Classification: Biological Sciences: Cell biology

Title: Combined hydrogels that switch Human Pluripotent Stem cells from self-renewal to differentiation

Authors: James E. Dixon[a], Disheet A. Shah[a], Catherine Rogers[a], Stephen Hall[b], Nicola Weston[c], Donal McNally[d], Chris Denning[e] and Kevin M. Shakesheff[a1]

Author affiliation: Wolfson Centre for Stem Cells, Tissue Engineering, and Modelling (STEM), Centre of Biomolecular Sciences, [a]School of Pharmacy, [b]Medical Engineering Unit, [c]School of Mechanical, Materials and Manufacturing Engineering, [d]Faculty of Engineering, [e]School of Medicine; University of Nottingham, Nottingham, NG7 2RD, UK. [1]To whom correspondence may be addressed. Email: kevin.shakesheff@nottingham.ac.uk

Corresponding author:

Kevin M. Shaksheff

Wolfson Centre for Stem Cells, Tissue Engineering, and Modelling (STEM)

Centre for Biomolecular Sciences

University of Nottingham

University Park, Nottingham

NG7 2RD, UK

Tel: +44 (0) 115 82 32003

Fax: +44 (0) 115 84 68002

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Short Running Title: Human Pluripotent Stem cell Combined Hydrogels.

Abstract

The ability of materials to define the architecture and micro-environment experienced by cells provides new opportunities to direct the fate of human pluripotent stem cells (HPSCs) (Robinton DA, et al (2012) Nature 481:295-305). However, the conditions required for self-renewal verses differentiation of HPSCs are different and a single system that efficiently achieves both outcomes is not available (Giobbe GG, et al. (2012) Biotech Bioeng 109:3119-3132). We have addressed this dual need by developing a hydrogel-based material that uses ionic decrosslinking to remove a self-renewal permissive hydrogel (alginate) and switch to a differentiation-permissive micro-environment (collagen). Adjusting the timing of this switch can preferentially steer the HPSC differentiation to mimic lineage commitment during gastrulation to ectoderm (early switch) or mesoderm/endoderm (late switch). As an exemplar differentiated cell type, we showed that directing early-lineage specification using this single system can promote cardiogenesis with increased gene expression in high-density cell populations. This work will facilitate regenerative medicine by allowing *in situ* HPSC expansion to be coupled with early lineage-specification within defined tissue geometries.

Significance Statement

Stem cell micro-environment has been identified as an important modulator of plasticity, self-renewal and differentiation. This paper details the development of a hydrogel system tailored to promote human pluripotent stem cell (HPSC) self-renewal with a simple chemical micro-environmental switch to direct differentiation. Furthermore the timing of switching post-hydrogel fabrication can promote specific lineage differentiation as *in vivo*. This system highlights the role of micro-environment on fate choices of pluripotent cells and demonstrates it may be tailored to control differentiation *in vitro*. Importantly this approach may improve the generation of fully differentiated tissues, as demonstrated for cardiogenic differentiation. Our combination of hydrogels allows dense tissue structures to be produced from HPSCs using a single step process inaccessible to any current methodology.

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HPSCs comprise human embryonic stem cells (HESCs) and human induced pluripotent stem cells (HiPSCs) (1). The ability to couple expansion and differentiation of these cells underpins current efforts in regenerative medicine (2, 3). Initial efforts to direct the fate of HPSCs by recapitulating the developmental process of gastrulation, (employing spontaneous differentiation of embryoid bodies, termed EBs) have been refined to allow directed differentiation in two- and three-dimensions (3D) (4). This includes coupling bioreactor expansion of HPSCs in 3D aggregates with differentiation to neural lineages (5). The differentiated cells from these processes can be harvested and then used to seed geometrically complex scaffolds. However, this two stage process could be better controlled and streamlined by *in situ* HPSC expansion and differentiation within a single template. Furthermore *in situ* tissue development more closely recapitulates embryogenesis (6) and could produce tissue with authentic cellular complexity and physiology (7).

To date natural (8, 9) and synthetic (10) materials have been developed to retain the self-renewal phenotype of HPSCs. We (11) and others (12) have shown hydrogel systems can instruct cell behaviour by providing cell-adhesive or non-adhesive micro-environments. Extracellular matrix (ECM) hydrogels, such as collagen, have fibrous microstructures (13) and are suitable for cell adhesion, growth and migration (14). This is unlike hydrogels such as alginate, which are non-adhesive, nano-porous and prevent migration. Collagen (type-I) is crosslinked by neutralising acidity and leads to fibril formation, whereas alginate gels are formed or disaggregated by regulating divalent cation availability; usually Ca^{2+} in the form of $CaCl_2$ (15).

Importantly HPSC self-renewal and triggering of gastrulation-like differentiation requires different culture and micro-environmental conditions (2). Therefore the development of hydrogel systems that allow the modification of the structural and adhesive micro-environment after the initial crosslinking would be ideal to control cell behaviour (16-18). A previous study explored this concept and used alginate switching from cross-linked to uncross-linked states to demonstrate non-adhesive-to-adhesive tailoring of the micro-environment in the presence of somatic cell lines. This switch affected attributes such as rate of solute transport, gel mechanics, cell adhesion, morphology and migration (12). Here we describe the development of a system that can direct HPSC fate from self-renewal to differentiation using alginate crosslinked/decrosslinked-state as a micro-environmental switch (Fig. 1*A*).

Results

Creation of a switching hydrogel system.

We optimised gelation of our hydrogel system to aid fabrication into complex geometries. Using CaCO$_3$ and D-glucono-δ-lactone (19) we created a delayed setting formulation for alginate. CaCO$_3$ and GDL addition allows alginate-containing suspensions, with or without cells, to be moulded into complex geometric shapes before gelation. Using these approaches, we produced free-standing, uniform and transparent crosslinked alginate gels within 5-10 minutes and complete crosslinking within 30 minutes at 37 °C (1.2 % alginate w/v crosslinked with 34 mM CaCO$_3$ and 42 mM GDL). We confirmed complete and uniform crosslinking of alginate by dry/wet and rehydrated/wet weight comparisons of intact gels (Table. S1) or gel slices (Fig. S1).

By combining collagen into this slow setting alginate gel, we were able to construct combined matrices (alginate/collagen), which had the same gelation properties as alginate-only gels (Table. S1, Fig. S1). We demonstrated that these combined hydrogels could be decrosslinked and the alginate component efficiently removed, a process we term 'switching'. Switching converts the environment from alginate-dominated to collagen-dominated (Fig. 1*A*) and relies on Ca^{2+} ion chelation using EDTA/sodium citrate-based treatment (12, 20). The material properties of this hydrogel system also allowed application in bioprinting technologies which retained geometry after switching (Fig. 1*B*).

We determined that the switching process resulted in >85% removal of alginate but >80% retention of collagen in combined hydrogels (Fig. 1*C*). We also observed (Fig.1*D*) (22) that collagen fibril formation only occurred after alginate removal was complete even when several days post-fabrication (Fig. S2). Efficient removal of alginate was confirmed by 488nm confocal microscopy (Fig. 1*E*) and environmental SEM (E-SEM) (Fig. S3). Alginate removal occurred with <10 minutes of chelation corroborated by assessing the dry/wet and rehydrated/wet weights, which showed hydration characteristics changed during switching (Fig. S1).

Furthermore the combination hydrogel mechanical properties changed from ~21.37 ± 5.37 kPa (alginate-only being ~19.37 ± 6.98 kPa) to ~4.87 ± 1.64 kPa (collagen-only being ~6.28 ± 2.83 kPa) representing a switch from alginate- to collagen-dominated character. Using ultrasound we were also able to demonstrate a density and bulk mechanical change after switching relative to acoustic impedance of the material (Fig. S4). Overall these data demonstrates that alginate crosslinking prevented the majority of collagen fibril formation. Switching does not lead to an extensive loss of collagen, triggers fibrillogenesis and changes the bulk mechanical properties of the hydrogel.

To test the compatibility of the switching process with HPSC culture we treated HPSCs maintained on tissue-culture plastic with the chemical regime used to switch the hydrogels (Fig. S5). We thought this to be a valid test as monolayers of HPSCs are considered to be exquisitely sensitive to changes in culture conditions (21). Treatments required for switching were compatible with survival, proliferation and alkaline phosphatase (AP) activity of HPSCs (Fig. S5).

We tested how HUES7 HESCs responded when seeded within composite hydrogels formulated as disc shapes (Fig. 2) or injection moulded to 3D structures, such as tubes (Fig. S6). Disc constructs were thin enough (~25 µm) for adequate mass-transport of nutrients/metabolic by-products (11) and were loaded with high cell densities (up to 5×10^6 HUES7 cells/ml) in MEF-conditioned medium (termed CM) (23). After 21 days HPSCs were uniformly distributed and sustained metabolic and AP activity. Similar levels of metabolic and AP activity were seen in alginate-only hydrogels but were considerably reduced in collagen-only hydrogels (Fig. S7*A*). Combination hydrogels increased in size (diameter of 3.21 ± 0.34 verses 4.38 ± 0.57 mm on day 0 and 14, respectively) as HPSCs proliferated.

Optimisation of switching hydrogels for HPSC self-renewal

As poly(vinyl alcohol) (PVA) and the extracellular matrix Matrigel^{TM} have been shown to have positive effects on maintenance of HESCs in monolayers (4, 25), we tested whether these substances could further facilitate HPSC pluripotency in the combined hydrogels (Fig. S7*B*).

Firstly we determined that addition of PVA and Matrigel^{TM} did not prevent the alginate-mediated inhibition of collagen fibrillogenesis in combined hydrogels (Fig S2*A*). Inclusion of PVA at 1

mg/ml and Matrigel™ at 25% v/v enhanced expression of stem cell markers (*OCT4*, *NANOG* and alkaline phosphatase) in the absence of differentiation marker expression (*T* for mesoderm and *SOX17* for endoderm) (Fig. S7*B* and 2*A*) and therefore enhanced the self-renewal phenotype of HPSCs. Therefore Matrigel™ and PVA were included in subsequent hydrogel formulations. Microscopy of cells within optimised composite hydrogels showed a rounded morphology with an average cell diameter of 10 ± 0.32 µm consistent with published sizes for undifferentiated cells (24) (Fig. S7*C*). Cell proliferated as isolated aggregates until they impinged on their neighbours and the aggregates merged (Fig. S7*C* and S8).

We next assessed the effect on cell behaviour, self-renewal and differentiation of conversion to the collagen-rich switched-form. We seeded cells on top of gels to more clearly observe differences in cell-matrix interaction (Fig. S9). HPSCs on alginate-only or unswitched composite gels were rounded, had lower perimeter length, cell body area and were loosely attached (Fig. S9*A*), while cells on collagen or switched composite gels were adherent (Fig. S9*B*).

Gene expression by QPCR of HPSCs cultured in CM for 21 days within switched combined hydrogels showed significant down-regulation of pluripotency markers (*p*<0.05 for both *OCT4* and *NANOG*). Decreases in expression were observed one day post-switching with expression completely lost by day 6 for *OCT4* and day 12 for *NANOG* (Fig. 3*A*). Conversely, differentiation markers (*T* for mesoderm and *SOX17* for endoderm) were up-regulated directly after switching (Fig. 2*A*). Therefore the micro-environment within switched combined gels has a dominant effect and 'primes' differentiation of HPSCs even in pluripotency-maintaining CM conditions.

As expected, substitution of CM for differentiation-inducing medium (termed DIFF medium; Fig. 2*B*) accelerated down-regulation of *OCT4* and *NANOG* in switched hydrogels (Fig. S10).

Furthermore, DIFF media induced differentiation without switching demonstrating unswitched combination gels cannot override extrinsic influences from the culture medium. The switching of combined hydrogel micro-environment had a more profound effect on HPSC fate than changing media conditions alone. Furthermore if these parameters were changed together there was a combined synergistic effect to more efficiently induce HPSC differentiation (Fig. 2*C* & *D*).

The effect of switching on early HPSC lineage specification.

As the switching of combined hydrogels from alginate- to collagen-dominated character had profound influence on HPSC self-renewal, we assessed if the timing of switching between states could direct early lineage-specification during differentiation (Fig. 3). Previously it has been suggested that the micro-environmental history of HPSCs may skew the induction of specific lineages as for embryonic gastrulation (26). Here, when switching hydrogels, we simultaneously swapped CM culture media to DIFF media to promote the priming of differentiation by switching (Fig. 3*C* & *D*). We determined that *NANOG* and *OCT4* are rapidly down-regulated upon switching at any time post-gelation ($p<0.01$ seven days post-switching) (n=6) (Fig. 3*A*). We confirmed that switch timing can direct the efficient induction of the specific germ-layers (mesoderm, endoderm, and ectoderm) as assessed with QPCR (Fig. 3*B*). We demonstrated that early switching generates more ectodermal-differentiation (peaking with the switch on day 3 with *SOX1* and *OTX2* expression) (~81 and ~642 -fold increase over monolayer cultures, respectively; $p<0.05$), whereas mesodermal- (*T* and *HAND1* expression; $p<0.01$) and endodermal-commitment (*SOX17* and *GATA4* expression; $p<0.005$) was highest with a day 5 switch (~92/~893- and ~4750/~6454 -fold increase over monolayer cultures, respectively) (n=6)

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(Fig. 3*B*). Therefore we show that early HPSC lineage commitment can be skewed by the time of switching micro-environmental states in combined hydrogels. This indicates that with further optimisation this approach may allow precise fine-tuning HPSC germ-layer differentiation.

Employing switching hydrogels to create terminally differentiated tissues from HPSCs

We wanted to determine if switching of germ-layer specification could improve the generation of terminally differentiated tissues *in situ* which is the ultimate goal for any regenerative medicine application (Fig. 4*A*). Firstly we used a transcription-factor driven method by directly programming gene-regulatory networks (23). We transduced HUES7 HESCs with *GATA4/TBX5*-lentiviruses, loaded cells into combined hydrogels and varied the time of switching (Fig. 4*B*). This approach induced mesoderm (*T;* $p<0.01$), cardiac mesoderm (*NXK2.5*; $p<0.05$) and a terminal cardiac marker (*MYH6*; $p<0.001$). While cardiac differentiation was apparent in unswitched hydrogels, switching promoted cardiogenesis (~48,000-fold relative to non-programmed monolayer cultures). Day of switching also influenced their developmental position within the lineage, with cells towards a cardiac progenitor (day 9 switch) or specified cardiomyocyte identity (day 5 switch) (Fig. 4*B*).

To test whether cardiomyocyte differentiation could be induced within switched combined hydrogels by non-transgenic methods, we used a protocol (27) that relies of sequential addition of growth factors (BMP4 and FGF2; Fig. S11). This induced significant up-regulation of cardiac markers *NKX2.5* and *MYH6* relative to switched hydrogels seeded with HPSCs cultured in CM or with HPSCs following the transcription-factor driven protocol ($p<0.05$) (Fig. 4*B* and *C*). Growth factor induction of cardiogenesis in the switched hydrogels also enhanced expression of

cardiac markers relative to a conventional EB differentiation protocol ($p<0.01$). The optimal time to generate mature cardiomyocyte gene expression (*MYH6*) was achieved by day 7 switching (~150,000 -fold over non-programmed monolayer cultures) and enhanced cardiac mesoderm gene expression was achieved by day 5 switching (~12,000 -fold over non-programmed monolayer cultures) ($p<0.05$) (n=6). By comparison, in unswitched combined hydrogels expression of cardiac markers was ~12-fold less efficient for transcription-factor mediated differentiation and ~8-fold less for growth-factor mediated differentiation ($p<0.05$) (n=6) (Fig. 4*B* and *C*). These experiments demonstrate that extrinsic programming of HPSCs along with control of micro-environmental to direct specification can more efficiently produce terminally differentiated cell types.

Discussion

HPSCs represent an attractive approach to generate any genetically matched tissue type (2). However with unlimited potential, HPSCs are the furthest developmentally from differentiated tissues. Therefore efficient and faithful control of expansion and differentiation must be achieved (21, 28). Previous work has used pre-fabricated chitosan/alginate scaffolds to maintain HPSC self-renewal (8). This approach requires chemical modification, heating and lyophilization to create chemically bonded chitosan-alginate and produce porous sponges.

During all stages of embryogenesis but especially at gastrulation/lineage commitment (between day 14-16 post-ovulation) changes in 3D micro-environments affect cell migration, growth, apoptosis and identity (6). HESCs are derived from pre-implantation embryos (29) but resemble the pluripotent cells of an older pre-gastrulation epiblast-stage embryo (30). Therefore, we

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hypothesised that by manipulating HPSC culture micro-environment, it may be possible to efficiently direct cell fate towards the cell-type of choice (3, 31, 32). We combined existing technologies using a mixture of two natural hydrogels with divergent influence on cells (33). By tailoring these gels for initial self-renewal of HPSCs we were able to achieve high cell densities ($\sim 2\times10^7$/ml; Fig. S7*B*) before switching the properties of the hydrogel to promote early lineage-specification. The switching process has no direct negative effect on self-renewal of HPSCs and can be completed within 40 minutes with non-toxic chemicals (Fig. S5). In contrast to other studies (12), our system also has the advantage that construct geometry was retained after removal of alginate (Fig.1*B*). We performed an extensive study of proliferation and differentiation using germ-layer specification and cardiomyogenesis as an exemplar. By using previously published methods to direct differentiation either by growth-factor regimes (27) or by transcription-factor regulation (23), we demonstrated efficient cardiac differentiation depending on the timing of the switch thereby showing that micro-environmental control significantly influences the cell fate outcomes.

Our material may be optimised with matrices that stimulate specific cell outcomes (35). For example, further work could directly replace collagen with decellularized ECM (dECM) gels to more closely recapitulate the micro-environment of the target tissue (e.g. heart dECM for cardiac differentiation) (36). Mechanistically, it is likely that cell adhesion, degradation of the hydrogel and elasticity all influence the switching in cell behaviour.

This study has demonstrated that the self-renewal and differentiation of HPSCs can be controlled *in situ* within a single combination scaffold system. Furthermore our work demonstrates that combined hydrogels when switched yields *in situ* tissue development which more closely recapitulates gene expression observed during embryogenesis (6, 7) and the process of lineage

commitment in gastrulation. Tailoring micro-environmental changes as well as growth factor- and small molecule-directed manipulation of cells will be an important parameter when devising methods to produce human engineered tissues for regenerative medicine applications.

Materials and Methods. Detailed information is provided in *SI Materials and Methods*. Combined hydrogels (1.2% w/v alginate/2mg/ml collagen type-I) were crosslinked by $CaCO_3$ and GDL (34 mM and 42 mM, respectively). Switching (removal of alginate) was achieved by firstly stabilizing gels with L-lysine and chelating Ca^{2+} with sodium citrate and EDTA (200mM and 30mM, respectively). HUES7 cells were grown on Matrigel^{TM} in feeder-conditioned media (CM) before fabrication of hydrogels. Hydrogels were grown in CM or in DIFF media (DIFF; DMEM media containing fetal calf serum previously described (25)). Viability assays used AlamarBlue^{TM} and Live/Dead assays. Microscopy of DiI cell staining used macroconfocal analyses to assess HPSC growth in hydrogels. Cell proliferation and collagen fibrillogenesis was assessed by scanning and environmental scanning electron microscopy (SEM and ESEM). Mechanical and gel property analyses employed protein and 1,9-dimethyl methylene blue (DMMB) assays for collagen and alginate, respectively. Wet/dry weight assessments and ultrasound of hydrogels were used to assess switching efficiency and gel mechanics, respectively. Assessment of pluripotency was achieved by alkaline phosphatase (AP) staining/assays and quantitative real-time PCR (QPCR). HUESs were transduced with *GATA4/TBX5* lentiviruses to direct cardiogenesis as previously described (23). Growth-factor cardiac differentiation was as previously described using FGF2 and BMP4 (27).

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Competing Financial Interests. The authors declare no competing financial interests.
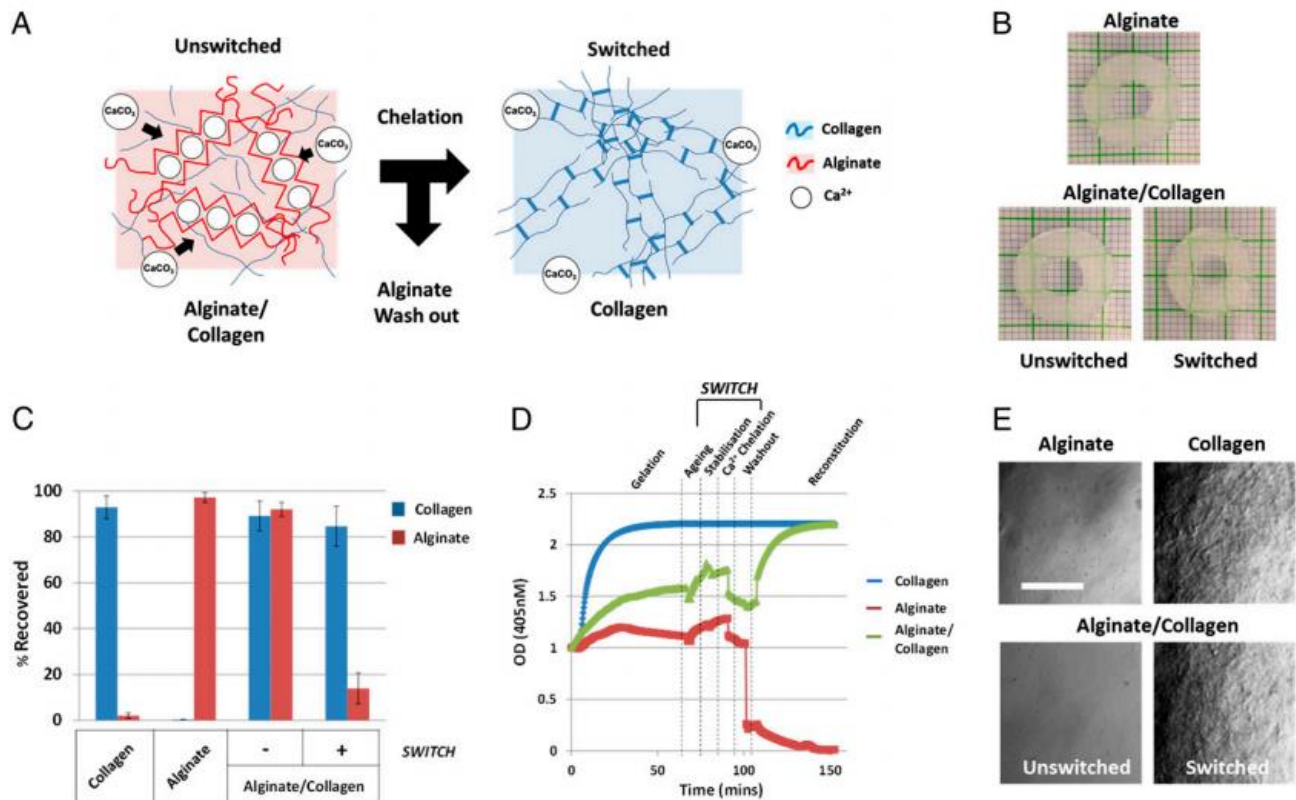
Fig. 1. *(A)* Alginate serves as structural modulator to prevent the adhesive and fibrous network created by collagen. Upon switching (chelation of Ca^{2+} ions) alginate is removed and collagen fibres are generated forming an adhesive microenvironment. *(B)* 3D printed gels retain geometry when switched by chelation (grid; 1mm). *(C)* Quantitation of alginate and collagen before and after switching (n=3). *(D)* Spectroscopy measuring collagen fibre character during cross-linking and switching of hydrogels. Alginate prevents complete collagen network formation until chelation and wash-out. (n=6) *(E)* Confocal images of collagen fibres with transmission imaging. Collagen fibre formation is inhibited until alginate is removed (Bar is 20µm).
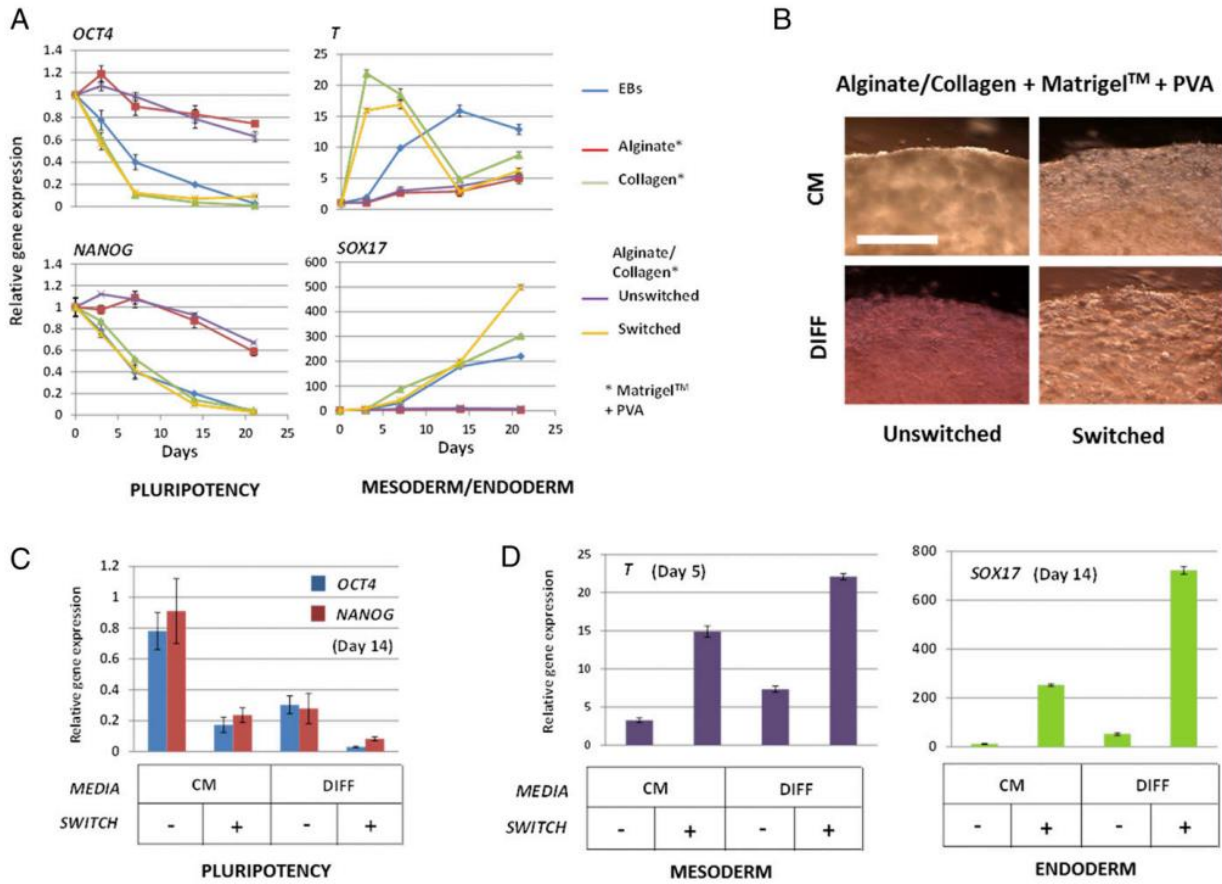
Fig. 2. Switching HPSC self-renewal to differentiation. *(A)* Quantitative gene expression of pluripotency markers *OCT4* and *NANOG* in HUES7 HESCs cultured in hydrogels (containing Matrigel^TM and PVA) compared to those grown as conventional monolayers or as embryoid bodies (EBs, (23)). Relative expression levels for constructs over a 21 day period cultured in HPSC maintenance media (Conditioned media; CM). (Bars are S.E.) (n=6). *(B)* Light microscopy of HUES7 HESCs cultured in composite hydrogels (containing Matrigel^TM and PVA) with or without switching at day 0 (post-crosslinking of hydrogel) cultured in CM or Differentiation media (DIFF, (23)). HUES7 cells within unswitched hydrogels cultured in CM retained HESC morphology and proliferated to fill the gel volume which was inhibited if cultured in DIFF media. HUES7 cells in switched hydrogels, especially those cultured in DIFF rapidly lose HESC morphology and proliferation. Images were taken at 14 days post-

crosslinking (Bar is 50µm). *(C-D)* Quantitative gene expression analyses for the culture conditions described in *(B)*. *(C) OCT4* and *NANOG* (at day 14) and *(D) T* (at day 5) and *SOX17* (at day 14) gene expression is shown. (Bars are S.E.) (n=6).
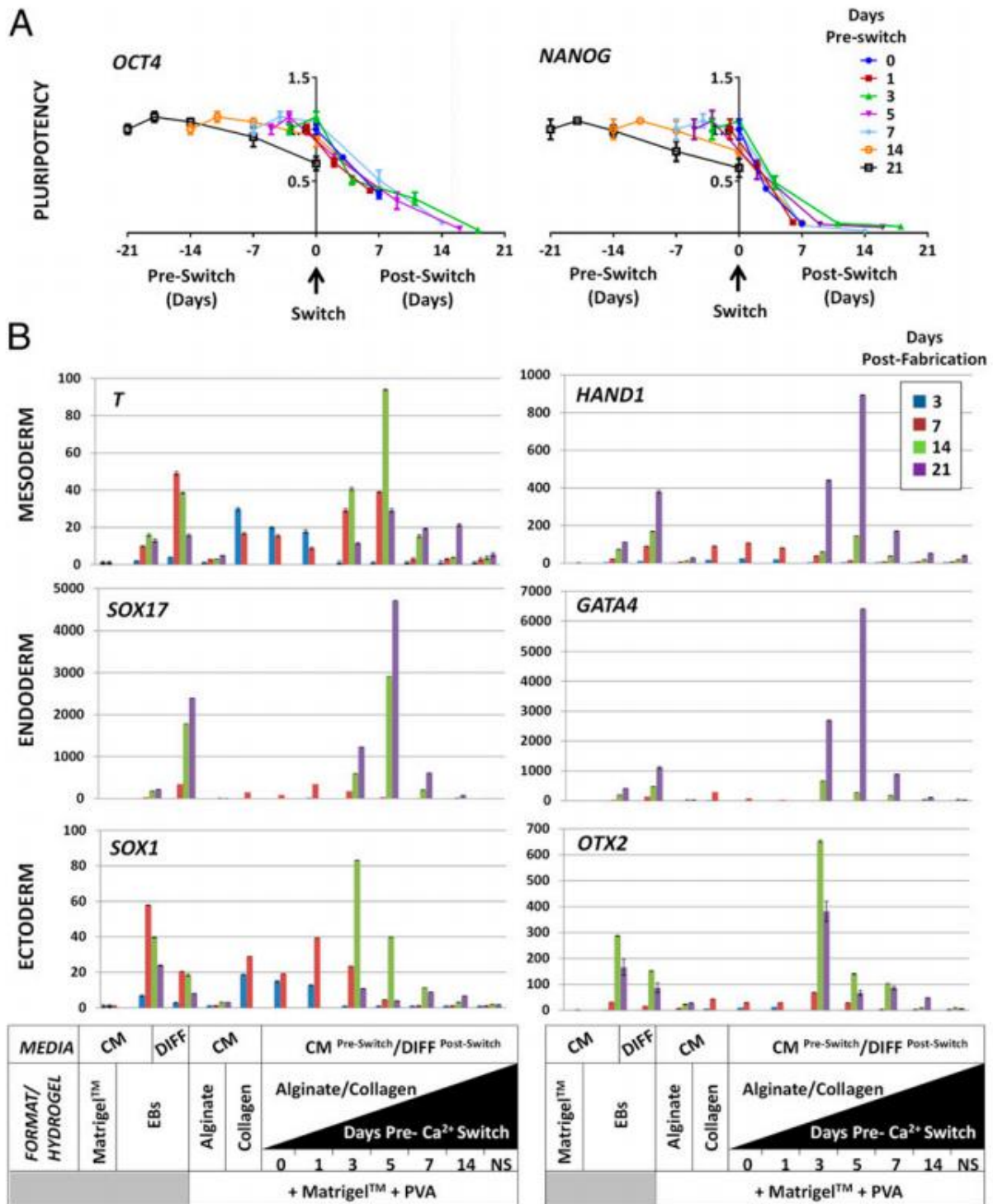
Fig. 3. Switch timing influences HPSC fate. *(A)* Quantitative gene expression of pluripotency markers *OCT4* and *NANOG* in HUES7 HESCs cultured with a variable switching time.

Switching was undertaken on day 0 post cross linking or at either day 1, 3, 5, 7, 14 or not switched within the 21 day culture period. HUES7 HESCs cultured in CM with longer time before switching retained pluripotent gene expression at higher levels. Early switching caused rapid down-regulation both *OCT4* and *NANOG* (Bars are S.E.) (n=6). *(B)* Quantitative gene expression characterisation of mesodermal- (*T* and *HAND1*), endodermal- (*SOX17* and *GATA4*) and ectodermal-(*SOX1* and *OTX2*) differentiation with variable switching time. The timing of the switch appears to direct the differentiation process with very early switching generating more ectodermal- and later switching generating more endodermal- gene expression. (Bars are S.E.) (n=6).
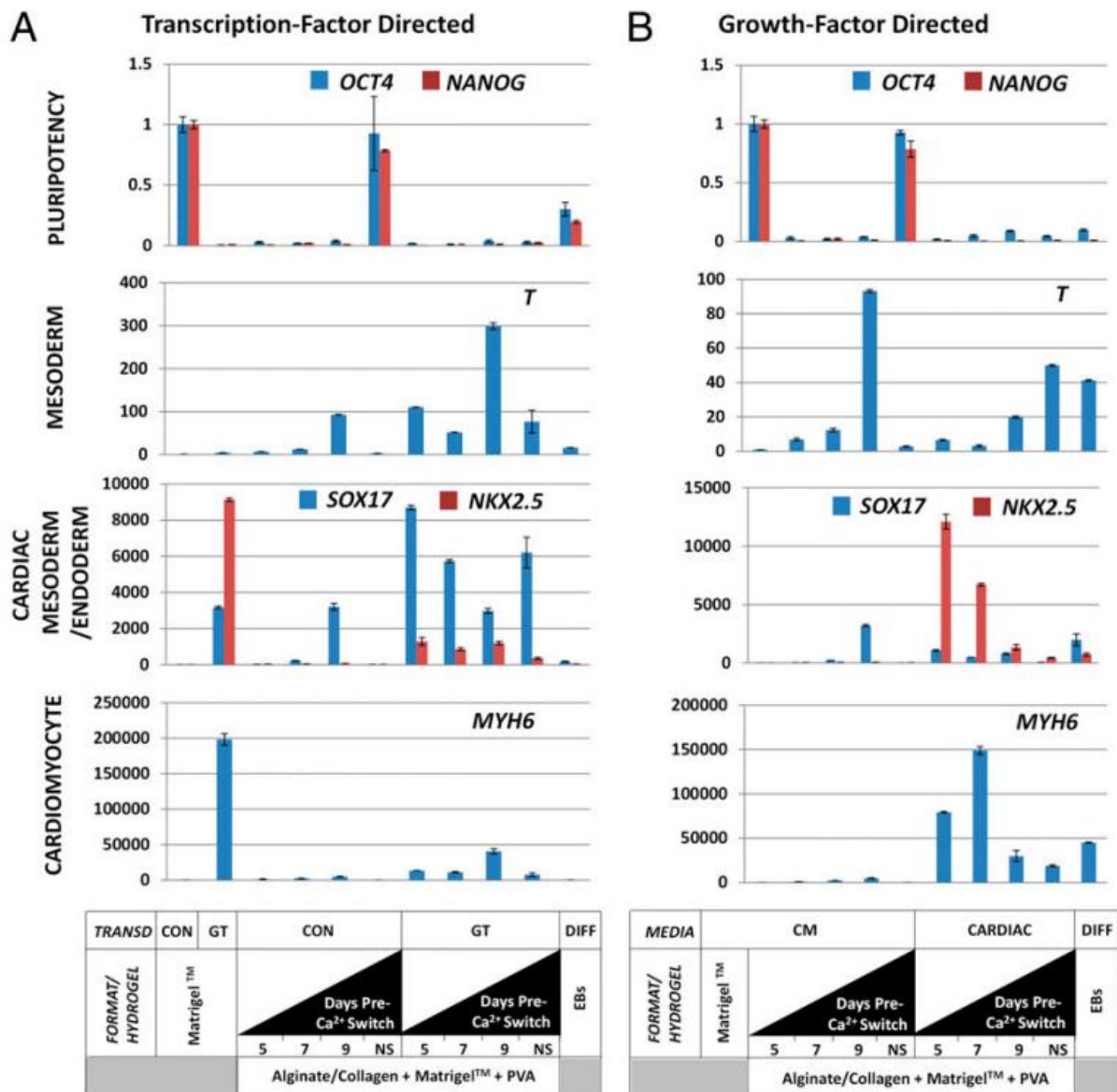
Fig. 4. Promoting directed cardiac differentiation of HPSCs by transcription- or growth-factors using optimal switching time. *(A)* Quantitative gene expression of transcription-factor driven differentiation after 21 days culturing with variable switching time (day 5, 7, 9 or no switch). Analyses employed pluripotency- (*OCT4* and *NANOG)*, mesoderm- (*T, BRACHYURY*), endoderm- (*SOX17*), cardiac mesoderm- (*NXK2.5*) and terminally differentiated cardiomyocyte- (*MYH6*) markers. Control- transduced (CON) constructs (*eGFP*-expressing lentivirus) possessed

no significant cardiac differentiation, whereas those transduced with *GT* significantly up-regulated cardiac mesoderm and cardiomyocyte markers ($p<0.05$). This was more efficient for cardiac mesoderm than for terminal differentiation ($p<0.05$) when compared to monolayer differentiation as previously described (23). Switching time significantly affected specification and terminal differentiation with 7 days prior to switching the most efficient at specifying cardiac mesoderm ($p<0.05$) (Bars are S.E.) (n=6). *(B)* Quantitative gene expression characterisation of growth-factor driven differentiation. Control constructs cultivated in CM media showed some differentiation depending on switching time but no significant cardiac gene expression. Hydrogels cultivated with the growth-factor regime showed significant cardiac gene expression, even in unswitched constructs ($p<0.05$). Those switched at day 7 or 9 showed the highest specification and terminal differentiation beyond that produced by the transcription-factor directed system. (Bars are S.E.) (n=6).