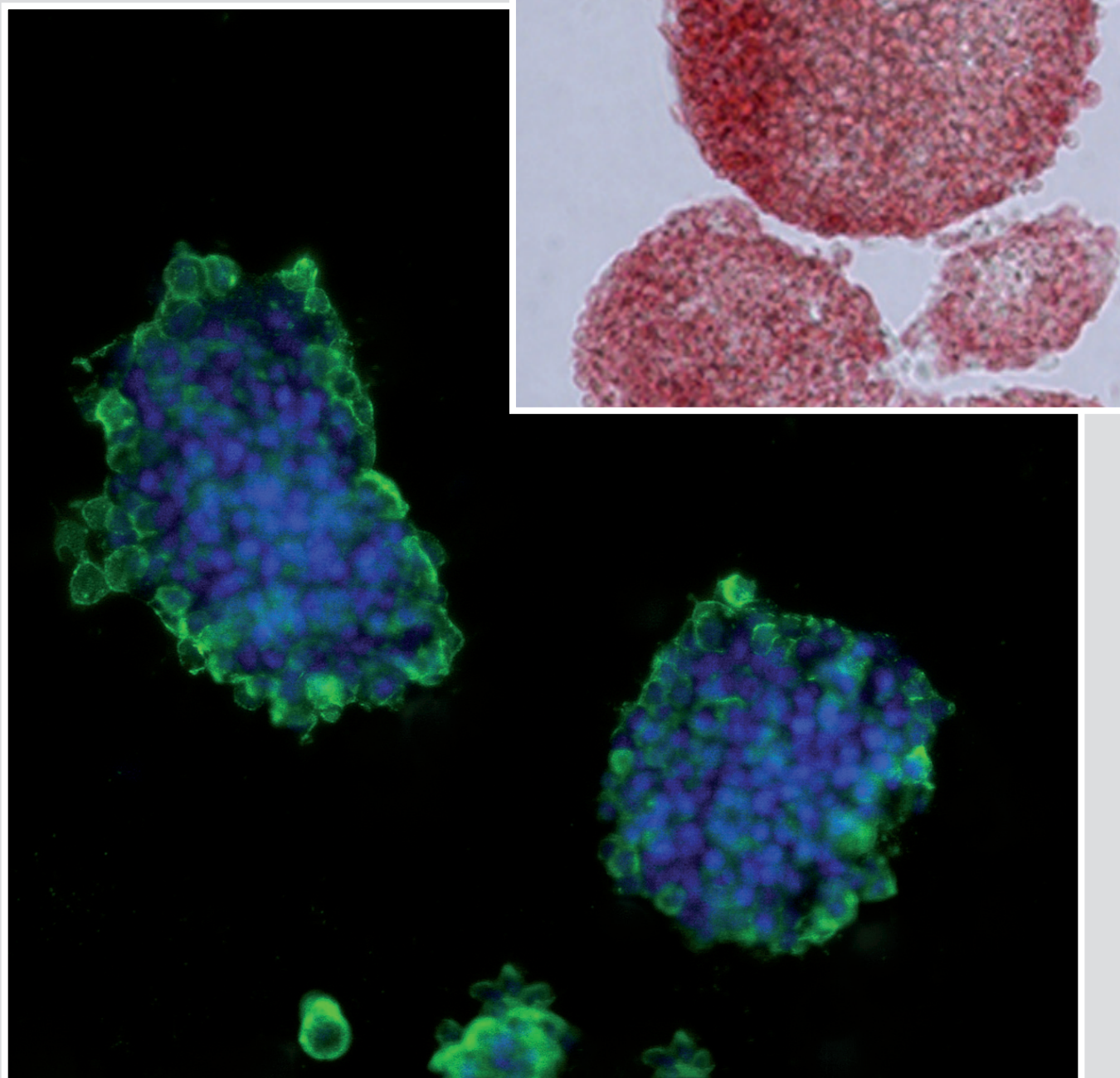


Application Note

Improved Cultivation and Differentiation of Embryonic Stem Cells



1. Introduction

1.1. Stem Cells

Stem cells are characterized by two properties: They are unspecialized cells capable of replicating themselves to form other identical stem cells and they also have the remarkable potential to give rise to differentiated cells (figure 1).

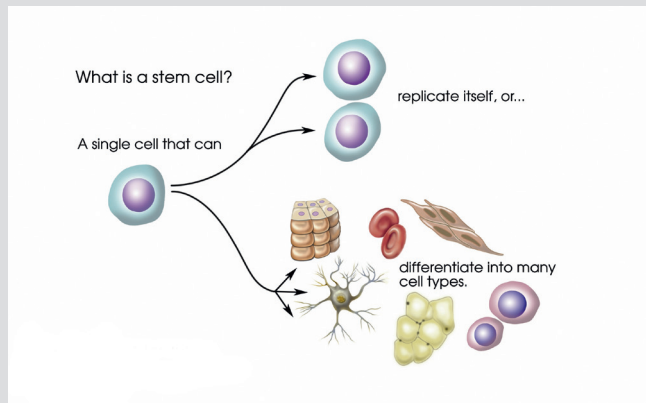


Figure 1: Characteristics of stem cells
(Source <http://www.nationalacademies.org/stemcells>)

Given their unique regenerative abilities, stem cells offer new potentials for cell based therapies and reparative medicine to replace diseased cells or destroyed tissue. The use of bone marrow transplants containing stem cells, which can form new hematopoietic cells, is already well established. However, the therapeutic provision of pancreatic cells for diabetes patients, the substitution of neuronal cells after stroke or the regeneration of a heart muscle after a heart attack is still under development. Depending from where the stem cells originated, they have a clear hierarchy in their capacity to differentiate into specialized cells which is defined as stem cell potency.

Totipotent (also known as omnipotent) stem cells can differentiate into embryonic and extra-embryonic cell types and can construct a complete, viable organism. These are the earliest cells produced from the fusion of an egg and a sperm cell and the first subsequent divisions.

Pluripotent stem cells are descendants of totipotent cells and can differentiate into the cells derived from any of the three germ layers of the developing embryo:

The ectoderm which differentiates into the nervous system and the epidermis, the mesoderm giving rise to bone, muscle and connective tissue and the endoderm building up the digestive and respiratory system as well as the thymus. Based on their origin these pluripotent stem cells are also called embryonic stem cells (ESCs).

Multipotent stem cells are adult stem cells which are found in most organs of the adult body including for example liver, lung, heart, intestine, skin, muscle and brain. Although adult stem cells cannot be expanded in culture indefinitely, their use does not pose the ethical problems involved in the use of embryonic stem cells. As the cells can be generated directly from a patient, the application of such cells might circumvent immune rejections, making adult stem cells a safer option for cell based therapies.

Restricting this approach is the adult stem cells limited potency in differentiation which refers only to those cells which are closely related to the tissue they have been generated from.

1.2. Pluripotent Embryonic Stem Cells

Embryonic stem cells are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation in vitro^{1,2}. They are isolated from the inner cell mass of a blastocyst and can be cultivated on plastic disposables in vitro (figure 2).

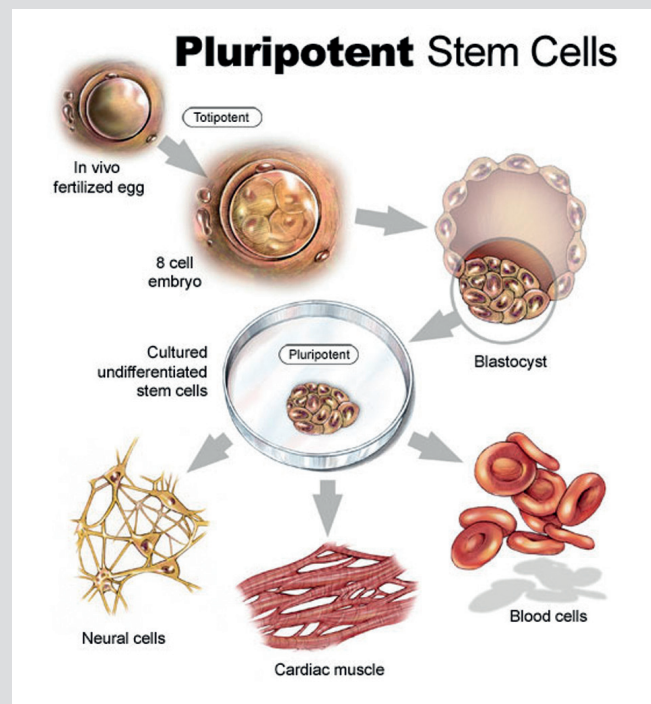


Figure 2: Preparation of pluripotent embryonic stem cells
(Source <http://stem-cell-treatment-now.info/stem-cell-education.html>)

The importance of embryonic stem cells rests in their lack of specialization. These basic cells are present in the earliest stages of developing embryos and are able to convert into virtually any type of cell and tissue in the body. As such, an understanding of their unique attributes can lead to in-depth knowledge about early human development. Furthermore, embryonic stem cells can offer a prospective limitless source of cells and tissue due to their self-renewing potential. Based on this feature embryonic stem cells have gained enormous importance over the past decades in medical science. Cell therapeutic approaches for example aim to replace damaged or diseased cells and tissues by embryonic stem cells. But due to limited knowledge in stem cell research with the first human embryonic stem cells being isolated approximately twenty years ago³ there is still an extensive need for basic scientific investigation.

It is still necessary to understand the complexity of human diseases as well as stem cell maintenance and differentiation.

1.3. Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are artificially derived from non-pluripotent cells, typically adult somatic cells that have been genetically reprogrammed to an embryonic stem-cell like state by inducing a forced expression of certain genes. IPS cells are believed to be similar to natural pluripotent stem cells such as embryonic stem cells but the full extent of their relation to natural pluripotent stem cells is still being assessed. Mouse iPS cells were first reported in 2006³, whereas human iPS cells were originally generated in late 2007^{4,5}. IPS cells demonstrate important characteristics of pluripotent stem cells, including expression of specific stem cell markers, teratoma formation and differentiation into various tissues upon stimulation. Therefore iPS cells are a useful tool for drug development and in vitro disease modeling as they allow researches to analyze pluripotent stem cells without the controversial use of embryos.

1.4. General Cultivation of Embryonic and iPS cells

The traditional embryonic and/or iPS cell culture can be subdivided into the phases of maintenance and expansion, embryoid body (EB) formation and the terminal differentiation into the desired cell lineage. Each phase represents obstacles which have to be faced.

In general propagation of an explanted cell in vitro can be difficult. In vivo cells of a multi-cellular organism are embedded in the three-dimensional structure of the extracellular matrix (ECM) of adjacent cells. In addition to providing structural support, the ECM also comprises a wide range of cellular growth factors and mediates biochemical signals which essentially influence cellular proliferation and survival^{6,7}.

Cultivation of cells in vitro mainly refers to a two-dimensional culture on plastic surfaces lacking the vital signals provided by the connective tissue. Embryonic stem cells are a very sensitive cellular system with a high risk of spontaneous differentiation under inappropriate culture conditions. Due to this, they are usually co-cultured with “feeder” cells derived from mouse or human during maintenance and expansion. These feeder cells provide secreted factors such as leukaemia inhibitory factor (LIF), extracellular matrix, and cellular contacts for the preservation of stem cells in the undifferentiated state. However feeder cells may possess a potential risk of cross-contamination such as passing animal pathogens or retroviruses to human embryonic stem cells hindering clinical application of these cells. Although feeder-free systems^{8,9} have been reported in the last couple of years, these approaches often require the addition of feeder conditioned medium carrying a similar risk of pathogen contamination as well as batch to batch variations.

The major aim therefore is to develop chemically defined media and conditions to eliminate the risk of infection from animal components as well as to reduce lot-to-lot variability resulting in consistent and comparable cellular behaviour and facilitating eventual use in clinical applications.

A possible method to initiate differentiation of embryonic stem cells is the so called “embryoid body formation”, where ESCs spontaneously form tissue like spheroids in suspension culture¹⁰.

This process has been shown to recapitulate aspects of early embryogenesis, including the formation of a complex three-dimensional arrangement and the development of the three embryonic germ layers.

Final differentiation of embryonic stem cells can either be achieved by returning embryoid bodies to adherent culture conditions or by directed stimulation of ESCs by specific reagents (see also chapter 3.3 Neuronal Differentiation).

2. Material and Methods

2.1. Material, Reagents and Culture Media

Item	Manufacturer	Cat.-No.
ES-D3 (mouse embryonic stem cell)	ATCC	CRL-1934
STO (mouse embryonic fibroblast)	ATCC	CRL-1503
EmbryoMax® ES Cell Qualified Gelatine Solution 0,1%	Millipore	ES-006-B
Dulbecco's MEM Medium (1x)	Biochrom AG	F0435
EmbryoMax® ES Cell Qualified Nucleosides	Millipore	ES-008
L-Alanyl-L-Glutamine	Biochrom AG	K0302
MEM-amino acids w/o L-Glutamine	Biochrom AG	K0363
Penicillin/Streptomycin-Solution	Biochrom AG	A2213
EmbryoMax® ES Cell Qualified Fetal Bovine Serum	Millipore	ES-011-B
β-Mercaptoethanol, liquid, cell culture tested	Sigma-Aldrich	M7522
ESGRO, mLIF Medium Supplement 1x10 ⁶ units	Millipore	ESG1106
Trypsin/EDTA-Solution in PBS (0,05% / 0,02%)	Biochrom AG	A2213
Vector Red Alkaline Phosphatase Substrate Kit	Linaris	ESA5100
Formalin Solution	Sigma-Aldrich	HT501128
Anti-Oct-4 antibody	abcam	ab19857
Anti-SSEA-1 antibody	Cell Signaling	MC480
Anti-βIII tubulin antibody	Sigma-Aldrich	T857
Anti-nestin antibody	Sigma-Aldrich	N5413
Anti-GFAP antibody	Sigma-Aldrich	G4546
Alexa Fluor® 488 Goat-anti-rabbit-IgG	Invitrogen	A11008
AlexaFluor® 546 Goat-anti-mouse-IgG	Invitrogen	A11003
DAPI	Sigma-Aldrich	T8532
Cytosine β-D-Arabinofurano-side, Hydrochloride	Sigma-Aldrich	C6645
All trans-Retinoic acid	Sigma-Aldrich	R2625
Mitomycin C	Sigma-Aldrich	M4287
96well CELLSTAR® Cell Culture Microplate; TC	Greiner Bio-One	655180
96well CELLSTAR® Cell Culture Microplate; Suspension	Greiner Bio-One	655185
96well Advanced TC™ Cell Culture Microplate	Greiner Bio-One	655980
96well CELLCOAT® Cell Culture Microplate; PDL	Greiner Bio-One	655940
96well CELLSTAR® Cell Culture Microplate; TC µClear®	Greiner Bio-One	655090
96well Advanced TC™ Cell Culture Microplate; µClear®	Greiner Bio-One	655986
96well CELLCOAT® Cell Culture Microplate; PDL, µClear®	Greiner Bio-One	655946

2.2. Murine Embryonic Stem Cell Cultivation

The clonal embryonic stem cell line ES-D3 is derived from blastocysts of a 129S2/SvPas mouse. The cells spontaneously differentiate into embryonic structures in the absence of a feeder layer or conditioned medium. They can be maintained in the undifferentiated state by frequent subculture on confluent inactivated feeder layers. ES-D3 cells have been cultivated in the following media:

-LIF-Medium:

DMEM	400 ml
Nucleosides (100x)	1x (1:100 → 5 ml for 500 ml)
L-Alanyl-L-Glutamine (200mM)	2mM (1:100 → 5 ml for 500 ml)
Pen./ Strep.-Solution (100x)	1x (1:100 → 5 ml for 500 ml)
MEM- amino acids (50x)	1x (1:50 → 10 ml for 500 ml)
FBS ESC qualified	15% (75 ml for 500 ml Medium)

+LIF-Medium:

(stable 2 weeks at 4°C)

-LIF-Medium	100 ml
β-Mercaptoethanol (1M)	0.1 mM (1:10.000 → 10 µl for 100 ml)
ESGRO mLIF	1x10 ⁶ U/ml (1:1000 → 110 µl for 100 ml)

Depending on the experimental setup ES-D3 cells were either pre-cultivated on mytomycin C inactivated STO cells using standard cell culture flasks or directly seeded on 96well cell culture microplates with the indicated surfaces or coatings (see chapter 3. Results).

2.3. Alkaline Phosphatase Staining

Alkaline phosphatase (AP) staining was performed corresponding to the manufacturer's instruction. In brief the Vector® Red substrate working solution was prepared immediately before use:

To 5 ml of 100 mM Tris-HCl buffer (pH 8.2 - 8.5) two drops of Reagent 1 were added and mixed well. Thereafter in consecutive mixing steps, two drops of Reagent 2 and two drops of Reagent 3 were added to the solution.

Note: The reagents are supplied in dropper bottles. It is not recommended to pipette reagents directly from the bottles as drop volumes of each component may be different due to solvent characteristics. Proper concentrations of substrate components in the working solution are assured only by using the drop dispensers.

Media was gently aspirated from the cultivated ESCs and cells were washed once with PBS. Thereafter cells were incubated, protected from light, for 30 minutes with freshly prepared AP staining solution. Red stained cell colonies were microscopically evaluated after removal of AP staining solution and two washing steps with PBS.

2.4. Immunocytochemistry

All immunocytochemistry experiments have been performed in 96 well microplates. Media was gently aspirated from the cultivated ESCs and cells were washed once with PBS. Cells were fixed in 4% formalin solution for 20 minutes. After two PBS washing steps, the cells were permeabilised with 0.1% Triton X₁₀₀ in PBS for 5 minutes if intracellular antigens were stained. After removal of the permeabilisation solution cells were washed twice with PBS. To block unspecific binding sites, cells were incubated with blocking solution (5% goat serum in PBS) for one hour at room temperature. Thereafter primary antibodies, diluted 1:500 in blocking solution were added for an over night incubation at 4°C. The next day cells were washed three times with PBS and the appropriate secondary Alexa Fluor® antibody, diluted 1:1000 in blocking solution, was incubated for one hour at room temperature. If a nuclear counter stain was necessary cells were incubated with a DAPI staining solution (10µg/ml in PBS) after removal of the secondary antibody and three consecutive washing steps.

3. Results

As long as murine embryonic stem cells are cultivated under controlled conditions they remain undifferentiated and express specific markers like OCT3/4 and SSEA-1. Stem cells can be cultivated either with a feeder layer of inactivated fibroblast or STO cells as well as feeder free. The latter being more suitable if cells are intended for therapeutic approaches as there will be no contamination with feeder cells after cell harvest. Essential for the undifferentiated state of murine ES cells is the addition of LIF (leukaemia inhibitory factor). LIF is normally expressed in the trophectoderm of the developing embryo. As embryonic stem cells are derived from the inner cell mass at the blastocyst stage, removing them from the inner cell mass also removes their source of LIF.

Therefore the addition of LIF keeps these cells in their pluripotent state. If embryonic stem cells are allowed to clump together to form embryoid like bodies, they begin to differentiate spontaneously after a certain cultivation time. This is an ineffective way of differentiation as it leads to a mixture of cells. Therefore scientists try to establish protocols which reliably direct the differentiation of ES cells into specific cell types. This process is accompanied by a change of morphology and can be influenced also by the surface effect of the disposable used. Based on this information and the importance of embryonic stem cell research, it was our aim to establish and analyse embryonic stem cell cultivation on GBO surfaces: In the first experimental setup, murine embryonic stem cells were ought to propagate successfully on the surfaces listed below without spontaneous differentiation:

- A feeder layer of inactivated STO cells (intended as a type of gold standard for an undifferentiated cultivation of stem cells)
- Gelatine coated dishes as this procedure is also mentioned as a possible coating in various publications
- The CELLSTAR® TC surface
- The CELLSTAR® Suspension surface to facility spheroid culture
- The novel Advanced TC™ surface

Figure 3 displays embryonic stem cells after two days in vitro cultivated either with the addition of LIF or without. The latter is a very important control as only healthy stem cells would spontaneously differentiate after the withdrawal of LIF as well as it also implies the possibility to analyse if a specific surface might supersede the addition of LIF. As expected embryonic stem cells cultivated with LIF show a tendency to remain in cell clumps or aggregates. As long as these have very sharp borders the cells can be defined as undifferentiated.

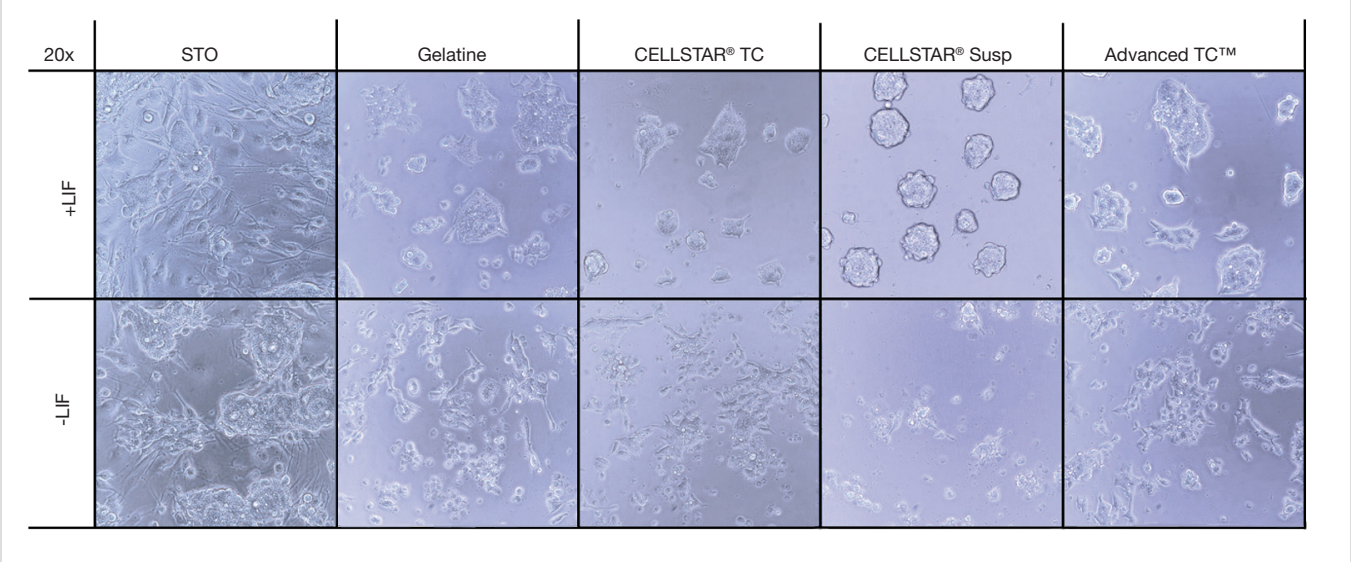


Figure 3: Morphological analysis of embryonic stem cells cultivated on various surfaces after two days in culture (magnification 20x).

If cellular aggregates start to dissolve and single cells migrate into the culture as well as begin to change their morphology and flatten, the cells start to differentiate. This is visible in the lower panel for the cells cultivated without LIF proving correct experimental settings.

After four days in culture, cellular aggregates increased in size (figure 4). During this time it is very important, that the aggregates do not exceed a specific size as well as do not get in direct contact to each other as this can also stimulate embryonic stem cell differentiation.

Comparing the morphology of ESCs on all analysed cell culture surfaces indicates that embryonic stem cell aggregates display sharp borders when cultivated on inactivated STO cells, the CELLSTAR® TC and suspension surface as well as on the novel Advanced TC™ polymer modification when LIF is added. Without LIF, ESCs are highly differentiating indicating a healthy stem cell population. Interestingly a high proportion of differentiating cells can also be detected on the gelatine surface even in the LIF containing culture. This can be verified by figure 5 and 6 visualizing the alkaline phosphatase staining at these time points.

Certain individual cells found in some of the ESC aggregate are negative for AP expression. This indicates that already after this short incubation time cell spontaneously start to differentiate on gelatine coated plates. Without LIF cells migrate into the culture and display a more differentiated morphology and reduced AP staining intensity. On the CELLSTAR® TC surface a comparable picture to stem cells cultivated on the STO feeder layer can be generated indicating that as long as LIF is added to the culture embryonic stem cells can be kept undifferentiated on this surface. This is a major advantage as it makes the feeder layer dispensable and thinking about therapeutic approaches it makes harvest and purification of stem cells much easier. Comparable to the CELLSTAR® TC surface, the Advanced TC™ polymer modification provides a high number of undifferentiated cell aggregates with sharp borders mirroring optimal culture conditions for the propagation of these cells. The CELLSTAR® suspension surface facilitates spheroid culture conditions while keeping cells in a highly undifferentiated state displayed by the intense red staining.

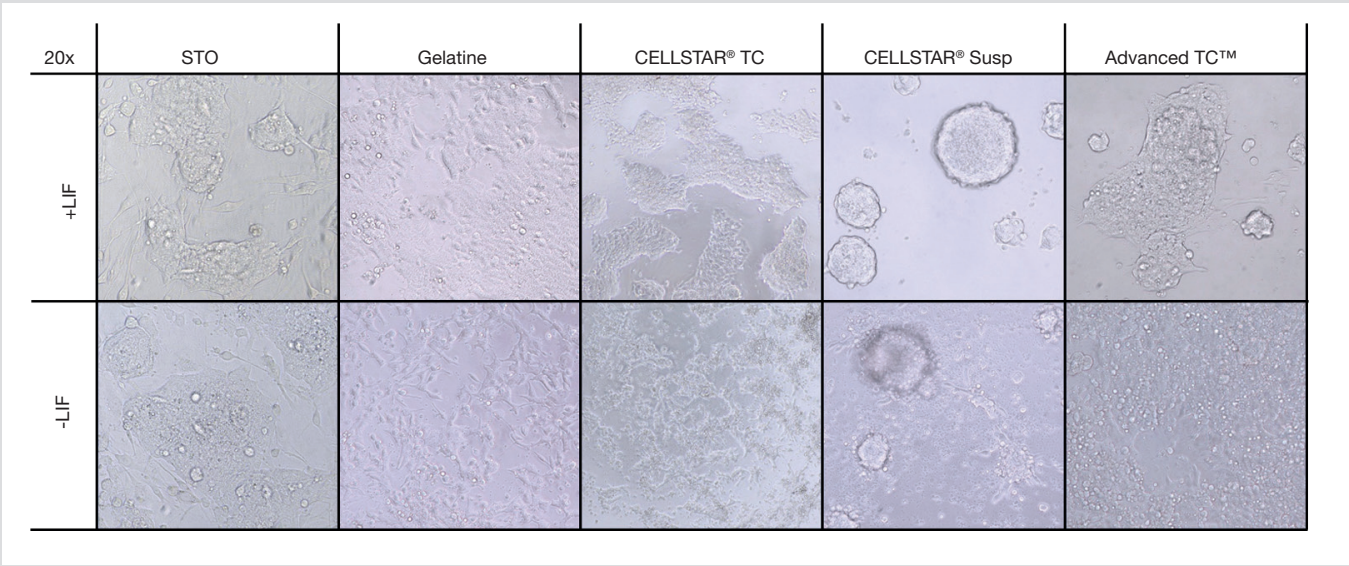


Figure 4: Morphological analysis of embryonic stem cells cultivated on various surfaces after four days in culture (magnification 20x).

3.1. Alkaline Phosphatase Staining

As embryonic stem cells express high levels of membrane alkaline phosphatase (AP) the staining of this molecule can be used to analyse the differentiation state of these cells in more detail. Figure 5 displays AP staining of murine embryonic stem cells cultivated for two days on the indicated surfaces: ESCs cultivated on inactivated STO cells with LIF express high levels of AP and grow in compact cellular aggregates indicating high level of undifferentiation. Even without LIF ESC clusters rather keep a condensed morphology presumably because the inactivated feeder layer provides growth factors and other molecules keeping the cells in an undifferentiated state. As speculated on the unstained pictures ESCs cultivated on a gelatine coating showed first signs of spontaneous differentiation already after two days even when LIF was added to the media.

If LIF is not included in the media, cells start to differentiate and possibly get lost during the staining procedure because they are unable to attach to the suspension surface. Results after four days of cultivation resemble the findings described before (figure 6). Again embryonic stem cell aggregates cultivated on an inactivated STO feeder layer in LIF containing media display a very intense red staining and sharp borders. ESCs on the gelatine coated plates express only low levels of AP leading to a faded staining and a high degree of differentiation with and without LIF. On the CELLSTAR® TC and also on the Advanced TC™ surface embryonic stem cells display a very intense AP staining comparable to cells cultivated on a feeder layer. Therefore these surfaces are a most favourable alternative enabling pure embryonic stem cell cultures while assuring optimal culture conditions and avoiding spontaneous differentiation.

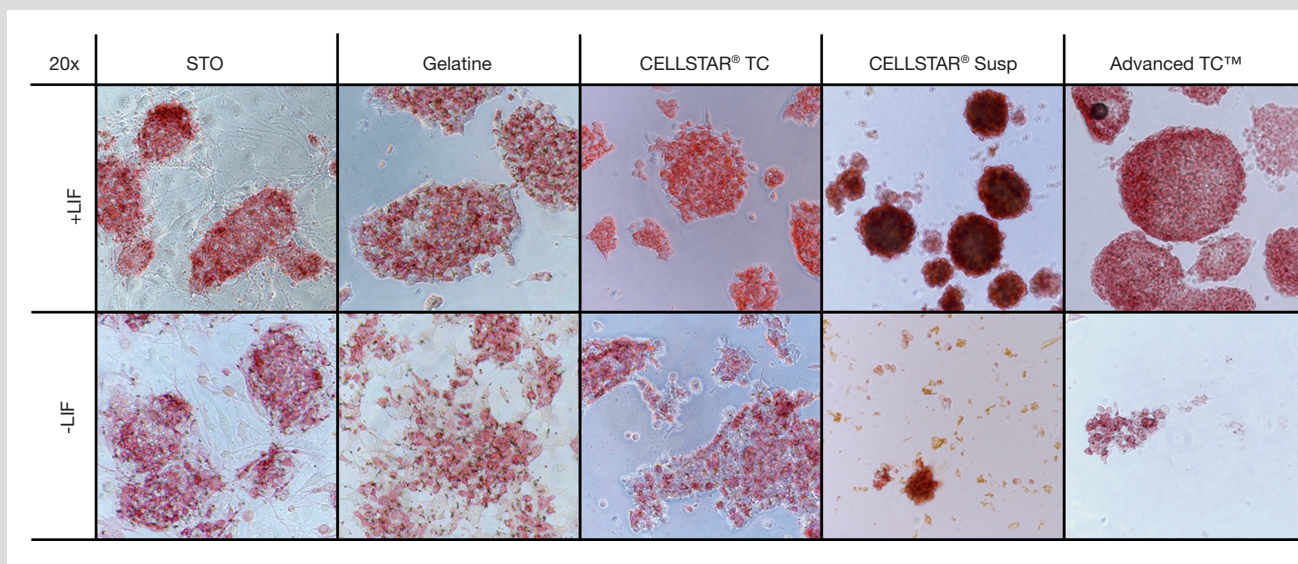


Figure 5: AP staining of embryonic stem cells cultivated on various surfaces after two days in culture (magnification 20x).

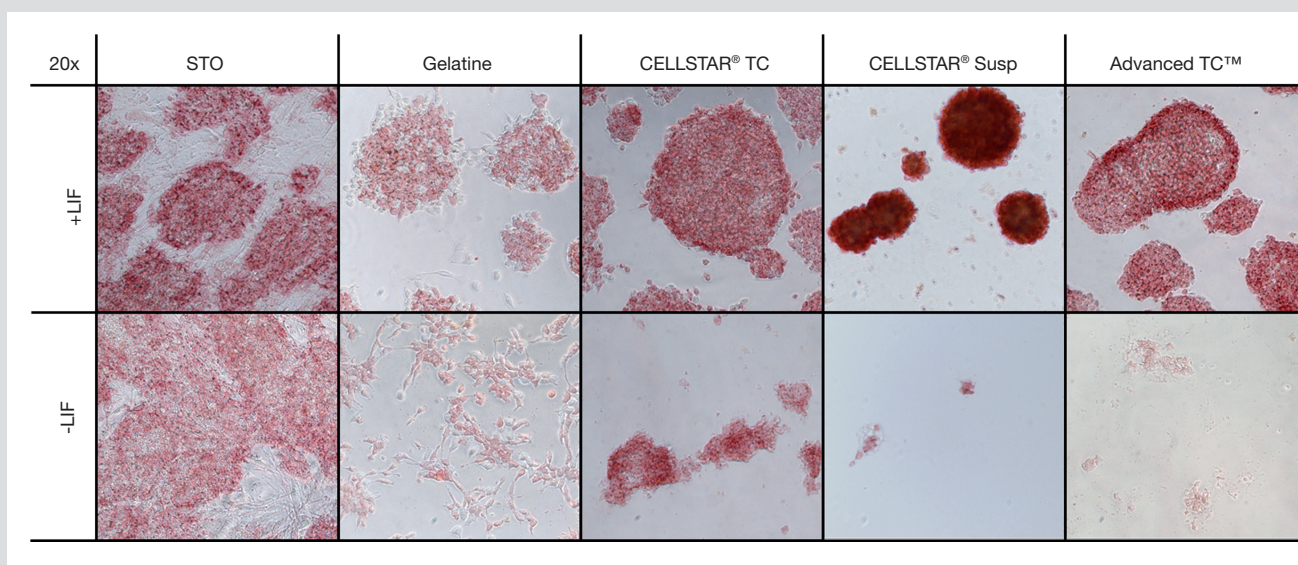


Figure 6: AP staining of embryonic stem cells cultivated on various surfaces after four days in culture (magnification 20x).

3.2. Immunocytochemistry of Embryonic Stem Cell Specific Markers

Beside alkaline phosphatase expression it is very important that embryonic stem cells express specific markers of undifferentiation. For murine embryonic stem cells one of these is the stage-specific embryonic antigen 1 (SSEA-1). SSEA-1 is expressed on the surface of murine embryonic stem cells and is down regulated following differentiation. Therefore it is a well established marker protein for the analysis of differentiation. Analysing first murine embryonic stem cells cultivated on inactivated STO cells by immunocytochemistry confirmed the results obtained by the AP staining (figure 7, left picture). The SSEA-1 was stained using a rabbit-anti-SSEA-1 and a secondary Alexa Fluor®488 coupled goat-anti-rabbit antibody (green). Cell nuclei were stained with DAPI in blue.

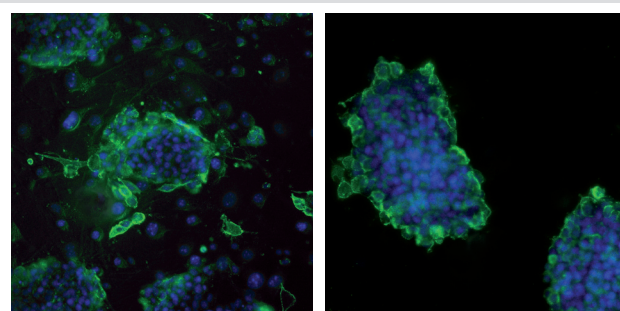


Figure 7: Immunocytochemistry of murine embryonic stem cells, cultivated on inactivated STO cells (left picture) or the Advanced TC™ surface (right picture). SSEA-1 is stained in green, while cell nuclei are displayed in blue (magnification 40x).

Murine embryonic stem cells found in the cellular aggregates show no sign of differentiation and specifically express the membrane bound SSEA-1 protein. No staining is detectable in the cytoplasm of these cells. As some cells display blue nuclei but no SSEA-1 staining these cells most probably correspond to STO feeder layer cells underneath the stem cell population. However based on this staining it can not be excluded that some of these cells might also refer to differentiated embryonic stem cells. Hence it is obvious that co-cultivation of feeder cells and ESCs does not only hinder clinical application but also complicates general analysis. This problem can be solved by cultivating the cells on the CELLSTAR® TC or the Advanced TC™ surface. Embryonic stem cells exhibited the same membrane specific SSEA-1 staining without the interfering DAPI staining of SSEA-1 negative cells (displayed exemplarily for Advanced TC™ in figure 7, right picture). This indicates that murine embryonic stem cells can be successfully expanded in an undifferentiated state on feeder-free surfaces like CELLSTAR® TC or Advanced TC™ as well as cells can be analysed and harvested without any interference or cross contamination of non-ESCs.

To verify the embryogenic state of the cultivated cells a second marker for undifferentiated stem cells has been analysed. Octamer-binding transcription factor 4 (Oct-4) is a transcription factor that is critically involved in the self-renewal of undifferentiated embryonic stem cells. It is initially active as a maternal factor in the oocyte and remains active in embryos throughout the preimplantation period. Therefore Oct-4 expression is associated with an undifferentiated phenotype and as such, it is frequently used as a marker for undifferentiated embryonic stem cells.

In contrast to SSEA-1, Oct-4 is expressed specifically in the cytoplasm of the cells. The analysed embryonic stem cells, cultivated on STO feeder layers, the CELLSTAR® TC and the Advanced TC™ surface displayed positive and antigen specific signals confirming the results of the SSEA-1 staining and proving differentiation had not occurred.

Equivalent to the first fluorescent analysis, the same obstacle was faced for ESCs cultivated on STO feeder layers: Depending on the staining result it could not be exactly defined if DAPI positive, but Oct-4 negative cells refer to the STO feeder layer cells or to differentiated embryonic stem cells. Only a feeder-free setup like the Advanced TC™ polymer modification (results depicted in figure 8) guaranteed a comprehensible result.

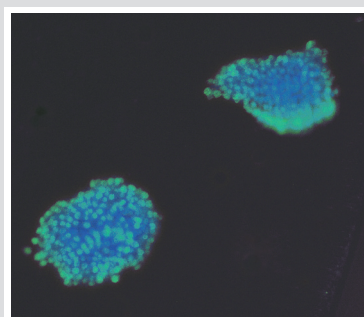


Figure 8: Immunocytochemistry of murine embryonic stem cells, cultivated on the Advanced TC™ surface. Oct-4 is stained in green, while cell nuclei are displayed in blue (magnification 40x).

From these first findings it can be concluded, that embryonic stem cells can be effectively cultivated on GBO surfaces like CELLSTAR® TC and Advanced TC™. Cells can be kept in an undifferentiated state and express relevant marker proteins like alkaline phosphatase, Oct-4 and SSEA-1. The expression level are comparable to ESCs cultivated on feeder layers whereas specificity is substantially increased as no non-embryonic cells can interfere with the staining result.

Based on the AP staining (figure 5 and 6) a gelatine coating as a “feeder-cell-free” system can not be recommended in this context. In summary Greiner Bio-One provides two surfaces (CELLSTAR® TC and Advanced TC™) for the cultivation and expansion of embryonic stem cells which can be directly utilized and do not require any pre-treatment or coating thus simplifying experimental procedures.

Aside from the propagation of embryonic stem cells the differentiation of these cells is of major importance for their therapeutic application. Embryonic stem cells can differentiate in vitro into endodermal, mesodermal and ectodermal cell types. In conjunction with their unrestricted self renewal this pluripotency provides a basis for generating unlimited numbers of defined somatic cell types for biomedical applications such as transplantation therapy, compound screening and transgenic disease modelling. One of the most promising application fields for such a cell therapy approach are neurological disorders like Parkinsons disease, stroke and multiple sclerosis. Under normal circumstances, the nervous system is incapable of healing itself. In the case of disease or injury, patients can be left with impaired motor function, paralysis or other disorders. Stem cells, however, can be used to create new neurological cells and tissue, and in theory could be used to repair damaged cells and restore normal function.

Therefore the neuronal differentiation of murine embryonic stem cells has been analysed with the following concept.

3.3. Neuronal Differentiation

Among many factors used to induce neuronal ESC differentiation, retinoic acid (RA) plays a pivotal role¹¹ and has been shown, depending on the experimental setups, to give rise to different neuronal cells¹²⁻¹⁵. As the majority of these approaches use intermediate embryoid body formation ahead the neuronal differentiation causing a mixture of spontaneously and directed differentiation a novel neuronal differentiation concept was designed. The objective was to establish a simple but efficient protocol leading to a high proportion of differentiated neurons while facilitating also the analysis of surface effects (figure 9).

Murine embryonic stem cells were first expanded in LIF containing media on STO feeder layers. Thereafter cells were transferred onto various GBO surfaces including CELLSTAR® TC, CELLSTAR® Suspension, Advanced TC™ and CELLCOAT® PDL. After 24 hours, differentiation was induced by the addition of retinoic acid for 72 hours. After 48 hours cytosine arabinoside (CAR) was applied to half of the respective cultures and incubated for the last 24 hours of RA activation.

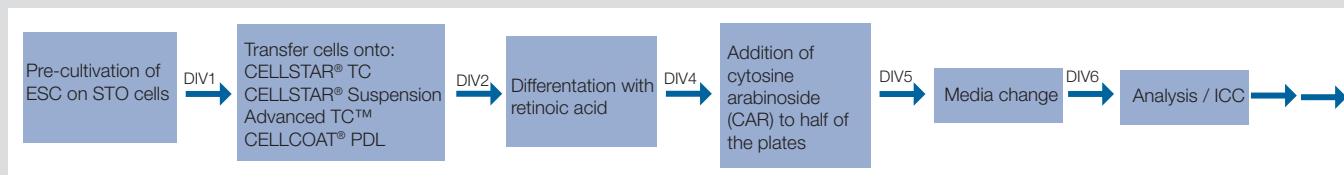


Figure 9: Concept of neuronal differentiation (DIV = days in vitro).

Cytosine arabinoside is an antimetabolic agent. Its mode of action is due to its rapid conversion into cytosine arabinoside triphosphate, which damages DNA when the cell cycle holds in the S phase. Additionally CAR also inhibits DNA and RNA polymerases and nucleotide reductase enzymes needed for DNA synthesis. Rapidly dividing cells, which require DNA replication for mitosis, are therefore most affected and die in culture. Addition of CAR hence supports differentiation of cells into neurons as these do not proliferate once they are differentiated. A possible contamination of non neuronal cells, induced by spontaneous or undirected differentiation can be eliminated by the addition of CAR.

One day before the addition of RA individual cell aggregates with sharp borders were visible on the indicated Greiner Bio-One surfaces representing optimal embryonic stem cell culture conditions, depicted exemplarily in figure 10 for the Advanced TC™ polymer modification.

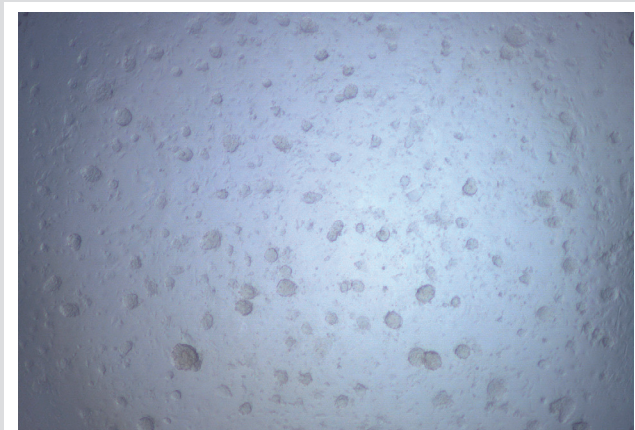


Figure 10: Initial culture situation before RA addition on the Advanced TC™ polymer modification (magnification 2.5x)

As ESCs still proliferate during RA incubation, stem cell aggregates increased in size until day four but simultaneously cells started to differentiate and migrate out of the aggregates. On day five, the borders of the aggregate dissolved and single cells exhibiting a neuronal morphology could be identified optically (data not shown). The induction of differentiation could be determined also by the fading of the AP staining. Especially on the CELLSTAR® TC and Advanced TC™ surface murine embryonic stem cells displayed only a minimal AP staining (data not shown). To clearly identify the neuronal phenotype immunocytochemical analysis with the following antibodies was performed: Nestin was used as a marker for neuronal precursor cells, neuronal BIII tubulin for the identification of differentiated neurons and GFAP as an indicator for non-neuronal glia cells (figure 11).

Nestin is a type VI intermediate filament protein expressed in dividing cells during the early stages of development in the central and peripheral nervous system¹⁶. Upon differentiation, nestin becomes downregulated and is replaced by tissue-specific intermediate filament proteins. Therefore nestin is a suitable marker protein for the intermediate phase of embryonic stem cell transition into neuronal cells.

BIII tubulin is a microtubule element of the tubulin family found almost exclusively in neurons¹⁷. Monoclonal antibodies directed towards this protein facilitate identification of neuronal cells, separating for example neurons from glial cells, which do not express BIII tubulin.

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is expressed in the central nervous system in glia cells¹⁸. It is involved in many cellular functioning processes, such as cell structure and movement, cell communication, and the blood brain barrier. As GFAP is not expressed in neurons it is an ideal marker to separate non-neuronal glia cells from differentiated neurons. Based on the literature and the experimental setup with the retinoic acid addition on the second day after cell transfer, the expected expression pattern referred to a maximum expression of nestin at day three to four, an increasing expression of BIII tubulin from day five on and day six for GFAP respectively (figure 11).

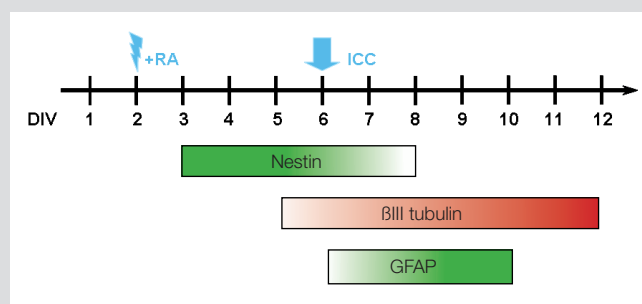


Figure 11: Expected expression pattern of the marker proteins nestin, BIII tubulin and GFAP (RA = Addition of retinoic acid; ICC = Immunocytochemistry)

To facilitate the detection of all marker proteins immunocytochemical (ICC) analysis was initiated on the sixth day of cultivation in vitro.

In general, more differentiated neurons could be detected when CAR was added on the last day of retinoic acid incubation. While all adherent cell surfaces (CELLSTAR® TC, Advanced TC™ and CELLCOAT® PDL) led to a good ICC staining result, cells on the CELLSTAR® Suspension plates got lost during the staining procedure.

Thus only the results of the upper mentioned adherent surfaces will be displayed.

Double staining for β III tubulin and nestin revealed a high number of neurons on the CELLSTAR® TC and the Advanced TC™ surface whereas the differentiation was less prominent on the CELLCOAT® PDL surface (figure 12). Based on the morphology of the cells and the high proportion of neuronal interconnections the state of differentiation was maximal when cells were cultivated on the Advanced TC™ polymer modification (figure 12, Advanced TC™). To determine the amount of non-neuronal glia cells and the selectivity of the applied neuronal differentiation protocol, cultures were analysed by a β III tubulin and GFAP double staining.

A slight GFAP staining, mainly located in cellular aggregates, could be detected on CELLCOAT® PDL and CELLSTAR® TC plates (figure 13) but no morphologically defined glia cell could be identified throughout the cultivation process up to day 8 on all analysed surfaces (data not shown). As expected no GFAP-positive cells could be identified when CAR was added on the final day of RA incubation (data not shown). As the antimetabolic agent inhibits cellular proliferation of all non-neuronal cells (see also p.9) glia cells could not survive under these conditions. Hence the addition of CAR supports the selective differentiation of cells into neurons.

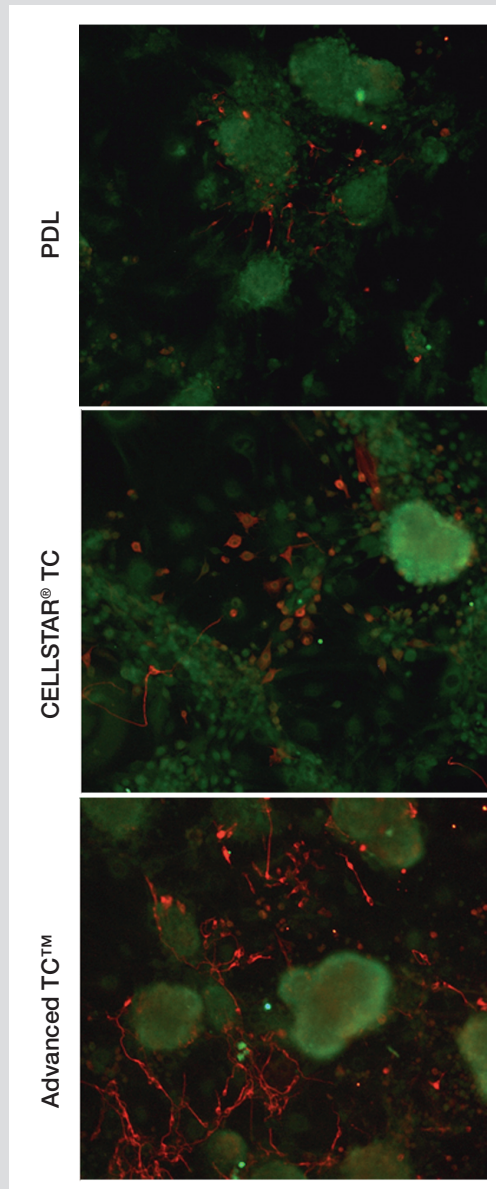


Figure 12: Nestin (green) and β III tubulin (red) double staining of murine embryonic stem cells differentiated by retinoic acid and CAR-selection (magnification 10x).

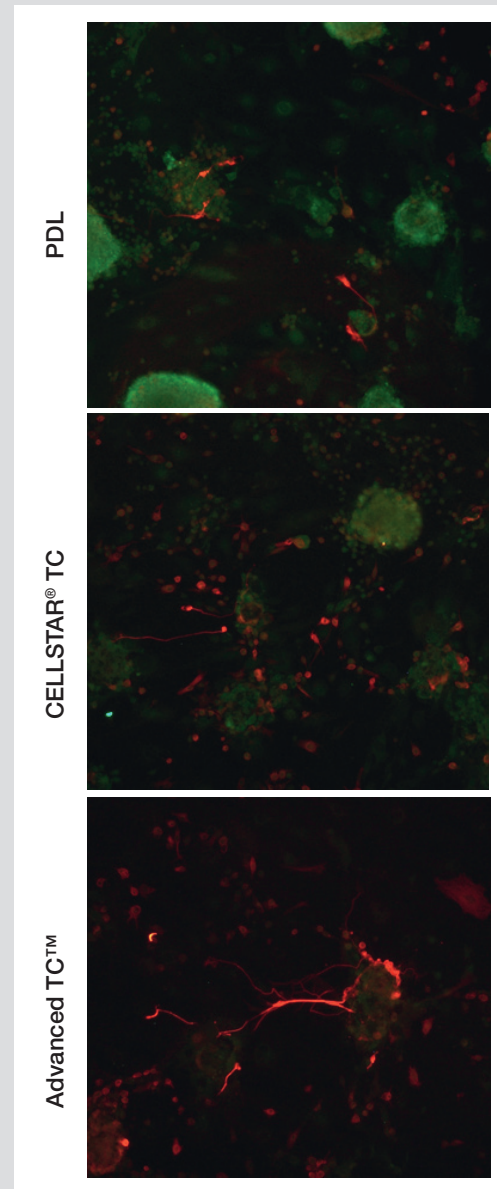


Figure 13: GFAP (green) and β III tubulin (red) double staining of murine embryonic stem cells differentiated by retinoic acid without CAR-selection (magnification 10x).

The highest number of differentiated neurons could be achieved when murine embryonic stem cells were cultivated on the Advanced TC™ polymer modification (figure 14).

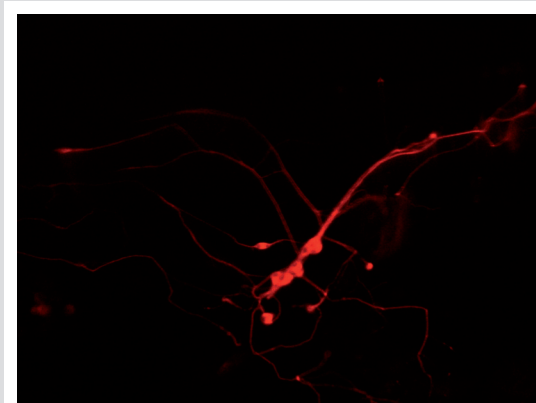


Figure 14: GFAP (green; no staining detectable) and βIII tubulin (red) double staining of murine embryonic stem cells differentiated by retinoic acid and CAR-selection on the Advanced TC™ polymer modification (magnification 20x).

As the pre-cultivation of ESCs on feeder layers is a laborious and complex process and feeder cells possess a potential risk of cross-contamination hindering clinical application we wanted to evaluate if the described experiment could be performed without the usage of STO cells. Therefore ESCs were cultivated on the Advanced TC™ surface and then either transferred to a CELLCOAT® PDL plate or kept in the original plate for retinoic acid induced differentiation. Similar to the first experimental setup cells were stained for βIII tubulin, nestin and GFAP starting from day six and monitored for a total of ten days. The results obtained were comparable to the pre-cultivation experiment and a high number of differentiated neurons could be detected forming a neuronal network; exemplarily depicted in figure 15 for the Advanced TC™ polymer modification.

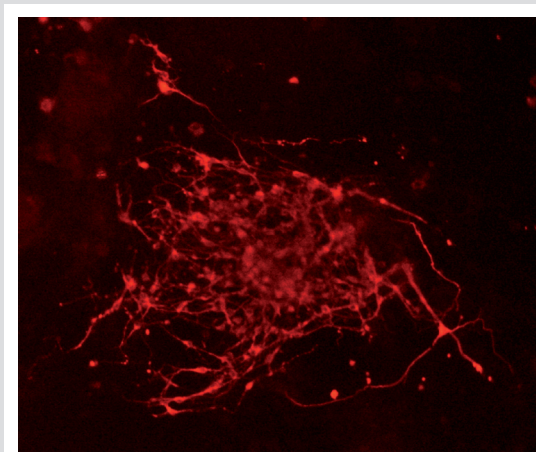


Figure 15: GFAP (green; no staining detectable) and βIII tubulin (red) double staining of murine embryonic stem cells ten days after induced differentiation and CAR-selection on the Advanced TC™ polymer modification without any pre-cultivation on STO feeder cells (magnification 20x)

4. Conclusion and Discussion

4.1. Maintenance of Murine Embryonic Stem Cells

The results displayed in this application note, illustrate that adherent embryonic stem cells can be effectively cultivated and expanded on GBO surfaces like CELLSTAR® TC and Advanced TC™. Embryonic stem cells remain pluripotent determined by the high expression of alkaline phosphatase, the surface marker SSEA-1 and the transcription factor Oct-4. The expression levels are comparable to ESCs cultivated on feeder layers whereas specificity is substantially better as no non-embryonic cell can interfere with the staining result. Therefore these surfaces are a most favourable alternative enabling pure embryonic stem cell cultures while assuring optimal culture conditions and avoiding spontaneous differentiation as well as excluding any risk of cross-contamination and pathogen spreading possibly caused by feeder cells.

In contrast to adherent cell culture conditions, spheroid culture for embryoid body formation could be achieved on the CELLSTAR® suspension surface. No specific non-binding or low-attachment surfaces were required for the analysed cells. In summary Greiner Bio-One provides two surfaces (CELLSTAR® TC and Advanced TC™) for the cultivation and expansion of adherent murine embryonic stem cells which can be directly utilized and do not require any pre-treatment or coating simplifying experimental procedures as well as the CELLSTAR® suspension surface facilitating non-adherent, spheroid embryonic stem cell culture.

4.2. Neuronal Differentiation of Murine Embryonic Stem Cells

Effective and direct neuronal differentiation could be achieved by the addition of retinoic acid and selection with cytosine arabinoside on the CELLSTAR® TC and CELLCOAT® PDL surface without the need of embryoid body formation. Beside the positive effect of the novel Advanced TC™ cell culture surface during the propagation of murine embryonic stem cells, the polymer modification also supported neuronal differentiation and led to the most selective cellular transformation. Even without the addition of CAR mainly neuronal cells could be identified on the Advanced TC™ surface. In summary these results emphasise the capability of Advanced TC™ as a powerful tool for embryonic stem cell research facilitating cultivation and neuronal differentiation of murine embryonic stem cells on a non biological, xeno-free surface.

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