Human trophoblast lineage characterisation with stem cell models : a dive into human placental development

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A dive into human placental development

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RÉSUMÉ EN FRANÇAIS

Cette thèse, intitulée « Étude du trophoblaste humain au moyen de modèles de cellules souches » - « Une plongée dans la placentation humaine » (en Anglais : « Human trophoblast lineage characterisation with stem cell models » - « A dive into human placentation »), est l'aboutissement de trois années de recherche doctorale sur la lignée trophoblastique à l'origine du placenta (partie fœtale) et sur les processus régissant sa formation. Elle s'articule autour du développement et de la caractérisation d'un nouveau modèle cellulaire, les cellules souches trophoblastiques humaines induites (hiTSCs) par reprogrammation de cellules somatiques, ou converties (hcTSCs) à partir de lignées pluripotentes (hiPSCs) de patients.

Ses objectifs, multiples, s'étendent de la compréhension des mécanismes moléculaires associés à la spécification du trophoblaste, dans une démarche de biologie fondamentale, à la mise en place d'un outil de recherche clinique à visée thérapeutique, pour le traitement des maladies placentaires et de l'infertilité, reconnue problème de santé mondial par l'Organisation Mondiale de la Santé (OMS).

Dans un premier temps (partie *Introduction*), nous avons resitué notre étude dans le contexte de l'évolution. Nous avons vu que l'émergence des Mammifères Placentaires est en lien direct avec l'apparition de la lignée trophoblastique, conséquence d'événements successifs d'intégration et d'exaptation de gènes rétroviraux, les syncytines, à l'origine du syncytiotrophoblaste. D'autres modifications génomiques ont accompagné cette évolution, comme la perte des vitellogénines ayant

entraîné la réduction du vitellus, contribuant également à la transition du développement embryonnaire externe *in ovo* (trait conservé par les Monotrèmes/Protothériens) au développement interne *in utero* (partiel chez les Marsupiaux/Métathériens, poursuivi dans la poche marsupiale, et complet chez les Euthériens).

Nous avons ensuite rappelé les principales étapes de la morphogénèse du placenta et leurs mécanismes moléculaires potentiels, souvent déduits des expériences génétiques dans l'embryon de souris, en comparant ces observations aux résultats obtenus chez l'homme dans un contexte normal ou pathologique.

Malgré certaines similitudes entre les deux espèces, comme l'implication de l'axe aPKC-YAP/TAZ-GATA3 dans la spécification du trophectoderme, nous avons vu que des différences notoires nécessitent d'étudier spécifiquement le développement embryonnaire humain. Un exemple parmi d'autres est le délai d'expression de *CDX2* et son caractère transitoire chez l'homme par rapport à la souris dans la lignée trophoblastique. Cela implique d'utiliser des modèles cellulaires du trophoblaste, afin de mener des expériences à grande échelle *in vitro*, avant de valider les résultats dans l'embryon humain, qui reste un matériel biologique relativement rare, soumis à la limite légale des 14 jours de culture.

Parmi les différents modèles présentés, nous avons souligné les nombreux avantages des cellules souches trophoblastiques humaines (hTSCs), dérivées pour la première fois par Okae, *et al.* (2018) à partir de *villi* chorioniques du premier trimestre

ou de blastocystes préimplantatoires. Contrairement aux lignées immortalisées à partir de choriocarcinomes ou aux autres cellules du placenta, les hTSCs sont tout à la fois génétiquement stables, capables de s'auto-renouveler indéfiniment et de générer les principaux types cellulaires trophoblastiques différenciés que sont le syncytiotrophoblaste et les trophoblastes extravilleux. Cela étant, le répertoire génétique des lignées d'hTSCs primaires est restreint par le fait qu'elles sont dérivées d'embryons FIV surnuméraires ou de placentas issus d'avortements, ce qui limite leur intérêt pour l'étude des maladies placentaires causées par des mutations. En d'autres termes, la nécessité de dériver les hTSCs à partir de ces tissus relativement peu accessibles rend difficile la génération de lignées pour des fonds génétiques spécifiques.

Le parallèle entre cette situation et les cellules souches embryonnaires humaines (dérivées dès 1998) avant la découverte des hiPSCs (2007) nous est apparu flagrant. Dès lors, le besoin de disposer d'hTSCs de patients a été un facteur déterminant pour le lancement de notre projet en 2018.

Deux observations majeures ont aussi largement contribué à initier cette étude. Premièrement, nous avons révélé par analyse transcriptomique que les quatre facteurs de transcription OCT4, SOX2, KLF4 et MYC (OSKM, ou facteurs de Yamanaka) sont tous très fortement exprimés dans l'embryon humain, avant la première spécification qui sépare le trophectoderme de la masse cellulaire interne. Deuxièmement, nous avons mis en évidence des événements de différenciation

trophoblastique spontanés dans les intermédiaires de reprogrammation, au cours de l'induction de la pluripotence naïve humaine.

Forts de ce constat, nous avons alors cherché à générer des cellules souches trophoblastiques humaines induites (hiTSCs) par reprogrammation de fibroblastes de patients avec les facteurs OSKM, ce à quoi nous sommes parvenus en quelques mois.

Nous avons ensuite voulu savoir si le potentiel de produire des hTSCs était spécifique aux intermédiaires de reprogrammation exprimant les transgènes, ou s'il était retenu par les hiPSCs établies, une hypothèse soutenue par la persistance d'événements rares de différenciation trophoblastique dans des lignées naïves. Pour déterminer cela, nous sommes partis de différents types de cellules souches pluripotentes : les naïves, donc, modèle de l'épiblaste préimplantatoire, les amorcées (ou « primed » en Anglais), équivalent de l'épiblaste postimplantatoire, et les pluripotentes à potentiel étendu, initialement décrites comme proches des blastomères, mais dont nous avons montré qu'elles sont en fait globalement similaires aux amorcées à l'échelle du transcriptome. Nous avons transféré ces cellules dans le milieu hTSC mis au point par Okae *et al.* (2018), et avons observé la formation d'hTSCs converties (hcTSCs) à partir de naïves et de pluripotentes à potentiel étendu, mais pas à partir des cellules amorcées. Nous en avons conclu que cela reflète une restriction progressive du potentiel de l'épiblaste au cours du développement péri-implantatoire chez l'homme.

Ces résultats, confirmés par analyse transcriptomique et validation fonctionnelle du potentiel de différenciation en syncytiotrophoblaste et en trophoblaste extravilleux, sont à l'origine d'un article publié dans Cell Reports en 2020, qui constitue la section *Résultats* (*Results*) de cette thèse.

Outre la nouveauté des hi/cTSCs et leur intérêt pour la recherche fondamentale ou clinique, un aspect important de cette étude a été la comparaison de ces cellules à l'embryon humain péri-implantatoire. Pour cela, nous avons tout d'abord effectué une analyse par réduction de dimensionnalité appelée UMAP (pour « Uniform Manifold Approximation and Projection » en anglais) sur des données de *single-cell RNA-Seq* d'embryons humains, du troisième au quatorzième jour de développement. Ce type d'analyse répartit les cellules dans l'espace selon leur proximité transcriptomique. En projetant l'annotation des jours embryonnaires sur la UMAP, nous avons confirmé qu'elle récapitulait précisément la temporalité du développement péri-implantatoire. Nous avons ensuite utilisé Monocle 3 pour déterminer les *clusters* de cellules au sein de l'embryon, ce qui a produit 19 *clusters*. En nous basant sur les profils d'expression de gènes spécifiques et sur la littérature, nous avons pu annoter ces *clusters* qui forment plusieurs branches cohérentes : l'épiblaste, l'endoderme primitif, le trophectoderme/trophoblaste, le syncytiotrophoblaste et le trophoblaste extravilleux. Nous avons alors extrait les gènes spécifiques de ces *clusters* grâce à l'aire sous la courbe (AUC pour « Area Under the Curve » en anglais), puis nous avons comparé les profils d'expression des types cellulaires de l'embryon avec ceux de nos modèles *in vitro*. Cette analyse a révélé la proximité des h(i/c)TSCs avec le trophoblaste au huitième jour du développement, bien que des investigations complémentaires

incluant notamment des trophoblastes du placenta plus tardif soient nécessaires pour conclure sur le stade développemental exact de ces cellules.

Nous avons ensuite discuté ces résultats plus en détail dans la partie *Discussion*, au regard de nouvelles expériences menées après publication. Nous avons d'abord utilisé nos nouvelles lignées d'hi/cTSCs pour étudier plus à fond la biologie des cellules souches trophoblastiques humaines. Par tests de culture sur différents substrats, nous avons mis en évidence des besoins spécifiques de ces cellules en termes de matrice extracellulaire, en lien avec leur profil d'expression spécifique de gènes codant des protéines d'adhésion, telles que les intégrines, affinant ainsi notre compréhension de la niche des hTSCs.

À cette occasion, nous avons constaté une certaine hétérogénéité cellulaire parmi les populations d'h(i/c)TSCs, à des degrés variables entre les lignées ou au sein même des colonies. Certaines cellules exprimant à des niveaux très élevés des marqueurs spécifiques tels que NR2F2, nous avons émis l'hypothèse qu'elles pourraient représenter des cellules souches à haut potentiel. À l'inverse, d'autres cellules caractérisées par l'expression de gènes comme CGB pourraient correspondre à des progéniteurs déjà engagés dans une voie de différenciation, faisant écho à l'efficacité variable des différentes lignées à générer du syncytiotrophoblaste ou des trophoblastes extravilleux, malgré leur bipotentiel avéré. Un autre aspect de l'hétérogénéité des h(i/c)TSCs que nous avons révélé est la coexistence de sous-populations exprimant des marqueurs associés à des stades développementaux distincts, tels que CD24. En effet, ce dernier est très fortement exprimé dans le

trophectoderme de l'embryon humain préimplantatoire, de façon spécifique, ce qui suggère que les lignées d'h(i/c)TSCs pourraient contenir des cellules plus précoces, plus proches du trophectoderme que les autres ressemblant au trophoblaste mature (jour 8).

Nous nous sommes également intéressés aux mécanismes moléculaires contrôlant la plasticité entre les cellules souches pluripotentes naïves ou à potentiel étendu et les cellules souches trophoblastiques humaines. Nous avons d'abord rappelé que la plasticité de destin cellulaire est une propriété observée dans l'embryon, par exemple dans le cas de la compensation réciproque du pluriblaste et du trophectoderme chez les Marsupiaux en cas de dommage, ou de façon similaire lors de la reconstitution de l'ICM, après ablation, par les cellules du trophectoderme chez la souris et l'homme. Une hypothèse intéressante est que ce mécanisme de « réparation » embryonnaire pourrait conférer un avantage évolutif à ces espèces, et que la plasticité cellulaire observée *in vitro* en serait le reflet, ce qui reste à démontrer.

Ensuite, grâce à des approches complémentaires d'analyse en composantes principales (ACP) sur voies de signalisation (*pathway eigengenes*) et de WGCNA (pour « Weighted Gene Correlation Network Analysis » en anglais) générant des modules de gènes coexprimés, nous avons identifié de nouveaux processus biologiques associés à cette plasticité au cours de la spécification des hTSCs, dans les contextes de conversion de cellules pluripotentes et de reprogrammation de

cellules somatiques. Ces résultats, confirmés par analyse d'expression de gènes marqueurs et par *western blot*, montrent que certains modules, et certains gènes en particulier sont uniquement exprimés dans les intermédiaires de reprogrammation ou de conversion capables de produire des hTSCs. C'est notamment le cas de *TACSTD2* et *HAND1*, retrouvés dans le trophoblaste humain *in vivo*. À l'inverse, d'autres modules et certains gènes, pourtant spécifiques des hTSCs *in vivo*, sont systématiquement induits dans tous les modèles de conversion trophoblastique, y compris à partir des hPSCs amorcées (« primed »), ce qui est le cas de *NR2F2* par exemple. Ces observations suggèrent que des processus distincts agissent en parallèle pour spécifier les hTSCs. Considérés dans leur ensemble, nos résultats indiquent que la répression des voies de signalisation de NODAL et MTOR, et l'activation combinée de la signalisation médiée par BMP4 et le calcium convergent sur la coexpression de KLF4, GATA3 et GRHL3, trois facteurs de transcription possiblement impliqués dans l'épithélialisation à l'origine des hTSCs.

Enfin, nos travaux sur la plasticité des cellules souches pluripotentes naïves ont contribué au développement des premiers blastoïdes humains, un modèle de l'embryon formé à partir de ces cellules, qui récapitule des événements morphocinétiques et des fonctions clefs du blastocyste péri-implantatoire tels que la cavitation, l'implantation par le trophectoderme polaire, et l'ébauche de la cavité amniotique. En effet, les hiPSCs naïves ont le potentiel de générer non seulement un analogue de l'épiblaste, mais également de l'endoderme primitif et, comme l'ont suggéré nos résultats, du trophectoderme, avec la particularité de s'auto-assembler

en trois dimensions et de positionner correctement ces trois types cellulaires dans l'espace. Les blastoïdes humains ainsi formés peuvent être rapidement produits en très grandes quantités (70% d'efficacité, réplicats par centaines) à partir de n'importe quel fond génétique *a priori*. Non seulement cette capacité de production inégalée augmentera considérablement la reproductibilité expérimentale, mais encore ce nouveau modèle autorisera de nombreuses manipulations jusqu'alors très limitées dans l'embryon humain, comme l'édition de génome par exemple. Par ailleurs, à la différence des modèles cellulaires « classiques » du trophoblaste, les blastoïdes offrent la possibilité d'étudier l'interaction entre les compartiments embryonnaire et extra-embryonnaire, en particulier entre l'épiblaste et le trophectoderme polaire, que l'on pense être déterminante pour le succès de l'implantation de l'embryon dans l'endomètre.

Nous terminons cette thèse en proposant des perspectives à nos travaux, qui permettent la génération d'hi/cTSCs spécifiques de patients, accédant ainsi à des fonds génétiques variés, incluant notamment ceux associés aux dysfonctions placentaires. Nous pensons donc que ce modèle contribuera à améliorer notre compréhension de la placentation humaine, dans un contexte normal ou pathologique, avec des applications cliniques possibles pour le traitement de ces maladies, entre autres. Enfin, les protocoles de reprogrammation et de conversion que nous avons élaborés offrent l'opportunité d'étudier les mécanismes régissant la spécification des cellules souches trophoblastiques humaines, notamment grâce à la plasticité des hPSCs naïves mise en évidence par nos travaux et la mise au point du modèle

blastoïde humain. Ce dernier représente une avancée majeure dans notre domaine de recherche, et permettra de mieux comprendre le développement embryonnaire péri-implantatoire chez l'homme, avec un intérêt clair pour la médecine de la reproduction.

Do not go gentle into that good night,
Old age should burn and rave at close of day;
Rage, rage against the dying of the light.

Dylan Thomas - 1914-1953

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ABSTRACT

This thesis, entitled "Human trophoblast lineage characterisation with stem cell models", focuses on cell type specification events occurring in the early human embryo and related molecular mechanisms involved in the formation of the placenta.

These aspects are crucial for basic research and carry an elevated discovery potential, as major differences distinguish the embryonic development of humans from those of other model organisms, while the human embryo has remained largely unexplored so far.

Moreover, this topic is important for clinical research, as it may contribute to improving assisted reproduction techniques (ART) and treatment of placental dysfunctions, such as preeclampsia.

A big issue surrounding this field of research is the limited access to human embryos, as legal restrictions limit their culture *in vitro* (14-day rule) and the range of possible experiments. As a consequence, most processes driving the formation of the human placenta, initiated by day 5 in the trophectoderm and pursued during the first trimester of pregnancy, could not be investigated in depth. Therefore, this time window of development is a black box regarding the molecular mechanisms involved.

To overcome these limitations, various cellular models of the human

trophoblast have been developed *in vitro*, each having its own assets and drawbacks. Recently, successful derivation of human trophoblast stem cells, 20 years after their equivalent in the mouse, has been a breakthrough in this research area, allowing to study the complex molecular mechanisms regulating primary trophoblast progenitors in human.

However, despite these advances, an important aspect is missing: the access to patient specific trophoblast stem cells, notably those related to placental diseases.

Regarding the embryonic lineage, the discovery of iPSCs obtained by somatic cell reprogramming has allowed to generate numerous cell lines from patients, which have proven useful to investigate molecular mechanisms underlying diseases, as these cells are able to generate multiple differentiated cell types *in vitro*.

Therefore, we applied somatic cell reprogramming and cell fate conversion approaches to generate human induced/converted trophoblast stem cells (hiTSCs) from patients' fibroblasts or iPSC lines. After testing various culture conditions, we elaborated an original protocol for producing such cells.

We then characterised these induced stem cell lines using RNA-Seq for transcriptomic analysis, and tested their differentiation potential into syncytiotrophoblast and extravillous trophoblast cells.

Once we validated the trophoblastic identity of these cells, we further

investigated molecular mechanisms underlying cellular plasticity with embryonic lineages. We thereby revealed three processes associated with the establishment of human induced trophoblast stem cells: pluripotency gene silencing, induction of the trophoblast program from key transcription factors, and epithelialisation of hiTSC colonies, required for self-renewal.

We also uncovered properties of human trophoblast stem cells related to cellular heterogeneity within colonies, and requirements of specific extracellular matrix compounds and signalling molecules for these cells to self-renew or differentiate properly.

Finally, our work on naive pluripotent stem cell plasticity with the trophoblast lineage contributed to developing a stem cell-based model of the embryo, namely human blastoids. These 3D structures mimicking the early human embryo are produced in large amounts *in vitro*, thus opening avenues to large-scale experiments. Among others, they allow genetic manipulation required for validating presumptive mechanisms of embryonic development in humans.

In conclusion, our study provides new insights into the biology of human trophoblast stem cells and cell fate plasticity with pluripotent lineages. We have designed an original platform to generate patient specific induced trophoblast stem cell lines, that can be used for investigating processes of normal *vs* pathological development of the human placenta, with possible applications to improving IVF techniques and treatments of placental diseases.

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1 INTRODUCTION

1.1 Placenta: a keystone of Therians evolution

This story starts in the far, wild lands of Australia. There, living beings have evolved in an environment separated from the rest of the world, often yielding strange things, at least from a European perspective. Among the islanders, a timid creature is dabbling in the calm waters of Broken River. The layman would think of a beaver, others might see a duck, but biologist experts will unambiguously recognize a Platypus. Force is to admit that this rare animal is confusing, with a tail and the fur of a beaver crowned by a duck face, literally speaking, with a beak. People might think the overall appearance is a bit exaggerated, but the Platypus is kind of an eccentricity in the broad panel of evolution, and its most surprising trait is not physical, but about its mode of reproduction: this Mammal lays eggs. It sounds strange, as we all more or less have learnt, during our biology classes, that Mammals carry their offspring into their uterus, from fertilization in a fallopian tube up to parturition. Instead, Platypus embryonic development, which initially depends on uterine nutrition like other Mammals, rapidly switches to a yolk-based nutrition, as the yolk keeps growing, while a rigid shell is deposited around the conceptus, to later protect the egg from desiccation once it is laid. In fact, four other mammalian species from Australia and New Guinea, dubbed Echidnas, share this mode of reproduction with the Platypus. They are close cousins, and together these species form the order of Monotremes, which is about 166 million years old (Heider *et al*, 2011).

The peculiar embryonic development of Monotremes is in part permitted by meroblastic divisions of the zygote, a trait shared with Birds and Reptiles, yielding formation of the yolk, roughly a stock of maternal proteins and nutrients on which the conceptus relies for external development. Meanwhile, Monotreme embryos develop *in utero* until the 19-20 somite stage, before eggs are laid and hatch 10-12 days later. Resemblance with avian and reptilian development includes other features, such as a latebra connected to the yolk bed beneath the germinal disc and a striate zone beneath the *zona pellucida* (Carter, 2021). Even on the scale of genetics, Monotremes share similarities with Reptiles: their genome is dominated by LINE2 repeat elements, while Therians are mostly full of LINE1 (Zhou *et al*, 2021) ; nutritional stock of their yolk is predominantly made of vitellogenins, proteins encoded by the *vtg* gene family, abundant in other egg-laying Vertebrates. While Monotremes have conserved an active copy of *VTG2/VIT2* and an eroded sequence of *VTG1/VIT1*, homologues of *vtg* genes in viviparous Mammals have been degenerated by frame-shift mutations and premature stop codons (**Figure 1A**) (Brawand *et al*, 2008 ; Zhou *et al*, 2021). Consequently, Marsupials have little yolk, while it is almost missing in Eutherians (Selwood & Johnson, 2006).

Then, why are these Monotremes considered Mammals? Because of a unique feature, of utmost importance: lactation. Indeed, after they hatch from their eggs, the fragile Monotreme newborns are dependent on maternal nourishment to survive, which is provided by the colostrum and the milk at that time. This is likely the most fundamental trait common to all Mammals, which truly defines them. Colostrum and later milk from

the mother not only provide essential nutrients and antibodies, critical to protect immune naive neonates from pathogens, but they also have contributed to develop an intimate, strong link between the mother and the newborns, probably key to Mammals' spreading across the world. Genes encoding the major milk proteins are called caseins. They were present in the common ancestor of Mammals, prior to the loss of genes encoding vitellogenins, which suggests that the emergence of lactation contributed to the gradual loss of yolk-dependent nourishment during mammalian evolution (Brawand *et al*, 2008).

However, throughout the mainly external development of Monotremes, a key feature of most Mammals is missing: the placenta. Then why are we mentionning Monotremes in a thesis dedicated to placental development? To make a long story short, this is precisely the absence of this trait in Monotremes that makes them particularly suited for understanding how the placenta emerged during evolution, about 150 million years ago (Zhou *et al*, 2021).

The placenta is a unique organ, formed by the conceptus through differentiation of the trophoblast cell lineage into a complex interface with the maternal uterus. While other extraembryonic annexes, such as allantois, amnion and yolk sac are common to all Amniotes (comprising Reptiles, Birds and Mammals), the placenta is specific to Therians and has been key to the evolution from an oviparous to a viviparous embryonic development in these animals, allowing for longer development notably producing a larger fetal brain. Not only as individuals, but as species, this important head start given to the mammalian young might have been key to the radiation of

Placental Mammals worldwide. On the scale of embryonic development, the placenta has provided such a huge evolutionnary advantage to Therians that it has progressively superseded the yolk sac for nutritional functions. As a consequence, the yolk sac, critical for Monotremes' development, has regressed into a vestigial structure in Therians, despite a remnant role in primitive hematopoiesis and germline induction (Ross & Boroviak, 2020).

It has been suggested that the distinct mammalian groups, showing apparently gross developmental differences, are in fact close in terms of cell lineages, while macroscopic divergence results from differential timing of two mechanisms: cell adhesion and polarization in the embryo (Selwood & Johnson, 2006). In line with this, Monotremes seem to possess a trophoblast cell lineage, although they do not produce a placenta. At the 16-cell stage, 10 to 12 cells called marginal vitellocytes are pinched off the growing yolk, following the 4-5 central pluriblast cells (the progenitors of the fetus, equivalent to the epiblast of Eutherians). Vitellocytes are thought to be homologous to the pericytes of Birds and the meroblast of Reptiles. However, vitellocytes exclusively form a multinucleate syncytial structure surrounding the pluriblast called the germ ring, which ensures nutritional transport from the yolk to the embryo proper. Structural and functional similarities with the syncytiotrophoblast of Therians might indicate a common trophoblast lineage identity, although the germ ring in Monotremes utilises yolk instead of maternal blood as a source of nutrients. However, the molecular process of syncytialisation differs, possibly involving endoreplication in Monotremes, which could partly explain why these species do not generate placental structures (Niwa *et al*, 2008).

To get further insight, we are now considering another rare mammalian order: Marsupials. This order appeared between 143 and 178 million years ago (Luo *et al*, 2011 ; Phillips *et al*, 2009) and comprises the famous Koalas, Kangaroos and Oppossums, among others. Like Monotremes, Marsupials are mostly found in Australia. However, several species of Opossums are also found in South America, which might indicate a better adaptation of Marsupials relative to Monotremes. Notably, their mode of reproduction appears slightly more advantageous. Indeed, unlike Monotremes, Marsupials possess a placenta, although it is short-lived and underdeveloped compared to Eutherians. Therefore, although these animals give birth to altricial noenates, their basic placenta ensures a prolonged development *in utero* relative to Monotremes. As a consequence, Marsupials have been freed from completing external development within eggs, which has dramatically increased their offspring's survival. Instead, the fragile newborns keep growing inside a pouch, which promotes a larger fetal brain development (Roberts *et al*, 2016).

What happened to Marsupials? Nothing less than an extraordinary example of exaptation, an evolutionary process by which features acquire functions for which they were not originally adapted or selected. One day, a retrovirus infected the common ancestor of present Marsupials, but instead of being eliminated, its genetic material was integrated into germline DNA and vertically transmitted to the offspring (Cornelis *et al*, 2015). Among others, retroviruses have an envelope gene (*env*), encoding a precursor that is cleaved into a surface unit (SU) and a transmembrane protein (TM). SU binds a target cell through receptor interaction, while TM promotes virion entrance through the

fusion of its envelope with cell membrane (Lavialle *et al*, 2013). Often, TM also contains a short motif with immunosuppressive properties, allowing the virus to hide from the host's immune system. Therefore, integrated *env* gene should have initially promoted retroviral infection of the host. However, once captured in the offspring's genome, it has evolved into a captive gene called a syncytin, progressively redirected to the formation of a multinucleated placental layer: the syncytiotrophoblast. It is quite amazing how evolution has taken advantage of originally deleterious fusogenic and immunosuppressive viral activities. For the first time in the mammalian lineage, acquisition of syncytiotrophoblast has ensured direct transport of nutrients, waste and gases, along with immune tolerance between the developing conceptus and the maternal organism, truly connecting them.

The emergence of Eutherians, with a much more complex placenta, partly results from successive acquisitions of additional syncytin genes (**Figure 1B**). A phylogenomic reconstruction has revealed that these insertion events have occurred seven times independently during the evolution of Therians (Malik, 2012). This is a striking example of convergent evolution, which further illustrates the selective advantage of possessing a complex placenta. The oldest known syncytin gene is *Syncytin-Car1*, which integrated the genome of present Carnivora's common ancestor, about 60 million years ago (Cornelis *et al*, 2012). In contrast, the *syncytin-1* gene was domesticated about 25 million years ago and is specific to primates. In Marsupials, the *Syncytin-opo-1* gene was acquired about 20 million years ago (Cornelis *et al*, 2015). Remarkably, the diversity of syncytin genes mirrors the broad panel of placental

structures, which comprise the hemochorial, endotheliochorial and epitheliochorial subtypes, among others (Lavialle *et al*, 2013). In contrast to Marsupials and Eutherians, Monotremes do not have syncytin genes. From this perspective, the successive acquisitions of syncytin genes might have conferred a selective advantage upon Placental Mammals within diverse ecological niches.

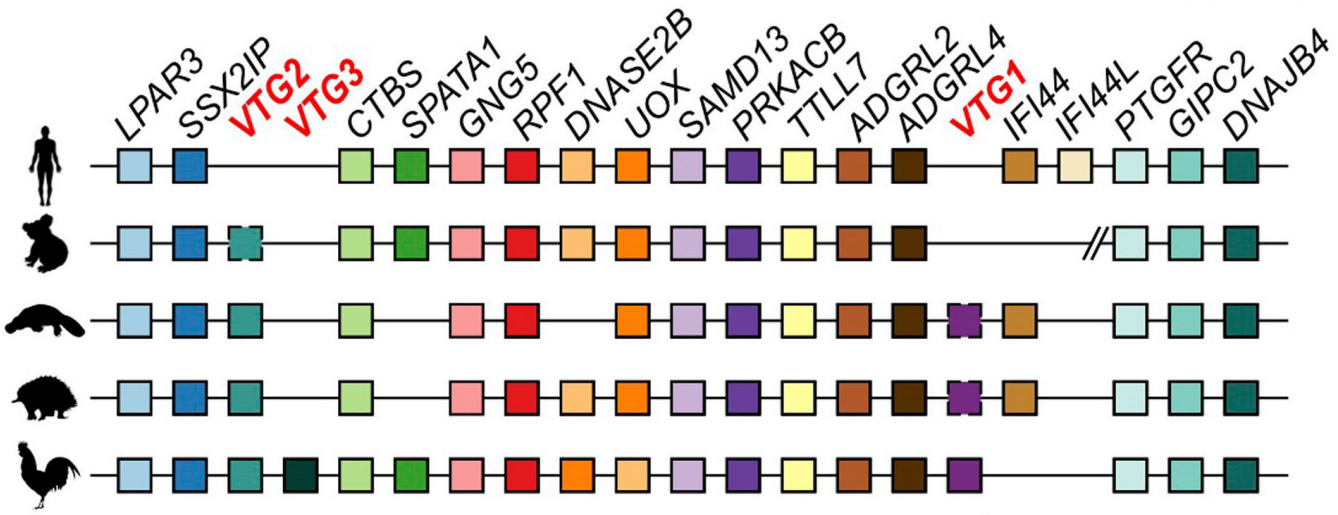
Another gene, *Peg10* (standing for *Paternally Expressed Gene 10*), is specific to Placental Mammals (Edwards *et al*, 2019 ; Ono *et al*, 2006). Like syncytins, it originates from retroviral integration and is critical for placental labyrinth and spongiotrophoblast formation in the mouse (Ono *et al*, 2006). *Peg10* is maternally imprinted by high methylation of CpGs, resistant to DNA demethylation waves in the early embryo, and thus only the paternal allele is expressed. On the scale of mammalian evolution, an hypothesis has emerged that paternal and maternal genomes are somehow in conflict for spreading to descendants. For both, genetic mixing advantageously promotes heterozygosity, dominance of "strong" alleles and reduces risks of genetic disorders. To this end, it seems beneficial for males to fertilize as many females as possible, and for females to reproduce with multiple partners. However, for a given offspring, the paternal involvement is rather low compared with the huge energetic cost for the pregnant female, especially in Placental Mammals. Therefore, paternal alleles of placental genes could opportunistically evolve to promote access of embryos to the maternal resources, which increases chances of successful development and transmission of the male genome. To counter it, maternal alleles could evolve conversely to limit energetic cost of each offspring, while promoting reproduction with multiple partners. In

line with this hypothesis, genomic imprinting seems to be unique to Placental Mammals, resulting from exaptation of retrotransposon silencing by DNA methylation (Suzuki *et al*, 2007).

In conclusion, the placenta has emerged during mammalian evolution, resulting from successive retroviral integration events, on the background of a preexisting trophoblast cell lineage. Its formation involves two major mechanisms related to infection by retroviruses: cell fusion and retrotransposon silencing by DNA methylation, which have been redirected to syncytialisation and genomic imprinting, respectively.

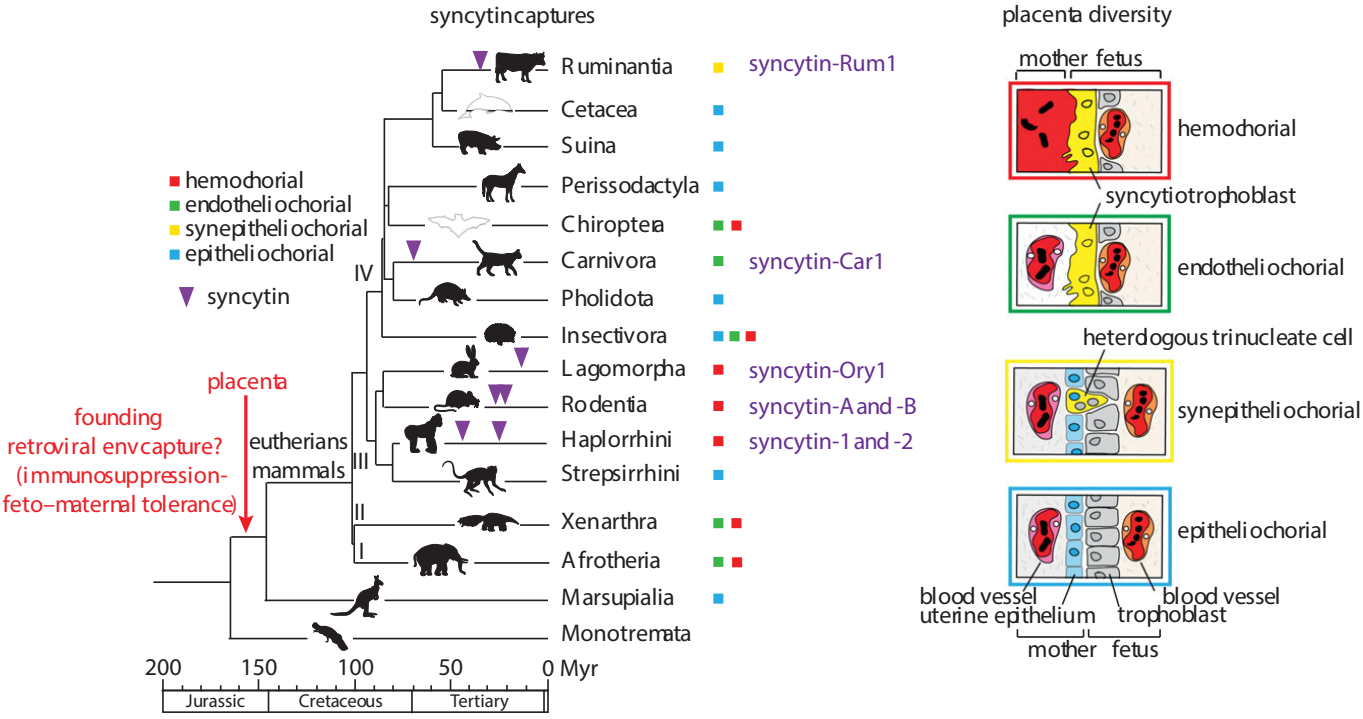
Figure 1: Genome evolution studies reveal the molecular basis of the emergence of placentation in Mammals.

A



Adapted from Zhou *et al*, 2021

B



Adapted from Lavialle *et al*, 2013

Figure 1. Genome evolution studies reveal the molecular basis of the emergence of placentation in Mammals.

(A) The progressive loss of vitellogenin genes (VTG1, VTG2, VTG3) accompanies the transition from an external to an in utero embryonic development, and from a dependence on the yolk to the direct consumption of maternal blood resources.

(B) Phylogenetic tree of Mammals, showing the multiple insertion events of retroviral syncytin genes during evolution. Estimated time of capture is indicated on the timeline below the graph (unit: million years). Roman numbers indicate the four major clades of Eutherians, globally characterised by distinct placental structures, illustrated on the right.

1.2 Ontogenesis of the human placenta

After considering the placenta on the scale of evolution, we will now focus on the main topic: the human placenta. As an introduction, we will recapitulate the main aspects of human embryonic development, which is prerequisite to this study.

Every human life starts with the formation of a zygote, resulting from the fertilisation of an oocyte by a spermatozoa (**Figure 2A**). This unique cell, capable of yielding a complete multicellular organism, is defined as totipotent. The zygote is protected from polyspermy and ectopic implantation by a thick layer called the *zona pellucida* (*ZP*), conserved through evolution while originally preventing interspecies cross-breeding, which can occur when oocytes are released into external environment, like aqueous medium. During three days, the zygote undergoes three successive cleavages forming blastomeres, while the embryo migrates through the fallopian tube (Niakan *et al*, 2012). This is followed by the compaction of the embryo (8- to 16-cell stage) which forms a morula, a stage characterised by a reduction in cellular size and internalisation of prospective ICM cells (Shahbazi, 2020). Subsequently, intercellular spaces start to fill with uterine liquid, first producing microlumens that ultimately coarsen to form a single cavity called the blastocoel. This results by day 5 in the formation of a blastocyst comprising an inner cell mass (ICM), from which the fetus and its immediate membranes will arise (*i.e* amnion, yolk sac, allantois, and mesodermal components of the placenta), and a peripheral layer of cells called trophectoderm (TE), which later produces the placenta.

Between days 6 and 7, the blastocyst hatches from the *ZP*, broken by internal pressure of blastocoel expansion, and implants into the uterine endometrium (**Figure 2B**). Meanwhile, the TE rapidly matures at the embryonic pole, mediating implantation, and by day 8 produces a multinucleate cellular expansion called the primitive syncytium (**Figure 2C**). The main function of these cells is to invade the maternal decidua, promoting endometrial erosion, further decidualization and embryo attachment, during the second week of development (Knöfler *et al*, 2019 ; Jaremek *et al*, 2021). The primitive syncytium rapidly surrounds the embryo, digesting uterine glands and capillaries called uterine sinusoids to form lacunae filled with maternal blood (**Figure 2D**). This process allows the active uptake of maternal nutrients and increases oxygen diffusion to the rapidly growing embryo. In the meantime, underlying cytotrophoblasts (CTBs) proliferate and form primary chorionic villi, which break through the primitive syncytium and connect to each other, ultimately surrounding the embryo and forming the cytotrophoblast shell by days 13-14 (**Figure 2E**).

This structure anchors the conceptus deeper into the decidua basalis, while chorionic *villi* further branch extensively, increasing exchange surface with maternal blood. Again, fusion of peripheral CTBs produces a multinucleate outer layer, forming the definitive syncytiotrophoblast. At the apex of chorionic villi, column CTBs differentiate into migrating cells called extravillous trophoblasts (EVTs), which invade deeper the maternal decidua, contacting and remodeling spiral arteries. At this stage, the syncytiotrophoblast entirely bathes in maternal blood, which defines the hemochorial placenta, one of the most connective placental organisations. Subsequently,

the extraembryonic mesoderm, originating from the primitive endoderm (Spencer Chapman *et al*, 2021), infiltrates chorion and forms secondary villi. Ultimately, the allantois-derived umbilical vessels fuse with the chorionic plate, penetrate chorion and form definitive tertiary villi. Altogether, these events yield a large network of fetal capillaries, which by the end of the first trimester constitute the definitive feto-maternal interface.

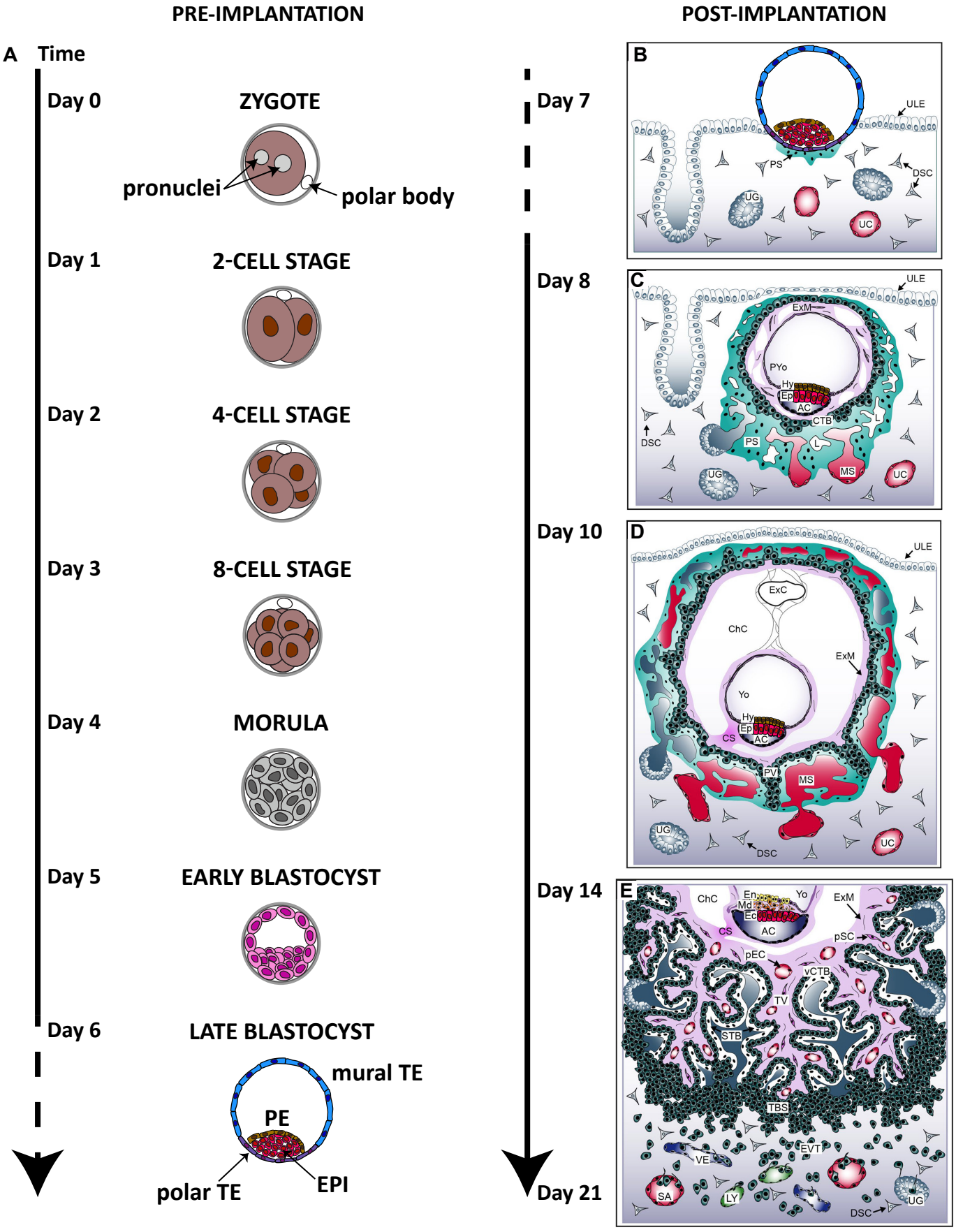
The mature placenta fulfills a wide range of functions, normally ensured by multiple organs in the adulthood, which illustrates the complexity of this organ. These include nutrient and oxygen intake, hormone secretion and metabolic waste elimination (the fetus excretes carbon dioxide, water, urea, hormones, and other waste product), among others (Burton & Fowden, 2015). The large syncytiotrophoblast surrounding the whole chorionic villous tree reaches 12-14 m^2 at term. It acts as a physical barrier to prevent entry of pathogens in the fetal circulation, which is facilitated by the absence of intercellular junctions throughout this layer. Only desmosomes at the basal side ensure the efficient transport of nutrients, while oxygen enters by diffusion. Also, ST surface is covered by microvilli that considerably increase the contact area, further facilitating exchanges. Moreover, contractile tonofilaments of cytokeratins, notably the pan-trophoblast KRT7, scatter through the syncytioplasm, anchored to basal desmosomes, promoting fluidic movements that increase diffusion of nutrients.

Throughout early steps of placental development, a pool of trophoblast stem cells and proliferative progenitors, residing in the CTB layer of the chorionic villi, ensure

organ growth, proper organisation and homeostasis (Chang & Parast, 2017 ; Hemberger *et al*, 2010; Li & Parast, 2014; Nandi *et al*, 2018 ; Okae *et al*, 2018). However, from the mid second trimester onward, the placenta progressively changes. Villous cytotrophoblasts cease to proliferate but continue to fuse, thus the CTB compartment decreases while the ST thickens. At some point, the cytotrophoblast layer is so diminished that it can no longer renew the syncytiotrophoblast. Markers of senescence and inflammation are observed in the myometrium, decidua and cervix, which coincides with normal pregnancy term. These properties of the ageing placenta, no longer able to sustain maternal immune tolerance of the fetus, contribute to parturition (Menon, 2019).

In conclusion, the extremely complex but yet finely controlled formation of the human placenta largely contributes to the success of gestation.

Figure 2: Anatomical description of human peri-implantation development, with a focus on placental ontogenesis (first three weeks).



Adapted from Niakan *et al*, 2012

Adapted from Knöfler *et al*, 2019

Figure 2. Anatomical description of human peri-implantation development, with a focus on placental ontogenesis (first three weeks).

(A) Pre-implantation window of human embryonic development. From top to bottom: fertilisation of the oocyte by the sperm cell (day 0) ; the totipotent zygote undergoes a series of cleavages, yielding the 2-cell (day 1), 4-cell (day 2), and 8-cell stages (day 3), while blastomeres are supposedly still totipotent ; on day 4, the embryo undergoes compaction, yielding the morula with differential apico-basal polarity between outer cells (polar) and inner cells (apolar), at the origin of TE *vs* ICM specification ; by day 5, fluid-filled cavities fuse and cavitation of the embryo produces the blastocyst, composed of an inner cell mass encircled by a trophectoderm layer surrounding the blastocoel (cell types at this stage are supposed to be specified but not committed yet in humans) ; by day 6, the first three lineages rapidly emerge, comprising the epiblast (EPI), that will generate the fetus and the amniotic ectoderm, the trophectoderm (TE), that will produce the fetal part of the placenta, and the primitive endoderm (PE) yielding the yolk sac.

(B) By days 6-7 post-fertilisation, the embryo implants into the uterine endometrium, through the polar trophectoderm (the presumptive precursor of mature trophoblast lineages). At the site of implantation, the primitive syncytium formation is visible.

(C) The embryo next burrows into the uterine wall, and is progressively encircled by the primitive syncytium, which starts disrupting endometrial sinusoids for supplying the growing conceptus with maternal blood resources (nutrients, oxygen...).

(D) At this stage (around days 9-10), the conceptus bathes in maternal blood lacunae, and proliferative cytotrophoblasts form primary villi that breaks through the primitive syncytium.

(E) By days 13-14, chorionic villi connect to each other, forming the trophoblastic shell that surrounds the embryo. Meanwhile, the extraembryonic mesoderm, supposedly originating from the primitive endoderm in humans, pushes through the primary chorionic villi and forms secondary villi. From week 3 to 7, allantois-derived umbilical vessels then fuse with the chorionic plate, penetrate the loose connective tissue core and connect up with embryonic blood vessels, establishing the embryonic blood circulation. Ultimately, the tertiary chorionic villi are composed of three trophoblast layers: villous cytotrophoblasts (including the proliferative compartment of hTSCs), the multinucleated syncytiotrophoblast (the site of hormone secretion and exchanges with the maternal blood), and extravillous trophoblasts that invade the decidua, anchor the conceptus, and further remodel the spiral arteries.

Abbreviations: ICM: Inner Cell Mass ; EPI/Ep: epiblast ; PE/Hy: primitive endoderm/hypoblast ; TE: trophectoderm ; ULE: uterine luminal epithelium ; PS: primitive syncytium ; UC: uterine capillaries ; UG: uterine glands ; DSC: decidual stromal cell ; AC: amniotic cavity ; PYo: primitive yolk sac ; ExM: extraembryonic mesoderm ; MS: maternal blood sinusoids ; L: lacunae ; CTB: cytotrophoblasts ; Yo: yolk sac ; ChC: chorionic cavity ; ExC: exocoelomic cyst (remnant of the transient primitive/primary yolk sac) ; CS: connecting stalk ; PV: primary chorionic villi ; Ec: ectoderm ; Md: mesoderm ; En: endoderm ; (Ec, Md, and En form the three germ layers during gastrulation, from day 14 to 21) ; TV: tertiary chorionic villi ; pEC: placental endothelial cells ; pSC: placental stromal cells ; vCTB: villous cytotrophoblast ; TBS: trophoblastic shell ; STB: syncytiotrophoblast ; EVT: extravillous trophoblasts ; SA: spiral arteries (maternal) ; VE: venous vessels (maternal) ; LY: lymphatic vessels (maternal).

1.3 Molecular mechanisms of placentation

The anatomical description of embryonic stages and placental tissue sections has provided valuable knowledge of the developmental timing and sequence of events during human placentation. By contrast, our comprehension of the molecular mechanisms governing these processes is lamentably poor. This situation is largely imputable to a combination of two factors: the limited access to human embryos for research, and the even more limited range of experiments authorized on the human embryo.

As a consequence, presumptive molecular mechanisms of placental development have been extrapolated mostly from the mouse embryo, which represents a limitless source of experiments, notably for genetic manipulation. Despite some similarities with human (*e.g.* hemochorial organisation, discoid shape...), the mouse placenta has important differences: two layers of syncytiotrophoblast, a single cotyledon, and a labyrinth instead of chorionic villous tree, among others, which are likely to come out of species-specific molecular processes. As an example, the *Eomes* gene is required for the proper maturation of mouse TE into TSCs, while it is poorly expressed in human trophoblast and seems dispensable for this purpose (McConnell *et al*, 2005 ; Okae *et al*, 2018 ; Russ *et al*, 2000). In this section, we summarise molecular mechanisms uncovered in the mouse, evaluating how far they could apply to human placentation.

Onset of TE specification deciphered at the molecular level

Initiation of TE cell specification in the mouse relies on morphokinetics events of embryo compaction, differentially positioning outer and inner cells within the morula. An asymmetric distribution of apical proteins and mechanical constraints yield apico-basal polarity in outer cells. Within apical domains, proteins such as aPKC-Par6-Par3 forming a complex bind and sequestrate components of the Hippo pathway, like Amot, Lats1/2 and Nf2. Their function is thus inhibited and the Hippo pathway is turned off in these prospective TE cells. Consequently, dephosphorylation of Yap1 and Taz/Wwtr1 frees them from cytosolic retention by 14-3-3 proteins, and provokes their translocation to the nucleus. In turn, they associate with Tead4, binding and activating target genes of the TE program, notably *Cdx2* and *Gata3* (Manzanares *et al*, 2013).

These events have been monitored by immunostaining, hierarchy being evaluated by genetic manipulation in the mouse embryo. Either knockdown (KD) of *Pard6b* or knockout (KO) of aPKC subunit genes (PKClambda-/-:PKCzeta-/-) disrupts the aPKC-Par6-Par3 complex, turning on Hippo pathway in outer cells, which yields phosphorylation and cytosolic restriction of Yap1. Therefore, the TE program is not initiated in these cells, illustrated by the reduction of *Cdx2* expression (Alarcon, 2010 ; Hirate *et al*, 2013). Disruption of apico-basal polarity is made obvious by the basolateral marker *Scribble* relocating all along the plasma membrane in these cells, thus resembling inner cells.

Neither loss of *Yap1* nor that of *Wwtr1/Taz* impacts TE formation

pre-implantation, but development is arrested by E8.5 in *Yap1-/-* embryos, showing defects in chorioallantoic fusion, yolk sac vasculogenesis, and embryonic axis elongation, while *Wwtr1/Taz-/-* mutants either die by the age of weaning (unknown cause, 35-50%) or have minor skeletal defects and renal cysts in the adulthood, for those surviving (Hossain *et al*, 2007 ; Morin-Kensicki *et al*, 2006). However, combined deletion of *Yap1* and *Wwtr1/Taz* provokes embryonic lethality prior to the morula stage, highlighting the critical yet redundant function of these genes in the onset of the TE program (Nishioka *et al*, 2009). *Yap1* and *Wwtr1/Taz* are homologues of the Drosophila Yorkie gene and result from gene duplication. Redundancy might seem to be a waste of energy, but given that genetic drift has fixed both genes during mammalian evolution, it rather suggests an important compensatory mechanism, safeguarding early embryonic development.

To refine the analysis of Yap1 function during this period, a dominant negative isoform has been engineered. Its transcriptional activation domain was replaced by a repression domain, and serine-112 by alanine, which impairs the phosphorylation of Yap1 at this site, thus promoting nuclear translocation and activity. Overexpression of dominant negative Yap1 by RNA injection into 2-cell-stage mouse embryos significantly reduces *Cdx2* expression pre-implantation, in a dose-dependent manner. Consistently, the overexpression of *Lats2*, which phosphorylates Yap1 and Wwtr1/Taz, greatly reduces nuclear translocation of Yap1 and *Cdx2* expression in outer cells (Nishioka *et al*, 2009).

As mentioned previously, Yap1 and Wwrt1/Taz physically interact with Tead4 through a C-terminal domain highly conserved in TEAD proteins. Phenotypically, most

Tead4-/- mutants fail to form a blastocoel and die before the blastocyst stage, without implanting. Functionally, embryonic stem cells can be derived from these embryos but not trophoblast stem cells (Nishioka *et al*, 2008 ; Ralston *et al*, 2010 ; Yagi *et al*, 2007). Such embryos show an almost complete silencing of *Cdx2* and *Gata3* (Nishioka *et al*, 2008 ; Ralston *et al*, 2010 ; Yagi *et al*, 2007). Nonetheless, detecting low levels of Cdx2 and Gata3 in some rare mutants, which initiate blastocoel formation, could mean that Tead4 maintains rather than induces the TE program (Ralston *et al*, 2010 ; Stamatiadis *et al*, unpublished). However, this faded expression might simply relate to a transcriptional leakage, or basal transcription. When *Tead4* deletion is performed post-implantation, embryos develop correctly, which strongly supports the specificity of Tead4 function in TE specification. In line with it, conditional expression of constitutively active Tead4 (Tead4VP16ER) yields a strong, ectopic expression of Cdx2 in inner cells, despite high levels of ICM genes, further supporting robustness of TE program initiation by Tead4 (Nishioka *et al*, 2009). Finally, *Tead4* is required for inducing mitochondrial oxidative phosphorylation (OXPHOS) in TE cells, producing more ATP to fulfil energetic demand of these rapidly expanding cells. By contrast, ICM cells remaining globally undifferentiated maintain a glycolytic metabolism. In TE cells, *Tead4* enters mitochondria and promotes the expression of mtDNA-encoded genes of the electron transport chain components. This is known as the metabolic switch of the embryo, which is required for normal development in the mouse. If *Tead4* is deleted by KO, blastocyst cavitation is compromised and mutant embryos die pre-implantation (Kumar *et al*, 2018).

Globally, disrupting genes involved in apico-basal polarity or Hippo signalling

specifically impairs TE formation in the mouse pre-implantation embryo, affecting neither total cell number nor developmental timing.

A recent embryological study suggests that TE initiation program is partly conserved between mouse and human (**Figure 3A**). In the human morula, outer cells also acquire an apico-basal polarity, illustrated by the apical location of aPKC (atypical protein kinase C). TEAD4 is ubiquitous at this stage, while nuclear YAP1, WWTR1 and GATA3 are confined to outer cells, similar to the mouse embryo (Gerri *et al*, 2020). Treatment of human embryos pre-compaction with CRT0276121, a potent aPKC inhibitor, and TRIM-Away protein depletion of aPKC both disrupt apico-basal polarity, which in turn prevents nuclear translocation of YAP1 and GATA3 expression in outer cells, ultimately impairing TE formation (Gerri *et al*, 2020). The onset of the TE program in human could also be monitored by single-cell ATAC-Seq, revealing rapid changes in chromatin accessibility pre-implantation. Open sites are initially enriched in binding motifs of 8-cell-stage specific transcription factors, comprising DUXA/DUX4 and ZSCAN4. However, they become rapidly dominated by GATA and TEAD binding motifs at the morula stage, accompanied by an increase in activity scores of GATA3 and TEAD4, both associated with TE specification in the mouse (Gerri *et al*, 2020 ; Liu *et al*, 2019).

Nonetheless, some differences regarding TEAD4 have been reported between mouse and human. While nuclear Tead4 is observed from the 4-cell stage in the mouse, it is not until the 16-cell stage in human. Also, while Tead4 is rapidly silenced in the mouse

ICM, it is maintained in inner cells of the human blastocyst. Importantly, *TEAD4* KO in the human embryo leads to *CDX2* downregulation, similar to the mouse, but surprisingly does not affect *GATA3* expression (Stamatiadis *et al*, unpublished). These observations suggest that TEAD4 not only has distinct timing and pattern of expression, but also a slightly different mode of action on TE lineage specification in human.

Interplay between ICM and TE programs

Given that blastomeres are initially equivalent but rapidly produce distinct lineages, a process supposedly relying on few actors (only a few TFs are restricted to TE by the blastocyst stage), a model of reciprocal repression of ICM and TE programs has been proposed. Supporting this view, disruption of TE fate drivers in the mouse often yields expression of ICM related genes in outer cells. As an example, deletion of *Cdx2* yields ectopic expression of *Pou5f1 / Oct4* and *Nanog* in the presumptive TE (Strumpf *et al*, 2005).

However, the direct repression of TE genes by ICM specific transcription factors is not obvious. Some have reported that Cdx2 is ectopically expressed in the ICM of *Pou5f1-/-* mutants, but this expression is low and only concerns rare cells, adjacent to the mural TE, whose identity is not clear (Frum *et al*, 2013). Others have also observed that inner cells isolated from these mutant embryos could generate trophoblast but not pluripotent cell colonies *in vitro*. They have interpreted that Pou5f1 acts as a repressor of

TE genes in the ICM (Nichols *et al*, 1998). However, there is no direct evidence of such mechanism. Similarly, it has been reported that *Nanog* deletion yields ectopic expression of Cdx2 in the mouse embryo, but the corresponding immunofluorescence only shows a weak signal in two cells at the margin of the ICM, while the global morphology is clearly abnormal (Chen *et al*, 2009).

In fact, another study has refined this view, by carefully considering successive developmental stages of the *Pou5f1-/-* mutant embryos. It appears that Cdx2 and Gata3 are properly restricted to the TE by E3.5, but are clearly detectable in the ICM of E4.25 mutants (Ralston *et al*, 2010). These observations might indicate that adjacent TE cells have colonised the defective ICM, or that progressive loss of ICM gene circuitry yields a molecular context permissive to TE program initiation. This process could be triggered by the second wave of epithelialisation in the embryo, which normally produces primitive endoderm (PE), but could instead generate TE in the absence of ICM related genes.

Using a complex episomal system, another study has concluded that *Pou5f1/Oct4* represses *Cdx2* in mESCs (Niwa *et al*, 2005). However, the reverse has been observed in mTSCs, in which episomal *Pou5f1* further promotes *Cdx2* positive autoregulation. Therefore, effects are context-dependent, and these results do not demonstrate a direct repressive mechanism of *Pou5f1* on *Cdx2*. Instead, it seems that loss of pluripotency circuitry following *Pou5f1* deletion yields a cellular state permissive to *Cdx2* induction and TE program onset, in line with observations in the embryo.

Interestingly, a communication between ICM and TE compartments seems to be mediated by paracrine signals. For instance, if the ICM is experimentally removed from early mouse embryos, polar TE proliferation is compromised, and trophoblasts rapidly differentiate into giant cells called primary TGCs (Ansell & Snow, 1975). Also, the deletion of *Pou5f1* hampers cavitation of the blastocyst, without affecting cell death or total cell number, suggesting a positive yet indirect effect of this gene on TE swelling, likely through *Fgf4* expression (Frum *et al*, 2013 ; Nichols *et al*, 1998).

In the human embryo, *Pou5f1* KO yields global downregulation of many pluripotency related genes, including *KLF17*, *DPPA5*, *ETV4*, *TDGF1*, *VENTX*, along with *Nanog*, which is completely silenced in mutant embryos. A more surprising result is the concomitant alteration of trophectoderm-associated genes, such as *Cdx2*, *HAND1*, *DLX3*, *TEAD3*, *PLAC8* and *PPARG*, which appear globally down-regulated, or even completely silenced, like *GATA2*, in the mutant embryos. Interestingly, not only *Pou5f1*-null cells are affected, but also neighbour wild-type and heterozygous cells in mosaic embryos, which suggests both autocrine and paracrine signals mediating ICM maintenance and communication with TE, respectively, similar to the mouse (Fogarty *et al*, 2017). This view is further supported by the expression of genes involved in ICM signalling pathways, such as *Fgf4*, and the exclusive expression patterns of receptors in ICM (*e.g.* *FGFR1/2*) or TE compartment (*e.g.* *FGFR4*) (Meistermann *et al*, 2021). These observations contrast with the mouse embryo, in which *Pou5f1* deletion does not affect immediately the expression of other ICM genes, such as *Nanog*, nor TE associated genes, like *Cdx2* (Fogarty *et al*, 2017 ; Frum *et al*, 2013). This suggests a wider role of

POU5F1 in the human embryo, possibly involved in both ICM and TE specification events. Another difference with the mouse is the overlapping expression of some ICM genes, notably *Pou5f1* and *KLF4*, with TE-related ones in the trophectoderm of early human blastocysts, which could indicate a retarded commitment to the TE fate (Meistermann *et al*, 2021 ; Niakan *et al*, 2013).

Altogether, these observations support that master TE fate driver genes, such as *Cdx2*, act in outer cells of the morula as repressors of ICM associated genes, initially ubiquitous, such as *Nanog* and *Oct4* (Strumpf *et al*, 2005). This is a prerequisite for the TE program onset in those cells. Meanwhile, ICM genes maintain undifferentiated inner cells, whose major role at this stage is to transmit the pluripotent genome throughout subsequent developmental progression. This dual mechanism is at the root of embryonic and extraembryonic lineage segregation. While this process is completed by E3.5 in the mouse, it seems delayed in human, only achieved by day 6. Once ICM and TE are established, they communicate with each other through paracrine signals, which are required for successful embryonic development.

TE maturation

The above-mentioned molecular mechanisms ultimately converge to the establishment of a specific transcription factor core network in TE cells. Many studies have considered two master TE fate drivers: Cdx2 and Gata3.

Cdx2-/- mutant mice embryos still initiate but fail to complete blastocoel expansion and collapse, thus not hatching from the *ZP*, and die without implanting (Chawengsaksophak *et al*, 1997 ; Strumpf *et al*, 2005 ; Tamai *et al*, 1999). Mutants also fail to downregulate ICM associated genes, such as *Pou5f1 / Oct4* and *Nanog*, in outer cells of prospective TE, and ectopic expression is associated with subsequent cell death (Strumpf *et al*, 2005). Moreover, disruption of TE epithelial integrity is observed in *Cdx2-/-* mutants, illustrated by a loss of integrin alpha 7 in these cells.

It has been observed that the onset of *Cdx2* expression depends on Tfap2c transcription factor, among others. Indeed, depletion of *Tfap2c* in mouse embryos by RNA interference largely prevents *Cdx2* expression (Cao *et al*, 2015). Effect of *Tfap2c* on *Cdx2* pre-implantation seems to be mediated by distinct, complementary mechanisms: first, depletion of *Tfap2c* yields downregulation of *Pard6b*, thus affecting PKCzeta location and cell polarity, which in turn prevents nuclear translocation of Yap1 in outer cells ; secondly, it binds an intronic enhancer of *Cdx2*, which promotes its expression.

Importantly, disruption of neither *Cdx2* nor *Tfap2c* affects the expression of *Gata3* in the mouse embryo (Ralston *et al*, 2010). This observation suggests independent mechanisms of *Cdx2* and *Gata3* expression onset, although both require *Tead4*.

Gata3 and *Gata2* genes are highly similar in sequence and yield structurally close transcription factors. *Gata3* deletion by conditional KO in the E0.5 mouse embyro only partially affects blastocyst maturation, while *Gata2* KO has no obvious effect

pre-implantation. However, combined deletion of *Gata3* and *Gata2* largely hampers blastocyst expansion, yielding implantation failure in all mutant embryos. Therefore, these GATA genes have a redundant but yet important function in TE formation (Home *et al*, 2017). When KO is induced post-implantation at E5.5, while *Gata3* and *Gata2* are confined to the trophoblast compartment (normally until E10.5), individual knockouts do not affect global phenotype, but double KO (DKO) completely blocks placental development and all embryos die by E9.5. In these mutants, trophoblast genes are severely deregulated, suggesting a redundant but yet central place of *Gata3* and *Gata2* in mouse trophoblast TF core circuitry. The fact that *Gata3* and *Gata2*, which are paralogues, are redundant in this process, suggests an evolutionary compensation mechanism safeguarding placental development.

Both *Cdx2* and *Gata3* are highly expressed in the pre-implantation human embryo (Meistermann *et al*, 2021 ; Niakan *et al*, 2013). However, *Cdx2* expression onset is delayed relative to the mouse, only expressed from the early blastocyst stage in human. By contrast, *GATA3* is already detected in outer cells of the human morula, preceding *Cdx2* expression (Gerri *et al*, 2020). Moreover, *GATA3* is maintained throughout trophoblast maturation, while *Cdx2* rapidly turns off after TE specification (Meistermann *et al*, 2021). Also, *Gata3* clearly precedes *GATA2* expression in human, while both are expressed from the 2-cell stage in the mouse (Gerri *et al*, 2020 ; Home *et al*, 2017). Therefore, whether functional redundancy of these genes pre-implantation is conserved between mouse and human is questionable and should be addressed.

Altogether, these results refine our comprehension of *Cdx2* and *Gata3* complementary functions in TE specification and maturation. It seems that Cdx2 chiefly acts as a repressor of ICM genes, while Gata3 establishes the TE program. TE specification appears delayed in human in comparison to the mouse. Moreover, while both are supposed to drive TE specification, the role of GATA3 might be predominant in human.

Establishment of the proliferative trophoblast

Once TE is established, the nascent trophoblast lineage undergoes rapid maturation, fulfilling two major functions: establishing a proliferative progenitor cell pool for organ growth ; producing differentiated cell types, specialised in many diverse placental functions.

Post-implantation, the mouse blastocyst elongates forming the egg cylinder, with proliferative polar TE cells producing the extraembryonic ectoderm (ExE). These compartments are considered to be sites of mouse trophoblast stem cells, which can be routinely isolated and cultured from polar TE outgrowths of E3.5 blastocysts or from E6.5 ExE explants (Hemberger *et al*, 2020 ; Tanaka *et al*, 1998 ; Uy *et al*, 2002). In contrast, cells of the mural TE can induce decidualisation, produce primary TGCs, but do not proliferate and fail to sustain developmental progression. This is rescued when ICM cells are added to mural TE vesicles before transfer to pseudopregnant mice

(Gardner & Johnson, 1972 ; Gardner *et al*, 1973). These observations further support the importance of inductive signals from the ICM to sustain TE proliferation (Christodoulou *et al*, 2019).

Proliferative cells in the ExE specifically express a set of transcription factors, comprising Cdx2, Eomes, Elf5, and Esrrb. As mentioned previously, *Cdx2* initially expressed in outer cells of the morula is later restricted to TE cells of the blastocyst, and ultimately retained at E6.5 in the ExE. There, *Cdx2*, *Eomes* and *Elf5* expression patterns finely overlap (Donnison *et al*, 2015). *Eomes* expression started pre-implantation in the TE is still low at E3.5, but is rapidly up-regulated post-implantation and reaches high levels at E5.0 in ExE. After E7.25, it progressively decreases but is retained in the chorionic ectoderm, possibly the last site of mTSCs (Uy *et al*, 2002). Deletion of *Eomes* in the mouse embryo provokes arrest at the blastocyst stage, because TE cells fail to produce mature trophoblast lineages (Russ *et al*, 2000). *Elf5* deletion yields a complete loss of ExE by E6.5, which impairs further trophoblast development and results in embryonic death (Donnison *et al*, 2005). Likewise, *Esrrb/Estrrb* is highly expressed in ExE at E5.5, and remains in the chorionic ectoderm at E7.5. It declines after that, but is still detectable in the free margins of the chorion at later stages. *Esrrb* deletion in the mouse embryo impairs placental formation, yielding fetal growth retardation and lethality at E10.5. In mutants, chorionic development is blocked but TGCs are produced in excess, possibly equivalent to primary TGCs originating from the mural TE, supporting that this is by default differentiation path of TE cells loosing proliferation capacity (Luo *et al*, 1997). Moreover, while *Cdx2*, *Eomes*,

and *Esrrb*/*Errb* are highly expressed in mouse TSCs, they are rapidly down-regulated upon differentiation, which suggests that they promote mTSC self-renewal (Tanaka *et al*, 1998).

Other transcription factors are central in the gene network of trophoblast progenitors. For instance, combined deletion of *Gata3* and *Gata2* in E5.5 mouse embryos downregulates important TSC-related genes, including *Cdx2*, *Eomes* and *Elf5*, which impairs placental development (Chakraborty & Ain, 2018 ; Donnison *et al*, 2015 ; Home *et al*, 2017). Similarly, conditional KO of *Tfap2c* in ExE at E6.5 blocks the junctional zone (JZ) and labyrinth formation (Sharma *et al*, 2016).

In human, the trophoblast lineage also arises from the polar TE lining the ICM. This observation reinforces notion of paracrine signals from the ICM promoting TE proliferation, which seem to be conserved in human. Despite some similarities, like *Tfap2c* and *Gata3* widespread across mouse and human placentas, major differences in molecular signatures are observed between both species. For instance, *TACSTD2* is specifically expressed by trophoblast stem cells in human, while the *Trop-2* orthologue is common to cytotrophoblasts and syncytiotrophoblasts in the mouse. Similarly, *Peg10* exhibits high expression levels in all layers of the mouse placenta (Shiura *et al*, 2021), but is specific to trophoblast stem cells in human. Other genes are exclusively associated with stemness in the human placenta, such as *NR2F2* and *LRP2* (**Figure 3B-C**). Conversely, *Cdx2* is maintained in mouse trophoblast stem cells but is rapidly down-regulated after human TE specification, no longer expressed in the trophoblast

lineage after that (Okae *et al*, 2018). Similar patterns have been observed for *Eomes* and *Elf5*, both poorly expressed across the human placenta, although hypomethylation of *Elf5* promoter is a conserved hallmark of the trophoblast lineage (Lee *et al*, 2016 ; Meistermann *et al*, 2021 ; Okae *et al*, 2018 ; Vento-Tormo *et al*, 2018).

These observations suggest that major differences distinguish the molecular core circuitry of trophoblast progenitors in human from that of the mouse.

Trophoblast differentiation

The balance between self-renewal and differentiation of TSCs is crucial to the formation of mature placental structures. For instance, the loss of trophoblast stemness upon combined deletion of *Gata3* and *Gata2* at E5.5 is accompanied by the up-regulation of genes associated with differentiation, which indicate that differentiated cell types are rapidly produced upon exit from trophoblast stemness. In spite of that, placental maturation is compromised and all mutants die by E9.5 (Chakraborty & Ain, 2018 ; Donnison *et al*, 2015 ; Home *et al*, 2017). It shows that a stable stem cell pool is critical for completing placental development, by sustaining continuous differentiation.

Meanwhile, alternative gene networks come into play to produce specialised cell types. Chorionic *villi* formation starts by E7.5, initiating labyrinth development, which coincides with the induction of *Gcm1*. This so-called "chorion-specific transcription factor" plays an important role in specifying the syncytiotrophoblast. In *Gcm1-/-*

mutants, branching morphogenesis is blocked and syncytiotrophoblast differentiation fails, although progenitor cells are not affected, and all embryos die by E10.5 (Anson-Cartwright *et al*, 2000). More precisely, *Gcm1* is critical for producing the SynT-II layer but seems dispensable for SynT-I, globally unaffected in mutants. Of note, *Cebpa* and *Synb*, both markers of SynT-II, are down-regulated upon *Gcm1* deletion, thus supposedly being expressed downstream of this gene (Simmons *et al*, 2008). In line with this, a similar phenotype has been observed in *Cebpa/b-/-* mutant embryos, in which labyrinth branching morphogenesis is severly altered, resulting in embryonic death before E13.5, while spongiotrophoblast and parietal TGCs remain normal. Of note, the presence of either *Cebpa* or *Cebpb* rescues fetal development (although *Cebpa* is haploinsufficient for survival of the newborns after birth), possibly indicating a compensatory mechanism of these closely related transcription factors (Bégay *et al*, 2004).

As mentioned previously, formation of the multinucleated syncytiotrophoblast results from the fusion of mononuclear progenitors, largely mediated by syncytins. In the mouse, *Syna* and *Synb* are the two main syncytin genes, both essential for mouse placental development. *Syna* is specifically expressed in the SynT-I layer, while *Synb* is expressed in SynT-II. The former is coexpressed with *HAND1*, while the latter is downstream of *Gcm1*. Expression of these genes appears starting by E8.5 in distinct precursor lineages in the chorion, early during labyrinth morphogenesis (Simmons *et al*, 2008). Deletion of *Syna* in the mouse provokes embryonic lethality at different timepoints, from E11.5 to E14.5 when all embryos are dead. Mutant embryos show

growth retardation (up to 25% body weight reduction) and look paler due to a poor vascularisation, notably visible at the fetal side of the placenta and surface of the yolk sac. Transplacental passage is also affected, reduced by 15% (Dupressoir *et al*, 2009). While the spongiotrophoblast and parietal TGCs are not affected, the labyrinth is reduced. It is characterised by Ki67+ proliferative aggregates, squeezing fetal blood vessels, suggesting that progenitors have accumulated upon the blockade of SynT-I differentiation due to *Syna* deletion. *Synb* KO has milder effects than *Syna* deletion and some neonates are viable, with very little growth retardation. In these mutants, up-regulation of *Gjb6* at the interhemal layer suggests a compensatory mechanism safeguarding molecular transport when syncytialisation of SynT-II is compromised. However, deletion of *Synb* yields dilatation of maternal lacunae, provoking abnormal blood stasis in the labyrinth, and maternal blood sinusoids are considerably enlarged by E18.5 (Dupressoir *et al*, 2011). Moreover, combined deletion of *Syna* and *Synb* results in a phenotype more severe than that of *Syna*-/- mutants, accompanied by an earlier embryonic lethality occurring between E9.5 and E10.5. These observations suggest complementary functions of SynT-I and SynT-II in transplacental passage, mediated by *Syna* and *Synb*, respectively.

PPARG is another transcription factor specifically expressed in the mouse placenta from E8.5 onward, while it is not detected in embryonic tissues until E13.5. Deletion of *PPARG* results in embryonic death at E10.0, due to placental dysfunction, which has been finely demonstrated by tetraploid complementation that rescues development *in utero* (Barak *et al*, 1999 ; Barak *et al*, 2008). In mutants, placental

compartments are correctly distributed, however, trophoblast precursors within the labyrinth fail to engage terminal differentiation into syncytiotrophoblast. A partner of *PPARG*, *Rxra*, is also critical for mouse placental development. If deleted, conceptuses fail to form a normal labyrinth, which appears disorganised and thickened, partly because some trophoblast progenitors accumulate, unable to differentiate into mature cells (*e.g.* syncytiotrophoblast and glycogen cells). In the placental compartment, mutants show edema, abnormal stasis of maternal blood, signs of disruption of fetal vessels and die before E16.5, likely from a combination of extraembryonic cues and cardiac defects (Kastner *et al*, 1994 ; Sapin *et al*, 1997). This phenotype is further aggravated by the combined deletion of *Rxrb*, a co-partner of *Rxra*. Indeed, these double mutants die between E9.5 and E10.5, due to placental defects, characterised by the absence of a labyrinth and failed branching morphogenesis, as allantoic capillaries do not penetrate the chorionic plate (Wendling *et al*, 1999).

A similar phenotype has been observed for *Ovol2*-deficient mice, which also die between E9.5 and E10.5, showing severe defects in placental labyrinth formation, along with defective vascularisation and heart morphogenesis (Unezaki *et al*, 2007). Some have reported that *Ovol2* deletion does not impair specification of major trophoblast lineages, and that important genes remain unaffected at E9.5, such as *PL1* (*placental lactogen-1*), *Tpbpa/4311* and *Gcm1*, markers of TGCs, spongiotrophoblast, and SynT-II, respectively. However, these observations have been contradicted by a recent study, showing downregulation of these genes upon the loss of *Ovol2* (Jeyarajah *et al*, 2020). In fact, after initiating chorio-allantoic fusion normally, the branching process is rapidly

blocked in mutants and very few fetal vessels are observed in the chorion at E10.5, suggesting that *Ovol2* is sustaining rather than driving this process. Interestingly, *Ovol2* is induced upon differentiation of mouse trophoblast stem cells *in vitro*, and represses TSC genes, comprising Esrrb, Eomes and Id2. Therefore, it seems that *Ovol2* function is to silence the trophoblast stemness program, bringing differentiation and branching morphogenesis to completion.

Similarly to the mouse, *Gcm1* is specifically associated with differentiated tissues of the human placenta and is required for ST formation (Baczyk *et al*, 2009 ; Chiu & Chen, 2016). If *Gcm1* is depleted with siRNA and antisense oligonucleotides (ASOs), cytotrophoblasts of villous explants cannot regenerate the ST layer after trypsinisation. *CEBPA* and *CEBPB* too are found in the human placenta, expressed in ST and EVT *in vivo* (Bamberger *et al*, 2004). However, they are considered specific to ST *in vitro*, and their exact role has not been identified yet in human. By contrast, *ERVW-1*, the human orthologue of *Syna*, and *ERVFRD-1*, orthologue of *Synb*, are both highly up-regulated in ST. Moreover, *ERVFRD-1* (along with *ERVMER34-1*, another syncytin gene) is detected early in the primitive syncytium, suggesting a robust, conserved mechanism of their fusogenic activities in syncytiotrophoblast differentiation (Roberts *et al*, 2021). In line with this, both are important for the fusion of trophoblasts into multinucleated ST-like cells *in vitro*, as demonstrated by siRNA and ASO experiments (Frendo *et al*, 2003 ; Vargas *et al*, 2009).

Consistent with observations in the mouse, *PPARG* and *Rxra* are specifically

expressed in human ST. *PPARG* is required for CT fusion into ST cells, as demonstrated by siRNA experiments and point mutation (E352Q) in its ligand-binding domain (Shoaito *et al*, 2020). Moreover, the human ST is a place of intense production of placental hormones, critical for sustaining pregnancy (acting on the decidua, the corpus luteum or maternal glycaemia, among others), and PPARgamma/RXRalpha heterodimers appear central to this process. Notably, they bind to responsive elements in the regulatory region of the *CGB* gene cluster, located on human chromosome 19, thus promoting hCGbeta expression. They also promote expression of genes encoding other important ST hormones, such as human placental lactogen (*CSH2*), human placental GH (*GH2*), and leptin (*LEP*) (Tarrade *et al*, 2001).

In human, *OVOL1* is thought to have functions in placental development, equivalent to those of *Ovol2* in the mouse. Of note, *OVOL1*, the orthologue of human *OVOL1*, and *Ovol2* are paralogues, showing high degree of redundancy. *OVOL1* is induced upon ST differentiation *in vitro* and represses genes supposedly maintaining CT in a progenitor state, including *TP63*, *MYC*, *ID1* and *ASCL2* (Renaud *et al*, 2015). Conversely, depletion of *OVOL1* by shRNA during differentiation assay does not affect much the expression of ST related genes, such as *Gcm1*, *ERVW-1* and *ERVFRD-1*, still robustly induced, in line with results in the mouse.

Altogether, these results suggest conserved mechanisms of syncytiotrophoblast formation between mouse and human. The core gene network seems to be centred on key transcription factors: *OVOL1*, acting as a repressor of stemness, *Gcm1* activating

ST specific genes, notably fusogenic syncytins, and the *PPARG/Rxra* complex, promoting expression of placental hormone genes (**Figure 3D**). However, some differences are observed between the two species, such as placental hormone production, mainly attributed to TGCs in the mouse. Moreover, functions of these genes in the human embryo have remained speculative so far, extrapolated from *in vitro* studies.

Extravillous trophoblasts (EVT) represent the other major trophoblast lineage in human. Major functions comprise invading the maternal decidua and anchoring the conceptus. EVT also remodel spiral arteries, increasing blood flow to the intervillous space, and promote fetal tolerance of the maternal immune system. These processes are mediated by EMT-like transition producing migratory and invasive EVT, and specialised genes such as *MMP2* (a matrix metalloproteinase digesting ECM) and *HLA-G*, a trophoblast specific member of the major histocompatibility complex in human (MHC).

Cellular counterparts of EVT in the mouse are supposed to comprise spongiotrophoblast and TGCs, possibly originating from common progenitors in the EPC, regulated by distinct gene circuitry as compared with the syncytiotrophoblast (Hemberger *et al*, 2020 ; Simmons & Cross, 2005). For instance, the transcription factor *ASCL2/Mash2* is crucial to the formation of the spongiotrophoblast (SpT). Indeed, *Ascl2-/-* mutants die around E9.5/E10.5, from placental failure, associated with a complete absence of this cellular layer (Guillemot *et al*, 1994). Conversely, parietal TGCs (P-TGCs) overexpand upon deletion of *ASCL2*, supporting that it is key to the

balance between SpT and TGC fate decision. Wider repercussions on placental development comprise the absence of diploid progenitors in the EPC, a reduced chorionic ectoderm and a poorly vascularised labyrinth layer. Of note, chimeras with tetraploid WT embryos, which contribute exculsively to the extraembryonic compartment, rescue the normal phenotype, supporting that *ASCL2* is dispensable in the embryo proper at this stage. Strikingly, ratios of genotype transmission are biased only in the mutant female progeny, evidencing that *ASCL2* is maternally expressed, while the paternal allele is silenced. In fact, imprinting at this *locus* is established after E7.5, specifically in the trophoblast lineage (Guillemot *et al*, 1995). This observation is in line with the hypothesis of maternal and paternal genomes competing each other in the placenta. Indeed, *ASCL2*, by regulating the SpT/TGC fate decision, might be central to controlling invasion of the decidua and access to maternal resources.

EVT differentiation is thought to be driven by low oxygen tension at distal sites of the hemochorial placenta. A major transcription factor complex connecting hypoxia with gene expression is the so-called Hypoxia-Inducible Factor HIF, a heterodimer composed of HIFalpha and HIFbeta (ARNT) subunits. A phenotype similar to that of *ASCL2*-/- mutants has been observed in *Arnt*-/- and *Hif1alpha*-/-*Hif2alpha*-/- mutant mice, characterised by TGC overexpansion to the detriment of spongiotrophoblast, and disruption of the labyrinth containing fewer fetal blood vessels. Consequently, these embryos exhibit defective placental vascularisation and poor decidual invasion. *In vitro*, *Arnt*- or *Hifalpha*-null mTSCs fail to generate spongiotrophoblasts, instead differentiating exclusively into multinucleated

syncytiotrophoblast, and it has been shown that HIF positively regulates *ASCL2/Mash2* expression (Cowden Dahl *et al*, 2005).

In human, *ASCL2* is expressed in cell columns, located distally on top of anchoring villi, containing EVT progenitors (Alders *et al* 1997 ; Varberg *et al* 2021). In striking contrast to the mouse, *ASCL2* is not imprinted in human. Initially, observations were contradictory. Indeed, lack of *ASCL2/HASH2* expression in some androgenetic moles (trophoblastic tumors containing haploid male genome only), could indicate this gene is also imprinted in human (Alders *et al* 1997). However, all of placental stages analysed during normal development (weeks 12-39) displayed biallelic expression patterns (Miyamoto *et al*, 2002). This difference might result from distinct levels of intra-brood conflict between the two species, mice being more prone to multiple paternity than humans. *In vitro*, differentiation of human trophoblast stem cells into EVT is accompanied by the induction of *ASCL2* expression. Knockdown of *ASCL2* by shRNA impairs EVT cell differentiation, as indicated by failed EMT-like transition and disrupted gene expression profiles, including those of *MMP2* and *HLA-G* (Varberg *et al* 2021).

Similar to the mouse, the HIF complex is associated with the first stages of EVT differentiation in human. Up-regulation of *HIF1A* expression accompanies the transition from EGFR+ CT progenitors into HLA-G+ proximal column EVTs (pcEVTs). Moreover, culture of villous CTs in low oxygen directs differentiation toward EVTs to the detriment of ST. Disruption of *Arnt/HIFbeta* mediated by shRNA

hampers this process, demonstrating that an intact HIF complex is required (Wakeland *et al* 2017)

Altogether, these results suggest conserved mechanisms of EVT formation. Occurring at the distal part of the hemochorial placenta, it appears to be driven by hypoxia, mediated by the HIF complex and regulated by ASCL2 (**Figure 3D**). Mirroring TGC function in the mouse, human EVTs too produce important placental hormones, such as the human placental lactogen (hPL), also called the chorionic somatomammotropin hormone 1 (*CSH1*). However, some differences are observed between the two species: while *Gcm1* is involved in syncytiotrophoblast but not TGC specification in the mouse, it is required for both ST and EVT formation in human, highly expressed in these cellular compartments (Baczyk *et al*, 2009).

In conclusion, besides the above-mentioned genes, whose role in development has been demonstrated by genetic manipulation, a myriad of trophoblast markers have been identified. Although precise mechanisms are still unclear, and the list is not exhaustive, it gives a global view of molecular events regulating human placentation.

Figure 3: Presumptive molecular mechanisms of human placentation.

Time

A

Day 4

MORULA

aPKC

YAP1/WWTR1

GATA3

Day 5

EARLY BLASTOCYST

TEAD4

CDX2

B

Day 7
(IMPLANTATION)

LATE BLASTOCYST

GATA2/3

FGF4

FGFR4

POU5F1

NR2F2

GCM1

ERVFRD-1

ERVMER34-1

ULE

DSC

PS

UC

UG

polar TE

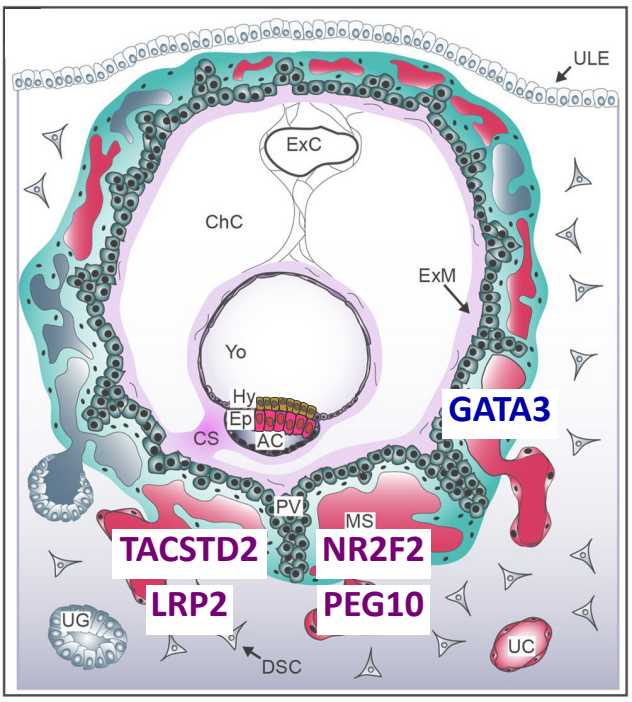
mural TE

EPI

PE

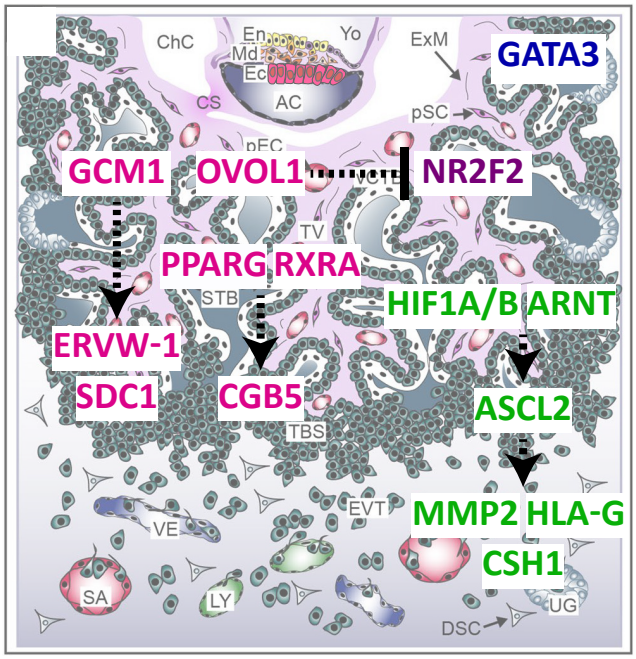
C

Day 10



D

Day 14



Day 21

Figure 3. Presumptive molecular mechanisms of human placentation.

(A) Onset of TE specification: at the morula stage, aPKC localised in apical domains of outer cells contributes to turning off the Hippo pathway, thus releasing YAP1/WWTR1 that translocate into the nucleus and activate the TE master gene *GATA3* (day 4). This is followed by the induction of *CDX2* by TEAD4 at the early blastocyst stage (day 5). The complementary functions of GATA3 and CDX2 then ensure the proper establishment of the TE compartment.

(B) TE maturation: at the embryonic pole of the late blastocyst, the polar TE (presumptive precursor of mature trophoblast lineages) acquires a specific molecular signature characterised by *NR2F2* expression, distinct from that of mural TE cells (days 6-7). This likely responds to signals from the epiblast, possibly mediated by FGF4. Shortly after implantation, part of the polar TE differentiates to form the primitive syncytium, a process supposedly driven by GCM1, which activates fusogenic syncytin genes.

(C) Proliferative trophoblast formation: by day 10, chorionic villi emerge that will host the trophoblast stem cell niche during the first trimester of development. Putative hTSCs are characterised by the expression of specific markers, comprising *NR2F2*, *PEG10*, *TACSTD2*, and *LRP2*, besides pan-trophoblast genes like *GATA3*.

(D) Trophoblast differentiation: OVOL1 is supposed to repress the trophoblast stemness program, while GCM1 activates membrane and syncytin genes to form the multinucleated syncytiotrophoblast, that will produce high amounts of placental hormones downstream of PPARG/RXRA. Conversely, in response to hypoxia, the HIF complex promotes *ASCL2* expression that in turn activates the EVT program.

Abbreviations: ICM: Inner Cell Mass ; EPI/Ep: epiblast ; PE/Hy: primitive endoderm/hypoblast ; TE: trophectoderm ; ULE: uterine luminal epithelium ; PS: primitive syncytium ; UC: uterine capillaries ; UG: uterine glands ; DSC: decidual stromal cell ; AC: amniotic cavity ; PYo: primitive yolk sac ; ExM: extraembryonic mesoderm ; MS: maternal blood sinusoids ; Yo: yolk sac ; ChC: chorionic cavity ; ExC: exocoelomic cyst (remnant of the transient primitive/primary yolk sac) ; CS: connecting stalk ; PV: primary chorionic villi ; Ec: ectoderm ; Md: mesoderm ; En: endoderm ; (Ec, Md, and En form the three germ layers during gastrulation, from day 14 to 21) ; TV: tertiary chorionic villi ; pEC: placental endothelial cells ; pSC: placental stromal cells ; vCTB: villous cytotrophoblast ; TBS: trophoblastic shell ; STB: syncytiotrophoblast ; EVT: extravillous trophoblasts ; SA: spiral arteries (maternal) ; VE: venous vessels (maternal) ; LY: lymphatic vessels (maternal).

Pictures representing pre- and post-implantation human embryos are adapted from Niakan *et al*, 2012 and Knöfler *et al*, 2019, respectively.

1.4 Defective placental development alters human reproduction

In many ways, the placenta is a unique organ in human. It accumulates a large amount of genomic aberrations, clonaly inherited from the early bottleneck that segregates embryonic and extraembryonic lineages (Coorens *et al*, 2021). By contrast, the pluripotent epiblast should be devoid of chromosomal abnormalities, ensuring the transmission of a stable genome throughout developmental progression of the prospective fetus. Distinct requirement and stringency of genomic integrity somehow mirrors the opposite functions of TE and EPI. Indeed, the trophoblast lineage is rapidly produced to mediate implantation and sustain pregnancy, but on the scale of human life, the placenta is a transient organ with a short lifespan. Conversely, the embryo proper needs to keep free from mutations, notably during germline formation, and later in the adulthood, to prevent malignant transformation.

However, some mutations can severely affect placental development, with negative repercussions on pregnancy outcome. In this section, we describe major placental diseases, from their origin to the consequences on human reproduction.

Infertility

The industrial revolution, initiated by the mid-eighteenth century, has brought unprecedented technological progress to mankind. However, a side effect was the

production of thousands artificial, completely new chemicals, released in the air, water and soil, that life had never experienced throughout evolution. While comfort and medical advances, resulting from overproduction, have yielded exponential demographic increase worldwide, these contaminants have impacted human health at the same time (not to mention repercussions on biodiversity, human activity provoking the sixth mass extinction of species).

On the scale of human reproduction, endocrine disruptors have largely contributed to infertility, which is defined as the inability to conceive or maintain a pregnancy to the point of a live birth (World Health Organization, 2020). Fertility worldwide has dropped by nearly 1 percent per year for the last sixty years, and today 1 in 6 couples is diagnosed as infertile in western countries, such as the USA or France. The use of assisted reproductive technologies (ART) is therefore increasing by 5–10% per year (Ravitsky and Kimmins, 2019). In France, 3% of births result from IVF.

This is combined with the fact that human pregnancy is by nature inefficient compared with other Mammals (only 30% of naturally fertilised embryos result in live births). Some evidence has pointed to a possible connection between the high oxygen consumption of the fetal human brain and oxidative stress of the placenta, often associated with poor pregnancy outcome (Burton & Fowden, 2015 ; Duhig *et al*, 2016).

Nowadays, infertility is considered to be a public health problem by WHO (Joffe *et al*, 2010 ; Macklon *et al*, 2002). Biological causes of failed fertilisation are mainly of

paternal origin, related to declining sperm counts, decreasing by 1.4% per year on average, with an overall decline of 52.4% between 1973 and 2011 in western countries (Levine *et al*, 2017 ; Liu & Baker, 2000). However, when the cause of infertility is downstream, either one of these factors is likely to be involved: chromosomal abnormalities provoking fetal death, or defective placental development. Consistently, a large panel of trophoblast dysfunctions are accountable for miscarriage, whose frequency is increasing by about 1% a year in the USA, concerning 1/3 to 1/2 pregnancies.

Preeclampsia

Preeclampsia has many possible causes, but globally, it is characterised by an inflammatory context at the end of the first gestational trimester in human, possibly yielding pregnancy loss. Overall, preeclampsia is predominant in "developing" countries, and concerns about 4.6% of live births worldwide (Wang *et al*, 2021). However, important variations are observed between regions (*e.g* 1.0% in the eastern Mediterranean and 5.6% in Africa). In total preeclampsia accounts for $\sim$ 10 to 15% of maternal deaths, and 20% of preterm births (premature neonates represent 7% of total births) (Valero *et al*, 2018).

Dysfunctions of the syncytiotrophoblast have been associated with preeclampsia. Some structures called syncytial sprouts often break away from the syncytiotrophoblast and enter the maternal circulation. This process, related to normal trophoblast proliferation and formation of new villi, prevails in the first half of

pregnancy. However, the end of pregnancy (¿ 32 weeks) is characterised by a switch towards apoptotic syncytial knots, indicating syncytiotrophoblast "ageing". Syncytial sprouts continuously pass through the uterine veins and accumulate in the capillary bed of maternal lungs. The deported trophoblast material is thought to promote tolerance of the conceptus by the maternal immune system. However, aberrant forms of syncytial knots, referred to as wave-like apoptotic and aponecrotic knots, have been associated with severe IUGR and preeclampsia, respectively. In these pathological situations, the necrotic and proinflammatory trophoblastic debris induce endothelial cell activation of maternal blood vessels, which can provoke uncontrolled, deleterious immune reaction, often observed in preeclamptic placentas (Raguema *et al*, 2020 ; Tomas *et al*, 2011). Maternal macrophages abnormally targeting the syncytiotrophoblast are supposedly involved in this process. Moreover, aberrant syncytial knots can obstruct chorionic vessels, yielding hypertension, edematous damages, fetal artery thromboses and occlusion (Burton & Jones, 2009).

Some genes, such as *GRP78* and *alpha2M maternal circulating protein*, are involved in preeclampsia, through deregulation of ST fusion. Deficiency in Vitamin D (¡20ng/ml) is also associated with this disease. Moreover, a study has evidenced that a mutation of *STOX1*, a DNA binding protein, is linked to the prevalence of preeclampsia in the Dutch population (Van Dijk *et al*, 2005). The activity of *STOX1*, expressed in invasive EVTs, is regulated by PI3K-Akt-FOX signalling. Upon activation of this pathway, STOX1 is translocated into the nucleus, binds DNA and promotes polyploidisation of EVT cells. However, this process is defective due to *STOX1*

mutation, and invasion of the maternal decidua by EVTs remains incomplete, resulting in preeclampsia.

Finally, preeclamptic placentas are characterised by higher stiffness. Indeed, they are of 7 kPa tension, approximately, reaching 17.4 kPa in necrotic tissues, contrasting with the 1.3 kPa tension measured in healthy 3rd trimester placentas.

Therefore, preeclampsia is a pluricausal placental disease, often resulting from ST and EVT lineage dysfunctions.

Infectious diseases

Infections are another source of placental defects in human. Although most microorganisms are not able to cross the ST barrier, some viruses can, which are referred to as TORCH (Toxoplasmosis, syphilis, varicella-zoster, parvovirus B19, Rubella, Cytomegalovirus, and Herpes virus). They often induce a type-I interferon (IFN-I) response in the placenta, potentially deleterious to the conceptus. For instance, a defense mechanism involving *IFITM2* (an IFN-induced transmembrane protein) blocks syncytin activity in the ST to prevent viral entry. However, it also impairs fusion of CT and thus the renewing of the ST layer. Consequently, ST enters aponecrosis, often yielding intrauterine growth restriction (IUGR), preeclampsia, congenital anomalies or even fetal demise (Buchrieser *et al*, 2019).

Placental tumors

Similar to those of somatic tissues, some placental tumors are benign, while others are malign. Their prevalence is low (180 cases per year in France), but the panel is large, comprising: hydatiform and invasive moles, trophoblastic tumors at implantation site, epithelioid trophoblastic tumors and choriocarcinomas (1 in 40,000 pregnancies in western countries). Interestingly, some ethnic groups are more prone to develop one or another tumor type, supporting genetic causes (Gockley *et al*, 2016).

Choriocarcinomas are probably the worst trophoblastic tumors, very aggressive and highly prone to metastases. Gestational choriocarcinomas arise from a trophoblast stem or progenitor cell, subject to malignant transformation. The neoplastic cytotrophoblast further progresses into intermediate trophoblasts and syncytiotrophoblast-like cells, somehow mimicking aspects of peri-implantation blastocyst development (Bishop & Edemekong, 2021 ; Mao *et al*, 2007). Deregulation of specific genes (but no mutation) has been associated with choriocarcinoma onset, including: *TP53*, *MDM2*, *NECC1*, *EGFR*, *DAB2*, *RASA1* (*Ras GTPase-activating protein gene*), *CDH1*, *HIC-1*, *P16*, *TIMP3* and *HLA-G*. Of note, these tumors seem to redirect gene functions to inactivate locally and escape the maternal immune system.

1.5 Cellular models of the human placenta

Prolific investigation of placental diseases and limited access to human embryos both contributed to developing cellular models of the trophoblast lineage *in vitro*. In the next paragraphs, we are going to mention the major ones, discussing pros and cons.

Primary cells

As mentioned previously, during the first trimester of development, the human placenta is composed of three major cell types: villous cytotrophoblasts (CTs), the syncytiotrophoblast (ST), and extravillous trophoblasts (EVTs). These cells can be isolated from aborted or term placentas. To this end, chorionic *villi* are dissected, CT and EVT separated by trypsin and DNase digestion (reduces viscosity of the digests, promotes single cell separation), sedimentation, filtration and fractionation on discontinuous Percoll gradient (Handschuh *et al*, 2006). CTs are cultured directly on plastic dishes, in DMEM with 10% inactivated fetal calf serum (FCS) supplemented with 2 mM glutamine, usually to be analysed within 2 hours. EVTs are cultured on Matrigel, otherwise under similar conditions, usually for 48 hours. ST cells are obtained by 72 hours upon spontaneous differentiation of CTs.

These cells, collected from primary tissues, are the closest to normal trophoblasts found *in vivo*. However, they have a short lifespan and cannot be maintained *in vitro* more

than a few days, thus precluding large-scale experiments.

Immortalised trophoblast cell lines

By contrast, immortalised cell lines, usually derived from choriocarcinomas, proliferate unlimitedly and thus are easily expandable. They are suitable to chemical screening, gene editing or reporter line construction, among others, and have contributed to uncover many molecular mechanisms regulating the trophoblast lineage in human. The main ones comprise: BeWo, JEG-3, Jar, and HTR-8/SVneo cells.

However, these lines, resulting from malignant transformation, are very different from normal trophoblasts in many ways, having important drawbacks, notably chromosomal abnormalities and altered differentiation potential. For instance, BeWo cells, biased towards the villous lineage, can recapitulate syncytiotrophoblast but not extravillous trophoblast formation. Conversely, the HTR-8/SVneo line is restricted to extravillous lineages, and JEG-3 cells fail to undergo substantial fusion. These differences are associated with distinct cellular morphologies: BeWo cells form epithelioid colonies, while HTR-8/SVneo and Swan71 are individualised cells resembling extravillous trophoblasts (Apps *et al*, 2009 ; Bilban *et al*, 2010).

An explanation to the limited differentiation potential of these lines is that they probably originate from transformed progenitors rather than stem cells. Indeed, proliferative progenitors are supposedly plenty within a tissue while quiescent stem cells

are fewer and thus have less chances to undergo malignant transformation. However, trophoblast progenitors are committed to either villous or extravillous lineages, and their restricted potential may be retained in corresponding cell lines. Another possibility is that malignant transformation has abolished multilineage potential in these cells, through deregulation of stemness gene network.

BAP-treated pluripotent stem cells

Treatment of primed human pluripotent stem cells (hPSCs) with BMP4, A83-01 and PD173074 (BAP cocktail) induces trophoblast-related gene expression. However, a controversy surrounding identity of the differentiated cell lineages has been dividing biologists for about 10 years, yielding passionate debates. Hopefully, scientists are wise people who prefer words over brawls. The bone of contention partly arose from overlapping molecular signatures between distinct cell types and divergent conceptual view of lineage commitment. Some have reported that BAP-treated hPSCs produce trophoblasts, while others argue that these cells generate mesoderm (Amita *et al*, 2013 ; Bernardo *et al*, 2011 ; Wu *et al*, 2008).

However, using the BAP system, Krendl *et al* have shown that gene networks and molecular signatures are close to those of trophoblast lineages. We agree with this view, corroborated by in-house transcriptomic analyses. Moreover, combined RNA-Seq and ChIP-Seq assays identified *TFAP2A/C* and *GATA2/3* as core transcription factors

driving trophoblast specification in BAP-treated cells, in line with gene expression profiles of the human embryo (Krendl *et al*, 2017).

These cells, derived from stable hiPSC lines, offer the opportunity to study trophoblast specification in different contexts, specific to patients. However, they cannot be maintained more than two weeks as they rapidly cease to proliferate, and it is not clear whether they produce ST, EVT, or a mixture of mature trophoblasts.

Human trophoblast stem cells

Recently, the isolation of human trophoblast stem cells (hTSCs), 20 years after mouse TSCs, has been a breakthrough in our field of research (Okae *et al*, 2018). These cells can be derived from pre-implantation blastocysts and first trimester chorionic villi. They are genetically stable, match trophoblast progenitors *in vivo*, and can be expanded unlimitedly while producing *bona fide* ST and EVT cells. Not only do hTSCs fit stemness properties of long-term self-renewal and differentiation potential, but they also meet the four criteria defining human trophoblast identity: expression of trophoblast markers, specific HLA class I profile, DNA methylation of the *Elf5* promoter and expression of microRNAs (miRNAs) from the chromosome 19 miRNA cluster (C19MC) (Lee *et al*, 2016).

In the following section, we revisit milestones that have paved the way to this fundamental discovery.

First of all, the very notion of trophoblast stem cells was questionable. Indeed, stemness typically defines rare quiescent cells, emerged early in development and maintained throughout adult life, that retain the capacity to sustain tissue homeostasis and repair. It clearly applies to certain cell types, such as hematopoietic stem cells, that can repopulate the entire blood and immune systems over serial transplantation in successive generations. Conversely, placental progenitors are not supposed to self-renew longer than embryonic development lasts. In line with this, proliferation of trophoblasts drastically decreases by the end of pregnancy, yielding gradual reduction of the villous cytotrophoblast layer. However, this might be due to changing micro-environment during placental "ageing", while putative trophoblast stem cells could possibly be maintained *in vitro* under appropriate culture conditions.

Globally, two prerequisites were needed for achieving hTSC derivation: finding their niche in the placenta, and defining their needs on the molecular scale.

Attempt was made to derive hTSCs from the chorionic membrane, after stripping off *villi* (Genbacev *et al*, 2011). This compartment, along with the chorionic mesenchyme below, was indeed supposed to be a site of hTSCs. Such hypothesis was based on immunostaining for pluripotency (POU5F1/OCT4) and trophoblast-related markers (GCM1, KRT7/CK7), although POU5F1 signal in this case is intriguing and should not be detected in the human placenta. From these experiments, so-called trophoblast progenitor cells (TBPCs) were isolated that propagated for 25 passages, under culture conditions adapted from mouse TSC and BAP media (stimulation of FGF

and inhibition of TGF-beta signalling, respectively). Global gene expression was assessed, but despite some similarities with CT molecular signatures, transcriptomic profiles were heterogenous, indicative of differentiated cells rather than trophoblast progenitors. In addition, these cells expressed many genes associated with distant lineages, such as *GATA4*, a marker of the primitive endoderm.

In fact, it has been later demonstrated that hTSCs precisely reside at the basis of chorionic villi. Therefore, Genbacev *et al* had little chance of succeeding, so did approaches based more on knowledge from the mouse than on direct observations in human, as mTSCs and hTSCs rely on different signalling pathways to self-renew. Over-extrapolation can even be misleading, as it was the case for *Cdx2*, *Elf5* and *Eomes* expected patterns (Hemberger *et al*, 2010). Indeed, these genes are highly expressed in mouse TSCs, but globally absent from hTSCs.

Meanwhile, others have demonstrated regenerative capacity and bi-potential of first trimester villous cytotrophoblasts. They isolated chorionic *villi* and explants were further denuded by digestion with trypsin, to remove the syncytiotrophoblast layer and cell columns (Baczyk *et al*, 2006). Recovery was monitored by immunostaining for KRT7/CK7, and *de novo* formation of ST and EVT was accompanied by hCG and HLA-G expression, respectively (**Figure 4A**). Not only could CT produce differentiated lineages, but they also recapitulated placental tissue organisation. These results strongly supported putative stemness of these cells, further corroborated by Ki67/MKI67 expression and BrdU incorporation, indicative of proliferation. However, these cells

could not be maintained more than a few days *in vitro*, but this was due to inadequate culture conditions. Others later confirmed the presence of a proliferative trophoblast niche at the base of CT cell columns in first trimester placentas, characterised by the co-expression of Ki67 and ITGA2 (**Figure 4B**) (Lee *et al*, 2018).

However, much of the credit goes to Okae and colleagues, who first isolated and maintained *bona fide* hTSCs *in vitro* (**Figure 4C**) (Okae *et al*, 2018). Not only did they rely on previous reports to formulate effective working hypotheses, but they also performed transcriptome-wide analysis of major plancental lineages in human, to define trophoblast stemness signature. Applying functional enrichment analysis to these data, they identified candidate pathways active in proliferative CT, and found that they were similar to those regulating epithelial stem cells in other tissues. Therefore, they screened small molecules reported to maintain such cells *in vitro* and designed a culture medium suitable for hTSCs, globally based on the activation of WNT and EGF pathways, combined with inhibition of HDAC, Rho-kinase, and TGFbeta/NODAL signalling. These culture conditions enabled the formation of epithelioid colonies, successfully establishing hTSC lines. These were characterised by long-term self-renewal, unrestricted differentiation potential, and met the four criteria defining trophoblast lineage in human (Lee *et al*, 2016). Of note, the now conventional and widely used hTSC medium of Okae *et al* is sometimes referred to as ASECRiAV in this manuscript, in reference to its chemical formulation: A83-01, SB431542, hEGF, CHIR99021, ROCKi/Y27632, Ascorbic acid/Vitamin C and Valproic acid.

In our view, hTSCs represent the most suitable *in vitro* model for studying complex molecular mechanisms of placental development in human. However, despite these advances, an important aspect is missing: the access to patient-specific trophoblast stem cells, notably those associated with pathological situations. Indeed, all hTSC lines came from aborted placentas or surplus embryos, and the repertoire of genetic backgrounds is still very limited.

Figure 4: Evidence of the human trophoblast stem cell niche in vivo.

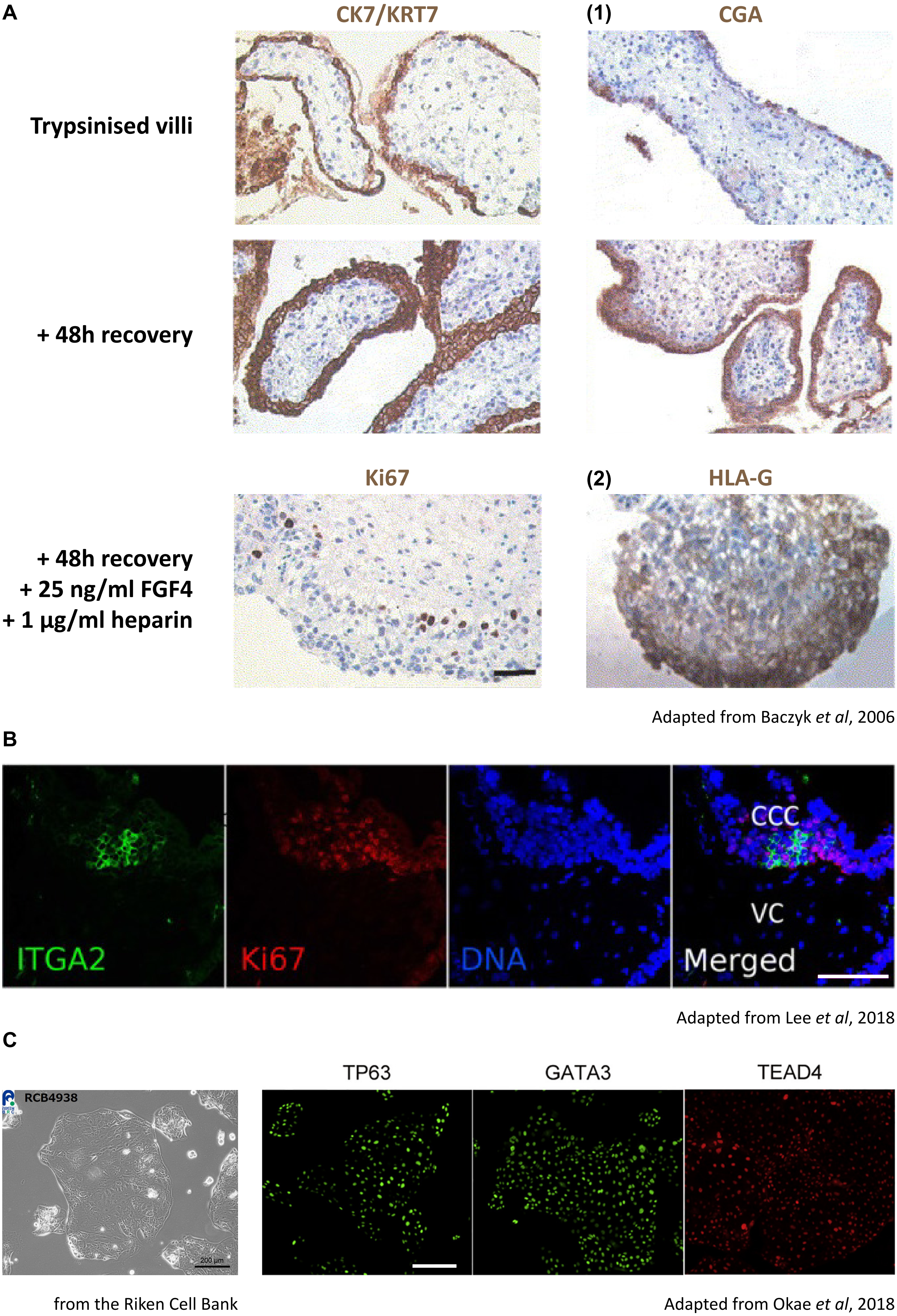

A
CK7/KRT7
(1)
CGA
Trypsinised villi
+ 48h recovery
Ki67
(2)
HLA-G
+ 48h recovery
+ 25 ng/ml FGF4
+ 1 µg/ml heparin
Adapted from Baczyk et al, 2006
B
ITGA2
Ki67
DNA
CCC
VC
Merged
Adapted from Lee et al, 2018
C
RCB4938
200 µm
TP63
GATA3
TEAD4
from the Riken Cell Bank
Adapted from Okae et al, 2018

Figure 4. Evidence of the human trophoblast stem cell niche *in vivo*.

(A) *In vitro* digestion assays, stripping explants of first trimester human chorionic villi, showing the regenerative capacity of rare, proliferative villous cytotrophoblasts (Ki67+). *De novo* formation of (1) syncytiotrophoblast (CGA+ / KRT7+) and (2) extravillous trophoblast (HLA-G+) lineages demonstrates the bi-potential of these cells. Scale bar: 50 µm.

(B) Tissue section of a first trimester placenta showing the localisation of proliferative (Ki67+) villous cytotrophoblasts *in vivo*, at the base of chorionic villi. This subpopulation is characterised by the expression of ITGA2. CCC: cytotrophoblast cell columns ; VC: villous core. Scale bar: 100 µm.

(C) Bright field picture (left) and immunostainings (right) to trophoblast lineage markers in primary hTSCs isolated *in vitro*, derived from the first trimester villous cytotrophoblast. Scale bars: 200 µm.

Human induced and converted trophoblast stem cells

Discovery of induced pluripotent stem cells (iPSCs), obtained by somatic cell reprogramming with Yamanaka factors (*i.e.* OCT4/POU5F1, SOX2, KLF4, MYC, abbreviated as OSKM), enabled to get patient-derived cell lines, very useful to investigate molecular mechanisms of diseases. Another approach has involved transdifferentiation, a sort of cell fate conversion between distant lineages, to produce cell types of interest from accessible sources of cells.

However, what applied to somatic tissues was not extended to the trophoblast lineage, somehow shelving research on placental dysfunctions in this field. Therefore, we decided to investigate cell reprogramming and fate conversion to generate trophoblast stem cells from patients, using either fibroblasts or iPSCs. After testing various culture conditions, we designed an original protocol for producing stable lines, which we referred to as human induced and converted trophoblast stem cells (hi/cTSCs).

The following section recapitulates results generated in this study, published in Cell Reports in 2020.

2 RESULTS

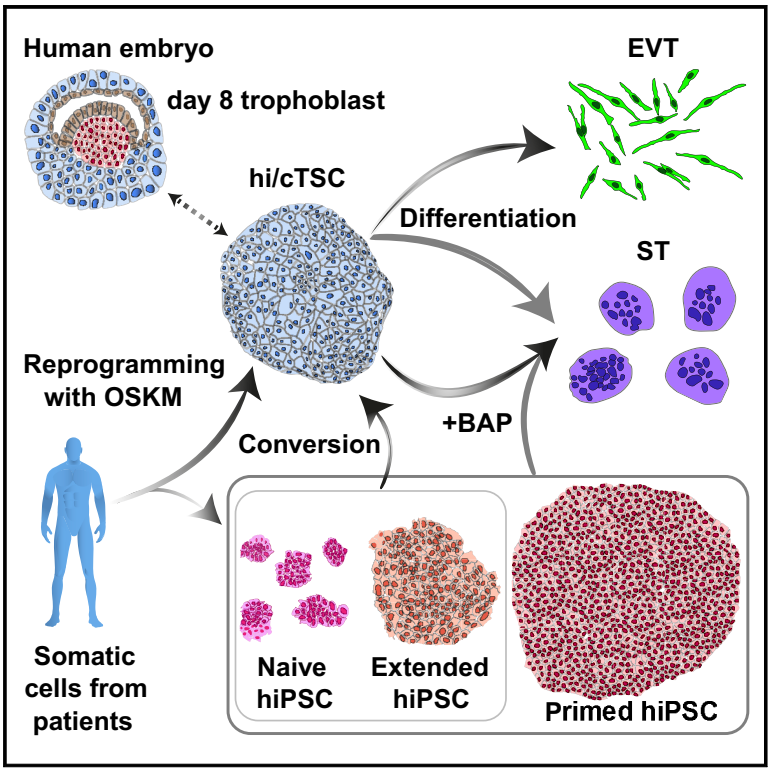
Castel G, Meistermann D, Bretin B, Firmin J, Blin J, Loubersac S, Bruneau A, Chevolleau S, Kilens S, Chariau C, Gaignerie A, Francheteau Q, Kagawa H, Charpentier E, Flippe L, François-Campion V, Haider S, Dietrich B, Knöfler M, Arima T, Bourdon J, Rivron N, Masson D, Fournier T, Okae H, Fréour T, David L. Induction of Human Trophoblast Stem Cells from Somatic Cells and Pluripotent Stem Cells. Cell Rep. 2020 Nov 24;33(8):108419. doi: 10.1016/j.celrep.2020.108419. PMID: 33238118.

Cell Reports

Article

Induction of Human Trophoblast Stem Cells from Somatic Cells and Pluripotent Stem Cells

Graphical Abstract



Highlights

- Reprogramming of patient somatic cells to induced hTSCs with OSKM
- Conversion of naive and extended hPSCs to hTSCs
- Comparison of models of the human trophoblast lineage
- h(i/c)TSCs are akin to day 8 trophoblasts of the human embryo

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In Brief

Castel et al. report the generation of patient-specific human induced trophoblast stem cells via two methods: (1) somatic cell reprogramming with OSKM and (2) conversion of naive and extended hiPSCs. Their findings open avenues to study placental diseases and relations between the trophoblast lineage, pluripotency, and the human embryo.

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November 24, 2020
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Article

Induction of Human Trophoblast Stem Cells from Somatic Cells and Pluripotent Stem Cells

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SUMMARY

Human trophoblast stem cells (hTSCs) derived from blastocysts and first-trimester cytotrophoblasts offer an unprecedented opportunity to study the placenta. However, access to human embryos and first-trimester placentas is limited, thus preventing the establishment of hTSCs from diverse genetic backgrounds associated with placental disorders. Here, we show that hTSCs can be generated from numerous genetic backgrounds using post-natal cells via two alternative methods: (1) somatic cell reprogramming of adult fibroblasts with OCT4, SOX2, KLF4, MYC (OSKM) and (2) cell fate conversion of naive and extended pluripotent stem cells. The resulting induced/converted hTSCs recapitulated hallmarks of hTSCs including long-term self-renewal, expression of specific transcription factors, transcriptomic signature, and the potential to differentiate into syncytiotrophoblast and extravillous trophoblast cells. We also clarified the developmental stage of hTSCs and show that these cells resemble day 8 cytotrophoblasts. Altogether, hTSC lines of diverse genetic origins open the possibility to model both placental development and diseases in a dish.

INTRODUCTION

During the first trimester of pregnancy, a subset of proliferative villous cytotrophoblasts (VCTs) ensures the development and homeostasis of the placenta. These cells self-renew and differentiate into all cell types of the trophoblast lineage. Therefore, they are considered to be human trophoblast stem cells (hTSCs).

Isolation of hTSCs has been a major issue in the fields of developmental biology and stem cell research. Mouse TSCs were derived in 1998, but hTSCs were isolated only recently (Okae et al., 2018), due to the difficulty to identify the compartment of these cells *in vivo* and the signaling pathways governing their self-renewal. Okae et al. (2018) successfully derived hTSCs from blastocysts and first-trimester VCTs. They designed a medium containing notably epidermal growth factor (EGF), NODAL/transforming growth factor β (TGF-β) pathway inhibitors, and a WNT pathway activator, which allowed prolonged culture of hTSCs. Herein, this medium is referred to as hTSC medium.

hTSCs cultured *in vitro* represent a pristine model to investigate the development of the trophoblast lineage. These cells generate all differentiated trophoblast cell types, comprising the syncytiotrophoblast (ST) and extravillous trophoblasts (EVTs) (Okae et al., 2018). The ST is the multinucleated outer layer of the trophoblast epithelium formed by cell-cell fusion of cytotrophoblasts. The unique structure of the ST facilitates diffusion of nutrients and gases between maternal blood and the fetus and protects the latter from pathogen entry. EVTs are migratory cells formed by epithelial-mesenchymal-like transition of cytotrophoblasts. EVTs invade the decidual stroma, remodel the spiral arteries, and participate to immune tolerance between

the developing conceptus and the mother through a unique pattern of histocompatibility leukocyte antigen (HLA) expression, notably HLA-G (Knöfler et al., 2019; Turco and Moffett, 2019).

hTSCs at the origin of these processes play a central role in the formation of the maternal-fetal interface, and abnormal hTSCs are likely to have dramatic consequences on placental development. This, in turn, can have post-natal outcomes and ultimately provoke chronic disease in the adulthood (Burton et al., 2016). However, we neither understand the nature and incidence of hTSC disorders nor the connection of hTSCs with placental diseases such as preeclampsia, fetal growth restriction, miscarriage, or choriocarcinomas. For this purpose, researchers need to access hTSCs of diverse genetic backgrounds associated with normal and pathological situations. So far, only a few hTSC lines have been isolated from surplus embryos donated to research and from aborted placentas, and we cannot access hTSCs from individuals who were born after placental complications (Ezashi et al., 2019). To overcome these issues, we need alternative methods to generate hTSCs from more accessible sources of cells.

Human induced pluripotent stem cells (hiPSCs), generated by somatic cell reprogramming, have the potential to differentiate into any cell type in the body and give access to patient-specific cells (Kilens et al., 2018; Takahashi et al., 2007). Cell fate conversion is another method to generate cell types of interest, representing a faster approach that does not involve generation of iPSC lines. Chemical compounds can be sufficient to achieve cell fate conversion, which avoids transduction of exogenous factors (Kim et al., 2020). We hypothesized that these reprogramming strategies, largely applied to embryonic lineages, could also give access to extraembryonic cells, including those of the placenta. It has been reported that primed hPSCs, corresponding to the post-implantation epiblast (EPI), acquire a trophoblast-like fate in response to BMP4, A83-01 (NODAL/TGF-β pathway inhibitor), and PD173074 (fibroblast growth factor [FGF] pathway inhibitor), known as BAP treatment (Amita et al., 2013). However, these cells share features with differentiated trophoblasts and do not self-renew, which limits their use to model the human placenta. Also, the BAP model is debated, as some claim that it produces mesoderm, but others amnion-like cells (Bernardo et al., 2011; Guo et al., 2020).

In this study, we applied OCT4, SOX2, KLF4, MYC (OSKM) reprogramming of somatic cells and conversion of pluripotent stem cells to generate human induced and converted TSCs (hiTSCs and hcTSCs, respectively), from patients with diverse genetic backgrounds. Comparison with isogenic hiPSCs, placental cell types (VCTs, VCT-ST cells, EVTs), and previously established hTSC lines confirmed that hi/cTSCs share similar differentiation potential and molecular signature with embryo- and placenta-derived hTSCs. This study paves the way to the production of patient-specific hiTSCs, with applications to obstetric medicine and the treatment of placental diseases.

RESULTS

Somatic Cell Reprogramming into hiTSCs

We recently achieved reprogramming of somatic cells with OSKM into induced naive hPSCs (hiNPSCs), the counterpart of the pre-implantation EPI in human (Kilens et al., 2018). At low passage in t2iLGö medium, in cells that were still expressing OSKM transgenes, we observed cobblestone-shaped morphology that was reminiscent of hTSCs, in accordance with the expression of trophoblast-associated transcription factor *GATA3* (Figures S1A and S1B). This suggested the occurrence of cells with dual potential to become either hTSCs or hiNPSCs, or a heterogeneous cell population. We thus investigated whether specific combinations of OSKM transgenes enabled to enrich the reprogrammed cells with hTSCs. In the majority of cases, cells stopped proliferating at early passages. Only specific stoichiometries yielded naive hPSC (hiNPSCs), but not hTSC lines (5:5:3 and 3:3:3 KOS, K, M multiplicity of infection). These results suggest that the stoichiometry of the OSKM reprogramming factors is not sufficient to reroute OSKM reprogramming toward hTSCs.

We thus hypothesized that the acquisition of the hTSC state might rather reside in environmental cues and repeated OSKM reprogramming in hTSC culture conditions. After 7 days, cells were transferred either in E7 medium that supports the early phase of reprogramming (Chan et al., 2009) or in hTSC medium. After seven additional days, we observed the formation of epithelial colonies in both conditions, although these colonies were more abundant in E7. These results suggest that E7 not only supports the early phase of reprogramming but also promotes a mesenchymal-epithelial transition and the survival of reprogramming intermediates. Clearly, after culture in E7 (from day 7 to 21), cells robustly supported a transition into hTSC medium. Upon additional culture (2 passages), we observed the rapid formation of cobblestone-shaped colonies, morphologically reminiscent of hTSCs (Figure 1A). These cell lines subsequently lost their transgenes (after 10–15 passages) and expressed the *GATA2* and *GATA3* genes associated with the trophoblast lineage (Gerri et al., 2020; Home et al., 2017; Krendl et al., 2017; Meistermann et al., 2019). By contrast, they did not express the pluripotency markers *NANOG* and *KLF17* (data not shown). These cells propagated unlimitedly, showing long-term self-renewal (>70 passages). Based on their morphology, gene expression profile, and culture condition requirement, we referred to these reprogrammed cell lines as hiTSCs (Table S1A).

Cell Fate Conversion of hNPSCs and hEPSs to hTSCs

To further study the plasticity between pluripotent and trophoblast fates, we tested the potential for primed hPSCs that represent the post-implantation EPI (Amit et al., 2000; Thomson et al., 1998), extended hPSCs (hEPSs) that stabilize a high-potency state (Yang et al., 2017), and hNPSCs that reflect the pre-implantation EPI (Guo et al., 2016; Kilens et al., 2018; Takashima et al., 2014) to respond to BAP treatment (Amita et al., 2013). Primed hPSCs were initially cultured in knockout serum replacement (KSR) + FGF2 or iPS-BREW, hEPSs in LCDM, and hNPSCs in t2iLGöY medium. Consistent with previous reports, primed hPSCs rapidly responded to BAP and transdifferentiated into large cell sheets morphologically reminiscent of trophoblasts. Interestingly, BAP culture could also induce similar morphological changes in hNPSCs and hEPSs, although these cell types are reflecting different states of pluripotency (Figure S2A). We confirmed the expression of trophoblast marker genes in all

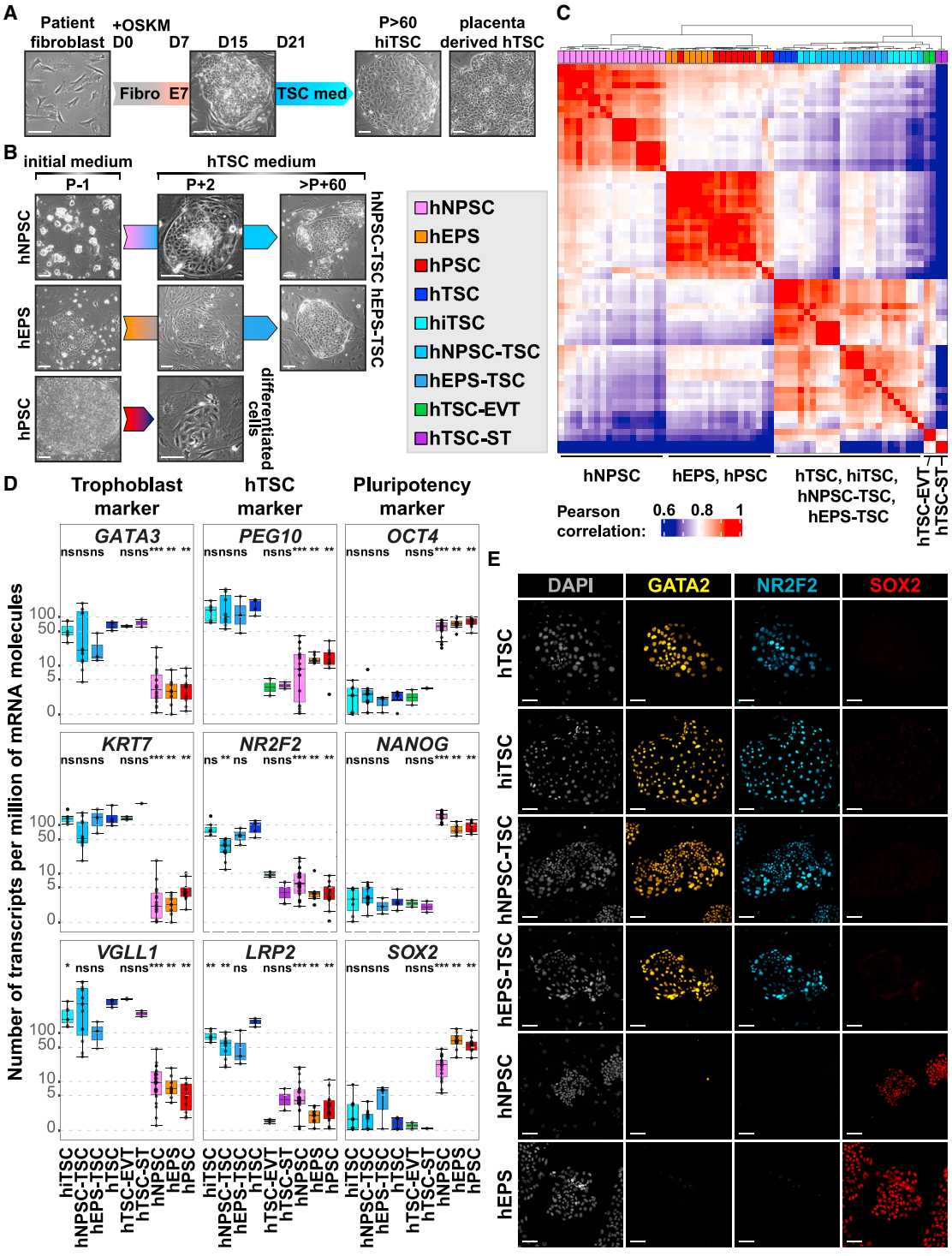

Figure 1. Generation of Human Induced Trophoblast Stem Cells by Reprogramming of Somatic Cells with OSKM and Conversion of Pluripotent Stem Cells

(A) Schematic representation of the reprogramming protocol. Phase contrast pictures show the changes in cell morphology. Placenta-derived hTSCs are shown as controls.

(B) Schematic representation of the conversion protocol. Phase contrast pictures show the changes in cell morphology.

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BAP-treated hPSCs, while we did not detect the expression of genes associated with other lineages, such as mesoderm or amnion (Figures S2B and S2C). These gene expression patterns were comparable to those of hiTSCs treated with BAP, but the cells rapidly stopped proliferating and could not be maintained beyond day 16. We concluded that BAP medium efficiently promoted the conversion of hPSCs to trophoblast-like cells independently from their initial state, but was not suitable for maintenance of self-renewing and expandable hTSCs.

Next, we repeated the similar experiment in hTSC medium. Primed hPSCs did not expand in the hTSC condition, and we observed elevated cell death from 48 to 120 h. Colony integrity faded after 1 or 2 passages and cells stopped proliferating, thus failing to establish hTSCs. Extended hPSCs also experienced an elevated cell death from 48 to 120 h, but few cobblestone-shaped colonies reminiscent of hTSCs emerged after 2 passages (7–14 days) that could be expanded. By sharp contrast, hNPSCs sustained moderate cell death (48–120 h), and numerous colonies similar to hTSCs rapidly emerged (7–14 days) and propagated unlimitedly (>50 passages, Figure 1B). These cells had lost expression of pluripotency markers *NANOG* and *KLF17* and gained expression of trophoblast-associated genes *GATA2* and *GATA3* (data not shown). Hereafter, these cell lines are referred to as hcTSCs (Table S1A).

Molecular Characterization of hiTSCs and hcTSCs

We conducted broad transcriptomic analyses to further characterize hiTSCs and hcTSCs in direct comparison with previously established hTSCs and the differentiated ST cells and EVTs (Okae et al., 2018).

Hierarchical clustering defined three groups of cells: (1) hNPSCs, (2) extended and primed hPSCs, and (3) trophoblasts. Both hiTSCs and hcTSCs clustered together with previously established embryo- and placenta-derived hTSCs to form the group of trophoblasts. This group further subdivided between h(i/c)TSCs, ST cells and EVTs. Pearson correlation analysis further confirmed the proximity of induced, converted, embryo-derived, and placenta-derived hTSCs. Surprisingly, extended and primed hPSCs showed a high degree of transcriptional similarity, despite relative differences in their potential to form hTSCs (Figure 1C). Principal component analysis (PCA) confirmed these observations and produced distinct clusters corresponding to the above-mentioned cell types (Figure S1C). This can suggest that hEPSs might contain rare subpopulations with higher potency comparable to hNPSCs or that the potential to form hTSCs might rely on discrete cellular properties shared between hNPSCs and hEPSs.

Further analysis confirmed that hiTSCs and hcTSCs expressed key trophoblast lineage markers. Notably, the expression levels of *GATA3*, *KRT7*, and *VGLL1* were similar to those found in previously established embryo- and placenta-derived hTSCs (absolute gene expression ranging from 10 to 300 transcripts per million [TPM]). We also identified genes associated with stemness of hTSCs, including *PEG10*, *NR2F2*, and *LRP2*. These were expressed at similar levels in hTSCs, hiTSCs, and hcTSCs, but not in the differentiated ST cells and EVTs (absolute gene expression ranging from 20 to 200 TPM in hTSCs; below 10 TPM in hTSC-ST cells and hTSC-EVTs) (Figure 1D). By contrast, hiTSCs and hcTSCs did not express pluripotency-associated markers such as *NANOG*, *SOX2*, or *OCT4* (*POU5F1*) (absolute gene expression below 10 TPM). We also confirmed that hi/cTSCs did not express genes associated with other lineages (Figure S1D). Globally, gene expression profiles of hi/cTSCs were comparable with those of embryo- and placenta-derived hTSCs, but different from those of hPSCs, which was confirmed by statistical analysis.

We finally analyzed hiTSCs and hcTSCs by immunofluorescence for the trophoblast markers NR2F2 and GATA2 that are expressed in the trophectoderm (TE) of human blastocysts (Meistermann et al., 2019). NR2F2 and GATA2 were highly expressed and localized in nuclei of all cells. Conversely, SOX2 was highly expressed in hPSCs but absent in hTSCs (Figure 1E). These expression patterns were comparable between hiTSCs, hcTSCs, and placenta-derived hTSCs. These results confirm that hi/cTSCs share similar expression profiles with previously established hTSCs.

Functional Validation of hi/cTSCs: Differentiation into EVTs and ST Cells

hTSCs are characterized by their ability to generate highly specialized trophoblast cell types that ensure the unique functions of the placenta. Notably, these cell lineages include the syncytiotrophoblast (ST) and EVTs. Therefore, we assessed the potential for hiTSCs and hcTSCs to differentiate into these cell types, using previously established protocols based on NRG1 for EVT, and forskolin for ST, differentiation (Okae et al., 2018). We directly compared this potential with those of embryo- and placenta-derived hTSCs, along with VCT, ST, and EVT cells isolated from human placentas.

Prior to differentiation assays, hTSCs were cultured either in hypoxia or normoxia to promote EVT or ST formation, respectively (Chng et al., 2010; Wakeland et al., 2017). Initially, we cultured hTSCs on mouse embryonic fibroblast (MEF) feeder

(C) Heatmap of Pearson correlation coefficients of hPSC, hEPS, hNPSC, hiTSC, hcTSC, and hTSC lines along with ST cells and EVTs differentiated from hTSCs. Correlations are determined from the comparison of the 2,770 most over-dispersed genes of the dataset (see STAR Methods). Samples are clustered from the Euclidean distance of correlations, by a hierarchical clustering using Ward's method.

(D) Gene expression levels of indicated lineage markers are shown for hPSC, hEPS, hNPSC, hiTSC, hcTSC, and previously established embryo- and placenta-derived hTSC lines. The differentiated ST cells and EVTs are included as controls. Expression levels are given as number of transcripts per million of mRNA molecules. In each boxplot, the top and bottom of the box represent the third and first quartile, respectively; the band represents the median (second quartile); and error bars show the interquartile range (IQR) (lower bound: Q1 – 1.5 × IQR; upper bound: Q3 + 1.5 × IQR). A Wilcoxon-Mann-Whitney statistical test was performed for each type of hPSC and hTSC, with embryo- and placenta-derived hTSCs taken as the reference group. Asterisks indicate statistical significance of the difference: *p value < 0.05, **p value < 0.01, ***p value < 0.001.

(E) Immunofluorescence images of hTSCs, hiTSCs, hcTSCs, hNPSCs, and hEPSs stained for trophoblast-associated transcription factors GATA2 and NR2F2 and pluripotency-associated transcription factor SOX2. Nuclei were stained with DAPI.

Scale bars, 100 μm.

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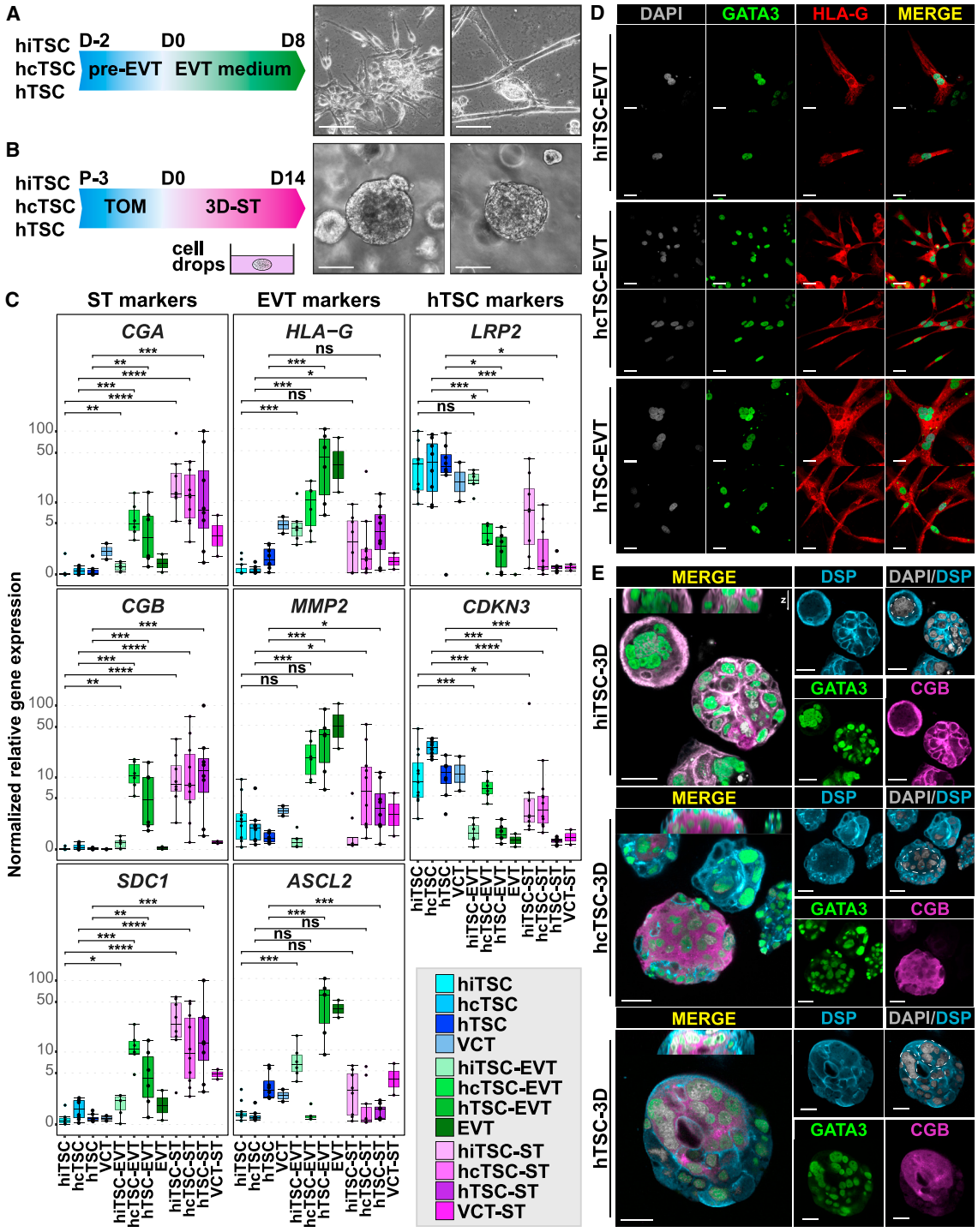

Figure 2. Induced and Converted hTSCs Can Differentiate into Extravillous Trophoblasts and the Syncytiotrophoblast

(A) Schematic representation of the EVT differentiation protocol (left). Bright-field pictures of the EVT progeny of h(i/c)TSCs (right).

(B) Schematic representation of the 3D-ST differentiation protocol (left). Bright-field pictures of the 3D-ST structures derived from h(i/c)TSCs (right).

(C) qRT-PCR quantification of markers associated with ST cells (*CGA*, *CGB*, *SDC1*), EVTs (*HLA-G*, *MMP2*, *ASCL2*), and hTSCs (*LRP2*, *CDKN3*). In each boxplot, the top and bottom of the box represent the third and first quartiles, respectively; the band represents the median (second quartile); and error bars show the IQR (lower bound: Q1 − 1.5 × IQR; upper bound: Q3 + 1.5 × IQR). A Wilcoxon-Mann-Whitney statistical test was performed for each type of hTSC and the differentiated cell progeny. Asterisks indicate statistical significance of the difference: *p value < 0.05, **p value < 0.01, ***p value < 0.001.

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layers that promoted undifferentiated proliferation compared with other matrices. However, we observed that MEFs impaired the formation of ST cells and EVTs so we screened for other matrices to adapt hTSCs prior to differentiation assays. Fibronectin coatings were efficient at maintaining hTSCs over time, and laminin 521 could further enhance proliferation (Figure S3A). For an efficient differentiation, we adjusted the protocols of Okae et al. (2018) as follows: EVT differentiation was enhanced by complementing the medium with IWR-1, which promoted the accumulation of EVT progenitors, as previously described (pre-EVT medium; Haider et al., 2018). For ST and EVT differentiation, we adjusted cell density at seeding from 0.8 to 6.0 × 10^4 cells/cm^2 and the timing of treatment initiation from 0 to 6 h, depending on the lines (Figures 2A and 2B).

Upon optimized ST differentiation, cells upregulated the expression of *CGA*, *CGB*, and *SDC1*, which are not expressed in hTSCs (relative gene expression ranging from 10- to 1,300-fold change). By contrast, *HLA-G*, *MMP2*, and *ASCL2* were globally increased in the EVT differentiation condition (relative gene expression ranging from 10- to 700-fold change). Finally, *LRP2* and *CDKN3* predominantly expressed in hTSCs were downregulated upon differentiation (relative gene expression ranging from 2- to 70-fold change). Importantly, gene expression patterns were comparable with those of placental cells, which was confirmed by statistical analysis (Figure 2C).

Of note, some cell lines did not upregulate *MMP2* upon EVT differentiation, while others did not upregulate *ASCL2*. However, the clear expression of *HLA-G* unequivocally confirmed the EVT identity. This raises the possibility of distinct EVT populations, as previously described (Knöfler et al., 2019; Xiang et al., 2020), or it might reflect intrinsic cellular properties of cell lines. In addition to specific gene expression, EVTs can be reliably identified by dramatic morphological changes resulting in elongated shape (phase contrast images, Figure 2A). Immunostainings for GATA3 and HLA-G confirmed the identity of EVTs differentiated from hiTSCs, hcTSCs, and placenta-derived hTSCs (Figure 2D).

In addition, ST cells can be identified by two important characteristics: the production of human chorionic gonadotropin (hCG) and the fusion of cells that form multinucleated syncytia. After 6 days of forskolin treatment, β-hCG secretion was increased in h(i/c)TSCs, with a mean secretion superior to 3.9 × 10^4 mIU/mL. By contrast, in those conditions, hPSC lines globally did not secrete β-hCG. In line with transcriptomic analysis, both hPSC and hTSC lines secreted β-hCG when treated with BAP (Figure S3D).

Finally, it has been reported that the 3D culture of hTSCs promotes the formation of ST cells (Haider et al., 2018; Okae et al., 2018). Based on trophoblast organoid formation protocols, we embedded hTSCs as single cells in a semi-solid environment made of Matrigel, fibronectin, and laminin 521. Cells were subsequently cultured in human trophoblast organoid medium (TOM) with small modifications (Turco et al., 2018). Within a week, we observed the formation of 3D structures that grew to ∼200 μm in diameter after 2 weeks (Figure 2B). These structures contained multinucleated syncytia expressing desmoplakin (DSP) and CGB, and typically containing 6–10 nuclei (Figure 2E). In the majority of cases, fusion of cells occurred in the center of the 3D structures, as previously observed with placenta-derived trophoblast organoids (Haider et al., 2018; Turco et al., 2018). Over time, these placental organoids further grew until they reached confluence within the drops of Matrigel. These larger structures also contained multinucleated ST cells (Figure S3C). Further optimization of culture conditions is needed to determine whether this system will allow expandable culture of hTSC-derived trophoblast organoids. We concluded that optimized differentiation protocols facilitate the formation of functional ST- and EVT-like cells and 3D self-organization from h(i/c)TSC lines. This confirmed the potential for hiTSCs and hcTSCs to form complex placental-like tissues including multiple differentiated and functional cell types.

Dynamics of Cell Fate Conversion from hPSCs into hTSCs

To further understand the conversion process of hPSCs into hTSCs, we projected our cellular models on a PCA, which shows that samples cluster according to their fate. PC1 and PC2 accounted for 18 and 11% of variance, respectively. We identified five main clusters: (1) hNPSCs, (2) hEPSs/hPSCs, (3) hTSCs/hiTSCs/hcTSCs, (4) EVTs, and (5) ST cells (Figure 3A). PC3 accounted for 9% variance and segregated hNPSCs from other cells, thus confirming the particularity of naive pluripotent stem cells, reflecting the early EPI, and capable of efficiently generating hTSCs (Figures 3B and 3C).

We further analyzed the progression of cells during cell fate conversion. All types of hPSCs treated with BAP showed rapid and dramatic transcriptomic changes and became close to differentiated trophoblasts by day 6. Despite some partial overlap with hTSCs, they formed a distinct group, closer to the differentiated EVTs and ST cells. Importantly, hTSCs treated with BAP acquired a similar state, supporting that these transcriptional changes relate to the differentiation of the trophoblast lineage (Figure 3D).

hNPSCs transferred to hTSC medium formed a separate cluster characterized by an intermediate transcriptome (P+2), followed by the acquisition of a hTSC molecular signature (∼P+3, Figure 3E). hEPSs had delayed transcriptional variations and remained globally unchanged until day 5 before transiting through an intermediate transcriptional state (P+2), ultimately acquiring a profile characteristic of hTSCs (P+3, Figure 3F). We concluded that hNPSCs and hEPSs transited through an intermediate transcriptional state before ultimately achieving a cell fate conversion into hTSCs. Whether this transcriptional progression reflects a developmental path remains to be investigated (Cinkornpumin et al., 2020). By contrast, primed hPSCs initiated similar transcriptional changes,

(D) Immunofluorescence images of EVTs differentiated from hiTSC, hcTSC, and placenta-derived hTSC lines stained for the trophoblast-associated transcription factor GATA3 and the extravillous trophoblast-specific surface marker HLA-G. Nuclei were stained with DAPI.
(E) Immunofluorescence images of 3D-ST structures derived from hiTSC, hcTSC, and hTSC lines stained for GATA3 and the membrane-associated protein desmoplakin (DSP) highlighting syncytia, along with the syncytiotrophoblast-associated marker CGB. Nuclei were stained with DAPI. Main images correspond to the x-y plane of a single z section. Related orthogonal y-z plane is shown above merged images.
Scale bars: 100 μm in (A) and (B) and 30 μm in (D) and (E).

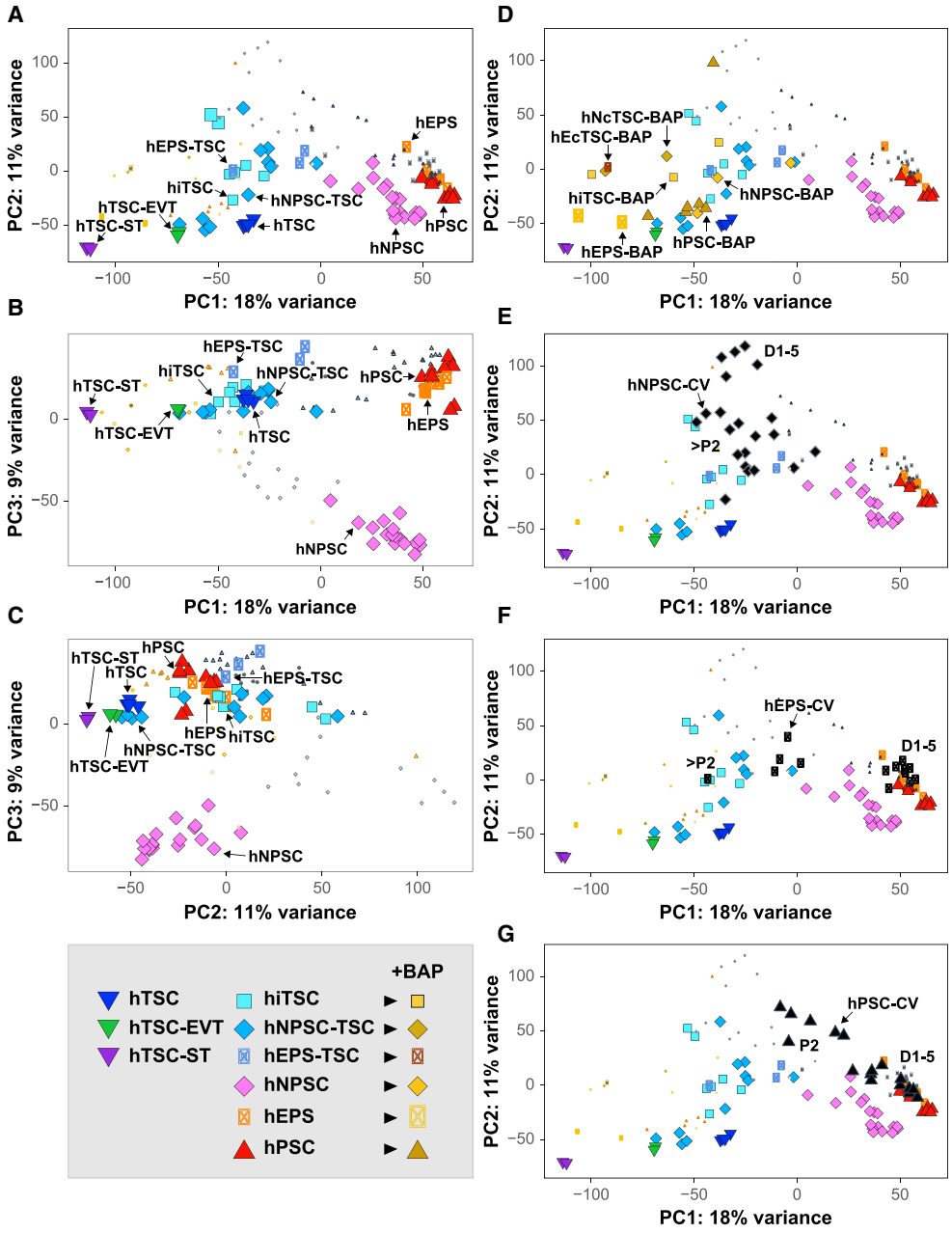

Figure 3. Transcriptional Specificities of h(i/c)TSCs and Intermediate Steps of Cell Fate Conversion

(A–G) PCA analysis of h(i/c)TSCs and hPSCs in maintenance, differentiation, or conversion media. PC1, PC2, and PC3 are displayed for differentiated cells, hPSC and hTSC lines (A–C). PC1 versus PC2 of BAP-treated cells (D), naive hPSCs converted into hTSCs (E), extended hPSCs converted into hTSCs (F) or primed hPSCs treated with hTSC medium (G) are highlighted in specific panels.

but the cell fate conversion was not completed and cells did not acquire hTSC signature (P+2, Figure 3G).

Globally, treatments of hPSCs with either BAP or hTSC medium converged to induce the acquisition of the trophoblast fate, but only hTSC culture condition supported the proliferation and self-renewal programs required to stabilize expandable stem cells. Understanding how external cues are integrated by cells to mediate these different outcomes will allow the identification of determinants of cell fate conversion, self-renewal, and proliferation of the native human placental progenitors.

Modulations of Signaling Pathway Signatures Underly the Conversion of hPSCs to hTSCs

We reasoned that signaling pathway variations upon conversion of hPSCs to hTSCs or differentiated trophoblasts might inform about the mechanisms of specification, self-renewal, and

differentiation of human trophoblast progenitors. We thus performed a clustering analysis to identify co-regulated genes and a pathway enrichment analysis.

Clustering of samples on differentially regulated pathways globally confirmed the sample clustering previously performed on differentially expressed genes. This segregated three main groups: (1) hPSCs, (2) hTSCs, and (3) differentiated trophoblasts. In line with the PCA results, BAP-treated cells (day 6) were akin to ST cells and EVTs and showed profiles of enriched pathways resembling those of differentiated trophoblasts. By contrast, hNPSC-TSCs and hEPS-TSCs shared similar pathway signatures with hiTSCs, embryo-derived, and placenta-derived hTSCs, while primed hPSCs transited in hTSC medium globally failed to acquire hTSC pathway signatures (Figure S4).

The clustering of pathways produced two main groups: (1) pathways associated with hPSCs and (2) pathways associated with trophoblasts. Pathways associated with hPSCs included glycolysis, cell cycle, and base excision repair, in line with previous studies on cell cycle regulation and metabolic state of hPSCs (Kilens et al., 2018). By contrast, hTSCs and differentiated trophoblasts globally shared common pathway signatures, clearly distinct from those of hPSCs.

Differentially regulated pathways included HIPPO, NOTCH, and ERBB pathways, which are main drivers of the self-renewing state of mouse TSCs (El-Hashash et al., 2010; Home et al., 2012; Nishioka et al., 2009; Rayon et al., 2014; Rivron et al., 2018b) and PPARG that is associated with mouse trophoblast proliferation and differentiation (Parast et al., 2009). These results complete previous studies suggesting conserved mechanisms of trophoblast development across mammals (Gerri et al., 2020; Hunkapiller et al., 2011; Saha et al., 2020; Schaiff et al., 2000), while they also highlight specific features of the human trophoblast lineage such as the steroid hormone biosynthesis pathway that was specifically associated with the ST in humans (Malassiné et al., 2003). Moreover, our analysis pointed to additional pathways including MTOR, estrogen, RAP1, and JAK/STAT signaling pathways that seem to be active in h(i/c)TSCs (Figures S4 and S5).

Regarding the MTOR signaling pathway, genes with positive contribution to the eigengene were dominantly expressed in hTSCs. They included *CASTOR1*, an inhibitor of mTORC1, and *PRR5*, a subunit of mTORC2. Also, trophoblast cells downregulated *RRAGD*, an activator of mTORC1. By contrast, hPSCs expressed *MTOR*, *ULK1*, and *SGK1*, which are effectors of mTORC1 signaling. These observations could indicate a switch from TORC1 in hPSCs to TORC2 activity in hTSCs during the cell fate conversion process. Interestingly, we also observed differences in MTOR pathway signatures between the distinct types of hPSCs. Along with other genes, the expression of *DEPTOR*, a regulator of MTOR signaling, was high in hNPSCs, moderate in hEPSs, and low in hPSCs, suggesting a differential regulation of the pathway between these cells associated with different degrees of trophoblast potential (Figure S5).

The estrogen signaling pathway seemed to be active in hTSCs, which is reflecting the response to placental hormones that is observed *in vivo*. This was accompanied with the expression of *CREB3* and *FOS* that can mediate the transcription of estrogen-responsive genes, and the upregulation of keratin genes, such as *KRT17*, *KRT18*, and *KRT19*, in line with the morphological changes associated with the formation of epithelial hTSCs (Figure S5). Conversely to hTSCs, hPSCs expressed *FKBP5* and *HSP90AB1*, which can form a complex that inhibits the translocation of the estrogen receptor to the nucleus (Baker et al., 2018).

hTSCs also expressed *RRAS*, *VAV3*, and *RAC2*, which are effectors of the RAP1 signaling pathway, along with *EGFR* and *GNAI1*, which can signal to RAP1 through receptor tyrosine kinase (RTK)- and G protein-coupled receptor (GPCR)-induced cascades, respectively. By contrast, hEPSs and hPSCs expressed *ID1*, which is inhibited by RAP1, and globally did not express actors of the RAP1 signaling pathway, suggesting that it is not active in these cells. However, hNPSCs expressed *RASGRP2*, which specifically activates RAP1, along with *FGFR3* and *LPAR2*, which encode for two receptors that can signal to RAP1 (Figure S5). These gene expression profiles suggest that the RAP1 signaling pathway might be active in hTSCs and hNPSCs, but is mediated through different input signals, while it is not active in hEPSs and hPSCs.

We also found that hTSCs expressed genes encoding receptors that can activate the JAK/STAT signaling pathway, including *IL10RB*, *CSF3R*, *CSF2RA*, and *IFNGR1*. By contrast, hEPSs and hPSCs expressed *SOCS3*, a member of the suppressor of cytokine signaling protein family that inhibits JAK/STAT signaling, while hNPSCs expressed *SOCS4* and *PTPN2*, which dephosphorylate JAK and STAT proteins thus inhibiting the signaling.

Interestingly, our analysis also pointed to gene expression profiles that suggested crosstalk between the identified pathways. For example, *SFN*, which is associated with cell cycle, was specifically expressed in hTSCs. When bound to KRT17, SFN regulates epithelial cell growth by stimulating the MTOR pathway (Kim et al., 2006). This suggests a potential crosstalk between the cell cycle, estrogen, and MTOR pathways in hTSCs (Figures S4 and S5).

Our analysis highlighted both conserved and divergent expression profiles of signaling pathway components underlying trophoblast specification and self-renewal. hTSC pathway signatures were globally milder than those of differentiated trophoblasts, and the switch between the self-renewing state (hTSC) and the differentiated state (BAP-treated cells) was mainly reflected by an accentuation of these same pathway signatures. This suggests that human trophoblast development is driven by a continuity rather than a sequential switch between different signaling activities and that the strength of the signaling activity correlates with the progression from a self-renewal to a differentiation program. This dampened signaling activity observed in the self-renewing state might reflect the minimal requirement of unspecialized human trophoblast progenitors.

A comprehensive list of pathway components and their contribution to pathway eigengenes can be found in Table S2G. Further knockout experiments are needed to determine how these pathways are modulated between hPSCs, hTSCs, and trophoblast lineages; yet, this analysis gives a global picture of hTSC pathway signatures and changes associated with cell fate conversion of hPSCs.

hTSCs Are Akin to Post-implantation Day 8–10 Cytotrophoblasts

An outstanding question regarding hTSCs is to understand which developmental stage these cells are reflecting. To address

this question, we compared the transcriptomes of hTSCs with single-cell RNA sequencing (scRNA-seq) data of human peri-implantation embryos (146 embryos; 6,838 cells), from day 3 to 14 (prolonged culture of human embryos for 9 days after blastocyst stage) (Petropoulos et al., 2016; Zhou et al., 2019). We projected all cells on a uniform manifold approximation and projection (UMAP) and highlighted sample annotation (Figure 4A, inset). UMAP recapitulated developmental time and fate, showing the succession of eight-cell stage, morula, early blastocyst, EPI, primitive endoderm (PrE), TE, and trophoblast (TB) cells from left to right. We noticed a cluster of trophoblasts with enrichment of apoptosis-related genes that we named "apoptotic trophoblasts" and the previously reported cluster of yolk-sac trophoblasts (Zhou et al., 2019). We further clustered cells on the UMAP, which yielded 19 clusters of cells. In particular, this distinguished clusters associated with the development of the trophoblast lineage: early, medium, and late pre-implantation TE, in line with our recent report (Meistermann et al., 2019); five post-implantation trophoblasts (trophoblast #1 to trophoblast #5); and pre-EVT, EVT, pre-ST, and ST (Figure 4A).

We used gene sets associated with each cluster, or gene sets recently associated with pre-implantation TE, post-implantation trophoblast, EVT, and ST to benchmark the transcriptional signature of h(i/c)TSCs (Xiang et al., 2020) (Figures S6A and S6B). Gene sets specific of each cluster highlighted the hierarchy of transcriptomic changes upon progression toward the trophoblast lineage (Figure S6A). Overall, comparison of transcriptional profiles of h(i/c)TSCs with peri-implantation scRNA-seq datasets pointed that h(i/c)TSCs are related to the trophoblast lineage from day 5 to 12 (Figures S6A and S6B). Curation of the list led us to propose markers of post-implantation trophoblasts that matched hTSC lines and distinguished them from ST cells, EVTs, and hPSC lines (Figure 5). The markers that are better associated with hTSCs are found in the gene sets specific of clusters trophoblast #1 and trophoblast #2, which are mainly composed of cells isolated from day 8 and day 10 embryos. Among those gene lists, we identified markers that have been assessed by immunofluorescence in human embryos: NR2F2, CDX2, GATA3, KRT7, and CCR7 (Deglincerti et al., 2016; Meistermann et al., 2019; Niakan and Eggan, 2013; Petropoulos et al., 2016). Projection of the expression of those markers on the UMAP led us to propose that hTSCs expressed genes associated with clusters trophoblast #1 and trophoblast #2. Indeed, hTSCs are expressing LRP2 and NR2F2, but not CDX2 (marker of medium TE) or EVT or ST markers (Figures 4B and 5). To further support our conclusion, we represented gene set expression across embryonic cell populations and stem cells as violin plots summarizing the heatmap (Figure 6). This representation allows to appreciate genes sets present or absent in each cell type. For example, hNPSCs are displaying high expression of EPI genes, moderate expression of early TE genes, and low expression of post-implantation trophoblast genes. hNPSCs are therefore the closest to EPI. h(i/c)TSCs were akin to the trophoblast #1 and trophoblast #2 clusters, while BAP-treated cells were closer to the trophoblast #5 and pre-ST clusters. Finally, ST and EVT cells derived from hTSC differentiation *in vitro* shared similar expression profiles with those of ST and EVT cells found in the embryo (Figure 6).

Altogether, our analysis points out markers that can distinguish developmental timing of TE and trophoblast cells and associate hTSCs with day 8–10 of development. Those markers notably include LRP2 and NR2F2.

DISCUSSION

In the present study, we generated hiTSCs from patients with diverse genetic backgrounds. We found that the OSKM system was not restricted to embryonic lineages, but was permissive to the trophoblast fate. Therefore, this system, largely accessible to researchers, is suitable for the parallel generation of isogenic hiTSCs and hiPSCs, which could greatly benefit the study of placental diseases. Comparison with primary placental cells and hTSC lines confirmed that hi/cTSCs were similar to embryo- and placenta-derived hTSCs. They recapitulated transcriptome, protein markers, and differentiation potential into EVT and ST cells.

In this study, we also revisited the relations between hPSCs and the trophoblast lineage. We used two different systems to evaluate the potential of hPSCs to generate hTSCs: BAP treatment and transition into hTSC medium. We assessed a broad spectrum of hPSCs, comprising hNPSCs, related to pre-implantation EPI; hEPSs, showing contribution to extra-embryonic lineages in chimeras; and primed hPSCs, related to post-implantation EPI. We found that all types of hPSCs produced differentiated trophoblasts, but not hTSCs, in response to BAP. By contrast, hNPSCs and extended hPSCs, but not primed hPSCs, converted to hTSCs, following transition into hTSC medium.

During the preparation and revision of this manuscript, other groups have reported conflicting evidence that the potential to generate trophoblasts is either higher in ground state or in expanded potential stem cells (Cinkornpumin et al., 2020; Dong et al., 2020; Gao et al., 2019). For example, Gao et al. (2019) claimed that expanded potential hPSCs (hEPSCs), but not hNPSCs (cultured in 5iLAF), can generate hTSCs. Dong et al. (2020) and Cinkornpumin et al. (2020) reported conversion from 5iLAF naive cells. Here, we report successful conversion into hTSCs from the two other main culture media used for hNPSCs (Takashima et al., 2014) and extended hPSCs (Yang et al., 2017). Our results indicate that the potential to engage the trophoblast lineage is common to all hPSCs. However, in our model, only hNPSCs and hEPSs completed cell fate conversion to hTSCs. We concluded that the potential to form hTSCs correlates with the proposed developmental time equivalent of the initial culture, with hNPSCs being the most potent state to form hTSCs. We do not exclude that primed hPSCs could generate hTSCs in another system, but this might rely on other pathways. Further investigations are needed to determine whether plasticity exists in the embryo, between the EPI and the TE, and how it is regulated upon developmental progression.

Another key point of this study was to determine the developmental counterpart of hTSCs in the embryo. To address this, we took advantage of scRNA-seq datasets of the human embryo during the peri-implantation development, from day 3 to 14. Our analysis revealed high complexity of the trophoblast lineage, divided in twelve clusters of cells, including TE, CTB, ST, and EVT. We compared molecular signatures and found that hTSCs resemble NR2F2+ day 8–10 CTB, which is clearly distinct from

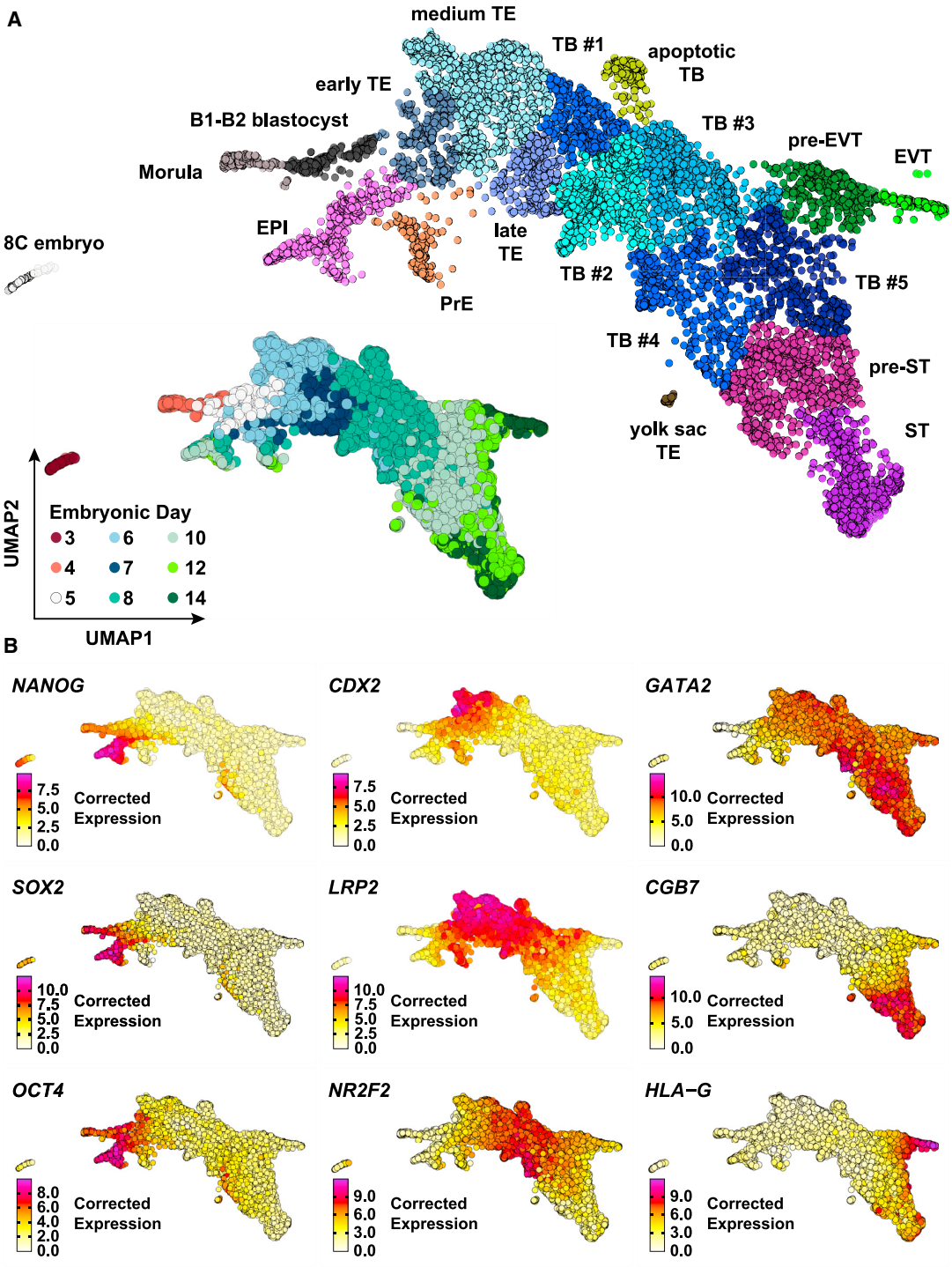

Figure 4. Transcriptomic Analysis of Human Development from Day 3 to 14

(A) UMAP representation of the combined scRNA-seq dataset from Petropoulos et al. (2016) and Zhou et al., (2019), covering eight-cell stage to "day 14" of human development. Cluster analysis revealed 19 clusters, indicated on the UMAP.

(B) Projection of the developmental day annotation on the UMAP.

(C) Projection of expression levels for selected genes on the UMAP. The scale of expression is logarithmic and is equivalent to *a log2*$(x+1)$ transformation of expression counts.

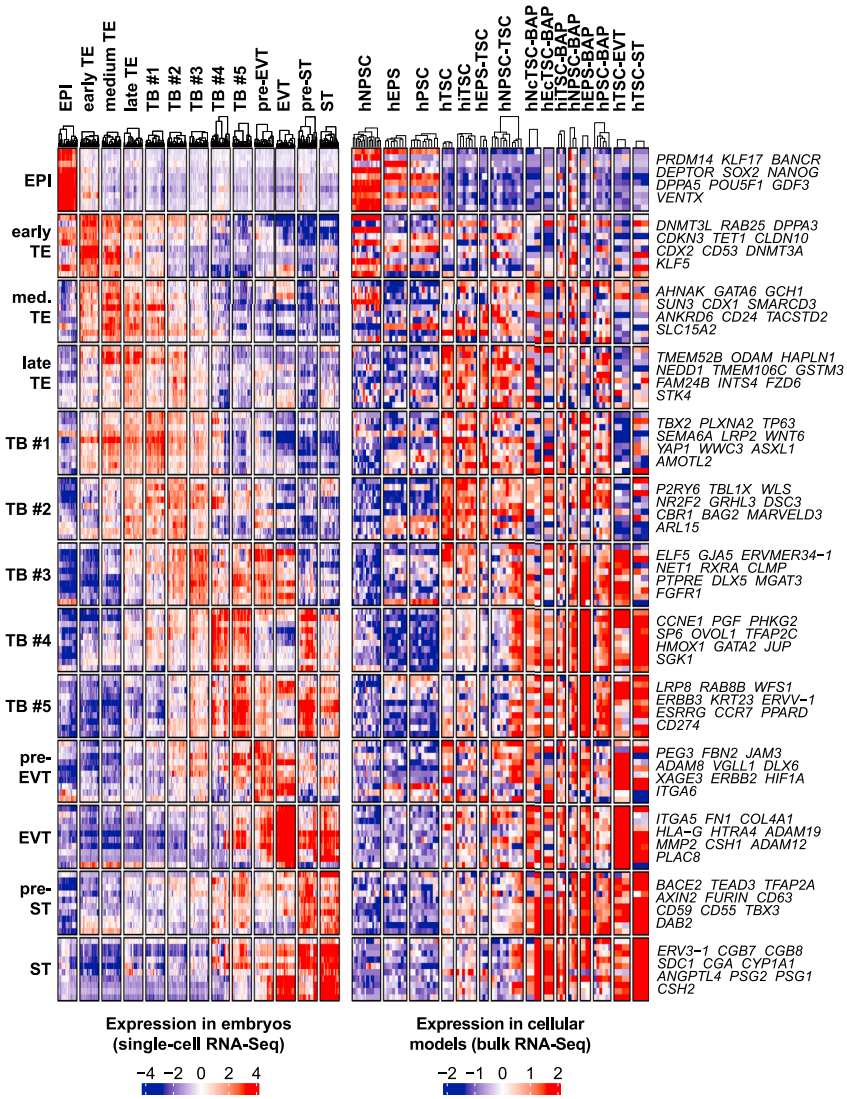

Figure 5. Developmental Matching of hTSCs with Peri-implantation Trophoblasts of the Human Embryo

Gene expression heatmaps of selected markers characterizing embryo cell clusters are shown for scRNA-seq embryo samples (left) or digital gene expression sequencing (DGE-seq) cellular model samples sequenced for this study (right). Expression is *Z* scored. A panel of 10 genes was selected among the best markers of each cell cluster, indicated at the right of the heatmaps. The entire list of markers is available in Table S2. On the left heatmap, 30 cells were randomly selected for each single-cell cluster.

CDX2+ day 5–6 TE. Therefore, hTSCs might be suitable to study early events of trophoblast lineage development, but maybe not for pre-implantation TE. To address this, we need to optimize culture conditions to isolate and maintain human trophectoderm stem cells (hTESCs). Recent studies suggest that this state exists in human, and CDX2+ hTSCs have been obtained, but these cells have not been compared with the human TE yet (Knöfler et al., 2019; Mischler et al., 2019).

Finally, generation of hiTSCs by reprogramming provides a welcome alternative to the derivation of hTSCs from embryos and placentas. This will enlarge the genetic repertoire of hTSC lines and give access to specific genetic backgrounds of interest. A next step is to generate hiTSCs from patients affected by placental disorders. With this strategy, we can now consider studies to investigate the role of genetics in placental development and diseases such as preeclampsia, intrauterine growth restriction, miscarriage, or choriocarcinomas. hiTSCs could also serve for screening new formulations of human embryo culture media, with potential applications to *in vitro* fertilization. New models of the embryo, such as blastoids, could benefit to this field of research (Rivron et al., 2018a). In this context, parallel derivation of isogenic hiTSCs and hiPSCs could provide a valuable source of cells. Overall, the assets of hiTSCs are comparable to the advantages of hiPSCs over hESCs in the field of human development and disease modeling.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead Contact
  - Materials Availability
  - Data and Code Availability

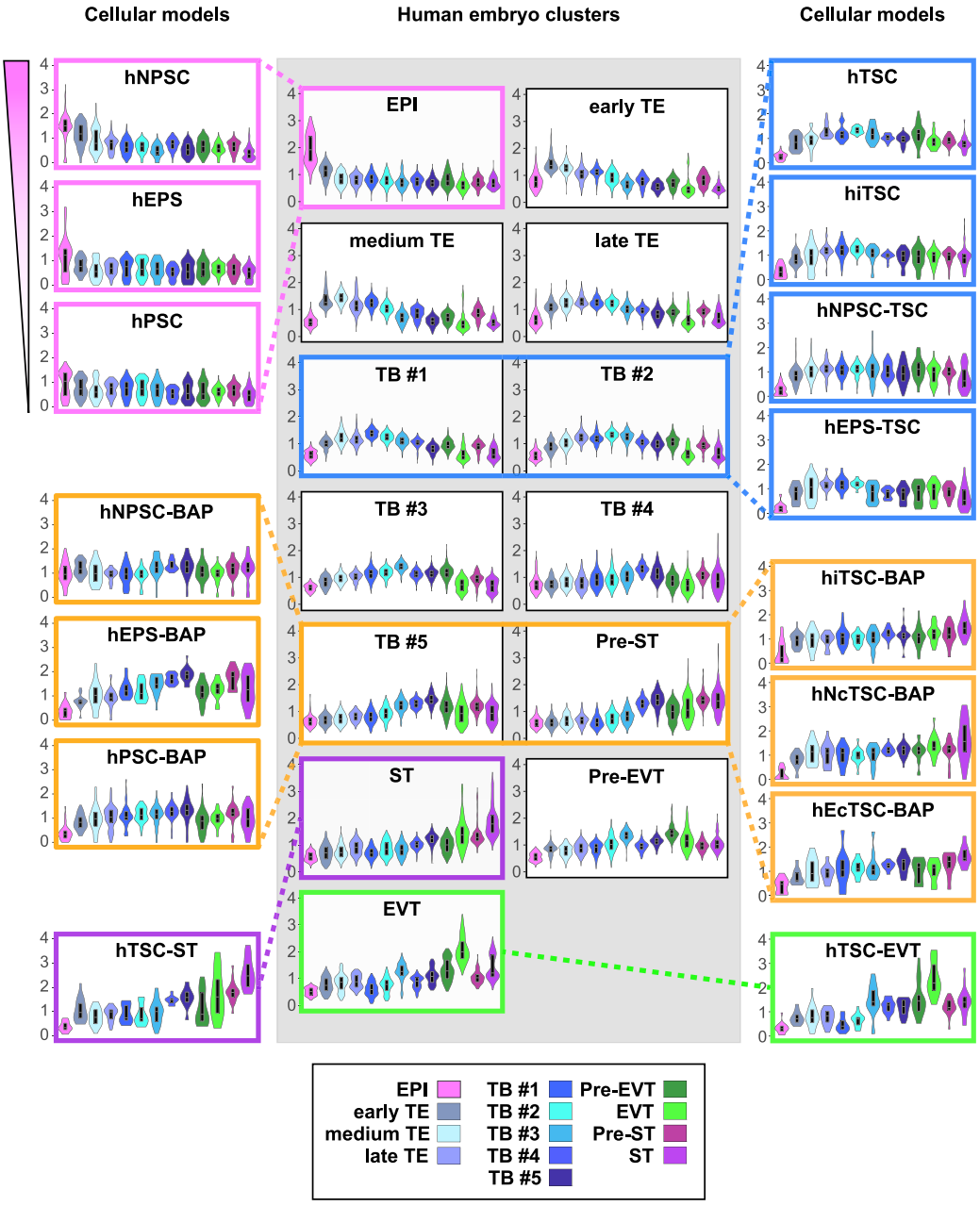

Figure 6. Expression Profiles of Human Embryo Gene Sets in hi/cTSCs

Violin plots summarizing the heatmaps of Figure 5. Gene sets characterizing embryo cell populations are plotted for single-cell clusters (middle, gray-shaded panels) or cellular models for this study (left and right). Within each plot, each violin/boxplot consists of the aggregation of gene sets' expression levels. Expression of each gene is scaled by the standard deviation prior to the aggregation.

- Immunostaining
- RNA extraction and qRT-PCR
- DGE-Seq data generation

- QUANTIFICATION AND STATISTICAL ANALYSIS
  - DGE-Seq data preprocessing
  - Transcriptomic analyses from DGE-Seq data
  - Transcriptomic analyses from single-cell RNA-Seq data
  - Statistical tests and group size

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.108419.

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AUTHOR CONTRIBUTIONS

G.C. and B.B. performed experiments, with the help of other authors. J.B. performed biochemical dosages. D.M., G.C., and S.C. performed bioinformatic analysis, with the help of E.C. G.C. and L.D. conceived the study and wrote the manuscript, with the input of all authors.

DECLARATION OF INTERESTS

D.M. is supported by FINOX forward grant initiative. G.C. and L.D. have a provisional patent filled on the generation of hiTSCs.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GATA2	Sigma-Aldrich	WH0002624M1; RRID:AB_1841726
GATA3	R&D	AF2605; RRID:AB_2108571
NR2F2	Abcam	ab211776
CGB	Abcam	ab9582; RRID:AB_296507
HLA-G	Abcam	ab52455; RRID:AB_880552
DSP	Abcam	ab71690; RRID:AB_1603776
SOX2	SCBT	sc-17320; RRID:AB_2286684
Donkey anti-rabbit-Alexa Fluor 488	ThermoFisher	A21206; RRID:AB_2535792
Donkey anti-mouse-Alexa Fluor 568	ThermoFisher	A10037; RRID:AB_2534013
Donkey anti-goat-Alexa Fluor 647	ThermoFisher	A21447; RRID:AB_141844
Biological Samples		
Villous cytotrophoblasts from 1st trimester human placenta	Thierry Fournier's lab	1536
Villous cytotrophoblasts from 1st trimester human placenta	Thierry Fournier's lab	1560
Extravillous trophoblasts from 1st trimester human placenta	Thierry Fournier's lab	1478
Extravillous trophoblasts from 1st trimester human placenta	Thierry Fournier's lab	1657
Chemicals, Peptides, and Recombinant Proteins		
Y27632 (ROCK inhibitor)	Axon Medchem	1683
Insulin-Transferrin-Selenium-Ethanolamine supplement (ITS-X)	GIBCO	51500-056
L-ascorbic acid	Sigma-Aldrich	A7506
hEGF	Miltenyi Biotec	130-097-751
CHIR99021	Axon Medchem	1386
A83-01	Axon Medchem	1421
SB431542	Axon Medchem	1661
valproic acid	Sigma-Aldrich	P4543
PD0325901	Axon Medchem	1408
mLIF	Miltenyi Biotec	130-095-779
Gö6983	Axon Medchem	2466
N2 supplement	GIBCO	17502048
B27 supplement	GIBCO	17504-001
B27 supplement minus vitamin A	GIBCO	12587010
human LIF	Miltenyi Biotec	130-108-156
(S)-(+)-Dimethindene maleate	Tocris	1425
Minocycline hydrochloride	Tocris	3268
IWR-endo-1	Axon Medchem	2510
human fibroblast growth factor 2	Peprotech	100-18B
human NRG1	CST	5218SC
Forskolin	Axon Medchem	2264
human R-Spondin-1	Peprotech	120-38
human HGF	Peprotech	100-39H

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
N-Acetyl-L-cysteine	Sigma-Aldrich	A9165
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A3059
Mitomycin C	Sigma-Aldrich	M4287
Critical Commercial Assays		
Elecsys free βhCG	Cobas/Roche	04854071
Deposited Data		
DGE-seq datasets generated by this study	This paper; ENA	https://www.ebi.ac.uk/ena/browser/view/PRJEB34037
Code generated by this study	This paper; GitLab	https://gitlab.univ-nantes.fr/E137833T/Castel_et_al_2020
Experimental Models: Cell Lines		
CT30	Okae et al., 2018; Riken BioBank	RCB4938
CT1	Okae et al., 2018	N/A
CT2	Okae et al., 2018	N/A
BL1	Okae et al., 2018	N/A
BL2	Okae et al., 2018	N/A
AV01	This paper	N/A
AV02	This paper	N/A
AV03	This paper	N/A
AV04	This paper	N/A
AV11	This paper	N/A
AV12	This paper	N/A
AV21	This paper	N/A
AV22	This paper	N/A
AV23	This paper	N/A
AV24	This paper	N/A
L8A2	Kilens et al., 2018	N/A
M2A8	Kilens et al., 2018	N/A
M8A9	Kilens et al., 2018	N/A
M8A15	Kilens et al., 2018	N/A
M2A18	Kilens et al., 2018	N/A
HNES1	Guo et al., 2016	N/A
EPS01	This paper	N/A
EPS02	This paper	N/A
H9 (WA09)	Thomson et al., 1998	N/A
L8K1	Kilens et al., 2018	N/A
L8B1	Kilens et al., 2018	N/A
Oligonucleotides		
Primer: CGA Forward: CAGAATGCACGCTACAGGAA	Eurofins Genomics	N/A
Primer: CGA Reverse: CGTGTGGTTCTCCACTTTGA	Eurofins Genomics	N/A
Primer: CGB Forward: TGTGCATCACCGTCAACA	Eurofins Genomics	N/A
Primer: CGB Reverse: TGCACATTGACAGCTGAGAG	Eurofins Genomics	N/A
Primer: SDC1 Forward: GGATGACTCTGACAACTTCTCC	Eurofins Genomics	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer: SDC1 Reverse: CTACAGCCTCTCCCTCCTT	Eurofins Genomics	N/A
Primer: HLA-G Forward: GCCAATGTGGCTGAACAAAG	Eurofins Genomics	N/A
Primer: HLA-G Reverse: TATGATCTCCGCAGGGTAGAA	Eurofins Genomics	N/A
Primer: MMP2 Forward: GGCACCCATTTACACCTACA	Eurofins Genomics	N/A
Primer: MMP2 Reverse: AACCGGTCCTTGAAGAAGAAG	Eurofins Genomics	N/A
The full list of primers used in this study can be found in Table S1B	N/A	N/A
Software and Algorithms		
Volocity	Quorum technologies	v6.5
Fiji	ImageJ	v1.52p
StepOne	Thermofisher	v2.3
R	Bioconductor	v4.0
Other		
scRNA-seq dataset from Petropoulos et al.	Petropoulos et al., 2016; ArrayExpress	https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3929/
scRNA-seq dataset from Zhou et al.	Zhou et al., 2019; GEO	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109555
scRNA-seq dataset from Xiang et al.	Xiang et al., 2020; GEO	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE136447

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr Laurent DAVID (laurent.david@univ-nantes.fr)

Materials Availability

There are restrictions to the availability of cell lines due to the lack of an external centralized repository for their distribution and our need to maintain the stock. We are glad to share cell lines with reasonable compensation by requestor for their processing and shipping. We may require a completed Materials Transfer Agreement if there is potential for commercial application.

Data and Code Availability

The datasets generated during this study (DGE-seq) are available at European Nucleotide Archive (ENA) https://www.ebi.ac.uk/ena/browser/view/PRJEB34037. The code generated during this study is available at GitLab https://gitlab.univ-nantes.fr/E137833T/Castel_et_al_2020.

The scRNA-seq dataset from Petropoulos et al. (2016) is available at ArrayExpress https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3929/. The scRNA-seq dataset from Zhou et al. (2019) is available at NCBI Gene Expression Omnibus (GEO) https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109555. The scRNA-seq dataset from Xiang et al. (2020) is available at NCBI Gene Expression Omnibus (GEO) https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE136447.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

For human somatic cell reprogramming into hiTSCs and hiEPSs, fibroblasts from healthy donors were used: BJ1, male fibroblasts are commercial BJ human neonatal fibroblasts extracted from normal human foreskin (Stemgent Cat# 08-0027); L71 from a 51-year-old healthy man; L80 from a 57-year-old healthy woman. Those fibroblasts were previously used to generate isogenic hiPSCs and hiNPSCs (Kilens et al., 2018). For conversion experiments, we used hiNPSC and hiPSC lines from Kilens et al., and H9 hESCs

(WA09 Lot WB0090) obtained from the WiCell Research Institute. hESCs were used under authorization RE17-007R from the French oversight committee, Agence de la Biomédecine. All cell lines used in this study are further described in Table S1A.

Human preimplantation embryos

Data analysis and transcriptomic modeling of human preimplantation development is detailed in Meistermann et al. (2019).

METHOD DETAILS

Experimental design

Biological replicates are indicated in each figure. Randomization and blinding were performed for qRT-PCR but not for other experiments. No data or samples were excluded from any of the experiments.

Tissue culture

All cell lines were cultured at 37 °C, under hypoxic (5% O_2, 5% CO_2) or normoxic conditions (20% O_2, 5% CO_2) as indicated. Culture medium was daily replaced. 10 μM Y27632 (Axon Medchem) was added to the culture medium upon cell seeding of human stem cells. PXX indicates passage number, and P+XX indicates that cells were converted for XX passages.

Human fibroblasts were cultured in fibroblast medium, composed of high glucose Dulbecco's modified Eagle's medium (DMEM) GlutaMAX-I (GIBCO) supplemented with 10% fetal bovine serum (FBS, Hyclone), 1% sodium pyruvate (GIBCO) and 1% non-essential amino acids (GIBCO).

Mouse embryonic fibroblasts (MEFs) were prepared from E13.5 pups that were decapitated, eviscerated, dissociated with 0.25% trypsin, 0.1% EDTA and plated in MEF medium [DMEM high glucose (Thermo Scientific ®), Glutamax 1:100 (GIBCO®), 0.5% of penicillin–streptomycin (Life Technologies)] on 0.1% gelatin-coated plates. MEFs were mitotically inactivated using 0.01mg/ml mitomycin C (Sigma-Aldrich®) to be used as feeder cells. MEF isolation was performed in compliance with the French law and under supervision of the UTE animal core facility, University of Nantes.

hiTSCs were cultured on MEF feeder cells in hTSC medium (Okae et al., 2018) [DMEM/F12 (GIBCO) supplemented with 0.1mM 2-mercaptoethanol (GIBCO), 0.2% FBS, 0.5% penicillin-streptomycin, 0.3% Bovine Serum Albumin (BSA, Sigma-Aldrich), 1% Insulin-Transferrin-Selenium-Ethanolamine supplement (ITS-X, GIBCO), 1.5 mg/ml L-ascorbic acid (Sigma-Aldrich), 50 ng/ml hEGF (Miltenyi Biotec), 2 μM CHIR99021 (Axon Medchem), 0.5 μM A83-01 (Tocris), 1 μM SB431542 (Tocris), 0.8 mM valproic acid (Sigma-Aldrich) and 5 μM Y27632]. hiTSCs could be passaged with TrypLE (15 min, 37°C, Life Technologies) every 4 days at a 1:3 to 1:4 split ratio or every 7 days at a 1:40 to 1:60 split ratio. hiTSCs were routinely cultured at 37°C in hypoxic conditions (5% O_2, 5% CO_2).

hNPSCs were cultured on MEF feeder cells in t2iLGöY medium (Takashima et al., 2014) [DMEM/F12 supplemented with 1% N2 (GIBCO), 1% B27 (GIBCO), 1% non-essential amino acids, 1% GlutaMAX (GIBCO), 0.1 mM 2-mercaptoethanol, 50 μg/ml BSA, 0.5% penicillin–streptomycin, 1 μM CHIR99021, 1 μM PD0325901 (Axon Medchem), 20 ng/ml mLIF (Miltenyi Biotec), 5 μM Gö6983 (Axon Medchem) and 10 μM Y27632] (Takashima et al., 2014). hNPSCs were passaged every 4 days at a 1:3 split ratio using TrypLE (5 min, 37°C, Life Technologies). hNPSCs were routinely cultured at 37 °C in hypoxic conditions (5% O_2, 5% CO_2).

hEPSs were cultured on MEF feeder cells in LCDM medium (Yang et al., 2017) [48% DMEM/F12 and 48% Neurobasal (GIBCO) supplemented with 0.5% N2 supplement, 1% B27 supplement minus vitamin A (GIBCO), 1% non-essential amino acids, 0.1 mM 2-mercaptoethanol, 0.5% penicillin-streptomycin, 5% knockout serum replacement (KSR, GIBCO), 10 ng/ml human LIF (Miltenyi Biotec), 1μM CHIR99021, 2 μM (S)-(+)-Dimethindene maleate (Tocris) and 2 μM Minocycline hydrochloride (Tocris), 1 μM IWR-endo-1 (Miltenyi Biotec) and 2 μM Y-27632] (Yang et al., 2017). hEPSs were passaged every 4 days at a 1:8 split ratio using TrypLE (5 min, 37°C, Life Technologies). hEPSs were routinely cultured at 37°C in normoxic conditions (20% O_2, 5% CO_2).

Primed hPSCs could be cultured on MEF feeder cells in KSR+FGF2 medium (Amit et al., 2000) [DMEM/F12 supplemented with 20% KSR, 1% non-essential amino acids, 1% GlutaMAX, 50 μM 2-mercaptoethanol, 0.5% penicillin-streptomycin and 10 ng/ml human fibroblast growth factor 2 (FGF2, Peprotech)]. 10 colonies were manually picked every 7 days for passage and seeded as small clumps (~200 μm) in a new 35mm dish. Primed hPSCs could also be cultured on MEF feeder cells in iPS-Brew medium (Miltenyi Biotec) and these cells were passaged every 4 to 6 days at a 1:8 to 1:25 split ratio using TrypLE (5 min, 37°C, Life Technologies). Primed hPSCs were routinely cultured at 37°C in normoxic conditions (20% O_2, 5% CO_2).

10μM ROCK inhibitor (Y-27632) was systematically added to the culture media for 1 day after cell passaging with TrypLE. All cell lines were tested negative for mycoplasma using the MycoAlert kit (LONZA, LT07-318).

Somatic cell reprogramming to hiTSC

Human adult fibroblasts were reprogrammed using the CytoTune-iPS 2.0 Sendai reprogramming kit (Life Technologies). Two days before infection, 3.0 to 4.0 × 10^4 fibroblasts were seeded per well on a 12-well plate, coated with Matrigel. At day 0, cells were infected with the three vectors: polycistronic Klf4-Oct4-Sox2, Myc and Klf4 at a 5:5:3 or 3:3:3 multiplicity of infection (MOI) respectively. At day 9 of infection, cells were dissociated with TrypLE (5 min, 37°C, Life Technologies) and seeded in 35mm dishes, on MEFs. On the following day, cells were transited into E7 reprogramming medium (STEMCELL Technologies). From day 21 onward, cells were transited into hTSC medium. Induced hTSC lines (hiTSC) were routinely cultured at 37°C in hypoxic conditions (5% O_2, 5%CO_2).

Somatic cell reprogramming to hiNPSCs, hiEPSs and hiPSCs was performed as described in previous reports (Kilens et al., 2018; Yang et al., 2017).

Conversion of hNPSC and hEPS to hcTSC

hNPSCs and hEPSs were dissociated with TrypLE (5 min, 37°C, Life Technologies) and seeded in 35mm dishes, on MEFs, at a density of 0.6 to 1.7 × 10^5 cells per dish. Cells were maintained in their initial medium supplemented with 10 μM Y27632 for 1 day. From day 2 onward, cells were transited into hTSC medium. Converted hTSC lines (hcTSC) were routinely cultured at 37°C in hypoxic conditions (5% O_2, 5% CO_2).

Primed hPSCs included in conversion experiments were initially cultured in KSR+FGF2 or iPS-BREW. 10 colonies were picked (KSR+FGF2) or cells were passaged with TrypLE (iPS-BREW) and seeded at a density of 0.5 to 1.25 × 10^5 cells per dish in 35mm dishes coated with MEFs for conversion assays.

Differentiation of hi/cTSC to EVT and ST

After at least 15 passages, cells were collected for differentiation assays.

Prior to differentiation into ST and EVT cells, h(i/c)TSCs (initially cultured on MEFs) were transited to fibronectin for at least 3 passages.

EVT differentiation

2-4 days before passage, h(i/c)TSCs were transited into pre-EVT medium [DMEM/F12 supplemented with 0.1mM 2-mercaptoethanol, 0.5% penicillin-streptomycin, 0.3% BSA, 1% ITS-X supplement, 4% KSR, 7.5 μM A83-01 (Tocris), 2.5 μM Y27632, 5 μM IWR-endo-1 (Miltenyi Biotec)]. Then, cells were passaged with TrypLE to a density of 0.8 to 3.0 × 10^4 cells/cm^2. Before treatment was initiated, cells were placed in differentiation basal medium [DMEM/F12 containing 0.1 mM 2-mercaptoethanol, 0.5% Penicillin-Streptomycin, 0.3% BSA, 1% ITS-X], supplemented with 10 μM ROCK inhibitor (Y27632). Within 6 hours, timing depending on the lines, cells were transited into EVT medium (Okae et al., 2018) [Differentiation basal medium, supplemented with 100 ng/ml NRG1, 7.5 mM A83-01, 2.5 mM Y27632, 4% KnockOut Serum Replacement, 2% Matrigel]. At day 3, medium was replaced with the EVT medium containing 0.5% Matrigel, without NRG1. Typically, EVT formation was observed by day 4-5. At day 6, medium was replaced with the EVT medium containing 0.5% Matrigel, without NRG1 and KSR. Cells were collected on day 8 for subsequent analyses.

2D-ST differentiation

h(i/c)TSCs were passaged with TrypLE to a density of 2.0 to 6.0 × 10^4 cells/cm^2. Before treatment was initiated, cells were placed in differentiation basal medium [DMEM/F12 containing 0.1 mM 2-mercaptoethanol, 0.5% Penicillin-Streptomycin, 0.3% BSA, 1% ITS-X], supplemented with 10 μM ROCK inhibitor (Y27632). Within 3 hours, timing depending on the lines, cells were transited into ST medium (Okae et al., 2018) [Differentiation basal medium, supplemented with 2.5 mM Y27632, 2 mM forskolin, and 4% KSR]. Medium was replaced at day 3, and cells were analyzed at day 6.

3D-ST differentiation

Prior to 3D differentiation assay, h(i/c)TSCs were transited to trophoblast organoid medium (TOM) with small modifications (Turco et al., 2018) [DMEM/F12, 1X N2 supplement, 1X B27 supplement minus vitamin A, 1.25 mM N-Acetyl-L-cysteine, 1% GlutaMAX (GIBCO), 0.5% Penicillin-Streptomycin (TOM basal medium), supplemented with 500 nM A83-01, 1.5 μM CHIR99021, 80 ng/ml human R-spondin1, 50 ng/ml hEGF, 100 ng/ml hFGF2, 50 ng/ml hHGF, 2 μM Y-27632]. 0.4 to 1.0 × 10^5 cells passaged with TrypLE were embedded into 150μl drops comprising: 50μl Matrigel and 50μl PBS+/+ along with 960 ng fibronectin, 50 ng laminin521 and 50μl TOM basal medium. Drops were carefully deposited on sterile parafilm covered dishes and placed at 37°C for 20 minutes to solidify. Complete TOM supplemented with 10 μM ROCK inhibitor (Y27632) was further added to cover the drops. Medium was replaced every 3 days with TOM. The 3D structures emerged within a week and were collected on day14 for subsequent analyses.

MEF-BAP treatment

hPSC and hi/cTSC lines were dissociated with TrypLE and seeded on 35mm dishes coated with MEFs, at a density of 0.4 to 1.0 × 10^5 cells per dish. For primed hPSCs cultured in KSR +FGF2 medium, about 10 colonies were manually picked and seeded per dish. hPSCs and hi/cTSCs were maintained in their initial medium supplemented with 10 μM Y27632 for 1 day. The following day, initial medium was replaced with MEF-BAP (Amita et al., 2013) [DMEM/F12 supplemented with 20% KSR, 1% non-essential amino acids, 1% GlutaMAX, 50μM 2-mercaptoethanol (MEF-CM, conditioned for 24 hours on a MEF monolayer and filtrated with a 0.22 μm pore size filtration unit), 2 ng/ml human BMP4 (Miltenyi Biotec), 1μM A83-01 and 0.1 μM PD173074 (Axon Medchem)]. MEF-BAP medium was daily replaced. Supernatants and cells were collected at day 3 and 6 of differentiation for subsequent analyses.

Isolation of VCT, ST and EVT cells from human placentas

Isolation of placental cell types was conducted following previously published protocols (Handschuh et al., 2006). Briefly, chorionic villi were dissected from term placentas of healthy mothers. Mononucleated cytotrophoblasts (VCTs) and extravillous trophoblasts (EVTs) were isolated after trypsin-DNase digestion, sedimentation, filtration and discontinuous Percoll gradient fractionation. VCTs

were cultured for 2h in Dulbecco's modified Eagle's medium (DMEM) containing 10% decomplemented fetal calf serum (FCS), 2 mM glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin. EVTs were cultured on Matrigel in the same culture conditions for 48h. ST cells were obtained by further differentiation of VCTs for 72h in these conditions (spontaneous aggregation and fusion of VCTs).

β-hCG dosage

Cell culture supernatants were collected at days 0, 3 and 6 of forskolin and MEF-BAP treatments. Amounts of secreted β-hCG were measured by electrochemiluminescence immunoassay "ECLIA" (Elecsys free βhCG, Cobas/Roche®) on Cobas® e601 immunoassay analyzer, at the Clinical Biochemistry Laboratory, CHU Nantes, France.

Immunostaining

For immunofluorescence (IF) analysis, cells were fixed at room temperature using 4% paraformaldehyde for 15 min. Samples were then permeabilized for 60 min at room temperature with IF buffer [phosphate-buffered saline (PBS), 0.2% Triton, 10% FBS], which also served as a blocking solution. Samples were incubated with primary antibodies overnight at 4 °C. The following antibodies were used: anti-GATA2 (1:50 ; Sigma® WH0002624M1), anti-GATA3 (1:100, R&D® AF2605), anti-NR2F2 (1:100, Abcam® ab211776), anti-CGB (1:100, Abcam® ab9582), anti-HLA-G (1:100, Abcam® 52455), anti-DSP (1:200, ref: Abcam® ab71690), anti-SOX2 (1:500, SCBT® sc-17320). Incubation with secondary antibodies was performed for 2 h at room temperature along with 4′,6-diamidino-2-phenylindole (DAPI) nuclei staining. Confocal immunofluorescence images were acquired with A1-SIM Nikon® confocal microscope. Optical sections of 0.5-1 μm-thick were collected. Images were processed using Volocity® visualization software.

RNA extraction and qRT-PCR

Total RNA was extracted using RNeasy® columns and DNase-treated using RNase-free DNase (QIAGEN®). First-strand cDNAs were generated using 500ng of RNA, M-MLV reverse transcriptase (Life technologies®), 25μg/ml polydT (Ambion®) and 9.6μg/ml random primers (Roche®).

qRT-PCR were performed on a StepOne instrument (Applied Biosystems®) using power SYBR green PCR master mix, for genes listed in the primers table (Table S1B). For each sample, the ratio of specific mRNA level relative to *GAPDH* levels was calculated. Experimental results are shown as relative gene expression.

DGE-Seq data generation

For 3′ DGE profiling, RNA-sequencing protocol was performed according to our implementation of Soumillon et al. protocol (Kilens et al., 2018; Soumillon et al., 2014). Briefly, the libraries were prepared from 10 ng of total RNA. The mRNA poly(A) tails were tagged with universal adapters, well-specific barcodes and unique molecular identifiers (UMIs) during template-switching reverse transcription. Barcoded cDNAs from multiple samples were then pooled, amplified and tagmented using a transposon-fragmentation approach which enriches for 3′ends of cDNAs. A library of 350–800 bp was run on an Illumina® HiSeq 2500 using a Hiseq Rapid SBS Kit v2-50 cycles and a Hiseq Rapid PE Cluster Kit v2.

QUANTIFICATION AND STATISTICAL ANALYSIS

DGE-Seq data preprocessing

Read pairs used for analysis matched the following criteria: all 16 bases of the first read had quality scores of at least 10 and the first 6 bases correspond exactly to a designed well-specific barcode. The second reads were aligned to RefSeq human mRNA sequences (hg19) using bwa version 0.7.17. Reads mapping to several transcripts of different genes or containing more than 3 mismatches with the reference sequences were filtered out from the analysis. DGE profiles were generated by counting for each sample the number of unique UMIs associated with each RefSeq genes. DGE-sequenced samples were acquired from five sequencing runs. Sequenced samples with at least 50000 counts and 6000 expressed genes were retained for further analysis.

Transcriptomic analyses from DGE-Seq data

Samples were filtered out if the number of unique UMIs was inferior to 50000 and the number of expressed genes inferior to 6000; 165 samples passed those cutoffs. Samples were normalized using same strategy as described in the DESeq2 method (Love et al., 2014). For performing dimension reduction and clustering, samples were logged using a log2(x+1) transformation. The five different runs were merged using ComBat (Johnson et al., 2007) from the library "sva" in parametric mode, using technical replicates between batches as references for computing batch effects. Each gene expression of the corrected values was subtracted by the minimum of the gene expression before the batch correction. This step does not change the relative expression of genes; however, it permits an easier interpretation of the expression values as minimums cannot be less than zero. Finally, each set of technical replicates were merged. The resulting samples consist in the median of each gene for its set of technical replicates. A set of over-dispersed genes was determined for computing the correlation heatmap from Figure 1C and the PCA from Figures 3 and S1C. To pick these, the coefficient of variation of each gene from the normalized adjusted expression was fitted by the mean expression of each gene, using a LOESS method. Genes with a positive residual for the regression were marked as over-dispersed. This leads to a total of 2770 genes. Pathway eigengenes and their gene contribution were computed with the following steps: first, the gene sets corresponding to each

pathway were downloaded on the KEGG database. Pathways with at least 4 genes existing in our data were conserved. Second, a PCA was computed for each pathway, using the gene set of the pathway as the input of the PCA. The first component of each PCA was designated as "pathway eigengene." In heatmaps, unless otherwise stated, samples are clustered from the Euclidean distance of expression, by a hierarchical clustering using Ward's method. Genes are clustered from a co-expression distance (distance of biweight midcorrelation) between gene expressions, by a hierarchical clustering using Ward's method.

Transcriptomic analyses from single-cell RNA-Seq data

Datasets (Petropoulos et al., 2016; Zhou et al., 2019) were normalized and log transformed using scran (Lun et al., 2016), then merged with *fastMNN* from the R library "batchelor" (Haghverdi et al., 2018) with the parameter *k* set up to 420. UMAP was performed on all available genes with the R library "uwot." The n_neighbors parameter was set to its maximum (6838), and min_dist = 0.2. A first cell clustering was done using Monocle3 (Qiu et al., 2017), then, preimplantation clusters were re-annotated using clusters found in Meistermann et al. (2019). First, coordinates of centroids of these previous clusters were determined on the UMAP using Petropoulos dataset, then, cells were attributed to the cluster with the nearest centroid in term of Euclidean distance. To determine the markers of each clusters, genes with at least a mean 2 normalized count in DGE-Seq and 4 normalized count in single-cell RNA-Seq were kept for computing AUROCs. Genes with an AUC of at least 0.85 were designated as markers. Markers determined by Xiang et al. (2020) were also filtered, with a mean expression of at least 2 normalized counts in DGE-Seq data.

Statistical tests and group size

Data were processed using R. All statistical tests are indicated in the figure legends. Group size (n) is represented by dots projected on boxplots (Figures 1D, 2C, S1D, S2B, and S2C), vertically aligned dots in scatterplots (Figure S3D), vertical slices on heatmaps (Figures 1C, 5 [right], S4–S6 [right]), or specific shaped and colored dots projected on PCA (Figures 3A–3G and S1C) which correspond to biological replicates. For marker gene expression levels, significance ($p < 0.05$) between two groups was calculated using the unpaired two-samples Wilcoxon-Mann-Whitney test that was computed with the stat_compare_means() function from ggplot2 R package. The Wilcoxon-Mann-Whitney test was chosen for stringency, low propensity to produce false positive results and for well-fitting the data. Given our type of data, no specific method was used to determine whether the data met assumptions of the statistical approach.

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Supplemental Information

Induction of Human Trophoblast Stem Cells from Somatic Cells and Pluripotent Stem Cells

Gaël Castel, Dimitri Meistermann, Betty Bretin, Julie Firmin, Justine Blin, Sophie Loubersac, Alexandre Bruneau, Simon Chevolleau, Stéphanie Kilens, Caroline Chariau, Anne Gaignerie, Quentin Francheteau, Harunobu Kagawa, Eric Charpentier, Léa Flippe, Valentin François–Campion, Sandra Haider, Bianca Dietrich, Martin Knöfler, Takahiro Arima, Jérémie Bourdon, Nicolas Rivron, Damien Masson, Thierry Fournier, Hiroaki Okae, Thomas Fréour, and Laurent David

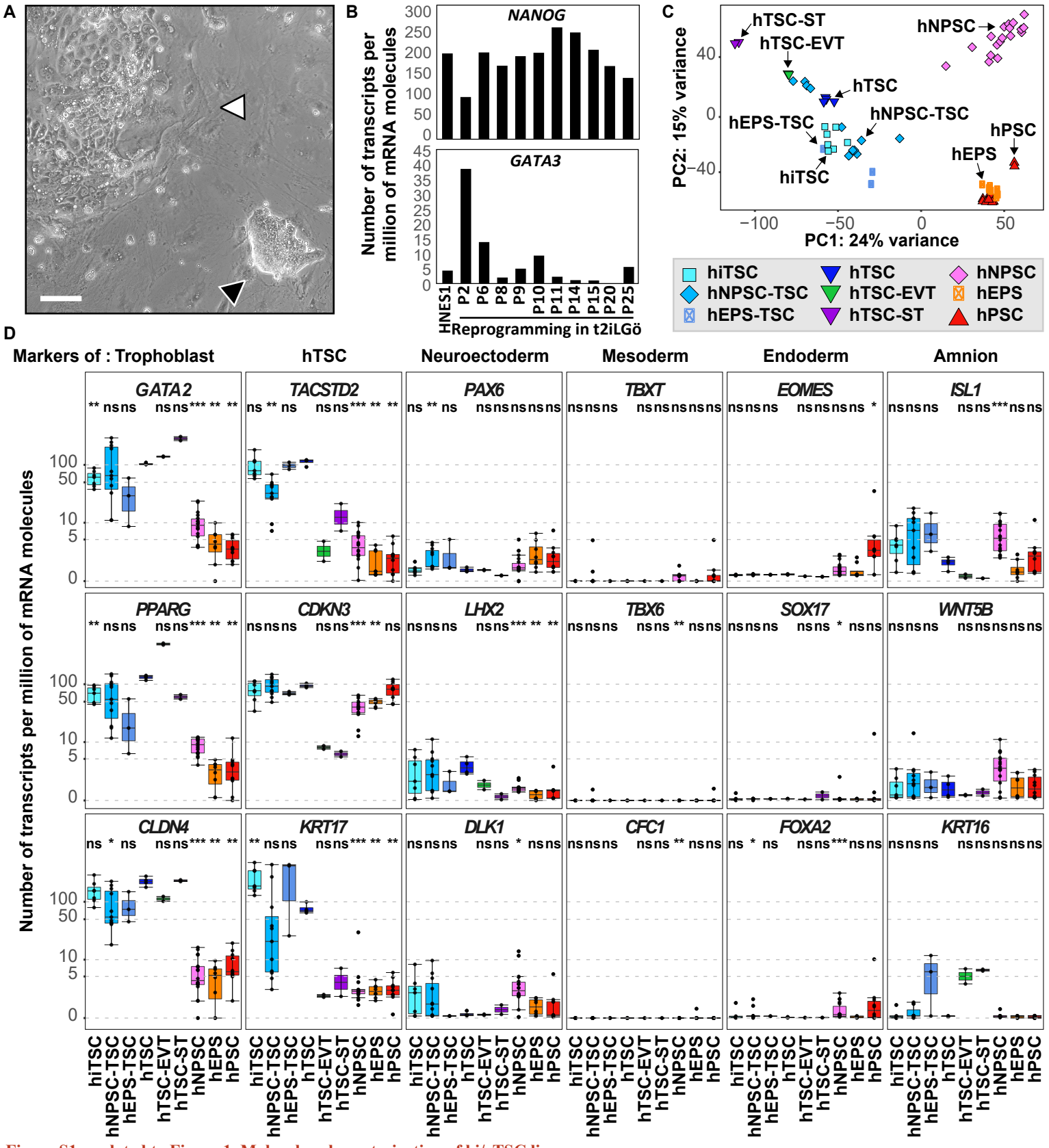


Figure S1 – related to Figure 1. Molecular characterization of hi/cTSC lines

(**A**) Bright field picture of cells undergoing naive pluripotent reprogramming (black arrow) concomitant to trophoblast-like colonies (white arrow). Scale bar: 100 µm. (**B**) Bar plots showing concomitant bulk gene expression of the pluripotency-specific gene *NANOG* and the trophoblast-associated gene *GATA3* in early intermediates during experiments of human somatic cell reprogramming towards naive pluripotency (P2 to P6). Absolute gene expression levels are given as number of transcripts per million of mRNA molecules. Under these culture conditions, *GATA3* expression is lost over passages while *NANOG* expression is stable. Data from (Kilens et al., 2018). (**C**) PCA analysis of hi/cTSCs, hNPSCs, hEPSs, and hPSCs. PC1 vs PC2 are displayed. Embryo- and placenta-derived hTSCs along with the differentiated ST cells and EVTs are included for comparison. (**D**) Gene expression levels of indicated trophoblast, germ layer or amnion lineage markers are shown for hiTSCs, hcTSCs, embryo- and placenta-derived hTSCs, along with hNPSCs, hEPSs and hPSCs. ST cells and EVTs are included for comparison. Expression levels are given as number of transcripts per million of mRNA molecules. Error bars show the interquartile range. A Wilcoxon-Mann-Whitney test was performed, with embryo- and placenta-derived hTSCs taken as the reference group. Stars indicate statistical significance of the difference: *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

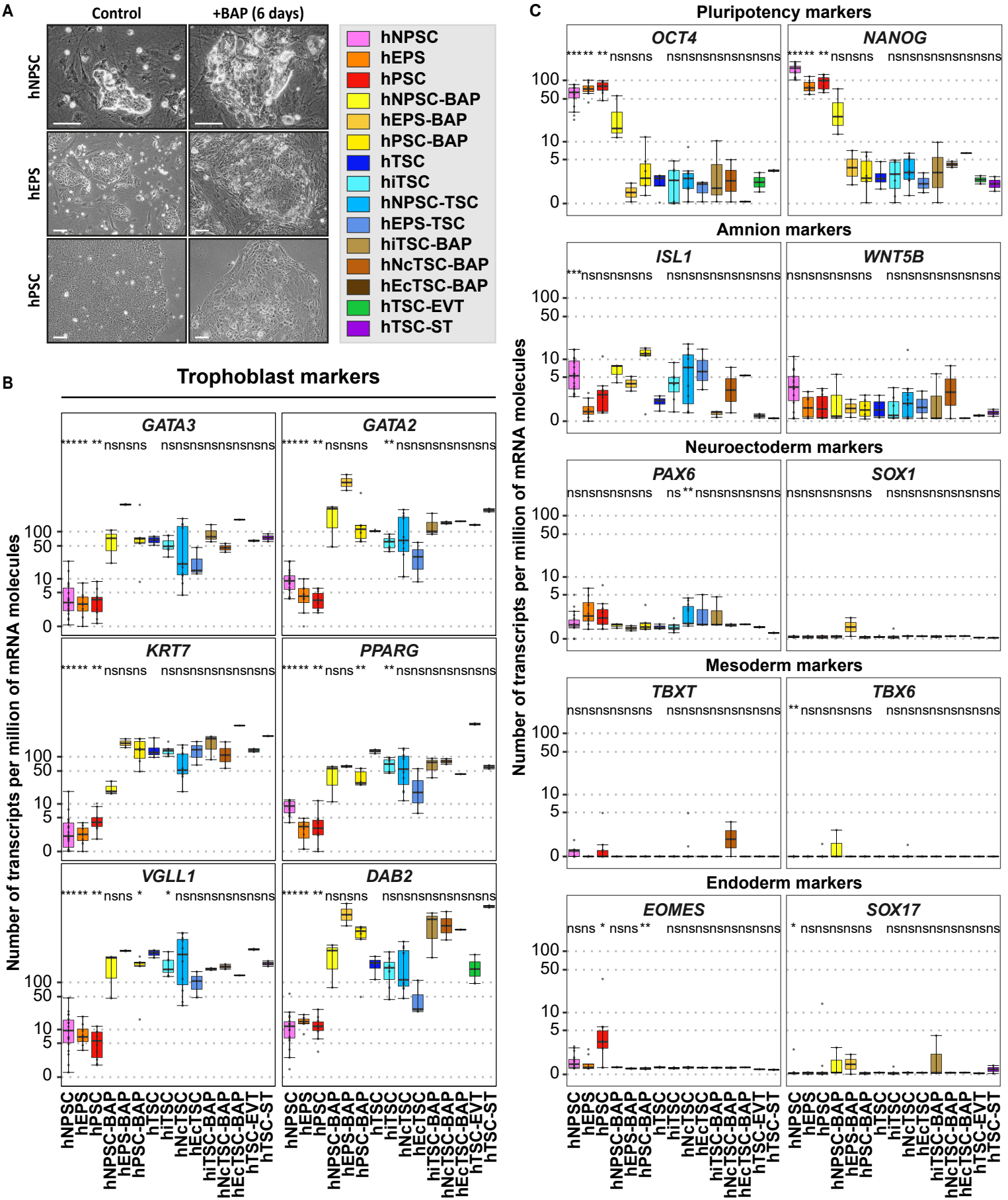


Figure S2 – related to Figure 1. Molecular characterization of BAP treated cells.

(**A**) Phase contrast images of hNPSCs, hEPSs and hPSCs treated with BAP, along with the relative controls. Scale bars: 100µm. (**B-C**) Gene expression levels of trophoblast (B) pluripotent, germ layer or amnion (C) lineage markers are shown for BAP-treated hNPSCs, hEPSs, hPSCs and hi/cTSCs along with the relative controls. Previously established hTSCs, ST cells and EVTs are included for comparison. Expression levels are given as number of transcripts per million of mRNA molecules. Error bars show the interquartile range. A Wilcoxon-Mann-Whitney test was performed, with embryo- and placenta-derived hTSCs taken as the reference group. Stars represent p-values: *p < 0.05; **p < 0.01; ***p < 0.001.

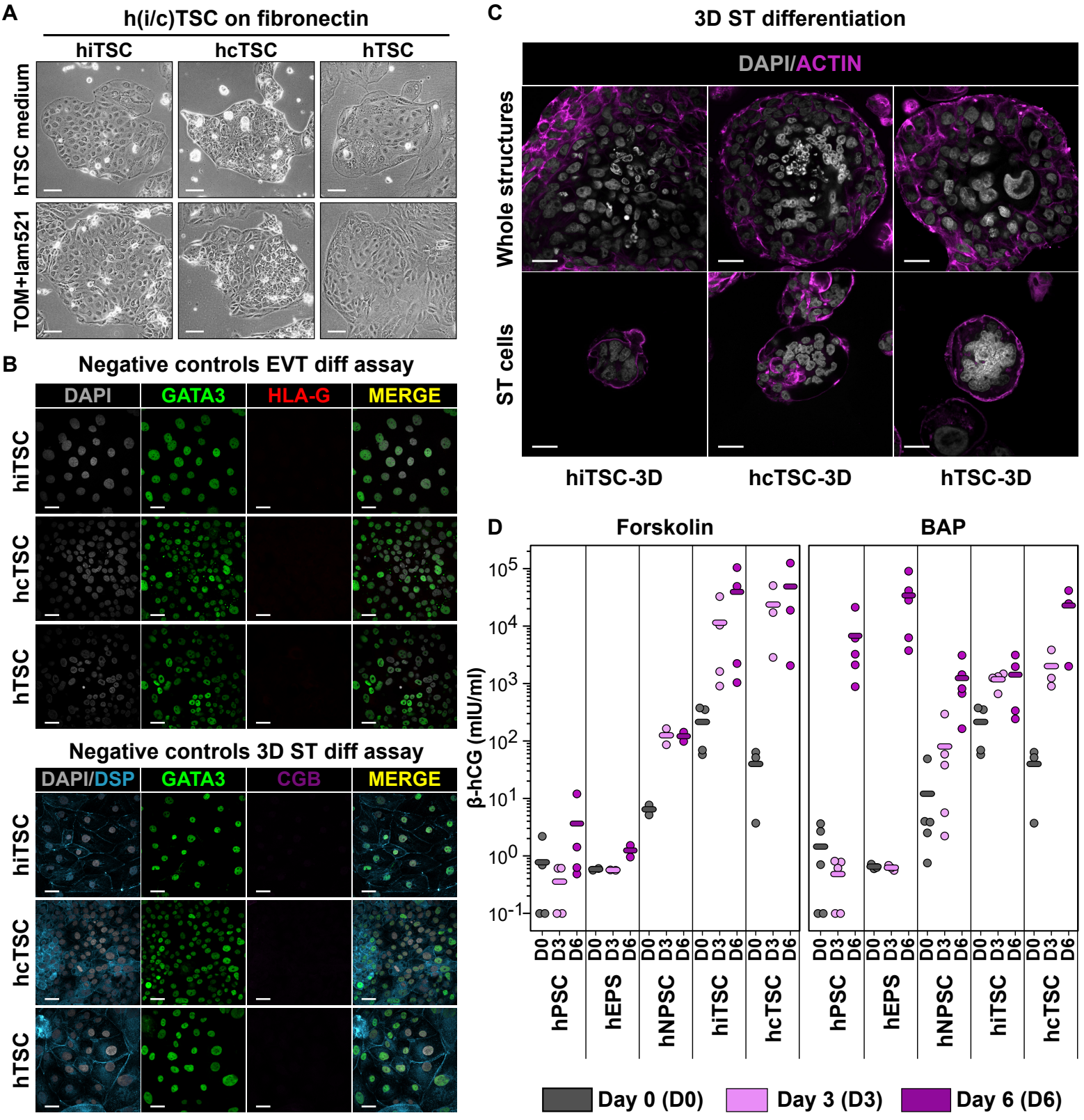


Figure S3 - related to Figure 2. Functional validation of hi/cTSC differentiation potential

(**A**) Brigthfield pictures of cells prior to differentiation. Top: h(i/c)TSCs were cultured on fibronectin in hTSC medium prior to EVT and ST differentiation assays. Bottom: h(i/c)TSCs were cultured on fibronectin and laminin521 in trophoblast organoid medium (TOM) prior to 3D-ST differentiation experiments. (**B**) Immunofluorescence images of undifferentiated h(i/c)TSCs stained for the trophoblast-associated transcription factor GATA3 and the extravillous trophoblast-specific surface marker HLA-G (top) or the syncytiotrophoblast-associated markers DSP and CGB along with GATA3 (bottom). Nuclei were stained with DAPI. (**C**) Immunofluorescence images of 3D-ST structures derived from hiTSC, hcTSC, and hTSC lines stained for ACTIN, highlighting the formation of syncytiotrophoblast cells (center of structures). Nuclei were stained with DAPI. (**D**) Scatter plots of secreted β-hCG levels in mIU/ml for indicated cell lines at day 0, 3, or 6 of ST differentiation assays. Left: results for forskolin treatment. Right: results for MEF-BAP treatment. Each biological replicate is represented by a dot. Bars represent averages.
Scale bars (A): 100 µm; (B-C): 30 µm.

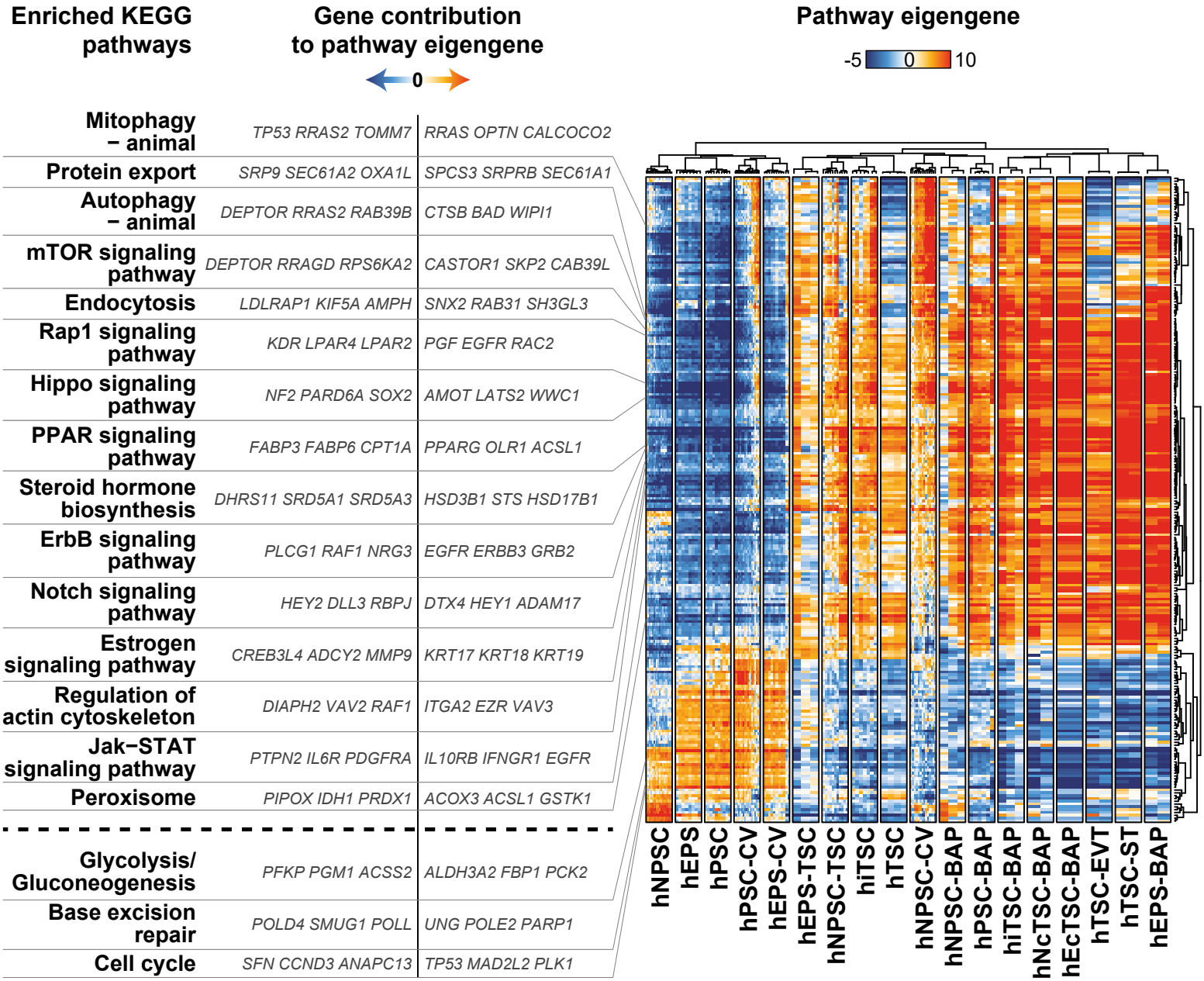


Figure S4 – related to Figure 3. Signaling pathway signatures of hi/cTSCs and hPSCs converting to the trophoblast lineage

Pathway expression of h(i/c)TSCs and hPSCs in maintenance, differentiation or conversion media. The entire KEGG database with the pathways that have at least 3 genes existing in our dataset is represented. The expression of each pathway is summarized in each sample by a score: the pathway eigengene. This score consists in a linear combination of gene expression from every gene of the pathway (see STAR Methods for more details). Genes can have a positive or a negative contribution in the linear combination. A selection of 3 genes with positive or negative contribution to the eigengene is represented for each selected pathway. For example, in the mTOR signaling pathway, when *DEPTOR* expression increases, the pathway eigengene decreases, while if *CAB39L* expression increases, the pathway eigengene also increases.

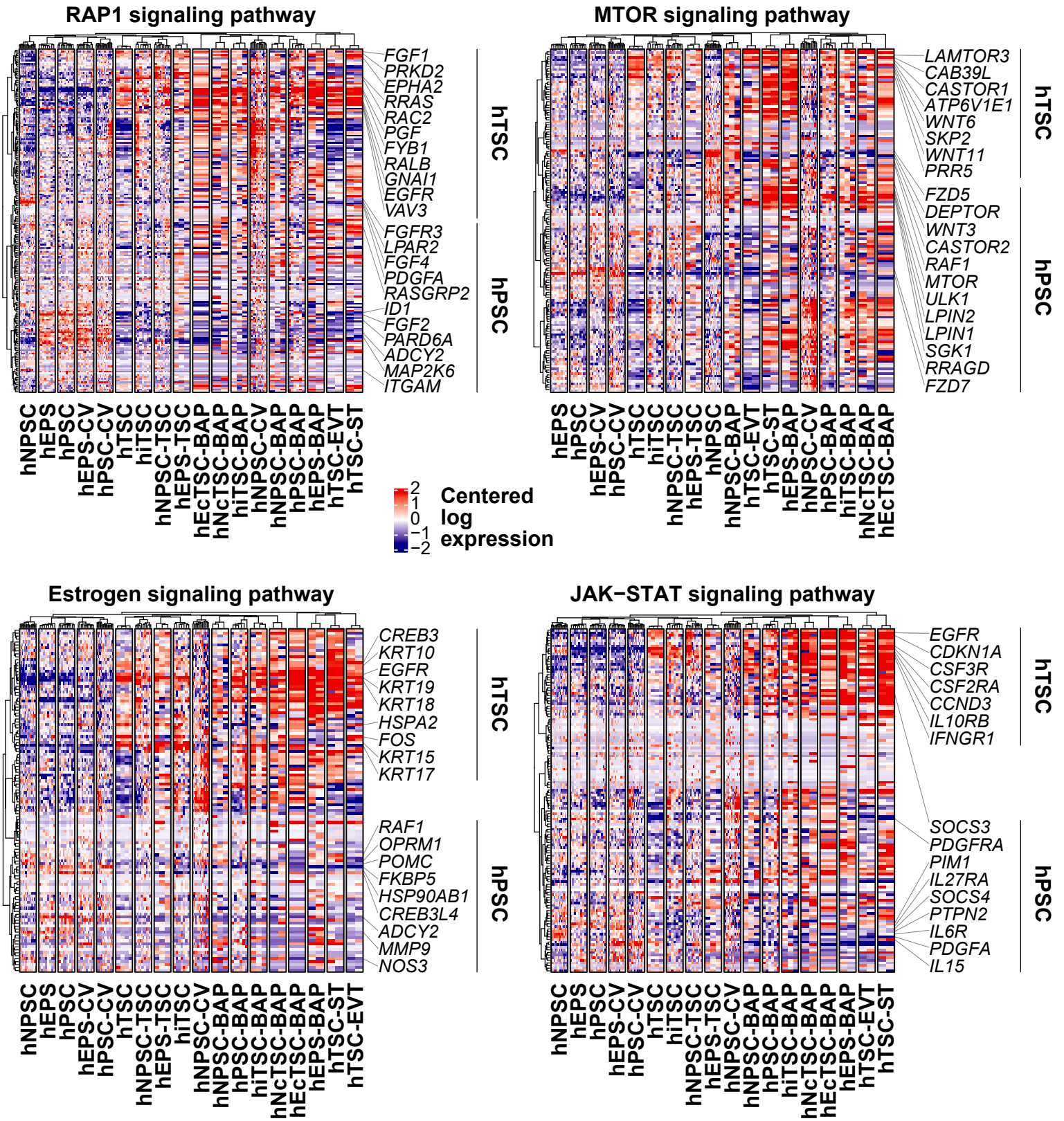


Figure S5 - related to Figure 3. h(i/c)TSC specific signaling pathway signatures

Expression profiles from a selection of 4 KEGG signaling pathways are shown across hPSCs, hTSCs and the differentiated EVTs and ST cells. These pathways were identified by the pathway eigengene analysis and comprise RAP1, MTOR, Estrogen and JAK-STAT signaling pathways that show distinct signatures across hPSCs and the trophoblast lineage.

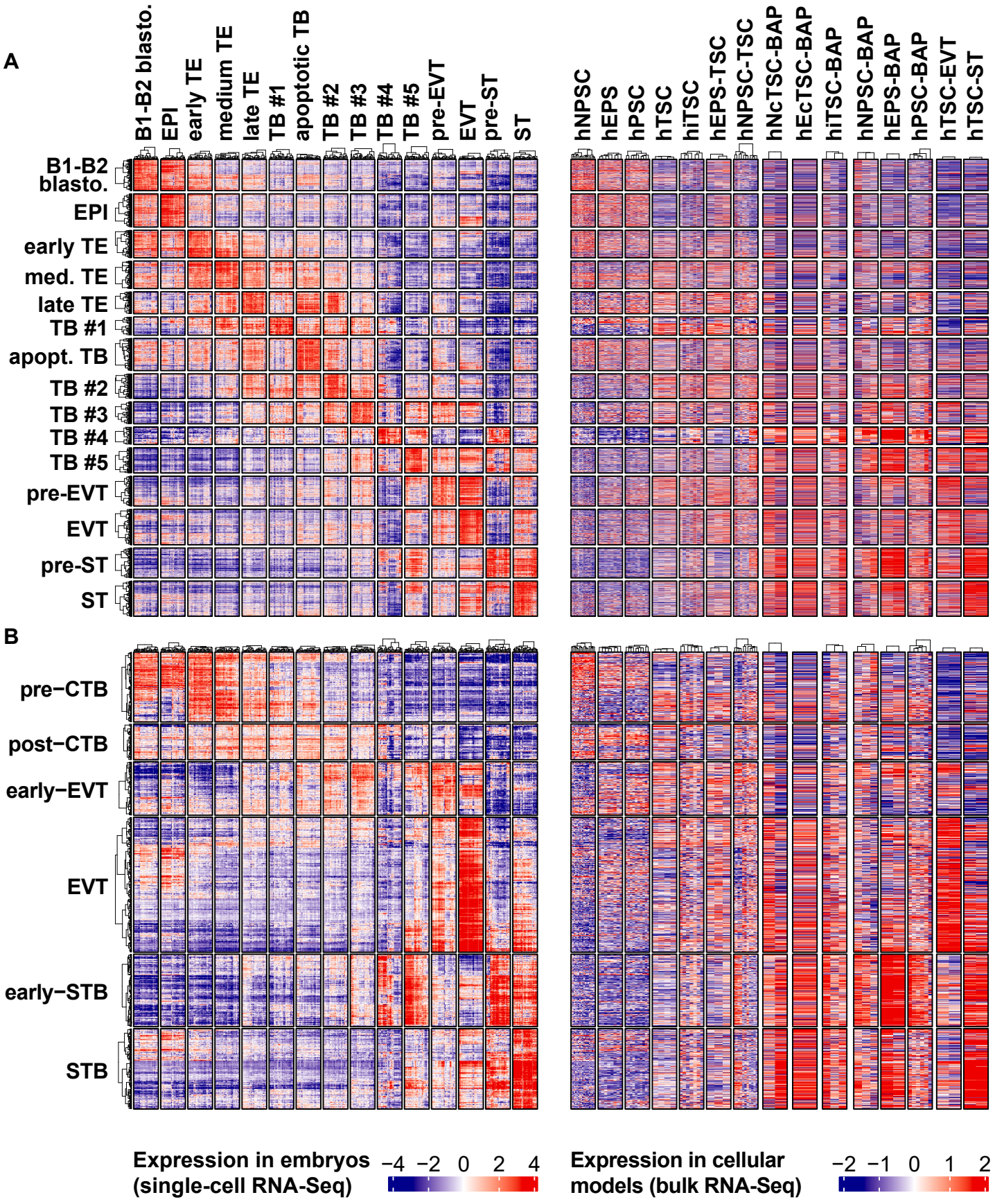


Figure S6 – related to Figure 5. Comparison of transcriptomic profiles between cell clusters of the human embryo and the cellular models

(**A-B**) Heatmap representation of genes associated with single-cell clusters (A) or gene sets (B, (Xiang et al., 2020)) for pre-implantation CTB, post-implantation CTB, early-EVT, EVT, early-STB and STB across scRNAseq peri-implantation human embryo clusters, hPSCs, h(i/c)TSCs and differentiated cells.

3 DISCUSSION

In the middle of 2018, a few months after the study of Okae *et al* was published, we generated patient-specific induced and converted trophoblast stem cells (hi/cTSCs), by somatic cell reprogramming of fibroblasts and cell fate conversion of naive and extended pluripotent stem cells. We characterised global gene expression and differentiation potential of these lines, confirming they were similar to primary hTSCs. We further investigated cell fate plasticity between pluripotent and trophoblast lineages, uncovering molecular mechanisms involved in this process.

In the following part, we discuss these results in more detail, providing additional data and considering the impact of our work in light of recent progress, notably the generation of the first human blastoids.

3.1 Somatic cell reprogramming into hiTSCs: erasing epigenetic barriers between embryonic and extraembryonic lineages

The notion of somatic cell reprogramming involves that epigenetic barriers (*e.g.* DNA methylation, histone marks...) established during development and lineage restriction are erased by key, master chromatin remodelers, so that specific genes of the early embryonic program are reactivated and establish pluripotency *de novo*. This is precisely the mode of action of Yamanaka factors OCT4/POU5F1, SOX2, KLF4 and

c-MYC.

First, OCT4/POU5F1 physically interacts with BRG1 to open the chromatin, thus driving the transition from a globally condensed state in somatic cells to a relaxed state in early reprogramming intermediates (Chen *et al*, 2020). Moreover, the chromatin-associated transcription factor UTF1, a direct downstream target of OCT4/POU5F1 and SOX2, is up-regulated in early reprogramming intermediates, globally preventing PRC2 (Polycomb Repressive Complex 2) binding, thus contributing to establish a transcriptionally active chromatin (Buganim *et al*, 2012 ; Galonska *et al*, 2014 ; Jia *et al*, 2012).

In turn, it seems that c-MYC and KLF4 further open the chromatin so that OCT4/POU5F1 and SOX2 can more easily bind to their targets and activate the embryonic program (Yamanaka, 2007). Among others, KLF4 can interact with P300 (a histone acetyltransferase), thus promoting the deposition of acetyl functional groups on lysine residues of histones, a mark of transcriptionally active chromatin (Evans *et al*, 2007). It also directly binds to epithelial genes and activates their transcription, thus promoting the mesenchymal-epithelial transition (MET) (Chen *et al*, 2020).

Finally, *PARP1* and *TET2* were found to be essential for somatic cell reprogramming, ensuring the active DNA demethylation in early intermediates (Doege *et al*, 2012).

Interestingly, hTSCs have their DNA globally hypomethylated, in that resembling early reprogramming intermediates and naive h(i)PSCs (Leitch *et al*, 2013 ; Okae *et al*, 2018 ; Yagi *et al*, 2019). Despite this, it was largely admitted until recently that strong epigenetic barriers, separating embryonic and extraembryonic lineages, impaired transdifferentiation of ESCs or somatic cells into trophoblasts, and *vice versa*. However, this idea has been challenged, notably by the evidence that hPSCs can produce trophoblast-like cells in response to BAP treatment (Amita *et al*, 2013).

Consistently, we observed rare, spontaneous differentiation of naive reprogramming intermediates into cells resembling trophoblasts and expressing *GATA3* (article, Figure S1A-B). This observation, combined with single-cell RNA-Seq data analysis of pre-implantation embryos, suggested that OSKM transcription factors were also permissive to trophoblast fate induction in human. Indeed, they are all expressed at high levels in cells of the embryo before ICM and TE segregation (**Figure 5**).

Figure 5: Ubiquitous expression of Yamanaka factors OSKM in the human embryo, prior to ICM vs TE specification.

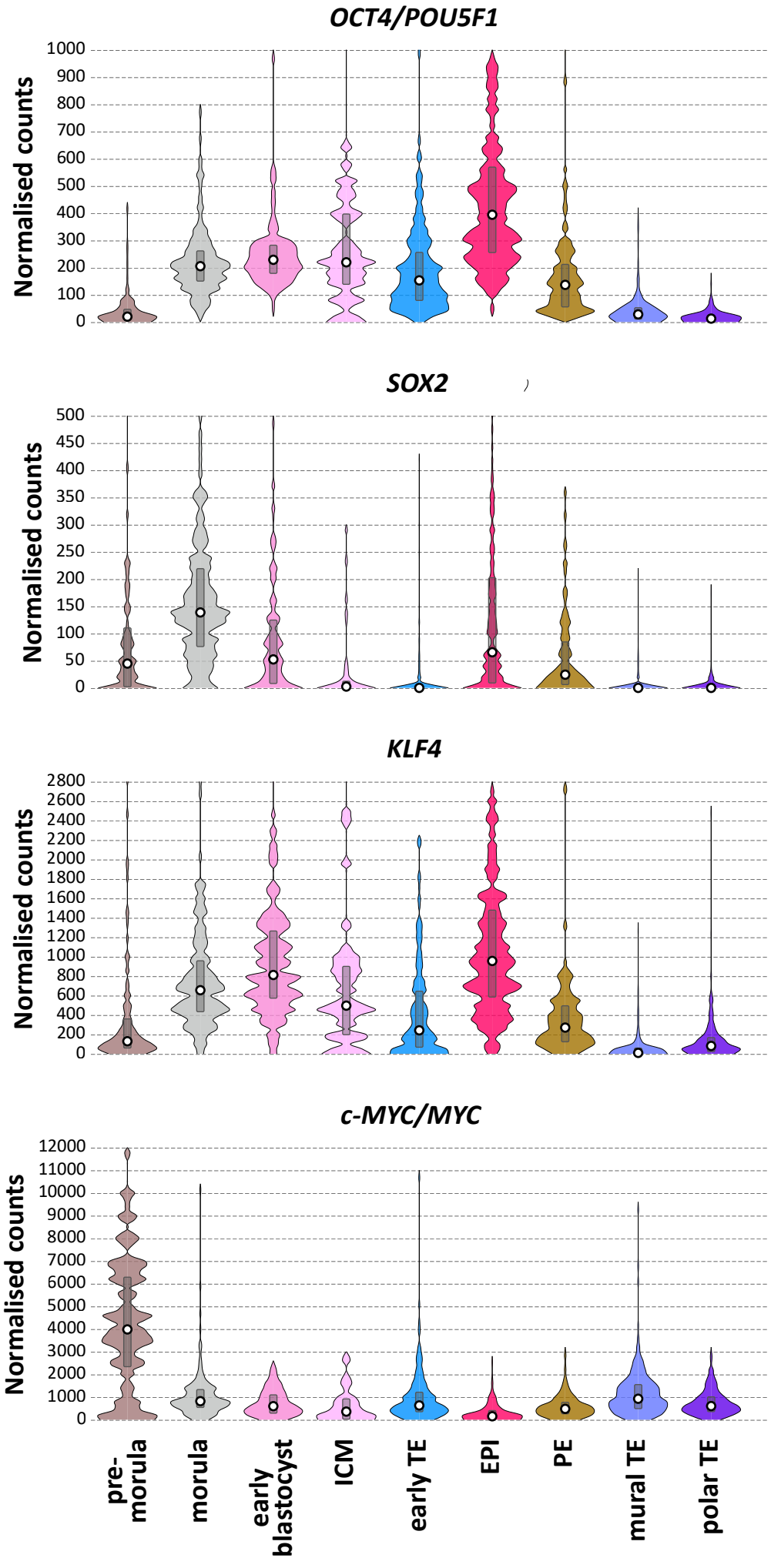

OCT4/POU5F1
Normalised counts
1000
900
800
700
600
500
400
300
200
100
0
SOX2
Normalised counts
500
450
400
350
300
250
200
150
100
50
0
KLF4
Normalised counts
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
c-MYC/MYC
Normalised counts
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
pre-morula
morula
early blastocyst
ICM
early TE
EPI
PE
mural TE
polar TE

Figure 5. Ubiquitous expression of Yamanaka factors OSKM in the human embryo, prior to ICM *vs* TE specification.

From top to bottom: violin plots of *OCT4/POU5F1*, *SOX2*, *KLF4*, and *c-MYC/MYC* expression levels and frequency in cell types of the pre-implantation human embryo.

Therefore, we combined OSKM reprogramming strategy with hTSC culture conditions. A week after infecting fibroblasts of patients with SeV particles, we transited cells into mTeSR E7 medium used to promote mesenchymal-epithelial transition (MET). Two weeks later, early reprogramming intermediates were switched to hTSC medium and readily formed epithelioid colonies (within a few days), reminiscent of hTSCs (**Figure 6A**). We could propagate these cells over passages, unlimitedly, yielding stable, long-term self-renewing hiTSC lines from one female and two male patients.

People say all roads lead to Rome, but some are quite sinuous. It was the case here, investigating routes to hiTSC reprogramming. Although our protocol has proven robust in the end, it came out of multiple attempts and failures. For instance, we tested four different culture media (T2iLGö, CHIR+Gö, ASECRiA, ASECRiAV) of which only that of Okae *et al* turned out to be appropriate. We tried different timing of transition into final media, from day 8 (directly from huFibro medium) to day 21, after culture in E7. We also tested two matrices: collagen IV and MEF feeders (mouse embryonic fibroblasts), and found that MEFs largely promoted hiTSC self-renewal while preventing differentiation.

At some point, we faced problems, partly because somatic cell reprogramming comprises an early stochastic phase (David & Polo, 2014). Consequently, some unexpected cell fate decisions can occur in reprogramming intermediates. Usually, alternative lineages are transient and specific culture conditions allow to select cells of interest. However, it is possible that "contaminating" cell types adapt to these

conditions, compromising the establishment of homogeneous cell lines. We experienced this situation in one out of three assays: a proliferative population of mesenchymal-like cells emerged beside presumptive hiTSC colonies. We hypothesized these cells could have undergone malignant transformation. They neither expressed trophoblast associated genes, like *GATA3*, nor pluripotency markers, such as *Nanog*. We spent much time trying to eliminate these cells, through rigorous morphology-based selection, and finally succeeded by picking hiTSC colonies out of the bulk population (**Figure 6B**). Furthermore, it can happen sometimes that reprogramming intermediates fail to eliminate transgenes, which occured once during this study (**Figure 6C**). Despite similarities with trophoblasts in terms of gene expression, such lines cannot be considered as induced trophoblast stem cells, and thus were excluded from subsequent analyses.

Due to time constraints relative to publication schedule and the necessary prioritisation of experiments, we could not investigate in depth the epigenetic modifications underlying the process of hiTSC reprogramming. However, we did observe that hiTSCs had low DNA methylation levels, similar to those of primary hTSCs, and a concomitant study of Cinkornpumin *et al.* (2020) provided valuable insight into these aspects, in a context of inter-lineage conversion. First of all, global DNA methylation levels are much lower in the placenta than in somatic tissues, and they are especially low in hTSCs (Robinson & Price, 2015 ; Okae *et al*, 2018). The notion of "gatekeeper" genes has thus emerged, *i.e* that key placental genes, whose promoters are methylated in somatic cells, has to be demethylated and reactivated for enabling reprogramming into

hiTSCs.

Importantly, analogous methylation in the mouse is a critical obstacle to complete conversion of ESCs into TSCs (Cambuli *et al*, 2014). However, major differences in epigenetic dynamics seem to distinguish human from mouse development. For instance, X chromosome inactivation (XCI) in the mouse is produced by two successive waves, the first one consisting in imprinted paternal X inactivation (Xi), retained in the placenta, while the second round results in random XCI, after the paternal X (Xp) has been reactivated in the inner cell mass (Patrat *et al*, 2009 ; Starmer & Magnuson, 2009). By contrast, it seems that only one wave of random XCI occurs in humans (Okamoto *et al*, 2011 ; Petropoulos *et al*, 2016 ; Vallot *et al*, 2017).

This inter-species difference possibly originates from distinct evolutionary mechanisms of reproduction, and degree of parental genome competition, that is supposedly higher in the mouse (multiple pregnancies) than in humans. Among others, it might differentially affect reprogramming outcomes in the two systems. Indeed, it seems to be an additional barrier to reprogramming in the mouse, that would necessitate to reproduce the very early imprinted Xp inactivation for generating *bona fide* iTSCs, which has not been observed during reprogramming so far (Panda *et al*, 2020). Conversely, embryonic and extraembryonic lineages in human seem to share the same XCI process, which could facilitate the production of hiTSCs.

Consistently, Cinkornpumin *et al.* (2020) have shown that naive hESCs are

largely permissive to epigenetic remodeling, and acquire a trophoblast-like DNA methylation landscape upon conversion. It includes the demethylation of specific promoters, such as those of *Elf5*, hCG and STB fusion genes, that are further reactivated in these cells (Cinkornpumin *et al*, 2020). Nonetheless, some placental-specific traits could not be reproduced, notably regarding imprinted *loci*, and further research is needed for completing trophoblast conversion, on the scale of the epigenome.

During the routine culture of hiTSCs, we also reconsidered some aspects of trophoblast stemness. Regarding culture conditions, all laboratories have their habits (different matrices, passage frequency, split ratios...). For instance, Okae *et al* initially used collagen IV to isolate primary hTSCs, but the Rikken institute, currently supplying these lines, now recommends to use iMatrix (laminin511).

We observed that hiTSCs have a high level of sensitivity to matrices. In our hands, MEFs clearly promoted long-term self-renewal. After testing different combinations of ECM compounds, we found that fibronectin (optionally combined with laminin521) was the most appropriate for trophoblast stemness maintenance in feeder-free conditions, while collagen IV induced cell differentiation.

We wondered what coud be the molecular basis of hiTSC dependence on ECM. We thus investigated ECM receptor and ligand expression in these cells. Lee and colleagues have shown that *ITGA2* is specifically expressed in a proliferative pool of

CTs in the first trimester human placenta (Lee *et al*, 2018). In our transcriptomic analyses, we did observe a preferential expression of *ITGA2* in hiTSCs, down-regulated in differentiated cells (**Figure 7A**). By contrast, *ITGB1*, the most abundant beta-integrin and co-partner of many integrin-alpha subunits (at least ten), was ubiquitous. In fact, beta1-integrin subunits are produced in excess, retained in the endoplasmic reticulum, only addressed to the plasma membrane after heterodimerisation with alpha subunits, which determine specific integrin profiles (Kechagia *et al*, 2019).

We observed a switch of integrin expression patterns upon differentiation of hiTSCs, suggesting the following combinations of receptors: ITGA2/B1, ITGA6/B1, ITGA2/B5 and ITGA6/B5 in hiTSCs ; ITGA1/B1 and ITGA5/B1 in EVTs ; ITGA1/B1, ITGA5/B1, ITGAV/B1, ITGA1/B3, ITGA5/B3 and ITGAV/B3 in ST. Because stemness is a fine-tuned balance of self-renewal and differentiation, integrins expressed in hiTSCs could mediate either one or the other. It has been shown that ITGA2 phosphorylates MST1 which in turn activates YAP signalling, and globally that mechanical forces mediated by integrins promote YAP/TAZ nuclear translocation and activation (Elosegui-Artola *et al*, 2017 ; Kechagia *et al*, 2019). Given the role of YAP in TE specification, it is possibly retained in hiTSCs, further supported by signalling pathway signatures.

We considered additional gene lists from curated pathways related to ECM (KEGG_ECM_RECEPTOR_INTERACTION ; NABA_ECM_AFFILIATED). We observed specific expression patterns associated either with stem or differentiated cell

types, likely mediating different interactions with ECM (**Figure 7B**). Not only did these cells express distinct receptors, but they also produced a variety of extra-cellular proteins that can modify the micro-environment. For example, *COL4A1*, initially used by Okae and colleagues to derive hTSCs, was largely up-regulated in EVTs, similar to *COL4A2* (confirmed by immunostaining). Other ECM-related genes were typical of differentiated trophoblasts, such as *SDC1/3* (syndecans), *MUCL1* (Mucin-like protein 1, with protective functions in epithelia), and *LGALS13/14* (Placental protein 13-like), markers of ST cells. Of note, both ST and EVTs expressed high levels of *C1QTNF6/CTRP6*, a complement regulator, indicative of their immuno-modulating functions (Murayama *et al*, 2015). By contrast, *LAMA1* was specific to hiTSCs, suggesting a role in self-renewal. Interestingly, *HMMR*, a receptor for hyaluronic acid involved in ERK activity, was also up-regulated in hiTSCs, consistent with enhanced proliferation, in contrast to terminally differentiated cells. Some ECM genes were instead ubiquitous, like *FN1* (fibronectin), despite a subtle up-regulation in EVTs fitting with previous reports. This contributed to our choice of fibronectin-rich matrices for hiTSC maintenance and differentiation assays.

Considering the core gene network of trophoblast stemness, we discovered that a few genes were specific to hiTSCs, comprising *NR2F2*, *LRP2*, *TACSTD2* and *Peg10* (article, Figures 1D and S1D). Of note, NR2F2 co-operates with GATA3 and FOXA1 to activate target genes of ESR1/ERalpha (Estrogen receptor), and LRP2 is a receptor for estrogen, leptin, SHH and lipoproteins. *TACSTD2*, an important paralogue of *EPCAM*, is acting as a growth factor receptor and a calcium signal transducer, already associated

with the trophoblast lineage, when the orthologue of *Peg10* in the mouse is crucial for placental development, as mentioned above. Therefore, trophoblast stemness in human might rely on additional signalling pathways, including those of estrogen, leptin and Sonic hedgehog, and others more related to metabolism, such as lipid trafficking or calcium flux.

In the meantime, Liu *et al* also managed to generate hiTSCs, which corroborated our results (Liu *et al*, 2020). Altogether, we demonstrated that inter-lineage barriers, originating from epigenetic marks established in somatic cells (Cinkornpumin *et al*, 2020), are erased by OSKM reprogramming, yielding a cellular state permissive to the induction of pluripotency and trophoblast stemness, without affecting genome stability (karyotype validation). In line with this, we found that global DNA methylation levels were low in hiTSCs, similar to naive h(i)PSCs and placental tissues, in contrast with primed h(i)PSCs and somatic cells. Moreover, hiTSCs reactivated typical genes of the placenta, such as *Peg10* and the *CGB* cluster, that are methylated in somatic lineages (Cinkornpumin *et al*, 2020 ; Rahat *et al*, 2017). Building on this success, we used hiTSC lines to investigate mechanisms of trophoblast lineage specification in human.

Figure 6: Reprogramming strategy and associated morphological changes.

A

PATIENT FIBROBLASTS

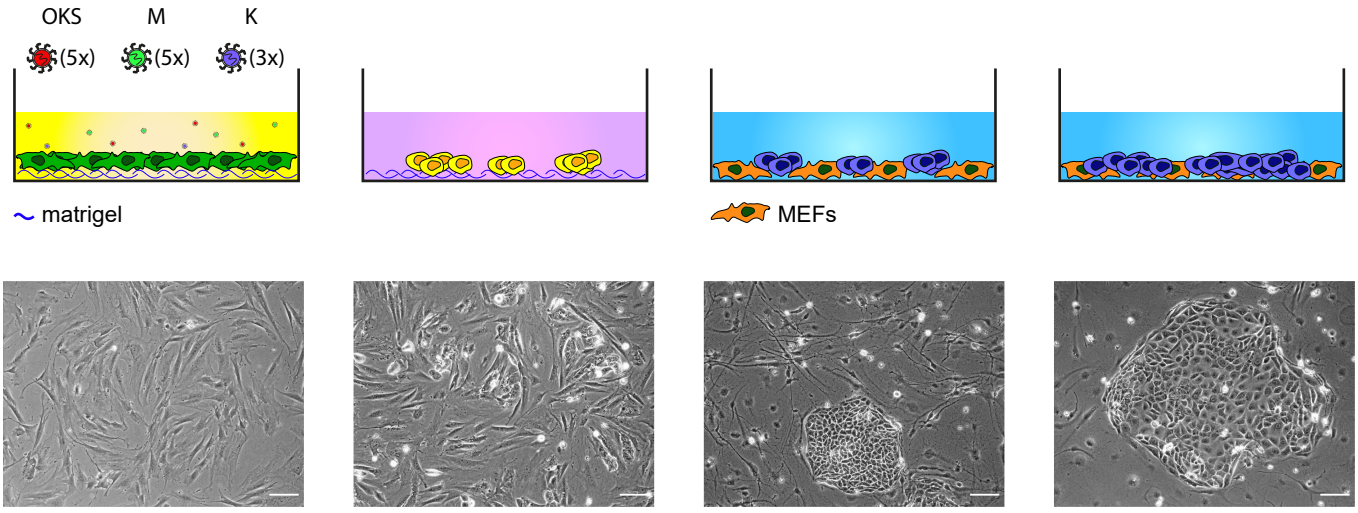
PATIENT hiTSCs

Day 0: SeV infection

Day 7: E7 medium

Day 21: hTSC medium

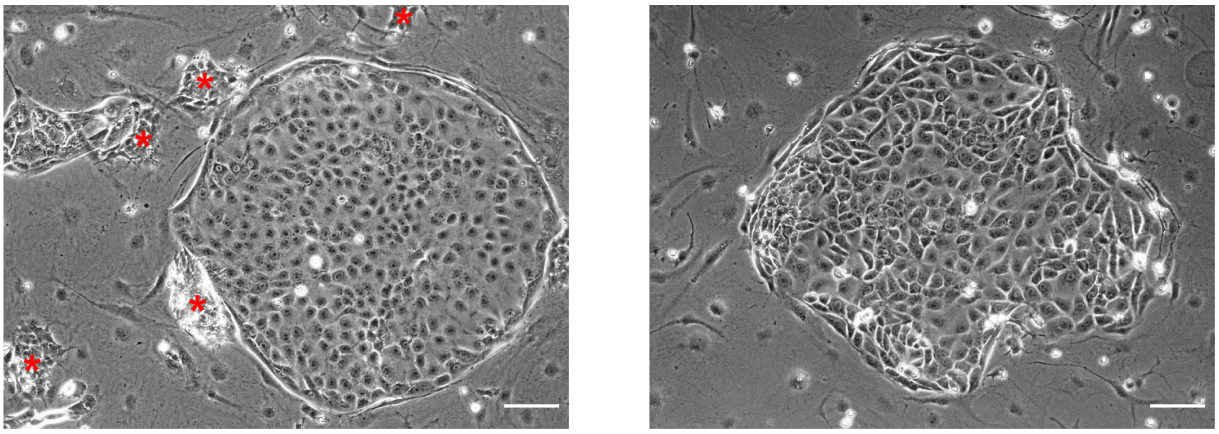
Day 70: SeV-free line



B

BEFORE CLEANING

AFTER CLEANING



C

EARLY PASSAGE

LATE PASSAGE

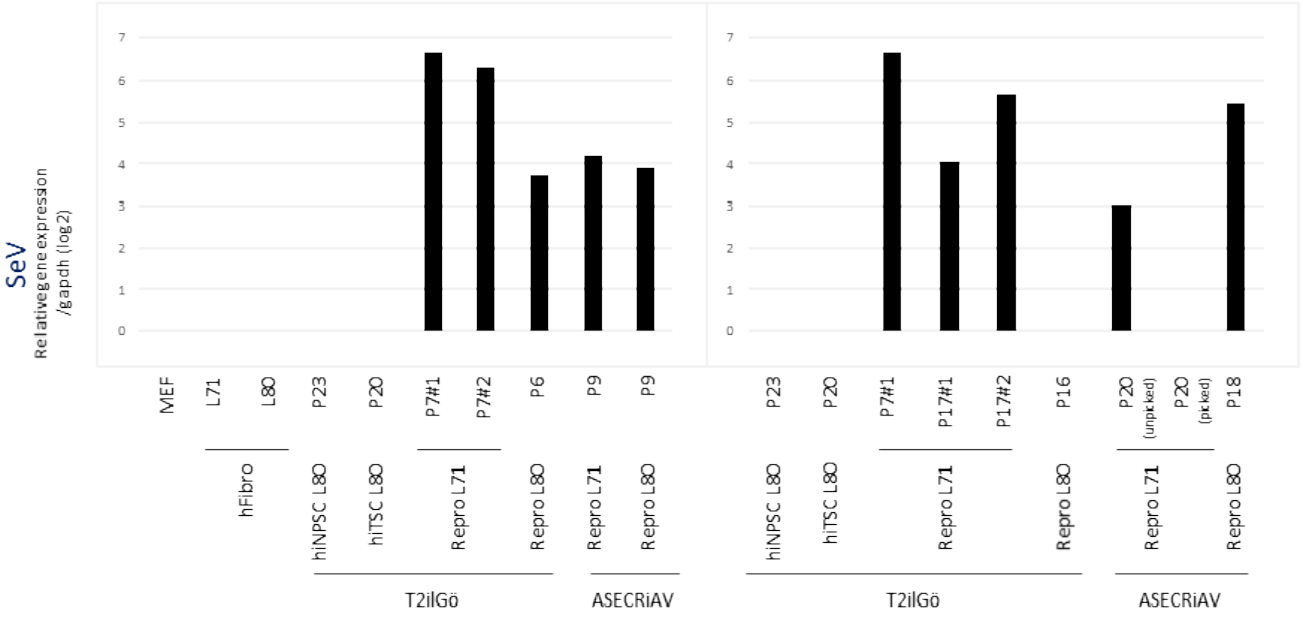


Figure 6. Reprogramming strategy and associated morphological changes.

(A) Top: a scheme describing hiTSC reprogramming protocol. Bottom: bright field images showing the morphological changes paving hiTSC reprogramming steps ; from left to right: adult fibroblasts before SeV infection (day 0), early reprogramming intermediates undergoing EMT (day 7), hiTSC-like colony formation (day 28, P3), a transgene-free long term self-renewing hiTSC line (day 86, P19).

(B) Left: a bright field image showing diverse cell populations in some reprogramming intermediates (day 63, P12) ; red asterisks indicate mesenchymal-like cells to be eliminated. Right: picture of a hiTSC colony from the same line, homogenous after cleaning.

(C) qRT-PCR to SeV constructs, showing the loss of transgenes in L71 (AV01) but not in L80 repro ASECRiAV cells.

Scale bar: 100 µm.

Figure 7: ECM-related gene profiles distinguish between trophoblast stem and differentiated cells.

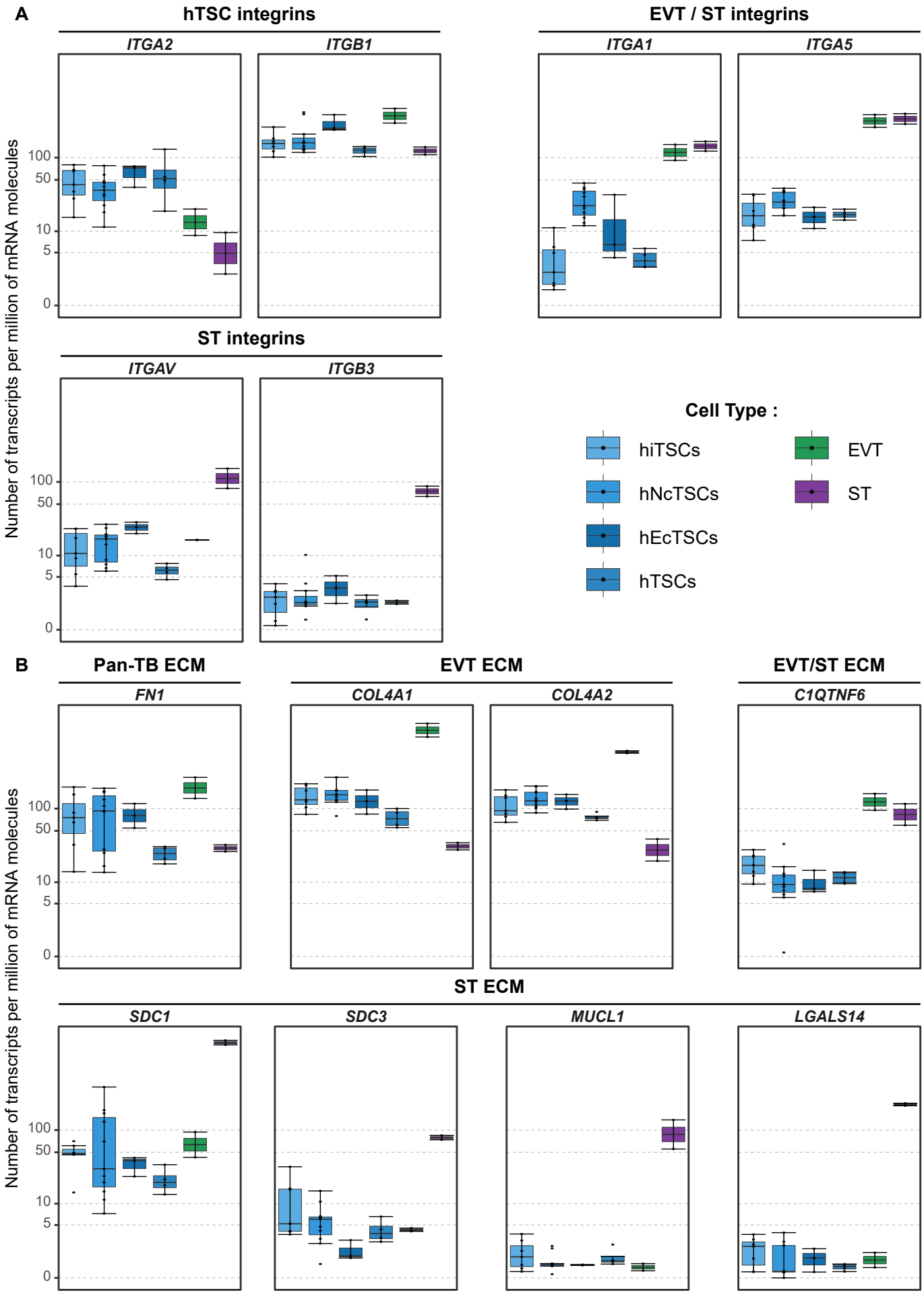

A
hTSC integrins
EVT / ST integrins
ITGA2
ITGB1
ITGA1
ITGA5
ST integrins
ITGAV
ITGB3
Number of transcripts per million of mRNA molecules
100
50
10
5
0
Cell Type :
hiTSCs
hNcTSCs
hEcTSCs
hTSCs
EVT
ST
B
Pan-TB ECM
EVT ECM
EVT/ST ECM
FN1
COL4A1
COL4A2
C1QTNF6
ST ECM
SDC1
SDC3
MUCL1
LGALS14

Figure 7. ECM-related gene profiles distinguish between trophoblast stem and differentiated cells.

(A) Gene expression levels of integrins in h(i/c)TSCs, ST and EVTs.

(B) Additional ECM-related genes, with specific expression patterns among trophoblast lineages.

Data: bulk RNA-Seq (DGE-Seq) ; unit: number of transcripts per million of mRNA molecules (UPM).

3.2 Differentiation of hiTSCs into ST and EVT cells

As mentioned previously, two main properties define stemness: long-term self-renewal, and the potential to produce mature, specialised cell types. Therefore, we conducted functional validation of hiTSCs, testing their capacity to differentiate into syncytiotrophoblast (ST) and extravillous trophoblast cells (EVTs).

Responsiveness to differentiation cues was variable among hiTSC lines. We tested diverse combinations of signalling molecules and timing of treatments, in order to increase differentiation efficiency. In the case of ST formation assays, we observed elevated cell death upon the addition of forskolin, compromising syncytialisation despite inducing many specific genes. We thus decreased forskolin concentration, but still observed apoptosis, suggesting a very deleterious effect of this compound on trophoblast survival. This was even the case with very different media, like one we adapted from Gupta *et al*, made of Forskolin, hEGF, hLIF, A83-01 and ROCKi (Gupta *et al*, 2016). We wondered whether the removal of cytokines from hTSC medium could promote ST differentiation *per se*, which happens spontaneously when CTs are derived *in vitro* (Handschuh *et al*, 2006). However, it also resulted in massive cell death, so did the addition of forskolin to complete hTSC medium, confirming that either cytokine depletion or forskolin alone was detrimental.

We then tested effects of individual cytokines, ending with a minimal cocktail permissive to cell survival (A83-01, SB431542 and CHIR99021), after eliminating those

supposed to hamper ST formation. However, although survival was rescued, cells did not engage ST differentiation anymore. We also tried cAMP (cyclic adenosine monophosphate) in replacement of forskolin, as both can activate the PKA pathway involved in ST differentiation (Orendi *et al*, 2010). Here again, we obtained mixed results. We tried out alternative media, such as IVC-1 and IVC-2 used for *in vitro* culture of pre-implantation human blastocysts, which form primitive syncytium upon attachment to the dish (Deglincerti *et al*, 2016). Under these conditions, ST related genes were induced, including *CGA* and *CGB*, but cells failed to fuse.

Recent studies on human trophoblast organoids have revealed the spontaneous formation of multinucleated ST cells in their center (Haider *et al*, 2018 ; Turco *et al*, 2018). Therefore, we investigated whether hiTSCs could form similar 3D structures. We cultured cells in trophoblast organoid medium (TOM), embedded in semi-solid drops of matrigel, supplemented with fibronectin and laminin521. Cell clumps appeared within a few days, and after 2-week growth, structures of about 200µm in diameter were collected for immunostaining. In some of them, high levels of hCG-beta (CGB) were detected in central, multinucleated cells. We confirmed their syncytiotrophoblastic identity by qRT-PCR to specific genes, including *SDC1*, *CGB* and *CGA*, the alpha subunit of HCG, complementary to CGB. This protocol was suitable for primary hTSCs too, suggesting culture conditions were both smoother and more efficient than forskolin or cAMP to induce ST differentiation.

Concerning EVT differentiation, we also observed elevated cell death upon the

addition of NRG1. However, if omitted, small epithelioid colonies formed, instead of migrating and invasive EVT cells. We could improve cell survival by delaying treatment initiation, or adapting cell density at seeding, but efficiency was still low. In the human trophoblast organoid system, Haider and colleagues have shown that fine-tuned WNT signalling controls EVT formation. A first wave, following the loss of paracrine signals (from proximal, proliferative CTs) and reduced WNT activity, regulates EVT specification in distal cell columns (NOTCH1+ progenitors). A second wave, characterised by autocrine induction of WNT signalling, promotes maturation into terminally differentiated EVTs, expressing HLA-G (Haider *et al*, 2018).

We tested different concentrations of CHIR99021, a WNT pathway activator (GSK3 inhibitor), prior to differentiation assays, without visible effects. However, the use of IWR-1 (inhibitor of WNT response 1) in combination with A83-01 and ROCKi, for 2 days before treatment with NRG1, clearly promoted EVT formation. This could be due to the accumulation of EVT progenitors, in line with previous results (Haider *et al*, 2018). During these experiments, we could further refine our comprehension of EVT specification. As an example, we observed these cells systematically emerged between days 4 and 5, suggesting that key mechanisms are finely regulated in a time-controlled manner.

Due to the time constraints associated with article submission procedure, we could not perform RNA-Seq of all differentiated cells, and we limited their validation to qRT-PCR analysis. After publication, we carried on these experiments and merged the

new dataset with previous ones (**Figures S 1 and S2**), performing additional transcriptomic analyses of hiTSC differentiated progeny. Pearson correlation heatmap and PCA confirmed that these cells clustered with ST and EVT derived from placentas or primary hTSCs differentiated *in vitro* (**Figures 8 and 9A**). Despite limited induction of *HLA-G* and *ASCL2* in bulk hiTSC-EVTs (**Figure 9B**), these results globally supported that hiTSC differentiation into ST and EVT lineages was successfully achieved.

Altogether, based on previous studies, we designed new protocols for generating ST and EVT cells from hiTSCs. Of course, further improvements are welcome, notably to increase differentiation efficiency, a prerequisite to using these cells for large-scale experiments.

Figure 8: Pearson correlation heatmap of stem and differentiated cell lineages.

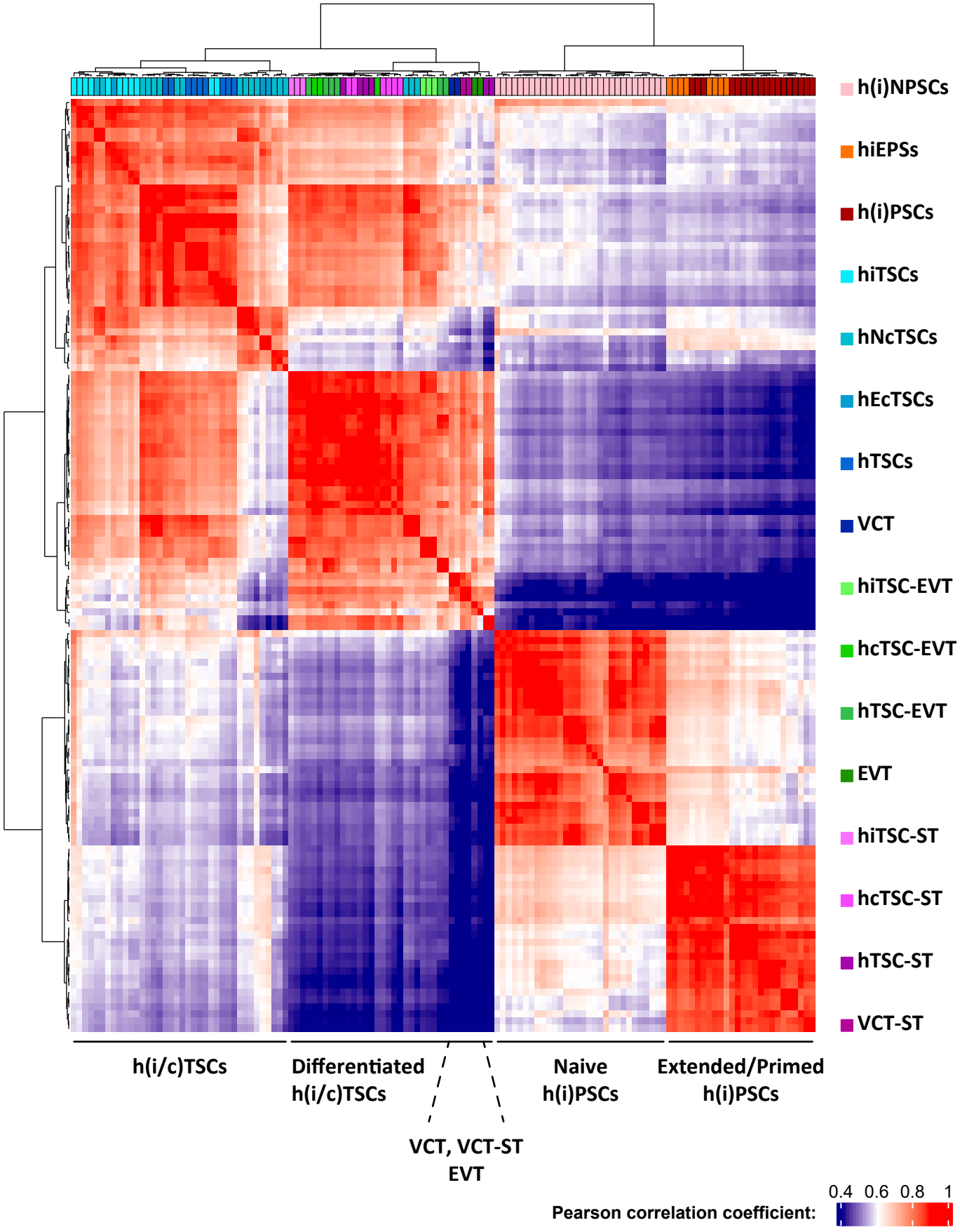

h(i)NPSCs
hiEPSs
h(i)PSCs
hiTSCs
hNcTSCs
hEcTSCs
hTSCs
VCT
hiTSC-EVT
hcTSC-EVT
hTSC-EVT
EVT
hiTSC-ST
hcTSC-ST
hTSC-ST
VCT-ST
h(i/c)TSCs
Differentiated h(i/c)TSCs
Naive h(i)PSCs
Extended/Primed h(i)PSCs
VCT, VCT-ST EVT
Pearson correlation coefficient:
0.4 0.6 0.8 1

Figure 8. Pearson correlation heatmap of stem and differentiated cell lineages.

Pearson correlation heatmap of cell types of interest, including new transcriptomic data of hi/cTSCs differentiated into ST and EVT cells. Hierarchical clustering distributes samples into four main groups, recapitulating cell lineages. From left to right: (1) primary, induced and converted hTSCs ; (2) ST and EVT derived from h(i/c)TSCs, along with primary villous cytotrophoblast (VCT), syncytiotrophoblast (VCT-ST), and extravillous trophoblast (EVT) cells derived from the placenta ; (3) Naive h(i)PSCs ; (4) Extended and primed h(i)PSCs.

Figure 9: PCA and specific gene expression of stem and differentiated cell lineages.

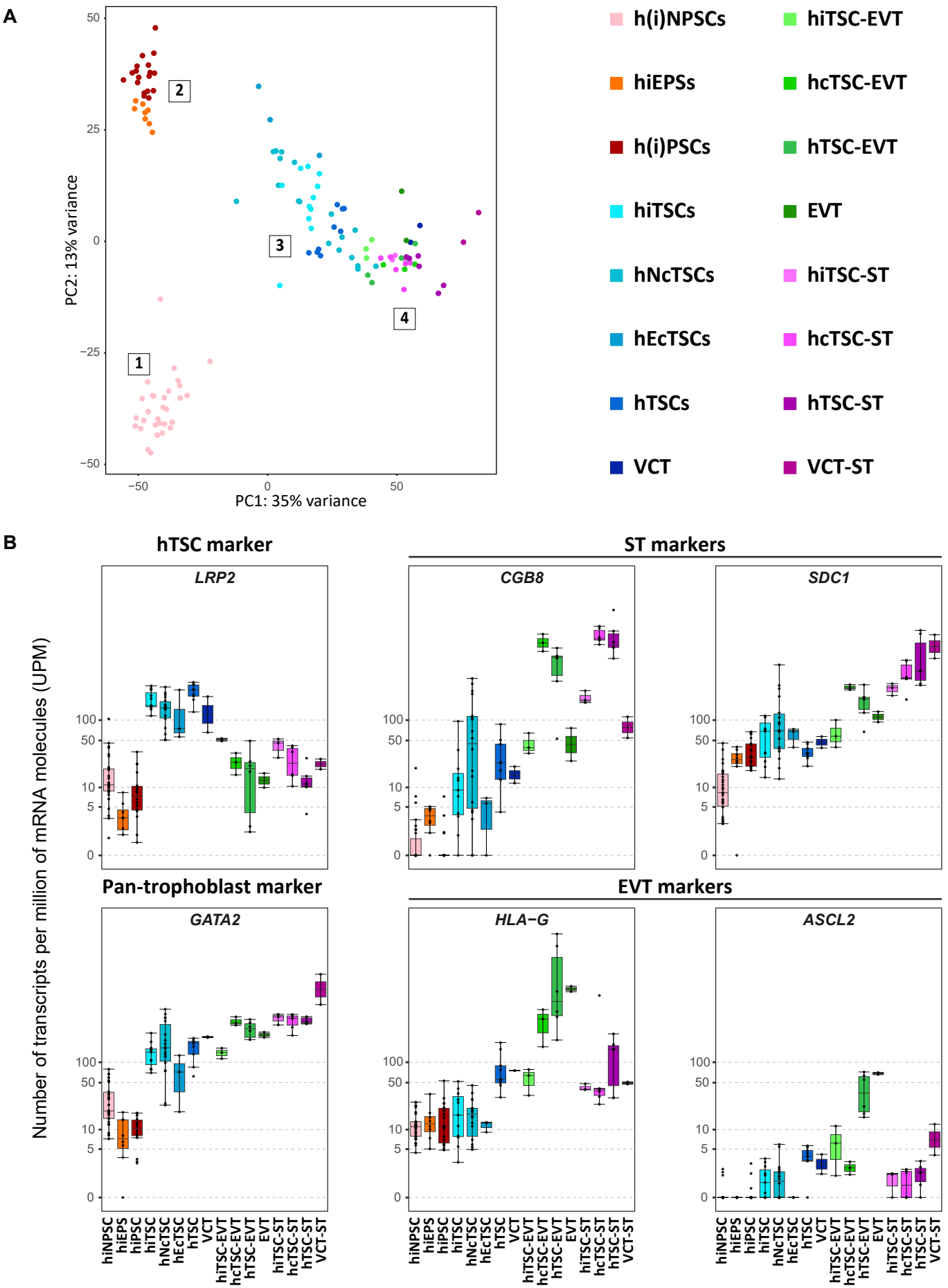

A
PC2: 13% variance
PC1: 35% variance
1
2
3
4
h(i)NPSCs
hiEPSs
h(i)PSCs
hiTSCs
hNcTSCs
hEcTSCs
hTSCs
VCT
hiTSC-EVT
hcTSC-EVT
hTSC-EVT
EVT
hiTSC-ST
hcTSC-ST
hTSC-ST
VCT-ST
B
Number of transcripts per million of mRNA molecules (UPM)
hTSC marker
LRP2
ST markers
CGB8
SDC1
Pan-trophoblast marker
GATA2
EVT markers
HLA−G
ASCL2
hiNPSC
hiEPS
hiPSC
hiTSC
hNcTSC
hEcTSC
hTSC
VCT
hiTSC-EVT
hcTSC-EVT
hTSC-EVT
EVT
hiTSC-ST
hcTSC-ST
hTSC-ST
VCT-ST

Figure 9. PCA and specific gene expression of stem and differentiated cell lineages.

(A) PCA of selected cell types after sample merging, including new transcriptomic data of hi/cTSCs differentiated into ST and EVT cells. Samples form four main groups, recapitulating cell lineages: (1) Naive h(i)PSCs (bottom-left) ; (2) Extended and primed h(i)PSCs (upper-left) ; (3) primary, induced and converted hTSCs (middle) ; (4) ST and EVT derived from h(i/c)TSCs, along with primary villous cytotrophoblast (VCT), syncytiotrophoblast (VCT-ST), and extravillous trophoblast (EVT) cells derived from the placenta (right).

(B) Boxplots showing gene expression levels of specific lineage markers. Data: bulk RNA-Seq (DGE-Seq) ; unit: number of transcripts per million of mRNA molecules (UPM).

3.3 Cellular heterogeneity of hiTSCs

The observation that hiTSC lines respond unequally to differentiation cues, despite similar transcriptomic profiles, points to alternative mechanisms controlling their commitment into mature lineages.

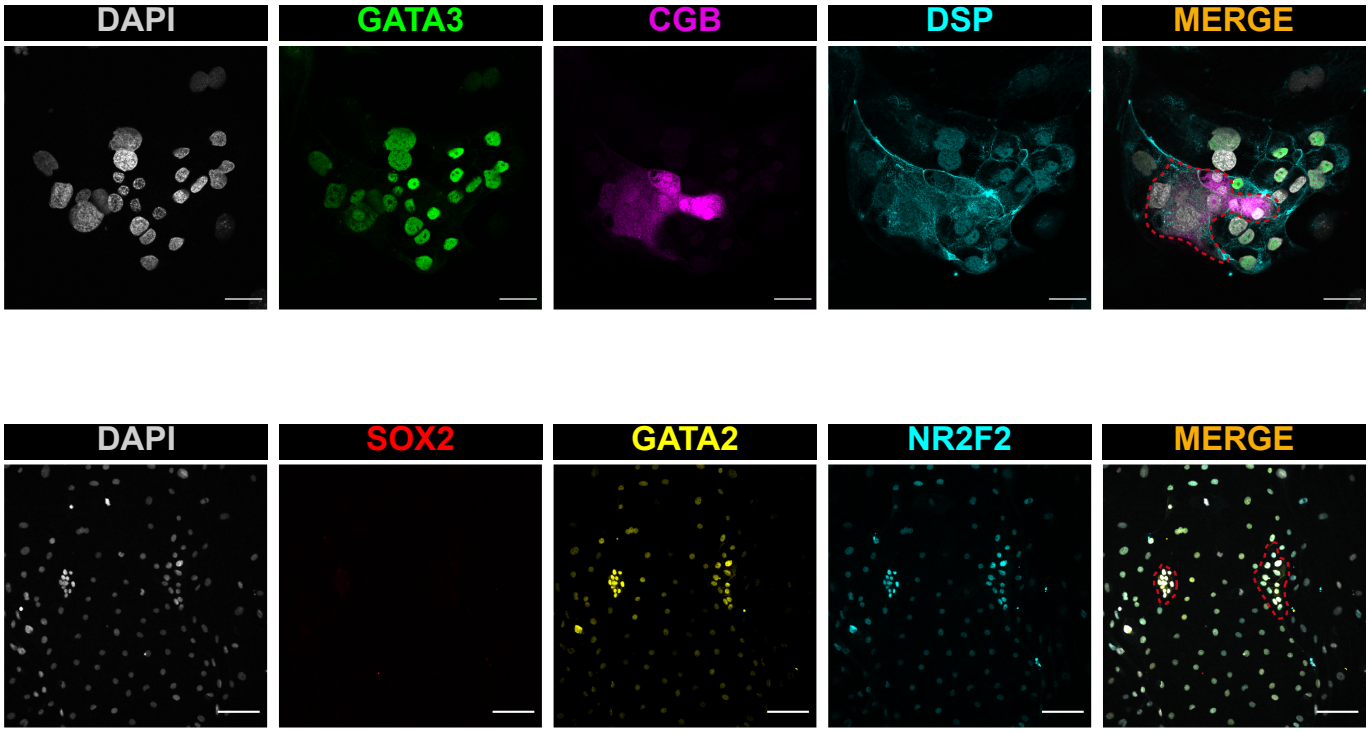
This could be due to epigenetic and post-transcriptional modifications, differential signalling or even metabolism. It might also relate to cellular heterogeneity among hiTSCs, as some lines were quite homogeneous, while others clearly contained different subpopulations of cells within colonies (**Figure 10A**). These were characterised by bigger nuclei and markers of ST and EVT (*e.g.* *CGB*). Increasing nuclear size could indicate endoreplication (or endomitosis), a process associated with TGC formation in the mouse. By contrast, high expression levels of *NR2F2* in groups of smaller cells are certainly indicative of stem cell "islets" within colonies. Therefore, some mature cells could be more prone to engage differentiation than immature ones, and relative contents might determine differentiation efficiency of hiTSC lines. Of note, similar heterogeneity has been observed in mouse TSCs, in which Cdx2-high cells exhibit higher potential than Cdx2-low ones (Frias-Aldeguer *et al*, 2020).

Furthermore, like pluripotent stem cells, hiTSCs might capture a wide spectrum of stemness, some residing in a more "naive" or "ground" state, while others are "primed" or "capacitated" and poised for differentiation (Rostovskaya *et al*, 2019). In line with this, we evidenced by flow cytometry some distinct hiTSC subpopulations, differentially

expressing markers of early TE or mature TB. Notably, we observed interesting patterns of CD24, a surface marker of the early human TE (see the ANNEXE), with a majority of CD24-high, presumptive "ground" stem cells, beside a CD24-low population, possibly residing in a "primed" state (**Figure 10B**).

Figure 10: Cellular heterogeneity among h(i/c)TSCs.

A


hiTSCs (AV03)
DAPI
GATA3
CGB
DSP
MERGE
DAPI
SOX2
GATA2
NR2F2
MERGE

B

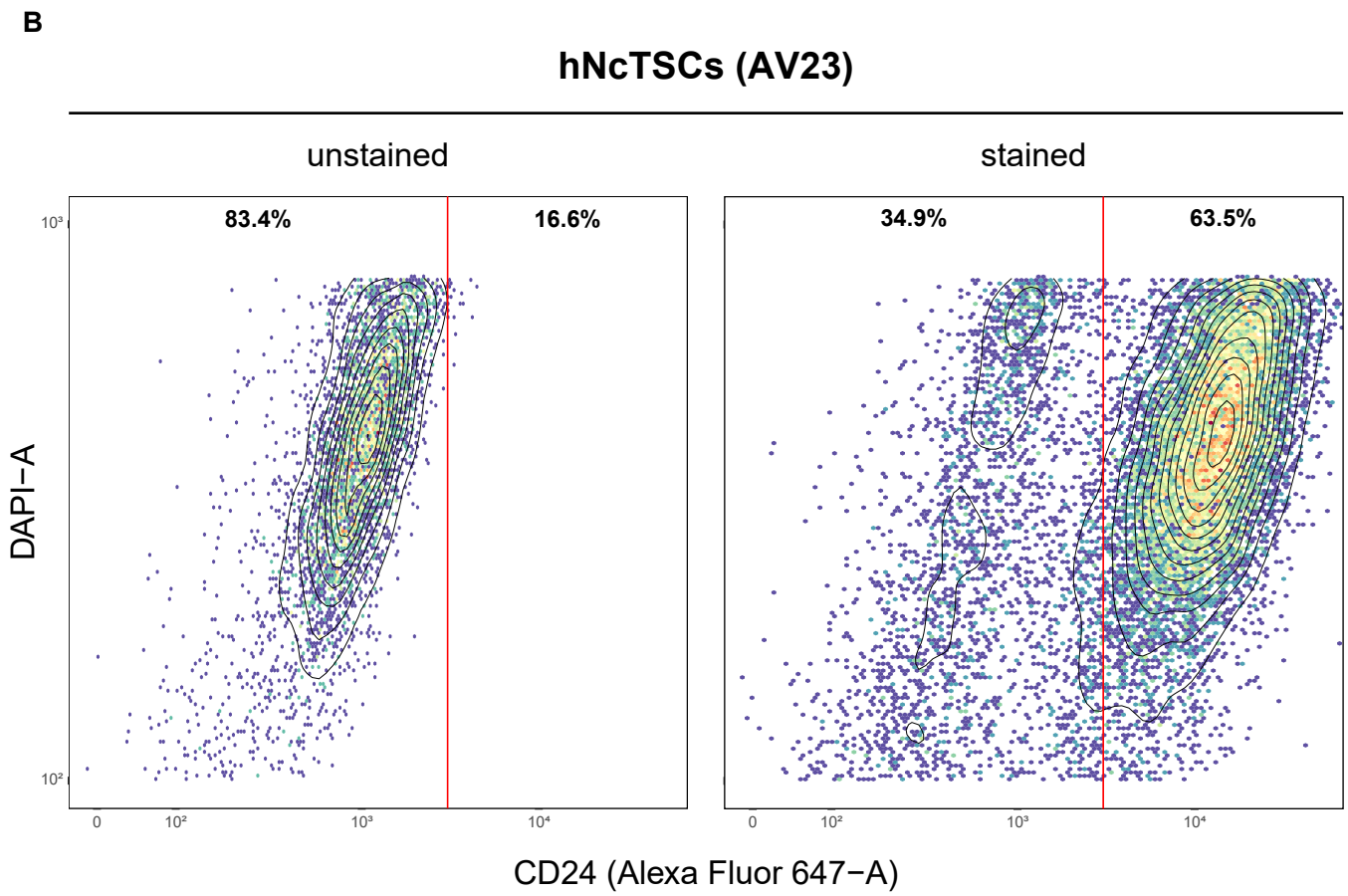

hNcTSCs (AV23)
unstained
stained
83.4%
16.6%
34.9%
63.5%
DAPI−A
CD24 (Alexa Fluor 647−A)

Figure 10. Cellular heterogeneity among h(i/c)TSCs.

(A) Immuno-staining for indicated genes in hiTSCs. Top: rare cells within colonies are multinucleated, expressing hCGbeta (CGB), a marker of ST (dashed line) ; scale bar: 30µm. Bottom: some islets of small cells express high levels of trophoblast core transcription factors, such as GATA2, and NR2F2, specifically associated with stemness (dashed line) ; scale bar: 100µm.

(B) Flow cytometry analysis of hNcTSCs. Left: unstained cells are used to control autofluorescence ; Right: cells stained for CD24 are clearly divided into two main subpopulations of CD24-high (63.5%) and CD24-low cells (34.9%).

3.4 Cell fate plasticity between embryonic and extraembryonic lineages

Naive hPSCs have unrestricted potential to generate hTSCs

As mentioned previously, we observed events of spontaneous trophoblast differentiation in some early naive reprogramming intermediates. We thus hypothesized that naive hiPSCs (hiNPSCs) could retain the potential to produce trophoblast stem cells. Supporting this, we observed rare trophoblast differentiation in established hiNPSCs too, confirmed by immunostaining (**Figure 11**).

Therefore, we tested the transition of naive hiPSCs into hTSC culture medium. Despite some cells entering apoptosis at initial steps, cellular morphology progressively changed, and cobblestone-shaped colonies reminiscent of hTSCs emerged within 3 passages ($\sim$ 15 days). We could expand these cells unlimitedly over passages, yielding stable, converted hTSC lines (hcTSCs) from three patients.

In the meantime, similar results were obtained by other groups, reinforcing our views (Cinkornpumin *et al*, 2020 ; Dong *et al*, 2020 ; Guo *et al*, 2021)

Extended hPSCs retain the potential to yield hTSCs

We wondered whether this property was specific to naive hiPSCs or was instead common to all pluripotent stem cells. We thus repeated the experiment with extended and primed hiPSCs.

Despite initially enhanced cell death, extended hiPSCs too produced converted hTSCs in our hands. This result was consistent with the original study of Yang *et al*, showing these cells are contributing to extraembryonic lineages in mouse chimeras (Yang *et al*, 2017). Our observation also echoed a report of hTSCs derived from expanded potential stem cells, cultured under close but not identical conditions, compared with extended hiPSCs (Gao *et al*, 2019).

Primed hPSCs cannot readily produce hTSCs

By contrast, we could not generate converted hTSCs from primed hiPSC lines. Instead, we found these cells rapidly lost colony integrity, producing individualised, differentiated trophoblasts, within a single passage.

We obtained comparable results with BAP-treated hiPSCs, although epithelioid morphology was maintained slightly longer (2 passages). In this regard, our observations contrast with others', stating that primed hPSCs produce amnion under similar culture conditions (Guo *et al*, 2021 ; Zheng *et al*, 2019). These cells, indeed, appeared close to

the human amniotic epithelium (AME), on the scale of global gene expression. However, it is worth mentioning that they were compared to rare, presumptive AME cells of human embryos cultured *in vitro*, that have not been fully characterised (Guo *et al*, 2021 ; Xiang *et al*, 2020). In our opinion, the debate remains open, and categorical statements should be avoided, in the absence of a proper reference *in vivo*. Recent single-cell RNA-Seq data of an entire, gastrulating human embryo ($\sim$ 16-19 dpf) might help resolve these questions in the near future (Tyser *et al*, 2020).

Altogether, our results suggest that naive hPSCs have the highest potential to generate hTSCs. This observation fits with the canalisation of developmental pathways, a concept formulated by Waddington, assuming that development follows a preferred path, made of sequential cell fate decisions (Waddington, 1942). Such events are accompanied by robust regulatory processes, safeguarding the normal course of development, so that environmental perturbations cannot disrupt this progression. As a consequence, differentiation potential is gradually restricted. The very notion of cell lineages predicts that the more distant two cell types are on the scale of development, the more refractory they are to inter-lineage conversion, due to stronger epigenetic barriers.

In this perspective, naive hPSCs, equivalents of the pre-implantation epiblast, are developmentally close to cells permissive to the first lineage decision, when ICM and TE fates bifurcate. Therefore, it is consistent that TSC fate induction is easier in naive than in more advanced hPSCs. The fact that extended hPSCs retain the potential to

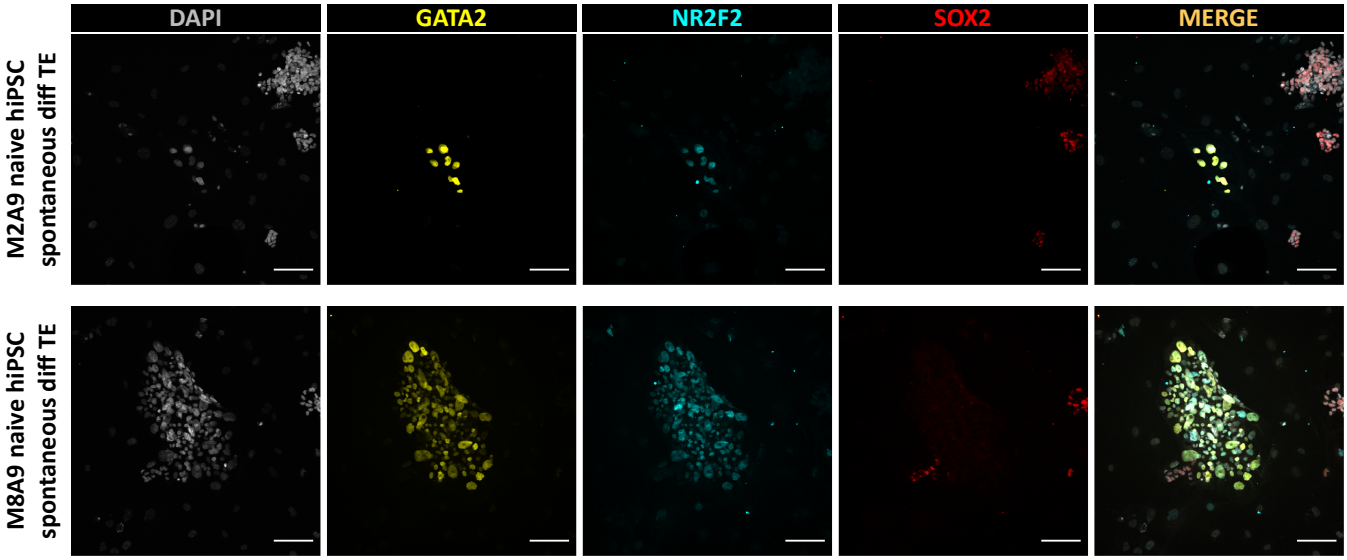
produce hTSCs, however with reduced efficiency, could indicate these cells are intermediate between naive and primed states, However, the fact that hEPSs are similar to primed hPSCs on the scale of the global transcriptome suggests that differences in potential rather originate from alternative cellular properties. We hypothesized that such traits could include distinct epigenetic landscapes or metabolic states. However, we observed that hEPSs had elevated levels of global DNA methylation, in that much closer to primed than to naive h(i)PSCs or h(i/c)TSCs, largely hypomethylated, even though we could not rule out distinct patterns at specific *loci* (data: DNA Mass spectrometry, not Bisulfite-Seq). Regarding OXPHOS metabolism, preliminary data suggested that hEPSs have a distinct metabolic status from those of primed and naive cells, further supported by differential expression of specific genes like metallothioneins, but it has to be confirmed by new assays.

Another possibility is that hEPSs contain rare sub-populations of naive-like cells, which could preferentially contribute to form converted hTSCs. In line with this, a TE progenitor-like sub-population was observed in naive hPSCs, expressing key TE genes such as *ABCG2*, *CLDN4*, *VGLL1*, *GATA2*, and *GATA3* (Messmer *et al*, 2019). Consistently, we observed that critical TE genes are detected late during hEPS conversion, as compared with naive conversion, suggesting that they are not induced in the bulk population, but rather in rare cells that are progressively selected. This hypothesis is further reinforced by higher contents of differentiated trophoblasts, reminiscent of primed conversion, beside hcTSC-like colonies at early steps of hEPS conversion, while naive conversion is homogeneous and more efficient.

Interestingly, hTSCs derived from naive hiPSCs also formed smaller colonies, with reduced cytoplasmic content, compared with those originating from extended hiPSCs. It suggests that some properties, inherited from parent cells, are resistant to lineage conversion, which can participate to distinct behaviours between lines, such as variable responsiveness to differentiation signals. Despite that, all converted hTSCs globally had similar gene expression patterns and proliferation rates.

Figure 11: Naive hiPSCs are prone to form rare TE-like cells spontaneously.

A


DAPI
GATA2
NR2F2
SOX2
MERGE
M2A9 naive hiPSC spontaneous diff TE
M8A9 naive hiPSC spontaneous diff TE

B

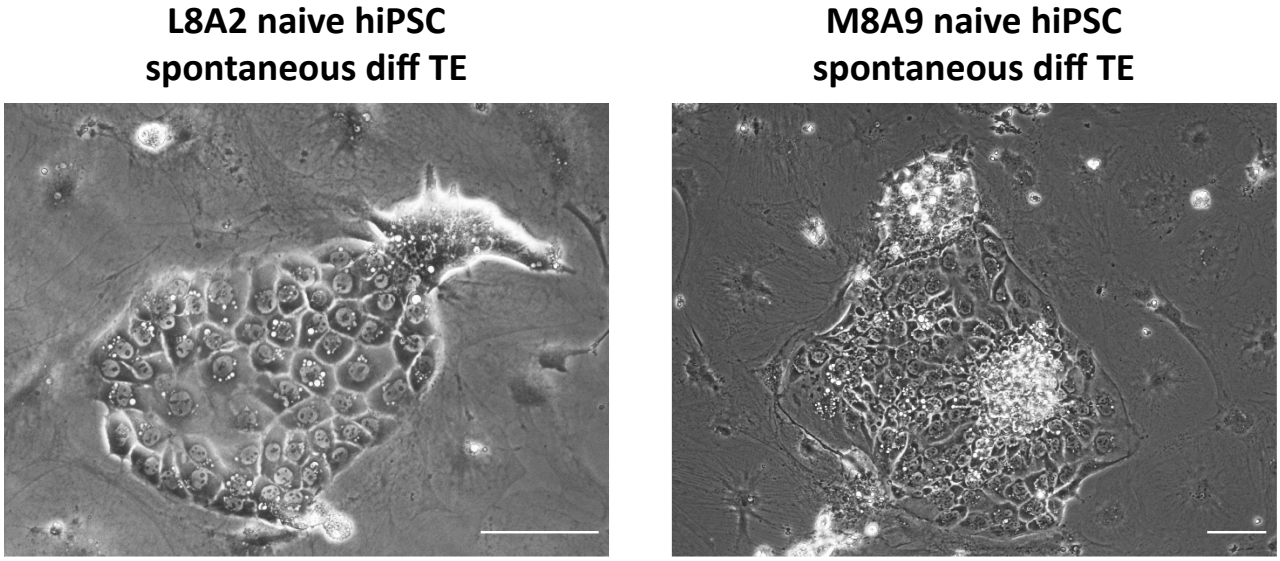

L8A2 naive hiPSC spontaneous diff TE
M8A9 naive hiPSC spontaneous diff TE

Figure 11. Naive hiPSCs are prone to form rare TE-like cells spontaneously.

(A) Immunofluorescence images (confocal slices) of naive hiPSCs showing spontaneous differentiation into trophectoderm-like cells, as evidenced by expression patterns of key lineage markers: SOX2 (pluripotent), NR2F2 and GATA2 (trophoblast).

(B) Bright field pictures showing morphological changes associated with TE formation from naive hiPSCs, comprising the acquisition of an epithelioid cobblestone-shape phenotype.

Scale bars: 100 µm.

Role of cell fate plasticity in development

An interesting point of view is to consider cell fate plasticity as a natural, regulatory mechanism acquired through evolution to compensate for accidental cell loss. This property has been observed in Monotremes and Marsupials, in which pluriblast and trophoblast lineages can repopulate each other in response to damage (Selwood & Johnson, 2006). Another characteristic of the marsupial embryo could involve cell fate plasticity: the absence of an ICM in the early unilaminar blastocyst, exclusively made of trophoblast, which only later produces the pluriblast (Frankenberg *et al*, 2013 ; Renfree *et al*, 2010).

Experiments in the mouse have shown that the ICM, deprived of TE cells by immuno-surgery, is able to reconstitute an entire blastocyst, comprising EPI, PE and TE, a property retained up to the nascent blastocyst stage (E3.0, 32-cell) (Wigger *et al*, 2017). However, the potential to produce TE cells is lost by the late blastocyst stage (64-cell), when ICM becomes heterogeneous, containing pre-EPI, pre-PE and bipotent cells. This could explain why mouse ES cells, usually derived from the epiblast of late blastocysts, are regarded as reluctant to engage trophoblast fate conversion.

In human, De Paepe *et al* have shown that TE cells are specified, but not yet committed up to the fully expanded blastocyst stage. As a consequence, they can reconstitute a complete embryo, including both ICM and TE compartments, if small clumps are reintroduced into an empty *ZP* (De Paepe *et al*, 2013). Conversely, ICM cells

alone cannot repopulate TE, but it has been attributed to insufficient cytoplasmic content rather than to fate barriers. In line with this, the capacity of TE cells to reconstitute an entire blastocyst depends directly on the number of injected cells. It has been evidenced recently that the ICM of the late human blastocyst (day 6) does retain the potential to form TE cells, although not an entire embryo, under specific culture conditions *in vitro* (Guo *et al*, 2021).

Cell fate plasticity may constitute a selective advantage, promoting the survival of the offspring by allowing compensatory mechanisms to occur between closely related lineages. On the other hand, this process should not disturb developmental progression, which could explain why cellular plasticity is gradually restricted through lineage commitment. The observation that naive and extended hPSCs are capable to convert into hTSCs, while primed hPSCs are refractory to this process, matches this view.

Molecular mechanisms of cell fate plasticity

Gene expression patterns, signalling pathway activities and other biological processes differ between cell types, determining their functions. The majority of them were identified in contexts very different from embryonic development (*e.g.* cancer, ageing), and the query of databases to investigate mechanisms of trophoblast specification turned out to be pointless. However, many of these processes are controlled by core gene networks, driven by transcription factors. Therefore, we applied WGCNA,

an unsupervised analysis of gene expression covariance, to this specific subset of genes, in order to decipher the molecular mechanisms regulating hTSC fate decision.

We found that *ESRRG* and *HAND1* modules were correlated with early reprogramming intermediates of both naive hiPSCs and hiTSCs (**Figure 12A**). Of note, the *ESRRG* module comprised *TFAP2A*, *Rxra*, *SATB1* (a key regulator of mouse TS cells), and *TCF7L1*, transiently activated in the embryo around the first lineage specification (see the ANNEXE). These genes are highly expressed in either TE or TB cells *in vivo*. We refined the analysis and found they were progressively induced, and maintained throughout hiTSC reprogramming, while their expression was transient in early naive intermediates.

During naive conversion, we also found *ESRRG* and related genes, such as *Rxra*, contributing to the *MSX2* module associated with late intermediates, shortly before hcTSC lines were established (**Figure 12B**). We thus hypothesized that naive hiPSCs, in this context, could initially dedifferentiate into cells similar to early reprogramming intermediates. Interestingly, kinetics of extended hiPSC conversion into hcTSCs appeared slightly different. A first wave, characterised by *TFAP2A* module induction, was followed by a second one, with genes like *ESRRG* forming the *SP6* module, correlated with the late phase. These results meet the delay observed during tissue culture, suggesting that extended hiPSCs, more distant from hTSCs than naive hiPSCs on the scale of developmental progression, might need additional steps to complete conversion.

We observed that genes of the *TCF4* module were transiently up-regulated at early phases of naive conversion. Their expression correlated with the repression of pluripotency associated genes, preceding the onset of the trophoblast program. However, while most of these genes were not detected in the embryo and thus could be artefacts of *in vitro* culture, some of them, like *TCF7L2*, were highly expressed in the human TB. This module contained many genes related to WNT signalling. Considering the conversion of extended hiPSCs, the *GATAD2B* module closely resembled the *TCF4* module of naive conversion, suggesting common mechanisms despite timing differences.

We then decided to include more genes in WGCNA, to get a better view of biological processes underlying naive and extended conversion into hcTSCs, while primed hiPSCs could not produce such cells. Notably, contrary to the former, the latter failed to induce the *CYB5R1*, *GSTM3*, *STXBP6* and *C15orf48* modules, normally activated by day 10 in naive and extended intermediates (**Figure 13A**). In-depth analysis revealed that the *CYB5R1* module comprised many cytokeratin genes, notably *KRT7*, a known trophoblast marker, and *KRT18*, expressed from the morula stage forward in the TE lineage, suggesting that primed intermediates did not complete epithelialisation. This module also contained *CLDN4* and other important trophoblast genes, such as *TACSTD2* and *STARD10*.

Of note, the *STXBP6* module gathered genes associated with BMP signalling (including *BMP4* and *NOG*), a pathway promoting the conversion of primed hPSCs into mature trophoblasts, also contributing to epithelialisation. Consistently, we observed a

peak of *BMP4* expression around day 10 of conversion, concurrent with the induction of *NOG*, suggesting that a negative feedback loop controls this process. Of note, *BMP4* was constantly expressed, at high levels, throughout naive and hiTSC reprogramming. Interestingly, we observed the transient expression of *HAND1*, a known transcription factor and direct target of BMP signalling (Okubo *et al*, 2021 ; Zheng *et al*, 2021), in conversion and reprogramming intermediates that yielded hi/cTSCs, but not in those which failed to produce such cells (**Figure 13B**). *HAND1* is critical to mouse placental development (Riley *et al*, 1998), also detected in the human TE pre-implantation, but lost in the first trimester placenta (Knöfler *et al*, 1998 ; Knöfler *et al*, 2002 ; Soncin *et al*, 2015). Another target of BMP signalling detected in the human TB, *MSX2*, was highly expressed in naive and extended conversion intermediates. Altogether, these results point to a transient BMP signalling wave playing a key role in trophoblast specification.

In primed hPSCs stimulated with BMP4, *MSX2* represses *SOX2* and up-regulates *NODAL*, directly binding to their promoters and inducing mesoderm fate (Wu *et al*, 2015). However, the concomitant addition of A83-01 blocks NODAL signalling, resulting in trophoblast conversion instead. We thus investigated genes related to the NODAL pathway, as they might contribute to hTSC fate specification. *NODAL* is naturally expressed in pluripotent stem cells and contributes to maintaining pluripotency in co-operation with FGF2. (Wu *et al*, 2015). Consistently, it was expressed at high levels throughout naive hiPSC reprogramming, while it remained very low between hiTSC intermediates and stable lines. The robust expression of *LEFTY1* and *LEFTY2*, direct targets of NODAL signalling, indicated the pathway was active in

these cells (even though they act as endogenous inhibitors). In line with this, we observed high expression levels of *TDGF1*, a co-receptor for NODAL, in naive but not hiTSC reprogramming intermediates.

Altogether, these observations suggest that the combined stimulation of BMP signalling and repression of NODAL pathway is key to inducing the trophoblast fate, consistent with the outcomes of BAP treatment. However, we showed that naive hiPSCs treated with BAP produced terminally differentiated trophoblasts rather than hTSCs, and thus we tested different combinations of small molecules to identify the processes that induce and maintain such cells. Among others, we designed a medium containing A83-01, BMP4, CHiR, MEKi, and ROCKi. Of note, CHiR and MEKi were added for sustaining hTSC self-renewal (Okae *et al*, 2018), and for maintaining cells in a pre-implantation state (Di Stefano *et al*, 2018 ; Guo *et al*, 2021), respectively, in order to generate putative hiTESCs (induced trophectoderm stem cells).

Naive hiPSCs transited into that medium showed a clear up-regulation of trophoblast-related genes, but failed to establish converted lines. Looking at gene expression, we found that treated cells successfully induced the *MSX2* module, that comprised *GRHL1*, *Tfap2c*, *GATA2*, *DLX5/6*, and *Gcm1*, thus confirming the activation of the BMP pathway. By contrast, they globally failed to up-regulate a few hTSC-specific genes, notably *TACSTD2*, similar to primed conversion intermediates. Furthermore, while they swiftly repressed NODAL signalling upon treatment initiation, it gradually recovered, at least *NODAL* expression, suggesting a resistance or selection

mechanism yielding naive hiPSCs unresponsive to treatment. This was further supported by the persistence of pluripotency associated genes (**Figure 14**).

Nevertheless, we could generate induced hTSC-like cells by somatic cell reprogramming in 2i + SB431542 + A83-01 + ROCKi (2iSAY), which we confirmed by transcriptomic analyses. These cells formed cobblestone-shape colonies, with a peculiar morphology reminiscent of the trophectoderm, that could be maintained over 10 passages. However, they ultimately entered terminal differentiation, and failed to establish stable lines (**Figures 14-15**).

In our view, an important difference between these treatments and conversion or reprogramming in hTSC medium is the addition of epidermal growth factor (hEGF) in the latter, which could be required for the maintenance of stable hi/cTSC lines. During reprogramming experiments, we observed that *KLF4* was the only Yamanaka factor whose expression was maintained in hiTSC intermediates, stable until late stages. Conversely, the other TFs, more specific of pluripotency, were rapidly down-regulated in these cells. Of note, *KLF4* is involved in the formation of many diverse epithelial tissues. Its functions during somatic cell reprogramming mainly concern the mesenchymal-epithelial transition (MET) of parent fibroblasts. In this context, KLF4 activates epithelium-related genes, such as *CDH1*, directly binding to promoter sequences. Moreover, the efficiency of hiPSC reprogramming relies on a fine-tuned balance between the activities of KLF4 and POU5F1, somehow playing a dual role. As an example, KLF4 inhibits while POU5F1 promotes *MGARP* expression (Tiemann *et al*, 2014).

Going through transcriptomic data mining of human peri-implantation embryos, we observed that *KLF4*, that we initially associated specifically with hiPSC reprogramming, was in fact expressed at very high levels in a subset of cytotrophoblasts comprising presumptive hTSCs (see the ANNEXE). Some studies even suggested that KLF4 activates *PSG5*, a trophoblast-specific gene, directly binding to its promoter (Blanchon *et al*, 2001 ; Blanchon *et al*, 2006 ; Li *et al*, 2019). Consistently, we observed sustained expression of *PSG5* throughout hiTSC reprogramming, also high in early naive intermediates, but down-regulated at later stages when levels of *KLF4* decrease.

Therefore, we hypothesized that the process of epithelialisation, involving KLF4, was important for establishing hTSCs. In this regard, we investigated expression profiles of cytoskeleton, plasma membrane and ECM associated genes. We observed that cytokeratin genes, notably *KRT7* and *KRT18*, were expressed at much higher levels in hiTSCs than in hiNPSCs. Similar patterns were observed for a majority of claudins, notably *CLDN4*, and the cell-surface receptor *TACSTD2*. By contrast, gene expression related to tight junction proteins, cadherins, and integrins was comparable between these cell types, with the notable exception of *ITGA2* up-regulated in hiTSCs (as described above). These results were consistent with the evidence of epithelialisation in the human TE, characterised by the expression of *KRT18* in outer cells, from the morula stage onwards (Gerri *et al*, 2020 ; Meistermann *et al*, 2021).

The analysis of single-cell ATAC-Seq data of human embryos revealed an enrichment in GATA TF binding motifs at the *KRT18 locus*, in open chromatin regions,

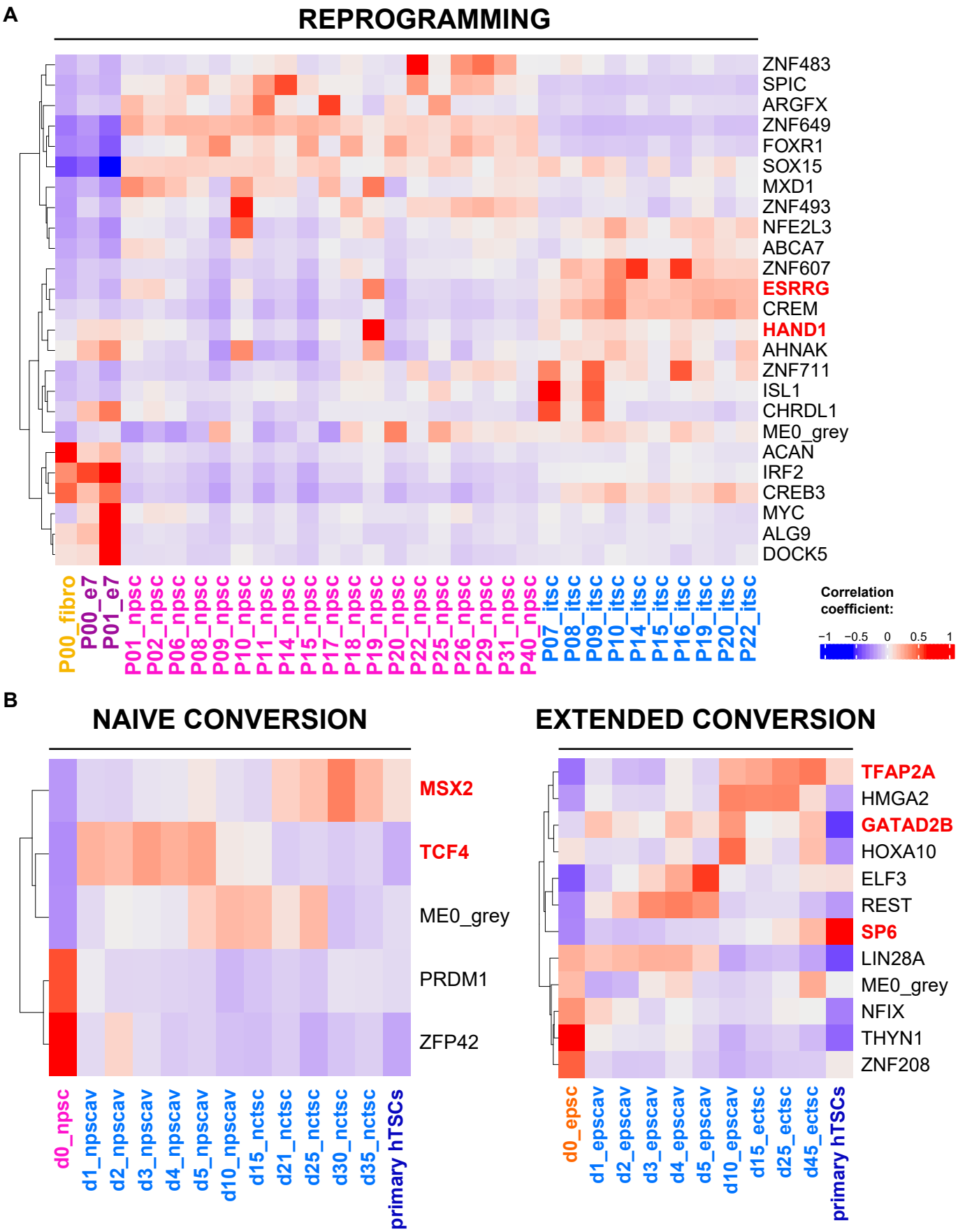
suggesting this gene might be directly activated by GATA3 at the onset of TE specification. Similarly, an enrichment in GATA3 binding motifs was also observed upstream of *GRHL2* (*Grainyhead-like transcription factor 2*), an important paralogue of *GRHL3* which is critical for epithelial morphogenesis and trophoblast branching in the mouse embryo (Gerri *et al*, 2020 ; Liu *et al*, 2019 ; Walentin *et al*, 2015). *GRHL2* is ubiquitous in the human morula, progressively restricted to the TE by the blastocyst stage. It is known for activating *CLDN4* and *RAB25*, thus contributing to the formation of tight junctions.

In the mouse, orthologues of *KLF4*, *GRHL3*, and *GATA3* regulate keratinocyte differentiation by controlling the expression of an overlapping set of genes (de Guzman Strong *et al*, 2006 ; Miyai *et al*, 2016 ; Patel *et al*, 2006 ; Yu et al, 2006). Moreover, *KLF4* is essential to the formation of epidermal barriers during mouse embryonic development (Segre *et al*, 1999). We observed that *KLF4*, *GATA3* and *GRHL3* were all robustly expressed at early stages of reprogramming, but *GATA3* and *GRHL3* later decreased in naive intermediates, while they remained high in hiTSCs (**Figure 13C**). Importantly, *KLF4* and *GATA3* are both controlled by the epidermal master regulator TP63, specifically expressed in hTSCs (Candi *et al*, 2006 ; Sen *et al*, 2012). Therefore, the rapid down-regulation of *TP63* during naive reprogramming could explain the concomitant loss of *GATA3* in these cells (while transgenic *KLF4* is still expressed).

Considering these results, we propose that NODAL signalling cascade represses *GATA3* and *GRHL3* during naive hiPSC reprogramming, thus preventing co-operation

with *KLF4*. In this context, KLF4 interacts preferentially with POU5F1 and SOX2, directed to the maintenance of pluripotency associated genes (Dhaliwal *et al*, 2019 ; Wei *et al*, 2009). In contrast, during hiTSC reprogramming, inhibition of the NODAL pathway by A83-01 maintains the expression of *GATA3* and *GRHL3*, thus interacting with *KLF4* to establish trophoblast stem cells. Our observations suggest that this process is mediated by epithelialisation and expression of key genes, such as *TACSTD2*, reinforced by BMP4 signalling. Importantly, one or several additional factors are needed to ensure long-term self-renewal of these cells, possibly comprising hEGF, that remains to be demonstrated.

Figure 12: WGCNA reveals gene modules associated with the divergence between pluripotent and trophoblast stem cell fates.


A
REPROGRAMMING
ZNF483
SPIC
ARGFX
ZNF649
FOXR1
SOX15
MXD1
ZNF493
NFE2L3
ABCA7
ZNF607
ESRRG
CREM
HAND1
AHNAK
ZNF711
ISL1
CHRDL1
ME0_grey
ACAN
IRF2
CREB3
MYC
ALG9
DOCK5
P00_fibro
P00_e7
P01_e7
P01_npsc
P02_npsc
P06_npsc
P08_npsc
P09_npsc
P10_npsc
P11_npsc
P14_npsc
P15_npsc
P17_npsc
P18_npsc
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P29_npsc
P31_npsc
P40_npsc
P07_itsc
P08_itsc
P09_itsc
P10_itsc
P14_itsc
P15_itsc
P16_itsc
P19_itsc
P20_itsc
P22_itsc
Correlation coefficient:
−1
−0.5
0
0.5
1
B
NAIVE CONVERSION
MSX2
TCF4
ME0_grey
PRDM1
ZFP42
d0_npsc
d1_npscav
d2_npscav
d3_npscav
d4_npscav
d5_npscav
d10_npscav
d15_nctsc
d21_nctsc
d25_nctsc
d30_nctsc
d35_nctsc
primary hTSCs
EXTENDED CONVERSION
TFAP2A
HMGA2
GATAD2B
HOXA10
ELF3
REST
SP6
LIN28A
ME0_grey
NFIX
THYN1
ZNF208
d0_epsc
d1_epscav
d2_epscav
d3_epscav
d4_epscav
d5_epscav
d10_epscav
d15_ectsc
d25_ectsc
d45_ectsc
primary hTSCs

Figure 12. WGCNA reveals gene modules associated with the divergence between pluripo-tent and trophoblast stem cell fates.

(A) Heatmap of WGCNA gene module correlation with naive and hiTSC reprogramming intermediates.
(B) Module correlation heatmap of naive and extended conversion.

Samples are organised according to cell types, from left to right: fibroblasts (yellow), E7 (purple), naive (pink), extended (orange), and hi/cTSC (blue) reprogramming or conversion intermediates. Otherwise, samples are ordered according to increasing days or passages.

Figure 13: Specific molecular mechanisms are critical for establishing hi/cTSCs.

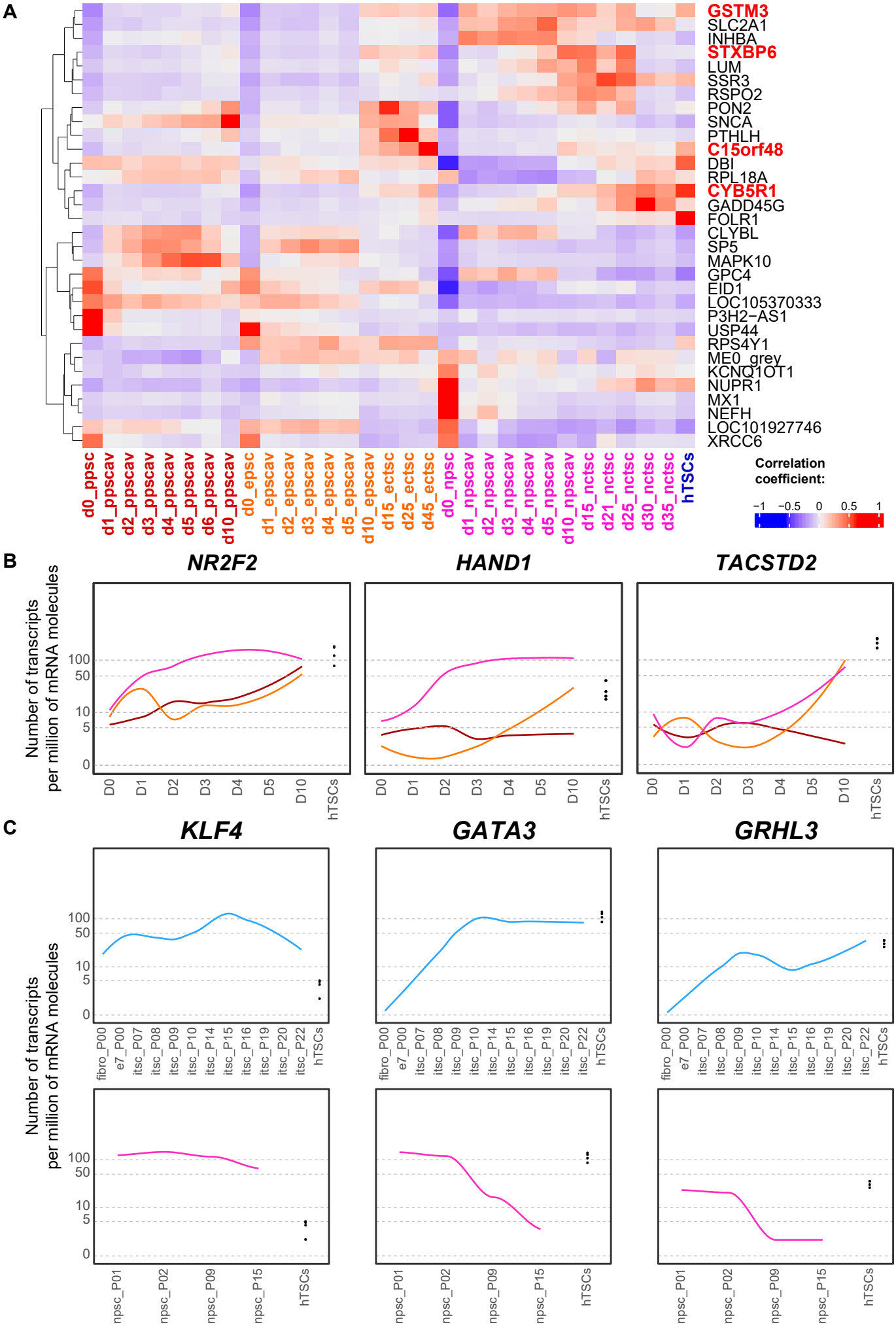

A
GSTM3
SLC2A1
INHBA
STXBP6
LUM
SSR3
RSPO2
PON2
SNCA
PTHLH
C15orf48
DBI
RPL18A
CYB5R1
GADD45G
FOLR1
CLYBL
SP5
MAPK10
GPC4
EID1
LOC105370333
P3H2−AS1
USP44
RPS4Y1
ME0_grey
KCNQ1OT1
NUPR1
MX1
NEFH
LOC101927746
XRCC6
d0_ppsc
d1_ppscav
d2_ppscav
d3_ppscav
d4_ppscav
d5_ppscav
d6_ppscav
d10_ppscav
d0_epsc
d1_epscav
d2_epscav
d3_epscav
d4_epscav
d5_epscav
d10_epscav
d15_ectsc
d25_ectsc
d45_ectsc
d0_npsc
d1_npscav
d2_npscav
d3_npscav
d4_npscav
d5_npscav
d10_npscav
d15_nctsc
d21_nctsc
d25_nctsc
d30_nctsc
d35_nctsc
hTSCs
Correlation coefficient:
−1 −0.5 0 0.5 1
B
NR2F2
HAND1
TACSTD2
Number of transcripts per million of mRNA molecules
100
50
10
5
0
D0 D1 D2 D3 D4 D5 D10 hTSCs
C
KLF4
GATA3
GRHL3
Number of transcripts per million of mRNA molecules
fibro_P00 e7_P00 itsc_P07 itsc_P08 itsc_P09 itsc_P10 itsc_P14 itsc_P15 itsc_P16 itsc_P19 itsc_P20 itsc_P22 hTSCs
npsc_P01 npsc_P02 npsc_P09 npsc_P15 hTSCs

Figure 13. Specific molecular mechanisms are critical for establishing hi/cTSCs.

(A) Heatmap of gene module correlation with conversion intermediates, showing different naive, extended, and primed outcomes. Samples are organised according to cell types, from left to right: primed (red), extended (orange), naive (pink) conversion intermediates.

(B) Expression levels (linear regression) of key genes during conversion of naive (pink), extended (orange), and primed (red) hiPSCs.

(C) Comparison of key gene expression in naive (pink) and hiTSC (blue) reprogramming intermediates.

Expression data: bulk RNA-Seq (DGE-Seq) ; unit: number of transcripts per million of mRNA molecules (UPM).

Figure 14: Combined NODAL repression and BMP signalling promote trophoblast fate specification.

hiTSC reprogramming
2iSAY reprogramming
Naive ASECRiAV
Naive 2iABY
Primed ASECRiAV
GATA3
TACSTD2
GRHL1
BMP4
MSX2
NODAL
LEFTY1
NANOG
Number of transcripts per million of mRNA molecules
1000
500
100
50
10
5
0
hFibro
E7 P0
hiTSC P8
hiTSC P9
hiTSC P10
hiTSC P14
hiTSC P16
hiTSC P22
hTSCs
hFibro
E7 P0
E7 P01
2iSAY P4
2iSAY P6
hTSCs
hiNPSCs
hiNPSC-cAV d1
hiNPSC-cAV d2
hiNPSC-cAV d3
hiNPSC-cAV d4
hiNPSC-cAV d5
hiNPSC-cAV d10
hTSCs
hiNPSCs
hiNPSC 2iABY d6
hiNPSC 2iABY d14
hiNPSC 2iABY d18
hTSCs
hiPSCs
hiPSC-cAV d1
hiPSC-cAV d2
hiPSC-cAV d3
hiPSC-cAV d4
hiPSC-cAV d5
hiPSC-cAV d10
hTSCs

Figure 14. Combined NODAL repression and BMP signalling promote trophoblast fate specification.

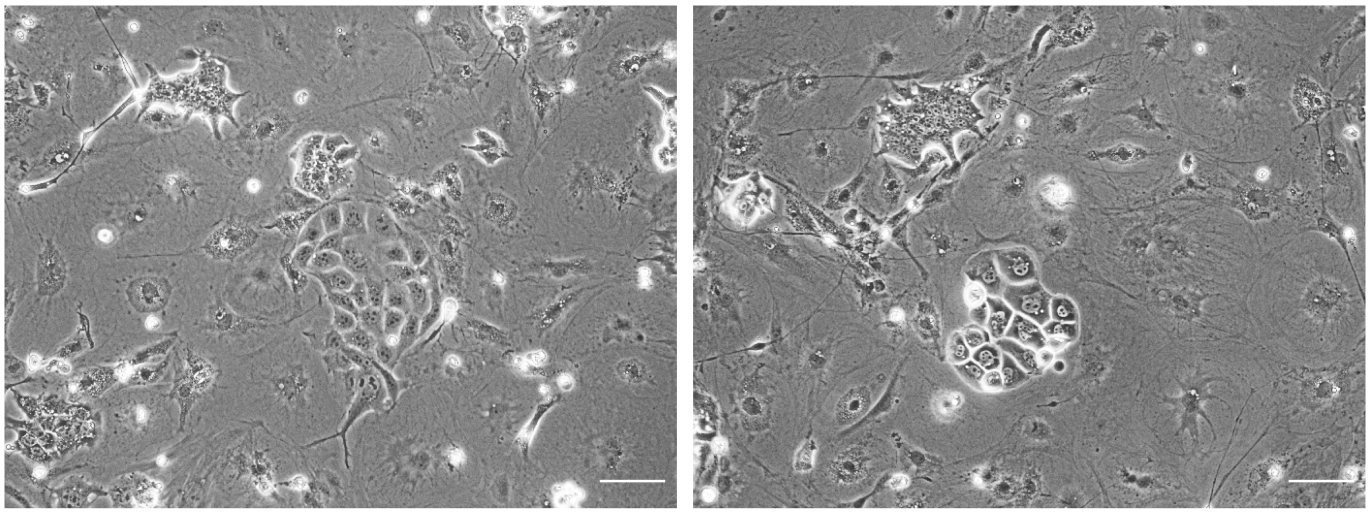
Plots showing regressed expression of lineage markers and genes of NODAL and BMP pathways, during naive and primed conversion, treatment of naive cells with 2iABY, and 2iSAY or hiTSC reprogramming. Only the first ten days of naive conversion are shown, to facilitate the comparison with primed conversion intermediates, that do not grow beyond this stage.

Expression data: bulk RNA-Seq (DGE-Seq) ; unit: number of transcripts per million of mRNA molecules (UPM).

Figure 15: Morphological changes associated with the acquisition of trophoblast fate upon NODAL repression and BMP stimulation, in naive hiPSCs and reprogramming intermediates.

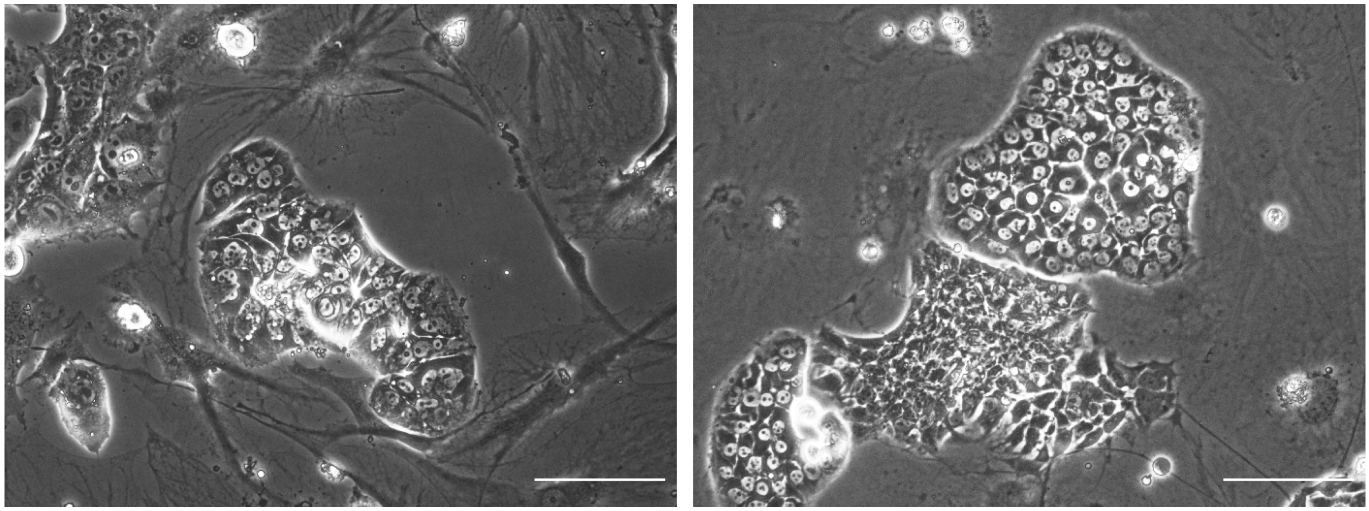
A **L8A2 naive hiPSCs treated with 2i + A83-01 + BMP4**

Day 4 Day 19



B **BJ1 2iSAY reprogramming**

Passage 2 Passage 9



C **L80 hiTESCs**

Passage 15 Passage 16

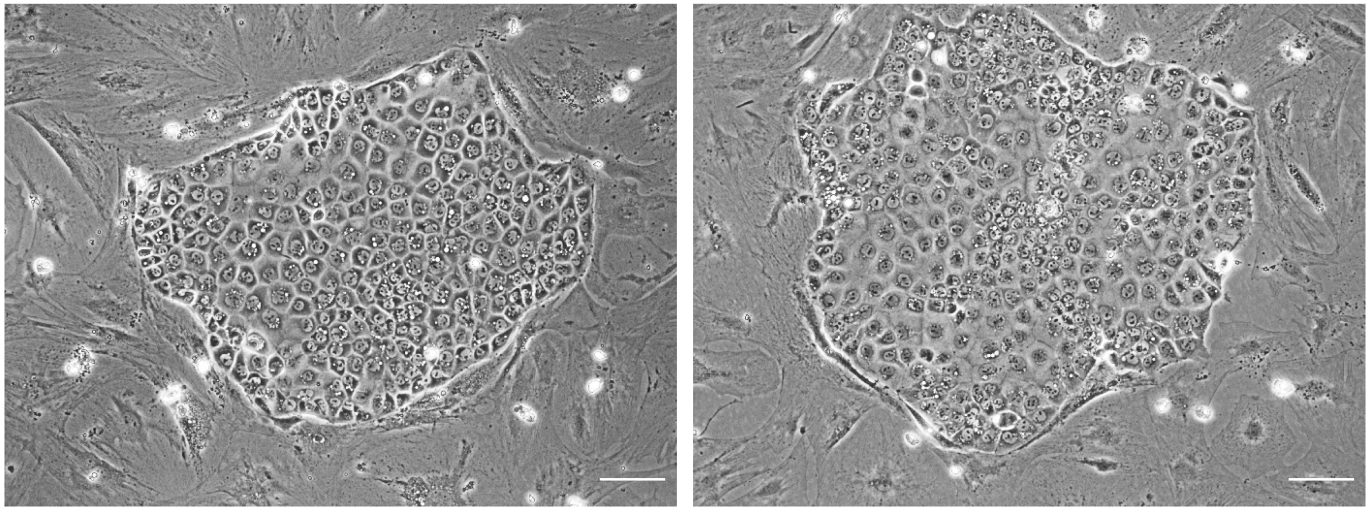


Figure 15. Morphological changes associated with the acquisition of trophoblast fate upon NODAL repression and BMP stimulation, in naive hiPSCs and reprogramming intermedi-ates.

(A) Bright field pictures showing the emergence of TE-like cells upon treatment of naive hiPSCs with 2i + A83-01 + BMP4 (+ ROCKi).

(B) Bright field images showing TE-like cell formation during reprogramming in 2iSAY medium.

(C) Bright field pictures of a presumptive hiTESC (induced trophectoderm stem cell) line, included as control of typical TE cellular phenotype (unpublished).

Scale bars: 100 μm.

Pathway signature analysis revealed many other signalling activities that were modulated upon trophoblast formation. Among these, we focused on the MTOR pathway, that showed lineage specific profiles. Moreover, MTOR is known to be pivotal for sensing and transducing a wide variety of signals, such as nutrients, amino acids or oxygen levels, coupling metabolism with gene expression, protein synthesis, proliferation and cellular growth, all processes supposedly involved in TE specification. Interestingly, we found that *DEPTOR*, an endogenous inhibitor of MTOR, was specifically expressed in naive hiPSCs, which was confirmed by immunostaining (**Figure 16A-B**). This gene is known for regulating MTOR activity in mESCs, maintaining the ground state stemness, while it is lost upon differentiation of these cells, a mechanism apparently conserved in humans (Agrawal *et al*, 2014 ; Bulut-Karslioglu *et al*, 2016). We thus investigated MTOR activity in embryonic and extra-embryonic lineages by Western Blot (WB) to different members of MTORC1/2 complexes and their targets (**Figure 16C**). While MTOR was phosphorylated in all cell types, we observed that P70S6K and AKT, direct targets of MTORC1, were phosphorylated only in pluripotent lineages, notably in naive hiPSCs, suggesting that the MTOR pathway is active in these cells despite *DEPTOR* expression, that likely results from a negative feedback loop. In line with this, the knock-down of *DEPTOR* by siRNA transfection over five days showed very little effects on lineage marker gene expression.

Although we cannot exclude that prolonged depletion of *DEPTOR* could produce more effects, these results suggest that *DEPTOR* is a good marker but not a driver of lineage specification in the embryo, and the regulation of its expression is not

sufficient to influence cell fate. However, the almost black-and-white patterns of MTOR activity between pluripotent and trophoblast stem cells reinforced our hypothesis that the regulation of this pathway could be involved in TE specification, possibly coupling the metabolic switch of the embryo to specific gene expression, and further investigation should be conducted to address this.

Finally, we were intrigued by the apparent correlation between the expression of *TACSTD2*, the so-called Tumor-associated calcium signal transducer 2, also known as *TROP2* (*Trophoblast cell surface antigen 2*), and the successful establishment and maintenance of h(i/c)TSCs. Of note, it has been shown that the loss of function of *TACSTD2* impairs epithelial barrier function, notably through altered expression and subcellular localisation of CLDN4, in gelatinous drop-Like corneal dystrophy (Nakatsukasa *et al*, 2010), processes that are supposedly involved in TE formation. We thus wondered whether calcium-mediated signalling could also contribute to TE specification. In line with this, we observed specific up-regulation of genes related to this pathway in hi/cTSCs, such as *CAST* and *CAPN2* (**Figure 17**), genes of the calpain/calpastatin system, that are critical for placental integrity and survival of the mouse embryo (Takano *et al*, 2011). We found these observations promising, and more experiments are to be conducted, such as Fura-2 assay, to determine the importance of calcium-mediated signalling in the establishment of hi/cTSCs.

Altogether, our results suggest that, besides canonical pathways like the YAP/TAZ mediated induction of *Cdx2* and *GATA3*, hTSC fate induction and

maintenance rely on specific combinations of signals, including NODAL inhibition, activation of the BMP4 pathway, MTOR modulation and calcium-mediated signalling, converging to establishing stable lines of h(i/c)TSC colonies.

Figure 16: Specific expression of DEPTOR in naive hiPSCs and the pre-implantation epiblast.

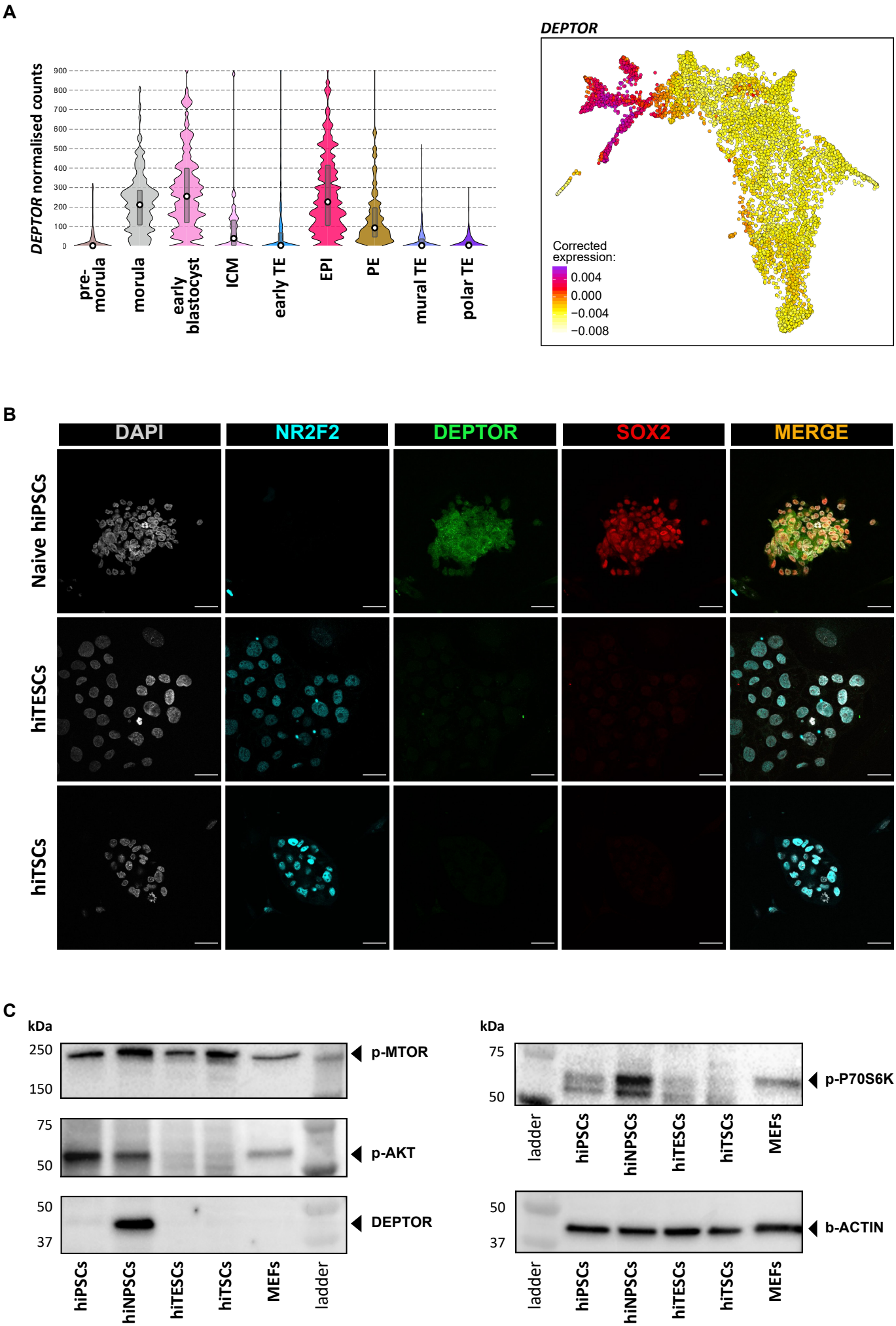

A
DEPTOR normalised counts
900
800
700
600
500
400
300
200
100
0
pre-morula
morula
early blastocyst
ICM
early TE
EPI
PE
mural TE
polar TE
DEPTOR
Corrected expression:
0.004
0.000
−0.004
−0.008
B
DAPI
NR2F2
DEPTOR
SOX2
MERGE
Naive hiPSCs
hiTESCs
hiTSCs
C
kDa
250
150
p-MTOR
75
50
p-AKT
50
37
DEPTOR
hiPSCs
hiNPSCs
hiTESCs
hiTSCs
MEFs
ladder
kDa
75
50
p-P70S6K
50
37
b-ACTIN
ladder
hiPSCs
hiNPSCs
hiTESCs
hiTSCs
MEFs

Figure 16. Specific expression of *DEPTOR* in naive hiPSCs and the pre-implantation epiblast.

(A) From left to right: violin plots of *DEPTOR* expression levels and frequency in cell types of the pre-implantation human embryo ; projection of *DEPTOR* expression onto the UMAP of human peri-implantation development. *DEPTOR* is induced at the morula stage and retained in EPI and PrE, but is immediately lost upon TE specification.

(B) Immunostaining to DEPTOR, NR2F2 (hTSC marker), and SOX2 (pluripotency marker) in naive hiPSCs, putative hiTESCs, and hiTSCs. DEPTOR is specifically found in naive hiPSCs and bright dots are visible, suggesting a localisation confined in subcellular compartments, possibly lysosomes within MTORC1/2 complexes. Scale bars: 30µm.

(C) Western blots to DEPTOR and other actors of the MTOR pathway, showing their phosphorylation status. Beta-actin (housekeeping gene) is used as a loading control.

Figure 17: Calcium-mediated signalling could be involved in hi/cTSC establishment and maintenance.

Naive reprogramming
hiTSC reprogramming
Naive conversion
Extended conversion
TACSTD2
CLDN4
CAPN2
CAST
Number of transcripts per million of mRNA molecules
1000
500
100
50
10
5
0
hFibro
E7 P0
hiNPSC P7
hiNPSC P8
hiNPSC P9
hiNPSC P11
hiNPSC P15
hiNPSC P18
hiNPSC P19
hiNPSC P20
hiNPSC P26
hTSCs
hFibro
E7 P0
hiTSC P8
hiTSC P9
hiTSC P10
hiTSC P14
hiTSC P16
hiTSC P22
hTSCs
hiNPSCs
hiNPSC-cAV d1
hiNPSC-cAV d2
hiNPSC-cAV d3
hiNPSC-cAV d4
hiNPSC-cAV d5
hiNPSC-cAV d10
hNcTSC d15
hNcTSC d21
hNcTSC d25
hTSCs
hiEPSs
hiEPS-cAV d1
hiEPS-cAV d2
hiEPS-cAV d3
hiEPS-cAV d4
hiEPS-cAV d5
hiEPS-cAV d10
hEcTSC d15
hEcTSC d45
hTSCs

Figure 17. Calcium-mediated signalling could be involved in hi/cTSC establishment and maintenance.

Regressed expression plots of calcium-signalling associated genes during naive hiPSC and hiTSC reprogramming, and during naive or extended hiPSC conversion into hcTSCs.

Expression data: bulk RNA-Seq (DGE-Seq) ; unit: number of transcripts per million of mRNA molecules (UPM).

3.5 hi/cTSCs and the human embryo

Developmental staging of hi/cTSCs

Even though primary hTSCs can be derived from pre-implantation blastocysts, these cells, similar to their counterparts in chorionic villi, resemble post-implantation trophoblasts, and global gene expression is clearly distinct from that of TE cells, either mural or polar. Conventional hTSCs are thus useful to study post-implantation events, but they are supposedly limited regarding early development.

Significant efforts are made currently to derive human trophectoderm stem cells (hTESCs) corresponding to the pre-implantation TE. Mischler *et al* claim to have obtained such cells after hESC differentiation, first with BMP4, followed by a transition into a culture medium called TM4 (mTeSR E6 supplemented with CYM5541, A83-01, FGF10, and CHIR99021) (Mischler *et al*, 2021). Notably, they have used CYM5541, an agonist of S1PR3 that promotes YAP signalling. They have generated CDX2+ epithelioid colonies expressing TE related genes, such as *GATA3*, *KRT7*, *Tead4* and *Tfap2c*, after silencing the pluripotency core network. After transiting into the conventional hTSC medium, cells could differentiate into ST and EVT. Although promising, these putative hTESCs should be characterised further, with transcriptome-wide analyses and comparison to the human embryo. From our observations in the peri-implantation embryo and one in-house presumptive hiTESC line, we consider that such cells should additionally express specific markers, including

CD53 and *ODAM*.

The protocol of Guo *et al*, reporting treatment of naive hESCs with A83-01 and PD0325901 (MEK inhibitor), produced cells that were similar to the pre-implantation TE, on the scale of global gene expression (Guo *et al*, 2021). However, these cells did not proliferate and could not be maintained more than a few days.

Although more research is needed before we can establish *bona fide* hTESC lines, recent studies on cell fate plasticity, including our own, have contributed to the development of the first human blastoids, a stem cell-based model of the early embryo.

Blastoids or the pristine model of the human embryo

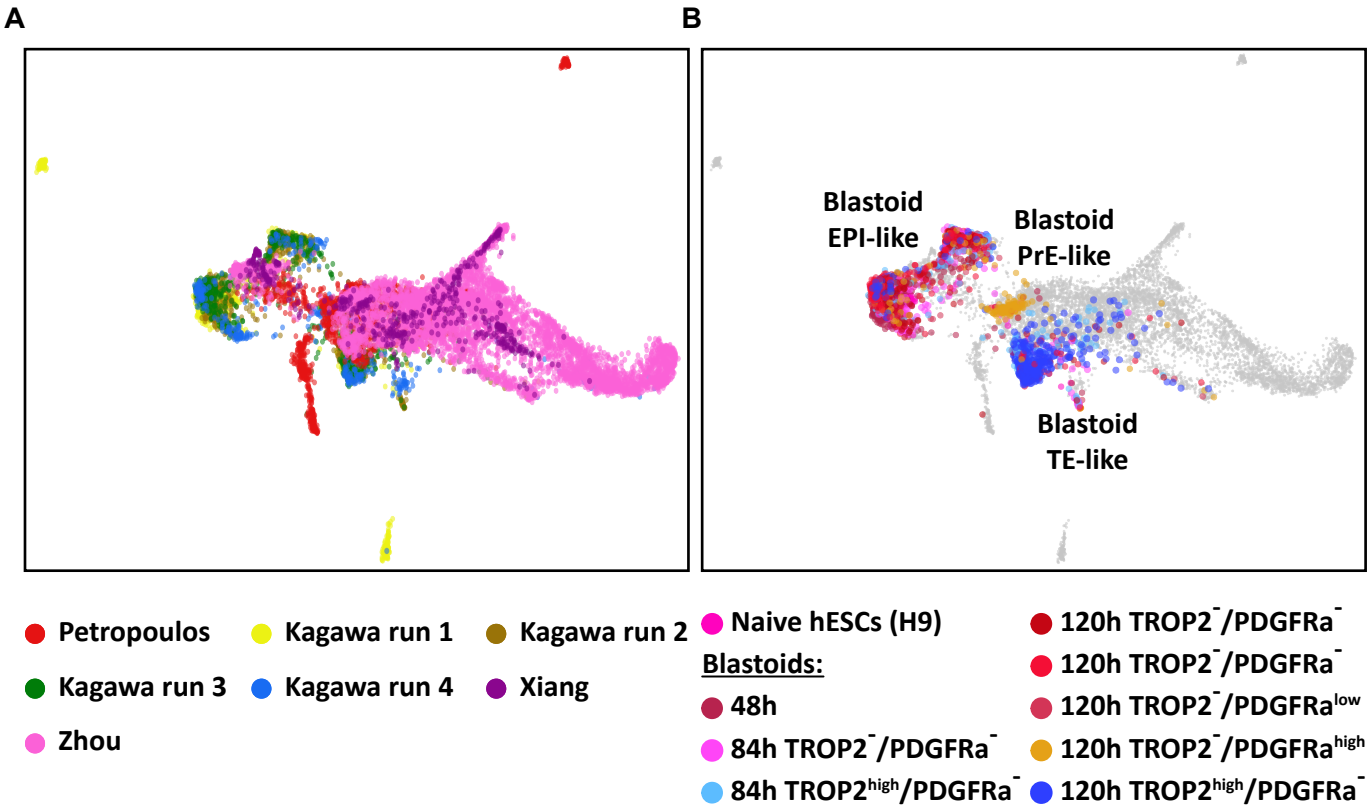
Human blastoids are 3D structures, mimicking the embryo, which are derived from naive or extended hPSCs. Under defined culture conditions in microwells, they can self-assemble, adopting a blastocyst-like conformation and forming ICM- and TE-analogs.

To confirm this observation, we performed unsupervised transcriptomic analyses of single-cell RNA-Seq data of blastoid and embryo cells. Among others, a UMAP analysis showed that naive cells and their derivatives within blastoids are equivalent to peri-implantation embryo cells, forming analogs of EPI, PE, and TE lineages (**Figure 18**).

Importantly, blastoids have been carefully benchmarked against the peri-implantation human embryo (Kagawa *et al*, 2021 ; Liu *et al*, 2021 ; Sozen *et al*, 2021 ; Yanagida *et al*, 2021 ; Yu *et al*, 2021). It has been evidenced that they recapitulate morphokinetic and molecular events of early development, comprising: cavitation, blastocoel expansion, gene expression patterning and lineage allocation (EPI, PE and TE). Notably, the formation of the proliferative, NR2F2+ polar TE is conserved, lining the ICM-analog. Not only do blastoids mirror the embryo structurally, but they also ensure key functions, such as pseudo-implantation onto endometrial cells (stimulated with hormones), at the embryonic-like pole.

Hereafter, this model is going to unleash the production of embryo-like structures, with stable genetic backgrounds, increasing experimental reproducibility. This opens avenues to unlimited, large-scale assays, such as genetic manipulation to validate molecular mechanisms of early development. Finally, blastoids provide an ethically safe alternative to the use of human embryos for research.

Figure 18: Cell fate plasticity of naive hPSCs is the root of human blastoid formation.


A
B
Blastoid
EPI-like
Blastoid
PrE-like
Blastoid
TE-like
Petropoulos
Kagawa run 1
Kagawa run 2
Kagawa run 3
Kagawa run 4
Xiang
Zhou
Naive hESCs (H9)
Blastoids:
48h
84h TROP2-/PDGFRa-
84h TROP2high/PDGFRa-
120h TROP2-/PDGFRa-
120h TROP2-/PDGFRa-
120h TROP2-/PDGFRalow
120h TROP2-/PDGFRahigh
120h TROP2high/PDGFRa-

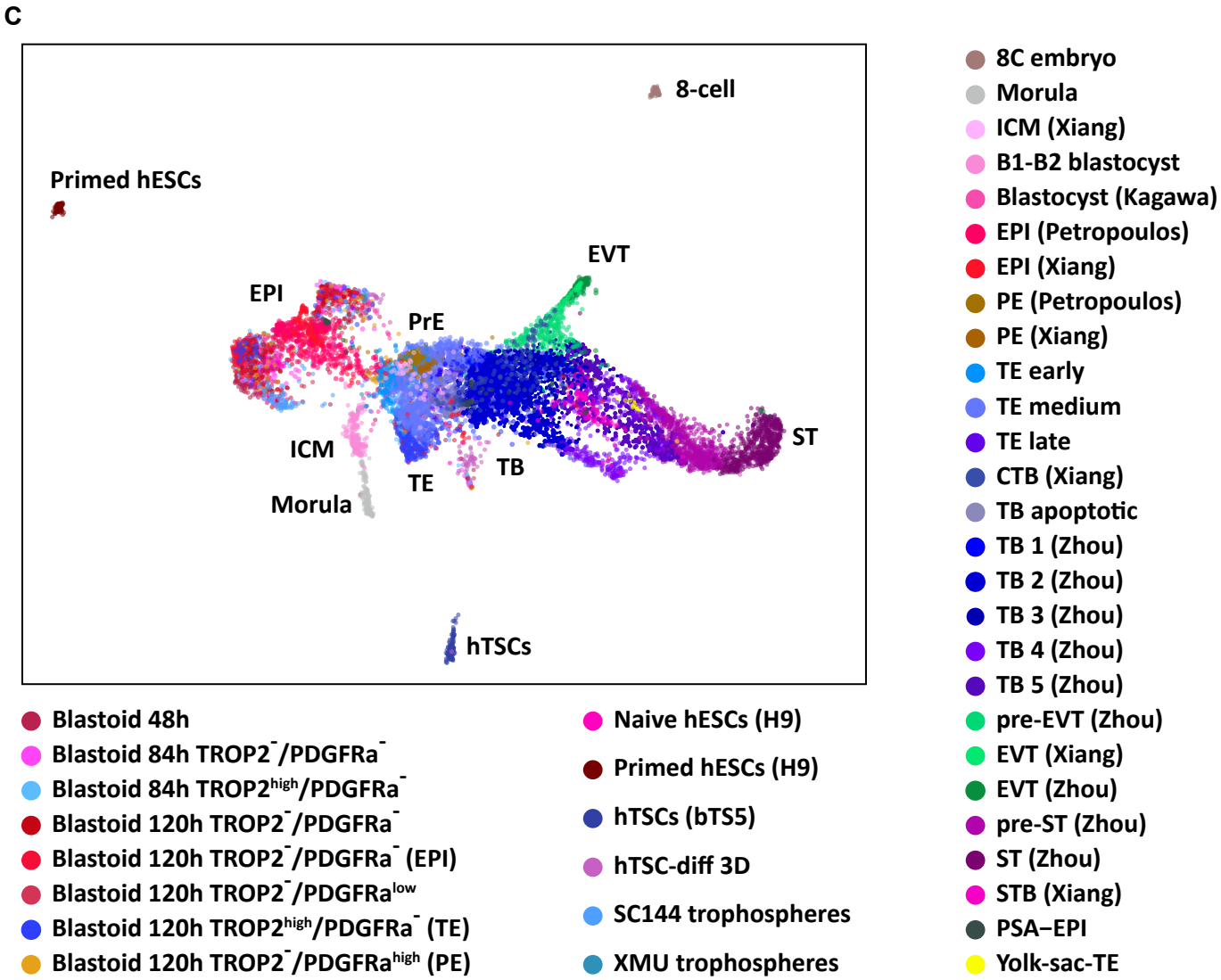

C
8-cell
Primed hESCs
EVT
EPI
PrE
ICM
TE
TB
ST
Morula
hTSCs
Blastoid 48h
Blastoid 84h TROP2-/PDGFRa-
Blastoid 84h TROP2high/PDGFRa-
Blastoid 120h TROP2-/PDGFRa-
Blastoid 120h TROP2-/PDGFRa- (EPI)
Blastoid 120h TROP2-/PDGFRalow
Blastoid 120h TROP2high/PDGFRa- (TE)
Blastoid 120h TROP2-/PDGFRahigh (PE)
Naive hESCs (H9)
Primed hESCs (H9)
hTSCs (bTS5)
hTSC-diff 3D
SC144 trophospheres
XMU trophospheres
8C embryo
Morula
ICM (Xiang)
B1-B2 blastocyst
Blastocyst (Kagawa)
EPI (Petropoulos)
EPI (Xiang)
PE (Petropoulos)
PE (Xiang)
TE early
TE medium
TE late
CTB (Xiang)
TB apoptotic
TB 1 (Zhou)
TB 2 (Zhou)
TB 3 (Zhou)
TB 4 (Zhou)
TB 5 (Zhou)
pre-EVT (Zhou)
EVT (Xiang)
EVT (Zhou)
pre-ST (Zhou)
ST (Zhou)
STB (Xiang)
PSA–EPI
Yolk-sac-TE

Figure 18. Cell fate plasticity of naive hPSCs is the root of human blastoid formation.

(A) UMAP of merged single-cell RNA-Seq data including human blastoids, peri-implantation embryos and cellular models. The different runs of origin are highlighted, that show efficient merging after batch correction with fastMNN (Mutual Nearest Neighbours).

(B) Human blastoid cells are highlighted on the UMAP, showing the spontaneous formation of EPI-, PE-, and TE-analogues.

(C) Projection on the UMAP of cell type annotation. Abbreviations: 8C: 8-cell stage embryo ; ICM: Inner Cell Mass ; EPI: Epiblast ; PE: Primitive Endoderm ; TE: Trophectoderm ; CTB: Cytotrophoblast ; TB: Trophoblast ; EVT: Extravillous trophoblast ; ST/STB: Syncytiotrophoblast ; PSA-EPI: Primitive streak anlage epiblast (Xiang) ; Yolk-sac-TE: Yolk-sac-trophectoderm (Zhou) ; SC144 trophospheres: blastoids treated with SC144, an inhibitor of GP130 (IL6 receptor), forming trophospheres devoid of EPI-analogue ; XMU trophospheres: blastoids treated with XMU-MP-1, an inhibitor of the Hippo kinases MST1/2, also forming trophospheres devoid of EPI-analogue.

4 PERSPECTIVES

The human embryo is fascinating, carrying such a high potential, able to produce a complete, new living organism. Critical processes occur during the embryonic development, such as the formation of the germline or the patterning of the body, among many others. They exhibit an extremely high reproducibility, and yet some tiny differences distinguish between individuals, making everyone unique. In itself, understanding how these mechanisms interact together, how they build tissues and organs, is worth dedicating a life to research. However, the human embryo remains poorly understood, and more than ever, we need alternative models to investigate it further.

In this regard, our work represents both a conceptual advance and a technical progress, demonstrating the biological and technical feasibility of somatic-to-extraembryonic cell reprogramming. This strategy now enables the generation of induced trophoblast stem cell lines from patients, notably useful to investigate placental dysfunctions. Moreover, our study sheds new light onto cell fate plasticity between embryonic and extra-embryonic lineages, and onto the potential of naive and extended pluripotent stem cells to produce hTSCs. It also provides further insight into the biology of human trophoblast stem cells, their needs, and mechanisms of stemness, regulating the fine-tuned balance between self-renewal and differentiation.

This thesis was the occasion to recapitulate our main results and go further

into detailed analyses that could not be developed elsewhere. We discussed the cellular heterogeneity observed among hi/cTSCs, and proposed that trophoblast stemness might be "plural", capturing diverse developmental stages of lineage progression, mirroring in this regard the spectrum of pluripotency. We could also explore in-depth molecular mechanisms underlying hiTSC reprogramming and conversion. Our results suggest that trophoblast stem cell specification relies on three main parameters: the repression of pluripotency gene circuitry and related pathways, such as NODAL signalling ; the induction of trophoblast programs, centred on core transcription factors (GATA3, NR2F2, TFAP2C, GHRL1/2/3), and stem cell markers (TACSTD2, PEG10) ; and the completion of epithelialisation, required for establishing hTSC colonies (induction of KRT genes, stable intercellular junctions). We found that such processes could be controlled by key transcription factors, comprising KLF4, GATA3 and GRHL3.

Moreover, we carefully benchmarked h(i/c)TSCs against the human embryo (on the scale of global gene expression) and showed these cells correspond to post-implantation proliferative trophoblasts (TBs). Our results participate in clarifying the assets and limitations of this model for investigating stage-specific developmental processes. They point out the need for alternatives, such as trophectoderm stem cells (hTESCs), in order to study the first lineage specification in human. In this regard, the recent development of human blastoids, to which our results on cell fate plasticity have contributed, is promising.

Finally, we tried to put things into perspective, taking a step back to consider

these results more widely, on the scale of mammalian evolution. Comparison with other species, notably the mouse, has revealed both conserved and divergent mechanisms governing placental development. In our opinion, the reflection on fundamental biology is complementary to clinical research, as science and society need both for progressing.

In conclusion, we hope that hi/cTSCs from patients will be useful to investigate molecular mechanisms of normal *vs* pathological development of the human placenta. Of course, further improvements are needed for using these cells to model complex placental structures *in vitro*, but we have confidence that they will bring progress to basic research, IVF techniques, and treatments of placental diseases.

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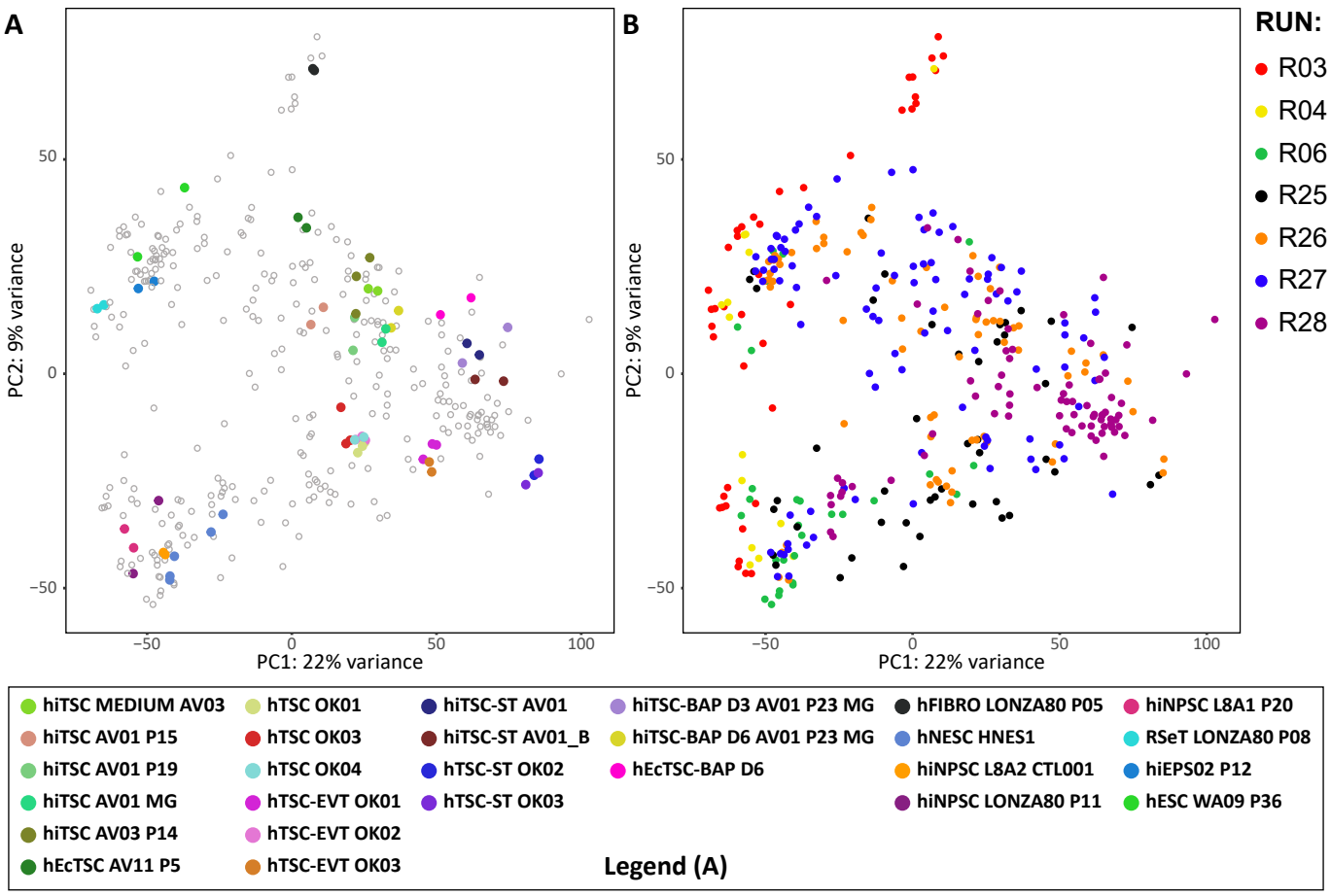
1 SUPPLEMENTAL INFORMATION

Supplementary Figure 1: S1 - related to Figure 9. Principal Component Analysis of merged DGE-Seq datasets.


A
B
RUN:
R03
R04
R06
R25
R26
R27
R28
PC2: 9% variance
PC1: 22% variance
50
0
−50
−50
0
50
100
hiTSC MEDIUM AV03
hiTSC AV01 P15
hiTSC AV01 P19
hiTSC AV01 MG
hiTSC AV03 P14
hEcTSC AV11 P5
hTSC OK01
hTSC OK03
hTSC OK04
hTSC-EVT OK01
hTSC-EVT OK02
hTSC-EVT OK03
hiTSC-ST AV01
hiTSC-ST AV01_B
hTSC-ST OK02
hTSC-ST OK03
hiTSC-BAP D3 AV01 P23 MG
hiTSC-BAP D6 AV01 P23 MG
hEcTSC-BAP D6
hFIBRO LONZA80 P05
hNESC HNES1
hiNPSC L8A2 CTL001
hiNPSC LONZA80 P11
hiNPSC L8A1 P20
RSeT LONZA80 P08
hiEPS02 P12
hESC WA09 P36
Legend (A)

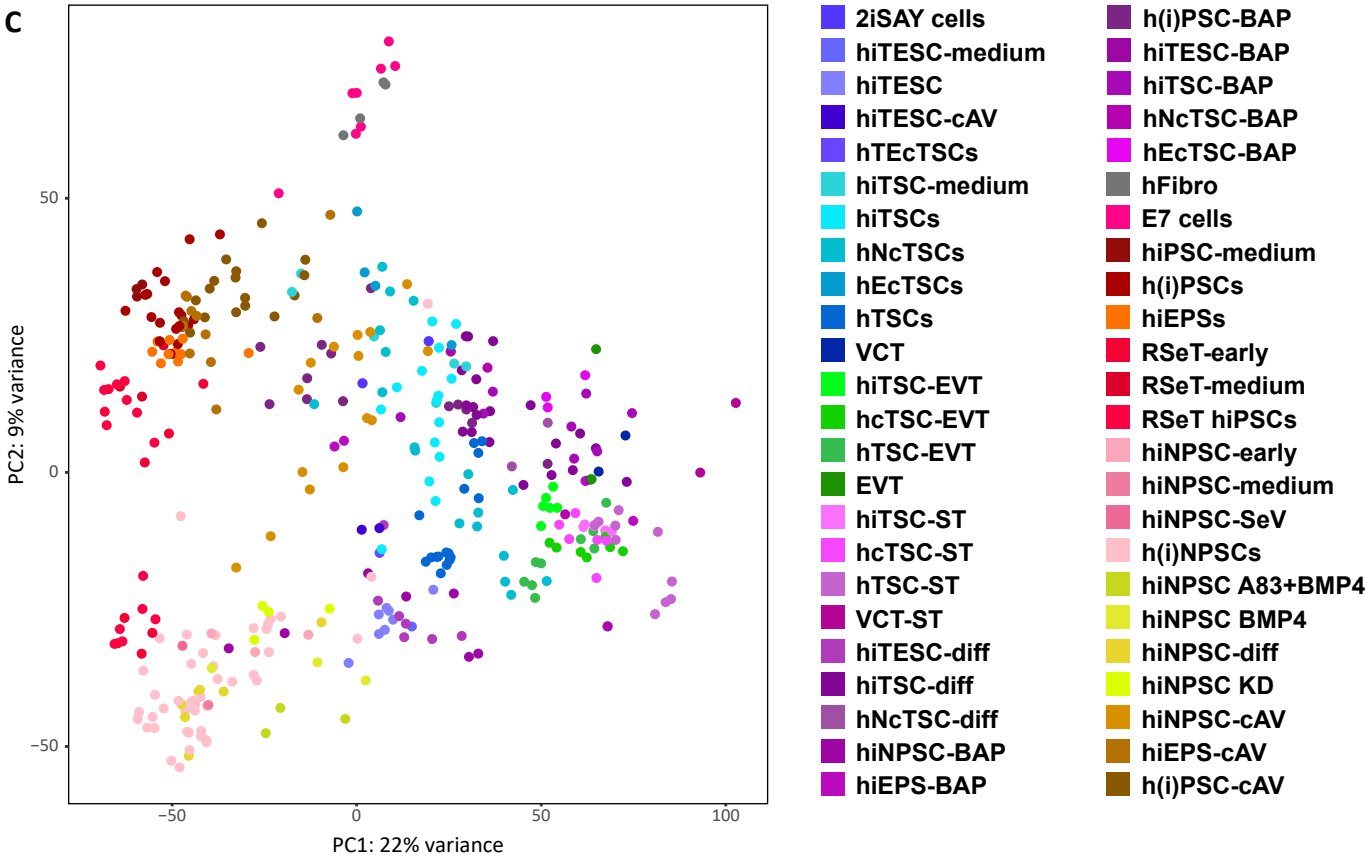

C
PC2: 9% variance
PC1: 22% variance
50
0
−50
−50
0
50
100
2iSAY cells
hiTESC-medium
hiTESC
hiTESC-cAV
hTEcTSCs
hiTSC-medium
hiTSCs
hNcTSCs
hEcTSCs
hTSCs
VCT
hiTSC-EVT
hcTSC-EVT
hTSC-EVT
EVT
hiTSC-ST
hcTSC-ST
hTSC-ST
VCT-ST
hiTESC-diff
hiTSC-diff
hNcTSC-diff
hiNPSC-BAP
hiEPS-BAP
h(i)PSC-BAP
hiTESC-BAP
hiTSC-BAP
hNcTSC-BAP
hEcTSC-BAP
hFibro
E7 cells
hiPSC-medium
h(i)PSCs
hiEPSs
RSeT-early
RSeT-medium
RSeT hiPSCs
hiNPSC-early
hiNPSC-medium
hiNPSC-SeV
h(i)NPSCs
hiNPSC A83+BMP4
hiNPSC BMP4
hiNPSC-diff
hiNPSC KD
hiNPSC-cAV
hiEPS-cAV
h(i)PSC-cAV

Figure S1 - related to Figure 9. Principal Component Analysis of merged DGE-Seq datasets.

PCA of merged datasets for controlling the batch correction performed with ComBat algorithm: (A) technical replicates, highlighted with colours, are closely distributed within the reduced space, after batch correction ; (B) the different DGE-Seq runs, with partly overlapping biological diversity of samples, are highlighted with colours and are largely interspersed ; (C) the various cell lineages, consistently distributed, are highlighted according to the following colour code:

- **2iSAY cells**: hiTSC-like cells (unpublished)
- **hiTESC-medium**: hiTESC reprogramming intermediates
- **hiTESC**: human induced trophectoderm stem-like cells (unpublished)
- **hiTESC-cAV**: intermediates of hiTESC conversion into hTSCs
- **hTEcTSCs**: hTSCs derived from human induced trophectoderm stem-like cells
- **hiTSC-medium**: hiTSC reprogramming intermediates
- **hiTSCs**: human induced trophoblast stem cells
- **hNcTSCs**: converted human trophoblast stem cells derived from naive hiPSCs
- **hEcTSCs**: converted human trophoblast stem cells derived from extended hiPSCs
- **hTSCs**: primary hTSCs derived from human pre-implantation blastocysts and first-trimester placentas
- **VCT**: villous cytotrophoblasts derived from term human placentas
- **hiTSC-EVT**: extravillous trophoblast-like cells derived from hiTSCs
- **hcTSC-EVT**: extravillous trophoblasts derived from converted hTSCs
- **hTSC-EVT**: extravillous trophoblasts derived from primary hTSCs differentiated in vitro
- **EVT**: extravillous trophoblasts derived from term human placentas
- **hiTSC-ST**: syncytiotrophoblast derived from hiTSCs
- **hcTSC-ST**: syncytiotrophoblast derived from converted hTSCs
- **hTSC-ST**: syncytiotrophoblast derived from primary hTSCs differentiated in vitro
- **VCT-ST**: syncytiotrophoblast derived from primary VCTs differentiated in vitro
- **hiTESC-diff**: mixture of mature and differentiated trophoblasts derived from hiTESCs
- **hiTSC-diff**: mixture of mature and differentiated trophoblasts derived from hiTSCs
- **hNcTSC-diff**: mixture of mature and differentiated trophoblasts derived from hNcTSCs
- **hiNPSC-BAP**: hiNPSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hiEPS-BAP**: hiEPSs treated with BMP4 + A83-01 + PD173074 (BAP)
- **h(i)PSC-BAP**: h(i)PSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hiTESC-BAP**: human induced trophectoderm stem-like cells treated with BMP4 + A83-01 + PD173074 (BAP)
- **hiTSC-BAP**: hiTSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hNcTSC-BAP**: hNcTSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hEcTSC-BAP**: hEcTSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hFibro**: human fibroblasts prior to reprogramming
- **E7 cells**: early reprogramming intermediates cultured in mTeSR E7 medium
- **hiPSC-medium**: primed reprogramming intermediates
- **h(i)PSCs**: human (induced or embryonic) primed pluripotent stem cells
- **hiEPSs**: human induced extended pluripotent stem cells
- **RSeT-early**: early reprogramming intermediates cultured in RSeT medium
- **RSeT-medium**: reprogramming intermediates cultured in RSeT medium
- **RSeT hiPSCs**: hiPSCs cultured in RSeT, intermediate state between naive and primed pluripotency
- **hiNPSC-early**: hiNPSC early reprogramming intermediates
- **hiNPSC-medium**: hiNPSC reprogramming intermediates
- **hiNPSC-SeV**: hiNPSC-like cells showing persistence of SeV particles (OSKM transgenes)
- **h(i)NPSCs**: human (induced or embryonic) naive pluripotent stem cells
- **hiNPSC A83+BMP4**: hiNPSCs treated with A83-01 + BMP4
- **hiNPSC BMP4**: hiNPSCs treated with BMP4
- **hiNPSC-diff**: hiNPSCs submitted to differentiation assays (LDN, SB431542, Activin A...), not covered by this study
- **hiNPSC KD**: hiNPSCs involved in DEPTOR KD assay (siRNA anti-DEPTOR and scramble)
- **hiNPSC-cAV**: intermediates of hiNPSC conversion into hTSCs
- **hiEPS-cAV**: intermediates of extended conversion into hEcTSCs
- **h(i)PSC-cAV**: intermediates of primed conversion into trophoblast-like cells

Supplementary Figure 2: S2 - related to Figure 8. Pearson correlation heatmap of merged DGE-Seq datasets, including all samples.

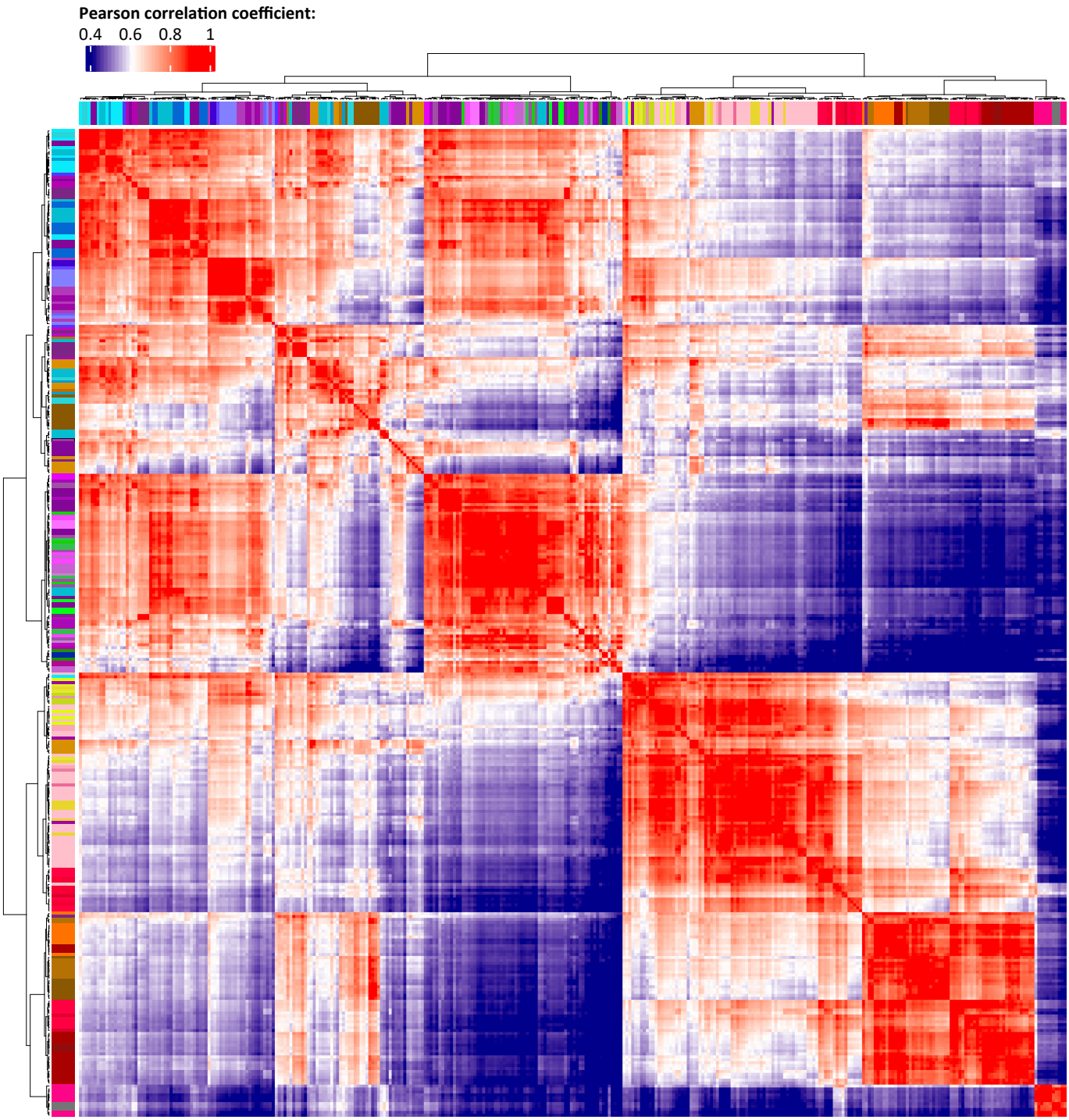

Pearson correlation coefficient:
0.4 0.6 0.8 1

Figure S2 - related to Figure 8. Pearson correlation heatmap of merged DGE-Seq datasets, including all samples.

Pearson correlation heatmap of all merged samples. Hierarchical clustering recapitulates cell types, the two main branches separating extra-embryonic (left) and embryonic (right) lineages. The colour code below corresponds to the cell type annotation on top of the heatmap:

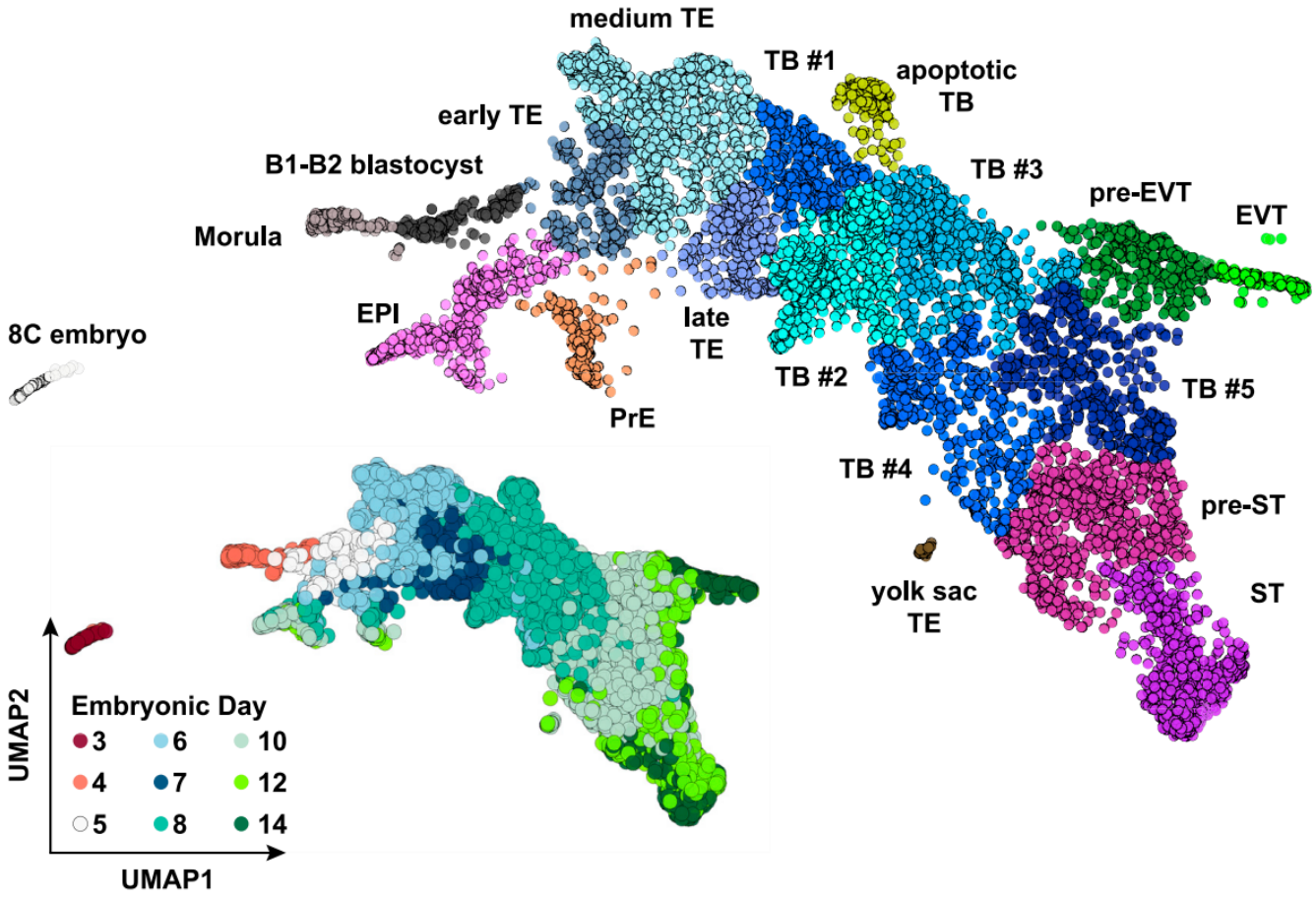
- **2iSAY cells**: hiTSC-like cells (unpublished)
- **hiTESC-medium**: hiTESC reprogramming intermediates
- **hiTESC**: human induced trophectoderm stem-like cells (unpublished)
- **hiTESC-cAV**: intermediates of hiTESC conversion into hTSCs
- **hTEcTSCs**: hTSCs derived from human induced trophectoderm stem-like cells
- **hiTSC-medium**: hiTSC reprogramming intermediates
- **hiTSCs**: human induced trophoblast stem cells
- **hNcTSCs**: converted human trophoblast stem cells derived from naive hiPSCs
- **hEcTSCs**: converted human trophoblast stem cells derived from extended hiPSCs
- **hTSCs**: primary hTSCs derived from human pre-implantation blastocysts and first-trimester placentas
- **VCT**: villous cytotrophoblasts derived from term human placentas
- **hiTSC-EVT**: extravillous trophoblast-like cells derived from hiTSCs
- **hcTSC-EVT**: extravillous trophoblasts derived from converted hTSCs
- **hTSC-EVT**: extravillous trophoblasts derived from primary hTSCs differentiated in vitro
- **EVT**: extravillous trophoblasts derived from term human placentas
- **hiTSC-ST**: syncytiotrophoblast derived from hiTSCs
- **hcTSC-ST**: syncytiotrophoblast derived from converted hTSCs
- **hTSC-ST**: syncytiotrophoblast derived from primary hTSCs differentiated in vitro
- **VCT-ST**: syncytiotrophoblast derived from primary VCTs differentiated in vitro
- **hiTESC-diff**: mixture of mature and differentiated trophoblasts derived from hiTESCs
- **hiTSC-diff**: mixture of mature and differentiated trophoblasts derived from hiTSCs
- **hNcTSC-diff**: mixture of mature and differentiated trophoblasts derived from hNcTSCs
- **hTSC-diff**: mixture of mature and differentiated trophoblasts derived from primary hTSCs
- **hiNPSC-BAP**: hiNPSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hiEPS-BAP**: hiEPSs treated with BMP4 + A83-01 + PD173074 (BAP)
- **h(i)PSC-BAP**: h(i)PSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hiTESC-BAP**: human induced trophectoderm stem-like cells treated with BMP4 + A83-01 + PD173074 (BAP)
- **hiTSC-BAP**: hiTSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hNcTSC-BAP**: hNcTSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hEcTSC-BAP**: hEcTSCs treated with BMP4 + A83-01 + PD173074 (BAP)
- **hFibro**: human fibroblasts prior to reprogramming
- **E7 cells**: early reprogramming intermediates cultured in mTeSR E7 medium
- **hiPSC-medium**: primed reprogramming intermediates
- **h(i)PSCs**: human (induced or embryonic) primed pluripotent stem cells
- **hiEPSs**: human induced extended pluripotent stem cells
- **RSeT-early**: early reprogramming intermediates cultured in RSeT medium
- **RSeT-medium**: reprogramming intermediates cultured in RSeT medium
- **RSeT hiPSCs**: hiPSCs cultured in RSeT, intermediate state between naive and primed pluripotency
- **hiNPSC-early**: hiNPSC early reprogramming intermediates
- **hiNPSC-medium**: hiNPSC reprogramming intermediates
- **hiNPSC-SeV**: hiNPSC-like cells showing persistence of SeV particles (OSKM transgenes)
- **hiNPSC-ery**: hiNPSCs derived from erythroblasts
- **h(i)NPSCs**: human (induced or embryonic) naive pluripotent stem cells
- **hiNPSC A83+BMP4**: hiNPSCs treated with A83-01 + BMP4
- **hiNPSC BMP4**: hiNPSCs treated with BMP4
- **hiNPSC-diff**: hiNPSCs submitted to differentiation assays (LDN, SB431542, Activin A...), not covered by this study
- **hiNPSC KD**: hiNPSCs involved in DEPTOR KD assay (siRNA anti-DEPTOR and scramble)
- **hiNPSC-cAV**: intermediates of hiNPSC conversion into hTSCs
- **hiEPS-cAV**: intermediates of extended conversion into hEcTSCs
- **h(i)PSC-cAV**: intermediates of primed conversion into trophoblast-like cells

2 ANNEXE

Annexe 1. Projection of gene expression onto the UMAP of human peri-implantation development.

The first panel indicates developmental stage and lineage allocation of individual cells from human embryos, cultured *in vitro* until day 14, that were analysed by single-cell RNA-Seq and used to compute the UMAP.

Next panels show the gene expression of key lineage markers projected onto the UMAP.


medium TE
TB #1
apoptotic TB
early TE
B1-B2 blastocyst
TB #3
pre-EVT
EVT
Morula
EPI
late TE
8C embryo
TB #2
TB #5
PrE
TB #4
pre-ST
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Embryonic Day
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5
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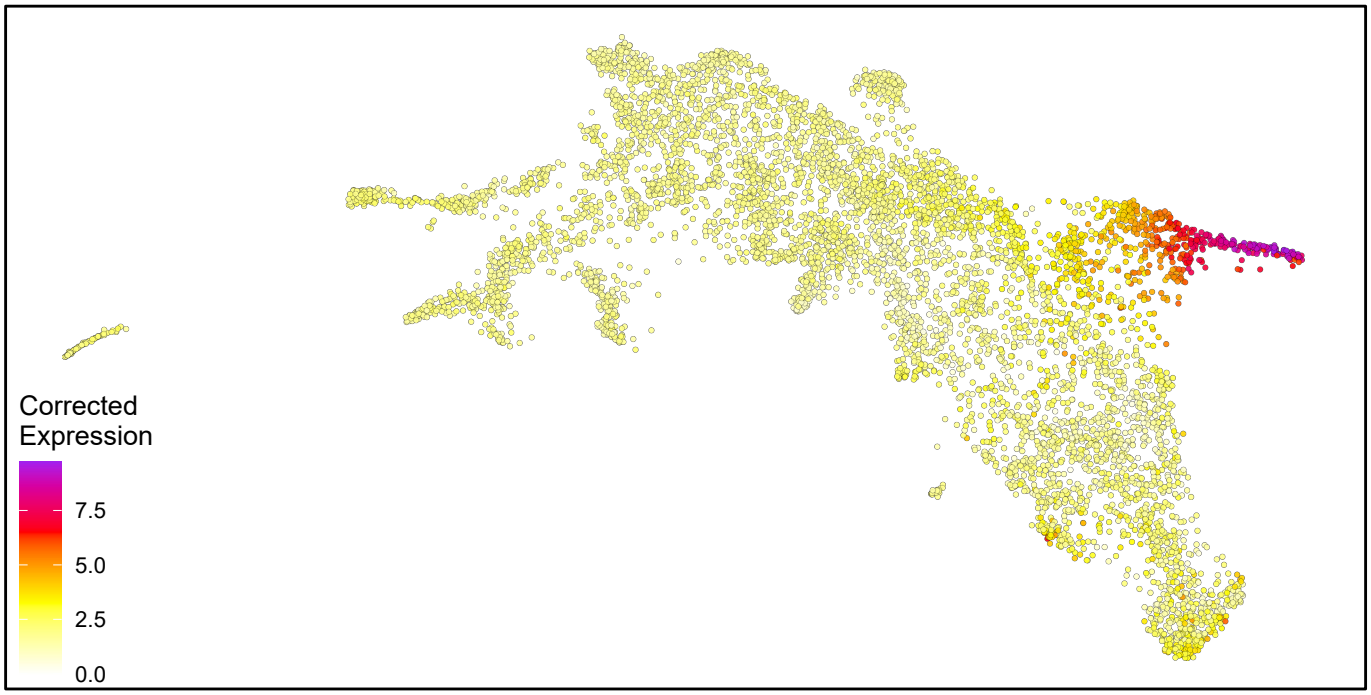
7
8
10
12
14
UMAP2
UMAP1

ASCL2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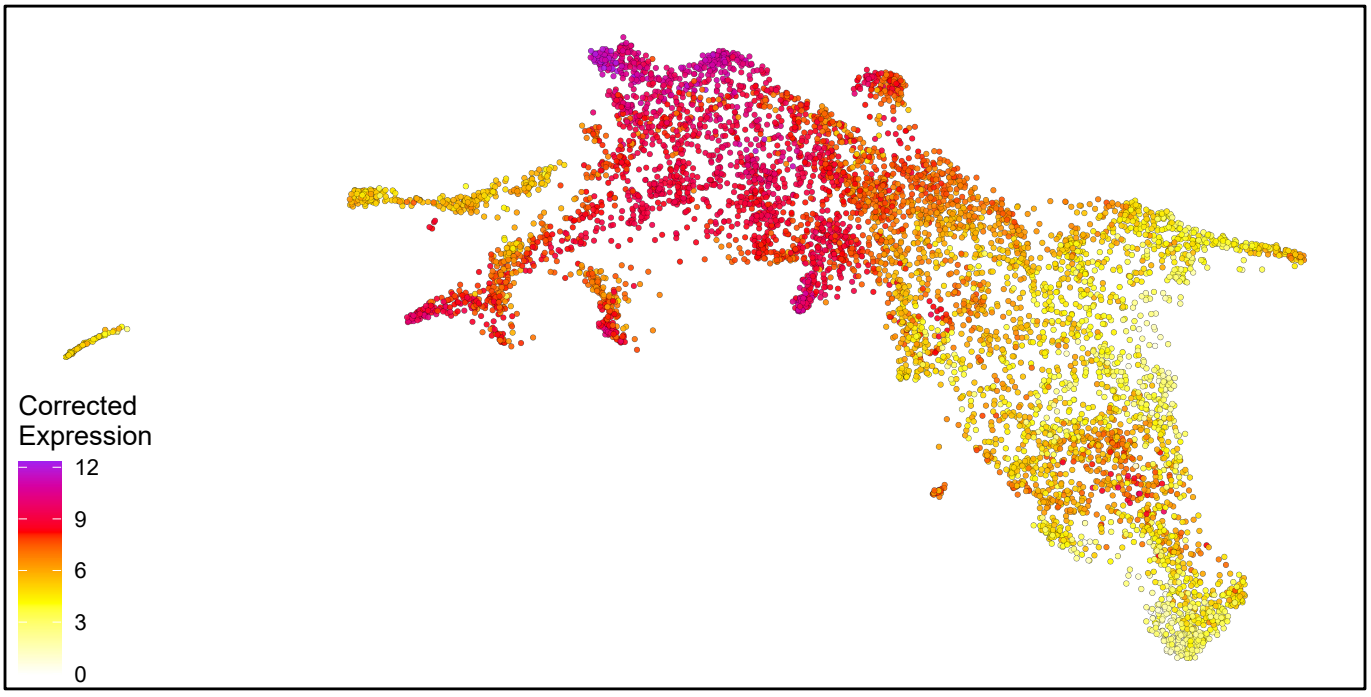
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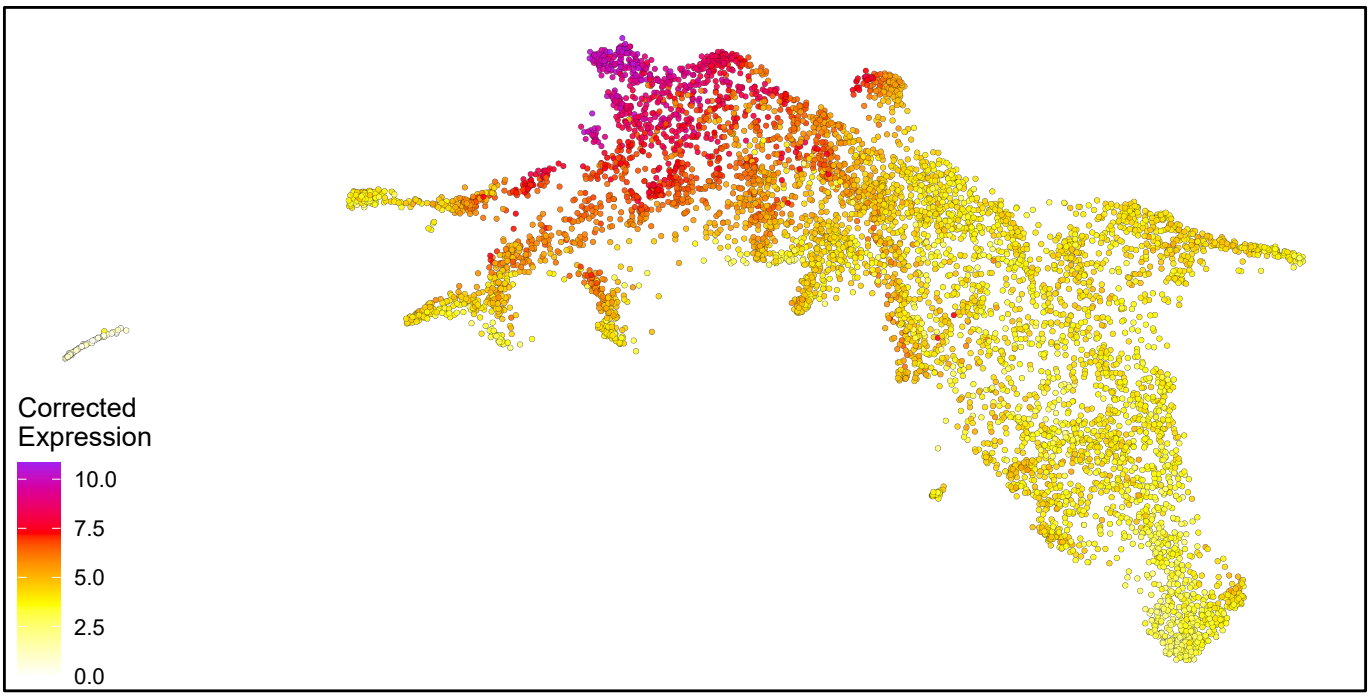
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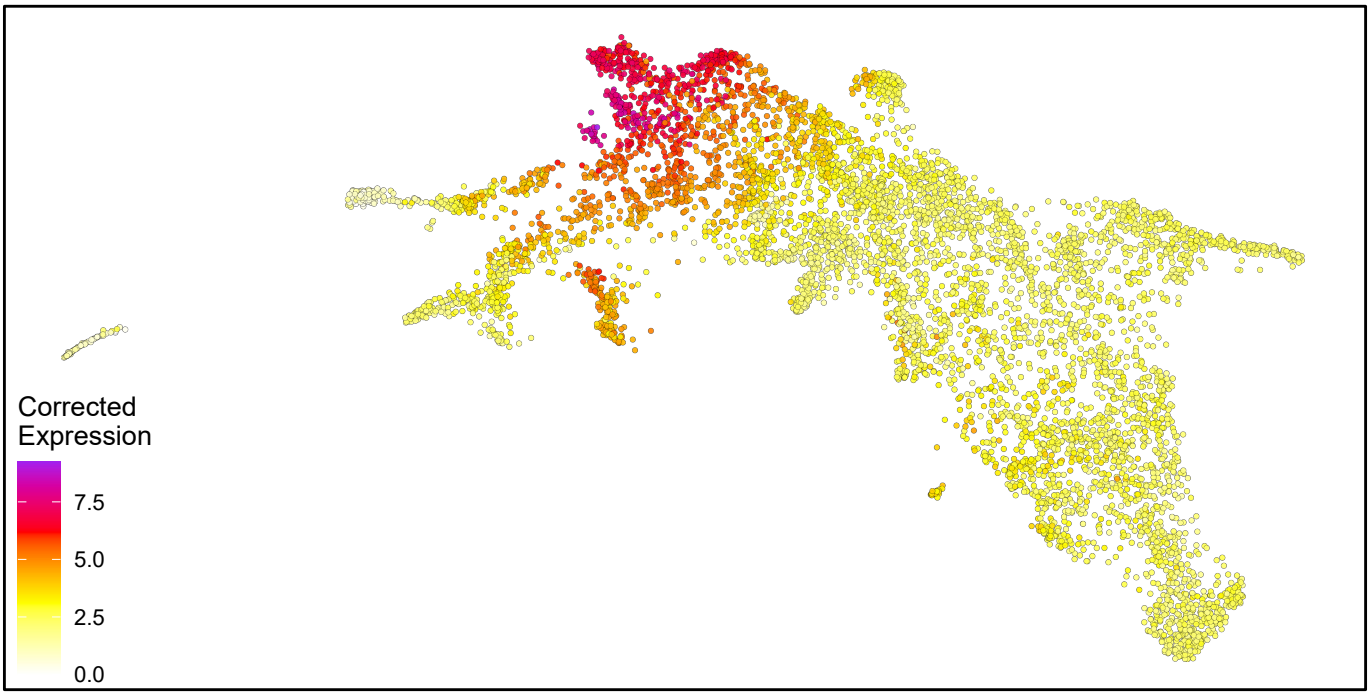
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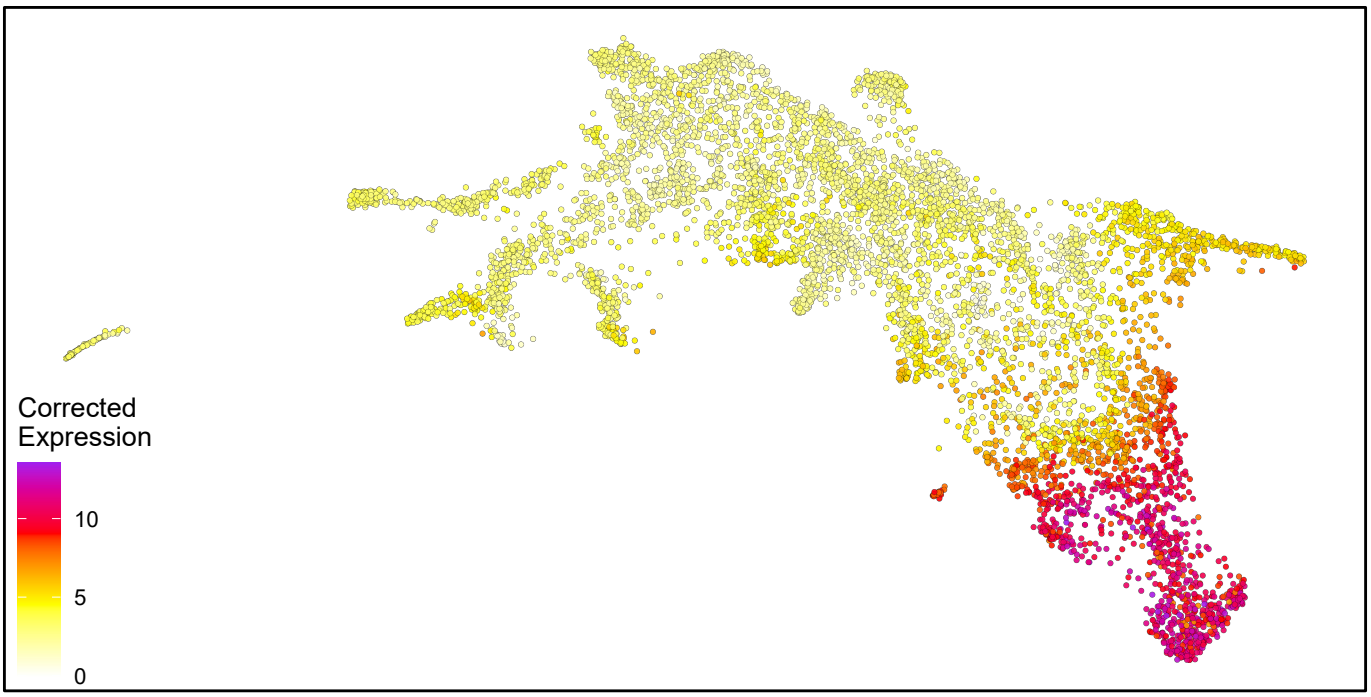

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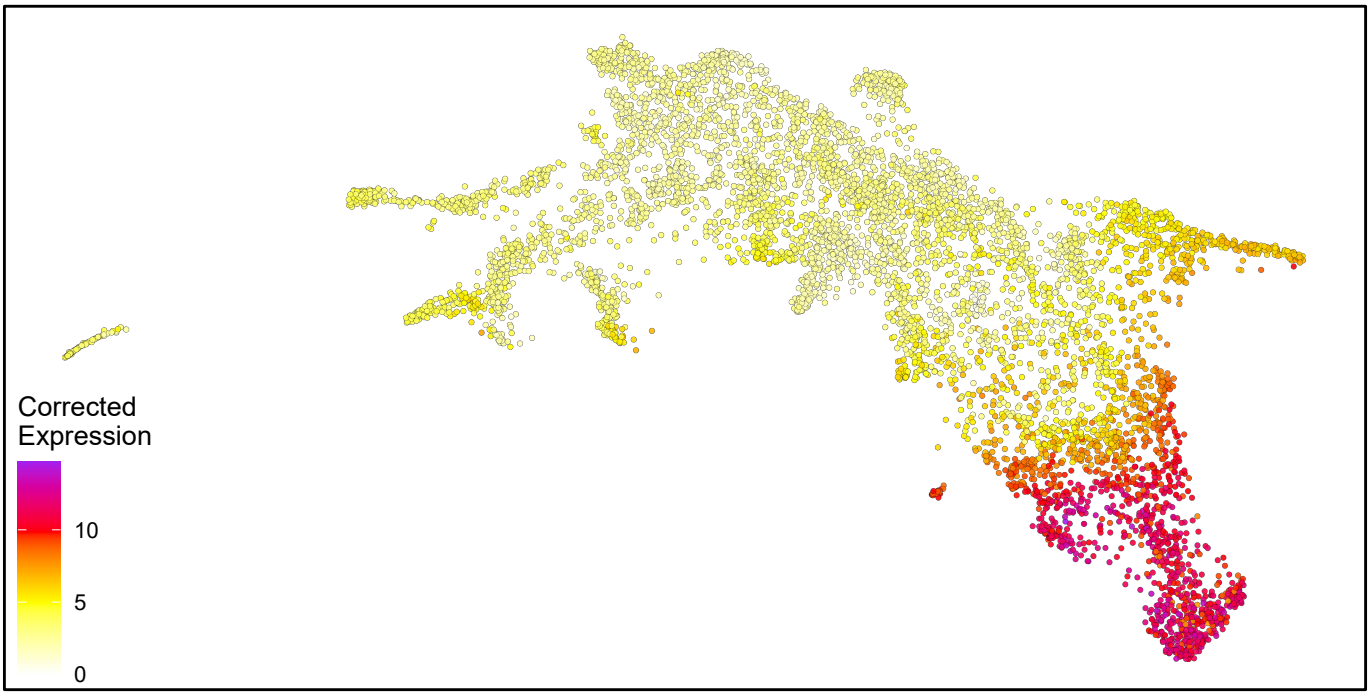

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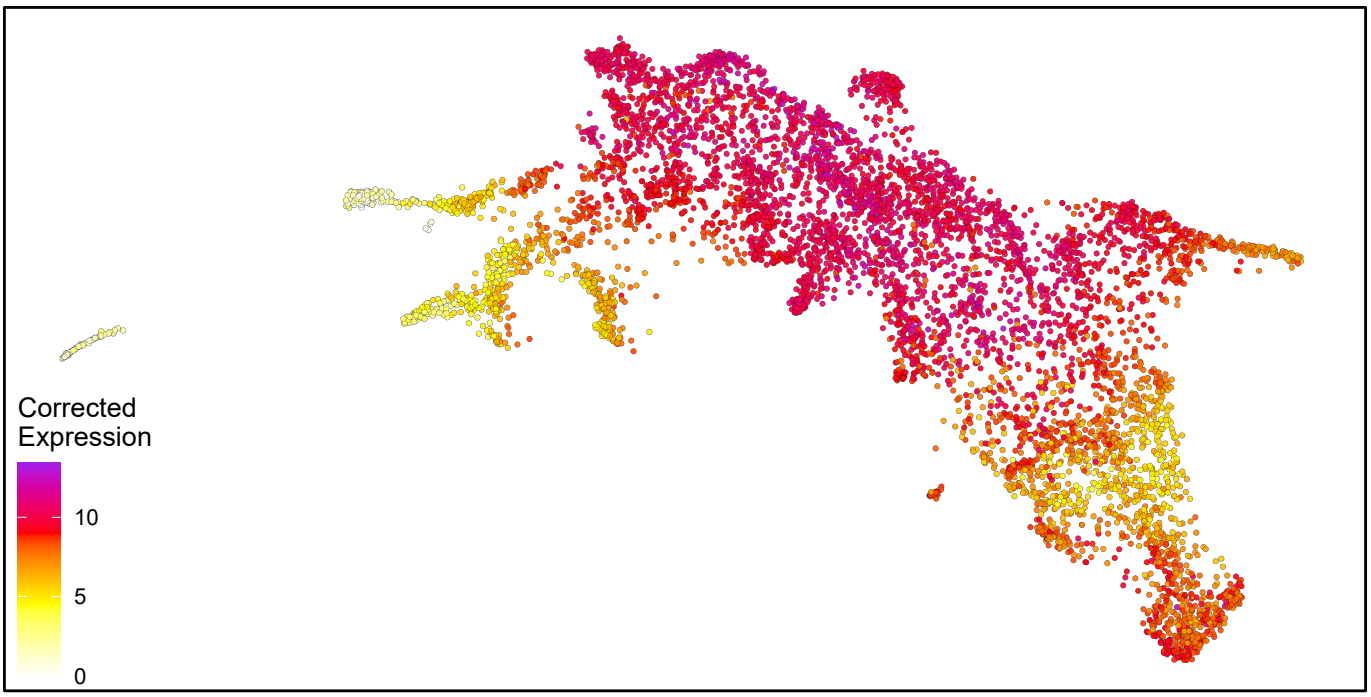

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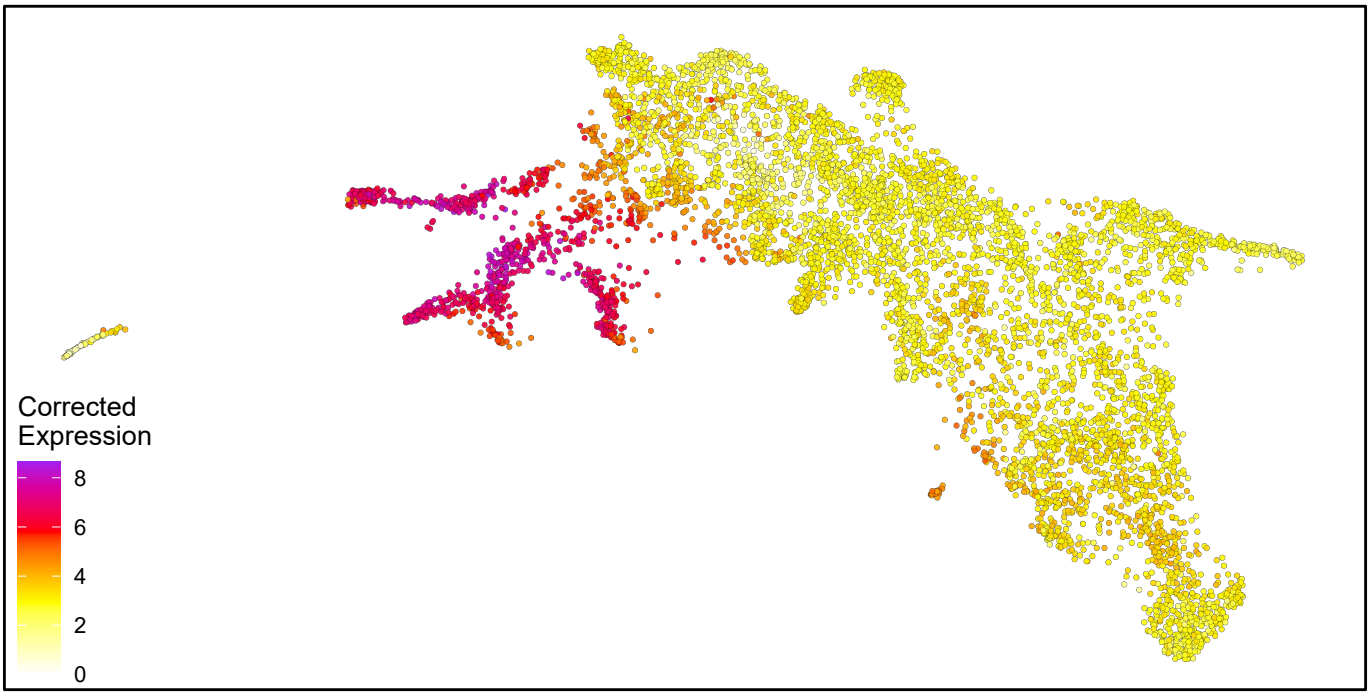

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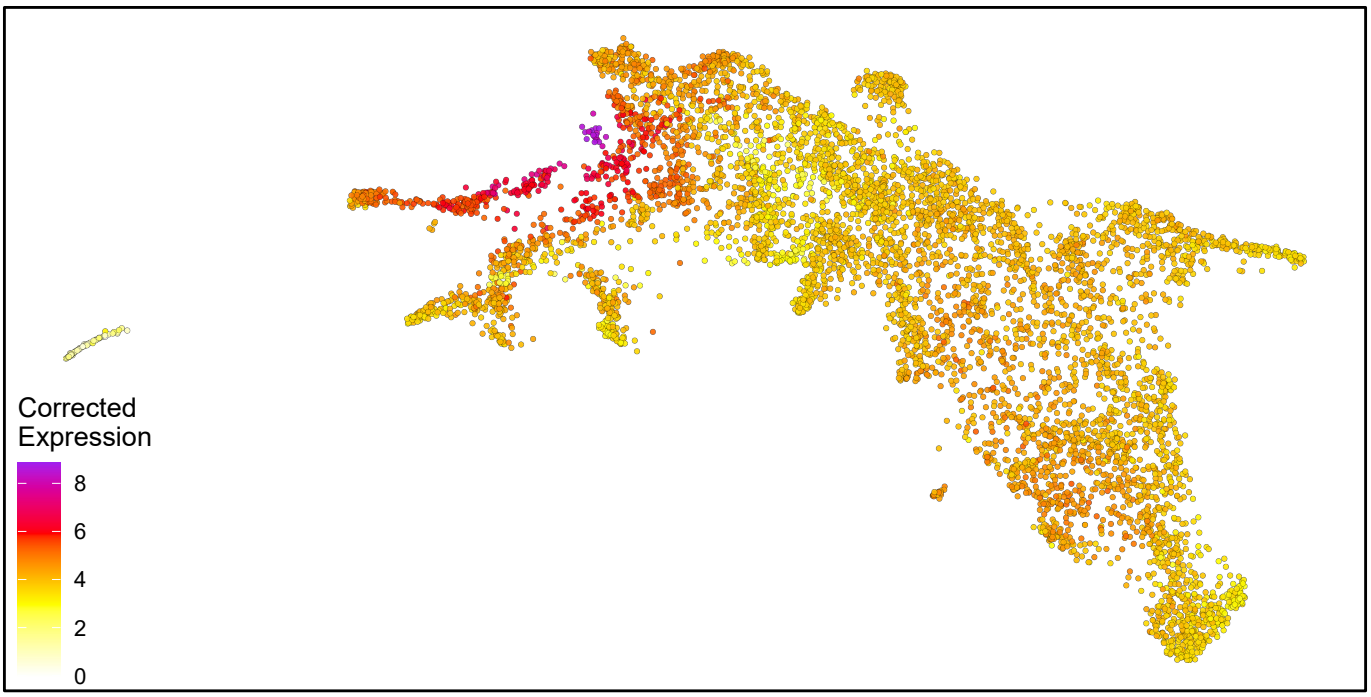

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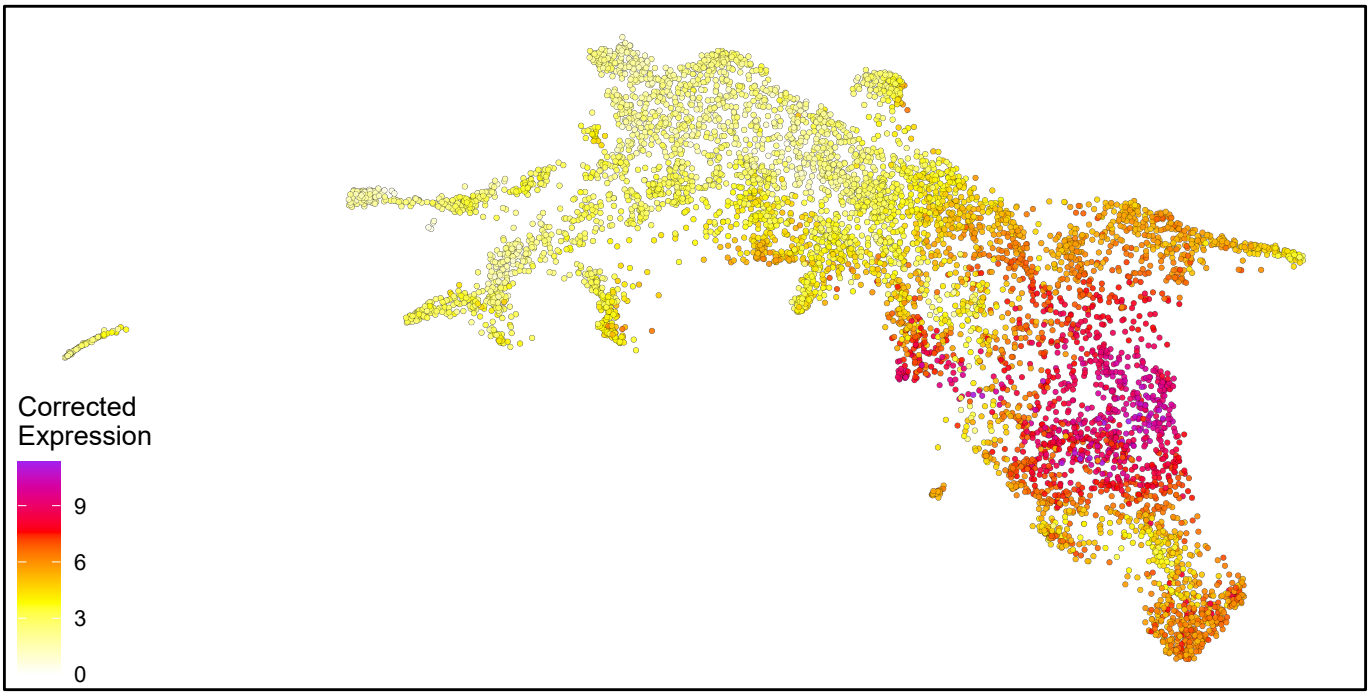

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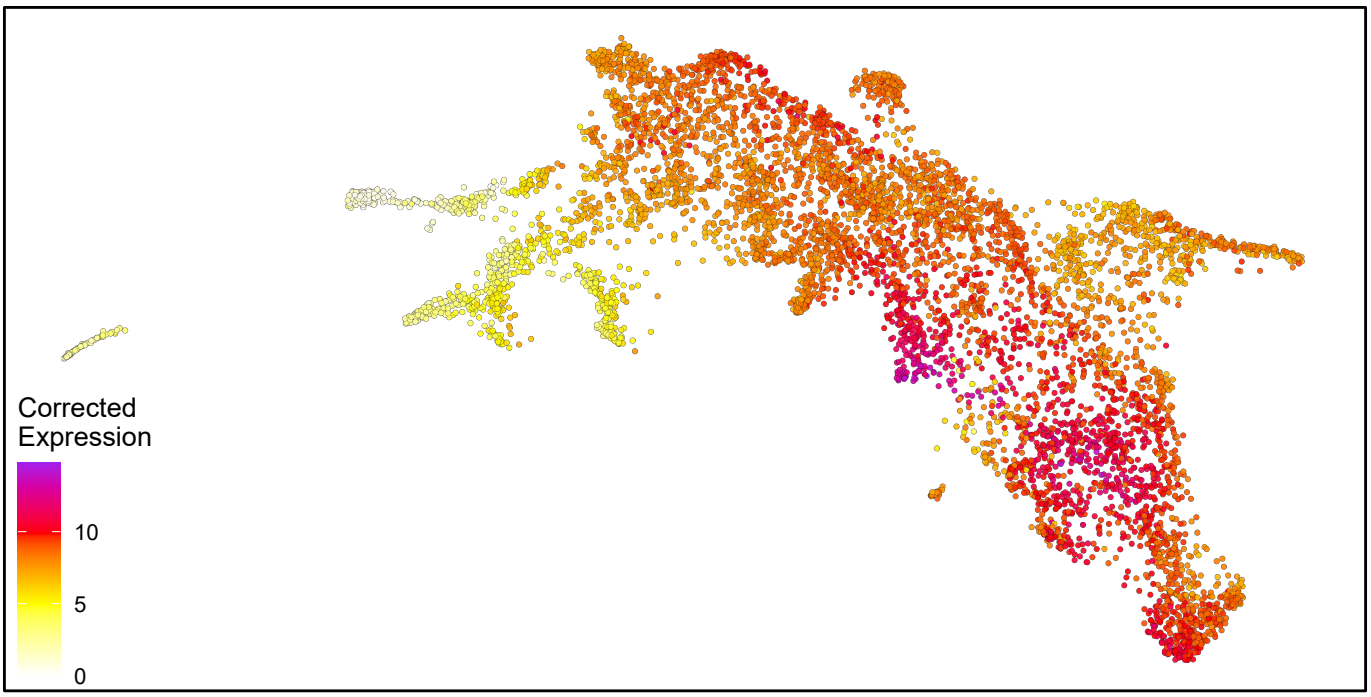

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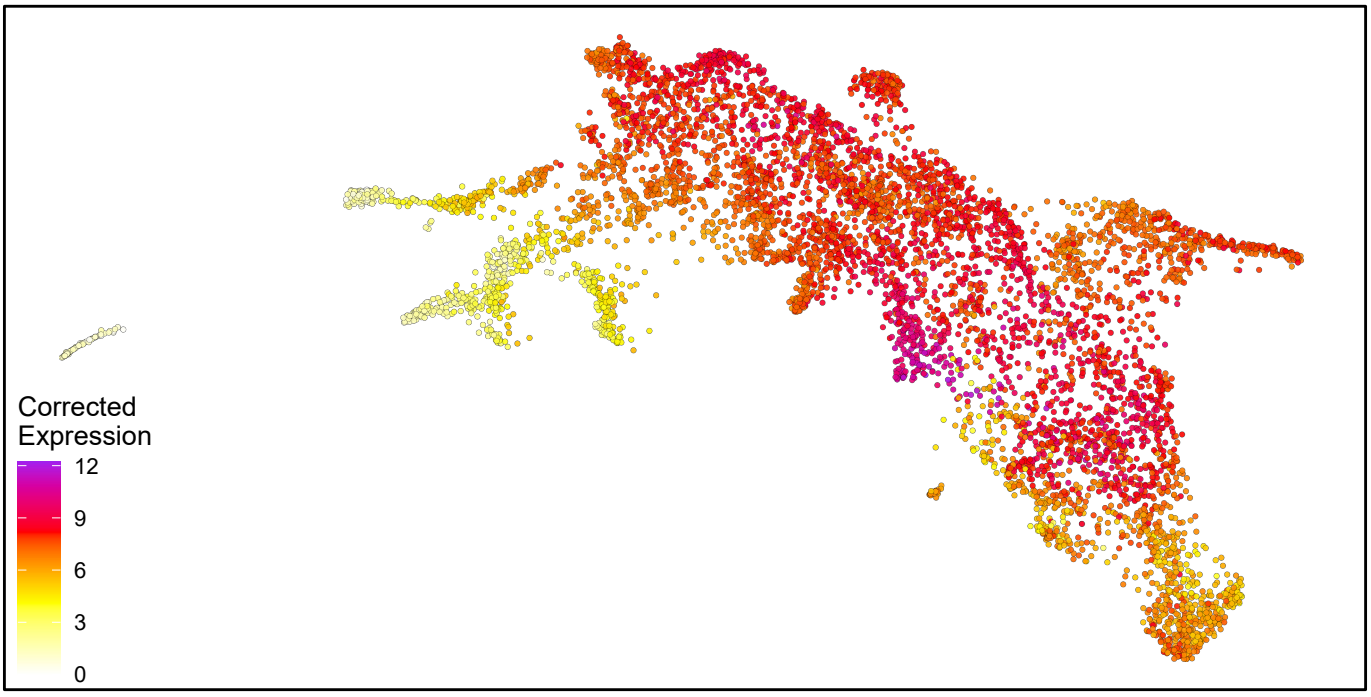

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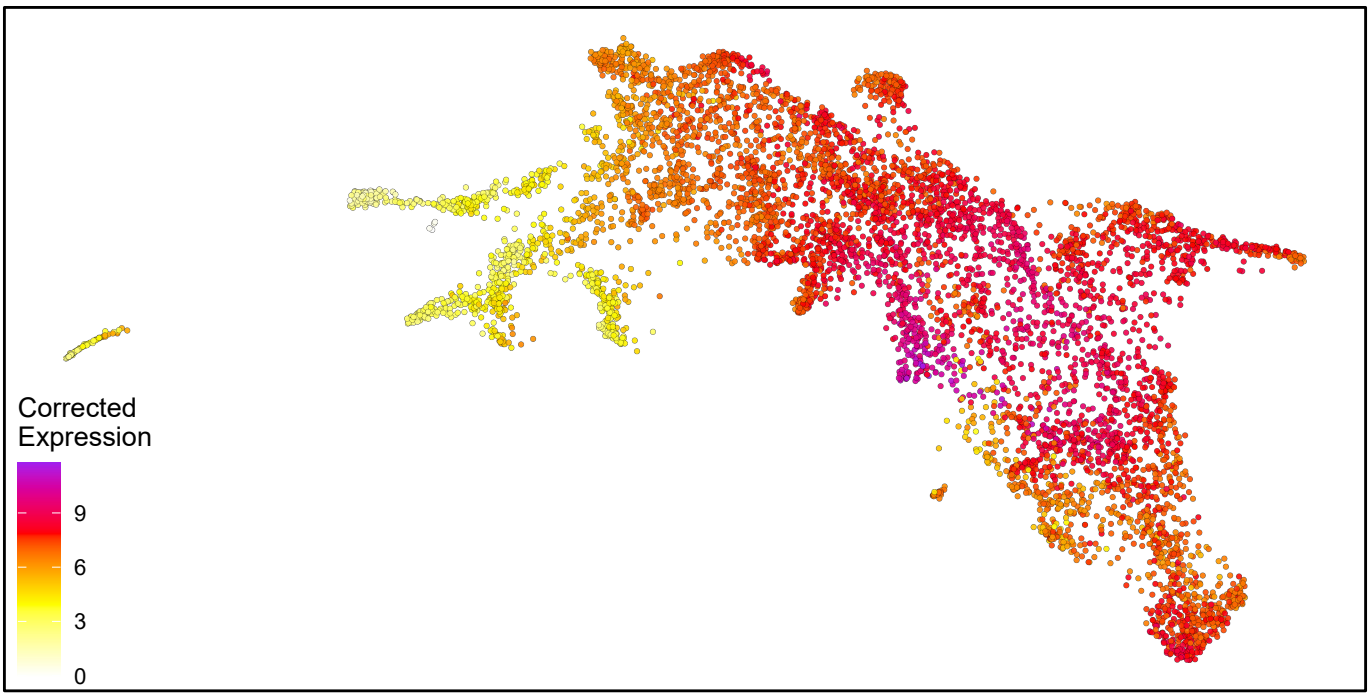
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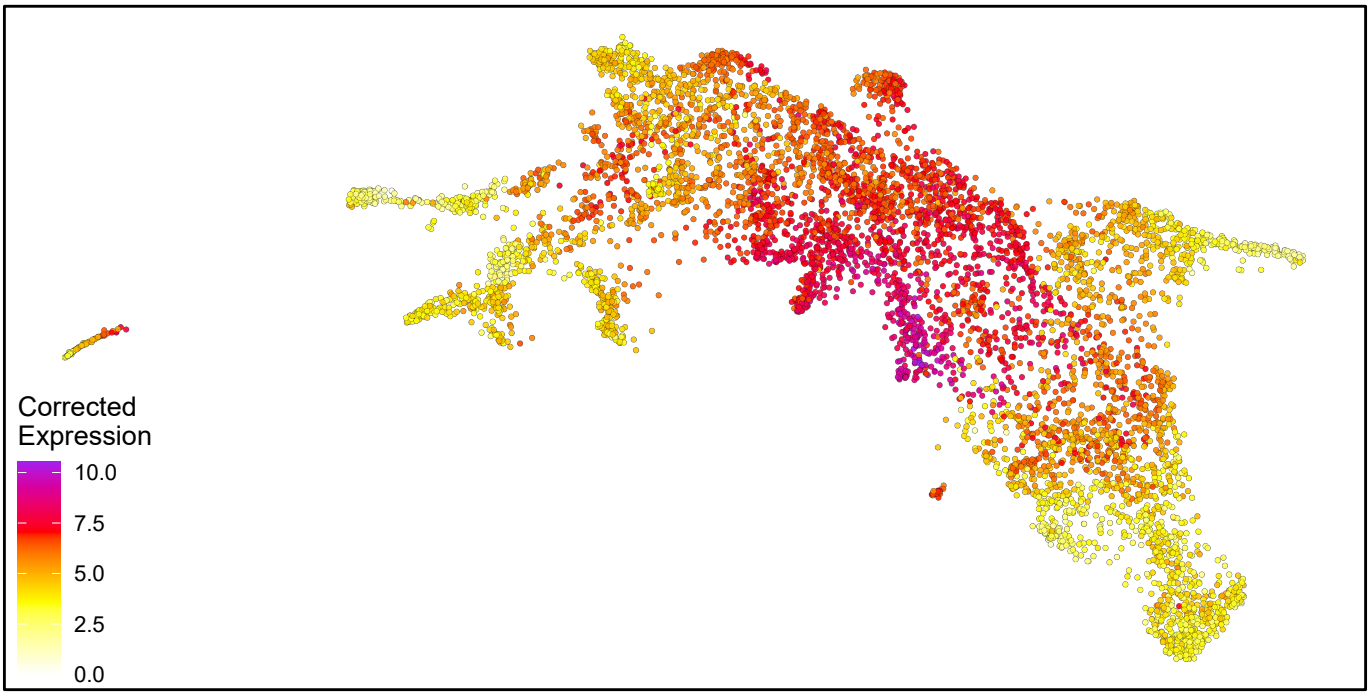
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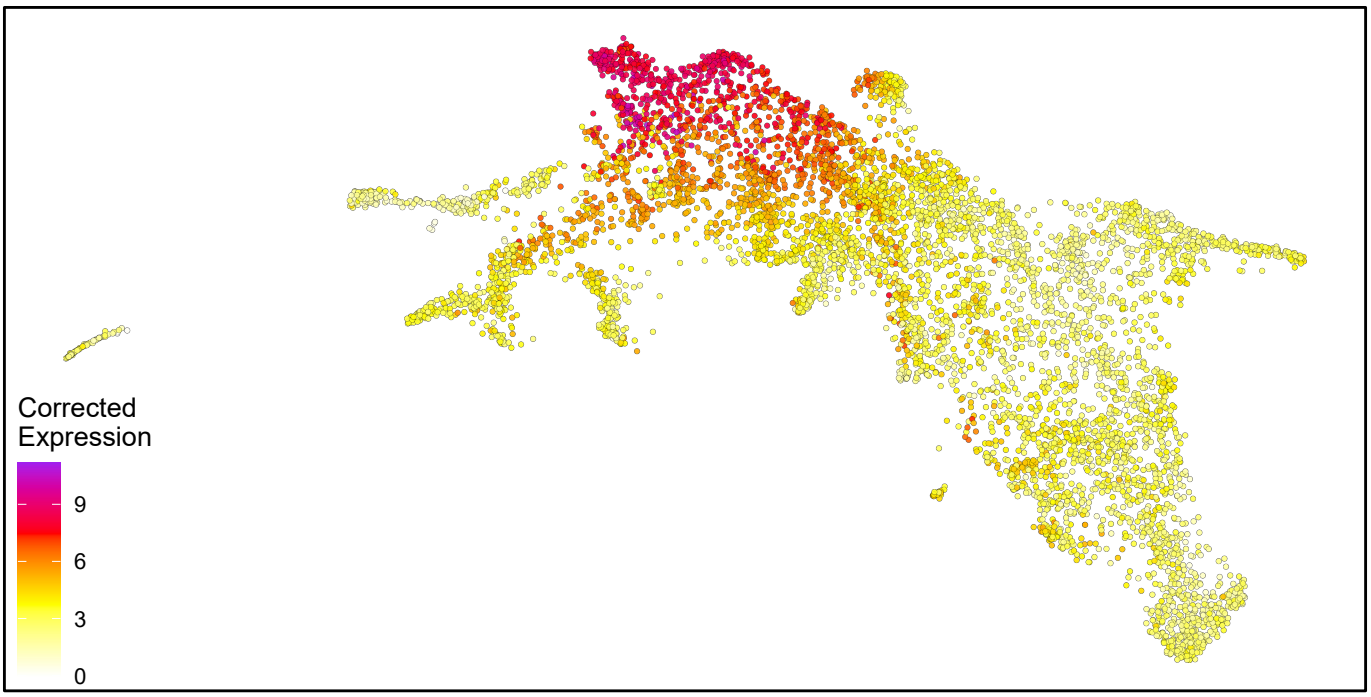
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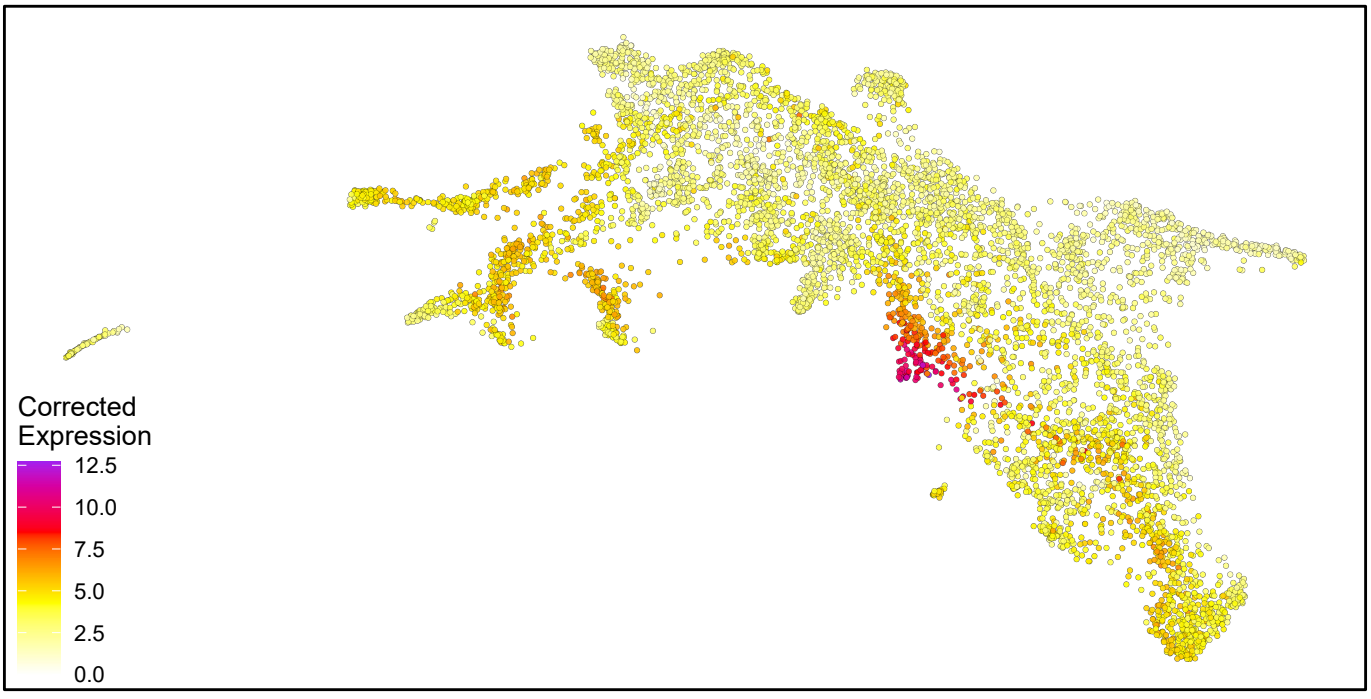
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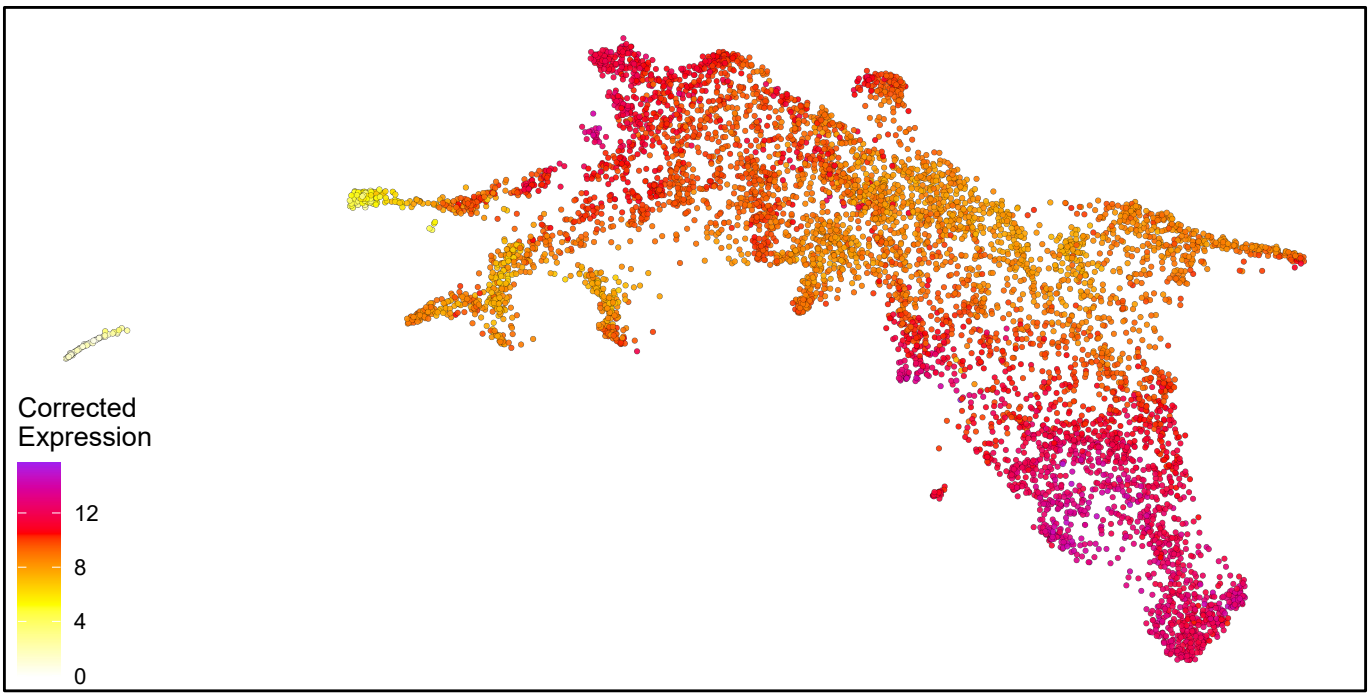
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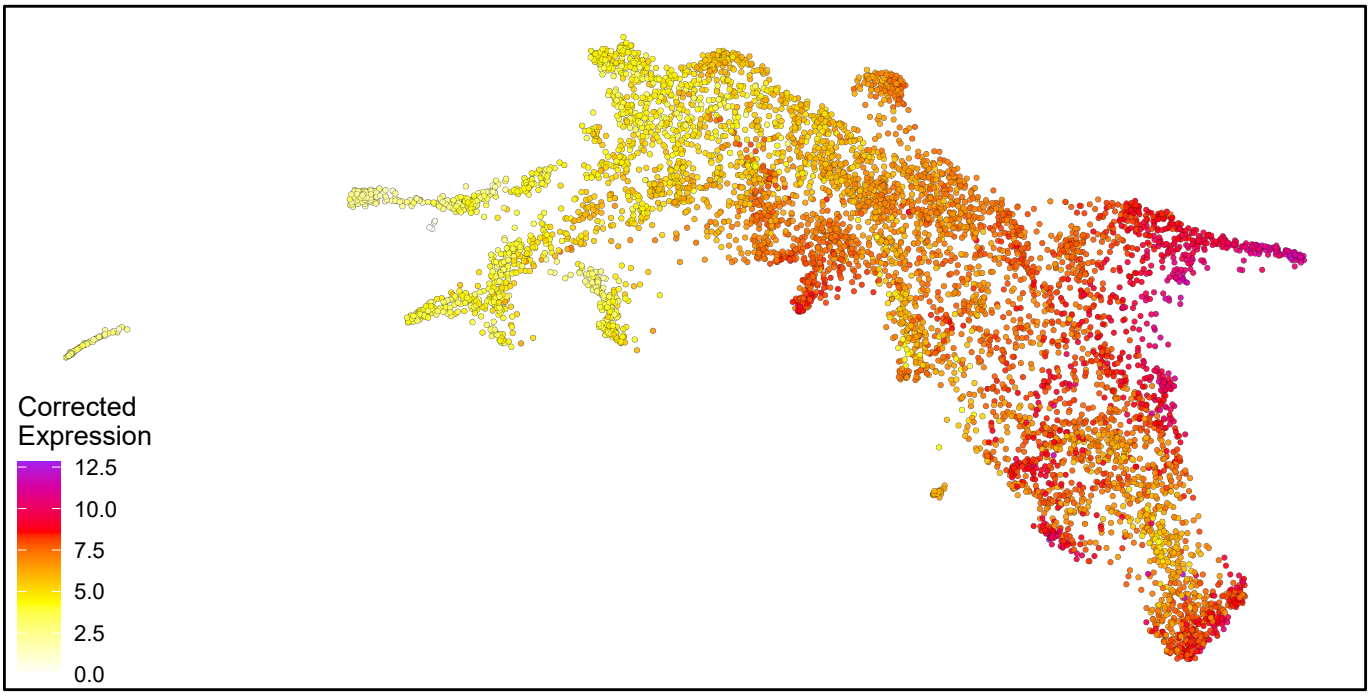
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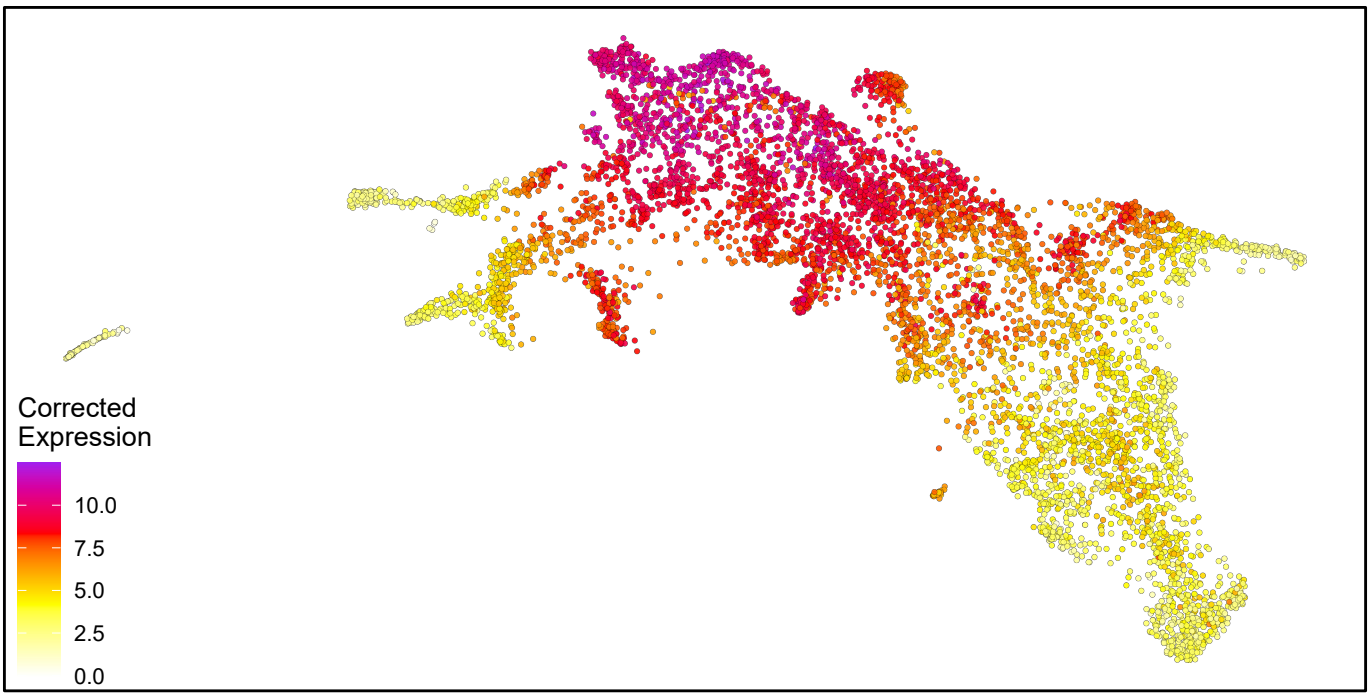
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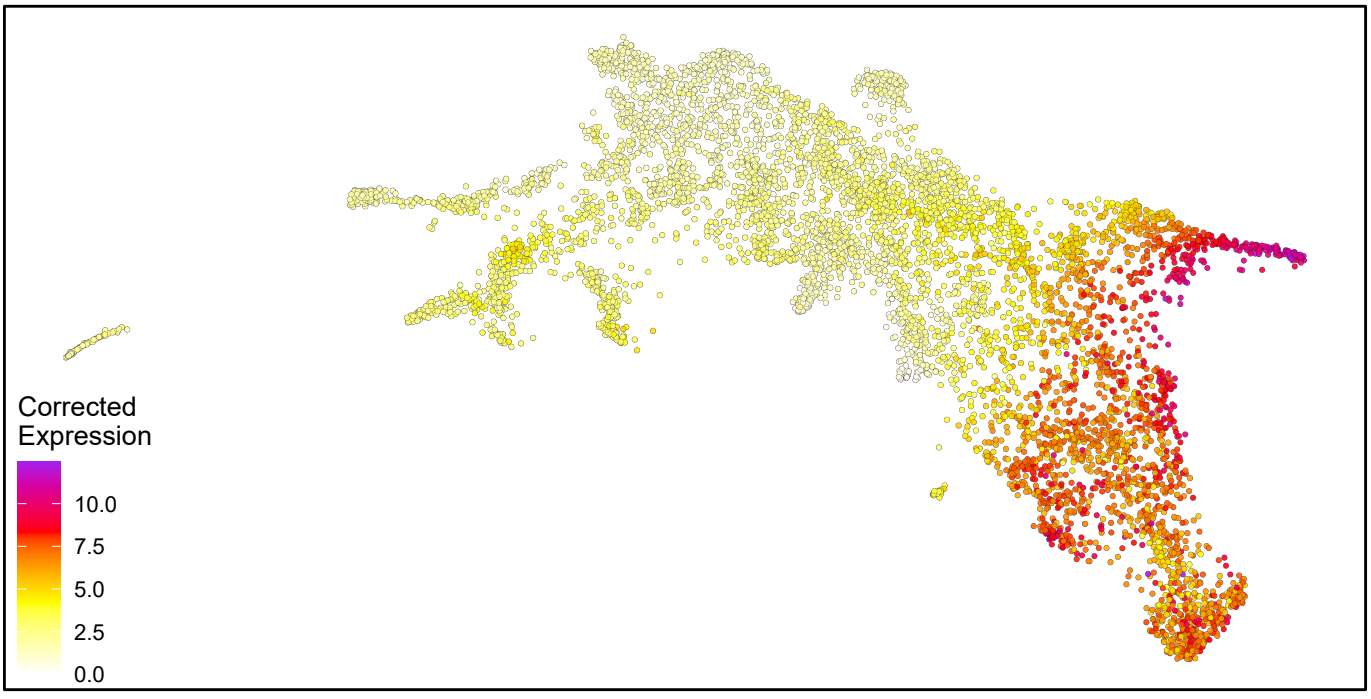
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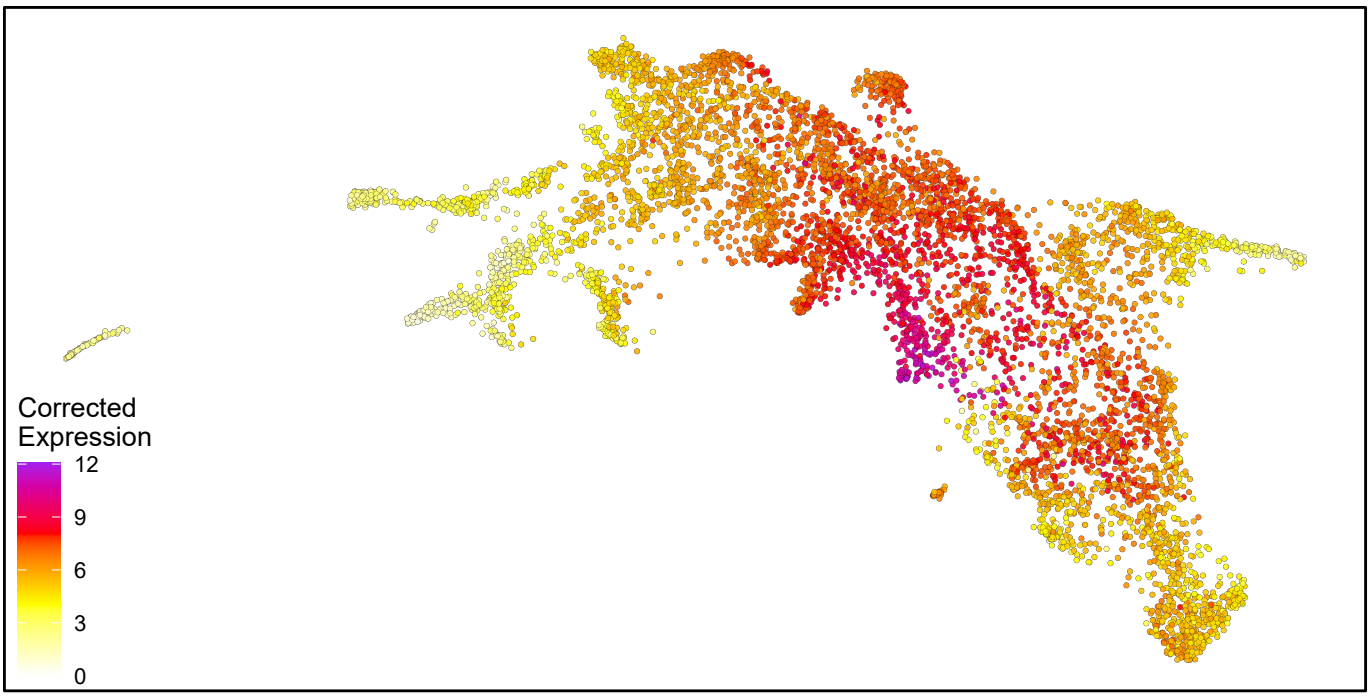

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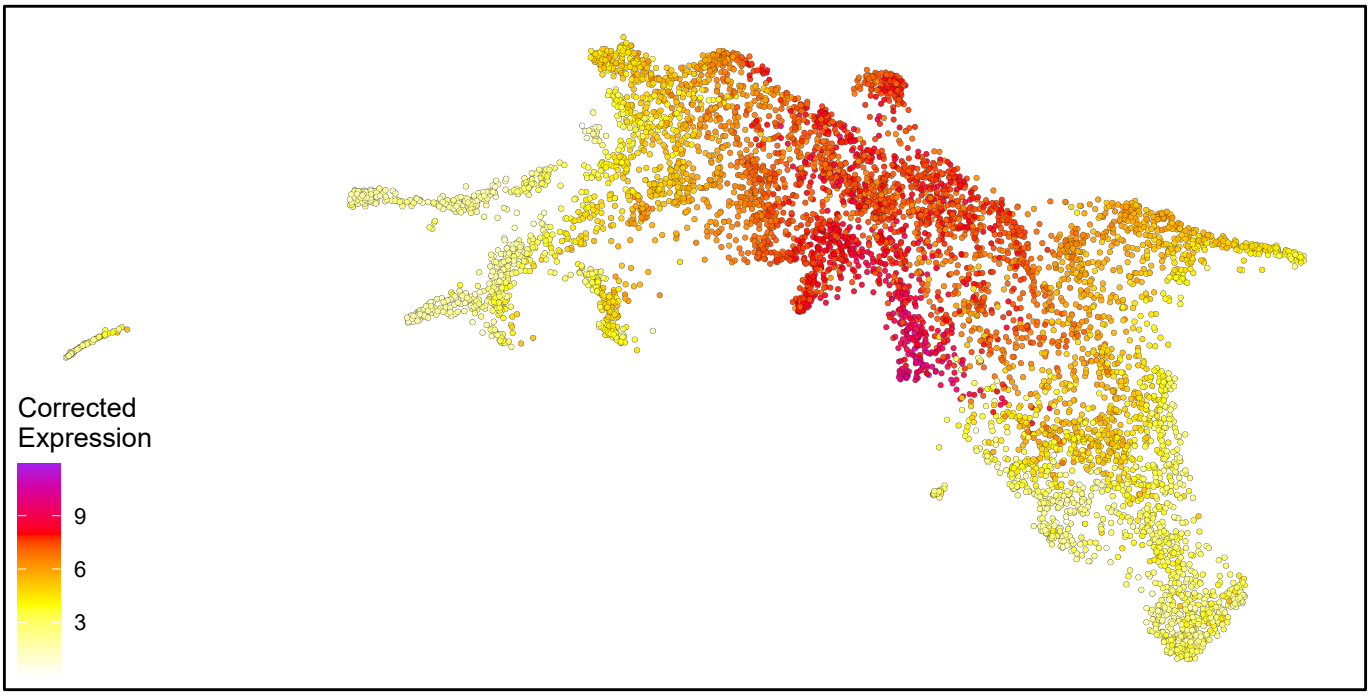

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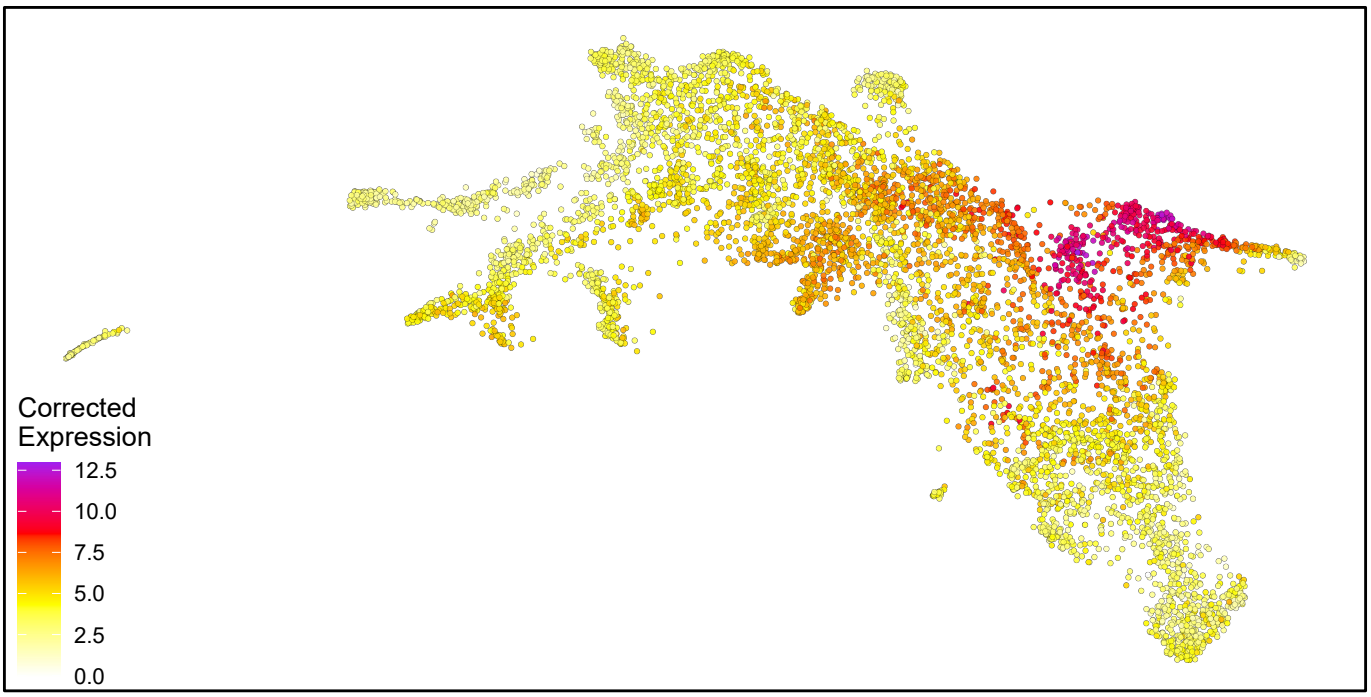

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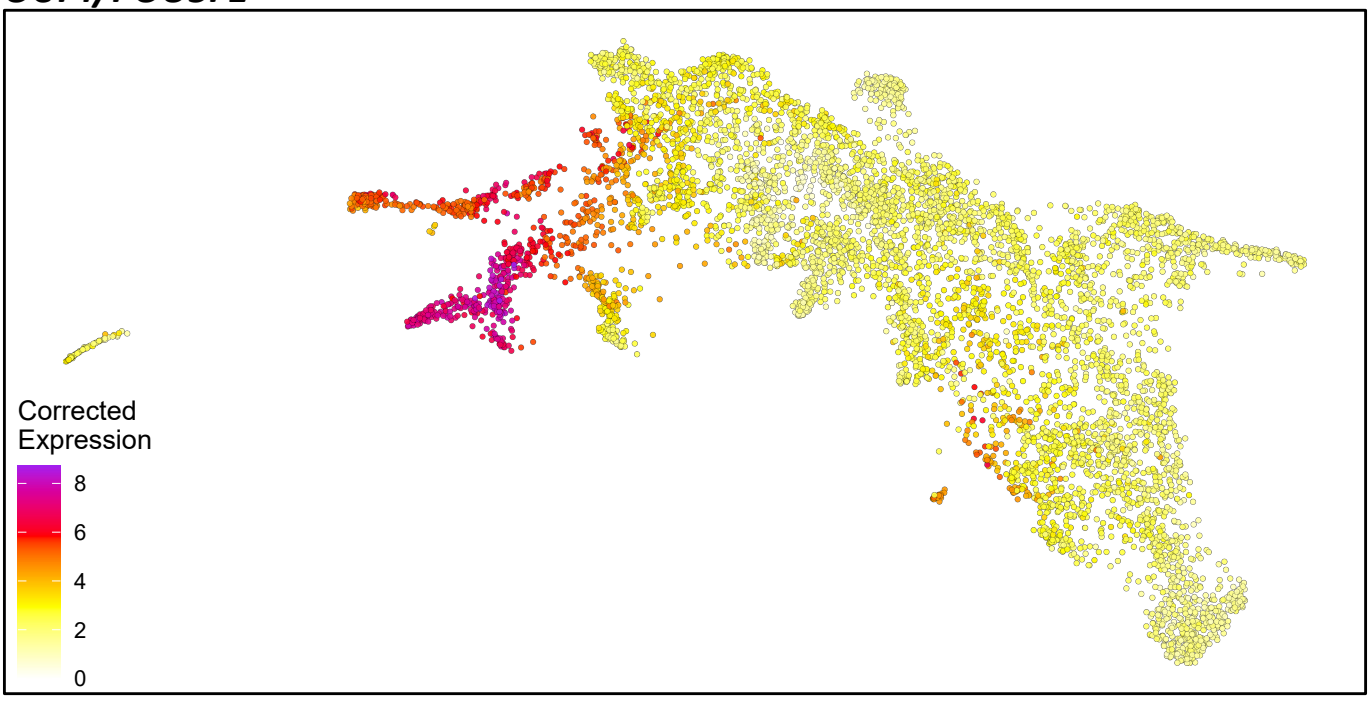

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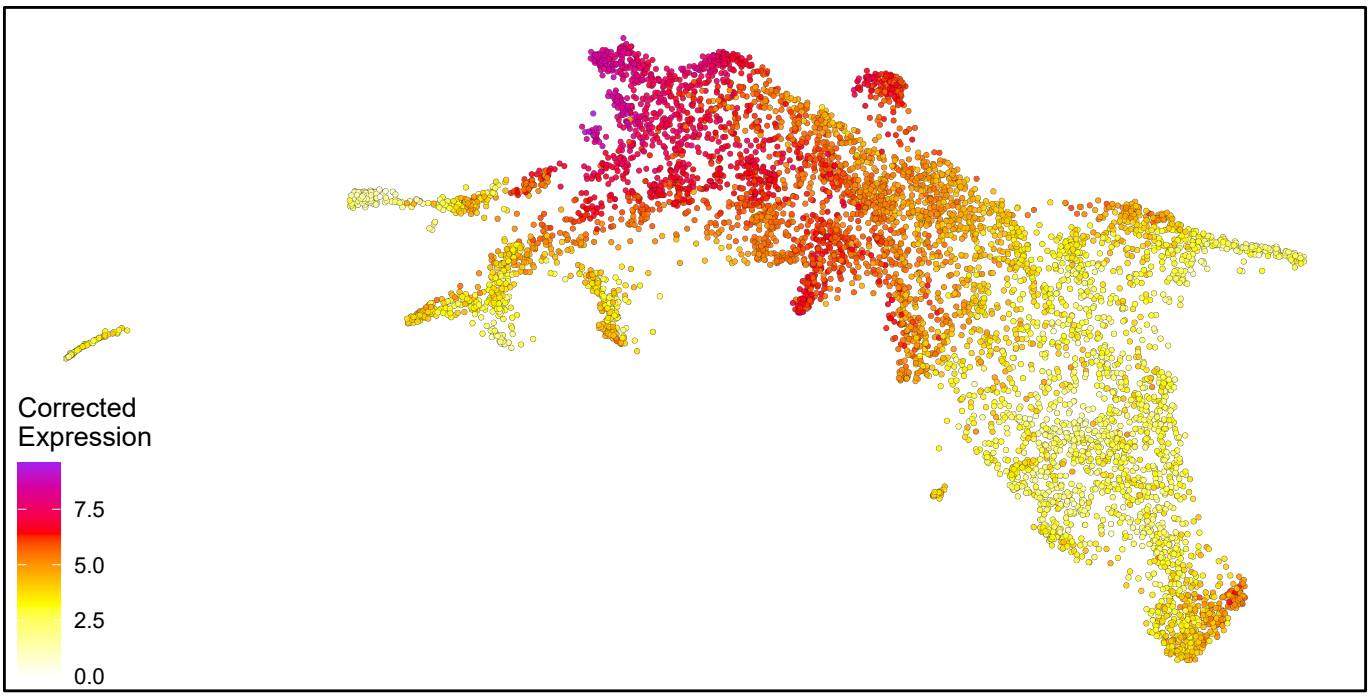
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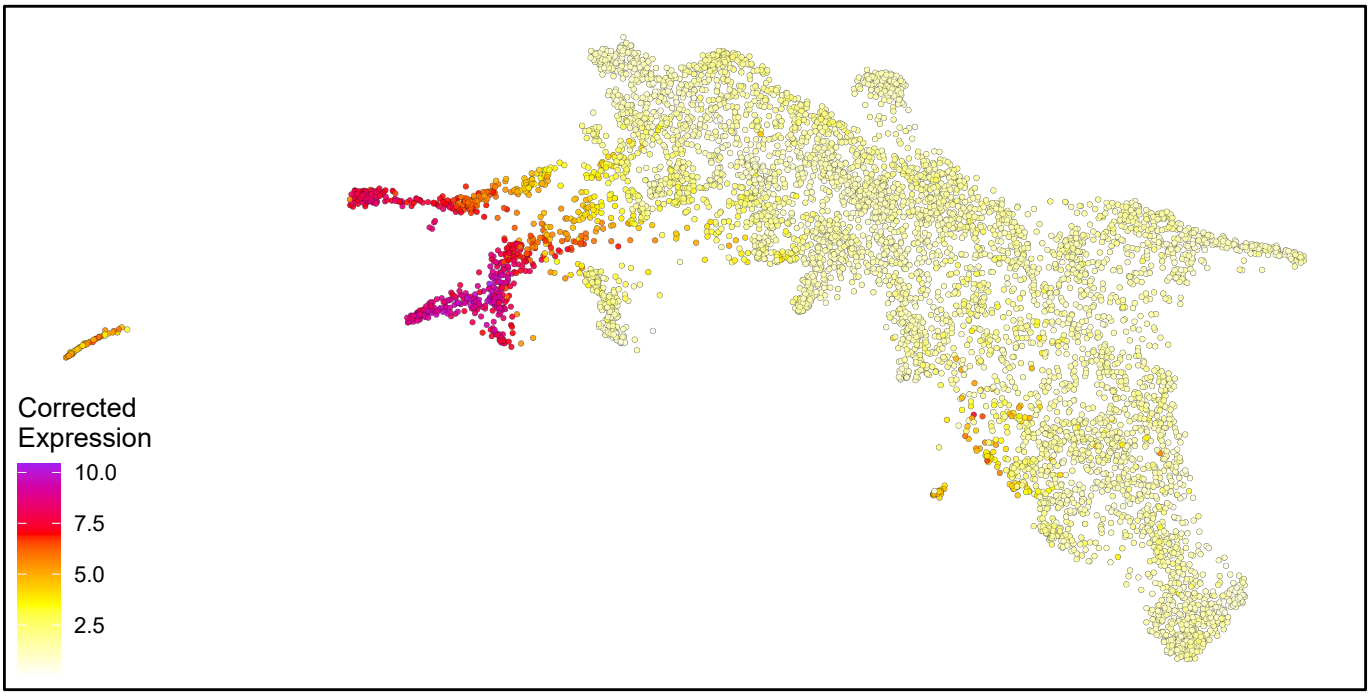
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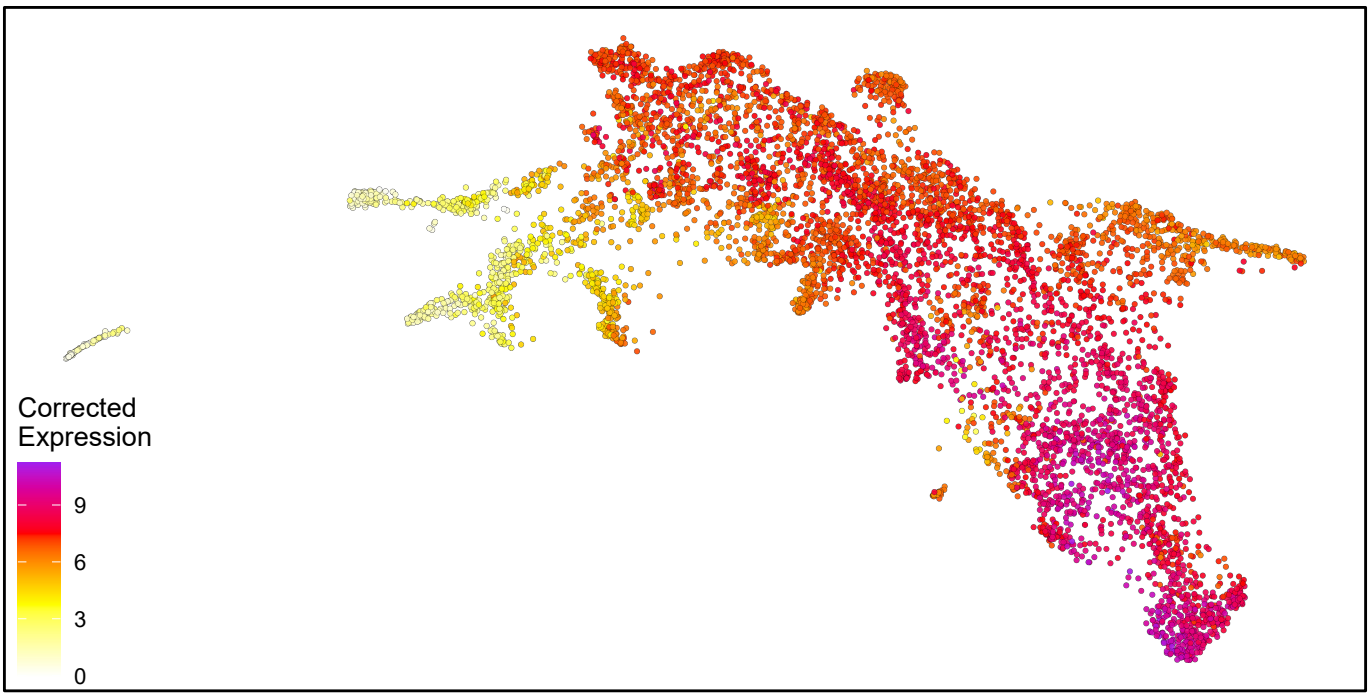

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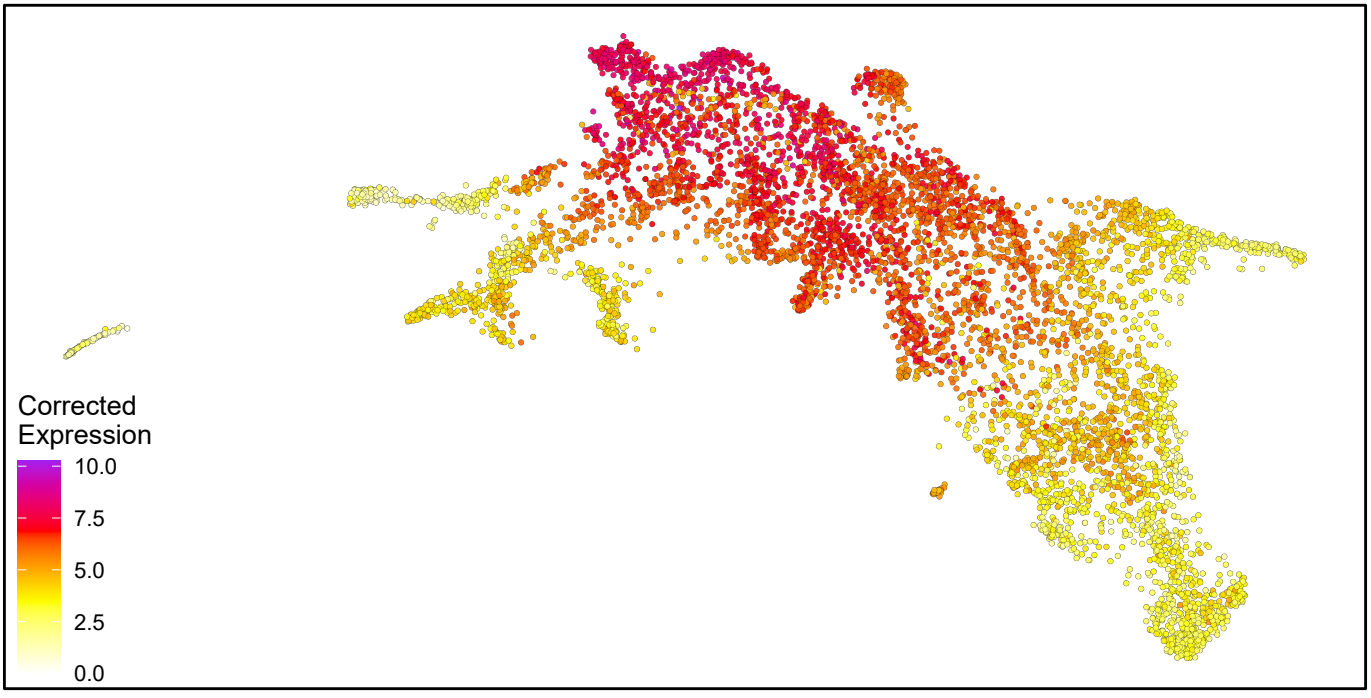

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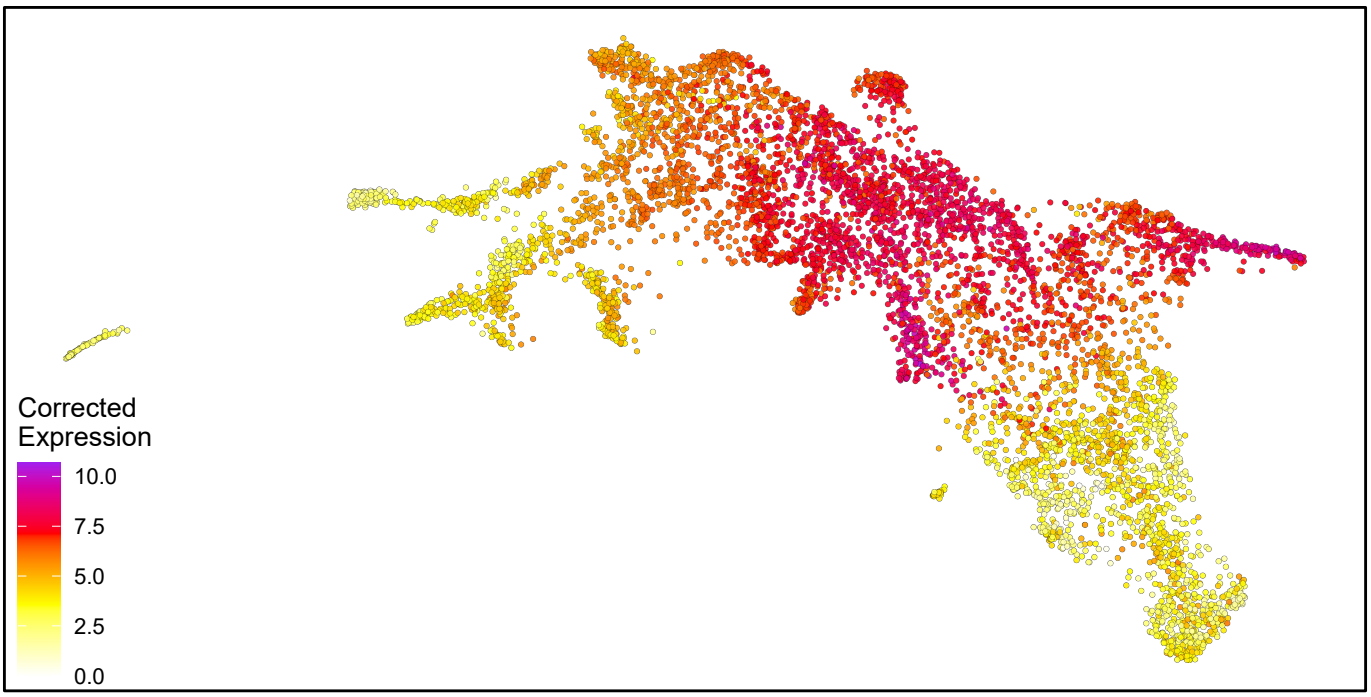

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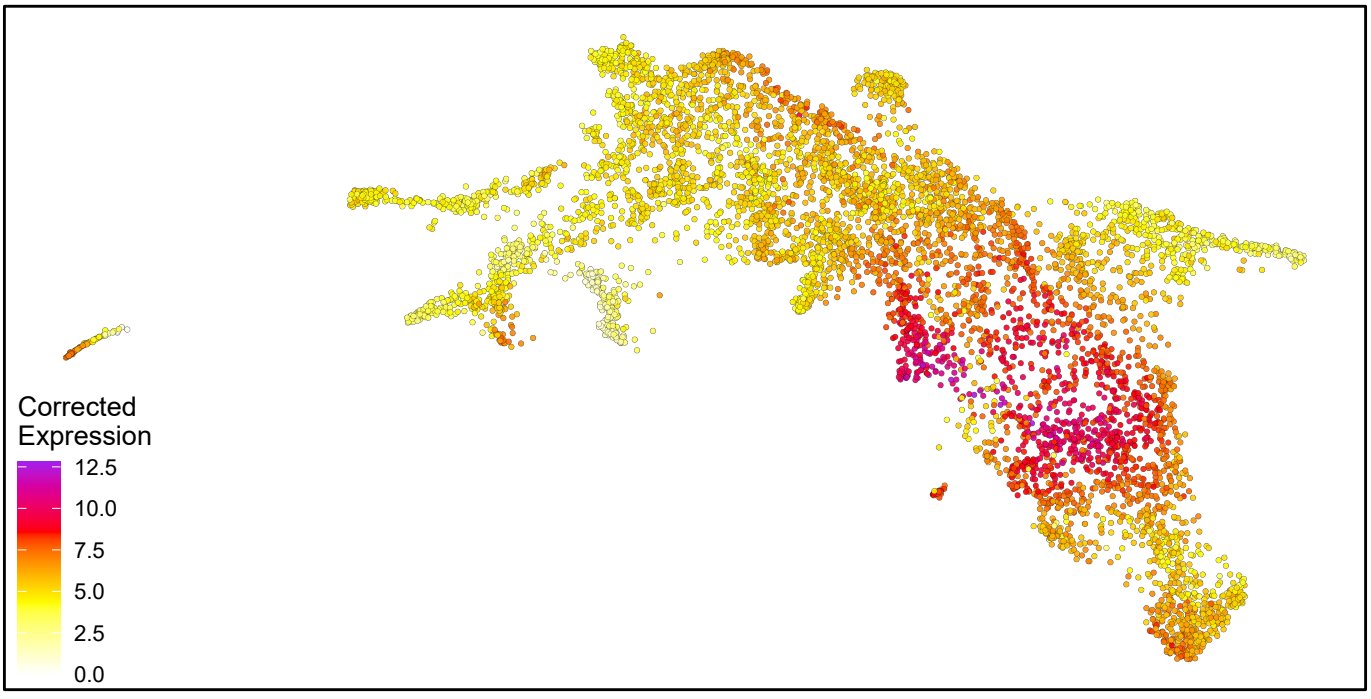

MRC Human Genetics Unit

THE UNIVERSITY of EDINBURGH

24 November 2021

Re: **Report for the PhD project of Gaël Castel**

In this PhD project, the background to the field of study has been very well researched and is presented with a clear and engaging narrative. First, different types of mammals are described and their distinct features identified. In particular, the nature and extent of the trophoblast lineage is compared between the sub-classes of mammals, since the main aim of the project is to characterise, generate and differentiate human trophoblast stem cells on several genetic backgrounds. Much of the molecular characterisation of trophoblast induction, differentiation and function performed to date has utilised mouse embryos, so this work is discussed accordingly. There is some overlap between the mouse and human systems, but the human data are quite sparse, hence the need for an *in vitro* model. There is a brief section comparing gene expression in the inner cell masses and trophectoderms of mouse versus human embryos; some of the concepts and mechanisms involved are outlined. Imprinting is discussed and the hypothesis that maternal versus paternal control may be involved in propagation is presented. The various types of trophoblast cells are described; how these may be modelled and the cell lines currently available for this purpose are outlined. Trophoblast-derived tumours, which are relatively common in human, but not in mice, are also well described in the background section.

At the start of this PhD project, no trophoblast stem cell lines had been derived directly from human embryos, despite considerable effort from several labs, but in 2018 the first report of hTSCs derived from both placental tissue and preimplantation embryos was published, thus providing the template for culture conditions to be utilised for this project. Human fibroblasts and existing hESC lines were selected as starting material, which were reprogrammed using the well-defined OKSM transcription factors. Introduction of TSC culture conditions during reprogramming could divert differentiation of the cells from

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pluripotency to trophoblast. Two male and one female fibroblast lines were thus derived for analysis. Considerable effort was made to assess the effects of the culture regime and optimise production of TSCs by testing different substrates and culture media. To overcome problems of heterogeneity, cells were physically selected, based upon their morphology, either by removal of unwanted cell types or picking and replating colonies exhibiting TSC morphology. It was also important to ensure that the reprogramming transgenes were silenced; cell lines in which transgenes were still expressed were discarded. Molecular analysis of the cell lines and the differentiation process required removal of feeder cells. Therefore, various substrates were tested. A combination of Fibronectin and Laminin 521 was chosen. Since one of the main aims of the project was to produce each of the three trophoblast derivatives and investigate the differentiation process, furhter culture optimisation was then required. Various combinations were used and the derivative cells assessed for identity via RT-PCR. Single cell RNA-sequencing was performed on cultured human embryos and a UMAP constructed to provide the molecular embryonic template onto which the derived cells could be mapped. This analysis identified the derived cell lines as day 8 trophectoderm.

Establishment of the protocol for deriving TSC lines from adult cells and existing pluripotent stem cell lines opens the possibility to generate trophoblast material from diverse genetic backgrounds and from patients carrying genetic defects in trophoblast production, which is likely to have clinical relevance. The work reported in this project has been published in a well-respected peer reviewed journal with the candidate as sole first author.

The thesis demonstrates a significant body of work utilising multiple skills and disciplines. The background has been well researched and the work is presented in a logical, engaging and informative manner. In my opiniton, it is of a high standard and a suitable PhD project.

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Report for Gaël Castel's PhD 24 November 2021
Vincent Pasque, Rapporteur

Introduction
The introduction showed that the candidate has a good understanding of the topic and is aware of the literature in this area. He began with a description of the evolution of placenta development in therians, then reviewed the knowledge on placenta development in mouse and human. He then provided a history of discoveries about how the trophectoderm and trophoblast are specified in development at the cellular and molecular levels. He carefully summarised the diseases related to placental development. Castel also explained the cellular models that have been developed to study placenta development and highlighted their strengths and weaknesses. The introduction was pleasant to read. Additional references to back up key statements could have been presented. Gaël did not include figures.

Results
The result section starts with the main publication from the candidate, a very nice manuscript published in Cell Reports. In this work the candidate explains how he was able to reprogram human somatic cells into induced trophoblast stem cells (TSCs) as well as to convert human naïve pluripotent stem cells into trophoblast stem cells. These discoveries, along with those of others last year, have changed the way we think about pluripotency and early human embryo development and opens up the way to future experiments to better understand early human embryogenesis. Several elegant experiments were carried out to understand the plasticity and potential of several pluripotent stem cell types including naïve, extended, and primed human pluripotent stem cells. The work is one of the first to demonstrate the unrestricted lineage potential of human naïve pluripotent stem cells. This is an excellent manuscript.

Additional results chapters and discussion
An historical account of the work leading to the main manuscript is provided, as well as a discussion of the results. Additional references could have been provided to back up key claims ie. on pages 79, 83, 84. An interesting discussion on matrices is provided. The differentiation of human TSCs into different cell types is presented and discussed. Additional, unpublished results were also presented. A general discussion on cell fate plasticity between embryonic and extraembryonic lineages is provided. The results of the candidate as well as that of others were discussed.

The molecular mechanism that might underlie cell fate plasticity are discussed. Finally, a discussion of the work in the context of the early human embryo is provided. Novel stem cell embryo models including blastoids are discussed. A conclusion section is provided.

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3000 LEUVEN, BELGIUM

General assessment of the thesis

The work is of high current interest and presented in a good way. It was a great pleasure to read. It is clear Gaël has made an important contribution to the field and understands the subject well. I support the submission of the thesis.

Sincerely,

Vincent Pasque

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DOCTORAT BIOLOGIE BRETAGNE SANTE LOIRE

UNIVERSITÉ DE NANTES

Titre : Etude du trophoblaste humain au moyen de modèles de cellules souches

Mots clés : trophoblaste · placenta · embryon humain · cellules souches · reprogrammation

Résumé : Cette thèse s'intéresse aux événements de spécification des types cellulaires dans l'embryon humain et des mécanismes moléculaires qui leurs sont associés, dans le cadre de la formation du placenta.

Une problématique entourant ce sujet est l'accès restreint aux embryons humains, ainsi qu'aux cellules souches trophoblastiques de patients, notamment pour l'étude des maladies placentaires.

Nous avons donc cherché à appliquer les méthodes de reprogrammation et de conversion cellulaires pour générer des cellules souches trophoblastiques humaines induites (hiTSCs) à partir de fibroblastes ou d'iPSCs de patients. Nous avons confirmé l'identité trophoblastique de ces cellules par analyse transcriptomique et validation fonctionnelle de leur potentiel de différentiation en syncytiotrophoblaste et trophoblaste extravilleux.

Nos travaux ont également mis en évidence la plasticité des cellules souches pluripotentes humaines naïves et à potentiel étendu avec la lignée trophoblastique, contribuant ainsi au développement des premiers blastoïdes humains.

En conclusion, les hiTSCs de patients permettront d'étudier les mécanismes spécifiquement associés au développement normal ou pathologique du placenta, avec des applications possibles à l'amélioration des conditions de culture des embryons FIV et au traitement des maladies placentaires.

Title: Human trophoblast lineage characterisation with stem cell models

Keywords: trophoblast · placenta · human embryo · stem cells · reprogramming

Abstract: This thesis focuses on cell lineage specification events occurring in the human embryo and related molecular mechanisms involved in the formation of the placenta.

An issue surrounding this field of research is the limited access to human embryos and trophoblast stem cells from patients, notably those related to placental diseases.

Therefore, we applied somatic cell reprogramming and cell fate conversion approaches to generate human induced trophoblast stem cells (hiTSCs) from patients' fibroblasts or iPSC lines. We then characterised these cells by transcriptomic analysis and tested their differentiation potential into syncytio-trophoblast and extravillous trophoblast cells.

Our work also revealed cellular plasticity of human naive and extended pluripotent stem cells with the trophoblast lineage, which contributed to developing the first human blastoids, a stem cell-based model of the embryo.

In conclusion, hiTSCs from patients can be used to investigate molecular mechanisms of normal *vs* pathological development of the human placenta, with possible applications to improving IVF techniques and treatments of placental diseases.