denovoMATRIX
ENABLING BIOLOGY IN VITRO

Application note No. 570

Growth factor stabilization by myMATRIX coatings

Abstract

Growth factors are part of the cellular microenvironment and are stabilized by the cell surrounding extracellular matrix (ECM) components such as glycosaminoglycans (GAGs). Here, we analyzed the effect of growth factor stabilization by myMATRIX cell culture coatings. Our ECM-mimetic coatings, which contain GAGs, tripled the half-life of the growth factor basic fibroblast growth factor (FGF2) in comparison to other cell culture coatings. This stabilization supported the maintenance of stemness and promoted the survival of neural precursor cells (NPCs), especially when applying restricted feeding regimes. These results show that myMATRIX coatings improve growth factor stability and allow for reduced growth factor replenishment which reduces costs and simplifies stem cell culture.

Introduction

The cellular microenvironment is an organized combination of extracellular matrix (ECM), cells, and interstitial fluid that regulates cell fates through physical, mechanical, and biochemical mechanisms defining cell identity and guiding cell development. The ECM is a key component of the cellular microenvironment and is composed of various polysaccharides and proteins assembled into an organized meshwork. Cells are in tight connection with the ECM since it provides essential cues for their integrin binding, cell growth, and survival. Some of these signals are provided by growth factors bound to glycosaminoglycans (GAGs), a family of linear polysaccharides, in the ECM. Since most of the sugars are sulphated or carboxylated, GAGs are highly negatively charged. The negatively charged moieties bind to the positively charged basic amino acids of growth factors. This interaction controls the range of action, diffusion through the ECM, lifetime, and signalling activity of the growth factors. For example, the sulphated polysaccharide chains of the GAG Heparin bind to FGFs resulting in growth factor oligomerization which allows for cell surface receptor activation (Spivak-Kroizman et al., 1994).

In vitro recreation of the cellular microenvironment is of particular importance for cellular agriculture (clean meat) or regenerative medicine i.e. cell and tissue therapy. These applications require the optimization of culture time, efficiency, and yield. Since in vitro culture of cells usually aims for a minimalistic recreation of the ECM, the lack of other molecules residing in the cellular microenvironment such as GAGs, can hamper efficient and reliable cell manufacturing.

Given the importance of GAGs in growth factor function, the denovoMATRIX technology (myMATRIX) aims to mimic the ECM with cell culture matrices that include complex polysaccharide molecules. We have previously shown that this technology improves mesenchymal stromal cell culture for cell therapy applications (Thamm et al., 2020).

In this study, we focus on the beneficial effect of our myMATRIX coatings on the stabilization of FGF2,

a growth factor present in a plethora of cell culture media. The myMATRIX technology is able to significantly stabilize FGF2 in vitro. This leads to enhanced proliferation and stemness of mouse neural progenitor cells (NPCs).

Results

myMATRIX coatings triple the half-life of the temperature-sensitive growth factor FGF2

In order to understand the effect of GAG mimetics present in myMATRIX coatings on growth factor stabilization, we sought to determine the half-life of FGF2 (Fibroblast growth factor-2/basic FGF) in a commercially available pluripotent stem cell (PSC) medium. Cell culture plasticware was coated with either a myMATRIX coating, a laminin matrix, or Matrigel, filled with medium, and placed in a humidified incubator at 37°C. At different time points, the medium was sampled and the FGF2 content was determined using an ELISA (Fig. 1A). After 24 h on myMATRIX coating, the concentration of FGF2 dropped to 55% of the initially applied concentration (Fig. 1B). On Matrigel and the laminin matrix, we observed a more rapid degradation of FGF2 that was significantly different from the reduction on the myMATRIX coating. After 24 h, the concentration was reduced to 20 % and 16 % of the input, respectively. Using a non-linear fit, we estimated the half-life of FGF2 on myMATRIX. With 28 h it was more than 3 times higher than its half-life on Matrigel or the laminin matrix (about 9 h). Our data is in line with previous observations that determined the half-life of native form FGF2 to 4.7–13.7 h through western blot (Shiba et al., 2003). Beyond 48 h, the input concentration was reduced to 27% on myMATRIX, 8% on Matrigel, and 4% on the laminin

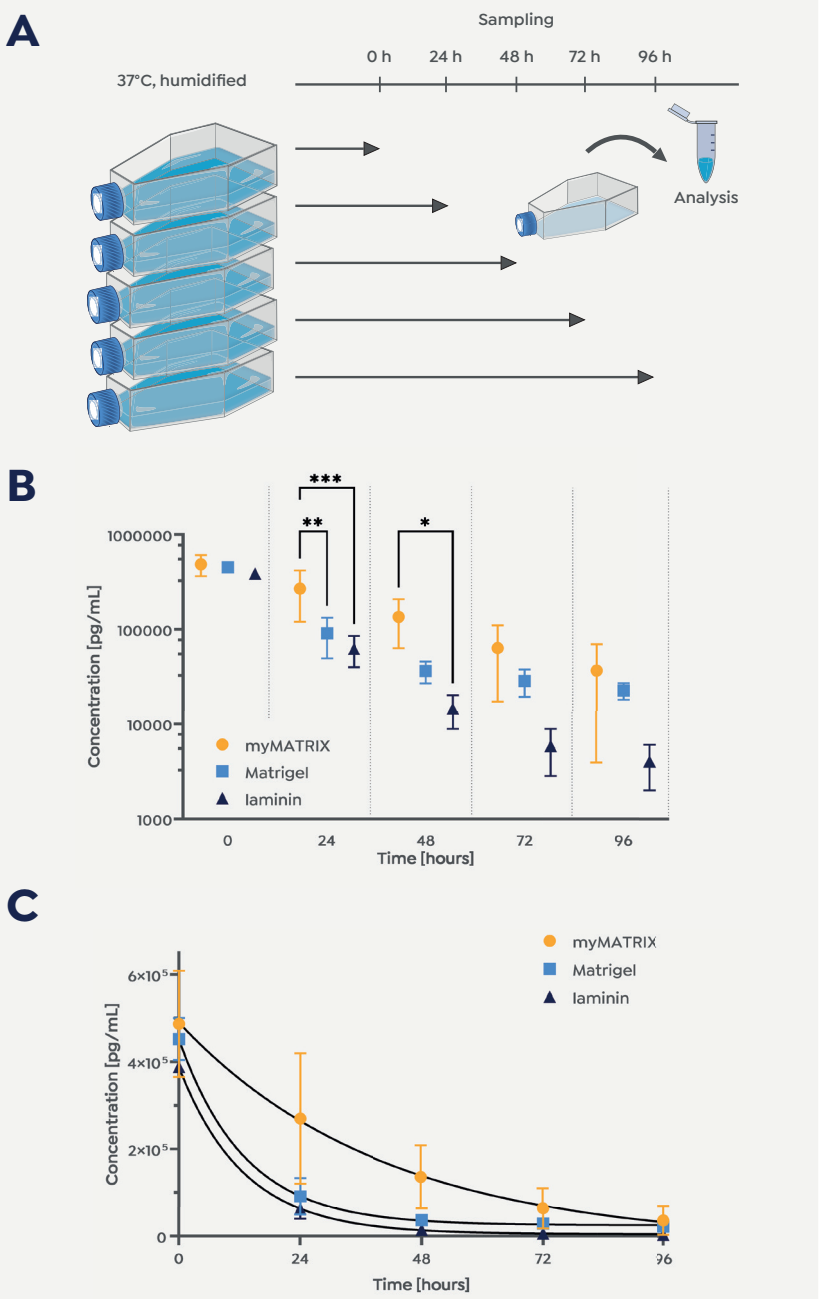

Figure 1. Stabilization of FGF2 by cell culture coatings. **A)** Sampling scheme of stabilization experiment. **B)** FGF2 stabilization in PSC medium by myMATRIX coating compared to Matrigel and a laminin matrix. Two-way-ANOVA Bonferroni multiple comparison tests (0.05, 95% confidence interval; shown is Mean±SEM, n=3). **C)** Half-life determination through non-linear fit using one-phase exponential decay equation: Y=(Y0 - plateau)*exp(-K*X) + plateau. (Half-life of myMATRIX: 28.2 h; Matrigel: 9.0 h; laminin matrix: 8.8 h).

matrix compared to the input concentration. These results indicate that the myMATRIX coating can significantly prolong the lifetime of temperature-sensitive growth factors.

Half-life prolongation of FGF2 through myMATRIX coatings provides growth advantage

To investigate the impact of growth factor stabilization by myMATRIX coatings on cells, we assessed the survival and stemness of mouse neural precursor cells (mNPCs) after restricted feeding regimes. Neural precursor cells rely on FGF2 for self-renewal and maintenance and thus usually require a refreshment of FGF2 every 2 days. Cells were seeded either on a mNPCs-optimized myMATRIX coating or a mNPCs-specific laminin matrix in a medium containing 10 ng/ml FGF2. After a culture period of 4 days mNPCs were fixed, stained, imaged, and counted. During their culture, cells received either a medium change after 48 h (+medium exchange) or not (-medium exchange). mNPCs that did not receive any FGF2 showed no proliferation (Fig. 2A, -FGF2). Cells that received a medium change after 48 h on myMATRIX or on the laminin matrix proliferated normally and showed an increase of about 8- and 3-fold, respectively. Therefore, the number of cells was markedly increased by approximately 2.4-fold on myMATRIX compared to the laminin matrix. When FGF2 was not replenished after 48 h, the difference in cell number increased to 3.5-fold (Fig 2A, C). Further, cells started to narrow indicating signs of neuronal differentiation as induced by the lack of active FGF2 with higher number of particules accumulated at cells on laminin surfaces (Fig. 2C).

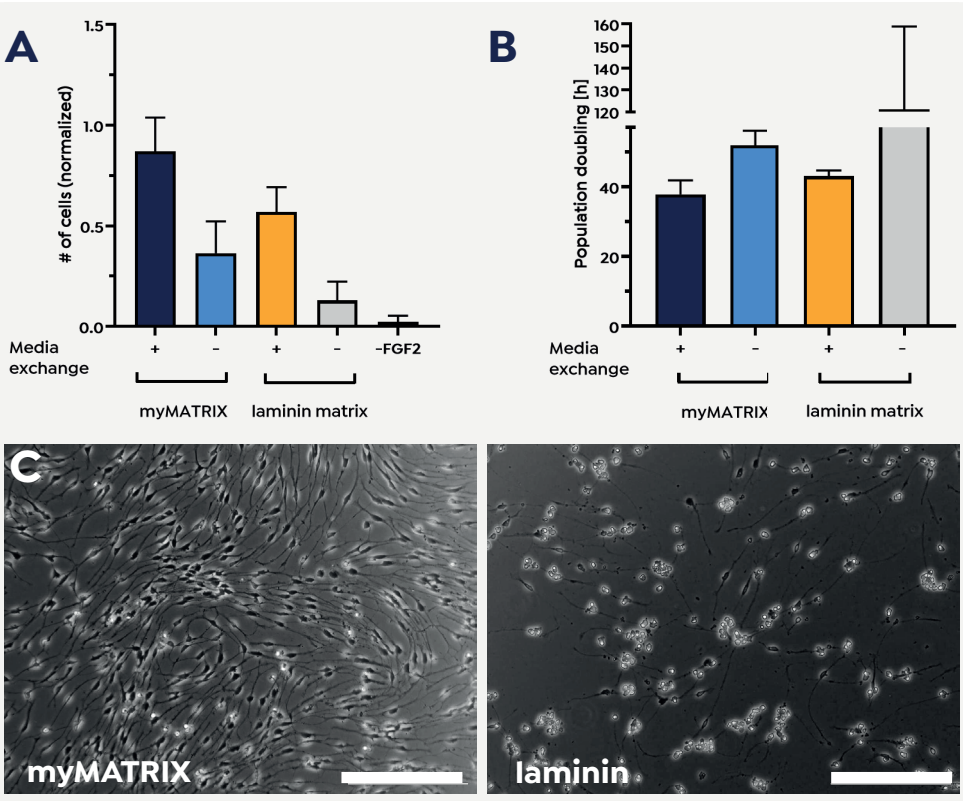

Figure 2. Proliferation of mNPCs during restricted feed regimes. A) Fold increase in the number of mouse neural precursor cells (mNPC) with and without a medium change. **B)** Doubling times of mNPC on different matrices with different feeding regimes. (n=3, Mean+/- SD); **C)** Phase-contrast images of mNPC cultured on myMATRIX and laminin without media exchange after 96 hours in culture. Scale bar: 200 µm

It is likely the consequence of the FGF2 stabilization by the myMATRIX coating allowing for cell proliferation to continue. This is further illustrated by the faster doubling time of mNPCs on myMATRIX of 38 h (+medium exchange) and 52 h (-medium exchange) compared to the laminin matrix with 43 h (+medium exchange) and 121 h (-medium exchange) (Fig. 2B). In sum, robust NPC proliferation is maintained during restricted feeds.

NPCs remain their stemness on myMATRIX coatings without FGF2 replenishment

NPCs have multilineage potential and can differentiate into neurons, astrocytes, and oligodendrocytes. Their stemness state is dependent on FGF2 and

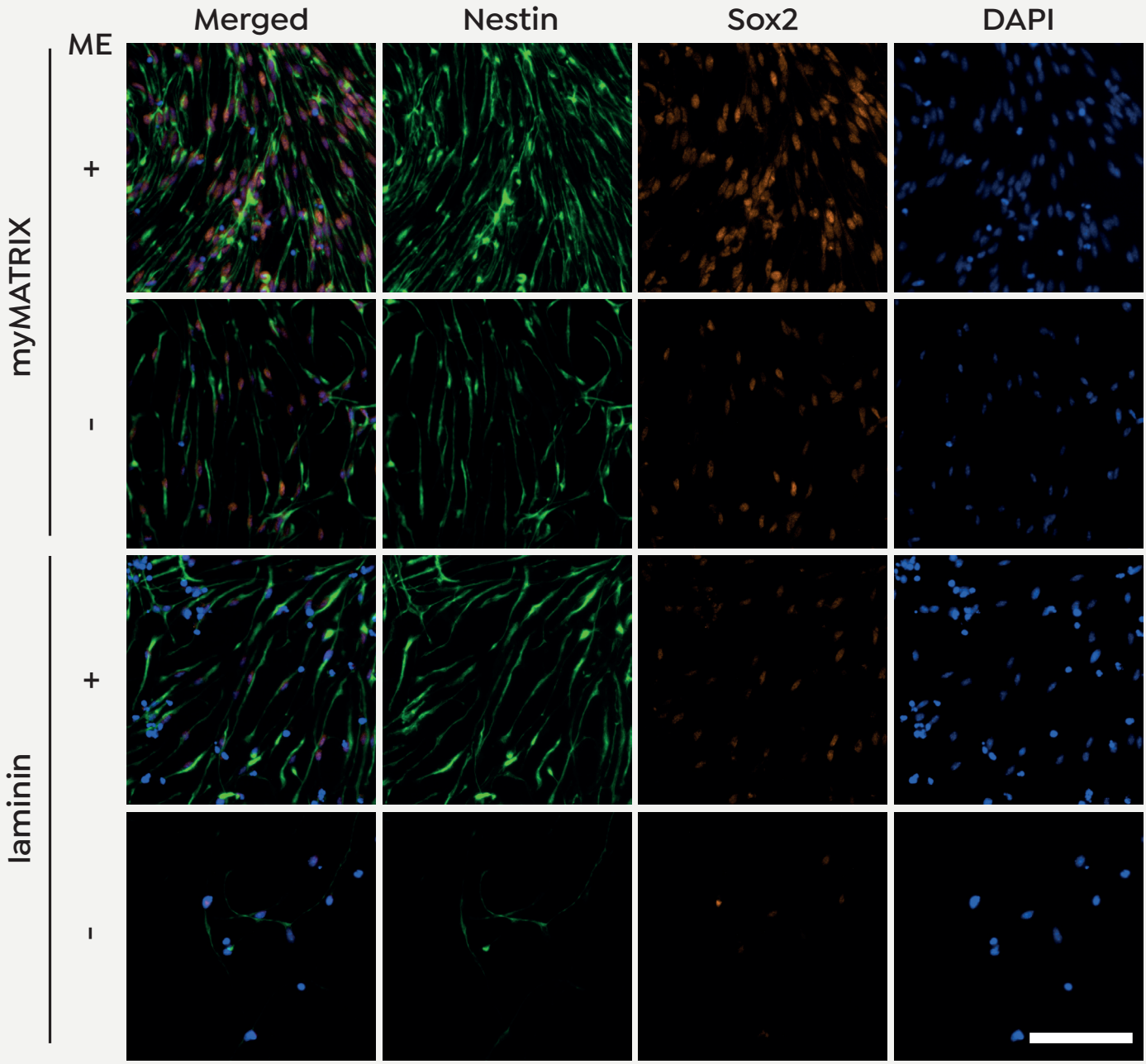

Figure 3. Stemness maintenance of mNPCs after FGF2 withdrawal. mNPCs were cultured for 4 days in FGF2-containing medium with and without medium exchange (ME) after 2 days. Cells were then fixed and stained for the stemness markers DAPI, Nestin and Sox2. Scale bar is 100 µm.

susceptible to culture conditions. In order to assess the stemness of NPCs that have been subjected to different feeding regimes, cells were stained for markers of neuronal differentiation (microtubule-associated Protein 2, Map2ab) and stemness (Nestin and Sox2) (Soltani et al., 2005). mNPCs that were cultured with bi-daily medium change show expression of neuronal markers Nestin and Sox2 in the majority of cells (Fig. 3, + conditions). We observed a stronger expression of

Sox2 and Nestin in mNPCs grown on myMATRIX coatings compared to cells grown on a laminin matrix. Without FGF2 replenishment after 48 h, cells start to lose the expression of Nestin and Sox2 on both matrices. Cells grown on myMATRIX still express, although to a lesser extent, stemness markers, whereas NPCs grown on the laminin matrix lost the expression of Nestin and Sox2 almost completely. In addition, the neuronal differentiation marker Map2ab was largely absent in mNPCs cultured on myMATRIX coating, whereas cells grown on the laminin surface exhibit neuronal differentiation, irrespective of the feeding regime (Fig.4). In conclusion, the myMATRIX coating promotes the survival, proliferation, and identity of mNPCs in FGF2-rich and -low culture environments.

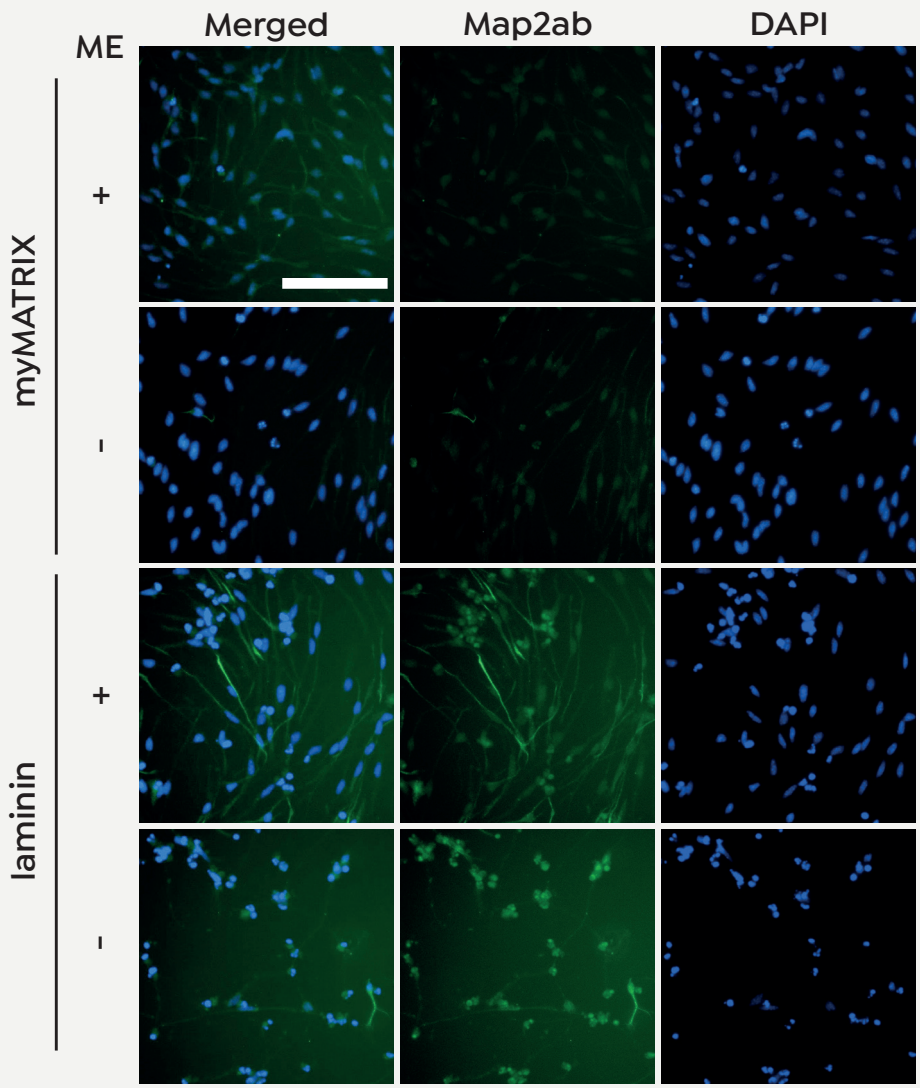

Figure 4. Expression of differentiation marker Map2ab in mNPCs after FGF2 withdrawal. mNPCs were cultured for 4 days in FGF2-containing medium with and without medium exchange (ME) after 2 days. Cells were then fixed and stained for the neuronal marker Map2ab. Scale bar is 100 µm.

Discussion

Growth factor stabilization through electrostatic interaction between the negatively charged sulfate and carboxyl groups of GAGs and the positively charged amino acids of the growth factors is an important role of GAGs as part of the microenvironment of the cell. The regulation of the availability, concentration, and activity of the growth factors through GAGs influences the maintenance of integrity, identity, and proliferation of cells. In vitro recapitulation of the microenvironment is therefore crucial to make cell culture efficient and reliable. Here, we tested the principle of growth factor stabilization in vitro using our myMATRIX coatings and found that they can prolong the half-life of FGF2 at least 3 times. This has several implications for research- and industrial-scale cell culture. FGF2 is the cost-driving component for the culture of primary cells and induced pluripotent stem cells (iPSCs) accounting for up to 90% of the total media costs. For applications such as cell therapy and clean meat, this implies massive cost-reduction especially for upscale cultures. Moreover, frequent or daily

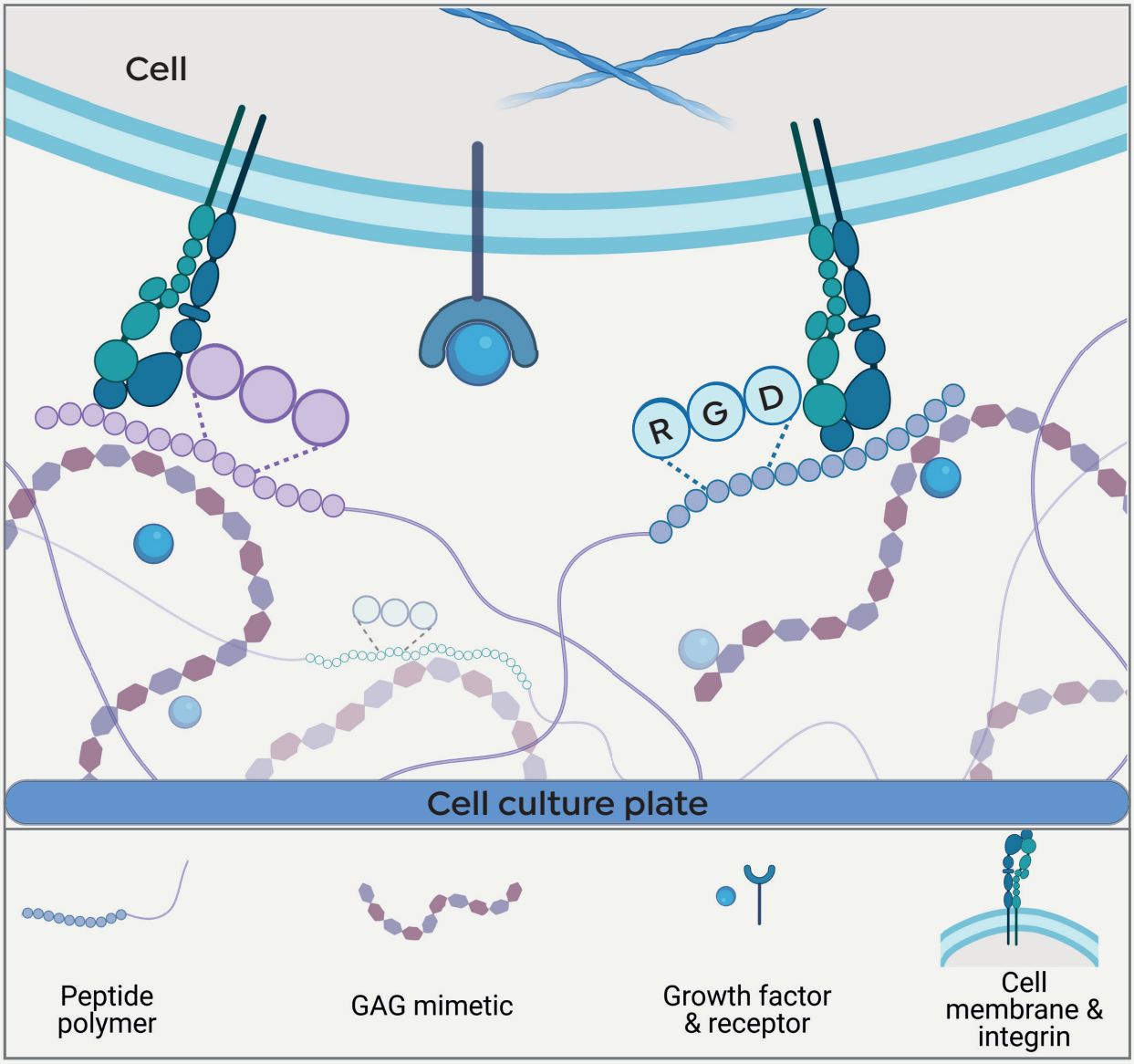

Figure 5. Model of myMATRIX mode of growth factor stabilization. denovoMATRIX biomaterials combine specific peptide polymers and polysaccharide chains of the glycosaminoglycan (GAG) type to recapitulate the key functions of the cell niche in vitro. While the peptides facilitate integrin binding and signaling, the glycosaminoglycan mimetics act as growth factor reservoirs and thus support cell adhesion and signaling.

replenishment of PSC medium is labor-intensive. Tripling the half-life of FGF2 allows for bi-daily or less frequent medium replenishment reducing labor and costs. The biological relevance of such an approach was tested by reducing FGF2 replenishment for neural precursor cells. mNPCs objected to FGF2 starvation on myMATRIX coating were able to maintain their stem cell state and moderate proliferation compared to a laminin surface that does not contain GAGs. Further experiments are required to establish whether the labile stem cell state of pluripotent stem cells can be maintained

in a restricted feeding regime similar to our mNPC experiments.
Since the principle of growth factor stabilization by GAGs is an established concept in vivo, the effect in vitro can be attributed to the presence of GAG mimetics in the coating rather than the peptide component. Moreover, it is likely that other growth factors such as members of the TGFß family are similarly supported providing a broader advantage of myMATRIX coatings for in vitro culture. We envisage the in vitro microenvironment represented by myMATRIX coatings as displayed in Figure 5. Integrin binding by the cells to the peptide component leads to anchorage of the cell to the plastic culture material. GAG mimetics present in the matrix serve as growth factor reservoirs and aid in the binding and biochemical signaling. The denovoMATRIX technology can support both aspects in a cell type- and application-specific manner.

Material Methods

In vitro growth factor stabilization

PSC cell culture medium (mTeSR™1, STEMCELL Technologies) was incubated for different time spans on Matrigel, a laminin matrix, or on myMATRIX coating (denovoMATRIX) in a humidified incubator at 37°C and 5% CO_2. An FGF2 ELISA-kit (Sigma-Aldrich, RAB0182) was used to measure the concentration of FGF2 following the manufacturer's instructions. Briefly, the samples and the protein standard were incubated on the ELISA plate for 2.5 h at room temperature (RT) with gentle orbital shaking. Then samples were aspirated and the plate was washed with washing buffer and incubated with the biotinylated detection antibody for one hour at RT with gentle orbital shaking. The antibody was detected by measuring the absorption of the colorimetric TMB reagent at 450 nm using an ENZO plate reader (ENZ-INS-A96).

NPC medium depletion experiment and characterization

Neural precursor cells were isolated from mouse hippocampus according to Babu et. al., 2009. 20.000 cells/cm^2 of mouse neural precursor cells (mNPCs) were cultured on myMATRIX, or on a laminin matrix. Cells were incubated at 37 °C for 4 days in a complete neuronal medium (neuronal basal medium (Life Technologies), 2% B27 (Life Technologies), 1% Pen/Strep (Life Technologies), 1% GlutaMAX (Life Technologies) ± 10 ng/ml FGF2 (Peprotech)) to reach approximately 80% confluency. Medium change was performed or not on day 2. Phase-contrast images were taken on day 2 and day 4 using the Lionheart FX (Biotek) microscope equipped with the software Gen5. On day 4, mNPCs were carefully fixed with 4% PFA (Sigma Aldrich) for 10 min, washed with PBS and stained with NucBlue (Life Technologies) for 10 min. The number of cells in each well was determined by counting the DAPI-stained nuclei using the Gen5 software. Immunofluorescence experiments were performed using Nestin (R&D systems) and SOX2 (Merck Millipore) and the differentiation marker Map2ab (Sigma Aldrich). Data analysis was performed with GraphPad Prism 9.3.1 and Fiji (ImageJ) software.

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