

WIS-NHSM Human Naïve Stem Cell Platform Approaches and Protocols

(HANNA LAB – Weizmann Institute of Science)

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This document details different validated compositions for expanding genetically unmodified human naïve pluripotent cells (ESCs and iPSCs) as described and based on *Gafni et al. Nature 2013*, including extensions, clarifications and improvements that will soon be published. The conditions support expansion of indefinitely stable genetically unmodified MAPK (P38, ERK, JNK) independent human naïve pluripotent cells (with at least one ground state epigenetic feature of H3K27me3 depletion over developmental genes).

We provide FGF/TGF β 1/Activin A **containing** and **free** compositions.:

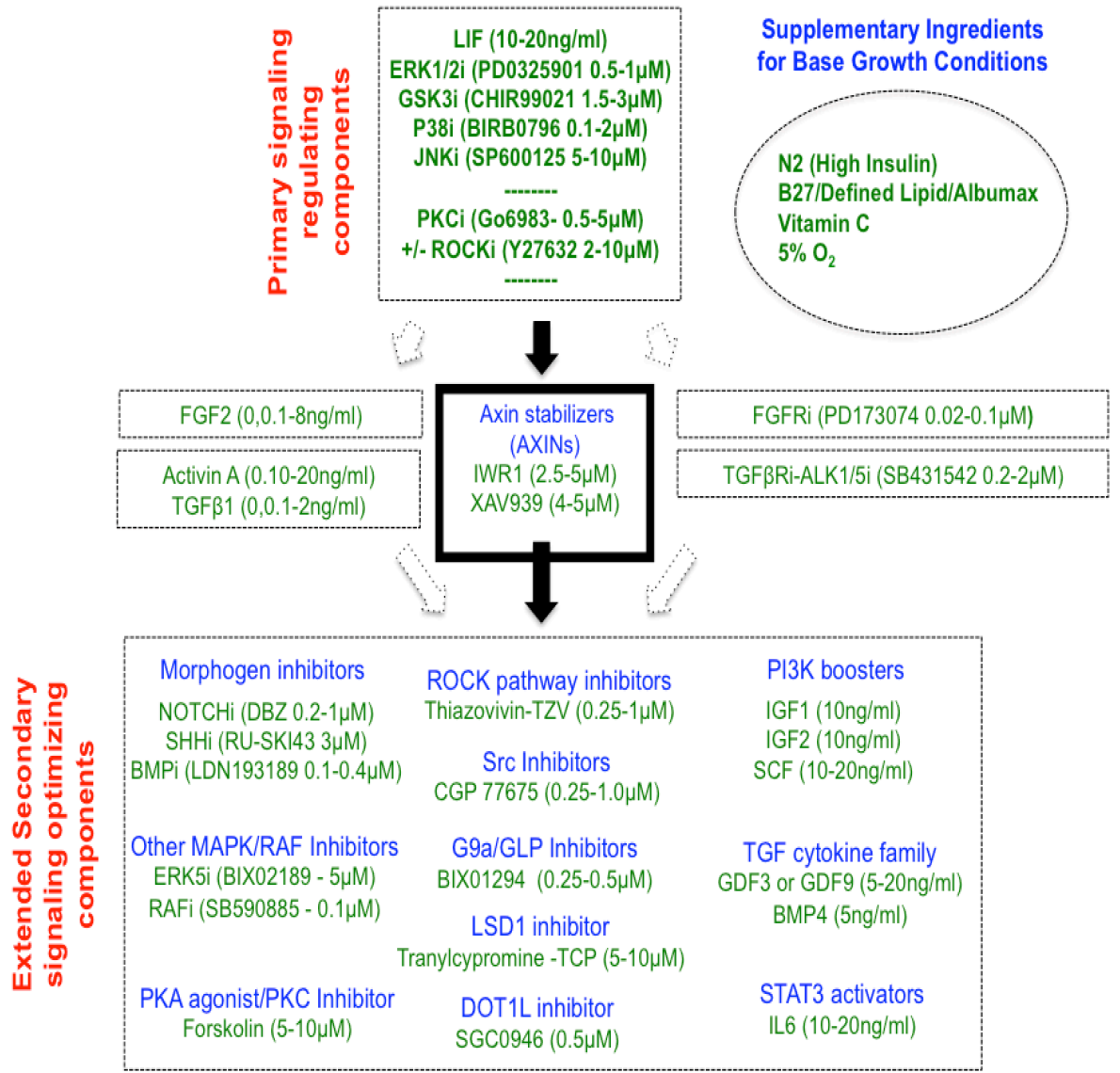
NHSM - Composition 1 is published in *Gafni et al. Nature 2013* (Composition 1A), and we now also introduced modifications (newer more specific inhibitors like BIRB0796 or optimized concentrations for aPKCi) and other clarifications based on questions from readers and users (Composition 1B).

ENHSM - Compositions 2, FGF/TGF free.

FGF/TGF/Activin A can support growth and survival at the expense of mild reduction in magnitude and kinetics of acquiring naïve characteristics. Notably, autocrine secretion of FGF and TGF/Activin A (and other GDFs) is notable in human naïve PSCs and support their growth rate. **PKCi and IWR1/XAV939 (Axin stabilizer) allow robust FGF/TGF free growth.**

The image below summarizes our approach strategy and ingredients of (1) Primary base Components, and (2) Secondary Optimizing Components that can be used to boost, consolidate and accelerate acquisition of naïve features of human pluripotent cells. Our conditions are most efficient thus far in naïve conversions and in long-term maintenance without feeders or transgene transfections and without high propensity for chromosomal abnormalities.

WIS-NHSM Compositions for Human Naïve Pluripotency by Cell Signaling Modulation



For conceptual explanations and summaries, please visit our Review Poster on: "Naïve and Primed Pluripotency Spectrum" published in Nature Reviews Molecular Cell Biology (2015)

<http://www.nature.com/nrm/posters/pluripotency/index.html>

<http://www.nature.com/nrm/posters/pluripotency/pluripotency.pdf>

Composition 1A: Basic NHSM composition (as Published in Gafni et al. Nature 2013) - (feeder and feeder free conditions):

WIS-NHSM media (Albumax I, vitamin C and N2 home made mix based +/- KSR):

- KO-DMEM (Invitrogen 10829-018) - 500ml
- Pen-strep 5ml (Biological Industries 03-033-1B)
- L-glutamine 5ml (Biological Industries - 02-022-1B)
- NEAA - 5ml (Biological Industries 01-340-1B)
- **5gr Albumax I (Invitrogen - 11020021) (dissolve in media bottle for 30 minutes in RT)**
- Insulin (Sigma I-1882) - add 6.25mg insulin per 1 bottle to give approximately additional 12.5microg/ml insulin)
- Apo-transferrin (Sigma T-1147), 100 µg/ml final concentration
- Progesterone (Sigma P8783), 0.02 µg/ml final concentration;
- Putrescine (SigmaP5780), 16 µg/ml final concentration
- Sodium selenite (Sigma S5261), add 5 µL of 3 mM stock solution per 500ml of medium.
- Optional and recommended: L-ascorbic acid 2-phosphate (Sigma A8960) (50 µg/ml final concentration)
- Optional: **5ml** of KSR (Invitrogen -10828-028)

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- 1) human LIF (in house produced or Peprotech # 300-05) - 10 µg total (~20ng/ml final concentration) (1 vial)
 - 2) FGF2 (Peprotech 100-18c or RnD systems -4114-TC-01M) 8ng/ml final (1 vial)
 - 3) TGFB1 - 1ng/ml final (Peprotech 100-21c-1000) (0.5 vial)
 - 4) Chir99021 (Axon 1386)- 3µM final (1 vial)
 - 5) PD0325901 (Axon 1408)- 1µM final (1 vial)
 - 6) p38i BIRB796 (Axon 1358)- 2µM final(1 vial)
 - 7) JNKi SP600125 (TOCRIS 1496)- 10µM final (2 vials).
 - **Secondary Optimizing Components:**
 - 8) PKCi (Go6983 - TOCRIS 2285)- 2µM final (**\$ 0.5-5µM titration range \$**)
 - 9) ROCKi Y27632 (Axon 1683)-10µM final**

Under the above conditions: Cells can be expanded on **Feeder/gelatin pre-coated plates, 0.2%Gelatin/Vitronectin**, BioLaminin 521 or **Matrigel**. Cells can be expanded preferably in 5% O2, or in 20% O2. PKCi boosts homogeneity and naivety of the cultures. ****On feeder cells permanent use of ROCKi is recommended. For Feeder free conditions, Permanent Y27632 ROCKi >2 µM CANNOT be used, but adding ROCKi 24h before and after splitting is preferable. Replace media every 24 hours.**

Composition 1B: Basic NHSM composition (Based on published in Gafni et al. Nature 2013) - (feeder and feeder free conditions):
WIS-NHSM media (Albumax I, vitamin C and N2 home made mix based +/- KSR):

- KO-DMEM (Invitrogen 10829-018) **or** DMEM/F12 (with NaHCO₃ and no HEPES – Biological Industries 06-1170-50-1A) **or** 1:1 mix of Neurobasal Medium (Invitrogen 21103-049) and DMEM/F12 (Biological Industries 06-1170-50-1A or Invitrogen 21331) - 500ml
- Pen-strep 5ml (Biological Industries 03-033-1B)
- L-glutamine 5ml (Biological Industries - 02-022-1B)
- NEAA – 5ml (Biological Industries 01-340-1B)
- **Sodium Pyruvate 3.5ml (Biological Industries 03-042-01B, 100mM stock solution)**
- **5gr Albumax I (Invitrogen - 11020021) (dissolve in media bottle for 30 minutes in RT)**
- Insulin (Sigma I-1882) - add 6.25mg insulin per 1 bottle to give approximately additional 12.5microg/ml insulin)
- Apo-transferrin (Sigma T-1147), 100 µg/ml final concentration
- Progesterone (Sigma P8783), 0.02 µg/ml final concentration;
- Putrescine (Sigma P5780), 16 µg/ml final concentration
- Sodium selenite (Sigma S5261), add 5 µL of 3 mM stock solution per 500ml of medium.
- Optional and recommended: L-ascorbic acid 2-phosphate (Sigma A8960) (50 µg/ml final concentration)
- Optional: **2.5ml** of KSR (Invitrogen -10828-028)

- 1) human LIF (in house produced or Peptotech # 300-05) – 10 µg total (~20ng/ml final concentration) (1 vial)
- 2) FGF2 (Peptotech 100-18c or RnD systems –4114-TC-01M) 8ng/ml final (1 vial)
- 3) Activin A (20ng/ml final Peptotech 120-14E)(2 vials)
- 4) Chir99021 (Axon 1386)– 1.5µM final (1/2 vial)
- 5) PD0325901 (Axon 1408)– 1µM final (1 vial)
- 6) p38i BIRB796 (Axon 1358)– 2µM final(1 vial)
- 7) JNKi SP600125 (TOCRIS 1496)– 5µM final (1 vial).
- **Secondary Optimizing Components:**
- 8\$) PKCi (Go6983 – TOCRIS 2285)- 2µM (2 vials)
- 9) ROCKi Y27632 (Axon 1683)-5µM final**

Under the above conditions: Cells can be expanded on **Feeder/gelatin pre-coated plates, 0.2%Gelatin/Vitronectin, Laminin-521**, Vitronectin only, or Matrigel. Cells can be expanded preferably in 5% O₂, or in 20% O₂. PKCi boosts homogeneity and naivety of the cultures. ****On feeder cells permanent use of ROCKi is recommended. For Feeder free conditions, Permanent Y27632 ROCKi >2 µM CANNOT be used, but adding ROCKi 24h before and after splitting is preferable. Replace media every 24 hours.

#Optional booster supplements: IWR1 SigmaAldrich - I0161) - 5µM final (1 vial), BMPi (LDN193189 – Axon 1509) 0.1µM (0.25 vial) and/or SRCi CGP77675 (Axon 2097) – 1.0µM final (1 vial).

Composition 2: Enhanced Naïve Defined/ XENO-Free FGF/TGF Free NHSM Composition – (feeder and feeder free conditions) (ENHSM – Unpublished yet): &&

Defined/XENO-Free WIS-NHSM media (B27, vitamin C and N2 home made mix based):

- 1:1 mix of Neurobasal Medium (Invitrogen 21103-049) and DMEM/F12 (Biological Industries 06-1170-50-1A or Invitrogen 21331) – total 470ml
- Pen-strep 5ml (Biological Industries 03-033-1B)
- L-glutamine 5ml (Biological Industries - 02-022-1B)
- NEAA – 5ml (Biological Industries 01-340-1B)
- **Sodium Pyruvate 3.5ml (Biological Industries 03-042-01B, 100mM stock solution)**
- 50µL of 50mM stock Beta-mercaptoethanol (1 vial)
- 10ml B27 supplement (Invitrogen 17504-044) or Xeno FREE B27 (Invitrogen A14867-01)
- Defined Lipid Concentrate (Invitrogen 11905-031)- **1ml**
- Insulin (Sigma I-1882) - add 6.25mg insulin per 1 (12.5microg/ml insulin)
- Apo-transferrin (Sigma T-1147), 100 µg/ml final concentration
- Progesterone (Sigma P8783), 0.02 µg/ml final concentration;
- Putrescine (SigmaP5780), 16 µg/ml final concentration
- Sodium selenite (Sigma S5261), add 5 µL of 3 mM stock solution per 500ml.
- L-ascorbic acid 2-phosphate (Sigma - A8960) (50 µg/ml final concentration)
- Optional and recommended: BSA (100X Fraction V 7.5% Solution Gibco 15260-037 - add 0.35ml per 500ml media bottle) **or** Human an Serum Albumin 10% solution (Biological industries 05-720-1B – add 0.3ml per 500ml media bottle)
- Optional and recommended: **2.5ml** of KSR (Invitrogen -10828-028)

Primary Cytokines + inhibitors:

- 1) human LIF (in house produced or Peprotech # 300-05) – 10 µg total (~20ng/ml final concentration) (1 vial)
- 2) *AXINs IWR1 (SigmaAldrich - I0161) – 5µM final (2 vials)*
- 3) Chir99021 (Axon 1386)– 1.5µM final (1/2 vial)
- 4) PD0325901 (Axon 1408)– 1µM final (1 vial)
- 5) p38i BIRB796 (Axon 1358)– 2 µM final (1 vial)
- 6) JNKi SP600125 (TOCRIS 1496)– 5µM final (1 vial).
- 7) PKCi (Go6983 – TOCRIS 2285)- **2µM** (2 vials)
- 8) *Activin A (20ng/ml Final – Peprotech 120-14E) (2 vials)*

Secondary Optimizing Components:

- 9) ROCKi Y27632 (Axon 1683)- 5µM final**
- 10) SRCi CGP77675 (Axon 2097) – 1.0µM final (1 vial)

Under the above conditions: Cells can be expanded on **Feeder/gelatin pre-coated plates** or **Laminin-521**, **Matrigel** or 0.2%Gelatin/Vitronectin coated plates. Cells can be expanded preferably in 5% O₂, or in 20% O₂. PKCi boosts homogeneity and naivety of the cultures. Cells can be expanded preferably in 5% O₂, or in 20% O₂. ****On feeder cells permanent use of ROCKi is recommended. For Feeder free conditions, Permanent Y27632 ROCKi >2 µM CANNOT be used, but adding ROCKi 24h before and after splitting is preferable. &&Compatible with changing media every 48 hours.**

Comments, explanations, FAQs and tips:

- To convert your already established primed hESCs and iPSCs, simply apply the media and start passaging the cells poly-clonally, as the naïve pluripotent cells take over within 2-3 passages. Sorting for pluripotency markers (e.g. SSEA4) at passage 2-3 can expedite the purification process for some cell lines, but is often not needed because our conditions are very rich. Acquisition and consolidation of most naïve features occurs within 7-14 days. **To get experience for the cells by new users, we recommend converting 2-3 lines simultaneously on feeder and feeder-free conditions.**

-\$ PKCi (Go6983 – TOCRIS 2285)- 0.5-5 μ M final concentration). Go6983 tends to be less stable and there is batch variability in its activity (we use TOCRIS lot # 2A1145248). Low stability at 4C (up to 7 days). It is possible to keep working stocks in -20 and add fresh every media change (thaw-freeze up to 3 times per vial). Recommended dose varies between batches (**recommended to initially try 2 μ M, but a range of 0.5 - 5 μ M has been found optimal for some batches, and titration down from 4 μ M by 1:2 dilutions is recommended (4,2,1,0.5 μ M)).** Go6983 has red fluorescence signal, so pre-washing of cells before analysis should be applied. **Remarkably, we note it down-regulates Mbd3 expression in pluripotent cells and during reprogramming.** PKCi also boosts naïve pluripotency gene expression (KLFs, and to much lesser extent ESRRB).

- **On Feeder cells, the cells are tolerant to PERMANENT 2-10 μ M ROCK inhibition (basically indefinitely without differentiating), and it improves their viability and naïve features. Permanent high concentration >2 μ M of Y27632 ROCKi CANNOT be used in feeder free condition maintenance. During iPSC reprogramming continuous 2-5 μ M Y27632 ROCKi is recommended (at least during first 5-7 days).

- The combination of GSK3 inhibitor (e.g. CHIR99021) and inducer/stabilizer of AXIN complex (abbreviated as AXINs) (e.g. IWR1 2.5-5 μ M final concentration or XAV939 4-5 μ M final) leads to a synergistic effect that induces cytoplasmic WNT/beta-catenin signaling at the expense of its nuclear localization effects, and thus reduces mesodermal gene expression patterns, reduces dependence on exogenous FGF2 and TGF, and boosts epithelial signature and pluripotency gene expression. (IWR1/CHIR or XAV939/CHIR combinations were described in Kim et al. Nature Communications 2013 DOI: 10.1038/ncomms3403). Remarkable positive effect during first 7 days of conversion of already established primed lines. We are currently exploring whether the effect is mediated via inhibiting YAP/TAZ nuclear localization, as the APC/AXIN cytoplasmic destruction complex may act as a decoy sink for YAP/TAZ (Piccolo group – Azzolin et al. Cell 2014).

- The use of SRC inhibitors has been previously described in mice to allow substrate independent and flexible maintenance of ESCs. We incorporate CGP77675 compound (0.25 - 1 μ M final concentration) as previously described. (Shimizu et al. Stem Cells 2012 2012;30:1394–1404: Dual Inhibition of Src and

GSK3 maintains Mouse Embryonic Stem Cells, Whose Differentiation is Mechanically Regulated by Src Signaling).

- B27 supplement (Invitrogen) alone can be minimal for maintenance of human naïve pluripotent cells, particularly without feeder cells. Lipid supplements from Albumax and/or **Chemically Defined Lipids** and/or Oleic acid is beneficial for robust human naïve stem cell expansion.

- *Instead of Albumax I: B27 **and** Defined lipid mix can be used (1- 4ml of Defined Lipid Concentrate per 500ml media (Invitrogen 11905-031). We are currently testing using Oleic, Pipecolic and Linoleic acid supplements only to get the beneficial effect of the defined lipid concentrate or B27 supplements (and reduce costs ☺ and improve viability and growth).*

- DMEM/F12-Neurobasal 1:1 mix (most optimal), or DMEM/F12 (with 2.4gr/lit NaHCO₃ and no HEPES – Biological Industries 06-1170-50-1A), can be used as a base medium instead of KO-DMEM as many human ESC/iPSC lines are already adapted to DMEM/F12 containing media.

- Cells can be passaged by 0.05% trypsinization as single cells or as small clumps (Biological Industries - 03-054-1). Reaction is stopped by FBS containing DME medium (nothing special - to eliminate traces of functional Trypsin) followed by centrifugation (3 min X1000 RPM). {Excellent outcome are also obtained by using Accutase, Dispase, or collagenase or TrypLE digestions}.

- ROCKi/PKCi are the most-unstable components in fully reconstituted media at 4C or 37C. Some may choose adding them fresh from -20 stored aliquots every media change. Condition 1: Replace media every 24 hours. **Conditions 2,3- replace media every 48 hours.**

- Cells are passaged every 4-5 days and reseeded at 1:5-1:8 ratios.

- Cells can be expanded in 5% O₂ or 20% O₂ conditions. **5% O₂ is preferable**, and shows increased survival, increased proliferation, increased nuclear TFE3 and reduced background cell death.

- Cells can be expanded on plates coated with 0.2% gelatin/irradiated or mitomycin C mouse feeder cells (we use DR4 routinely). **More optimal for our current gene targeting approaches and subsequent colony picking, and gives in general best results and wanted molecular changes.** Human irradiated HFFs can also be used. Overall, MEFs increase homogeneity of the cultures and improve naïve features like TFE3 nuclear localization and increase reduction kinetics in global DNA hypomethylation in Versions 1&3 (**notably all other published naïve protocols after our paper there is obligatory use of feeders, which severely biases comparisons**). Exogenous FGF2 is omitted if using feeders, particularly in Composition 2/3 variations (Because of IWR1/CHIR combo - Kim et al. Nature Communications 2013 DOI:

10.1038/ncomms3403). However, low dose FGF2 has a positive proliferative and survival effect.

- Composition 1: Cells can be expanded on 0.2% gelatin AND 1 µg/ml Vitronectin (in house made) coated plates (Mix gelatin solution with vitronectin, and use to coat wells – at least 1 hour at 37C) (coated plates can be left for up to 4 days in 37C and used for cells). The latter is also compatible with composition 2/3 and keeps cells undifferentiated, but the adherence of the cells on vitronectin is very low relatively to Matrigel or Laminin-521.

- Matrigel (GF reduced - BD) or Geltrex (Invitrogen) are also very favorable for feeder free expansion.

- Biolamina Laminin-521 is also optimal. Laminin-511 is also compatible, but we prefer Laminin-521.

- Media is relatively sensitive, no need for pre-heating (10 minutes at RT before use is enough, protect from light).

-# The use of JNKi SP600125 (TOCRIS 1496)– on its own ~5 µM final achieves partial JNK inhibition relative to 10 µM, but has less background toxicity, and therefore is recommended. High dose (10µM) SP600125 JNKi using also gives a previously reported side effect of enlarged nuclei.

-* While human naïve cells have 5-10% single cell survival without ROCKi, its inclusion at least in the first 12-24 hours after splitting boosts rates up to 70-90%.

- For p38 inhibition, we prefer BIRB796 (0.1-2µM) over SB203580 (10microM – Axon 1363) because it has less side effect of background toxicity and vacuolar cytoplasmic formation, but we see no molecular difference between the 2 conditions (on gelatin/vitronectin conditions). **SB203580 5microM leads only to a mild inhibition of DNMT3 expression (conditions used for our published gene array datasets), and therefore we recommend using SB203580 (10microM – Axon 1363) or preferably BIRB796 (0.1-2µM) (as validated in our published RT-PCR data).**

- %We note that the optional use of BMP pathway inhibitor (BMPi) LDN193189 (0.1-0.4µM final concentration– AXON Medchem 1509) can be tolerated by human naïve cells expanded in NHSM and further reduces the already low background differentiation in our feeder-free conditions. LDN 193189 inhibits BMP type I receptors ALK2 (IC50: 5 nM), ALK3 (IC50: 30 nM) subsequent SMAD phosphorylation. However, we note that replacing JNKi and p38i in our cocktail with BMPi leads to differentiation of human naïve pluripotent cells. **Further, aPKCi and p38i inhibition are critical components in down-regulation of DNMT3B/A in human cells (rather than ERKi alone).**

- **Compositions 2 and 3: FGFRi (PD173074 - AXON 1673) – up 0.05-1µM final (0.5 or 1 vial) can be used in Compositions 2- 3.**

- **Composition 3: TGFRI** (SB431542 AXON) – 2 μ M final (1 vial) can be permanently used in Composition 3 (particularly on MEFs) or during the first 2 days of iPSC reprogramming, but its permanent inhibition is not recommended, particularly on feeder free conditions, since it dramatically reduces growth rate. For TGFRI 0.25-0.5 μ M ALKi inhibitor A83-01 can be used instead (AXON MEDCHEM 1421). Not compatible with simultaneous use of FGFRI. (see below)

- Unlike for mouse naïve cells, **Use of both FGFRI and TGFRI together simultaneously for more than 5 days is very harsh for the human naïve cells and leads to extremely slow growth.** This is different than mouse ESCs, were they can robustly accommodate simultaneous inhibition of FGF and TGF β pathways!!

-RAF inhibitors like Sorafenib (0.1 μ M) or SB590885 (0.1 μ M) (both are highly selective for BRAF and CRAF) can be added on compositions 1,2 and 3, although no functional or molecular benefit has been observed so far to justify this.

- Please make sure you use HUMAN LIF and NOT mouse Lif (Human LIF works both on mouse and human cells, Mouse Lif works only on mouse cells).

- We do not use Glutamax (Invitrogen) for naïve ESC cultures. We prefer L-glutamine.

- If you prefer to use ready-made N2 supplement (Invitrogen - 17502-048) then we recommend providing additional human insulin (add 6.25mg insulin per 1 bottle to give approximately additional 12.5 μ g/ml insulin – gives total of 5+12.5 = 17.5 μ g/ml).

- Human insulin (Sigma I-2643) or from Prospec Bio (CYT-270) can be also used instead of Bovine Insulin (Sigma I-1882). Apo-Transferrin (ATF Prospec – PRO-325 - 5000MG) can be used instead of Sigma T-1147

- iPSC reprogramming from somatic cells is conducted in NHSM, ENHSM or GNHSM medium as indicated in detailed protocols for human naïve iPSC reprogramming are available also on our Website.

-For Composition 1: mTESR1 (Stem Cell Technologies- already contains TGF β 1 and FGF2) can be used as a base medium by merely adding missing cytokines and small molecules to support human naïve cells in WIS-NHSM. If assembling mTESR as a base from scratch (Ludwing and Thomson: *Curr. Protoc. Stem Cell Biol.* 2:1C.2.1-1C.2.16) for better control of cytokine and inhibitor content and/or generating FGF/TGF independent mix.

For Compositions 1/2: - We note that Activin A leads to better results in terms of upregulation of naïve pluripotency markers than TGF β 1.

Vial # indicated throughout the protocol is of course based on HANNA LAB internal aliquot stocks in our lab (which can change).

How should one approach adapting previously hESC/iPSC differentiation protocols?

We recommend to first test applying the differentiation protocol on human naïve cells, and/or simultaneously start with naïve cells, apply priming while the cells are adherent for 24-48 hours with primed human ESC medium, and then apply your the differentiation protocol.

Human Naïve Cell Handling Protocols:

-Freezing human naïve cells:

Solution 1: 20% DMSO and 80% FBS

Solution 2: Freshly made NHSM/ENHSM/GNHSM medium including 10microM Rocki.

Re-suspend cells in 1:1 solution mix and freeze in regular cryotubes at -80 in styro-foam boxes (for at least 1 day – up to 2 months). Then move vials to liquid nitrogen. No need to use special freezing devices throughout the process.

- Thawing human naïve cells:

Thaw vial at 37C and spin down in 10ml of 15% FBS supplemented DMEM (3 min at 1000RPM). Plate cells in NHSM/ENHSM/GNHSM containing 10microM ROCKi.

- DNA electroporation in human naïve PSCs:

200-50ug DNA can be used for electroporation 10”cm confluent plate of human naïve PSCs harvested and trypsinized into single cells (0.05% trypsin).

Electroporation parameters on human naïve PSCs on BIORAD Gene Pulser Xcell (with CE module): Square wave pulse protocol, Voltage: 250V, Pulse length 20ms, Number of pulses: 1, Pulse interval .0 (sec), 4mm cuvettes (BIORAD).

Preparing N2 stock components in-house:

Insulin (Prospec Bio CYT 270 = 1000mg)

- Prepare a 25mg/ml stock solution by dissolving 1000mg insulin in 40ml 0.1M HCl overnight at 4C. Sterile filter and store in 250µl individual aliquots in -80C (use 1 vial per 500ml bottle). Be careful, as insulin does not dissolve well.

Apo-Transferrin (ATF Prospec PRO-325 - 5000MG)

- Prepare a 100mg/ml stock by dissolving 5000mg A-T in 50ml dH₂O overnight at 4C. Sterile filter and store in -80C. Make 500µl aliquots (use 1 vial per 500ml bottle)

Progesterone (Sigma P8783, 25g)

- Prepare a 0.6mg/ml stock by dissolving 6mg Progesterone into 10ml Ethanol. Sterile filter and store in -80C. Make 17µl individual aliquots (use 1 vial per 500ml bottle)

Putrescine (Sigma P5780, 25g)

- Prepare a 160mg/ml stock by dissolving 1.6g Putrescine into 10ml dH₂O. Sterile filter and store. Make 50µl individual aliquots and store in -80C (use 1 vial per 500ml bottle)

Sodium Selenite (Sigma S5261, 25g)

- Prepare a 1.5mM stock by dissolving 2.59mg Na Selenite in 10ml dH₂O. Make 10µl individual aliquots and store in -80C (use 0.5 vial per 500ml bottle)

Hanna Lab - Human naïve PSC *in vitro* differentiation into PGCLCs **(Published in Irie/Weinberger et al. Cell 2015):**

1) Naïve human ESCs/iPSCs in ALL compositions indicated herein or (4i conditions and KSR based as indicated in Irie et al. Cell 2015, or NHSM, ENHSM or GNHSM), are either grown on irradiated mouse embryonic fibroblasts (MEFs) or feeder free vitronectin\gelatin coated plates or Laminin 521 coated plates.

2) The cells are trypsinized with 0.05% Trypsin + EDTA, and 300,000 cells/ 6-well are plated on 1ng/ml Fibronectin (F1141, Sigma) or vitronectin/gelatin coated plates (both work as equally well) in N2-KSR medium (250ml Neurobasal, 250ml DMEM-F12, N2 supplement, 1% KSR) medium with 8-10ng/ml bFGF (Peprotech), 1ng/ml TGF- β 1 (Peprotech) and ROCKi (10microM). This medium induces epiblast like (EpiLC) state. (Addition of B27 is optional – we do not see a meaningful benefit for including it) (Surani lab – plates 400,000 cells onto a 12 well plate – we see equal success of protocols) (20ng/ml Activin can be used instead of TGF β 1 and both give equivalent results.

3) After two days the EpiLC cells are trypsinized and moved to low-cell-binding 96-well plates (**145399 NUNC U bottom plates, NUNC Sphera 174925 U plates, or Corning 7007 U bottom plates**), at a density of 2500 cells/well, in 120ul PGC medium. PGC medium is comprised of GMEM, 15% KSR, 1% NNEA, Penicillin-Streptomycin, 1mM L-Glutamine, 5uM ROCKi and the following cytokines: 500ng/ml BMP4 (RnD), 20ng/ml LIF(Peprotech)(up to 1micrg/ml can be used) , 100 ng/ml SCF (Peprotech), 50ng/ml EGF (Peprotech). 50ul of fresh PGC medium was added to each well after two days. **The choice of plastic plate is extremely important as also seen with mouse (Saitou) differentiation protocol.**

4) Four or six days after the cells were moved to PGC medium, they can be separated using TripLE select (10x) (Invitrogen) for flow-cytometry, molecular analysis or tissue injection.

Hanna Lab - Human naïve iPSC microinjection protocol

1. Dissect oviducts of hormone primed and mated B6D2F1 females X B6D2F1 males, and extract zygotes (as routinely done with mouse micro-manipulation in our lab).
2. Culture zygotes for 2 days in KSOM medium droplets (Zenith Biotech KSOMaa Evolve cat # ZEKS-050) covered with mineral oil at 37C 20% O₂ or 5%O₂ incubator, until they develop to morula stage.
3. Grow human naïve cells to 70-90% confluence in (G)(E)NHSM medium on gelatin/vitronectin or gelatin/DR4 irradiated MEF coated plates.
4. The day before cell harvesting add 10 μ M ROCKi to the cells (in case not continuously used in the (G)(E)NHSM medium).
5. Trypsinize the cells for ~ 5 minutes with 0.05% trypsin, shake and pipette thoroughly to yield one cell suspension. Stop the reaction with DMEM+15% FBS and centrifuge at 1000RPM for 3 Minutes. Aspirate and discard medium.
6. Resuspended cells in 900 μ l (G)(E)NHSM medium, add 100 μ l filtered FBS (to reduce stickiness of cells) and 10 μ M ROCKi. Keep on ice until and during injections!!! It is preferable to inject the cells as soon as they are harvested.
7. Inject 5-12 human naïve cells to a mouse morula by using of Piezo (as routinely done with mouse ES injections). Include 10 μ M ROCKi also in M2 media throughout the injection period. (any drop that has naïve PSCs during injection should have ROCKi to increase cell survival during the process). (We use 15micron Piezo needles 15-15-MS for both mouse and human naïve injections).
8. After injection, incubate for 3-4 hours in KSOM droplets supplemented with 10 μ M ROCKi covered with mineral oil.
9. After 3-4 hours transfer the morulas to KSOM droplets (without ROCKi) covered with mineral oil, incubate over night. That way the morulas will develop into blastocyst.
10. The next day, most morulas should develop to blastocysts. Transfer 15-20 blastocysts to uterus of pseudo-pregnant B6D2F1 female mice.

Regarding human-mouse chimeric embryo data after micro-injection of human naïve GFP labeled iPSCs into host mouse Morulas (presented in Figure 4. and Extended Data Figure 10 – Gafni et al. Nature 2013), we now add previously obtained control experimental data that exclude issues of mouse cell contamination and/or auto-fluorescence. In **Figure 1** shown below, we validate co-localization of GFP expression and human-nuclear (HuNuc) antigen in E17.5 lung samples from chimeric E17.5 embryos, but not in un-injected mouse E17.5 embryo.

Figure 1. Generation of advanced cross-species chimaeric mice following naive human iPS cells microinjection into mouse morulas. Representative images showing integration of GFP-labeled human naive iPS-derived cells into different regions of an E17.5 embryonic lung. Lungs from hiPSCs-injected and un-injected E17.5 embryos were dissected, and paraffin sections were prepared and immuno-stained for anti-GFP (Green) and anti-human specific nuclear antigen (HuNuc) (Red, Anti-Human Nuclei Antibody, MP) antibodies. Specific detection and overlap between GFP and HuNuc were observed only in the lung sections of the hiPSCs-injected embryo derived sections (1st-2nd rows; 2nd row shows a zoom-in magnification of the region enclosed in square R1 in the 1st row). DAPI was used for counterstaining.

