Skeletal muscle in a dish: towards making skeletal muscle in vitro

Dahri, O.

chapter

06

General Discussion

General Discussion

Ouafa Dahri [1,2], Fanny Sage [1,2], Niels Geijsen [1,2]

[1] Department of Anatomy and Embryology, Leiden University Medical Center, Leiden 2333 ZC, The Netherlands

[2] The Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW), Leiden node, The Netherlands

Our understanding of skeletal muscle function and skeletal muscle disease has greatly advanced over the past few decades due to the development of sophisticated in vitro models. These models are powerful tools for researching stem cell-based regenerative medicine, testing novel treatments, and understanding disease mechanisms of muscular dystrophies.[1–5] The recreation of skeletal muscle to develop effective models in vitro will significantly benefit from close collaboration between developmental biology and tissue engineering. In this thesis, we investigated several aspects of recreating human skeletal muscle in vitro within these two fields. Starting from understanding the role of non-coding RNAs (ncRNAs) in induced pluripotent stem cell (iPSC) differentiation, followed by developing innovative materials for in vitro modelling and the testing of advanced three-dimensional (3D) models in the context of skeletal muscle disease. In this discussion, we evaluate the significance of our findings, discuss the limitations of our work and shine a light on the potential future directions.

In **Chapter 2** we investigated the role of ncRNAs and epigenetic memory during early germ layer differentiation, revealing a subset of ncRNA biotypes specific to mesoderm, endoderm, or ectoderm differentiation using multi-omics sequencing. Our study emphasized the importance of ncRNAs in regulating differentiation and identified potential targets for refining human iPSC-based differentiation protocols. Notably, transfer RNA (tRNA) fragments emerged in our analysis as an unexpected biotype, opening questions about their regulatory role in early germ layer differentiation. This research provides a robust multi-omics dataset that can inform future functional studies to validate the roles of identified ncRNAs, potentially improving the differentiation of iPSC-derived cell types. More specifically, the dataset provides insights into the ncRNAs in mesoderm differentiation, the germ layer from which skeletal muscle arises. Collectively, the findings can aid in the understanding of iPSC-derived skeletal muscle differentiation.

Since the discovery of iPSCs, the field of regenerative medicine and tissue engineering has undergone a revolution.[6] iPSCs have become the preferred cell type for in vitro modelling of many developmental processes and disease models due to their access to the human genome and ability to tailor to patient-specific biological questions. However, iPSCs display significant heterogeneity, both within and between lines, manifested in variations in gene expression, differentiation potential, and cellular behavior.s The genetic landscape of iPSCs is complex and largely unknown. In our study, we showed significant genetic and epigenetic differences between iPSC lines influenced by the original somatic cell type and the reprogramming method. The reprogramming process can introduce genetic mutations, which can sometimes be oncogenic. Besides, the epigenetic memory of donor cells can affect iPSC differentiation potential and stability.[7,8] This underscores a number of factors that influence iPSC differentiation and maintenance, as well as their generation. Understanding the factors that cause heterogeneity between iPSCs that affect differentiation can point us in the right direction when developing differentiation protocols for various cell types, such as skeletal muscle cells.

A key player in differentiation is the micro-environment, or niche, in which the iPSC cells reside. We aim to recapitulate the niche in vitro by mimicking extracellular matrix (ECM) components. The ECM allows for the facilitation of both structural support and can provide the cells with biochemical signals. It is known that specific ECM proteins, for example, laminin and fibronectin, are crucial for supporting iPSC growth and differentiation. Specific ECM proteins can be utilized to direct iPSC differentiation but can also be utilized to maintain pluripotency.[9] Two other important elements of the micro-environment that contribute to iPSC differentiation are soluble factors and cell-cell interactions. Soluble factors in the form of growth factors, transcription factors or cytokines can modulate iPSC behavior and can direct differentiation towards one or the other direction. Soluble factors are secreted by cells as a result of cell-cell interactions. Cell-cell interactions allow for cell communication that can initiate a cascade of events within the micro-environment.[10] Lastly, the physical properties of the culture substrate, such as stiffness and topography, can influence iPSC fate. Softer substrates mimic the natural stem cell niche better, promoting pluripotency, while stiffer substrates can induce differentiation.[11,12] To progress in the field, future research could benefit from sophisticated and dynamic culture systems that mimic parts of the in vivo niche.

The development of advanced sequencing methods has allowed us to gain detailed insights into factors that play important roles in differentiation. An example of this was shown in a recent study by Crist *et al.* Here, RNA bulk sequencing was leveraged to understand what factors within the micro-environment contribute to myogenic differentiation. In this study, human iPAX7 myogenic progenitors were transplanted into the muscles of non-dystrophic and dystrophic mice. Next, they analyzed the transcriptional profiles of the human grafts and compared them to in vitro-differentiated iPAX7 myotubes and human skeletal muscle samples. The transcriptional profiles revealed that human iPSC-derived skeletal myogenic progenitors produce mature myofibers after transplantation. To understand what factors from the micro-environment contributed to this maturity, the study combined human-specific reads with the results of the bulk RNA sequencing. They identified critical myogenic changes during the transition from in vitro to in vivo environments.[13] The study by Crist *et al.* highlights the power of RNA sequencing in identifying factors that can enhance in vitro differentiation.

Our study into ncRNAs in germ layer differentiation did not consider the micro-environment. This means that there might be factors that can significantly affect ncRNA dynamics, which are overlooked in our current set-up. Future research should incorporate advanced models such as human blastoids or human gastruloids. Human blastoids mimic pre-implantation blastocysts, while human gastruloids replicate some aspects of early embryonic development, including germ layer establishment. Both these more advanced models add a layer of complexity to the micro-environmental conditions of the culture system compared to the set-up presented in **Chapter 2.** The models contain multiple cell types at once, which can influence the differentiation dynamics and, therefore, offer us more physiologically relevant conditions for studying ncRNA dynamics during early germ layer differentiation.[14,15]

On the other hand, using real human embryos would offer the most accurate insights. However, such studies come with major ethical considerations and cannot be carried out easily. The advantage actual human embryos have over 3D models or animal models is the ability to investigate ncRNAs while maintaining the temporal and spatial dynamics of human embryonic development.

In addition to singular sequencing techniques, multi-omics approaches have gained more significance over time. This approach integrates multiple sequencing techniques such as genomics, transcriptomics, proteomics, epigenomics, and metabolomics. Multi-omics approaches have allowed us to understand the relationship of molecular factors that contribute to the complex human genetic landscape, for example, iPSCs.

Though multi-omics allows us to gain deeper insight into biology, these approaches face limitations. One of the main limitations occurs when integrating and interpreting large, complex datasets gathered with different sequencing techniques. As a result, the datasets have different scales, formats and dimensions. Currently, there are ways to normalize data gathered from different omics techniques, but there needs to be standardized protocols for multi-omics data analysis. Additionally, omics techniques are costly and resource-intensive as they require high-throughput sequencing machines and advanced computational infrastructure. Lastly, multi-omics studies often need more temporal and spatial resolution to capture dynamic changes in iPSC states. Our study circumvented this limitation by collecting data at different time points over an extended period. Emerging single-cell omics techniques attempt to address some of the current hurdles but still face challenges in sensitivity and coverage.[16]

Biological variability is another limiting factor for large-scale multi-omics approaches. To provide reliable conclusions, a large number of donors is required to account for this biological variability. However, obtaining sufficient sample sizes can be challenging. We used isogenic cell lines to control for genetic variability. This allowed us to work with the same genetic background, but our study was limited by an insufficient number of donors. As we only had one donor, the findings in **Chapter 2** cannot be generalized.

The study would, therefore, benefit from adding another set of isogenic iPSC lines from a different donor with the same cell of origin as the isogenic iPSCs used in our study. By adding one more donor, we could enhance the understanding of our study's findings. Preferable more than one donor would be added to future studies. Another way to control biological variation is by adding different iPSC clones from the same parental line. This can aid in drawing robust biological conclusions. Additionally, variability in iPSC reprogramming methods should be limited or avoided entirely in future research as it introduced additional variation in **Chapter 2**. In summary, a follow-up study should consider adding isogenic iPSCs from additional donors, all reprogrammed in the same way and should include more clones per iPSC line. Collectively, this should strengthen our findings from our study**.**

Chapter 3 focused on developing a novel magneto-active biocompatible material that demonstrated significant improvements in myoblast differentiation in 3D models. This magneto-conductive material for melt electrowriting (MEW) enhances muscle fiber alignment and maturation, mimicking the mechanical properties of in vivo skeletal muscle tissue. This advancement in material science offers the potential to more accurately represent the in vivo environment for in vitro skeletal muscle engineering.

The significance of the micro-environment in in vitro cell systems has been extensively documented. It is known that cells respond to their surroundings in a dynamic way. Therefore, the field of tissue engineering is constantly emerging with innovative approaches to bridging the gap between two-dimensional (2D) cultures and the complexity of the 3D nature of living organisms. As a result of these ad-

vancements, 3D culture systems that are increasingly becoming a standard culture method for a wide range of applications – from disease modeling to drug discovery. 3D cell culture systems are advancing due to the development of smart biomaterials. Smart biomaterials respond to external stimuli (such as temperature changes, light exposure, and magnetic stimulation). Upon stimulation, a smart biomaterial can dynamically change its properties. For example, a tissue can stiffen as a response to increased mechanical load, or cells can release growth factors upon light exposure.[17] This adaptability mimics parts of the dynamic nature of living tissues, where cells are constantly receiving and responding to signals from their environment. Another example of smart biomaterials are electroactive polymers. These polymers can change their physical properties in response to electrical stimulation, which is particularly beneficial for skeletal muscle engineering, as electrical signals play a crucial role in muscle contraction and function. By applying electrical stimuli, these polymers improve the alignment and contractility of muscle fibers, closely mimicking parts of in vivo muscle behavior.[18,19]

Besides the micro-environment, technologies such as electrospinning and MEW allow for great control over the macro-structure of engineered tissues. Skeletal muscles have different forms and shapes as they fulfill different functions in the human body. For example, the biceps have fusiform shaped fibers while the abdominal muscle contains parallel shaped fibers. The muscles around the eyes and mouth on the other hand have circular shaped fibers. Each muscle group plays a distinct role in movement and stability.[20] The resolution provided by electrospinning techniques gives us the flexibility to replicate these distinct shapes for in vitro muscle modeling. For example, on a recent study by Wang et al., electrospinning was utilized to create different macrostructures of muscle such as pennate muscle, orbicular muscle and rectus abdominis muscle.[21]

The magneto-active material for MEW that we developed in **Chapter 3** was successfully combined with PCL, a non-active material, into one scaffold. This proof-of-principle showed that combining magneto-active and non-active material could be used to mimic the muscle-tendon junction. Here, the skeletal muscle part could be engineered in a way that it is responsive to external magnetic stimulation while the tendon part remains static. MEW is superior to electrospinning in accurate fiber deposition and could, therefore, be leveraged to precisely any macrostructure of the different muscle phenotypes in vitro.[22]

However, to fully realize the potential of the magneto-active material developed in **Chapter 3**, a bioreactor capable of automating the sequential magnetic stimulation of engineered magnetoactive skeletal muscle tissues is required. Additionally, optimizing co-culture conditions with tendon cells is necessary to ensure proper integration and function. Finally, testing the system's compatibility with human iPSC-derived myogenic cells is crucial to enhance the clinical relevance of the model. These steps will help validate the model's applicability and pave the way for its use in skeletal muscle disease modeling.

Chapter 4 assessed different 3D skeletal muscle models on their ability to be used for disease modeling. The results in this chapter emphasized the potential of cantilever-based models in testing restored functionality. We also provided preliminary results from a vascularized skeletal muscle-on-a-chip model that showed promising differentiation efficiencies. With this chapter, we pioneered a systematic approach to selecting the appropriate 3D model tailored to specific experimental questions.

An important aspect in choosing the appropriate 3D model is the selection of the

suitable cell source. This depends largely on the specific biological question and application in mind. Human iPSC-derived myoblasts are often preferred due to their potential to model human-specific and patient-specific biology. However, these cells tend to be immature upon differentiation and require prolonged and extensive differentiation protocols. For the development of a robust and efficient 3D model, human-immortalized cell lines can be preferred over hiPSCs. Human immortalized myoblasts exhibit a more mature phenotype compared to hiPSC-derived myoblasts. Besides, myoblasts are readily available and only require expansion rather than long-term differentiation. Additionally, there is a wide availability of diseased immortalized cell lines, which opens the opportunity to also mimic diseases in 3D in vitro models.[23] For general biological questions, immortalized lines are appropriate and provide consistent, reliable results. However, when addressing patient-specific questions, iPSCs are necessary to capture the unique genetic and phenotypic characteristics of individual patients. Choosing the right cell source is often a tradeoff between genetic relevance on one side and cell maturity and differentiation efficiency on the other side. Hence, the cell source should be chosen with the objectives of the study in mind.

For example, in developing gene-editing therapies for Duchenne Muscular Dystrophy (DMD), immortalized lines may suffice when studying general mechanisms. However, if the biological question entails a patient-specific mutation for DMD, one might opt for hiPSC-derived myoblasts. For instance, exon skipping, a method that allows the production of a truncated functional dystrophin protein, is one such therapy that could be tested in a 3D model. Exon skipping targets specific "problematic" exons during mRNA processing and induces edits that cause the exon to be skipped during protein translation.[24,25] This can be achieved using CRISPR/Cas9 technologies or antisense oligonucleotides (AONs). CRISPR/Cas9 is a highly versatile and straightforward genome-editing technology that targets specific genomic DNA sites using a single guide RNA (sgRNA). In the context of DMD, the sgRNA directs the CRISPR/Cas9 complex to the "problematic" exon and allows for precise DNA edits.[26–28] AONs facilitate exon skipping by binding to specific sequences in pre-mRNA. This prevents the inclusion of the "problematic" exons during mRNA processing. In both cases, the reading frame of the dystrophin gene is restored, resulting in a partially functional protein that allows for moderate restoration of muscle function.[29]

A limitation of CRISPR technologies is the high cost associated with the synthesizing and validating of guide RNAs, especially when it comes to patient-specific mutations. Similarly, the synthesis of AONs can be costly as there is a need for extensive testing to ensure their efficacy and safety.[30] Additionally, translating the success of CRISPR technologies and AONs from in vitro studies to in vivo applications has proven challenging. CRISPR tools and AONs often show promising results in controlled 2D cell culture environments, but their efficacy and precision can diminish significantly when applied in vivo. The use of 3D models could aid in increasing the success rate of gene-based therapies in the context of DMD.

The utility of 3D models in developing gene-based therapies is underscored by the work presented in **Chapter 4**. Two models showed their ability to measure restored skeletal muscle tissue functionality and thereby provided a valuable readout that can aid in indicating the potential in vivo performance of the therapies. While the models presented are promising and could potentially reduce costs in gene-based therapy research, they are merely a proof of concept. Further development and validation are required to realize their potential fully. This has to include extensive in vivo studies and comparative analyses with traditional models.

From the two models in **Chapter 4** that were shown to be suitable for measuring restored skeletal muscle functionality, the tapered pillar microtissue model emerges as the most promising platform for future research. Although the Cuore cantilever-based model also demonstrated promising results, ensuring reproducibility within and between experiments in this platform is challenging. Variability in 3D model assembly and performance can lead to inconsistencies in output and inconclusive results. This complicates the validation of gene-based therapies and large-scale drug screenings. Transparency regarding the reproducibility of 3D models is crucial and often not reported. However, transparency can be achieved by establishing reproducibility metrics for published research. Detailed reporting on reproducibility will enhance reliability and will facilitate broader adoption of 3D models across different studies.

Another limitation we encountered with the Cuore cantilever-based model is the high number of cells required per engineered tissue. In this particular case, between 300,000 and 1,000,000 cells were needed to establish a skeletal muscle tissue. A high cell number translates into increased costs to establish the model and also limits its practical applications. High cell numbers are particularly challenging for testing substantial small molecule libraries (up to 30,000 per screen) for drug discovery or for the improvement of differentiation protocols. Scalability should, therefore, be taken into account when choosing a 3D model.

One of the emerging 3D model platforms is the so-called organ-on-a-chip (OOC). OOCs often incorporate multiple cell types into a single device and have multiple compartments to replicate the intricate communication between organ systems. For example, Gabbin et al. developed a model connecting heart and kidney organoids, demonstrating the potential of such systems to mimic inter-organ interactions.[31] Similarly, Fernandez-Costa et al. combined skeletal muscle and pancreatic islet cells on a chip to study the interplay between muscle exercise and insulin secretion, providing valuable insights into metabolic regulation.[32] Although few studies currently exist for skeletal muscle, it is a fast-paced and developing field. Further advancements are expected, potentially incorporating even more complex organ systems to enhance our understanding of the interaction of skeletal muscle with other organs.

In **Chapter 4**, we presented promising preliminary results of a vascularized skeletal muscle-on-a-chip model. As skeletal muscle is a highly vascularized tissue, we integrated vascular structures with skeletal muscle tissues to enhance the physiological relevance of the 3D model. The future of OOC technology lies in strategically combining essential cell types tailored to specific research questions. The closer these models replicate the in vivo environment, the higher the likelihood that experimental therapies will succeed in clinical applications. Building on the preliminary results shown for the vascularized skeletal muscle-on-a-chip, further development and optimization of the model presented will be essential to achieve more accurate and reliable outcomes. Additionally, the question of complexity remains to be answered. Future research must determine the optimal number of required cell types to recapitulate in vivo conditions accurately.

Chapter 5 established reliable RNA staining techniques for skeletal muscle tissues and microtissues. The detailed staining protocol developed for RNA and protein enhancement allows the field to visualize gene and protein expression accurately. The developed method is a valuable tool that can be applied to both in vivo skeletal muscle and 3D in vitro skeletal muscle models.

Future Perspectives

The research described in this thesis offers novel perspectives on the field of developmental biology and regenerative medicine and provides insights that can enhance future development. One promising direction for future research is the integration of artificial intelligence (AI) to streamline complex multi-omics pipelines.[33] By automating and optimizing the analysis of high-dimensional datasets, AI can facilitate a more comprehensive understanding of the molecular mechanisms underlying germ layer differentiation. AI can also be employed to predict the roles of ncRNAs in early differentiation stages, offering new insights into their regulatory functions and potential therapeutic targets. Moreover, AI has the potential to revolutionize gene editing in 3D models by designing patient-specific guide RNAs. Efforts have already been made to improve the prediction of bacterial CRISPRi guide efficiency[34], and it is only a matter of time before these technologies are translated into the human context.

A similar methodology could be applied to drug screening or other therapies. This approach can enhance the efficiency and precision of high-throughput screening, allowing for more accurate modeling of genetic diseases and the development of personalized therapies. Incorporating AI into these processes not only accelerates research but also increases the reliability and reproducibility of the findings. Additionally, the use of AI would democratize access to advanced bioinformatics analyses, enabling researchers with limited bioinformatics backgrounds to conduct unbiased and sophisticated data analysis. This broader accessibility can lead to more diverse research contributions and accelerated scientific discovery.

Another future direction involves using 3D models to advance the maturation of human iPSC-derived myogenic cells. While immortalized cell lines are advantageous for general research and general drug screening, patient-specific iPSC-derived models are crucial for personalized medicine. OOC technologies can further increase the complexity of the microenvironment, promoting the enhanced maturation of iPSC-derived cells. Future studies should focus on refining iPSC differentiation protocols and reducing heterogeneity to enhance the maturity and functionality of these cells.

In conclusion, the advancements discussed in this thesis represent significant progress towards creating more accurate and functional models of human skeletal muscle tissues. By pursuing the outlined future directions, researchers can enhance the utility of these models, contributing to the development of effective therapies and bridging the gap between in vitro studies and clinical applications. Additionally, these advancements will deepen our understanding of human skeletal muscle biology.

References

1. Tejedera-Villafranca, A., Montolio, M., Ramón-Azcón, J. & Fernández-Costa, J. M. Mimicking sarcolemmal damage in vitro: a contractile 3D model of skeletal muscle for drug testing in Duchenne muscular dystrophy. *Biofabrication* **15**, (2023).

2. Cedillo-Servin, G. *et al.* 3D Printed Magneto-Active Microfiber Scaffolds for Remote Stimulation and Guided Organization of 3D In Vitro Skeletal Muscle Models. *Small* (2023) doi:10.1002/smll.202307178.

3. Gholobova, D. *et al.* Human tissue-engineered skeletal muscle: a novel 3D in vitro model for drug disposition and toxicity after intramuscular injection. *Sci Rep* **8**, 12206 (2018).

4. Wang, J. *et al.* Engineered skeletal muscles for disease modeling and drug discovery. *Biomaterials* **221**, 119416 (2019).

5. Rajabian, N. *et al.* Bioengineered Skeletal Muscle as a Model of Muscle Aging and Regeneration. *Tissue Eng Part A* **27**, 74–86 (2021).

6. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131**, 861–872 (2007).

7. Rouhani, F. *et al.* Genetic Background Drives Transcriptional Variation in Human-Induced Pluripotent Stem Cells. *PLoS Genet* **10**, e1004432 (2014).

8. Poetsch, M. S., Strano, A. & Guan, K. Human Induced Pluripotent Stem Cells: From Cell Origin, Genomic Stability, and Epigenetic Memory to Translational Medicine. *Stem Cells* vol. 40 546–555 Preprint at https://doi.org/10.1093/stmcls/sxac020 (2022).

9. Rodin, S. *et al.* Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in the defined and xeno-free environment. *Nat Commun* **5**, 3195 (2014).

10. Pieters, T. & van Roy, F. Role of cell-cell adhesion complexes in embryonic stem cell biology. *J Cell Sci* **127**, 2603–2613 (2014).

11. Sun, Y. *et al.* Hippo/YAP-mediated rigidity-dependent motor neuron differentiation of human pluripotent stem cells. *Nat Mater* **13**, 599–604 (2014).

12. Hayashi, Y. & Furue, M. K. Biological Effects of Culture Substrates on Human Pluripotent Stem Cells. *Stem Cells Int* **2016**, 1–11 (2016).

13. Crist, S. B. *et al.* The adult environment promotes the transcriptional maturation of human iPSC-derived muscle grafts. *NPJ Regen Med* **9**, 16 (2024).

14. Kagawa, H. *et al.* Human blastoids model blastocyst development and implantation. *Nature* **601**, 600–605 (2022).

15. Moris, N. *et al.* An in vitro model of early anteroposterior organization during human development. *Nature* **582**, 410–415 (2020).

16. Stein, C. M., Weiskirchen, R., Damm, F. & Strzelecka, P. M. Single-cell omics: Overview, analysis, and application in biomedical science. *J Cell Biochem* **122**, 1571–1578 (2021).

17. Khan, H. M. *et al.* Smart biomaterials and their potential applications in tissue engineering. *J Mater Chem B* **10**, 6859–6895 (2022).

18. Meira, R. M. *et al.* Ionic-Liquid-Based Electroactive Polymer Composites for Muscle Tissue Engineering. *ACS Appl Polym Mater* **1**, 2649–2658 (2019).

19. Chen, J., Dong, R., Ge, J., Guo, B. & Ma, P. X. Biocompatible, Biodegradable, and Electroactive Polyurethane-Urea Elastomers with Tunable Hydrophilicity for Skeletal Muscle Tissue Engineering. *ACS Appl Mater Interfaces* **7**, 28273–28285 (2015).

20. Frontera, W. R. & Ochala, J. Skeletal Muscle: A Brief Review of Structure and Function. doi:10.1007/s00223-014-9915-y.

21. Wang, Z. *et al.* 3D Printing-Electrospinning Hybrid Nanofibrous Scaffold as LEGO-Like Bricks for Modular Assembling Skeletal Muscle-on-a-Chip Functional Platform. *Advanced Fiber Materials* (2024) doi:10.1007/s42765-024-00433-5.

22. Brown, T. D., Dalton, P. D. & Hutmacher, D. W. Direct Writing By Way of Melt Electrospinning. *Advanced Materials* **23**, 5651–5657 (2011).

23. Mamchaoui, K. *et al.* Immortalized pathological human myoblasts: Towards a universal tool for the study of neuromuscular disorders. *Skelet Muscle* **1**, 1–11 (2011).

24. Howard, M. T. *et al.* Readthrough of Dystrophin Stop Codon Mutations Induced by Aminoglycosides. *Ann Neurol* **55**, 422–426 (2004).

25. Patterson, G., Conner, H., Groneman, M., Blavo, C. & Parmar, M. S. Duchenne muscular dystrophy: Current treatment and emerging exon skipping and gene therapy approach. *Eur J Pharmacol* **947**, 175675 (2023).

26. Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science (1979)* **339**, 819–823 (2013).

27. Kim, S. *et al.* Probing allostery through DNA. *Science (1979)* **339**, 816–819 (2013).

28. Jinek, M. *et al. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity*. https://www.science.org.

29. Goossens, R., Verwey, N., Ariyurek, Y., Schnell, F. & Aartsma-Rus, A. *DMD* antisense oligonucleotide mediated exon skipping efficiency correlates with flanking intron retention time and target position within the exon. *RNA Biol* **20**, 693–702 (2023).

30. Chan, L. & Yokota, T. Development and Clinical Applications of Antisense Oligonucleotide Gapmers. in 21–47 (2020). doi:10.1007/978-1-0716-0771-8_2.

31. Gabbin, B. *et al.* Heart and kidney organoids maintain organ-specific function in a microfluidic system. *Mater Today Bio* **23**, 100818 (2023).

32. Fernández-Costa, J. M. *et al.* Training-on-a-Chip: A Multi-Organ Device to Study the Effect of Muscle Exercise on Insulin Secretion in Vitro. *Adv Mater Technol* **8**, (2023).

33. Singh, M. *et al.* How has the AI boom impacted algorithmic biology? *Cell*

Syst **15**, 483–487 (2024).

34. Yu, Y. *et al.* Improved prediction of bacterial CRISPRi guide efficiency from depletion screens through mixed-effect machine learning and data integration. *Genome Biol* **25**, 13 (2024).