RoosterBio®
Radically Simplifying Use of MSCs

denovoMATRIX
ENABLING BIOLOGY IN VITRO

3D Manufacturing of Therapeutic hMSCs

with RoosterNourish™-XF & beadMATRIX

TABLE OF CONTENTS

1 **ABSTRACT**

INTRODUCTION

RESULTS

Clinically Relevant hMSC Proliferation on beadMATRIX in 3D Bioreactor Cultures

2 *beadMATRIX Preserves hMSC Identity and Proliferative Capacities in 3D Bioreactor Expansion*

4 *beadMATRIX Preserves Immunomodulatory Capacities of hMSCs in 3D Bioreactor Expansion*

CONCLUSION

5 **REFERENCES**

ABSTRACT

The production of standardized, well-characterized, and high-quality human mesenchymal stromal cells (hMSCs) in clinically sufficient quantities is an area of great interest in biomanufacturing. Providing tools that mimic the natural cellular microenvironment could enable hMSC manufacturing scale-up while lowering manufacturing costs, standardizing manufacturing protocols, and complying with safety regulations. Here, we established the beadMATRIX technology, a microcarrier-based technology by denovoMATRIX, as a new valuable tool for cell-based therapies. Expansion of RoosterBio hMSCs in 3D vertical-wheel bioreactors on the beadMATRIX in conjunction with RoosterNourish™-XF media demonstrated the potential to manufacture clinically relevant quantities of hMSCs. The hMSCs maintained their identity, differentiation, and immunomodulatory capacity. Thus, beadMATRIX will contribute to meeting the economic and quality goals of hMSC manufacturing processes.

INTRODUCTION

Cost-effective and standardized manufacturing processes of safe and therapeutically active cells are key for the translation of cell-based therapies into clinical applications. Although there has been significant progress in optimizing tools and protocols for anchorage-dependent cell expansion, 2D cell culture remains labor-intensive and restricted in surface-to-volume ratio. For large-scale clinical applications, many cells are needed and the inefficient surface area utilization of 2D monolayer culture makes it challenging to meet the needs of manufacturing therapeutic cell products. New tools and strategies for 3D cell culture in large bioreactors will effectively maximize accessible surface area by using spherical microcarriers. These microcarriers could enable biomanufacturing control as they operate in functionally closed environments and require less manual work than 2D monolayer culture.

Human mesenchymal stromal cells (hMSCs) are one of the most frequently used sources for cell-based therapies as they are potent modulators of the immune response and thus could be applicable for a wide variety of diseases. Importantly, their administration has proven safe in over 60 clinical trials (Wang et al., 2021). hMSCs have high proliferative capacities and their therapeutic potency is preserved after storage. To facilitate the production of hMSCs needed for clinical applications, 3D manufacturing of hMSCs on microcarriers has been established. Its successful implementation relies on a microcarrier that supports high cell attachment and viability.

Since hMSCs are anchorage-dependent cells, they require interaction with a substrate for cell survival and proliferation. In their natural microenvironment, the extracellular matrix (ECM) provides different glycosaminoglycans (GAGs) and diverse structural proteins to support hMSC attachment and signal transduction. Therefore, in vitro proliferation of hMSCs necessitates a solid substratum that offers attachment sites, especially in serum-free culture conditions. Different protein-, peptide-, and polymer-based substrates have been used to functionalize planar cell culture plasticware as well as microcarriers.

To functionally recreate the plethora of signals presented by the ECM, yet simplifying it to a minimal system, denovoMATRIX has developed biomatrices that combine synthetic biofunctional peptides with GAG mimetics, modular in composition and concentration (Thamm et al., 2020; Wieduwild et al., 2018). Using this system, denovoMATRIX designed a chemically defined biomatrix that supports microcarrier-based culture of hMSCs in serum-free culture conditions, called the beadMATRIX. In this study, we characterized the performance of cells grown in an optimized microenvironment provided by beadMATRIX using the highly productive RoosterNourish-XF medium in a bioreactor set up with extensive subsequent cellular characterization. The aim of this study was to demonstrate that the combination of RoosterBio hMSCs, RoosterNourish-XF medium, and denovoMATRIX beadMATRIX could support clinically relevant hMSC proliferation.

RESULTS

Clinically Relevant hMSC Proliferation on beadMATRIX in 3D Bioreactor Cultures

A high cell number is instrumental in facilitating the use of hMSC-based therapies. To produce the required amount of hMSCs, the development of new tools that closely mimic the natural microenvironment of hMSCs, like the beadMATRIX, may help to improve hMSC biomanufacturing. To investigate whether the beadMATRIX supports high cell proliferation, human bone marrow (hBM)- and umbilical cord (hUC)-

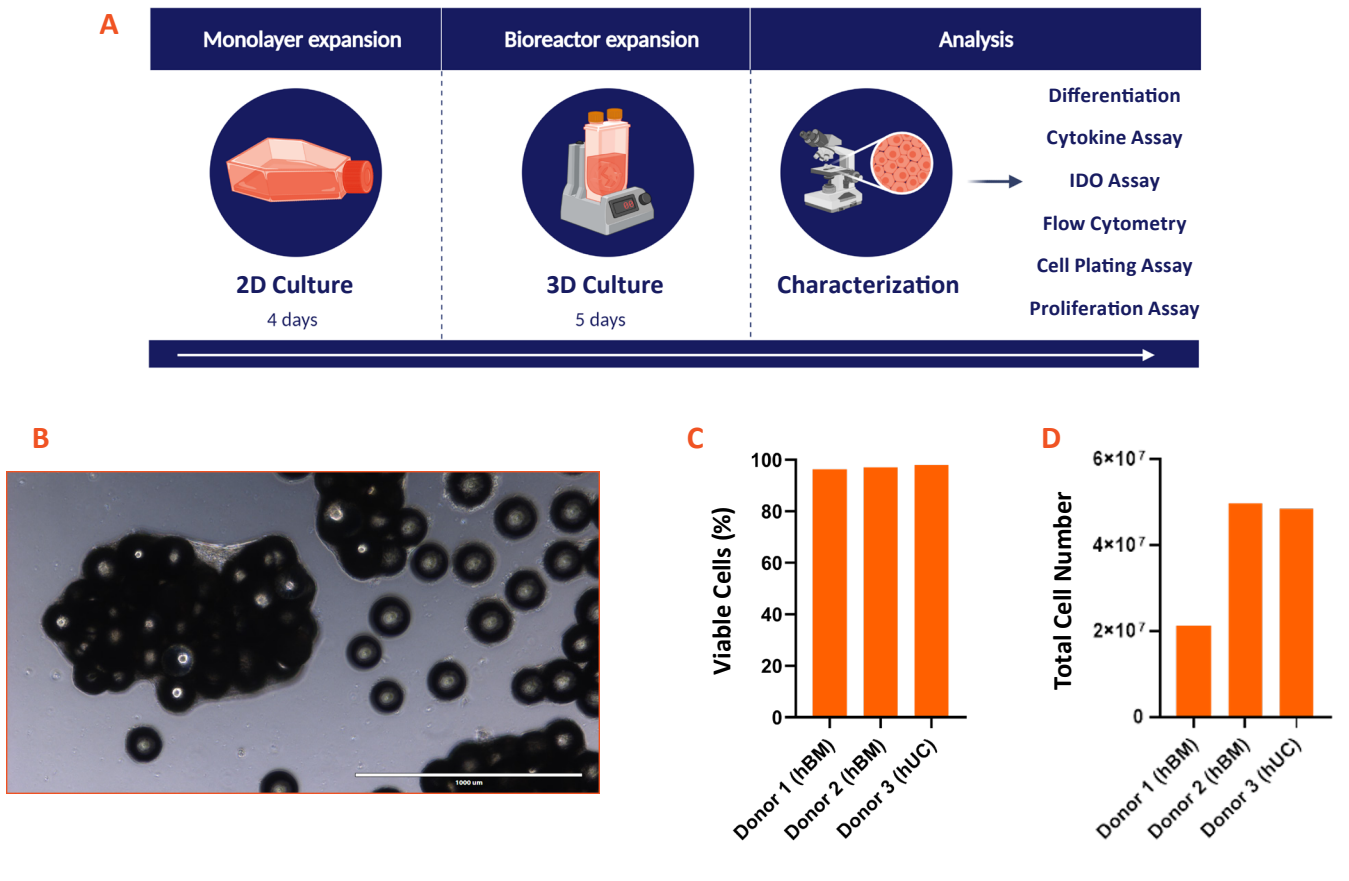
derived hMSCs were cultured according to RoosterBio's standard procedures. Cells were expanded in 2D prior to their propagation in 0.1 L Vertical-Wheel Bioreactors (PBS Biotech) using RoosterNourish™-XF medium (Fig. 1A). After the 3D expansion of hMSCs from three different donors (2x hBM and 1x hUC) for 5 days, cells were harvested to determine the final cell number and viability (Fig. 1B-D).

hMSCS of all three donors showed high viabilities. The final cell yield from the beadMATRIX carriers in combination with RoosterBio hMSCs and medium in the 0.1L Bioreactor was approximately 50 Mio cells for 2 out of 3 donors. Extrapolation of this data suggests that the beadMATRIX/ RoosterBio setup in a 20L Bioreactor would allow product developers to manufacture enough hMSCs for a Phase I clinical trial. Furthermore, the setup could also meet the demands in cell numbers for a Phase II clinical trial when employed in a 50L Bioreactor (Lembong and Rowley, 2021). These results indicated that the ECM-mimicking formulation of the beadMATRIX was beneficial for the 3D cell expansion of hMSCs.

beadMATRIX Preserves hMSC Identity & Proliferative Capacities in 3D Bioreactor Expansion

We first assessed the cellular fitness by determining plating efficiency and proliferation capacity from subsequent 2D cell culture. Briefly, cells were harvested from the bioreactor, seeded into 2D flasks, and either grown overnight for determination of plating efficiency or were grown for 5 days to determine cell density. hMSCs grown on beadMATRIX carriers in 3D had plating efficiencies > 95% for hBM-MSCs and > 65% for hUC-MSCs (Fig. 2A). Both hBM-MSCs and hUC-MSCs demonstrated high cell densities after a 5-day

Figure 1. Expansion of RoosterBio hBM- and hUC-MSCs on beadMATRIX Microcarrier.



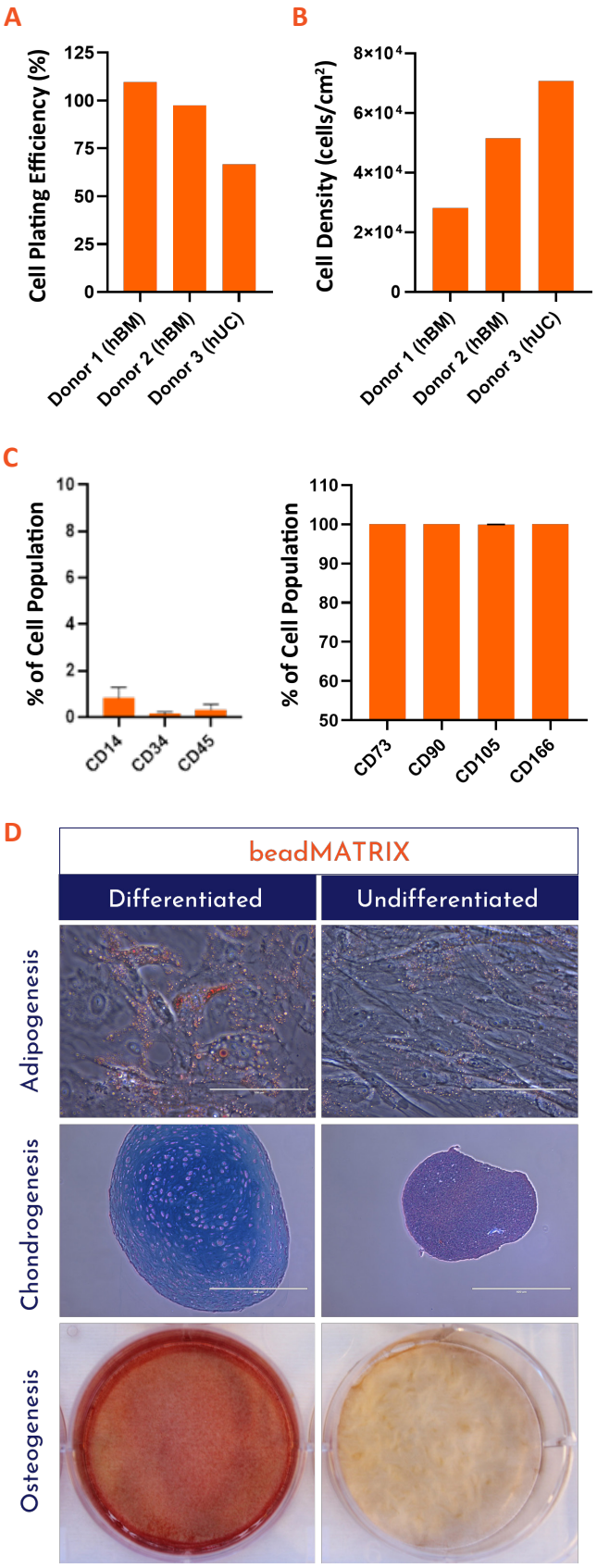
▲**Fig. 1 (A)** Graphical representation of the expansion procedure and subsequently performed analytical assays was created using BioRender.com. **(B)** Cells were grown in RoosterNourish-XF medium on beadMATRIX microcarriers. **(C)** Viability and **(D)** total cell number was determined after 5 days of expansion in Vertical-Wheel bioreactors using a Nucleocounter-200 cell counter.

2D culture period, indicating a high proliferative capacity of beadMATRIX-expanded hUC-MSCs despite lower initial attachment (Fig 2B).

Next, we examined the expression of hMSC-specific cell surface antigens. As defined by the International Society of Cellular Therapy, ≥ 95% of the hMSC population must express CD105, CD73, and CD90 (positive marker), and ≤ 2% can express CD45, CD34, CD14 or CD11b, CD79a, or CD19 and HLA class II (negative marker) measured by flow cytometry (Dominici et al., 2006). All analyzed cells from the bioreactor propagation were positive for CD73, CD90, and CD105. Additionally, CD166, previously identified as an additional positive marker to discriminate hMSCs from fibroblasts, was highly expressed (Brinkhof et al., 2020; Halfon et al., 2010). In contrast, less than 1.5% of the harvested cells expressed any of the three tested negative markers CD14, CD34, and CD45 (Fig. 2C).

hMSC identity is additionally determined by their multipotent differentiation potential (Dominici et al., 2006). Using commercially available differentiation media, hMSCs were induced towards adipogenic, chondrogenic, and osteogenic lineages. In parallel, an undifferentiating control was cultured in basal medium devoid of differentiation-stimulating supplements. Histochemical staining was performed to confirm the identity of the terminally differentiated cells. Adipocytes formed lipid droplets, which were stained with Oil red O (adipogenesis, left panel). For the evaluation of chondrogenic differentiation, cells were cultured in micromasses, fixed, embedded in paraffin, sectioned, and stained for extracellular proteoglycans using Alcian Blue (chondrogenesis, left panel). Osteogenic differentiation is characterized by the presence of Alizarin Red-stained calcium phosphate deposits (osteogenesis, left panel). We observed clear induction of adipogenesis, marked chondrogenesis, and strong osteogenesis compared to control cells for all conditions. This indicates that the beadMATRIX-expanded hMSCs have the required multipotent differentiation potential (Fig. 2D).

Figure 2. Characterization of hBM- and hUC-MSCs After Bioreactor Expansion.



▶**Fig. 2 (A)** Cell plating efficiency (CPE) of harvested cells from bioreactors was assessed after growing cells overnight in 2D flasks. The CPE is defined as the quotient of the number of cells recovered 1-day post plating by the number of cells plated times 100. **(B)** Cell density of hMSCs grown for 5 days in 2D cell culture flasks after bioreactor expansion. **(C)** Cell surface antigen expression of all three donors determined by flow cytometry analysis using three negative (CD14, CD34, and CD45) and four positive hMSC markers (CD73, CD90, CD105, and CD166). **(D)** Trilineage differentiation of 3D-expanded hMSC compared to undifferentiated control cells. Cells were stained for lipid droplets using Oil red O (adipogenesis), for proteoglycans using Alcian Blue (chondrogenesis), and for calcium phosphate deposits using Alizarin Red (osteogenesis).

beadMATRIX Preserves Immunomodulatory Capacities of hMSCs in 3D Bioreactor Expansion

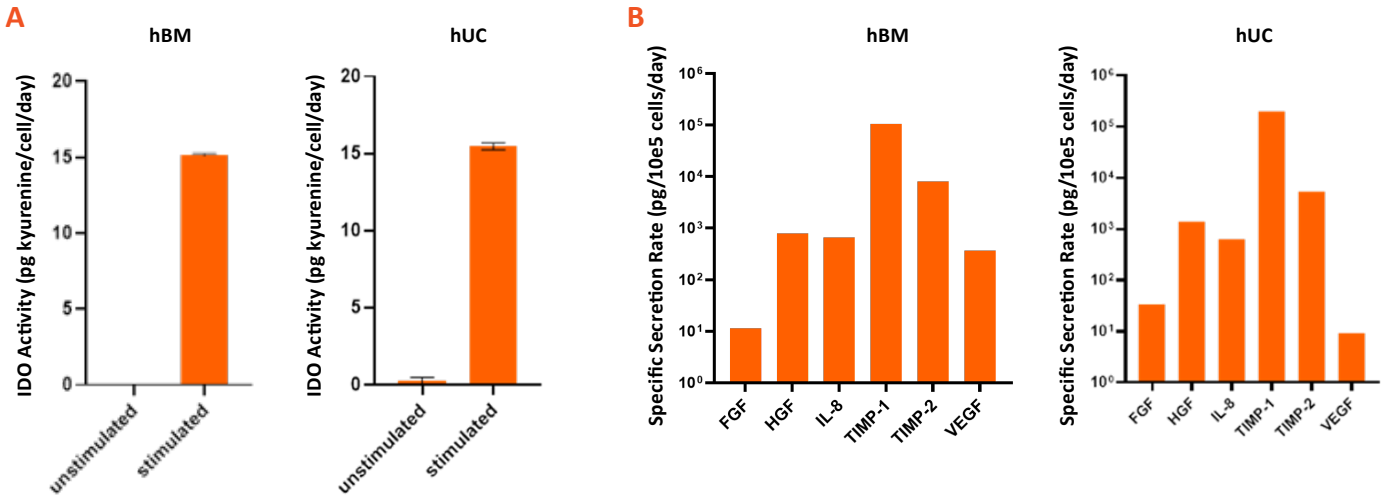
The immunomodulatory potential of beadMATRIX-expanded hMSCs was investigated using a functional Indoleamine 2,3, Dioxygenase (IDO) assay. In this assay, the expression of the immunomodulatory enzyme IDO is induced by the pro-inflammatory cytokine IFN-γ leading to the activation of the kynurenine pathway in which the amino acid tryptophan is converted into kynurenine and its derivatives. Ultimately, the depletion of tryptophan and the accumulation of kynurenine metabolites modulate the immune system by suppressing allogeneic T-cell activation and proliferation, as well as inducing regulatory T cell differentiation (Weiss, 2019). The kynurenine concentration in the supernatant can be measured 24 h post-stimulation using a spectrophotometric assay. After bioreactor expansion, hMSCs showed low basal IDO activity. After induction, hBM-MSCs and hUC-MSCs expanded on beadMATRIX both showed IDO activity indicating they still maintained immunomodulatory activity (Fig. 3A).

Lastly, we determined the angiogenic activity, another important potency parameter of hMSCs. Angiogenic activity is crucial for tissue regeneration and can be measured by the secretion of angiogenic cytokines such as FGF, HGF, IL-8, TIMP-1/2, and VEGF. 24 hours after cell seeding, a MultiPlex ELISA was performed to determine cytokine concentration in the medium. After bioreactor expansion, we observed the production of these angiogenic cytokines by hMSCs propagated on beadMATRIX microcarriers (Fig.3B).

CONCLUSION

Combining RoosterNourish™-XF medium with beadMATRIX microcarrier for the expansion of hMSCs in a bioreactor set-up enables a clinically relevant degree of hMSC proliferation while maintaining hMSC identity and potency. Harvested hMSCs showed high proliferation capacity, expression of hMSC-specific markers, significant trilineage differentiation potential, as well as strong immunomodulatory and angiogenic potency. Thus, the beadMATRIX technology is a new valuable tool for cell-based therapies and will contribute to meeting the economic and quality goals of cell manufacturing processes by maximizing lot size of therapeutically active hMSCs.

Figure 3. Immunomodulatory and Angiogenic Potency of hBM- and hUC-MSCs After Bioreactor Expansion.



▲**Fig. 3 (A)** Immunomodulatory activity measured by induction of IDO enzymatic activity. After incubation with IFN-γ for 24 hours, kynurenine concentration was measured in the medium using spectrophotometric assay and normalized to number of cells and days of incubation. **(B)** Angiogenic activity of hMSCs was determined by measuring the concentration of bFGF, HGF, IL-8, TIMP-1, TIMP-2, and VEGF in the medium after 24 hours of incubation with basal medium using MultiPlex ELISA. Cytokine concentrations were normalized to number of cells and days of incubation.

References

1. Brinkhof, B., Zhang, B., Cui, Z., Ye, H., & Wang, H. (2020). ALCAM (CD166) as a gene expression marker for human mesenchymal stromal cell characterisation. Gene: X, 5, 100031. https://doi.org/10.1016/j.gene.2020.100031
2. Dominici, M., Blanc, K. Le, Mueller, I., Marini, F. C., Krause, D. S., Deans, R. J., Keating, A., Prockop, D. J., & Horwitz, E. M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy, 8(4), 315–317. https://doi.org/10.1080/14653240600855905
3. Halfon, S., Abramov, N., Grinblat, B., & Ginis, I. (2010). Markers Distinguishing Mesenchymal Stem Cells from Fibroblasts Are Downregulated with Passaging. Https://Home.Liebertpub.Com/Scd, 20(1), 53–66. https://doi.org/10.1089/SCD.2010.0040
4. Lembong, J, & Rowley, J. (2021). Building Effective Multi-Year Process Development Programs I: Estimating hMSC Lot Size Ranges for Clinical Manufacturing Through Commercial Demand. https://www.roosterbio.com/blog/building-effective-multi-year-process-development-programs-i/
5. Thamm, K., Möbus, K., Towers, R., Segeletz, S., Wetzel, R., Bornhäuser, M., Zhang, Y., & Wobus, M. (2020). A Novel Synthetic, Xeno-Free Biomimetic Surface for Serum-Free Expansion of Human Mesenchymal Stromal Cells. Advanced Biosystems, 2000008. https://doi.org/10.1002/adbi.202000008
6. Wang, Y., Yi, H., & Song, Y. (2021). The safety of MSC therapy over the past 15 years: a meta-analysis. Stem Cell Research and Therapy, 12(1), 1–15. https://doi.org/10.1186/S13287-021-02609-X/FIGURES/6
7. Wieduwild, R., Wetzel, R., Husman, D., Bauer, S., El-Sayed, I., Duin, S., Murawala, P., Thomas, A. K., Wobus, M., Bornhäuser, M., & Zhang, Y. (2018). Coacervation-Mediated Combinatorial Synthesis of Biomatrices for Stem Cell Culture and Directed Differentiation. Advanced Materials, 30(22), 1–9. https://doi.org/10.1002/adma.201706100

RoosterBio®
Radically Simplifying Use of MSCs

RoosterBio accelerates human mesenchymal stem/stromal cell (hMSC) and exosome/extracellular vesicle product and process development to fuel the rapid commercialization of scalable regenerative cures. Our high-quality hMSCs, bioprocess media, genetic engineering tools, and exosome production solutions are paired with expert bioprocessing knowledge to progress therapeutic developers from concept to first-in-human testing and commercial manufacturing at reduced cost and increased productivity. With optimized, scalable processes, Type 2 Drug Master Files, and cGMP products, we have enabled therapeutic programs to traverse their path to clinical translation in under one year.

www.roosterbio.com
info@RoosterBio.com
+1 301 200 5366

5295 Westview Drive, Suite 275
Frederick, MD 21703

denovoMATRIX
ENABLING BIOLOGY IN VITRO

denovoMATRIX deploys a modular, biomimetic coating technology to enable high performance cell culture, bringing cell therapies to patients and accelerating the path to market for clean meat. The company leverages chemical synthesis, stem cell biology and design thinking to build a platform of biomaterials which address major life science challenges. By providing both cell-based therapy and clean meat companies with solutions, denovoMATRIX is at the forefront of innovation in cell manufacturing processes.

www.denovomatrix.com
mail@denovomatrix.com
+49 (0)351 8547-7890

Tatzberg 47/49
01307 Dresden, Germany