Austin Publishing Group

Biochemical Formulation of Chemically-Defined and Non-Xenogeneic Culture Media for Research and Clinical Applications of Human Stem Cells

Byron Baron[1]*

[1]Centre for Molecular Medicine and Biobanking, Faculty of Medicine and Surgery, University of Malta, Malta

***Corresponding author:** Byron Baron, Centre for Molecular Medicine and Biobanking, Faculty of Medicine and Surgery, University of Malta, Malta, Email: byron.baron@um.edu.mt

Published Date: August 25, 2016

ABSTRACT

Current methods of cell culture rely extensively on the use of growth media supplemented with sera and unknown animal-derived factors. The use of products containing unknown animal-derived components is being avoided in the development of reliable research models, and the presence of non-human components is not acceptable for therapeutic applications. The development of media composed of chemically-defined and non-xenogeneic compounds is thus desirable for reproducibility in research and a necessity for stem cell therapeutics. The use of non-xenogeneic media offers numerous advantages for controlling the propagation and differentiation of cells as well as preventing xeno-transfer of immunogens or pathogens. However, despite the development of media is highly desirable, the range and availability at present is quite limited. The approach to develop a chemically-defined medium consists of first identifying a suitable basal

medium from which the cells can receive the required nutrition and then the addition of signaling factors which promote cell growth, preserve pluripotency or induce and direct differentiation, depending on the requirements of the culture system. So far, a number of chemically-defined and non-xenogeneic media formulations have been successfully developed, but these tend to be undisclosed proprietary formulations and cell-lineage specific. It is thus important to know how to prepare the desired medium from its components, when and why to use specific components and how to modify the system to best suit the cells in culture.

Keywords: Biochemical formulation; Cell culture; Growth media; Human stem cells

INTRODUCTION

The drive for developing chemically-defined and non-xenogeneic (containing no non-human-derived component) media formulations arises from the promise of stem cell therapy for regenerative medicine, which requires the production of large numbers of human Stem Cells **(hSCs)** in a safe, efficient and reproducible manner in order to reach its full therapeutic potential. The two key attributes of both induced pluripotent and embryonic hSCs are: prolonged self-renewal and pluripotency i.e. the potential to differentiate into any cell type derived from the three primary germ layers, and both of these properties are affected by the constituents of culture media. Defined and reproducible culture systems must be employed in order to generate quantities of hSCs (and cells derived from them) that are able to sustain therapeutic applications [1].

This can only be achieved through the application of chemically-defined and non-xenogeneic systems. Chemically-defined means that all the components and their concentrations are known and reproducible [2,3], while non-xenogeneic refers to a formulation that is free of non-human-derived components, implying that factors are either of human origin or recombinant (made synthetically). For therapeutic applications, the presence of non-human components is not acceptable due to the possibility of xeno-transfer of immunogens or pathogens [4].

Thus, hSCs and cells derived from them, cultured in a chemically-defined and non-xenogeneic environment provide a means toward both patient-specific therapies and improved disease model development [5]. Moreover, in order for the area of hSC therapeutics to advance, it is important to focus research towards understanding and addressing key issues related to the reliance of hSC on intracellular signals and extrinsic factors for their survival, proliferation, and differentiation, which still require further elucidation [4].

The lessons learnt from hSC propagation and differentiation have been applied to other cell types and tissue systems, providing a better understanding of cellular properties as well as greatly affecting various research fields and questioning numerous basic practices of cell culture. However, most routine cell culture practices rely extensively on the addition of Fetal Bovine Serum **(FBS),** containing numerous unknown animal-derived factors, to basal growth media.

The development of media composed of chemically-defined, non-xenogeneic compounds is desirable in a research context to overcome several limitations, such as batch-to-batch variability of FBS, limited global supply of serum and potential xeno-contaminants [6-8].

The development and use of various chemically-defined, non-xenogeneic environments (including the Extracellular Matrix - **ECM**), which exclude the use of animal-derived products such as FBS and cytokines commonly used in traditional mammalian cell culture [9-13] offers clear advantages over using FBS in terms of consistency, control and understanding, particularly in the contexts of Cancer Stem Cells **(CSCs)** and even more so, organoid culture, for controlling the propagation and differentiation of cells to specific lineages or in cases where cellular organisation and differentiation should not be hindered or directed externally. Consequently, the use of cell culture-related products containing unknown animal-derived components is being avoided in the development of more reliable research models and improving reproducibility of results.

The development strategy for any chemically-defined medium consists of first identifying a suitable basal medium to provide cells with basic nutritional needs and then adding supplements and specific signaling factors to promote cell growth, preserve pluripotency or induce and direct differentiation, according to the objectives of the culture system. The origin of the selected factors will then determine whether the final formulation can be regarded as non-xenogeneic or otherwise.

At present, there are a number of commercially-available culture media that are sold as chemically-defined and non-xenogeneic, but because these have proprietary formulations, their exact composition, origin (and extraction method) of components (mainly proteins and growth factors), and concentrations are undisclosed, making optimisation for different cell types or culture systems extremely difficult. To improve culturing techniques it is important to understand when and why to use specific components and know how to prepare the desired medium from scratch, in order to be able to modify the system to best suit culture needs.

BASAL MEDIA

The transition towards chemically-defined, non-xenogeneic culture systems has necessitated the formulation of complete media, which are suitable for the propagation and differentiation of both hSCs and differentiated cells under serum-free conditions [9,14-21]. A basal medium formulation generally contains a mixture of amino acids, vitamins, inorganic salts and glucose, providing both nutrients and an optimal growth environment in terms of osmolarity and pH in a buffered solution to maintain cells. Basal media such as Dulbecco's Modified Eagle Medium **(DMEM),** Iscove's Modified Dulbecco's Medium **(IMDM),** Alpha Modification of Minimum Essential Medium **(αMEM)**, Ham's F-12 Nutrient Mixture **(HAM F12)** and Embryonic stem cell basal medium **(ESF)** or the combinations there of (e.g. Dulbecco's Modified Eagle Medium : Nutrient Mixture F-12 - DMEM/F12) have been the starting-point for the majority of both in-house and commercially-available culture media for chemically-defined, non-xenogeneic

systems. Roswell Park Memorial Institute 1640 medium **(RPMI-1640)** is similar in composition to DMEM except for the inclusion of reduced glucathione in the latter but it has not been applied so much in chemically-defined, non-xenogeneic preparations. The compositions of the five most commonly used commercially-available basal media in the formulation of chemically-defined and non-xenogeneic media are presented in Table 1.

Table 1: List of components making up the five most commonly used basal media in the formulation of chemically-defined and non-xenogeneic media (based on supplier information).

Basal Medium	Composition
DMEM	Amino acids: Glycine, L-Arginine hydrochloride, L-Cystine 2HCl, L-Glutamine, L-Histidine hydrochloride. H_2O, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine disodium salt dihydrate, L-Valine. Vitamins: Choline chloride, D-Calcium pantothenate, Folic Acid, Niacinamide, Pyridoxine hydrochloride, Riboflavin, Thiamine hydrochloride, i-Inositol. Inorganic Salts: Calcium Chloride ($CaCl_2$) (anhyd.), Ferric Nitrate ($Fe(NO_3)3.9H_2O$), Magnesium Sulfate ($MgSO_4$) (anhyd.), Potassium Chloride (KCl), Sodium Bicarbonate ($NaHCO_3$), Sodium Chloride (NaCl), Sodium Phosphate monobasic ($NaH_2PO_4.H_2O$). Other Components: D-Glucose (Dextrose).
IMDM	Amino acids: Glycine, L-Alanine, L-Arginine hydrochloride, L-Asparagine (freebase), L-Aspartic acid, L-Cystine 2HCl, L-Glutamic Acid, L-Glutamine, L-Histidine hydrochloride.H_2O, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine disodium salt, L-Valine. Vitamins: Biotin, Choline chloride, D-Calcium pantothenate, Folic Acid, Niacinamide, Pyridoxal hydrochloride, Riboflavin, Thiamine hydrochloride, Vitamin B12, i-Inositol. Inorganic Salts: Calcium Chloride ($CaCl_2$) (anhyd.), Magnesium Sulfate ($MgSO_4$) (anhyd.), Potassium Chloride (KCl), Potassium Nitrate (KNO_3), Sodium Bicarbonate ($NaHCO_3$), Sodium Chloride (NaCl), Sodium Phosphate monobasic ($NaH_2PO4.H_2O$), Sodium Selenite ($Na_2SeO_3.5H_20$). Other Components: D-Glucose (Dextrose), HEPES, Sodium Pyruvate, Lipoic Acid, Putrescine 2HCl, Thymidine.
*α*MEM	Amino Acids: Glycine, L-Alanine, L-Arginine, L-Asparagine.H_2O, L-Aspartic acid, L-Cysteine hydrochloride-H_2O, L-Cystine, L-Glutamic Acid, L-Glutamine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine. Vitamins: Ascorbic Acid, Biotin, Choline chloride, D-Calcium pantothenate, Folic Acid, Niacinamide, Pyridoxal hydrochloride, Riboflavin, Thiamine hydrochloride, Vitamin B12, i-Inositol. Inorganic Salts: Calcium Chloride ($CaCl_2.2H_2O$), Magnesium Sulfate ($MgSO_4$) (anhyd.), Potassium Chloride (KCl), Sodium Bicarbonate ($NaHCO_3$), Sodium Chloride (NaCl), Sodium Phosphate monobasic ($NaH_2PO_4.2H_2O$). Other Components: D-Glucose (Dextrose), Lipoic Acid, Sodium Pyruvate, Putrescine 2HCl, Thymidine.
HAM F12	Amino acids: Glycine, L-Alanine, L-Arginine hydrochloride, L-Asparagine.H_2O, L-Aspartic acid, L-Cysteine hydrochloride.H_2O, L-Glutamic Acid, L-Glutamine, L-Histidine hydrochloride.H_2O, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine disodium salt dihydrate, L-Valine. Vitamins: Biotin, Choline chloride, D-Calcium pantothenate, Folic Acid, Niacinamide, Pyridoxine hydrochloride, Riboflavin, Thiamine hydrochloride, Vitamin B12, i-Inositol. Inorganic Salts: Calcium Chloride ($CaCl_2$) (anhyd.), Cupric sulfate ($CuSO_4.5H_2O$), Ferrous sulfate ($FeSO_4.7H_2O$), Magnesium Chloride (MgCl2)(anhyd.), Potassium Chloride (KCl), Sodium Bicarbonate ($NaHCO_3$), Sodium Chloride (NaCl), Sodium Phosphate dibasic (Na_2HPO_4) (anhyd.), Zinc sulfate ($ZnSO_4.7H_2O$) Other Components: D-Glucose (Dextrose), Hypoxanthine Na, Linoleic Acid, Lipoic Acid, Putrescine 2HCl, Sodium Pyruvate, Thymidine.
ESF	Amino acids: Glycine, L-Alanine, L-Arginine, L-Arginine hydrochloride, L-Asparagine.H_2O, L-Aspartic acid, L-Cysteine hydrochloride.H_2O, L-Cysteine 2hydrochloride, L-Glutamic Acid, L-Glutamine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Hydroxyproline, L-Proline L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine. Vitamins: Biotin, Choline chloride, D-Calcium pantothenate, Folic Acid, Niacinamide, Pyridoxal, Pyridoxine hydrochloride, Riboflavin, Thiamine hydrochloride, Vitamin B12, i-Inositol, Para-aminobenzoic. Inorganic Salts: Calcium Chloride ($CaCl_2$) (anhyd.), Calcium nitrate ($Ca(NO_3)2$), Cupric sulfate ($CuSO_4.5H_2O$), Ferric Nitrate ($Fe(NO_3)3.9H_2O$), Ferrous sulfate ($FeSO_4.7H_2O$), Magnesium Chloride (anhyd.), Magnesium Sulfate ($MgSO_4$) (anhyd.), Potassium Chloride (KCl), Sodium Chloride (NaCl), Sodium Phosphate dibasic (Na_2HPO_4) (anhyd.), Sodium Phosphate monobasic (NaH_2PO_4) (anhydr.), Zinc sulfate ($ZnSO_4$-$7H_2O$), Sodium Selenite ($Na_2SeO_3.5H_20$). Other Components: D-Glucose (Dextrose), Hypoxanthine Na, Glutathione, Linoleic Acid, Lipoic Acid, Putrescine 2HCl, Sodium Pyruvate, Thymidine.

The noteworthy components of basal media are:

• Sodium pyruvate, which is an intermediate metabolite in the glycolysis pathway. It is frequently added to media formulations as an additional and easily accessible carbon and energy source (together with glucose). Biochemically it is involved in other critical metabolic pathways, including the Tricarboxylic Acid **(TCA)** cycle and amino acid metabolism so its addition by-passes the need for cells to synthesize it from glucose or amino acids.

• Thymidine and hypoxanthine, which are a deoxynucleoside and a purine derivative respectively. These act as intermediates in DNA synthesis. Their inclusion in media formulations allow the by-pass of *de novo* nucleotide synthesis and stimulate the initial cell growth.

• Glutathione, which is an essential antioxidant for the maintenance of a favourable cellular redox environment. It is key for the regeneration of sulfhydryl compounds and antioxidants, as a substrate for a number of seleno-proteins that convert peroxides to water or alcohols, as well as the maintenance of cysteine in its reduced form, thus keeping cysteine available as a substrate for the formation of proteins and glutathione itself.

• Lipoic and linoleic acid, which are non-essential fatty acids of importance as precursors for other biomolecules including prostaglandins, prostacyclins, thromboxanes, phospholipids, glycolipids, and vitamins. They are among a number of lipid options provided in media formulations. Their addition reduces the need for cells to synthesise them, improving cellular performance due to more efficient metabolism. Lipoic acid is particularly important because it is required for pyruvate metabolism, the synthesis of alpha-ketoglutarate (a TCA cycle intermediate), as well as glycine, leucine, isoleucine and valine (which are key amino acids), and additionally acting as an antioxidant through several mechanisms.

• Putrescine (tetramethylenediamine), which is produced by the breakdown of amino acids. It appears to be a necessary growth factor (together with related amines) for mammalian cell division.

SUPPLEMENTS

The traditional way of supplementing cells with numerous, often unidentified, (animal-derived) factors is through the use of FBS. This has been found to be inadequate in a multitude of contexts, with some stocks of FBS limiting the growth of cells or worse still, where the cells are dependent on specific factors which are not available in FBS. In this regard, hSC are among the worst affected. The use of FBS is not only inadequate for maintaining the undifferentiated characteristics of hSCs but can also lead to aberrant differentiation during culturing. This undesirable differentiation, during maintenance or direction along particular lineages is a key issue in both therapy and research, and how to limit such a process is a major consideration.

Supplements originating from feeder cells:

In early studies, hSCs were maintained by direct culture over layers of inactivated mouse Embryonic Fibroblast Feeder cells **(mEFs),** which secreted the factors required to support the hSCs, and this allowed the successful establishment of a number of hSC lines [22,23]. Optionally, conditioned medium from mEFs was added to medium supplemented with FBS [22,24]. The growth factors and cytokines secreted by mEFs providing insight into hSC biology (particularly signalling) and expanded the general understanding of the appropriate culture conditions for supporting self-renewal and pluripotency in hSC cultures [1].

However, this strategy exposed hSCs to xenogenic cells and biochemicals making them unsuitable for therapy due to the possibility of contamination by animal-derived infectious pathogens or immune rejection [1]. It is known that mEFs can contain viral particles capable of infecting humans [25]. The use of conditioned medium with FBS is also problematic as the latter could transmit prions or bovine viruses [26]. This also means that it is inappropriate to transition hSCs to a feeder-free and animal-protein free culture system at a late stage. A further hazard are bioactive molecules such as the non-human sialic acid Neu5GC [27] found in mEFs and animal-derived serum products, which can be metabolically incorporated on to the cell surface of hSCs [27] and result in an immune response following hSC transplantation, as humans have circulating antibodies against Neu5GC.

In order to eliminated the aforementioned risks, efforts were subsequently made to isolate autogenic (of human origin) feeder layers such as human foetal foreskin fibroblasts [28-31], adult epithelial cells [32], bone marrow cells [33,34] and placenta-derived feeder cells [35,36] as well as feeder cells derived from the hSC line being cultured themselves [37,38]. This also proved to be of greater benefit in a research context for understanding hSC biology. However, human feeder cells presented numerous difficulties in sourcing (due to variability in numerous parameters), derivation, preparation, preservation and eventual separation from co-culture making the process challenging, costly and time consuming [4].

Thus from a therapeutic perspective the potential hazards of xenogenic components is the major concern, since cells from a single hSC line might be used for numerous patients, presenting the risk of transmitting infectious pathogens to a large cohort of patients [1]. On the other hand, from a research perspective, the main issue is the varying composition of such supplementation and the unknown effect of xenogenic factors on signalling within human cells.

DEFINED SUPPLEMENTS

To improve on such culture conditions, one of the focuses of current stem cell research has been the design of feeder-free, non-xenogeneic culture systems with chemically-defined media formulations which can maintain stem cell characteristics [1,39]. Studies using feeder layers have contributed greatly to the understanding of *in vitro* hSC behaviour, characteristics and necessary culture conditions. A key approach to develop chemically-defined and non-xenogeneic media for hSC culture has been to use the signal transduction data from culturing on feeder layers to

identify and supply extrinsic growth factors which act on cascades related to the regulation of pluripotency genes [16,40], controlled through common genetic networks of transcriptional factors [41-43] centered on Nanog, Oct4 (Pou5f1) and Sox2, which eventually activate or repress genetic programs for hPSC self-renewal or differentiation [42,44,45] (Figure 1).

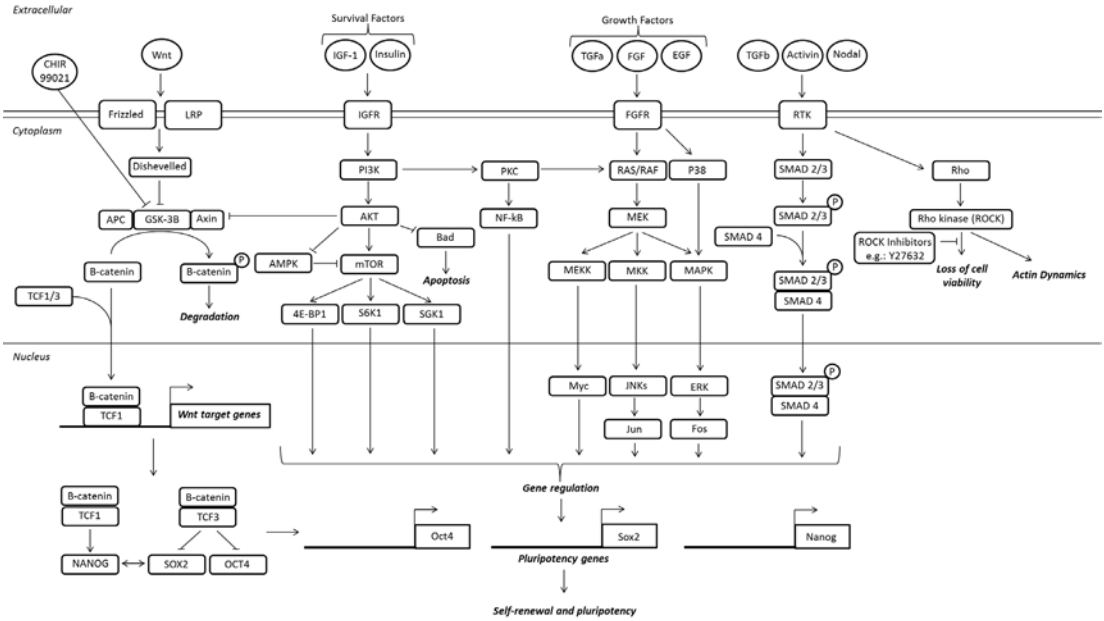

Figure 1: Signalling pathways for the maintenance of pluripotency and self-renewal in human stem cells.

The numerous components making up the various culture media used for maintaining hSCs, have been extensively compared through multiple experiments (for example as summarised by The International Stem Cell Initiative Consortium [19], which have determined that the most crucial factors are:

Albumin

This is the most controversial component of culture media, being attributed profound value in cell culture through numerous properties and functions. Albumin in serum serves a unique role as a carrier (binding and stabilising a range of biomolecules including proteins, vitamins, hormones, ions and drugs, improving their availability to cells), as well as functioning as a antioxidant through various mechanisms. It is known for binding and protecting fatty acids (such as linoleic, linolenic and oleic acid), divalent cations (primarily zinc and copper but also others), and antioxidant molecules (like cysteine and glutathione) from oxidation.

However, in order to produce non-xenogeneic media, Human Serum Albumin **(HSA)** has to be used as the protein source. The use of HSA makes such formulations too expensive and impractical for routine use, while still not being truly completely defined [46]. The main issue is the variation

between different batches of HSA due to extraction from different pools of donor sera. Moreover, different (generally unidentified) albumin-associated factors and plasma proteins, or pathogens and other contaminants, are co-purified with the HSA. The type and quantity of specific ligands bound by albumin results in observable differences in culture performance [39]. One way of reducing such variation and contamination issues is by relying on recombinant HSA for the development of chemically-defined and non-xenogeneic formulations. Moreover, the testing of pair wise interactions between different medium components showed that in the absence of 2-mercaptoethanol, BSA (or HSA) is no longer necessary for hSC culture [46].

Wingless-Related Integration Site (Wnt)

Wnt signalling has been indicated as having a major role in hSC self-renewal [47,48]. In embryonic development it is known to play a central role through its involvement in the formation of the body axis (called axis patterning), cell-fate specification through the induction of cell differentiation, and tissue development by regulating cell proliferation and migration [49,43]. However in media formulations, the use of recombinant Wnt3a to stimulate the Wnt pathway appeared to be insufficient to maintain hSCs undifferentiated and β-catenin-mediated transcriptional activity was upregulated during differentiation [50]. The difficulty in controlling Wnt signalling is compounded by the fact that it has been implicated in the specification of stem and progenitor cells along multiple lineages, which are often developmentally distant. Moreover, different hSC lines (of the same origin) exhibit disparate levels of endogenous Wnt activity. This gives a strong indication that the use of Wnt ligands to regulate hSCs should be finely customized [4].

Rho Kinase (ROCK) Inhibitors

ROCK is a key cytoskeleton regulation, affecting cell shape and movement through a non-canonical Wnt pathway. The addition of ROCK inhibitors, including HA100 and Y27632 [51,52] in medium formulations helps improves initial survival by preventing apoptosis in individual cells during the passaging phase [1] and supports a high cloning efficiency [42]. In one instance, the ROCK inhibitor Y27632 was combined with the Mitogen-Activated Protein Kinase **(MEK)** inhibitors PD98059 and PD0325901, the Glycogen Synthase Kinase **(GSK)** inhibitor CHIR99021 and the FGF Receptor **(FGFR)** tyrosine kinase inhibitor PD173074 to form a kinase-inhibitor cocktail for the culturing of single hSCs [53]. Blebbistatin (a myosin II inhibitor) is also often added to ROCK inhibitors to improve cell passaging [54].

Insulin

Insulin (and also insulin-like growth factors -IGFs) is considered to be an important growth factor in humans by acting through numerous pathways [55,56]. In media formulations, insulin is one of the factors that play a role in cell survival and proliferation [46,57].

Fibroblast Growth Factors (FGFs)

Possibly the most crucial factor in media formulations is some form of FGF. FGFs are key players in the processes of proliferation and differentiation of a wide variety of cells and tissues, performing multiple actions on a variety of cell types. For the purpose of maintaining hSCs in culture, most FGFs appear to be interchangeable [46]. The function of FGFs in media formulations include increasing cell survival, cloning efficiency, sustains cell proliferation, prevent differentiation and maintain hSC pluripotency [58-64].

The Transforming Growth Factor-Beta **(TGFβ)** super family-activated cascades and Receptor Tyrosine Kinase **(RTK)** signaling are two of the major signaling pathways downstream of FGF. It has been reported that the effect of FGFs on the self-renewal properties of hSCs is through the increased expression of TGFβ, Insulin-like Growth Factor 2 **(IGF-2)** and activin A (a protein complex of the TGFβ superfamily) [60].

Moreover, since suppression of Bone Morphogenetic Protein **(BMP)** signaling can prevent loss of hSC pluripotency [65,66], a number of BMP-antagonists (like noggin, dorsopmorphin and inhibitors of the Wnt signaling pathway) have been included to media formulations in order to work synergistically with FGFs to suppress spontaneous differentiation and maintain stemness properties [67]. In such cases, the concentration of FGFs appears to affect the ability of the BMP-antagonist noggin (a developmental protein) to support the growth of undifferentiated hSCs [66].

A number of small molecules have been identified which could be used to promote long-term hSC self-renewal without the need for exogenous FGFs in the medium. Such small molecules have the advantage that they can diffuse easily through multi-layer cellular configurations and have much longer degradation times [4]. In one study, 22 compounds were identified, that exhibited dose-dependent effects on Oct4, with four being Oct4 activators inducing self-renewal (namely flurbiprofen, gatifoxacin, sinomenine and theanine), and ten being Oct4 inhibitors inducing differentiation [68]. In a more recent study, 18 compounds were identified of which five (namely methotrimeprazine, trimipramine, trimeprazine, ethopropazine, and promethazine) were neurotransmitter antagonists [69]. Probably of greatest interest is however the identification of a compound **(ID-8)** that targets the dual-specificity tyrosine phosphorylation-regulated kinase **(DYRK).** ID-8 together with Wnt-3a allows hSC to proliferate and survive in an undifferentiated state without the need for exogenous FGF or TGFβ activation [70], by directly modulating the Wnt/β-catenin signaling pathway [71-73].

Transforming Growth Factor-Beta (TGFβ)

TGFβ signalling (including the activity of TGFβ, Nodal and Activin A) modulates cell fate decisions in the early embryo and is involved in maintaining the pluripotency of the inner cell mass [60,74-76]. This occurs through small body Size/Mothers against Decapentaplegic **(SMAD)** proteins [77,78], which are receptor-regulated intracellular transducers of extracellular TGFβ signalling that can act as transcription factors following phosphorylation. TGFβ is an important

growth factor in media formulations [79,80], (particularly important in the first 6-8 days) increased the number of hSC colonies, while presenting inhibitory effect at later stages of reprogramming [81]. The addition of TGFβ or Nodal to hSCs increases Nanog expression and lead to consistent long-term culture stability of hSCs, preventing sporadic differentiation after long-term passage [46]. In some cases hydrocortisone has been found useful in producing active effects with TGFβ [46].

Transferrin

Transferrin forms a glycoprotein by combining with two carbohydrate chains. It is added to cell culture formulations to provide iron to cells. It plays a crucial role in extracellular iron storage and transport as well as acting as an extracellular antioxidant. Transferrin can be replaced in culture formulations by iron chelators however these can cause oxidative stress and result in protein damage [82].

L-ascorbic acid

Ascorbic acid (vitamin C), or more accurately the ascorbate anion (at physiological pH), has proven to be an essential antioxidant for the growth and maintenance of cultured cells and promotes hSC proliferation and expansion [46]. It regenerates antioxidants and protects unsaturated fatty acids from peroxidation as well as being important in acetyl-coA synthesis and the formation of extracellular matrices. It is also possible that ascorbic acid in serum-free culture conditions leads to epigenetic modifications essential to both reprogramming and cell survival [81].

Selenium

This essential trace element forms the amino acid selenocysteine which is included into seleno-enzymes, which fulfil a number of antioxidant functions including protecting cells from various types of peroxides (e.g. glutathione reductase, thioredoxin reductase, glutathione peroxidase, and selenoprotein P). In media formulations it is important for sustained expansion [46]. The form and oxidation state of selenium in culture depends both on whether it is included as selenium, selenium dioxide or sodium selenite as well as the overall formulation chemistry.

COMMERCIALS

This insight has led to the development of numerous different chemically-defined and non-xenogeneic culture media formulations, either published by labs in academia [82] or commercially sold as proprietary compositions [39]. Among the most well-known and widely used chemically-defined and non-xenogeneic formulations available on the market are Knockout Serum Replacement **(KSR)** [85], Essential 8 (E8) [21], TeSR [86] and its successors TeSR2 and mTeSR [87], StemPRO [55], the Nutristem XF range, PSGro and *XVivo* 10 [14]. Other products sold as defined supplements include B27 [88,89]. However, most commercially available products have proprietary composition and may still contain animal-derived components such as Bovine Serum

Albumin **(BSA)** [4]. Component exclusion analysis demonstrated that the absence of several growth factors (TGFβ, LiCl, GABA and pipecolic acid) does not affect short-term cell survival and proliferation [21]. Moreover additives including chemically defined lipids, trace elements, glutathione, thiamine and l-glutamine had no positive effect [21].

CHALLENGES AND FUTURE PROSPECTS

Through all this development, it has been possible to greatly increase the consistency and safety of chemically-defined and non-xenogeneic media for culturing cells, most particularly hSCs, from various contexts to meet the desired quality for both research and clinical applications. In such a context, it is important to appreciate the fact that optimal culture supplementation will vary for different cell types. In general, the use of chemically-defined and non-xenogenic media produces doubling times comparable to or higher than those in FBS-containing medium, while maintaining or improving hSC characteristics such as expression of cell surface markers and differentiation capability [38,53,83,90-93], however growth rates differ, reflecting differences in the medium composition [92-96].

This necessitates the preparation of culture media of customised formulation to achieve the desired doubling time or differentiation path. Comparisons of different formulations have shown that the choice of medium plays a role in hSC proliferation, differentiation and time of cellular senescence [97,91]. Similarly it is important to understand that in some instances within the research sphere, such as in organoid cultures, a number of lineages of the same hSCs will have to be maintained.

Consequently, the complete reliance on recombinant supplements results in high costs which need to be taken into consideration, particularly in a research context where funding may be limited. This is magnified in situations where the cell doubling times may be limited by fluctuations in supplement availability (due to depletion or instability) requiring daily replenishment of media [1]. There is also an issue of cost associated with the automation of culture-related processes.

Moreover, the great majority of the development in this area of chemically-defined media and non-xenogeneic supplementation has been carried out in two-dimensional **(2D)** culture systems. It follows that with current trends in moving towards three-dimentional **(3D)** culture systems to better mimic the *invivo* situation and explore new facets of cellular architecture, signalling processes and organoid biology, translation of these culturing resources to 3D culture systems poses unique difficulties [4]. Optimisation of medium composition will be required for the 3D culture for each cell type, as growth rates vary from 2D to 3D cultures, and again between different 3D culture methods [39]. This is exacerbated by the fact that a variety of 3D culture systems rely on some sort of ECM, which plays a major role in signalling, factor release and cellular properties.

The microenvironment (generally in the form of a scaffold or hydrogel as ECM) plays a critical role in cell fate, through the release of soluble factors, cell-cell and/or cell-matrix interactions and mechanical stresses [4]. The self-renewal of hSCs is dependent on extracellular conditions and it is

also the case, that factors present in the ECM could cause spontaneous differentiation. Thus when considering ECMs for performing various types of hSC research or in large-scale production of hSC products for therapeutic applications, there is a similar need for relevant chemically-defined and non-xenogeneic matrices, for a more controlled environment [4]. The issues linked with non-xenogeneic ECM preparations have been reviewed elsewhere [98].

What is certain in this area of research is that there is still much to be learnt about the culturing of cells in chemically-defined and non-xenogeneic media, which will only come with a better understanding of cellular biochemistry, particularly of hSCs. This knowledge will no doubt be translatable to disease contexts and improve our understanding of the multitude of extracellular signals which current therapies might be ignoring. As a result, the development of chemically-defined and non-xenogeneic media, and similarly ECMs, will advance current culturing techniques for both research and therapy.

References

1. Desai N, Rambhia P, Gishto A. Human embryonic stem cell cultivation: historical perspective and evolution of xeno-free culture systems. Reproductive Biology and Endocrinology. 2015; 13: 9.
2. Van der Valk J, Brunner D, De Smet K, Fex Svenningsen A, Honegger P, et al. Optimization of chemically defined cell culture media–replacing fetal bovine serum in mammalian *in vitro* methods. Toxicol *in Vitro*. 2010; 24: 1053-1063.
3. Wang Y, Cheng L, Gerecht S. Efficient and scalable expansion of human pluripotent stem cells under clinically compliant settings: a view in 2013. Ann Biomed Eng. 2014; 42: 1357-1372.
4. Fan Y, Wu J, Ashok P, Hsiung M, Tzanakakis ES. Production of Human Pluripotent Stem Cell Therapeuticsunder Defined Xeno-free Conditions: Progress and Challenges. Stem Cell Rev and Rep. 2015; 11: 96-109.
5. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, et al. Disease-specific induced pluripotent stem cells. Cell. 2008; 134: 877-886.
6. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther. 2004; 9: 747-756.
7. Dimarakis I, Levicar N. Cell culture medium composition and translational adult bone marrow-derived stem cell research. Stem Cells. 2006; 24: 1407-1408.
8. Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells 2006; 24: 462-471.
9. Mallon BS, Park KY, Chen KG , Hamilton RS , McKay RD. Toward xeno-free culture of human embryonic stem cells. International Journal of Biochemistry and Cell Biology. 2006; 38: 1063-1075.
10. Lei T, Jacob S, Ajil-Zaraa I, Dubuisson JB, Irion O, et al. Xeno-free derivation and culture of human embryonic stem cells: current status, problems and challenges. Cell Research. 2007; 17: 682-688.
11. Vemuri MC, Timothy Schimmel, Pere Colls, Jacques Cohen. Derivation of human embryonic stem cells in xeno-free conditions. Methods in Molecular Biology. 2007; 407: 1-10.
12. Rajala K, Hakala H, Panula S, Aivio S, Pihlajamäki H, et al. Testing of nine different xeno-free culture media for human embryonic stem cell cultures. Human Reproduction. 2007; 22: 1231-1238.
13. Kehoe DE, Jing D, Lock LT, Tzanakakis ES. Scalable stirred-suspension bioreactor culture of human pluripotent stem cells. Tissue Engineering Part A. 2010; 16: 405-421.
14. Li Y, Powell S, Brunette E, Lebkowski J, Mandalam R. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. Biotechnol Bioeng. 2005; 91: 688-698.
15. Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol. 2006; 24: 185-187.
16. Chase LG, Firpo MT. Development of serum-free culture systems for human embryonic stem cells. Current Opinion in Chemical Biology. 2007; 11: 367-372.
17. Ludwig T, A Thomson J. Defined, feeder-independent medium for human embryonic stem cell culture. Curr Protoc Stem Cell Biol. 2007; Chapter 1: Unit 1C 2.

18. Furue MK, Na J, Jackson JP, Okamoto T, Jones M, et al. Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. Proc Natl Acad Sci U S A. 2008; 105: 13409-13414.

19. Akopian V, Andrews PW, Beil S, Benvenisty N, Brehm J, et al. Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. *In Vitro* Cell. Dev. Biol. Anim. 2010; 46: 247-258.

20. Valamehr B, Tsutsui H, Ho CM, Wu H. Developing defined culture systems for human pluripotent stem cells. Regenerative Medicine. 2011; 6: 623-634.

21. Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, et al. Chemically defined conditions for human iPSC derivation and culture. Nat Methods. 2011; 8: 424-429.

22. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998; 282: 1145-1147.

23. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. Nat Biotechnol. 2000; 18: 399-404.

24. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol. 2001; 19: 971-974.

25. Cobo F, Navarro JM, Herrera MI, Vivo A, Porcel D, et al. Electron microscopy reveals the presence of viruses in mouse embryonic fibroblasts but neither in human embryonic fibroblasts nor in human mesenchymal cells used for hESC maintenance: toward an implementation of microbiological quality assurance program in stem cell banks. Cloning Stem Cells. 2008; 10: 65-74.

26. Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, et al. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet. 2004; 363: 417-421.

27. Martin MJ, Muotri A, Gage F, Varki A, et al. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nature Medicine. 2005; 11: 228-232.

28. Amit M, V Margulets, H Segev, K Shariki, I. Laevsky, et al. Human feeder layers for human embryonic stem cells. Biology of Reproduction. 2003; 68: 2150-2156.

29. Hovatta O, Mikkola M, Gertow K, Strömberg AM, Inzunza J, et al. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Human Reproduction. 2003; 18: 1404-1409.

30. Choo AB, Jayanti P, Angela CP, Steve KW. Expansion of pluripotent human embryonic stem cells on human feeders. Biotechnology and Bioengineering. 2004; 88: 321-331.

31. Meng G, Liu S, Li X, Krawetz R, Rancourt DE. Extracellular matrix isolated from foreskin fibroblasts supports long-term xeno-free human embryonic stem cell culture. Stem Cells and Development. 2010; 19: 547-556.

32. Richards M, Chui-Yee Fong, Woon-Khiong Chan, Peng-Cheang Wong, Ariff Bongso. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nature Biotechnology. 2002; 20: 933-936.

33. Cheng LZ, Hammond H, Ye Z, Zhan X, Dravid G. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells. 2003; 21: 131-142.

34. Zhang K, Cai Z, Li Y, Shu J, Pan L, et al. Utilization of human amniotic mesenchymal cells as feeder layers to sustain propagation of human embryonic stem cells in the undifferentiated state. Cellular Reprogramming. 2011; 13: 281-288.

35. Miyamoto K, Hayashi K, Suzuki T, Ichihara S, Yamada T, et al. Human placenta feeder layers support undifferentiated growth of primate embryonic stem cells. Stem Cells; 2004; 22: 433-440.

36. Kim SJ, Chang Hee Song, Hwa Jung Sung, Young Do Yoo, Dong Ho Geum, et al. Human placenta-derived feeders support prolonged undifferentiated propagation of a human embryonic stem cell line, SNUhES3: comparison with human bone marrow-derived feeders. Stem Cells and Development. 2007; 16: 421-428.

37. Chen HF, Chuang CY, Shieh YK, Chang HW, Ho HN, et al. Novel autogenic feeders derived from human embryonic stem cells (hESCs) support an undifferentiated status of hESCs in xeno-free culture conditions. Hum Reprod. 2009; 24: 1114-1125.

38. Fu X, Toh WS, Liu H, Lu K, Li M, Hande MP, et al. Autologous feeder cells from embryoid body outgrowth support the long-term growth of human embryonic stem cells more effectively than those from direct differentiation. Tissue Eng Part C Methods. 2010; 16: 719-733.

39. Tan KY, Teo KL, Lim JF, Chen AKL, Reuveny S, et al. Serum-free media formulations are cell line-specific and requireoptimization for microcarrier culture. Cytotherapy. 2015; 17: 1152-1165.

40. Unger C, Skottman H, Blomberg P, Dilber MS, Hovatta O. Good manufacturing practice and clinicalgrade human embryonic stem cell lines. Human Molecular Genetics. 2008; 17: R48-R53.

41. Bibikova M, Laurent LC, Ren B, Loring JF, Fan JB. Unraveling epigenetic regulation in embryonic stem cells. Cell Stem Cell. 2008; 2: 123-134.

42. Chambers I, Tomlinson SR. The transcriptional foundation of pluripotency. Development. 2009; 136: 2311-2322.

43. Pera MF, Tam PP. Extrinsic regulation of pluripotent stem cells. Nature. 2010; 465: 713-720.

44. Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005; 122: 947-956.

45. Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, et al. Transcriptional regulation of nanog by OCT4 and SOX2. Journal of Biological Chemistry. 2005; 280: 24731-24737.

46. Chen G, Daniel R Gulbranson, Zhonggang Hou, Jennifer M Bolin, Victor Ruotti, et al. Chemically defined conditions for human iPSC derivation and culture. Nature Methods. 2011; 8: 424-429.

47. Sato N, Laurent Meijer, Leandros Skaltsounis, Paul Greengard, Ali H Brivanlou. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nature Medicine. 2004; 10: 55-63.

48. Fernandez A, Ian J Hugginsa, Luca Pernaa, David Brafmana, Desheng Lu, et al. The WNT receptor FZD7 is required for maintenance of the pluripotent state in human embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2014; 111: 1409-1414.

49. Boiani M, Scholer HR. Regulatory networks in embryo-derived pluripotent stem cells. Nature Reviews Molecular Cell Biology. 2005; 6: 872-884.

50. Dravid G, Ye Z, Hammond H, Chen G, Pyle A, et al. Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. Stem Cells. 2005; 23: 1489-1501.

51. Watanabe K, Ye Z, Hammond H, Chen G, Pyle A, et al. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat. Biotechnol. 2007; 25, 681-686.

52. Meng G, Liu S, Rancourt DE. Synergistic effect of medium, matrix, and exogenous factors on the adhesion and growth of human pluripotent stem cells under defined, xeno-free conditions. Stem Cells Dev. 2012; 21: 2036e48.

53. Tsutsui H, Valamehr B, Hindoyan A, Qiao R, Ding X, et al. An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells. Nat Commun. 2011; 2: 167.

54. Chen G, Hou Z, Gulbranson DR, Thomson JA. Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. Cell Stem Cell. 2010; 7: 240-248.

55. Wang L, Schulz TC, Sherrer ES, Dauphin DS, Shin S, et al. Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. Blood. 2007; 110: 4111-4119.

56. Bendall SC, Morag H Stewart, Pablo Menendez, Dustin George, Kausalia Vijayaragavan, et al. IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells *in vitro*. Nature. 2007; 448; 1015-1021.

57. Rhee YH, Choi M, Lee HS, Park CH, Kim SM, et al. Insulin concentration is critical in culturing human neural stem cells and neurons. Cell death & disease. 2013; 4: e766.

58. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol. 2000; 227: 271-278.

59. Klimanskaya I, Chung Y, Meisner L, Johnson J, West MD, et al. Human embryonic stem cells derived without feeder cells. Lancet. 2005; 365: 1636-1641.

60. Vallier L, Alexander M, Pedersen, RA. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. Journal of Cell Science. 2005; 118: 4495-509.

61. Xu RH, Peck RM, Li DS, Feng X, Ludwig T, et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods. 2005; 2: 185-190.

62. Xi J, Wang Y, Zhang P, He L, Nan X, et al. Human fetal liver stromal cells that over express bFGF support growth and maintenance of human embryonic stem cells. PLoS One. 2010; 5: e14457.

63. Park Y, Kim JH, Lee SJ, Choi IY, Park SJ, et al. Human feeder cells can support the undifferentiated growth of human and mouse embryonic stem cells using their own basic fibroblast growth factors. Stem Cells Dev. 2011; 20: 1901-1910.

64. Liu CX, Zhang RL, Gao J, Li T, Ren Z, et al. Derivation of human embryonic stem cell lines without any exogenous growth factors. Mol Reprod Dev. 2014.

65. Xu C, Rosler E, Jiang J, Lebkowski JS, Gold JD, et al. Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells. 2005; 23: 315-323.

66. Levenstein ME, Ludwig TE, Xu RH, Llanas RA, VanDenHeuvel-Kramer K, et al. Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem Cells. 2006; 24: 568-574.

67. Frank S, Zhang M, Scholer HR, Greber B. Small molecule-assisted, line-independent maintenance of human pluripotent stem cells in defined conditions. PLoS One. 2012; 7: e41958.

68. Desbordes SC, et al. High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. Cell Stem Cell. 2008; 2: 602-612.

69. Kumagai H, Placantonakis DG, Ciro A, Socci ND, Lee G, et al. Identification of small molecules that promote human embryonic stem cell self-renewal. Biochemical and Biophysical Research Communications. 2013; 434: 710-716.

70. Hasegawa K, Yasuda SY, Teo JL, Nguyen C, McMillan M, et al. Wnt signaling orchestration with a small molecule DYRK inhibitor provides long-term xeno-free human pluripotent cell expansion. Stem Cells Transl Med. 2012; 1: 18-28.

71. Cai L, Ye Z, Zhou BY, Mali P, Zhou C, et al. Promoting human embryonic stem cell renewal or differentiation by modulating Wnt signal and culture conditions. Cell Res. 2007; 17: 62-72.

72. Hasegawa K, Pomeroy JE, Pera MF. Current technology for the derivation of pluripotent stem cell lines from human embryos. Cell Stem Cell. 2010; 6: 521-531.

73. Miki T, Yasuda SY, Kahn M. Wnt/beta-catenin signaling in embryonic stem cell self-renewal and somatic cell reprogramming. Stem Cell Rev. 2011; 7: 836-846.

74. Beattie GM, Lopez AD, Bucay N, Hinton A, Firpo MT, et al. Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. Stem Cells. 2005; 23: 489-495.

75. James D, Levine AJ, Besser D, Hemmati-Brivanlou A. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development. 2005; 132: 1273-1282.

76. Chen S, Choo A, Chin A, Oh SK. TGF-beta2 allows pluripotent human embryonic stem cell proliferation on E6/E7 immortalized mouse embryonic fibroblasts. J Biotechnol. 2006; 122: 341-361.

77. Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH, Gelbart WM. "Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster". Genetics. 1995; 139: 1347-1358.

78. Savage C, Das P, Finelli AL, Townsend SR, Sun CY, et al. "Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components". Proc. Natl. Acad. Sci. USA. 1996; 93: 790-794.

79. Assoian RK, Frolik CA, Roberts AB, Miller DM, Sporn MB. Transforming growth factor-beta controls receptor levels for epidermal growth factor in NRK fibroblasts. Cell. 1984; 36: 35-41.

80. Roberts AB, Frolik CA, Roberts AB, Miller DM, Sporn MB, et al. Type beta transforming growth factor: a bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci. USA. 1985; 82: 119-123.

81. Lin T, Rajesh Ambasudhan, Xu Yuan, Wenlin Li, Simon Hilcove, et al. A chemical platform for improved induction of human iPSCs. Nat. Methods. 2009; 6: 805-808.

82. Galey JB. Potential use of iron chelators against oxidative damage. Advances in pharmacology. 1996; 38, 167-203.

83. Chung TL, Brena RM, Kolle G, Grimmond SM, Berman BP, et al. Vitamin C promotes widespread yet specific DNA demethylation of the epigenome in human embryonic stem cells. Stem Cells. 2010; 28: 1848-1855.

84. Gottipamula S, Muttigi MS, Kolkundkar U, Seetharam RN. Serum-free media for the production of human mesenchymal stromal cells: a review. Cell Prolif. 2013; 46: 608e27.

85. Garcia-Gonzalo FR, Izpisua Belmonte JC. Albumin associated lipids regulate human embryonic stem cell self-renewal. PLoS ONE. 2008; 3: e1384.

86. Ludwig TE, Mark E Levenstein, Jeffrey M Jones, W Travis Berggren, Erika R Mitchen, et al. Derivation of human embryonic stem cells in defined conditions. Nature Biotechnology. 2006a; 24: 185-187.

87. Ludwig TE, Veit Bergendahl, Mark E Levenstein, Junying Yu, Mitchell D Probasco, et al. Feeder-independent culture of humanembryonic stem cells. Nature Methods. 2006b; 3: 637-646.

88. Brewer GJ, Torricelli, JR, Evege, EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented neurobasal™, a new serum-free medium combination. Journal of neuroscience research. 1993; 35: 567-576.

89. Bottenstein J. Growth and differentiation of neural cells in defined media. In Cell Cultures in the Neurosciences. J. Bottenstein and G. Sato, editors. Plenum Press, New York. 1985; 3-43.

90. Jung S, Panchalingam KM, Rosenberg L, Behie LA. *Ex vivo* expansion of human mesenchymal stem cells in defined serum-free media. Stem Cells Int. 2012; 2012: 123030.

91. Rajaraman G, White J, Tan KS, Ulrich D, Rosamilia A, et al. Optimization and scale-up culture of human endometrial multipotent mesenchymal stromal cells: potential for clinical application. Tissue Eng Part C Methods 2013; 19: 80-92.

92. Crapnell K, Blaesius R, Hastings A, Lennon DP, Caplan AI, et al. Growth, differentiation capacity, and function of mesenchymal stem cells expanded in serum-free medium developed via combinatorial screening. Exp Cell Res. 2013; 319: 1409-1418.

93. Gottipamula S, Muttigi MS, Chaansa S, Ashwin KM, Priya N, et al. Large-scale expansion of pre-isolated bone marrow mesenchymal stromal cells in serum-free conditions. J Tissue Eng Regen Med. 2016; 10: 108-119.

94. Jung S, Sen A, Rosenberg L, Behie LA. Identification of growth and attachment factors for the serum-free isolation and expansion of human mesenchymal stromal cells. Cytotherapy. 2010; 12: 637-657.

95. Tarle SA, Shi S, Kaigler D. Development of a serum-free system to expand dental-derived stem cells: PDLSCs and SHEDs. J Cell Physiol. 2011; 226: 66-73.

96. Bray LJ, Heazlewood CF, Atkinson K, Hutmacher DW, Harkin DG. Evaluation of methods for cultivating limbal mesenchymal stromal cells. Cytotherapy. 2012; 14: 936-947.

97. Apel A, Groth A, Schlesinger S, Bruns H, Schemmer P, et al. Suitability of human mesenchymal stem cells for gene therapy depends on the expansion medium. Exp Cell Res. 2009; 315: 498e507.

98. Villa-Diaz LG, Ross AM, Lahann J, Krebsbach PH. Concise review: the evolution of human pluripotent stem cell culture: from feeder cells to synthetic coatings. Stem cells. 2013; 31: 1-7.