Heliyon 6 (2020) e04133

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

GDF5 induces TBX3 in a concentration dependent manner - on a gold nanoparticle gradient

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ARTICLE INFO

Keywords:
Cell biology
Developmental biology
Molecular biology
Biomolecules
Cell culture
Cell differentiation
Regenerative medicine
Spectroscopy
Stem cells research
GDF5
SOX9
Ln521
Nano gradient
iPSC
Chondrocyte differentiation
Limb budding zone
TGFb3
TBX3
TGFb1

ABSTRACT

Organs and tissues, such as cartilage and limbs, are formed during development through an orchestration of growth factors that function as morphogens. Examples of growth factors include growth differentiation factor 5 (GDF5) and transforming growth factors beta 1 and 3 (TGFβ-1 and TGFβ-3) which can specify creation of more than one cell type after forming a concentration gradient *in vivo*. Here, we studied the impact of morphogen gradients during differentiation of induced pluripotent stem cells (iPSCs) into the chondrocyte lineage. Cell budding zones, consisting of condensed cell aggregates, were observed only in gradients of GDF5. T-box transcription factor 3 (TBX3) was detected specifically in the budding zones (ranging from 500–1,500 particles/μm^2) of nuclei and cell vesicles. A homogenous density of GDF5 of 900 particles/μm^2 on a surface induced budding and expression of TBX3 after five days in iPSCs. Therefore, we conclude that a gradient of GDF5, as well as the specific homogenous density of GDF5, support the induction of TBX3 in iPCSs. Moreover, differentiation of iPSCs first on GDF5 gradient or homogenous surfaces for five days and then in a three-dimensional structure for five weeks resulted in pellets that expressed TBX3.

1. Introduction

Humans are multicellular creatures and, like all multicellular organisms, are formed from a single cell, the fertilized egg. A fundamental challenge during development is how to generate distinct cell types and organs from a single cell. A theory is that the patterning process achieved through morphogen gradients induces different cellular responses depending on the morphogen concentration and the resulting signals. Molecules belonging to the transforming growth factor beta (TGFβ) superfamily are considered to be morphogens. The TGF superfamily encompasses structurally related proteins, including 33 cytokines, such as TGFs, activins/inhibins, nodal proteins, and bone morphogenetic proteins (BMPs), some of which are growth differentiation factors (GDFs) (Horbelt et al., 2012). These proteins have been described in both vertebrates and invertebrates and are involved in the regulation of biological functions, such as proliferation, migration, inflammation, tissue repair, immune responses, cell differentiation, and apoptosis, in many different adult cell types and during early development (Tang et al., 2009; Bierie and Moses, 2010; Kasagi and Chen, 2013; Wang et al., 2014). Theories regarding morphogens are based on past research, e.g., on vertebrates such as the frog, *Xenopus laevis*. Related work has focused on how members of the TGFβ superfamily can act as morphogens in the induction of the mesoderm, and later give rise to a range of tissue types, including cartilage, notochord, muscles, and blood (Gurdon et al., 1998; Teleman and Cohen, 2000). Researchers have known for decades about the importance of particle gradients in early development and the significance of protein concentration for cell differentiation. Despite this, visualization of gradients and studies of responses to gradients remain few. So far, only a few reports – published almost twenty years ago – visualize a morphogen gradient, e.g., cellular responses to a

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https://doi.org/10.1016/j.heliyon.2020.e04133
Received 14 May 2019; Received in revised form 3 June 2019; Accepted 1 June 2020

decapentaplegic (Dpp) gradient *in vivo* in *Drosophila* wing imaginal disc (Teleman and Cohen, 2000). Reports of gradient visualization *in vitro* are still lacking. Therefore, we generated morphogen gradients *in vitro* on glass substrates, to be viewed through microscopy during differentiation of induced pluripotent stem cells (iPSCs) into the chondrocyte lineage.

1.1. Chondrogenesis

Cartilage arises through chondrocyte differentiation from mesenchymal stem cells and chondroprogenitor cells in early development. The process is initiated by mesenchymal condensation, entailing reduction of intercellular spaces, followed by continued differentiation. Chondrocytes will either remain resting, go through proliferation, followed by maturation and matrix production, be driven into hypertrophy, or undergo apoptosis and endochondral ossification to form bone tissue, whereby calcified hypertrophic cartilage is resorbed and replaced by bone (Goldring, 2012). Molecules such as GDF5, TGFβ-1, and TGFβ-3 are known to be essential during chondrogenesis (Goldring, 2012) and decreased levels of these morphogens are suggested to be involved in, e.g., diseases such as osteoarthritis (OA) (Tang et al., 2009; Bierie and Moses, 2010, van der Kraan et al., 2010; Wang et al., 2014).

1.2. The TGFβ family and the morphogenetic roles of its members

1.2.1. TGFβ

The members of the TGFβ superfamily are distinguished by their binding to different receptors on the cell surface, mediating signal transduction. All members have similar signaling cascades which, in combination with similar key molecules, form a highly interconnected signaling network (Horbelt et al., 2012). However, TGFβ signals through two different pathways: using the intracellular protein Smad, or using a Smad independent pathway, where signaling is mediated via ligand binding to two type II receptors that form a heterotetrameric complex with two type I receptors (Weiss and Attisano, 2013). In mammals, there are three types of TGFβ: TGFβ-1, TGFβ-2, and TGFβ-3 (van der Kraan et al., 2010). TGFβ-1 and TGFβ-3 have been shown to be important in early development in a number of diverse cellular processes, including cell proliferation, differentiation, migration, and apoptosis, and have also been shown to be involved in regulation of immune cells, for instance during inflammation (Tang et al., 2009; Bierie and Moses, 2010; Wang et al., 2014).

1.2.2. GDF5

GDF5, also known as bone morphogenetic protein 14 (BMP-14) or cartilage-derived morphogenetic protein 1 (CDMP-1), is a signaling molecule and member of the bone morphogenetic protein (BMP) family, which is part of the TGFβ superfamily and GDF5 controls chondrogenic cell growth and differentiation. It is also involved in cartilage, joint, bone, and limb bud formation through regulation of cartilage anabolic genes, such as *COL2A1* and *ACAN*, which code for collagen type II and aggrecan (Storm and Kingsley, 1999; Mikic, 2004). GDF5 has shown to enhance and support cell-to-cell adhesion and cellular condensation, an important process in the differentiation of chondrocytes (Francis-West et al., 1999; Coleman et al., 2013).

1.3. Limb bud formation

During limb formation in embryonic development, GDF5 is expressed in the condensed mesenchyme of the cartilage primordium. Later, during joint formation, GDF5 is expressed in the interzone region (Settle et al., 2003; van der Kraan 2018). It has been suggested that levels of GDF5 are linked to OA. Furthermore, mutations of GDF5 have been shown to result in abnormal joint development, a reduced limb phenotype in mice, shortening of bones and digits, and altered number of digits, brachypodism (Landauer, 1952; Gruneberg and Lee, 1973; Storm et al., 1994; Seemann et al., 2005). Mutations in *Gdf5* have also been linked to human skeletal dysplasias (Thomas et al., 1996; Polinkovsky et al., 1997), suggesting that *Gdf5* is involved in regulation of skeletal development in both mice and humans.

Transcription factor SOX9 (SOX9) is encoded by the *SOX9* gene. SOX9 is well-known to be an important regulating protein in chondrocyte differentiation, mesenchymal condensation, and for formation and secretion of cartilage structural proteins. SOX9 is expressed in chondroprogenitors and differentiated chondrocytes, but not in hypertrophic chondrocytes, indicating its importance in early cartilage development (Hino et al., 2014; Lefebvre and Dvir-Ginzberg, 2017). As previously described in relation to protein levels of GDF5, TGFβ-1, and TGFβ-3, mutations in the *SOX9* gene that lead to altered levels of SOX9 proteins are suggested to be linked to skeletal and degenerative diseases (Lefebvre and Dvir-Ginzberg, 2017). SOX9 is a chondrogenic marker and is therefore expected to be present during differentiation (Hino et al., 2014; Lefebvre and Dvir-Ginzberg, 2017).

T-box transcription factor 3 (TBX3) is encoded by the *TBX3* gene and is considered important for, and significantly expressed during, limb formation in early development (Sheeba and Logan, 2017). All T-box genes have a specific activity in embryonal developmental processes, e.g., by regulating the differentiation of mesoderm subpopulations in an embryo. Mutations, which have resulted in decreased protein expressions, have been found to cause developmental defects, such as ulnar-mammary syndrome (Bamshad et al., 1999).

1.4. Nanoparticles to create morphogen gradients

In this project, the impacts of GDF5 and TGFβ signaling in chondrocyte-derived iPSCs (c-iPSCs) were studied by using a gradient surface of either GDF5, TGFβ-1, or TGFβ-3. The gradient surface was created by coupling the morphogen to gold nanoparticles attached to a glass surface in a gradient pattern with increasing spacing between the particles which enabled screening for cell behavior and protein expression depending on morphogen density. The purpose of using gold nanoparticles was to enable binding of signaling molecules in a controlled and continuous gradient pattern to a stable substrate. As the cells were attached to a surface, the cell stimulations were comparable to those in matrix-bound cells *in vivo,* stimulated with local morphogen concentrations. The gradient surfaces were seeded with c-iPSCs, after being functionalized with the morphogen signaling molecules, followed by differentiation. Immunostaining was used to visualize morphogen-induced candidate proteins (SOX9 and TBX3) in c-iPSCs after five days of differentiation mainly with GDF5 stimulation. Cells on the gradient surfaces were stained with antibodies to determine if any specific morphogen density induced or decreased expression of SOX9 or TBX3. The choice of SOX9 and TBX3 was based on upregulation of expression in the presence of GDF5 in a microarray analysis (unpublished data).

2. Materials and methods

2.1. Preparation of gradient surfaces

Cline Scientific's Nano Gradients and Nano Surfaces (Cline Scientific AB, Sweden) were used as a tool in the differentiation process. The particle densities of the gradients were in the range 3–3,000 particles/μm^2, determined through scanning electron microscopy (SEM) using a Zeiss Ultra 55 operating at an accelerating voltage of 3.00 kV. Images were acquired in the secondary/backscattered electron mode using the In Lens detector. A functionalization protocol provided from Cline Scientific was utilized. Thus, thiolated streptavidin (SH-streptavidin, ProteinMods, USA) was applied to the nanoparticles. After sufficient incubation at room temperature, superfluous streptavidin was rinsed off with phosphate buffered saline (PBS) (Phosphate Buffered Saline, Amresco, USA). Thereafter, laminin 521 (Biolaminin 521 LN, BioLamina, Sweden) was applied to attach to the glass surfaces between the particles. After incubation (as above), superfluous laminin 521 was rinsed off with PBS.

Recombinant human GDF5 (R&D Systems, Bio-Techne, MN, USA), TGFβ-1 (R&D Systems, Bio-Techne, MN, USA), and TGFβ-3 (R&D Systems, Bio-Techne, MN, USA) were biotinylated to facilitate binding to streptavidin. The biotinylation was performed with a superfluous amount of biotin (EZ-Link™ Sulfo-NHS-LC-Biotin, Thermo Fisher Scientific, USA) to ensure that biotin was attached to all available sites of the protein. The biotin that was not attached was discarded using repeated wash and centrifugation, in accordance with Thermo Fisher's biotinylation protocol. The success of the biotinylation was checked using a HABA test (HABA/Avidin Reagent, Sigma Aldrich, USA). The HABA test confirmed that the number of biotin molecules attached to the proteins was within specified limits provided by the manufacturers. Lastly, biotinylated proteins with a known concentration, 40 nM in suspension, were attached to the streptavidin-coated gold nanoparticles and incubated at 4 °C overnight. As in the previous steps, superfluous proteins were rinsed off with PBS. After functionalization, the surfaces were stored at 4 °C until c-iPSCs were seeded and differentiated on the gradient surfaces.

After SEM analysis of the gradient surfaces, the particle density where the cell response was of interest was identified and this density was reproduced on new homogenous density (h.d.) surfaces (without a gradient pattern) (Nano Surfaces, Cline Scientific AB, Sweden). H.d. surfaces with a particle density higher or lower than the particle density of interest were used as controls. Gradient surfaces with only streptavidin and laminin 521 were used as references.

2.2. Cell culturing

We have previously shown that chondrocytes from autologous chondrocyte implantation donors can be reprogrammed into iPSCs using a footprint-free method based on mRNA delivery (Borestrom et al., 2014), a method that was used to generate c-iPSCs for this study. The iPSCs were derived from chondrocytes from an anonymous female donor. Newly thawed c-iPSCs were seeded for monolayer culturing in cell culture flasks (Corning™ Primaria™ Tissue Culture Flasks, Fisher Scientific, USA) which were coated specifically for c-iPSCs (DEF-CS 500 Coat-1, Cellartis, Sweden) and placed in a 37 °C humidity chamber at 90 % humidity and 5 % CO_2. Cell medium (Cellartis® DEF-CS™ 500 Basal Medium with Additives, Cellartis, Sweden), supplemented with additional growth factors, was changed every day and cell passage was performed every third day. The chondrocytes used for the pellet experiments were a combination from three anonymous male donors, provided with written consent. Chondrocytes were expanded in a monolayer in chondrocyte medium consisting of Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Thermo Fisher Scientific, USA) supplemented with 8 mM L-ascorbic acid (Sigma Aldrich, USA), 1 % penicillin-streptomycin (Sigma Aldrich, USA), and 10 % human serum, at 37 °C in 5 % CO_2 and 90 % relative humidity. The medium was changed three times per week. All handling of cells was performed in a sterile cell lab area.

2.3. Differentiation on gradient surfaces and h.d. surfaces

All gradient surfaces and h.d surfaces were seeded with 50,000 cells/cm^2 and incubated in 6 well plates at 37 °C in a humidity chamber for two hours. Thereafter, 2 ml maintenance medium was applied to each well. The next day, 2 ml of a previously published modified differentiation medium (Nguyen et al., 2017) (Table S1) was added, with differing composition depending on which morphogen the gradient surface was functionalized with. TGFβ-3 was added in the differentiation medium for gradient surfaces functionalized with TGFβ-1. TGFβ-1 was added to the differentiation medium for gradient surfaces functionalized with TGFβ-3. GDF5 gradient surfaces had both TGFβ-1 and TGFβ-3 added to the differentiation medium. To evaluate the effect of GDF5 immobilized on the gradient surface compared with having GDF5 free in solution, GDF5 was also added to the differentiation medium for some of the TGFβ-3 h.d. surfaces (see the results in Section 3.5). The differentiation medium was changed every second day during the five-day differentiation period.

2.4. c-iPSC analysis on gradient surfaces and h.d. surfaces

Previous studies performed using c-iPSCs and chondrocytes for differentiation on gradient surfaces showed that chondrocytes were particularly difficult to keep attached to the gradient surface after five days. Therefore, after five days of differentiation on the gradient surfaces in differentiation medium, the cells were fixed with Histofix™ (Histolab Products AB, Sweden), nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (ProLong™ Gold Antifade Mountant with DAPI, Thermo Fisher Scientific, USA), and the surfaces were mounted on glass slides. Cell analysis was performed using the fluorescence imaging microscope IN Cell Analyzer 6000 (IN Cell Analyzer 6000, GE Healthcare, United Kingdom), Eclipse 90i (Eclipse 90i, Nikon Instruments, Japan), and a confocal microscope (Nikon A1, Nikon Instruments, Japan).

2.5. Immunostaining of c-iPSC-seeded gradient surfaces and histological pellet sections

c-iPSCs on GDF5 gradient surfaces and h.d. surfaces were stained for SOX9 and TBX3. Histological pellet sections were stained for TBX3. TGFβ-3 h.d. surfaces, both with and without GDF5 in the medium, were used as controls. Paraffin-embedded histological sections were deparaffinized and dehydrated prior to immunostaining. Cells on surfaces and in pellet sections were permeabilized prior to immunostaining using 0.1 % tritonX-100 (TritonX-100, Sigma Aldrich, USA) in PBS and incubated at room temperature for 10 min. After permeabilization, cells were incubated in blocking medium (2 % BSA (bovine serum albumin, Sigma Aldrich, USA), 0.1 % tritonX-100, 100 mM glycine (Glycine, Riedel-de Haen AG, Germany) dissolved in PBS) for 15 min, followed by incubation in 500 μl of diluted specific primary antibody (Table S2) at 4 °C overnight. Next day, cells were rinsed and washed with PBS three times for 3 min. Thereafter, cells were incubated with blocking medium for 15 min, followed by incubation with a secondary antibody for 2 h in a humidity chamber (Table S2). After three rinsing/washing steps in PBS, each for 3 min, the cells and pellet sections were mounted with ProLong™ Gold Antifade Mountant media (ProLong™ Gold Antifade Mountant with DAPI, Thermo Fisher Scientific, USA) and imaged using fluorescence microscopy (Eclipse 90i, Nikon Instruments, Japan) and confocal microscopy (Nikon A1, Nikon Instruments, Japan).

2.6. Pellet cultures from GDF5 gradient surfaces

Gradients are involved in, and may sometimes trigger (Zecca et al., 1996), many different processes during early development and were therefore utilized to mimic the natural environment during chondrogenesis *in vivo*. GDF5 gradient surfaces and h.d. surfaces were used to differentiate c-iPSCs. After five days of differentiation, the cells were removed from gradient surfaces using trypsin-EDTA (0.05 %) (Thermo Fisher Scientific, USA) for further differentiation in a three-dimensional (3D) structure (pellet). The reaction was quenched with human serum and the cells were transferred into 15 ml conical tubes and centrifuged for 5 min at 1,200g in 2 ml differentiation medium with addition of both TGFβ-1 and TGFβ-3 (Table S1), to form 3D pellets. The pellets were stored with 200 μl differentiation medium in 96 well plates in a 37 °*C incubator* with 5 % CO_2 and were differentiated for five weeks. The medium was changed every third day.

To compare results from the c-iPSC pellets derived from GDF5 gradient surfaces and from those derived from h.d. surfaces, two control setups were employed. In one, control pellets were derived from chondrocytes using a GDF5 gradient surface and, in the other, pellets were derived without a gradient surface, but with 5 % human serum added to the differentiation medium. Chondrocyte pellets were differentiated for two weeks, since it has been shown that chondrocytes from OA donors start to express high levels of both collagen type II and proteoglycans after two weeks (Tallheden et al., 2005a). After the differentiation period, all pellets were fixated with Histofix™ (Histolab Products AB,

Sweden) overnight and then stored in 70 % ethanol. Thereafter, pellets were sent to HistoCenter (Mölndal, Sweden) for sectioning and staining with Alcian Blue van Gieson to visualize the extracellular matrix (ECM) composition of the pellets. The pellet compositions were analyzed using a light microscope (Eclipse 90i, Nikon Instruments, Japan).

3. Results

We used gradient surfaces to study how cells are affected by morphogens. Montages of SEM pictures, taken at different positions with 1 mm intervals along the surface and the gold nanoparticles, are used to present the data (Figure 1). The morphogens were attached to the gold nanoparticles in a gradient pattern using the linker streptavidin, with laminin 521 between particles to facilitate cell adhesion to the glass surface. After functionalization of the gradients, seeding, and differentiation of c-iPSCs, the cells on the gradient surfaces were observed using microscopy. Based on the cell response at a particular density on the gradient surfaces (e.g., bud formation), new surfaces with a corresponding homogenous density (h.d) were produced.

3.1. Laminin 521 control gradient surfaces

Previous experiments with glass surfaces have shown that c-iPSCs have an impaired capacity to attach to glass. Therefore, laminin 521 was attached to the gradient surface between the biomolecule-functionalized particles, successfully facilitating cell adhesion (Aumailley, 2013). Laminin 521 gradient surfaces (without morphogens) were used as controls to compare against the cellular responses from the morphogen gradient surfaces and the h.d. surfaces. Cells on all replicated laminin 521 gradient surfaces resulted in formation of condensed semi-circular structures consisting of cell-free cavities (Fig. S2).

3.2. GDF5 gradients and h.d. surfaces

Compared with results from laminin 521 control gradient surfaces, c-iPSCs differentiated on GFD5 gradient surfaces resulted in a distinct cell behavior with condensed cell clusters (termed budding zones) appearing at a density of 500–1,500 particles/μm^2 (Figure 2), determined using SEM (Figure 1A and Fig. S1). Larger cell masses were observed over the entire surface, while small cell budding clusters were seen in the budding zone. New h.d. surfaces with a particle density in the range of the budding zone were manufactured to create surfaces with a homogenous GDF5 density (Figure 3B). The same cell budding phenomenon observed on the gradient surfaces was also observed on these h.d. surfaces. For comparison, h.d. surfaces with a lower particle density were also produced (Figure 3A). On these h.d. surfaces, cells formed larger gatherings, but there were no indications of budding formations.

3.3. TGFβ-1 gradients and h.d. surfaces

In areas where the particle density was low, c-iPSCs on all replicated TGFβ-1 gradient surfaces formed semi-circular structures, a behavior comparable to that seen on control gradient surfaces with only laminin 521. At higher TGFβ-1 densities, this response was not observed. To compare the cell responses when exposed to different single morphogen densities, these were studied using h.d. surfaces. Separate surfaces with low or high particle density were produced. On low TGFβ-1 h.d. surfaces (600 particles/μm^2), cells tended to form the aforementioned semi-

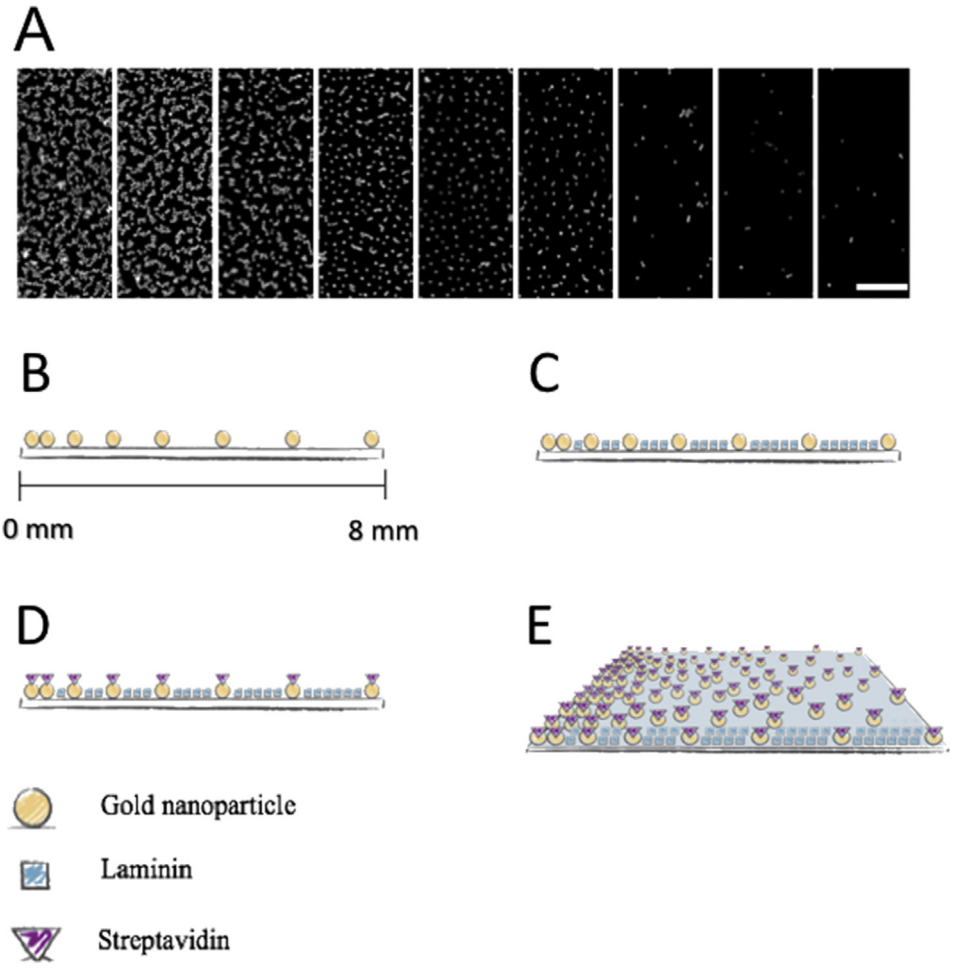

Figure 1. Images and schematic illustration of the gold nanoparticle gradient. (A) Montage of SEM pictures taken at different positions with a 1 mm interval along the gradient surface, scale bar 200 μm. The gradient is verified up to 8 mm on a 18 × 18 mm glass surface. (B) Gold nanoparticles (yellow) attached in a gradient pattern on a glass substrate. (C) Laminin 521 (blue) attached to the glass surface between particles. (D) Streptavidin (purple) attached to gold nanoparticles as a linker to the biotinylated biomolecule. (E) Gradient ready to be functionalized by, e.g., morphogens.

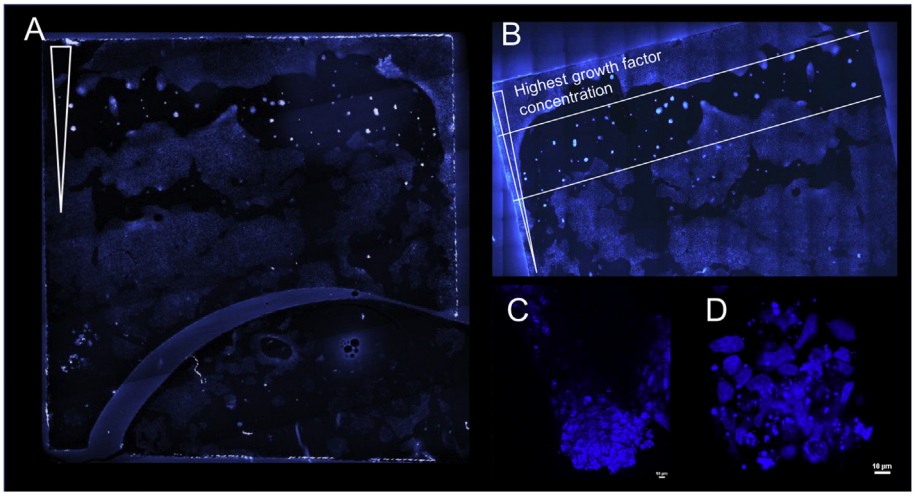

Figure 2. (A) GDF5 gradient surface, 18 × 18 mm, seeded with c-iPSCs visualized using high-throughput confocal IN CELL Analyzer 6000. The bar on the left upper side indicates the extent of the gradient, where the continuous density increase is shown with a marker on the left-hand side, with low GDF5 density at the bottom of the image and high at the top (the damaged lower left corner does not affect the gradient result). (B) Focusing on the budding cell clusters identified at a density range 500–1,500 particles/μm^2. Two white lines indicate the budding zone. (C–D) Close-up of buds identified in the budding zone. Scale bars 10 μm.

circular structures with cell-free cavities (Figure 3C). On high TGFβ-1 h.d. surfaces (2,000 particles/μm^2), the cells were evenly distributed (Figure 3D). No budding zone was observed.

3.4. TGFβ-3 gradients and h.d. surfaces

The c-iPSCs on TGFβ-3 gradient surfaces did not generate any visually identifiable cell response. To compare and confirm the results, h.d. surfaces with high and low morphogen densities were used. Cells were evenly distributed over the gradient surfaces and over the h.d. surfaces throughout differentiation, at both low (600 particles/μm^2) and high (1,900 particles/μm^2) density (Figures 3E & 3F). No budding zone or tendency to condense was observed.

3.5. Immunostaining analysis of SOX9 and TBX3 in c-iPSCs on gradient surfaces

The c-iPSC behavior resulting in a budding zone, found solely on GDF5 surfaces, was further studied using immunostaining for specific proteins. Immunostaining using selected antibodies (Table S2) revealed expression of SOX9 (Isotype control shown in Fig. S3 and control without primary antibody shown in Fig. S4) and TBX3 (Isotype control shown in Fig. S6 and control without primary antibody shown in Fig. S7) in c-iPSCs differentiated on GDF5 gradient surfaces. All cell aggregates showed high SOX9 expression (Figure 4). TBX3 expression was observed to be specifically located in the buds of the budding zone, primarily in the nuclei, but also in highly stained vesicles around the nuclei (Figure 5 and polyclonal antibody shown in Fig. S5). To validate the elevated levels of TBX3 observed in buds on GDF5 gradient surfaces, h.d. surfaces with a particle density corresponding to the particle density of the budding zones were used. For comparison, h.d. surfaces with GDF5 at low particle density, TGFβ-3 surfaces at budding zone density, TGFβ-3 surfaces at budding zone density with GDF5 added to the medium, and glass slides coated with Coat-1 without added biomolecules were used as controls. TBX3 expression on surfaces with the budding zone density was more upregulated compared with all controls (Figure 6, Row B). This finding confirms that TBX3 is more expressed at a specific GDF5 range, first found in the budding zone (Figures 5 and 6).

3.6. Histological analysis of micromass pellets

In order to study the role of the GDF5 gradient, cells were pre-differentiated on GDF5 gradient surfaces and GDF5 h.d. surfaces (one particle density from the budding zone range and one density with lower particle density as a control), before culturing as 3D micromass pellets. Histological sections of the pellets were stained with Alcian Blue van Gieson and revealed areas with a high amount of both blue-colored glycosaminoglycans (GAGs) and red/purple-colored collagen. The blue color was concentrated to one area of the pellets, while the red/purple color was seen around the blue area, particularly in the distinct edges. Two different chondrocyte pellets were used as controls. One had a GDF5 gradient surface and the same differentiation medium as the c-iPSC pellets (Table S1) (Figure 7D1 & 7D2) and the other was without a GDF5 gradient surface but had 5% human serum added to the medium (Figure 7E1 & 7E2). Both chondrocyte pellets (with and without GDF5 gradient) have a more compact structure than the c-iPSC pellets. The Alcian Blue van Gieson coloring indicated that the pellets generated from iPSCs is not homogenous but with areas similar in color to pellets derived from chondrocytes. Histological pellets from c-iPSCs stained positive for TBX3 using immunohistochemistry (Figure 7A3–7A6, 7B3, 7C3). TBX3

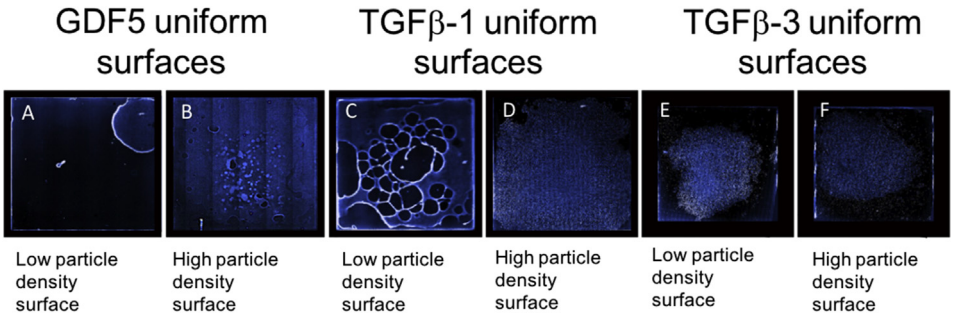

Figure 3. GDF5, TGFβ-1, and TGFβ-3 h.d. surfaces, 18 × 18 mm, visualized using high-throughput confocal IN CELL Analyzer 6000. (A) GDF5 surfaces with a density of 400 particles/μm^2 (control). No buds are identified. (B) GDF5 surface with a density in the range of the budding zone, 900 particles/μm^2. Buds are clearly identified as brighter colored clusters. (C) TGFβ-1 surfaces with a density of 600 particles/μm^2. Cell-free cavities were formed, a response comparable to that of cells on laminin 521 gradient surfaces (Fig. S2). (D) Evenly distributed cells on TGFβ-1 surfaces with a density of 2,000 particles/μm^2, (E) TGFβ-3 surfaces with a density of 600 particles/μm^2, and (F) a density of 1,900 particles/μm^2.

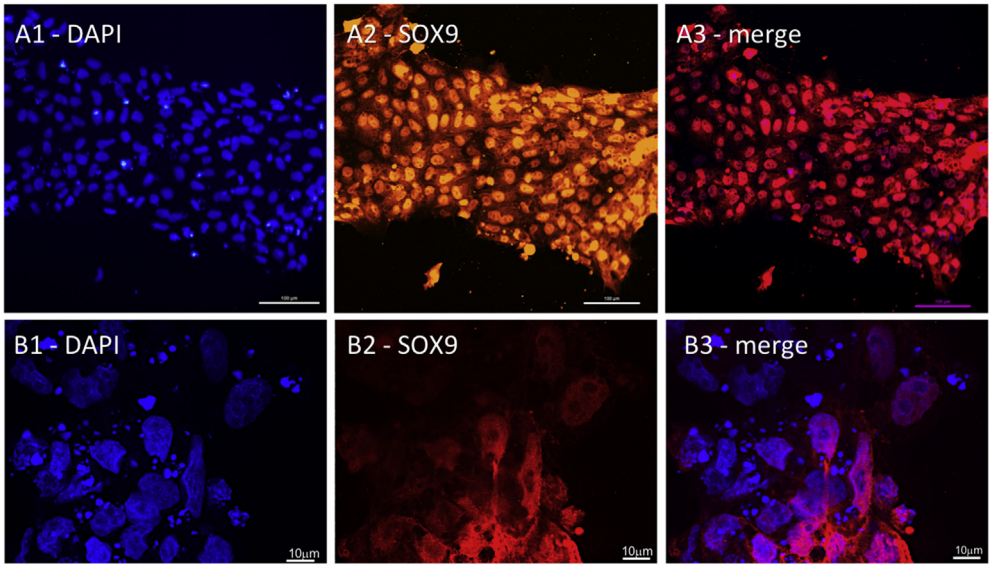

Figure 4. Immunostaining for SOX9 in c-iPSCs after five days of differentiation on a GDF5 gradient. (A1–A3) Images from the budding zone were acquired using fluorescence microscopy, 20X objective. Scale bars denote 100 µm. (B1–B3) Images from the budding zone were acquired using a confocal microscope, 40X objective. Scale bars denote 10 µm.

was specifically induced in the area of the pellets with upregulated amounts of GAG.

4. Discussion

Concentration gradients, in which local concentrations are used for regulation of cell behavior, are important for the function and structure for tissue generation (Zecca et al., 1996; Peret and Murphy, 2008). Such gradients occur naturally in the body and are essential inducers of many developmental and tissue-generating processes. The generation of iPSCs from somatic cells implies rejuvenation of adult cells into the blastula stage of development. Differentiation back into different types of tissue can be complex and the right concentration range of specific growth factors is key (Dakhore et al., 2018). Here, we used gold nanoparticle gradient surfaces to enable binding of GDF5, TGFβ-1, and TGFβ-3 in a defined and continuously inclining pattern, to study the impact of the change in density during differentiation into the chondrocyte lineage. As mentioned in the introduction, only a few reports visualize morphogen gradients *in vivo* and *in vitro* (Teleman and Cohen, 2000; Lagunas et al., 2013). Thus, the morphogen gradients visualized here, and the results of this study, are significant for understanding GDF5 function. Cell budding zones were observed in a specific density range, indicating GDF5's role in a condensing budding formation. No budding formations were found on TGFβ-1 or TGFβ-3 gradient surfaces.

4.1. Experiments on c-iPSCs-seeded gradient surfaces and h.d. surfaces

Biomolecule-functionalized gradient surfaces, made possible by the nanoparticle gradient technology, provide a controlled and defined tool for evaluation of different cellular responses. Importantly, it is the molecules linked to the gradient surface that affect the cells, providing a more precise stimulation than when molecules are added to a medium. In this study, the morphogens acted as internal controls against each other and different responses were successfully observed for each morphogen. This confirmed that the cells had been stimulated differently depending on which morphogen was present on the surface. The most obvious result was found when using the GDF5 gradient surfaces, where a budding zone within a specific particle density range showed that GDF5 affected cellular condensation.

It is important to point out that the number of molecules that the cells came in contact with on each surface was very precise and the molecules did not surround an entire cell as they would in a solution. To have the molecules bound to the surface provides a more defined stimulation of the cells cultured on the surface, which are thus in contact with the molecules, unlike when the molecules are added to a solution. The stimulation from the gradient can be compared to the environment *in vivo*, for example when chondrocytes are bound to the ECM, i.e., in contact with signaling molecules at local concentrations up to 1,000 times higher compared with in a solution (Horbett and Brash, 1987; Dee et al., 2002; Wijnands et al., 2018). Thus, it is difficult – and not necessarily relevant – to translate a molecular density attached to a surface into a concentration. The biologically relevant number of molecules presented to the cells via the ECM in nature is not fully elucidated. What is currently considered a relevant concentration in solution *in vitro* may very well be questionable compared with an *in vivo* setting, since it is completely dependent on how the molecules are presented to the cells (Dee et al., 2002). Biomolecules being surface-bound versus free in solution is just one example.

4.1.1. Laminin 521 control gradient surfaces

In this study, laminin 521 was mainly used as an adhesion-promoting protein (Aumailley, 2013), but it also has an iPSC-stimulating effect (Shibata et al., 2018). Such stimulating effects were in this case observed as cell-free cavities that covered the laminin 521 control gradient surfaces. However, it is clear that the morphogens, especially GDF5 and TGFβ-3, influenced the cells more. TGFβ-1 seemed to have a smaller effect on cells, in regard to the indications studied here, since the same cell behavior as for laminin 521 control gradient surfaces was found at lower molecular densities. However, it should be noted that the cells anywhere on the gradient surfaces, even at the lower particle density areas of the gradient, were exposed to much higher local concentrations than are usually used in cell medium.

4.2. GDF5 gradient surfaces and h.d. surfaces

GDF5 is a known morphogen that is involved in chondrogenesis and joint formation in early development (Storm and Kingsley, 1999) and was studied here in regard to the behavior of c-iPSCs after differentiation on a GDF5 gradient surface. We found that GDF5 at a specific density induced formation of condensed cell structures, here called the budding zone. It has previously been shown that there is a tendency of GDF5 to induce cell-to-cell adhesion (Francis-West et al., 1999), which supports

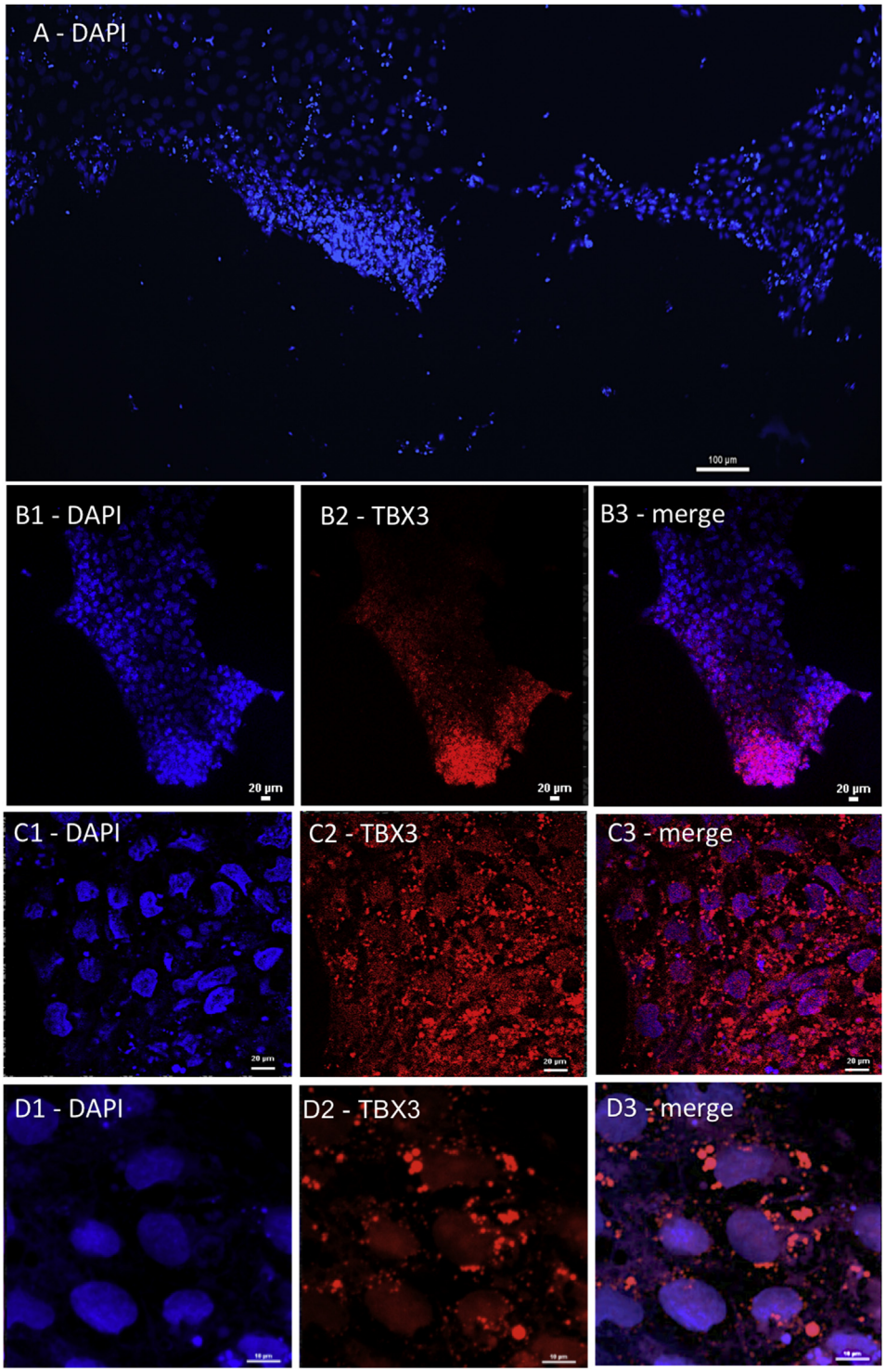

Figure 5. TBX3 are localized in the nuclei and in vesicles in the budding zone. TBX3 expression is visualized in c-iPSCs after five days of differentiation on a GDF5 gradient. (A) Budding zone on a GDF5 gradient. Scale bar 100 µm B1–B3 and C1–C3 show two different budding clusters on the GDF5 gradient. (D1-D3) Close-up of a budding zone. (B–C) Images were acquired with a confocal microscope, 40X objective. Scale bars 20 µm. (D) Images were acquired using a 60X objective. Scale bars 10 µm.

our findings that GDF5 has a role in cell clustering and budding. Using a Gdf5CreER knock-in murine model for lineage tracing during development showed that early Gdf5-positive interzone cells failed to mark all cells in the knee later during the differentiation and thereby indicated a loss of Gdf5 expression. Therefore, the authors hypothesized a model where influx of cells contributes to joint development. Early in development, Gdf5-expressing cells are located in the intermediate zone and overlap with cells that express Col2A1 and Sox9. Later during joint development, Gdf5-expressing cells completely cease with Col2A1 expression. Sox9 expression remains in most Gdf5-expressing cells even at later time points (Shwartz et al., 2016).

The results of this study were confirmed and repeated on specific h.d. surfaces, where the cells behaved in similar ways as observed on gradient surfaces, generating budding areas and a condensed structure. Cellular condensation, entailing a reduction of intercellular spaces, is an important part of the differentiation process during chondrogenesis and skeletogenesis. To achieve a condensation process, the composition of the cellular microenvironment is crucial

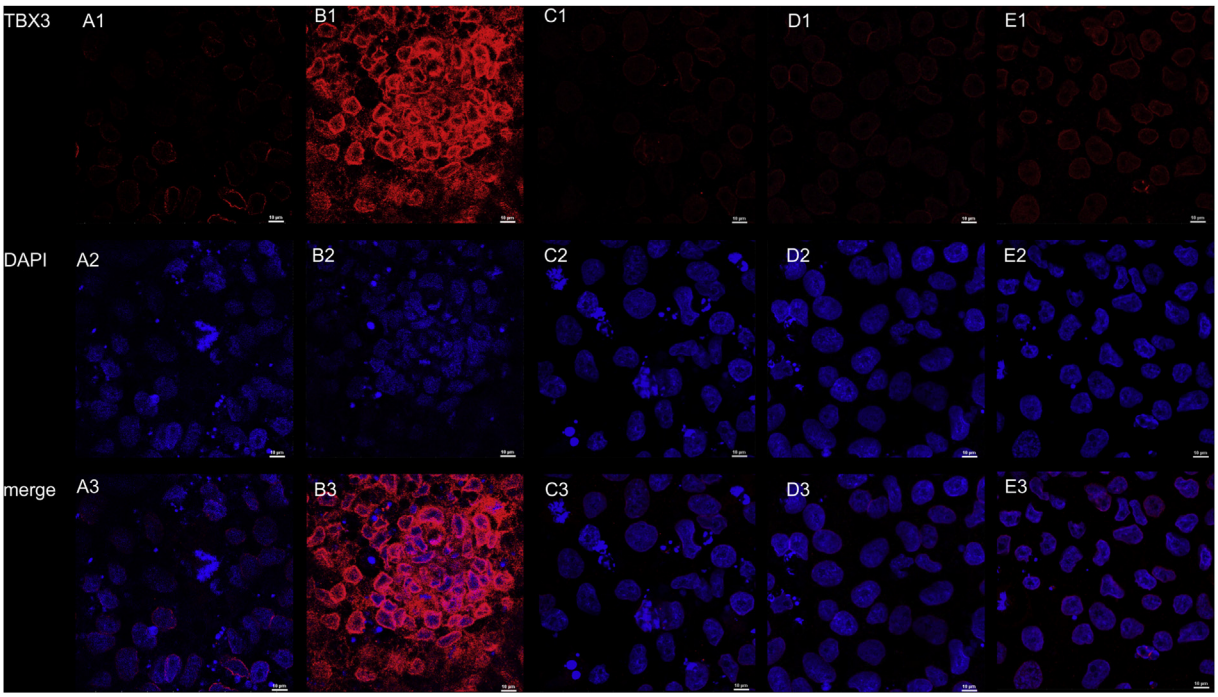

Figure 6. iPSCs on h.d. surfaces immunostained with TBX3 monoclonal (Table S2) antibody; images acquired using confocal microscopy. TBX3 expression is clearly upregulated on the GDF5 h.d. surface with a particle density in the range of the budding zone, as compared with on all other surfaces. (A) GDF5 surface with low particle density. (B) GDF5 budding zone surface. (C) TGFβ-3 surface with the same particle density as (B). (D) TGFβ-3 surface with the same particle density as (B), but with GDF5 (10 ng/ml) added to the differentiation medium. (E) Surface without biomolecules, coated with Coat-1 (DEF-CS 500 Coat-1, Cellartis, Sweden).

(Tacchetti et al., 1992). GDF5 is known to play a role in the formation of cartilage and joints (Storm and Kingsley, 1999). The appearance of cell clusters on both GDF5 gradient surfaces and h.d. surfaces provides important information for inducing condensation and budding formation during c-iPSC differentiation towards chondrocytes, as it indicates an early stage in the formation of condensed chondrogenic structures in c-iPSCs. These results suggest that the cell budding responses are dependent on specific GDF5 concentrations and that the density is essential to induce cellular clusters.

4.3. TGFβ-1 gradient surfaces and h.d. surfaces

It is suggested that both TGFβ and GDF5 induce condensation (Wang et al., 2014) c-iPSCs grown on TGFβ-1 gradient surfaces did not show condensation tendencies and did not respond in the same way as cells stimulated with GDF5, as no budding zone was observed on the TGFβ-1 gradient surfaces. TGFβ-1 is described as an inducer of chondrocyte differentiation and involved in, e.g., cell proliferation, condensation, and production of matrix proteins (Kim et al., 2014). Since TGFβ-1 is specifically connected to condensation, the lack of budding was rather surprising.

At lower TGFβ-1 densities along the gradient surface, cells tended to form another structure: smaller clusters in a semicircular shape. However, this is more likely due to the effect of laminin 521. As expected, based on the results on the gradient surfaces, cells on the low density h.d. surfaces formed cell-free cavities, while cells on high density h.d. surfaces were evenly distributed, and no cavities were formed.

As cells on all replicated laminin 521 gradient surfaces induced the same pattern seen at lower TGFβ-1 densities, it is most likely that low TGFβ-1 densities are not enough to override the effect of laminin 521. At higher densities, the effect of TGFβ-1 takes over and cell uniformity is evident. The specific processes that are taking place in the cells need to be further investigated.

4.4. TGFβ-3 gradient surfaces and h.d. surfaces

Results from gradient surfaces with TGFβ-3 did not show any indications of budding formation or condensed structures. Thus, the conclusion is that TGFβ-3 is not as relevant as GDF5 for condensation and to generate budding zones. TGFβ-3 is known to be important for other cartilage-generating processes, such as cell proliferation and differentiation (Tang et al., 2009; Bierie and Moses, 2010, van der Kraan et al., 2010; Wang et al., 2014). These processes were not further investigated in this study. The surfaces with high or low particle density showed no specific cell responses, like those observed primarily for GDF5, but also for TGFβ-1. In comparison with TGFβ-1, low densities of TGFβ-3 were not overridden by laminin 521 and no cavities were formed. No budding formation was observed for either TGFβ-1 or TGFβ-3. When comparing the three different proteins, GDF5, TGFβ-1, and TGFβ-3, it was clear that GDF5 induced condensation of cells to a higher extent than TGFβ-1 and TGFβ-3.

GDF5 and TGFβ-1 are both involved in cellular condensation (Francis-West et al., 1999; Coleman et al., 2013; Kim et al., 2014). However, cells formed condensed budding structures only on the GDF5 surfaces. TGFβ-3 has been shown to inhibit cellular condensation, which may correlate with the results in our *in vitro* setting (Jin et al., 2010). Thus, the similarities between TGFβ-1 and TGFβ-3 were unexpected, as was the lack of similarities between the results for TGFβ-1 and GDF5.

4.5. Immunostaining of SOX9 and TBX3 in c-iPSCs on GDF5 gradient surfaces

The *SOX9* gene is well-known to be important in, e.g., development of chondrocytes and formation of cartilage structural proteins (Akiyama et al., 2002; Hino et al., 2014; Lefebvre and Dvir-Ginzberg, 2017). Previously, it has been shown that higher GDF5 concentrations induce higher SOX9 expression (Ayerst et al., 2017). Here, a high SOX9 protein

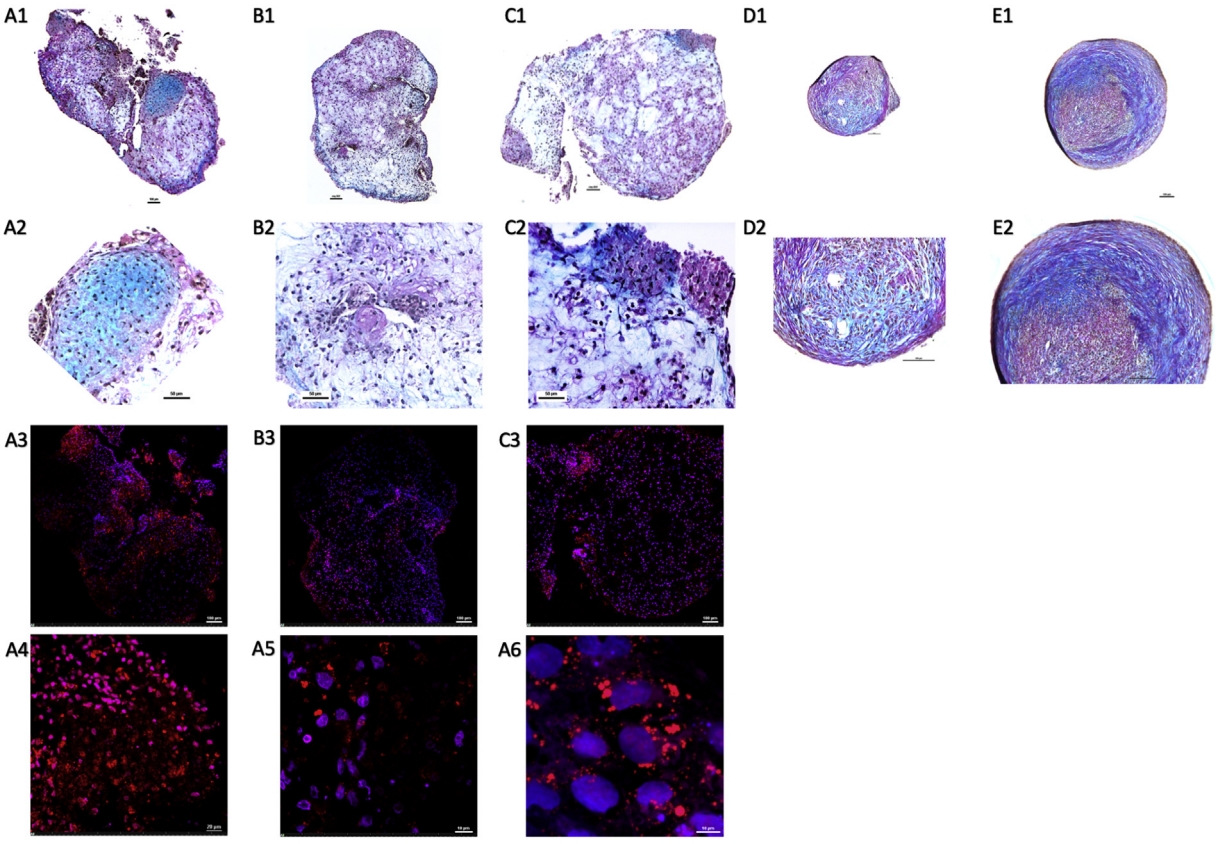

Figure 7. Histological sections of pellets generated from c-iPSCs (A–C) and chondrocytes (D–E). A1–A2 to E1–E2 were stained with Alcian Blue van Gieson, demonstrating the presence of proteoglycans (blue) and collagen (red/purple). A3–A6 & B3–C3 were immunostained for TBX3, which was clearly upregulated in the areas with higher amounts of GAG. (A1–A6) c-iPSCs pre-differentiated on a GDF5 h.d. surface with low particle density. (B1–B3) c-iPSCs pre-differentiated on a GDF5 h.d. surface with budding zone particle density. (C1–C3) c-iPSCs pre-differentiated on a GDF5 gradient surface. (D1–D2) Chondrocyte pellet pre-differentiated on a GDF5 gradient surface. (E1–E2) Chondrocyte pellet with 5 % human serum in the medium. (A1–E1, A3–C3, A5–A6) 100 μm scale bars. (A2–E2) 50 μm scale bars. (A4) 20 μm scale bars.

expression was observed in c-iPSCs differentiated on GDF5 gradient surfaces, which was to be expected since SOX9 is expressed both in chondrocyte progenitors and during chondrocyte differentiation (Hino et al., 2014; Lefebvre and Dvir-Ginzberg, 2017).

TBX3 was induced in the budding zone on the GDF5 gradient surfaces, a phenomenon that was not observed in cells stained for SOX9. This result was also confirmed when GDF5 h.d. surfaces were stained for TBX3 and compared with control surfaces with TGFβ-3 where this phenomenon was not found. This is in agreement with recent reports on the importance of TBX3 during limb formation (Sheeba and Logan, 2017) and preliminary microarray data (unpublished) show that TBX3 and SOX9 was upregulated by GDF5. A decrease of TBX3 in early development might lead to disruptions in limb development initiation and limb defects, which has caused it to be seen as one of the earliest limb initiation factors (Emechebe et al., 2016). Therefore, our findings of local TBX3 expression in the budding zone, i.e., the condensed buds on GDF5 gradient surfaces, could possibly be considered an indication of its expression in a situation resembling early limb bud development. Moreover, TBX3 expression was found to be upregulated in areas with a higher GAG production in pellet sections; high GAG production is also important for limb buds.

4.6. Differentiation on GDF5 gradient surfaces prior to pellet culturing

The gradient technology provides a tool for determining protein stimulations that drive cells towards specific responses. Experiments using the surfaces for high-quality culturing also confirmed the possibility to remove live cells from a gradient surface and form pellets for further culturing or differentiation. One advantage of using gradients to form pellets is the possibility to mimic the natural development of, e.g., cartilage, joints, and limbs, since concentration gradients can function as inducers to start a reaction important for development (Zecca et al., 1996). It has also been suggested that chondrocytes behave differently when cultured in a 3D pellet structure compared with in 2D culturing (Cheung et al., 2008). When chondrocytes are cultured in a 2D monolayer, they tend to produce more type I collagen but less type II collagen and aggrecan, compared with when cultured in 3D. The chondrocyte pellet culture system retains its phenotype better in 3D than in 2D culturing (Zhang et al., 2004). Condensation during embryonic development is a fundamental process in chondrogenesis, limb, and joint formation. The chondrocyte pellet structure mimics the environment during embryonic development and the high cell density promotes essential cellular signaling (Borestrom et al., 2014; Nguyen et al., 2017).

4.6.1. Micromass pellets

The pellet structures, in respect to proteoglycans and collagens, were visualized using Alcian Blue van Gieson staining, where the proteoglycan proteins were represented by covalently attached GAGs (Sophia Fox et al. 2009). The colorings of the c-iPSC pellets obtained from GDF5 gradient surfaces and h.d. surfaces were compared with chondrocyte control pellets. All indicated the presence of both GAGs (blue) and collagen (red/purple). This implies that the differentiation of the c-iPSC pellets, using the protocol including GDF5 gradients and surfaces, drives the cells towards the chondrocyte lineage, though the compactness and high cell quality of the pellets remains somewhat lacking and further research is needed. Moreover, the effect of the GDF5 setup is comparable to using

serum in the medium, as was done for the chondrocyte pellets. This can be due to the presence of multifunctional culture supplements, such as proliferating promoting growth factors, e.g., platelet-derived growth factor (PDGF) and TGFβ in the serum, providing a similar stimulation as GDF5. In addition, the serum has a high content of antioxidants, cytokines, and transporting proteins, and provides a buffering capacity (Tallheden et al., 2005b). The possibility that GDF5 (or molecules similar to GDF5) is included in the serum cannot be excluded due to the presence of an extremely high number of proteins. However, as serum has high variability in its composition (Adkins et al., 2002), the GDF5 gradient surfaces could provide a more defined and reproducible composition. A major drawback of using serum in the differentiation medium is the risk of pathogen contaminations (Karnieli et al., 2017), which could be avoided if replaced with GDF5 stimulation.

Further, our findings that a specific GDF5 density is supportive for TBX3 induction and budding formation in c-iPSCs differentiation may be utilized in future iPSC differentiation protocols or for unraveling disease mechanisms related to OA.

5. Conclusions

GDF5 gradient surfaces induced budding formation at a specific density (ranging from 500 to 1,500 particles/μm^2), an interesting finding in connection to, e.g., cartilage and limb formation. A surface with homogenous density in this range induced the same response. Neither TGFβ-1 nor TGFβ-3 induced such budding formation in this *in vitro* setting, even though both are involved in cartilage-generating processes. Further, GDF5 induced TBX3 in designated buds, as well as high SOX9 expression.

Pellets derived from either c-iPSCs or chondrocytes on GDF5 gradients or homogenous density surfaces produced GAG and collagens, comparable to chondrocyte pellets with serum, based on color. TBX3 was specifically induced in areas with upregulated amounts of GAG.

Declarations

Author contribution statement

Andreasson L., Evenbratt H., Simonsson S.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by the EU Horizon 2020 research and innovation program under grant agreement number 814558 project RESTORE and VR2015- 02560, granted to Stina Simonsson.

Competing interest statement

The authors declare the following conflict of interests: Andreasson L., Evenbratt H; [employees of Cline Scientific AB and hold stocks in Cline Scientific AB].

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e04133.

References

Adkins, J.N., Varnum, S.M., Auberry, K.J., Moore, R.J., Angell, N.H., Smith, R.D., Springer, D.L., Pounds, J.G., 2002. Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry. Mol. Cell. Proteomics 1 (12), 947–955.

Akiyama, H., Chaboissier, M.C., Martin, J.F., Schedl, A., de Crombrugghe, B., 2002. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. 16 (21), 2813–2828.

Aumailley, M., 2013. The laminin family. Cell Adhes. Migrat. 7 (1), 48–55.

Ayerst, B.I., Smith, R.A., Nurcombe, V., Day, A.J., Merry, C.L., Cool, S.M., 2017. Growth differentiation factor 5-mediated enhancement of chondrocyte phenotype is inhibited by heparin: implications for the use of heparin in the clinic and in tissue engineering applications. Tissue Eng Part A 23 (7-8), 275–292.

Bamshad, M., Le, T., Watkins, W.S., Dixon, M.E., Kramer, B.E., Roeder, A.D., Carey, J.C., Root, S., Schinzel, A., Van Maldergem, L., Gardner, R.J., Lin, R.C., Seidman, C.E., Seidman, J.G., Wallerstein, R., Moran, E., Sutphen, R., Campbell, C.E., Jorde, L.B., 1999. The Spectrum of Mutations in TBX3: Genotype/Phenotype Relationship in Ulnar-Mammary Syndrome. Am. J. Hum. Genet. 1550–1562.

Bierie, B., Moses, H.L., 2010. Transforming growth factor beta (TGF-beta) and inflammation in cancer. Cytokine Growth Factor Rev. 21 (1), 49–59.

Borestrom, C., Simonsson, S., Enochson, L., Bigdeli, N., Brantsing, C., Ellerstrom, C., Hyllner, J., Lindahl, A., 2014. Footprint-free human induced pluripotent stem cells from articular cartilage with redifferentiation capacity: a first step toward a clinical-grade cell source. Stem Cells Transl. Med. 3 (4), 433–447.

Cheung, W.H.C., K.H., Lee, K.M., Leung, K.S., 2008. Chondrocyte-pellet culture for cartilage repair research. In: A Practical Manual for Muscluloskeletal Research, pp. 165–175.

Coleman, C.M., Vaughan, E.E., Browe, D.C., Mooney, E., Howard, L., Barry, F., 2013. Growth differentiation factor-5 enhances in vitro mesenchymal stromal cell chondrogenesis and hypertrophy. Stem Cell. Dev. 22 (13), 1968–1976.

Dakhore, S., Nayer, B., Hasegawa, K., 2018. Human pluripotent stem cell culture: current status, challenges, and advancement. Stem Cell. Int. 2018, 7396905.

Dee, K.C., Puleo, D.A., Bizios, R., 2002. An Introduction to Tissue-Biomaterial Interactions.

Emechebe, U., Kumar, P.P., Rozenberg, J.M., Moore, B., Firment, A., Mirshahi, T., Moon, A.M., 2016. T-box3 is a ciliary protein and regulates stability of the Gli3 transcription factor to control digit number. Elife 5.

Francis-West, P.H., Abdelfattah, A., Chen, P., Allen, C., Parish, J., Ladher, R., Allen, S., MacPherson, S., Luyten, F.P., Archer, C.W., 1999. Mechanisms of GDF-5 action during skeletal development. Development 126 (6), 1305–1315.

Goldring, Mary B, 2012. Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. Ther. Adv. Muscoskel. Dis. 4, 269–285.

Gruneberg, H., Lee, A.J., 1973. The anatomy and development of brachypodism in the mouse. J. Embryol. Exp. Morphol. 30 (1), 119–141.

Gurdon, J.B., Dyson, S., St Johnston, D., 1998. Cells' perception of position in a concentration gradient. Cell 95 (2), 159–162.

Hino, K., Saito, A., Kido, M., Kanemoto, S., Asada, R., Takai, T., Cui, M., Cui, X., Imaizumi, K., 2014. Master regulator for chondrogenesis, Sox9, regulates transcriptional activation of the endoplasmic reticulum stress transducer BBF2H7/CREB3L2 in chondrocytes. J. Biol. Chem. 289 (20), 13810–13820.

Horbelt, D., Denkis, A., Knaus, P., 2012. A portrait of Transforming Growth Factor beta superfamily signalling: background matters. Int. J. Biochem. Cell Biol. 44 (3), 469–474.

Horbett, T.A., Brash, J.L., 1987. Proteins at Interfaces: Current Issues and Future Prospects, pp. 1–33.

Jin, E.J., Park, K.S., Kim, D., Lee, Y.S., Sonn, J.K., Jung, J.C., Bang, O.S., Kang, S.S., 2010. TGF-beta3 inhibits chondrogenesis by suppressing precartilage condensation through stimulation of N-cadherin shedding and reduction of cRREB-1 expression. Mol. Cells 29 (4), 425–432.

Karnieli, O., Friedner, O.M., Allickson, J.G., Zhang, N., Jung, S., Fiorentini, D., Abraham, E., Eaker, S.S., Yong, T.K., Chan, A., Griffiths, S., Wehn, A.K., Oh, S., Karnieli, O., 2017. A consensus introduction to serum replacements and serum-free media for cellular therapies. Cytotherapy 19 (2), 155–169.

Kasagi, S., Chen, W., 2013. TGF-beta1 on osteoimmunology and the bone component cells. Cell Biosci. 3 (1), 4.

Kim, Y.I., Ryu, J.S., Yeo, J.E., Choi, Y.J., Kim, Y.S., Ko, K., Koh, Y.G., 2014. Overexpression of TGF-beta1 enhances chondrogenic differentiation and proliferation of human synovium-derived stem cells. Biochem. Biophys. Res. Commun. 450 (4), 1593–1599.

Lagunas, A., Comelles, J., Oberhansl, S., Hortiguela, V., Martinez, E., Samitier, J., 2013. Continuous bone morphogenetic protein-2 gradients for concentration effect studies on C2C12 osteogenic fate. Nanomedicine 9 (5), 694–701.

Landauer, W., 1952. The genetic control of normal development in the chicken embryo. Ann. N. Y. Acad. Sci. 55 (2), 172–176.

Lefebvre, V., Dvir-Ginzberg, M., 2017. SOX9 and the many facets of its regulation in the chondrocyte lineage. Connect. Tissue Res. 58 (1), 2–14.

Mikic, B., 2004. Multiple effects of GDF-5 deficiency on skeletal tissues: implications for therapeutic bioengineering. Ann. Biomed. Eng. 32 (3), 466–476.

Nguyen, D., Hagg, D.A., Forsman, A., Ekholm, J., Nimkingratana, P., Brantsing, C., Kalogeropoulos, T., Zaunz, S., Concaro, S., Brittberg, M., Lindahl, A., Gatenholm, P., Enejder, A., Simonsson, S., 2017. Cartilage tissue engineering by the 3D bioprinting of iPS cells in a nanocellulose/alginate bioink. Sci. Rep. 7 (1), 658.

Peret, B.J., Murphy, W.L., 2008. Controllable soluble protein concentration gradients in hydrogel networks. Adv. Funct. Mater. 18 (21), 3410–3417.

Polinkovsky, A., Robin, N.H., Thomas, J.T., Irons, M., Lynn, A., Goodman, F.R., Reardon, W., Kant, S.G., Brunner, H.G., van der Burgt, I., Chitayat, D., McGaughran, J., Donnai, D., Luyten, F.P., Warman, M.L., 1997. Mutations in CDMP1 cause autosomal dominant brachydactyly type C. Nat. Genet. 17 (1), 18–19.

Seemann, P., Schwappacher, R., Kjaer, K.W., Krakow, D., Lehmann, K., Dawson, K., Stricker, S., Pohl, J., Ploger, F., Staub, E., Nickel, J., Sebald, W., Knaus, P., Mundlos, S., 2005. Activating and deactivating mutations in the receptor interaction

site of GDF5 cause symphalangism or brachydactyly type A2. J. Clin. Invest. 115 (9), 2373–2381.
Settle, Stephen H., Rountree, Ryan B., Abhishek, Sinha, Thacker, Abigail, Higgins, Kay, Kingsley, David M., 2003. Multiple joint and skeletal patterning defects caused by single and double mutations in the mouse Gdf6 and Gdf5genes. Dev. Biol. 254 (1), 116–130.
Sheeba, C.J., Logan, M.P., 2017. The roles of T-box genes in vertebrate limb development. Curr. Top. Dev. Biol. 122, 355–381.
Shibata, S., Hayashi, R., Okubo, T., Kudo, Y., Katayama, T., Ishikawa, Y., Toga, J., Yagi, E., Honma, Y., Quantock, A.J., Sekiguchi, K., Nishida, K., 2018. Selective laminin-directed differentiation of human induced pluripotent stem cells into distinct ocular lineages. Cell Rep. 25 (6), 1668–1679 e1665.
Shwartz, Y., Viukov, S., Krief, S., Zelzer, E., 2016. Joint development involves a continuous influx of Gdf5-positive cells. Cell Rep. 15 (12), 2577–2587.
Sophia Fox, A.J., Bedi, A., Rodeo, S.A., 2009. The basic science of articular cartilage: structure, composition, and function. Sports Health 1 (6), 461–468.
Storm, E.E., Huynh, T.V., Copeland, N.G., Jenkins, N.A., Kingsley, D.M., Lee, S.J., 1994. Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. Nature 368 (6472), 639–643.
Storm, E.E., Kingsley, D.M., 1999. GDF5 coordinates bone and joint formation during digit development. Dev. Biol. 209 (1), 11–27.
Tacchetti, C., Tavella, S., Dozin, B., Quarto, R., Robino, G., Cancedda, R., 1992. Cell condensation in chondrogenic differentiation. Exp. Cell Res. 200 (1), 26–33.
Tallheden, T., Bengtsson, C., Brantsing, C., Sjogren-Jansson, E., Carlsson, L., Peterson, L., Brittberg, M., Lindahl, A., 2005a. Proliferation and differentiation potential of chondrocytes from osteoarthritic patients. Arthritis Res. Ther. 7 (3), R560–568.
Tallheden, T., van der Lee, J., Brantsing, C., Mansson, J.E., Sjogren-Jansson, E., Lindahl, A., 2005b. Human serum for culture of articular chondrocytes. Cell Transplant. 14 (7), 469–479.
Tang, Q.O., Shakib, K., Heliotis, M., Tsiridis, E., Mantalaris, A., Ripamonti, U., Tsiridis, E., 2009. TGF-beta3: a potential biological therapy for enhancing chondrogenesis. Expet Opin. Biol. Ther. 9 (6), 689–701.
Teleman, A.A., Cohen, S.M., 2000. Dpp gradient formation in the Drosophila wing imaginal disc. Cell 103 (6), 971–980.
Thomas, J.T., Lin, K., Nandedkar, M., Camargo, M., Cervenka, J., Luyten, F.P., 1996. A human chondrodysplasia due to a mutation in a TGF-beta superfamily member. Nat. Genet. 12 (3), 315–317.
van der Kraan, P.M., Blaney Davidson, E.N., van den Berg, W.B., 2010. A role for age-related changes in TGFbeta signaling in aberrant chondrocyte differentiation and osteoarthritis. Arthritis Res. Ther. 12 (1), 201.
Wang, W., Rigueur, D., Lyons, K.M., 2014. TGFbeta signaling in cartilage development and maintenance. Birth Defects Res. C Embryo Today 102 (1), 37–51.
Weiss, A., Attisano, L., 2013. The TGFbeta superfamily signaling pathway. Wiley Interdiscip. Rev. Dev. Biol. 2 (1), 47–63.
Wijnands, S.P.W., Engelen, W., Lafleur, R.P.M., Meijer, E.W., Merkx, M., 2018. Controlling protein activity by dynamic recruitment on a supramolecular polymer platform. Nat. Commun. 9 (1), 65.
Zecca, M., Basler, K., Struhl, G., 1996. Direct and long-range action of a wingless morphogen gradient. Cell 87 (5), 833–844.
Zhang, Z., McCaffery, J.M., Spencer, R.G., Francomano, C.A., 2004. Hyaline cartilage engineered by chondrocytes in pellet culture: histological, immunohistochemical and ultrastructural analysis in comparison with cartilage explants. J. Anat. 205 (3), 229–237.