Rapid generation of functional mature pancreatic islet-beta cells from human pluripotent stem cells

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Method Article

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Abstract

Existing protocols for β-cell differentiation are inefficient and result in a mixed population of cells consisting of pancreatic progenitors and immature β-like cells that have limited glucose stimulated insulin secretion. Here, we describe a protocol to rapidly and efficiently generate functional and mature pancreatic islet β-cells from human pluripotent stem cells \(hPSCs) by recapitulating many aspects of pancreatic development in vitro. We first generate ~50% C-peptide+NKX6.1+ insulin producing cells in 20 days by differentiating hPSCs through the stages of endoderm, pancreatic progenitors and endocrine progenitors. These β-like cells are then isolated and re-aggregated to form islet sized enriched beta-clusters \(eBCs), mimicking the process of endocrine cell clustering that occurs during islet organogenesis. eBCs share physiological properties of human islets, including dynamic insulin secretion, calcium signaling, sulfonylurea response, and mitochondrial energization. The results of this protocol is detailed in the publication titled " Recapitulating endocrine cell clustering in culture promotes maturation of human stem cell-derived beta cells" in Nature Cell Biology. The whole protocol from hPSCs to eBCs takes 27 days.

Reagents

TrypLE Mouse embryonic fibroblasts \(MEFs) \(Gibco) PBS-/- \(Gibco) PBS+/+\(Gibco) Accumax \(Fisher #NC0443331) Rock inhibitor \(R&D #1254) Cell culture media MEF Media 100x MEM NEAA \(Life Tech #11140-050) FBS \(Thermo Sci #SH30071.03) 1x DMEM \(Life Tech #11995-065) hESC Media 100x Glutamax \(Life Tech #35050-061) 100x MEM-NEAA \(Life Tech #11140-050) 100x Beta-mercaptoethanol \(Millipore #ES-007-E) KSR \(Life Tech #10828-028) DMEM/F-12 \(Life Tech #11320-033) FGF-2 -10ng/ml \(R&D 233-FB-025/C) Day 0 Media hESC media Activin A- 10ng/ml \(R&D 338-AC/CF) HeregulinB -10 ng/ml \(Peprotech 100-03) Day 1 Media 100x Glutamax \(Life Tech #35050-061) 1x RPMI 1640 \(Mediatech #15-040-CM) FBS- 0.2% \(Corning/Fisher - #35-011-CV) ITS-1:5,000 \(Gibco) Activin A-100 ng/ml \(338-AC/CF) Human WNT3a- 50 ng/ml \(R&D -5036-WN-010/CF). Day 2 Media 100x Glutamax \(Life Tech #35050-061) 1x RPMI 1640 \(Mediatech #15-040-CM) FBS- 0.2% \(Corning/Fisher - #35-011-CV) ITS- 1:2,000 \(Gibco) Activin A -100 ng/ml \(338-AC/CF) Day 3 Media 100x Glutamax \(Life Tech #35050-061) 1x RPMI 1640 \(Mediatech #15-040-CM) FBS- 0.2% \(Corning/Fisher - #35-011-CV) ITS- 1:1,000 \(Gibco) TGFbi IV- 2.5 µM \(CalBioChem #616454) KGF- 25 ng/ml \(R&D- 251-KG-050/CF) Day 4-5 Media 100x Glutamax \(Life Tech #35050-061) 1x RPMI 1640 \(Mediatech #15-040-CM) FBS- 0.4% \(Corning/Fisher - #35-011-CV) ITS -1:1,000 KGF-25 ng/ml \(R&D- #251-KG-050/CF) Day 6-7 Media 100x Glutamax \(Life Tech #35050-061) 1x DMEM \(Life Tech #11960-051) B27- 1:100 \(Gibco) TTNPB - 3 nM \(Sigma-Aldrich#T3757) Day 8 Media 100x Glutamax \(Life Tech #35050-061) 1x DMEM \(Life Tech #11960-051) B27-1:100 \(Gibco) TTNPB - 3 nM \(Sigma-Aldrich#T3757) EGF- 50 ng/ml \(R&D #236-EG) Day 9-11 Media 100x Glutamax \(Life Tech #35050-061) 1x DMEM \(Life Tech #11960-051) B27- 1:100 \(Gibco) EGF- 50 ng/ml \(R&D #236-EG) KGF- 50 ng/ml \(R&D- #251-KG-050/CF) Day 12-20 Media 100x Glutamax \(Life Tech #35050-061) 1x DMEM \(Life Tech #11960-051) B27- 1:100 \(Gibco) NEAA- 1:100 \(Gibco) ALKi II- 10 µm \(Cayman Chemical #14794) LDN-193189 - 500

nM \(Fisher #04-0074-10) Xxi - 1 µm \(Millipore #565789) T3- 1 µM \(Sigma-Aldrich #T6397) Vitamin C - 0.5 mM \(Sigma-Aldrich#49752) N-acetyl Cysteine- 1 mM \(Sigma-Aldrich) Zinc sulfate- 10 µM \(Sigma-Aldrich) Heparin sulfate- 10 µg/ml \(Sigma-Aldrich#H3149) Day 20-27 Media 100x Glutamax \(Life Tech #35050-061) CMRL \(Life Tech #11530) 1:100 B27 \(or 10% FBS) \(Gibco) 1:100 NEAA \(Gibco) ALKi II -10 µm \(Cayman Chemical #14794) Vitamin C -0.5 mM \(Sigma-Aldrich#49752) T3- 1 µM \(Sigma-Aldrich #T6397) N-acetyl Cysteine -1 mM \(Sigma-Aldrich) Zinc sulfate- 10 µM \(Sigma-Aldrich) Heparin sulfate - 10 µg/ml \(Sigma-Aldrich#H3149)

Equipment

10cm Tissue culture dish \(Fisher #353003) 6 well suspension plate \(VVR #657185) AggreWell 400 \(Stem Cell Technologies) Panasonic \(PHC) MultiGas incubator \(MCO-19M\(UV)) Orbital Shaker \(EK scientific S2030-1000-B) Bin for Shaker \(Grainger #30255SCLAR) BD FACSAria II

Procedure

Plating MEFs MEFs are plated the day before passaging, on Mondays and Thursdays. 1. Coat 10 cm dishes with 5ml 0.1% Gelatin. 2. Distribute liquid evenly and incubate at room temperature for at least 30 min. 3. Take new cryovial of MEFs from liquid nitrogen and keep on dry ice until thaw 4. Warm the vial in a 37C water bath until approximately half the ice has thawed. 5. Slowly transfer the cells to a 15ml falcon tube containing 5ml warm MEF media. 6. Centrifuge cells at 1000 rpm for 3min 7. Aspirate gelatin from the 10 cm dishes. Count and distribute 570k MEFs/10 cm dish and place in incubator at 37C and 5%CO2. Maintenance of hPSC cultures hPSCs are cultured on a feeder layer of mouse embryonic fibroblasts \(MEFs). Cells are passaged twice a week on Tuesdays and Fridays. The cell line primarily used in this protocol is Mel1 INSGFP/W human embryonic stem cells \(hESCs). This cell line generates insulin-producing cells that express GFP, allowing us to track the differentiation and isolate insulin-producing cells for clustering into eBCs. 1. On the day of passaging, aspirate spent media from highly confluent hESC cultures on 10cm dishes. 2. Wash with 5ml PBS-/- 3. Add 2ml trypLE and incubate for 6-8 min 4. Once the cells dislodge, quench with 8ml hESC media 5. Centrifuge cells at 1000rpm for 3min 6. Take out MEF plates. Aspirate MEF media. 7. Distribute hESC cells at $4.3x10^{6}$ cells/10 cm on Tuesdays and $3.17x10_{6}$ cells/10cm on Fridays in 12ml hESC media \(Note: Add 10uM Rock inhibitor for cell lines that do not survive passaging). 8. Feed the cells everyday with hESC media. Seeding and Differentiation The protocol works for cell lines that can form spheres upon seeding. Cells are seeded for differentiation during passaging into suspension plates where they form 150-200um sized spheres. These spheres are then treated with differentiation media that pushes them through the various stages of pancreatic development. 1. To initiate differentiation, passage hESC cultures using TrypLE as described in the previous section. 2. Count and seed cells at 5.5x10^6 cells/well in 6-well suspension plates in 5.5ml day 0 media \(Note: Add 10uM Rock inhibitor for cell lines that do not survive passaging). 3. Place the plates in the incubator at 37°C and 5%CO2 on an orbital shaker at 100 rpm to induce 3D sphere formation. 4. After 24 hours check for sphere formation 5. Collect the spheres in 50 ml falcon tubes and allow them to settle

by gravity for 3min. 6. Wash once with PBS+/+. Aspirate PBS+/+ 7. Resuspend the spheres in day 1 media and distribute into fresh 6-well suspension plates at 5.5ml/well. Place it back on the shaker in the incubator. 8. Until day 3, aspirate 5 ml of spent media and replenish with 5.5 ml of the respective day media \(refer reagents). 9. From day 4 onwards, aspirate 4.5 ml of spent media and feed with 5ml of media prescribed for the respective day \(refer reagents). 10. On day 20, collect the spheres and incubate with Accumax for 8 minutes. \(Spheres from one well requires 1.5ml accumax). Flick the tube every 2 min to allow efficient dissociation. 11. Sort for Live GFPhigh cells on Aria II at low flow rates \(<3000 events/sec). 12. Collect the sorted cells and resuspend in day 20-27 media with 10uM Rock inhibitor at 1x10^6cells/1.5ml. 13. Dispense 1.5ml in each well of AggrewellTM-400 plates such that there are 1000cells/cluster. 14. Incubate the Aggrewell plates for 48 hours at 37C and 5%02 or until cells have aggregated into nicely round clusters. 15. Transfer the clusters into 6 well suspension plates in 3ml day 20-27 media and culture until the end of differentiation \(day 27) while refreshing the medium every third day. The clusters can be used until day 30.

Timing

The protocol for differentiation from seeding of hESCs until the formation of β-like cells take 20 days and additional 7 days after sorting and re-aggregation to generate functional mature β cells. If cryopreserved hESCs are to be used, it is recommended to start differentiation at least three passages after thaw.

Troubleshooting

hPSC cells do not passage well or form spheres: Add 10uM Rock inhibitor during passaging and seeding. Cells do not differentiate well: Media needs to be changed at the same time every day from day0 – day8. This is especially critical in the initial days day 0 to day 5. Lot of death on day 1: Density of clusters was too high on seeding/health of undifferentiated hESCs not good. Be gentle on the cells while dissociation and seeding. Cells do not differentiate into sufficient numbers of C-peptide+/NKX6.1+ cells at d20: Density of clusters was low in each well and was not sufficient for efficient induction of pancreatic endoderm. Pool wells on day 6 or day 9 of differentiation and keep the density high until the end of the protocol. Yield is low on FACS sorting: Cells need to be handled gently while dissociating for FACS at d20. Thaw fresh Accumax before every experiment. Limit the number of minutes the cells are incubating in Accumax and do not pipet the cells harshly.

Anticipated Results

Maintenance cells: hPSC passaged using our protocol are usually monolayer and are highly confluent on the day of passage \(Tuesdays and Fridays). They do not appear as colonies which are commonly observed in other hPSCs passaging techniques. Spheres at day 0 of differentiation: Cells aggregate into spheres sized 150-200um 24 hours after seeding. Spheres at day 2 of differentiation: Spheres are round and should be >90% double positive for SOX17/FOXA2 on evaluation by flow cytometry Spheres at day 4 of differentiation: Spheres are slightly fluffy and have uneven edges. There should be no indentations or

dark areas within the spheres. Spheres at day 11 of differentiation: Spheres are round and have smooth edges. They should be >80% double positive for PDX1/NKX6.1 on evaluation by flow cytometry. Spheres at d13 of differentiation: GFP \(Insulin+ cells) expression should start appearing in small proportion of cells within the spheres. Spheres at d20 of differentiation: >50% of cells in the spheres should express GFP, and ~40-50% of cells should be double positive for C-peptide/NKX6.1 on evaluation by FACS. eBCs at d27 of differentiation: 100-200 um sized clusters and >90% of cells should be positive for C-peptide and 80% double positive for C-peptide/NKX6.1

Figures

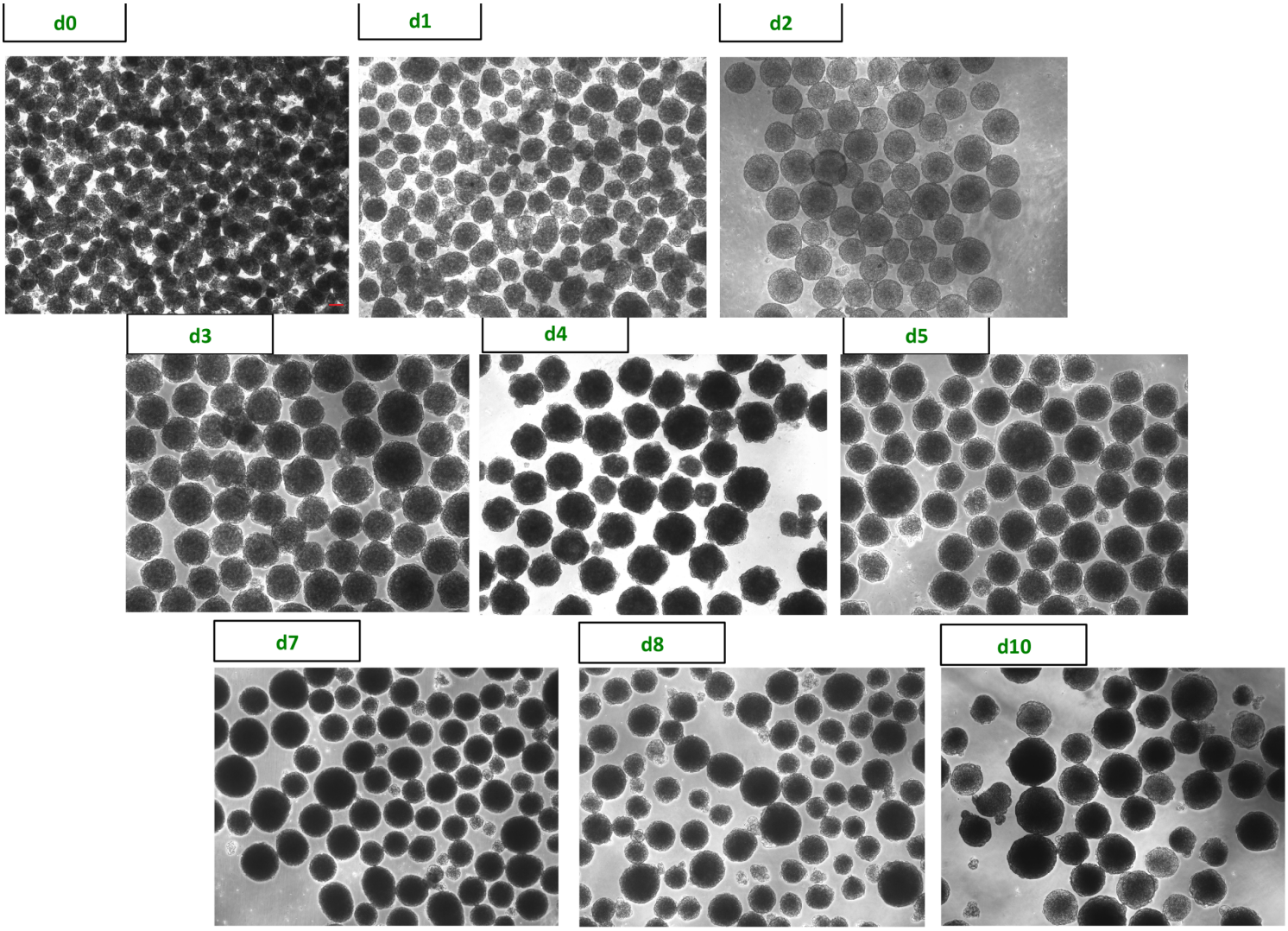

Figure 1

Morphology critical to directed differentiation Brightfield images of Mel1 INSGFP/W spheres from seeding, day 0 (d0) until day 12 (d12) of the differentiation protocol. Images show critical morphology of the spheres important to ensure correct directed differentiation to beta cells. Day 0 spheres are around 150-200um in size with slightly irregular shape. By day 2 (definitive endoderm), spheres are smooth and

round. On day 4, spheres have a fluffly appearance (foregut endoderm). The spheres should become progressively round and bigger with time (day 12- pancreatic endoderm). Scale bar, 100um.

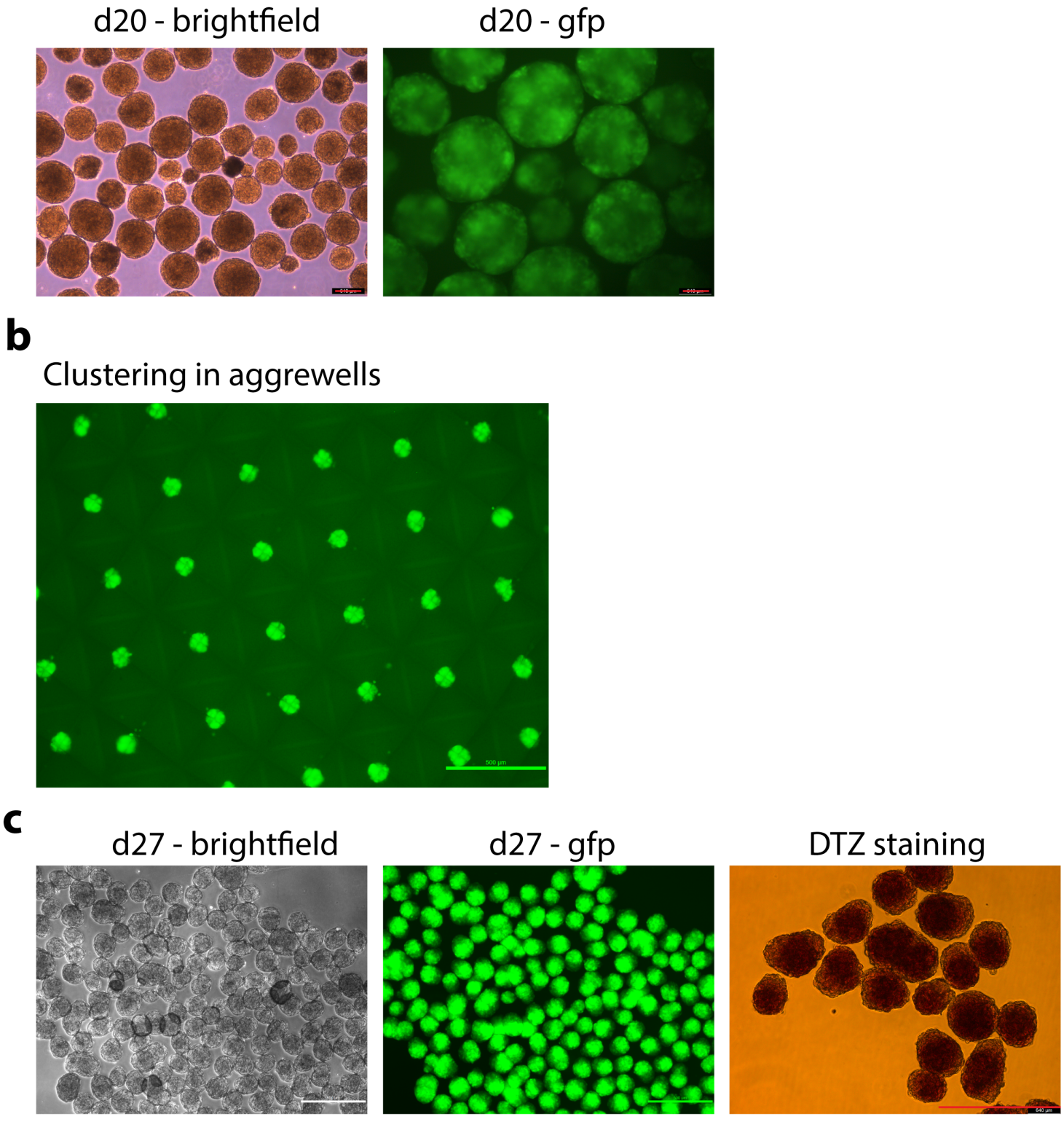

Figure 2

eBC generation (a) Brightfield and gfp images of day 20 (d20) spheres of Mel1 INSGFP/W cells. GFP indicates activation of insulin expression. Around 50% of cells are GFP+. Scale bar, 500um. (b) 48 hours after seeding sorted GFPhigh cells sorted in Aggrewell. Note cells have self-organized into clusters. Scale

bar, 500um. (c) Brightfield and gfp images day 27 (d27) eBCs. Also eBCs stain orange with Dithizone (DTZ) like human islets indicating enriched zinc in the insulin granules. Scale bar, 500um.