyo ocular immunology meeting on 1st September 2017.
- Kanewska, Anna (2017): Transient receptor potential channel expression in lacrimal gland, Meeting on the lacrimal gland at the Keio University, chair: Prof. Dr. Tsubota, 25th September 2017.
- Kanewska, Anna (2018): Expression and function of TRP-channels in the murine lacrimal gland, 46th Anatomical Colloquium at the Friedrich-Alexander University Erlangen Nürnberg on 8th March 2018.

2. Electromagnetic field preconditioning of neural stem cells for improved stem cell therapy after stroke

Jasmin Matuszak

Research Background

The second leading cause of death is stroke, resulting in around 5 million deaths per year all over the world [1]. At the moment only thrombolysis with tissue−plasminogen activator is a successful treatment for ischemic stroke, however it needs to be admitted within the short time of 4.5 h after occurrence of first symptoms, which is difficult for the majority of patients. Therefore, a lot of research is dealing with possible therapies for stroke. One promising concept is the regeneration of the damaged brain area by stem cell therapy. There are several *in vivo* studies using neural [2], embryonic [3] or mesenchymal stem cells [4] showing the feasibility of stem cell therapy. Although promising, only a small percentage of transplanted cells survives, proliferates and differentiates in the typical hostile environment of a stroke area [5]. It is known that electromagnetic fields (EMF) can have stimulatory as well as inhibitory influence on stem cells [6] and their therapeutic use was evaluated in clinical trials as well [7]. The main idea of this project is to us EMF for preconditioning of neural stem cells (NSC) before transplantation to improve stem cell survival and differentiation, for better regeneration of stroke damaged brain areas.

Project

The main goal of this project was to evaluate if the tested EMF settings have any beneficial effect on neural stem cells and therefore may improve stem cell transplantation.

During the project, murine neural stem cells from three different areas of the brain were used. For each experiment the brain of 4 male mice was dissected. The olfactory bulb, the subventricular zone and the hippocampus were isolated, as these are the only regions of the adult brain with stem cell niches. After dissociation, the single cell suspensions were cultured in neurobasal medium for around 1−2 weeks, until neuro-

【Keywords】 electromagnetic field (EMF); EMF preconditioning; neural stem cells; stroke; stem cell differentiation

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Period of Stay: From January 3, 2018 to December 30, 2018
Submission Date of Manuscript: March 29, 2019

spheres developed. The neurospheres were seeded in coated 6-well plates. Cells adhered overnight and were treated with EMF (1.4 mT, 50 Hz) for 7 days, whereas the control cells were kept in a normal incubator (37° C) without EMF. The total RNA was isolated and transcribed with reverse transcriptase into cDNA. This cDNA was used to perform a comparative quantitative real-time polymerase chain reaction (PCR) with SYBR-green.

We pursued two different experimental set-ups:

a) EMF preconditioning in undifferentiated NSC: NSC were cultured in medium with growth factors (P1). In this condition, cells are able to proliferate and maintain their stem cell like character, but cannot differentiate. After 7 days of EMF preconditioning the RNA was isolated immediately.

b) EMF preconditioning in differentiating neural stem cells: For the first 7 days of the experiment, the cells were cultured with P1 medium and one part of the cells was exposed to EMF. Afterwards the media was changed to media without growth factors (D1) allowing the cells to differentiate. The cells were kept for another 7 days at control conditions for differentiation. After 14 days in total, the RNA was isolated.

The total RNA was isolated and transcribed with reverse transcriptase into cDNA. This cDNA was used to perform a comparative quantitative real-time PCR with SYBR-green. As an endogenous control the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used. Measured values were normalized to endogenous control with the C_t method and afterwards normalized to control cells (37° C, no EMF).

Comparison of the C_T value of a target gene with that of the endogenous control gene allows the gene expression level of the target gene to be normalized to the amount of input RNA or cDNA.

During the project, a massive problem with the EMF system was detected. Because of a insufficient cooling system, the whole system heated up, with hot spots of over 40° C and in-well temperatures of 38° C.

Therefore, the first two sets of experiments were performed under heating conditions. They will be referred to as “old” or “heating” setting/condition. To analyze if the effect of the increased temperature was significantly influencing the experiment outcome, additional experiments were performed comparing the gene expression levels of the related genes at 37 and 38° C.

After improving the cooling system, temperatures of around 36.8° C under EMF were maintained throughout the whole experiment. This setting is described in this report as “new setting”.

Results and Discussion

Gene expression in NSC after 7 days of EMF preconditioning under "heating" conditions

a) Undifferentiated NSC: The results in Figure 1 display some significant changes in the gene expression of the tested genes after 7 days of EMF exposure for all areas. In the NSC derived from the olfactory bulb Olig2 and klf4 were significantly reduced. Both of those genes are expressed in very immature stem cells and progenitor cells. The decrease of their expression could be a sign of a possible acceleration of differentiation due to EMF. A similar result was observed for the NSC cultured from the subventricular zone, where SSEA−1, a marker for immature NSC, was decreased. Interestingly, a distinct increase of both marker typical for mature neurons, beta−III−tubulin and Map2, was measured for NSC from the hippocampus. This suggests the acceleration of differentiation and even maturation.

b) Differentiated NSC: Although gene expression levels in hippocampus derived cells were equally reduced, almost no changes could be observed for olfactory bulb or subventricular zone. In general, there were no significant changes in the gene expression levels in comparison to the EMF untreated control for any of the tested areas.

Although the gene expression levels shown in Figure 2 give a first idea that EMF probably had a distinct effect on the differentiation of NSC, it is unclear if this effect was mainly due to EMF or also due to the temperature increase of 1°C, which unfortunately were only detected after the performance of the first set of experiments. To analyze the possible effect of increased temperature on NSC, some additional experiments were performed (see Figure 4).

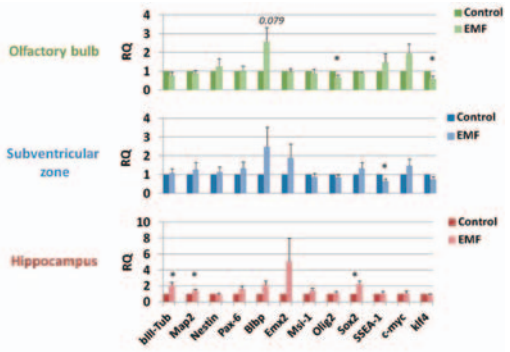

Figure 1: Gene expression of differentiation related genes in undifferentiated NSC. NSC were exposed to EMF (1.4 mT, 50 Hz) for 7 days under non−differentiating conditions. A RT−qPCR was used to determine the relative quantitation (RQ) values for the respective genes. Values are normalized to untreated control cells. n=7. Shown are mean ± SEM. * $p < 0.05$ vs. control with student's t−test.

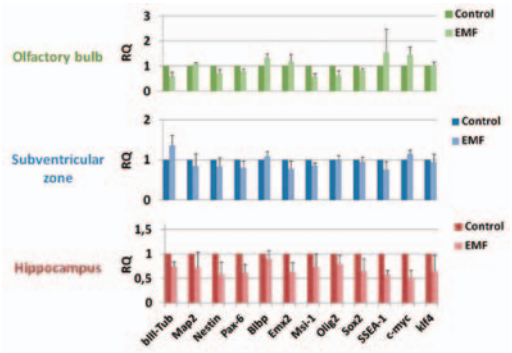

Figure 2: Gene expression of differentiation related genes in differentiated NSC. NSC were exposed to EMF (1.4 mT, 50 Hz) for 7 days under non-differentiating conditions. Afterwards EMF treatment was stopped and cells were cultured under differentiating conditions for additional 7 days. A RT-qPCR was used to determine the relative quantitation (RQ) values for the respective genes. Values are normalized to untreated control cells. n=7. Shown are mean ± SEM. *$p < 0.05$ vs. control with student's t-test.

The problem of insufficient temperature control within the EMF system

a) Improvement of the air cooling system: It is known that temperature is a crucial factor in nature for regulating biological processes. The essential key temperature in humans is around 37°C and scientific experiments *in vitro* are usually performed at defined conditions, such as 37°C, 5% CO_2 and 95% humidity.

During the first cascade of experiments performed within this project, a strongly enhanced evaporation in the well plate under EMF in comparison to the plate under control condition was observed. With a thermograph, several "hotspots" (areas with strongly increased temperatures) up to 42°C were identified. Additional in-well measurements with temperature sensors revealed temperatures over 38°C within the wells, corresponding to a temperature increase of more than 1°C in comparison to the baseline temperature of 37°C (Figure 3, "old setting"). After several adjustments of the cooling system and repeated measurements (data not shown) the temperature increase was avoided. The tubes connecting the cooling system with the magnetic coil were replaced by bigger tubes, allowing more cool air to reach the coil in shorter time and therefore more efficiently cool down the whole system. After application of an electromagnetic field of 1.4 mT, temperature changes were now stable in a range of ± 0.3°C within the wells.

b) Change in temperatures effects gene expression in neural stem cells: To address the possible effects of changes in temperature and inconsistent experiment set-up some additional experiments were performed, comparing the gene expression levels of differentiation related genes after exposure to 37°C and 38°C. The graph in Figure 4 shows that a few effects indeed were measured. Although for the majority of the genes no significant change in gene expression occurred, a distinct decrease was observed for β-III-tubulin in NSC from the hippocampus and subventricular zone. These findings ques-

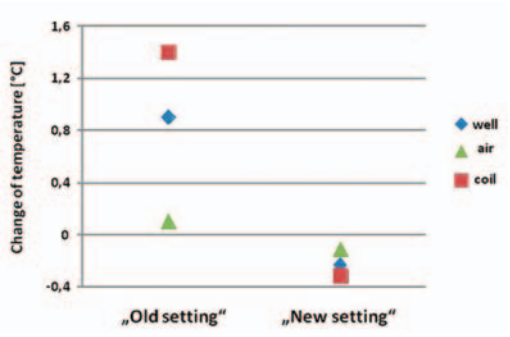

Figure 3: Change of temperature when using the EMF system before and after adjustment of the cooling system. Temperature was measured with highly sensitive sensors positioned at the electromagnetic coil, inside the well and in the middle of the incubator (air) with an electromagnetic field of 1.4 mT. Graph shows change in temperature (in comparison to baseline temperature of 37°C) 2 h after turning on the EMF with running cooling system. "Old setting" refers to the use of the not yet improved cooling system and "New setting" describes the system after adjusting the cooling system. n=1.

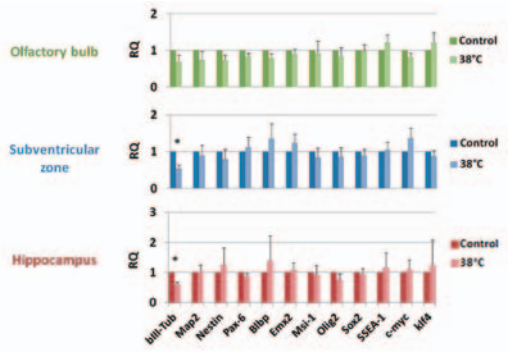

Figure 4: Gene expression of differentiation related genes in relation to temperature. NSC were exposed to 37°C or 38°C for 7 days under non-differentiating conditions. A RT-qPCR was used to determine the relative quantitation (RQ) values for the respective genes. Values are normalized to untreated control cells. n=7. Shown are mean ± SEM. * $p < 0.05$ vs. control with student's t-test.

tion the previous data obtained under "heating" conditions, because gene expression patterns for β-III-tubulin are exactly opposite comparing EMF+ "heating" and only "heating" (mimicked by 38°C). Whereas an increase of 1°C resulted in decreased β-III-tubulin expression, the combination of both EMF and temperature increase lead to an increase of the gene expression.

Gene expression in NSC after 7 days of EMF with "new setting"

a) Undifferentiated NSC: After repeating the experiments with proper temperature conditions, no significant changes in the gene expression for NSC from olfactory bulb were observed (see Figure 5). For the NSC from subventricular zone, a significant increase was

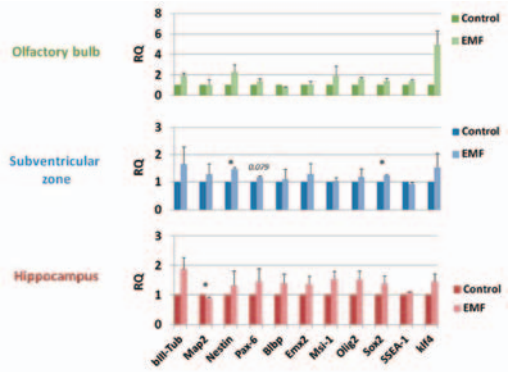

Figure 5: Gene expression of differentiation related genes in undifferentiated NSC ("New setting"). NSC were exposed to EMF (1.4 mT, 50 Hz) for 7 days under non-differentiating conditions. A RT-qPCR was used to determine the relative quantitation (RQ) values for the respective genes. Values are normalized to untreated control cells. n= 7. Shown are mean ± SEM. * $p < 0.05$ vs. control with student's t-test.

observed for SOX2 and nestin. Nestin is typically expressed in NSC and precursor cells. SOX2 is important for maintaining pluripotency of stem cells. The increased expression of both factors, also expressed at the same time in radial glial precursor cells, could be interpreted as a non-differentiating effect of EMF by preserving the stem cell or precursor state of the cell. In line with this goes the observed decreased expression of MAP2 in the NSC from hippocampus.

b) Differentiated NSC: The gene expression levels measured after 7 days of EMF and 7 additional days of differentiation with the "new setting" (see Figure 6) look similar to the previous observed results with the "old setting". For olfactory bulb a significant decrease in SOX2 was measured, although SOX2 is usually already lowly expressed in differentiated cells. Except that, no change in the gene expression pattern in NSC from other areas in comparison to control was observed. An explanation could be a time dependency, which means that possible effects of EMF are only exerting influence when EMF is actually applied or short time after.

Although it was observed that EMF influences many different cells in various ways, the presented results are less clear. The hypothesis of potential beneficial effects like acceleration of differentiation after EMF exposure on NSC for future stem cell therapy was not affirmed. Actually, an increase of nestin and SOX2 in subventricular zone NSC and a decrease of MAP2 in hippocampus derived NSC was measured, indicating a non-differentiating effect of the EMF. Of course, there are several potential reasons for these findings, like the EMF setting of 50 Hz and 1.4 mT. There are infinite possible variations of the magnetic field and frequency that can have very different effects on NSC. Maybe it is just a question of testing and a bit of luck to find the "one" combination that triggers the right gene expression patterns beneficial for stem cell therapy. Further,

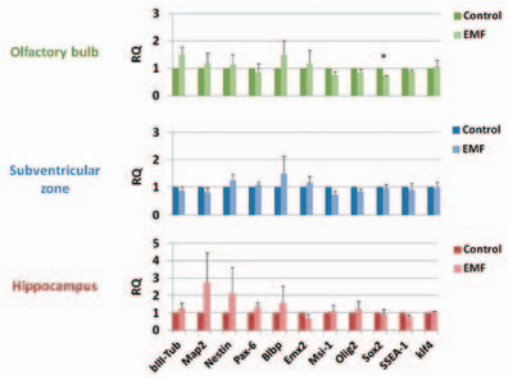

Figure 6: Gene expression of differentiation related genes in differentiated NSC ("New setting"). NSC were exposed to EMF (1.4 mT, 50 Hz) for 7 days under non-differentiating conditions. Afterwards EMF treatment was stopped and cells were cultured under differentiating conditions for additional 7 days. A RT-qPCR was used to determine the relative quantitation (RQ) values for the respective genes. Values are normalized to untreated control cells. n=7. Shown are mean ± SEM. * $p < 0.05$ vs. control with student's t-test.

the duration of EMF application can have a tremendous influence on the result outcome as well. From our experiments with differentiated cells, it seemed that one week after EMF almost no effect could be measured. An important task would be to examine in detail how long after preconditioning the EMF showed an effect. Important for further studies is the fact that with all tested EMF settings no significant decrease in the viability of NSC was observed (data not shown).

Although unintentionally, the occurred problems and mistakes during the project revealed how important it is to maintain proper experimental conditions during EMF and of course in general. Interestingly, the gene expression patterns observed with the wrong "heating" conditions were more beneficial for a potential use during stem cell therapy then during the corrected "new setting". Further, experiments only addressing temperature as a factor influencing the gene expression, resulted in partly contradictory results. It seems that not the increased temperature itself led to the differences observed between "old" and "new" setting, but the combination of EMF and temperature increase. This might be an interesting point to future investigations of potential factors for preconditioning of stem cells.

References

[1] Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Després JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jiménez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh

RW, Turner MB. American Heart Association Statistics Committee; Stroke Statistics Subcommittee. "Heart Disease and Stroke Statistics−2016 Update: A Report From the American Heart Association". *Circulation*. 2016 Jan 26;133(4):e38−360.

[2] Roitberg BZ, Mangubat E, Chen EY, Sugaya K, Thulborn KR, Kordower JH, Pawar A, Konecny T, Emborg ME. Survival and early differentiation of human neural stem cells transplanted in a nonhuman primate model of stroke. *J Neurosurg*. 2006 Jul; 105(1):96−102.

[3] Nagai N, Kawao N, Okada K, Okumoto K, Teramura T, Ueshima S, Umemura K, Matsuo O. Systemic transplantation of embryonic stem cells accelerates brain lesion decrease and angiogenesis. *Neuroreport*. 2010 Jun 2;21(8):575−579.

[4] Lee JS, Hong JM, Moon GJ, Lee PH, Ahn YH, Bang OY. A long−term follow−up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells*. 2010 Jun;28(6):1099−1106.

[5] Thored P, Arvidsson A, Cacci E, Ahlenius H, Kallur T, Darsalia V, Ekdahl CT, Kokaia Z, Lindvall O. Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells*. 2006 Mar;24(3):739−747.

[6] Maziarz A, Kocan B, Bester M, Budzik S, Cholewa M, Ochiya T, Banas A. How electromagnetic fields can influence adult stem cells: positive and negative impacts. *Stem Cell Res Ther*. 2016 Apr 18;7(1):54.

[7] Rohde CH, Taylor EM, Alonso A, Ascherman JA, Hardy KL, Pilla AA. Pulsed electromagnetic fields reduce postoperative interleukin−1β, pain, and inflammation: A double−blind, placebo−controlled study in TRAM flap breast reconstruction patients. *Plast Reconstr Surg*. 2015 May;135(5):808e−817e.

3. The topographical anatomy of the symphysis pubis—insights in the ligament's morphometry, the musculotendinous connections and vascular supply

Philipp Pieroh

Background

"Open book" injuries are rare occurring injuries of the pelvic ring and are defined by the symphyseal disruption and a widening or disruption of the sacroiliac joint [1]. In general, these injuries are treated with a plate osteosynthesis and depending on the posterior injury with sacroiliac screws or a triangular osteosynthesis. However, in 75% an implant failure occurs [2] but only 8% of patients require revision surgery [3]. Probably, an improved understanding of the anatomy of the symphyseal region may decrease the rate of implant failure and the related revision surgeries, e.g. by a more flexible osteosynthesis [4]. Even in inflammatory diseases of this region, namely symphysitis or osteitis pubis, the improved understanding of the anatomy might help to monitor the progress/regress of the disease. Currently, a grading using magnetic resonance imaging is not possible [5]. Besides traumatic ruptures of the symphysis or inflammatory processes, the symphyseal region is coming to the fore as a cause of pain, especially in sports medicine. Conflicting reports exist on the structure and location of the layers within this region [6, 7], complicating the interpretation of magnetic resonance tomography and possibly lead to differences to intraoperative findings. In addition, a "new" anatomical concept was recently presented [8]. These divergent data on the anatomy of the musculotendinous connections of the symphysis pubis underline the need for additional gross anatomical investigations to improve the understanding of the region and the treatment of patients. In addition, the vascular supply of the symphysis pubis was investigated.

Materials and Methods

Sixty formaldehyde–embalmed hemipelvises were obtained from 30 cadavers. During

【Keywords】 pelvis; symphysis pubis; pubic ligaments; vascular anatomy; groin pain

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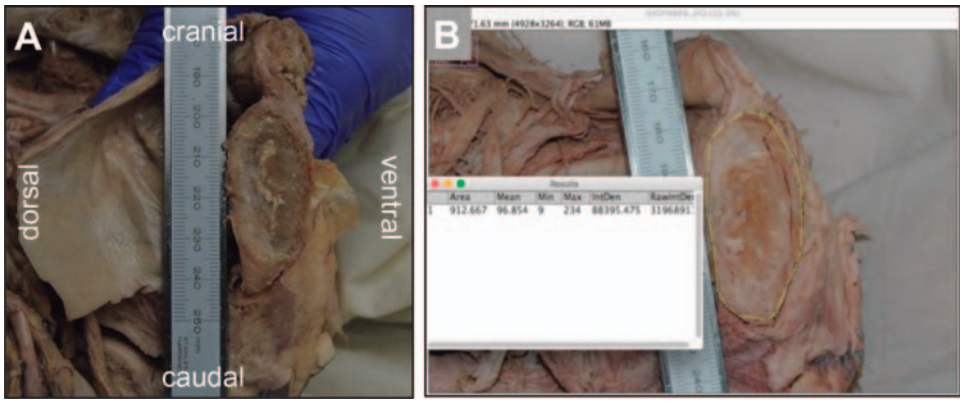

Figure 1: Measurement of the pubic ligaments. (A) Sagittal measurement of the symphysis in the hemipelvis. (B) Measurement of the photographs using ImageJ after previous normalization to the adjacent reference.

medical dissection course the medical students removed the surrounding soft tissue and a classic hemisection in the median plane was performed to receive two symmetric hemipelvises. Subsequently, a layered dissection was performed investigating the musculotendinous connections of the symphysis pubis. The following muscles were investigated: rectus abdominis, gracilis, adductor longus and brevis, obturatorius externus and internus. The extension of the symphysis, the area and thickness of the pubic ligaments (anterior, posterior, inferior, superior) were determined applying a ruler parallel to the sagittal plane of the pubic bone and taking a perpendicular photo (Figure 1). Measurements were performed using ImageJ (ImageJ 1.43, imagej.nih.gov/ij/).

Obtained data were confirmed during the preparation of 10 additional formaldehyde-embalmed, non-dissected cadavers. These cadavers were used to investigate the vascular anatomy of the symphysis pubis. Dissection was started with an anterior intrapelvic approach followed by a layered dissection identifying the vessels giving a vascular supply to the symphysis pubis. After all vessels were identified, the surrounding muscles were exposed beginning from the middle of the pectineal muscle, the periosteum was completely removed following a separation of the posterior and superior pubic ligament. Afterwards the pubic ligaments including their attached muscles were extracted and the muscles were step-step released from the "specimen" to get insights in the layered organization of the symphysial region.

Results

The pyramidal muscle and the inguinal ligament are connected and reach the superficial fibers of the adductor longus and rectus abdominis muscle as well as the anterior pubic ligament (Figure 2).

The adductor brevis muscle has a connection to both the superficial and the deep parts of the anterior pubic ligament. The adductor longus muscle (Figure 3) and rectus

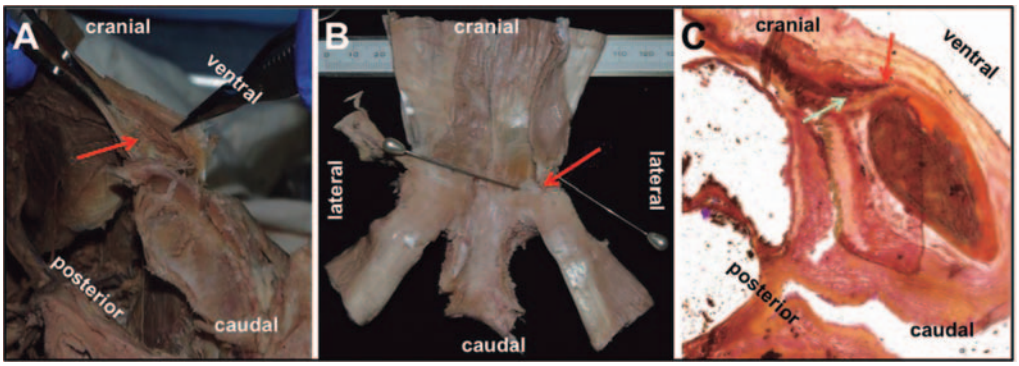

Figure 2: M. pyramidalis. (A) In the hemipelvis laterally mobilized (red arrow) without release of stronger fibers. (B) in the specimen mobilized with superficial attachment site (red arrow). (C) Plastinate sagittal view with red arrow marking the M. pyramidalis and M. rectus abdominis deep below (light green arrow).

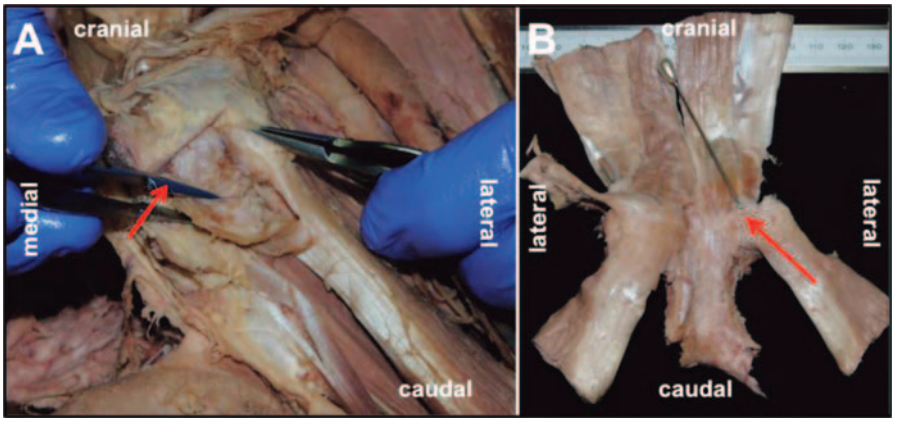

Figure 3: Adductor longus muscle. (A) In the hemipelvis, adductor longus muscle opened with connection to the anterior pubic ligament (red arrow). (B) Illustration of the connection between the adductor longus muscle and the anterior pubic ligament (red arrow) in the specimen.

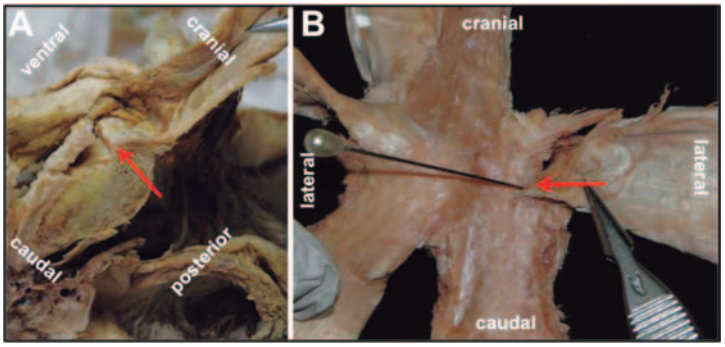

Figure 4: M. rectus abdominis. (A) In the hemipelvis attachment (red arrow) of the M. rectus abdominis. (B) In the specimen, the direct connection of the M. rectus abdominis to the anterior pubic ligament (red arrow) is visible.

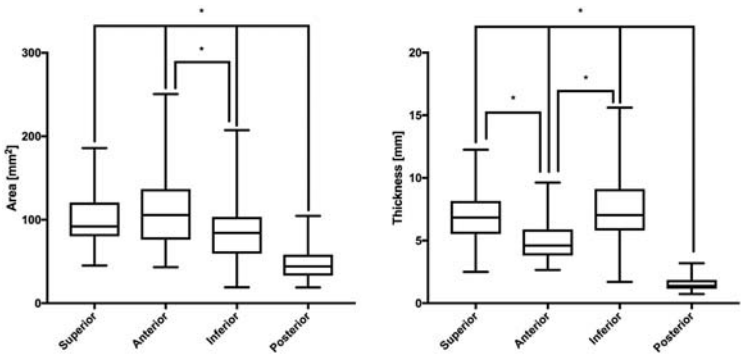

Figure 5: Measurements of area (left) and thickness (right) of the pubic ligaments in hemipelvises. * $p < 0.05$.

abdominis muscle (Figure 4) are directly connected to each other and to the anterior pubic ligament.

The obturatorius externus and internus had no direct connection to the pubic ligaments. The gracilis muscle is connected to the anterior and inferior pubic ligament. The posterior pubic ligament is connected to the medial pubovesical/puboprostaticum ligament. The posterior pubic ligament has the smallest thickness (1.51 ± 0.51 mm) and smallest area (45.60 ± 17.76 mm^2) of the pubic ligaments. The inferior pubic ligament (7.59 ± 3.03 mm) is thicker than the anterior pubic ligament (5.00 ± 1.58 mm, Figure 5). The area of the inferior pubic ligament (75.38 ± 27.75 mm^2) is smaller than that of the anterior pubic ligament (104.60 ± 38.91 mm^2) only in women. The anterior pubic ligament (5.23 ± 1.56 mm) is thinner than the superior pubic ligament (6.92 ± 2.19 mm) but only in men. The superior and inferior pubic ligaments are thicker in men than in women.

Arterial Supply

The superior part of the symphysis pubis got its arterial supply from a small artery running on the back of the inguinal ligament and originating from the inferior epigastric artery. Posteriorly, a vessel from the obturator artery supplies the symphysis pubis and the periosteum of the pubic bones. At the inferior portion, small branches from the dorsal penile or dorsal artery of the clitoris enter the inferior pubic ligament laterally. The anterior portion is the most complex regarding the course of the small arteries of the deep pudendal artery. Moreover, during the dissections multiple variations were detected.

Summary

Besides the function as anchor for several muscles also the thickness and area data of the anterior pubic ligament suggest a pivotal role for the stability of the symphysis pubis. Hence, apart from the superior and inferior ligament a (functional) reconstruction of the anterior pubic ligament should be considered. Our data reveal a direct connection of the

rectus abdominis, adductor longus and brevis and gracilis muscles to the pubic ligaments (anterior pubic ligament) as well as a superficial connection of the inguinal ligament and pyramidalis muscle. Furthermore, the vascular supply by multiple arteries supports the hypothesis that symphysitis pubis may occur hematogenous.

Acknowledgement

I would like to express my sincere gratitude to the Kanehara Foundation and the Yokochi Found offering me the opportunity to perform this project and granting me with this wonderful scholarship. I would like to thank me teacher in Japan, Masahiro Itoh who spend his time with me to discuss the project and introduces me in Japan. Furthermore, I would like to thank Zhong-Lian Li, Shin Kawata and Yuki Ogawa for the interminable help with the project and administrative affairs, the whole laboratory members as well as Michiyo Miyanaga and Toshiyuki Saito for their loving hospitality and Hanno Steinke, Christoph Josten and Faramarz Dehghani for their support.

References

[1] Pennal GF, Tile M, Waddell JP, Garside H. Pelvic disruption: assessment and classification. *Clin Orthop Relat Res.* 1980 Sep;151:12-21.

[2] Collinge C, Archdeacon MT, Dulaney-Cripe E, Moed BR. Radiographic changes of implant failure after plating for pubic symphysis diastasis: an underappreciated reality? *Clin Orthop Relat Res.* 2012 Aug;470(8):2148-2153.

[3] Morris SA, Loveridge J, Smart DK, Ward AJ, Chesser TJ. Is fixation failure after plate fixation of the symphysis pubis clinically important? *Clin Orthop Relat Res.* 2012 Aug;470(8):2154-2160.

[4] Kiskaddon EM, Wright A, Meeks BD, Froehle AW, Gould GC, Lubitz MG, Prayson MJ, Horne BR. A biomechanical cadaver comparison of suture button fixation to plate fixation for pubic symphysis diastasis. *Injury.* 2018 Nov;49(11):1993-1998.

[5] Larbi A, Pesquer L, Reboul G, Omoumi P, Perozziello A, Abadie P, Loriaut P, Copin P, Ducouret E, Dallaudière B. MRI in patients with chronic pubalgia: Is precise useful information provided to the surgeon? A case-control study. *Orthop Traumatol Surg Res.* 2016 Oct;102(6):747-754.

[6] Gamble JG, Simmons SC, Freedman M. The symphysis pubis. Anatomic and pathologic considerations. *Clin Orthop Relat Res.* 1986 Feb;203:261-272.

[7] Norton-Old KJ, Schache AG, Barker PJ, Clark RA, Harrison SM, Briggs CA. Anatomical and mechanical relationship between the proximal attachment of adductor longus and the distal rectus sheath. *Clin Anat.* 2013 May;26(4):522-530.

[8] Schilders E, Bharam S, Golan E, Dimitrakopoulou A, Mitchell A, Spaepen M, Beggs C, Cooke C, Holmich P. The pyramidalis-anterior pubic ligament-adductor longus complex (PLAC) and its role with adductor injuries: a new anatomical concept. *Knee Surg Sports Traumatol Arthrosc.* 2017 Dec;25(12):3969-3977.

Journal Articles Originated from the Study Period in Japan

- Kawata S, Marutani E, Hirai S, Hatayama N, Omotehara T, Nagahori K, Li ZL, Miyaso H, Pieroh P, Naito M, Itoh M (accepted) Spraying urea solution reduces formaldehyde levels during gross anatomy course. *Anat Sci Int*. In press.
- Yamamura S, Hayashi S, Li ZL, Kawata S, Pieroh P, Nagahori K, Omotehara T, Miyaso H, Itoh M. Investigations of cortical and cancellous clavicle bone patterns reveal an explanation for the load transmission and the higher incidence of lateral clavicle fractures in the elderly: a CT−based cadaveric study. *Anat Sci Int.* 2018 Sep; 93(4):479−486. PMID: 29654552.
- Shiboi R, Hayashi S, Kawata S, Li ZL, Pieroh P, Koga H, Takano Y, Inanami H, Itoh M. Anatomical relation between the accessory process and pedicle in the lumbar vertebrae. *Anat Sci Int.* 2018 Sep;93(4):430−436. PMID: 29427147.
- Pieroh P, Li ZL, Kawata S, Ogawa Y, Josten C, Steinke H, Dehghani F, Itoh M. The topography and morphometrics of the pubic ligaments. *Ann Anat*. 2021 Jul;236: 151698. doi:10.1016/j.aanat.2021.151698. Epub 2021 Feb 11. PMID: 33582299.
- Pieroh P, Li ZL, Kawata S, Ogawa Y, Josten C, Steinke H, Dehghani F, Itoh M. The arterial blood supply of the symphysis pubis − Spatial orientated and highly variable. *Ann Anat*. 2021 Mar;234:151649. doi:10.1016/j.aanat.2020.151649. Epub 2020 Nov 20. PMID: 33227373.

4. Immunohistochemical observations of neurovascular structures associated with the human sacrospinous ligament

Johann Zwirner

PART 1: MAIN STUDY

Introduction

Sacrospinous ligament (SSL) suspension is a surgical procedure to treat pelvic organ prolapse and can be performed either through a vaginal [1] or laparoscopic approach [2, 3]. Despite the high subjective (more than 84%) and objective (more than 67%) success rates of SSL suspension, especially postoperative perineal, gluteal or lower extremity pain [3, 4] forms an unpleasant side effect, which sometimes even necessitates the sutures to be released [5]. The entrapment of nerves adjacent to the SSL such as the pudendal nerve, inferior rectal nerve, inferior gluteal nerve, sacral nerve branches, posterior femoral cutaneous or the sciatic nerve [6, 7] seems to be the most obvious reason for a postoperative pain occurrence. Moreover, to avoid postoperative gluteal pain it was suggested to keep the depth of needle penetration during SSL suspension to a minimum [6], essentially assuming a nociceptive function of the SSL. Studies on the proprioception and nociception of ligaments are currently emerging [8, 9, 10, 11, 12, 13, 14] as these functions are suspected to be involved in the pathogenesis of pain syndromes [15]. Little is known about these qualities in the human SSL [16, 17] with missing qualitative and quantitative analyses according to the widely accepted Freeman and Wyke classification [18]. With the SSL being essential for the biomechanical integrity of the pelvic girdle [19] its proprioceptive function might be involved in various pelvic pain syndromes such as pelvic girdle pain, sacroiliac joint dysfunction or pubic symphysis dysfunction. A thorough analysis of the nociceptor distribution within the SSL might allow to define ‘safe zones’, which might decrease the likelihood of trapping these structures during SSL suspension and equally the number of women experiencing post-operative pain. The given study aims at a qualitative and quantitative immunohistochemical assessment of the

【Keywords】 free nerve endings; immunohistochemistry; pain; prolapse surgery; sacrospinous ligament

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neurovascular structures of the sacrospinous ligament. As previous studies of other ligament-associated mechanoreceptors indicated a polar distribution close to the bony attachments [13, 20, 21] with a potential positive influence on their sensitivity [22], we state the following hypothesis:

The density of mechanoreceptors of the central human SSL is significantly less compared to the medial and lateral polar areas, implicating that sutures in SSL suspensions should be placed centrally from a proprioceptive perspective.

Materials and Methods

Retrieval and handling of human sacrospinous ligaments

A total of 19 chemically unfixed right human SSLs were retrieved during forensic autopsy at the Institute of Legal Medicine, University of Leipzig, Germany. The cadavers were stored at 4° C after arrival at the mortuary. Study approval was obtained through the ethics committee of the University of Leipzig, Germany (protocol number 486/16-ek). The SSL retrieval was done as follows: Firstly, the surrounding connective tissue except for the coccygeus muscle was removed by blunt dissection. Secondly, the SSL was detached from the ischium laterally by sharply cutting off the tip of the ischial spine using a hammer and a gouge. Lastly, the SSL was carefully detached from the sacrum from medial to lateral using a scalpel. After retrieval, the SSLs were fixed in premixed 4% paraformaldehyde for 24 hours before being transferred to a self-mixed phosphate buffered saline solution (PBS; pH=7.4) for storage. When further processed, the SSLs were cut into 3 equispaced subsamples (medial, central and lateral) perpendicular to a line between the tip of the ischial spine and the proximodistal midpoint along the medial detachment of the SSL.

Histology, immunohistochemistry and scanning electron microscopy

The SSL subsamples were embedded in paraffin and cut into sections of 20 mm thickness using a rotary microtome (Leica RM 2235, Wetzlar, Germany). Cutting was performed in a transverse plane from superficial (coccygeus muscle facing side) to deep at 3 levels for each individual subsample. The mounted sections were deparaffinized using xylene and rehydrated with a decreasing ethanol series. For haematoxylin and eosin (H&E) staining, the sections were incubated as follows: Gill's haematoxylin for 4 min, tap water for 2 min, Scott's tap water for 2 min, tap water for 2 min, eosin for 30 s, tap water for 1 min and then dehydrated using an ethanol-xylene series. For the immunolabelings with anti-S100, anti-neurofilament (NF) and anti-von Willebrand Factor (vWF), the following protocol was used: sections were rinsed in PBS-Tween (PBS-T; pH=7.4) 3x7 min, 2%-hydrogen peroxide diluted in 60% methanol for 60 min, PBS-T for 10 min, blocker consisting of 10% bovine serum albumin (Serva, Heidelberg, Germany), 0.5% donkey serum (Jackson Immuno Research, West Grove, PA) and 0.3% casein (Carl Roth GmbH, Karlsruhe, Germany) in PBS-T for 60 min, primary rabbit antibody overnight at 4° C,

PBS-T for 3x7 min, secondary anti-rabbit-Ig-horseradish peroxidase-conjugated antibody, PBS-T 3x7 min, trishydroxymethylamin(Tris)-buffered hydrogen chloride for 5 min, diaminobenzidine substrate kit (Cell marque, Rocklin, CA), PBS-T for 2x7 min and finally left in distilled water for 2 min. The following primary antibodies were used: anti-S100-protein (diluted 1:500; BioGenex, Fremont, CA), anti-NF 200 (diluted 1:200; Sigma, Saint Louis, MO) and anti-vWF (diluted 1:400; DakoCytomation, Glostrup, Denmark). All stained sections were coverslipped with entellan (Merck, Darmstadt, Germany). Subsequent to the immunolabeling, the sections were counterstained using the following protocol: Gill's haematoxylin for 30 s, tap water for 2 min, Scott's tap water for 2 min, tap water for 1 min and finally dehydrated using an ethanol-xylene series. Negative controls without adding the primary antibodies were performed for the immunolabeled sections. All steps were performed at room temperature unless specified differently. Scanning electron microscopy was conducted on one lateral SSL subsample using a JEOL 6700F field emission scanning electron microscope (JEOL, Peabody, MA). Sample coating was performed in a K575X sputter coater with a 5-nm layer of gold palladium (Emitech Technologies, Kent, England).

Counting of neurovascular structures and data analysis

All sections were digitalized using a slide scanner. The mechanoreceptors were analysed according to the five specified types of the Freeman and Wyke classification [18]. Ruffini endings (type I), Pacini corpuscles (type II), Golgi-like endings (type III), free nerve endings (FNEs, type IV) and unclassifiable corpuscles (type V) were analysed in the anti-S100 and anti-NF immunolabeling. Vessels were identified using the anti-vWF immunolabeling. For counting of mechanoreceptors the fatty and ligamentous parts were manually outlined using ZEN lite 3.1 software (Carl Zeiss, Jena, Germany) and counted according to the following rules: Brown DAB-highlighted spots of less than 5 micrometers were counted as free FNEs in the S-100 stain. It was assessed whether these structures were associated with blood vessels by scanning the area of interest in the anti-vWF immunolabeling. Also, S-100-stained structures of more than 5 micrometers were counted to assess the number of larger nerve branches. Type I-III and V endings were assessed using the mentioned stains and confirmed by the anti-NF stain. Data evaluation was done separated for fat and ligaments to investigate the presence of mechanoreceptors per tissue type. The obtained data was statistically evaluated using Excel Version 16.15 (Microsoft Corporation, Redmond, WA) and GraphPad Prism version 7 (GraphPad So ware, La Jolla, CA). P-values equal to or smaller than 0.05 were considered to be statistically significant.

Preliminary Results

Mechanoreceptors of all types were predominantly found in fat islands associated with the SSL (Figure 1). The weight-bearing highly aligned collagens of the SSL only contained various FNEs, but did not surround any other receptor type according to the

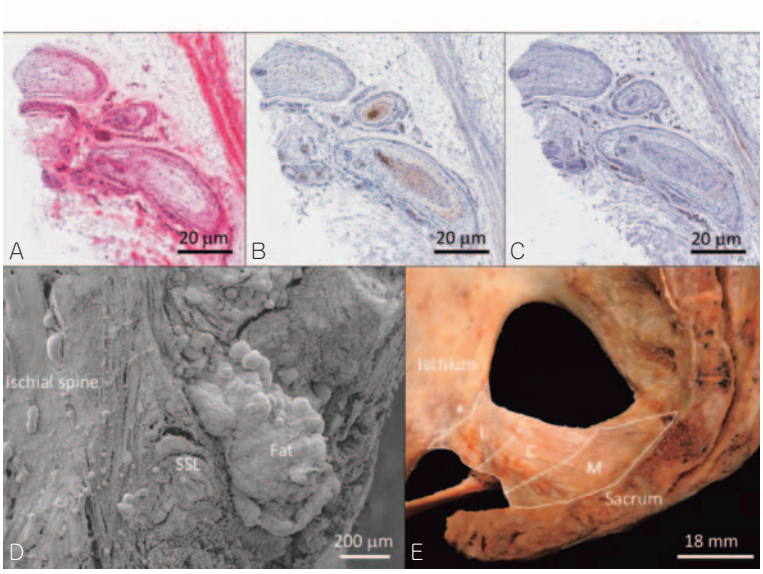

Figure 1: Impressions of the sacrospinous ligament and associated neuromechanics. Several Pacinian corpuscules are shown in H&E (A), anti-S-100 (B) and anti-vWF (von Willebrand Factor) stain (C). (D) shows a scanning electron microscopy image of the sacrospinous ligament and the ischial spine with related fat islands. (E) shows the sacrospinous ligament separated into the 3 different sub-regions (M, medial; C, central; L, lateral), which where compared in this study. White asterisk, ischial spine.

Freeman and Wyke classification. FNEs were almost exclusively associated with blood vessels. Detailed analyses of the mechanoreceptor density in various sub-regions of the SSL are currently being performed based on the obtained data.

Preliminary Conclusions

Mechanoreceptors of all types should be referred to as 'associated with' the SSL rather than calling them part of the ligament itself. The present study highlights the importance of fat as a 'space holder' or cushion for neurovascular structures.

PART 2: TIME IN JAPAN

Being the last student to receive the subsidy after 20 years the Yokochi Find was granted to emerging researchers, I felt deep gratitude when the committee informed me that my project had been approved. During my time at the Department of Clinical Anatomy of Tokyo Medical and Dental University (TMDU) I was surrounded by the outstanding team of Prof Keiichi Akita making life very enjoyable for me and supporting me wherever they could. In the future it will probably be very difficult to get a view out of my office that comes close to the one of the 18th floor of TMDU (Figure 2). Apart from studying I was able to dive deep into the Japanese culture taking a few days of holiday over New Year

Figure 2: Impressions of my study period at Tokyo Medical and Dental University (TMDU). The picture (A) shows the incredible view from Prof Akita's lab at the 18th floor of TMDU. (B) was taken at my welfare right before I left together with a couple of team members of Prof Akita's lab. (C) shows the venue, where the ceremony for body donors is held in Tokyo. (D) shows the TMDU building being a landmark close to Ochanomizu station.

2020 and begin a new decade full of exciting research challenges in Japan. I enjoyed the stunning mountains in Kamikochi, Osaka's delicious Okonomiyaki, the Golden Temple in Kyoto and the calming sunset in Nagasaki overlooking the harbor. After four months of studying in Tokyo there is barely a street I haven't walked or a food I haven't eaten and I definitely felt this special attraction that fascinates Europeans about the Land of the Rising Sun. Arigato!

Ceremony to honor body donors – a special experience

As an integral part of the body donation program, researchers and students around the globe honor the body donors with a special ceremony to acknowledge the irreplaceable gift that science received through their donation. It was a special experience for me to be part of this ceremony 9000 km from my hometown in Leipzig, Germany, and to observe the way respect is shown to the body donors in Japan.

Research in Prof Akita's lab – a German-Japanese success story

Soon after my arrival in Prof Akita's lab I realized that German culture influenced Japanese anatomists for a very long time. The library of Prof Akita's department includes a lot of anatomical treasures such as Walther Thiel's 'Photographischen Atlas der Praktischen Anatomie' ('Photographic Atlas of Practical Anatomy') or 'Studien der Anatomie des Nervensystems und der Bindegewebes' ('Anatomical Studies of the Ner-

Figure 3: Several examples of the library of the Department of Clinical Anatomy of Tokyo Medical and Dental University. (A) Several old German anatomical textbooks are shown. (B) The title page of 'Anatomical Studies of the Nervous System and Connective Tissue' of Gustav Retzius is shown. The table of contents of book is shown (C) as well as impressions of Pacini corpuscules (D) and handwritings (E).

vous System and Connective Tissue'). The latter was published in the last quarter of the 19th century by Gustav Retzius, the son of Anders Retzius, who gave his name to the retropubic space ('space of Retzius'). This book contained numerous impressive drawings and detailed descriptions of mechanoreceptors of various species including humans (Figure 3). It is remarkable that 150 years after these studies were performed on mechanoreceptors by Axel Key and Gustav Retzius still so much about their function and morphological environment remains unclear to date. Thanks to the Yokochi Fund and Prof Akita's team we were able to write another chapter of this story.

A little study on the side — biomechanics of the Achilles tendon-calcaneus-plantar fascia complex

Apart from the main study on the neuromechanics of the sacrospinous ligament, Prof Akita and I worked together on the topic of the structural continuity of the Achilles tendon (AT) via the outer calcaneus to the plantar fascia (PF), which is a basic morphological and functional consideration of the structures related to the clinically-relevant stretching treatment for plantar fasciitis patients. The study was based on plastination, histology and biomechanical materials testing (Figure 4). We found out that there is structural evidence for a morpho-logical connection between the AT and PF, which is in line with the concept of an integration and continuous remodeling of a tendinous AT-PF connection during life. Moreover, in this study a robust testing protocol for the AT-

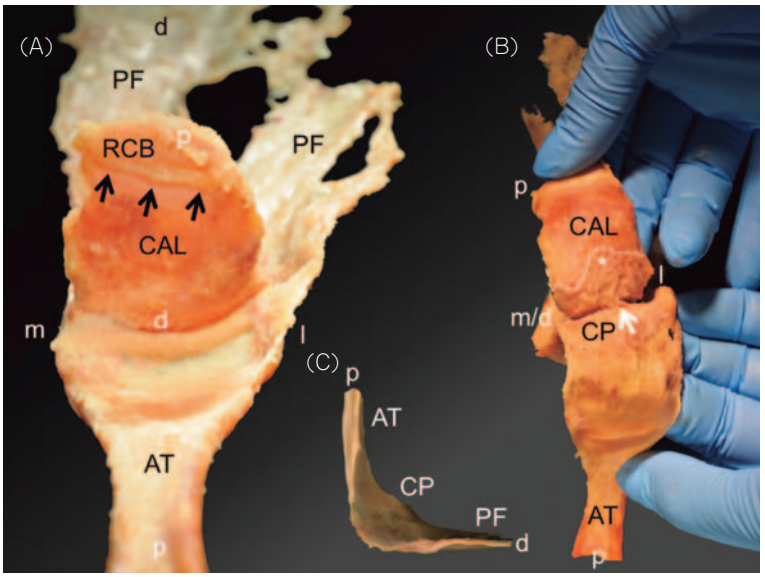

Figure 4: Macroscopic observations during separation of the Achilles tendon-calcaneus-plantar fascia complex (AT-CAL-PF complex) of the small morphological/biomechanical side study during my time in Prof Akita's lab. (A) AT–CAL–PF complex viewed from proximal to distal, (B) The calcaneal tuberosity (white arrow) is shown. (C) AT–calcaneal periosteum–PF complex after the calcaneus had been removed. AT, Achilles tendon; black arrows, transverse insertion line; CAL, calcaneus; CP, calcaneal periosteum; PF, plantar fascia; RCB, retrocalcaneal bursa; white asterisk, most proximal point of the calcaneal tuberosity; d, distal; m, medial; l, lateral; p, proximal.

calcaneus–PF complex was established that allows for a uniform tensile testing setup to be used for both soft and hard tissues. This led to a preliminary biomechanical map of the AT–calcaneus–PF complex from a materials testing perspective. This manuscript has recently been submitted to the journal of Clinical Anatomy (Zwirner et al., 2020 [refer to Publication Derived from This Study]).

References

[1] Richter K. The surgical anatomy of the vaginaefixatio sacrospinalis vaginalis. A contribution to the surgical treatment of vaginal blind pouch prolapse. *Geburtshilfe Frauenheilkd.* 1968;28:321–327.

[2] De Decker A, Fergusson R, Ondruschka B, Hammer N, Zwirner J. Anatomical structures at risk using different approaches for sacrospinous ligament fixation. *Clin Anat.* 2020 May;33(4):522-529. doi:10.1002/ca.23404. Epub 2019 May 29. PMID: 31087424.

[3] Wang Y, Wang D, Li Y, Liang Z, Xu H. Laparoscopic sacrospinous ligament fixation for uterovaginal prolapse: experience with 93 cases. *Int Urogynecol J.* 2011;22: 83–89.

[4] Unger CA, Walters MD. Gluteal and posterior thigh pain in the postoperative period and the need for intervention after sacrospinous ligament colpopexy. *Female Pelvic*

Med Reconstr Surg. 2014;20:208−211.

[5] Dangal G, Poudel R, Shrestha R, Karki A, Pradhan HK, Bhattachan K, Bajracharya N. Outcome of Sacrospinous Ligament Fixation of the Vault during Repair of Pelvic Organ Prolapse. *J Nepal Health Res Counc.* 2018;16:321−324.

[6] Florian−Rodriguez ME, Hare A, Chin K, Phelan JN, Ripperda CM, Corton MM. Inferior gluteal and other nerves associated with sacrospinous ligament: a cadaver study. *Am J Obstet Gynecol.* 2016 Nov;215(5):646.e1−646.e6. doi:10.1016/j.ajog.2016.06.025. Epub 2016 Jun 22. PMID: 27343565.

[7] Roshanravan SM, Wieslander CK, Schaffer JI, Corton MM. Neurovascular anatomy of the sacrospinous ligament region in female cadavers: Implications in sacrospinous ligament fixation. *Am J Obstet Gynecol.* 2007 Dec;197(6):660.e1−6. doi:10.1016/j.ajog.2007.08.061. PMID: 18060971.

[8] Cabuk H, Kusku Cabuk F, Tekin AC, Dedeoglu SS, Cakar M, Buyukkurt CD. Lower numbers of mechanoreceptors in the posterior cruciate ligament and anterior capsule of the osteoarthritic knees. *Knee Surg Sports Traumatol Arthrosc.* 2017;25:3146−3154.

[9] Gao F, Zhou J, He C, Ding J, Lou Z, Xie Q, Li H, Li F, Li G. A Morphologic and Quantitative Study of Mechanoreceptors in the Remnant Stump of the Human Anterior Cruciate Ligament. *Arthroscopy.* 2016;32:273−280.

[10] Hagert E, Garcia−Elias M, Forsgren S, Ljung BO. Immunohistochemical analysis of wrist ligament innervation in relation to their structural composition. *J Hand Surg Am.* 2007;32:30−36.

[11] Kholinne E, Lee HJ, Kim GY, Deslivia MF, Adikrishna A, Bin Z, Lee SJ, Rhyu IJ, Lim SJ, Hong HP, Jeon IH. Mechanoreceptors distribution in the human medial collateral ligament of the elbow. *Orthop Traumatol Surg Res.* 2018;104:251−255.

[12] Lobenhoffer P, Biedert R, Stauffer E, Lattermann C, Gerich TG, Muller W. Occurrence and distribution of free nerve endings in the distal iliotibial tract system of the knee. *Knee Surg Sports Traumatol Arthrosc.* 1996;4:111−115.

[13] Rein S, Hagert E, Hanisch U, Lwowski S, Fieguth A, Zwipp H. Immunohistochemical analysis of sensory nerve endings in ankle ligaments: a cadaver study. *Cells Tissues Organs.* 2013;197:64−76.

[14] Stecco C, Macchi V, Barbieri A, Tiengo C, Porzionato A, De Caro R. Hand fasciae innervation: The palmar aponeurosis. *Clin Anat.* 2018;31:677−683.

[15] Kiter E, Karaboyun T, Tufan AC, Acar K. Immunohistochemical demonstration of nerve endings in iliolumbar ligament. *Spine* (Phila Pa 1976). 2010;15:101−104.

[16] Barksdale PA, Gasser RF, Gauthier CM, Elkins TE, Wall LL. Intraligamentous nerves as a potential source of pain after sacrospinous ligament fixation of the vaginal apex. *Int Urogynecol J Pelvic Floor Dysfunct.* 1997;8:121−125.

[17] Varga E, Dudas B, Tile M. Putative proprioceptive function of the pelvic ligaments: biomechanical and histological studies. *Injury.* 2008;39:858−864.

[18] Freeman MA, Wyke B. The innervation of the knee joint. An anatomical and histo-

logical study in the cat. *J Anat.* 1967;101:505−532.
[19] Hammer N, Steinke H, Lingslebe U, Bechmann I, Josten C, Slowik V, Bohme J. Ligamentous influence in pelvic load distribution. *Spine J.* 2013;13:1321−1330.
[20] Cabuk H, Kusku Cabuk F. Mechanoreceptors of the ligaments and tendons around the knee. *Clin Anat.* 2016;29:789−795.
[21] Morisawa Y. Morphological study of mechanoreceptors on the coracoacromial ligament. *J Orthop Sci.* 1998;3:102−110.
[22] Takebayashi T, Yamashita T, Minaki Y, Ishii S. Mechanosensitive afferent units in the lateral ligament of the ankle. *J Bone Joint Surg Br.* 1997;79:490−493.

Publications Derived from This Study

- Zwirner J, Zhang M, Ondruschka B, Akita K, Hammer N. An ossifying bridge − on the structural continuity between the Achilles tendon and the plantar fascia. *Sci Rep.* 2020;10:14523.