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Chemically defined and xeno-free media enables the derivation of human extended pluripotent stem cell lines from discarded blastocysts with a high efficiency

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Abstract

Human extended pluripotent stem cells (hEPS) had been reported to be derived from discarded blastocysts, whereas the derivation method of hEPS cells was extremely complex in this protocol with feeder and animal serum conditions, which also limited the safety and homogeneity of hEPS. Here, we report an optimized, highly efficient protocol by utilizing chemically defined and xeno-free media for the derivation of human extended pluripotent stem cell lines from discarded blastocysts. With this method, we successfully isolated hEPS cell lines from discarded blastocysts with an efficiency of 46%. Chemically defined and xeno-free media simplified the process of hEPS cell isolation with a higher survival rate of cell aggregation passaging from outgrowth. To our knowledge, this is the first report of hEPS cells being efficiently derived from discarded blastocysts under chemically defined and xeno-free conditions.

Keywords Human extended pluripotent stem cells, Chemically defined, Xeno-free, Embryonic and extraembryonic differentiation capacity, Cell lines establishment efficiency

Introduction

hEPS are a type of extended pluripotent stem cells that possess the developmental potential of both embryonic and extraembryonic tissues [1]. EPS cells have been instrumental in advancing our understanding of the development of embryonic and extraembryonic tissues [2], as well as in the construction of functional early embryonic structures in vitro [3, 4]. The injection of human EPS cells into early-stage blastocysts of crab-eating macaques has resulted in the successful creation of chimeric human-monkey embryos, with the survival time extended to 20 days [5]. These studies underscore the broad application prospects and advantages of EPS cells.

Discarded human blastocysts are a vital and safe source of human EPS cells [6]. The main disadvantage

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of EPS cells is their limited proliferation capacity and the requirement for mouse-derived feeder layer cells and animal serum [7, 8]. Despite protocol optimization, this technique results in lower separation efficiency and reduced stability and safety of the cultured human EPS cells [8]. These concerns include the transfer of pathogens like viruses and mycoplasma, as well as the presence of non-human sialic acids like Neu5Gc, which can trigger immune responses. Additionally, the use of xenogeneic feeder cells can lead to contamination of the target cells, especially when they are used for clinical applications [9, 10]. Consequently, optimizing the culture condition for human EPS cells and identifying an efficient system for deriving EPS cells from discarded human blastocysts without xenogeneic feeder cells or animal serum is the core issue in obtaining high-quality EPS cell research.

In recent years, significant progress has been made in optimizing the culture condition for EPS cells, as evidenced by numerous previous articles. Through the screening of chemical small molecules, Gao et al. identified conditions for establishing porcine EPS cells, and similar culture conditions can transform traditional human embryonic stem cells (ESCs) into EPS cells [11]. Zheng et al. reported an advanced feeder-free culture condition that successfully converted traditional human ESCs and iPSCs into EPS cells [12]. Liu et al. enhanced the LCDM condition and established an in vitro culture system for EPS cells that is both well-defined and free of xenogeneic components, which they termed XF-free. They also developed a reprogramming method to induce somatic cells into XF-free human EPS cells using this system [13]. Hao et al. developed a novel culture medium, OCM175, to obtain EPS cells from human urine-derived cells [14]. These optimized culture conditions have all abandoned the reliance on mouse feeder layer cells and animal serum. However, whether the xeno-free and feeder-free culture conditions can enable the capture of extended pluripotent stem cells directly from the human discarded blastocyst has not been determined.

This article reports for the first time the establishment of human extended pluripotent stem cells from discarded blastocysts in a chemically defined and xeno-free culture system (XF-free hEPS), which can be stably passaged and expanded in vitro. XF-free hEPS cells express pluripotency markers and hEPS-specific markers, maintain a normal diploid karyotype after more than 20 passages, and have the ability to differentiate into cells from all three germ layers both in vitro and in vivo. Furthermore, the XF-free hEPS cells have a certain capacity for intra- and extraembryonic chimerism. The derivation of XF-free hEPS cells provides a new option for establishing hEPS cell lines that meet clinical application standards and will further promote the translational application of hEPS technology in the future.

Materials and methods

Collecting discarded human blastocysts

In this research, the embryos were donated by infertility couples from the Reproductive Medical Center of Peking University People's Hospital from October 2019 to December 2022. The donors signed the informed consent form after being thoroughly briefed on the study. All embryos were routinely cultured according to the sequential culture method until the 6th or 7th day, classified as discarded embryos, which were regarded as unsuitable for clinical transfer or freezing according to the Gardner criteria. To safeguard the privacy of the donors, all personal information was strictly confidential. No economic interests were involved in the donation process.

XF-hEPS cell line derivation and cultivation

Preparation of XF- hEPS cell derivation media

Taking 50 ml as an example, it was necessary to add 25 ml of DF12 and 25 ml of neurobasal + 10 μg/ml Insulin + 5.5 μg/ml Transferrin + 1 ng/ml selenium sodium, and 50 ng/ml ethanolamine + 10 ng/ml hLif + 1 μM ChIR + 2 μM (S)-(+)-dimethindene maleate + 2 μM minocycline hydrochloride + 5 μM Y27632 + (5–20) ng/ml Activin A + 5000×human catalase (liquid) + 100 μg/ml L-ascorbic acid-2-phosphate + 5% xeno-free KSR (which could be removed after XF-hEPS cells were expanded to the P_3 generation).

Primary isolation of XF-hEPS cells

(a) Zona pellucida removal treatment of blastocysts: Placed the blastocyst in GMOPS-PLUS medium prewarmed to 37 °C, using a Nikon ECLIPSE Ti microscope with infrared laser to cut off the junction between any two trophoblast cells connections, released the blastocyst cavity fluid and the entire blastocyst shrank and separated from the zona pellucida. Repeated pipetting of the embryo with a Pasteur pipette enabled the blastocyst to quickly "protrude" from the zona pellucida, which was then placed in a hypoxic incubator for culture. The duration of this procedure was not more than 2 min. (b) Blastocyst inoculation: One hour after zona pellucida removal, the blastocyst was transferred to the blastocyst inoculation well coated with laminin 521 and incubated at 37 °C, 5% CO_2 and 6% O_2; (c) Half of the liquid was changed on the 4th day, observed the outgrowth morphology and measured the area every day from the 4th to the 6th day when the growth rate of the outgrowth cell mass area slowed down or stopped growing, used Accutase enzyme for passaging; (d) Outgrowth passage and successful isolation of XF-hEPS cells: Accutase enzyme and centrifugation at 1600 rpm for 5 min were used to passage the outgrowth. The medium was changed every 2–3 days until XF-hEPS cells grew out. When the

clone grew to a sufficiently large size, it performed cell passage expansion and cryopreserved.

Pluripotency detection of XF-hEPS cells

The Pluripotency of XF-hEPS cells was detected for pluripotency Markers' expression, multiple differential abilities, and chimera capability.

Pluripotency markers expression detection The expression of pluripotent markers such as OCT-4, Sox2, Nanog, KLF4, FOXA2, GATA6, and TUJ1 in XF-hEPS cells were detected by immunofluorescence staining. XF-hEPS cells were washed with PBS and then fixed (for instance, using 4% paraformaldehyde). The cells underwent permeabilization treatment with 0.1% Triton X-100 to make the cell membrane permeable. To block non-specific binding sites, 5% BSA was used. The cells were incubated overnight with specific primary antibodies against OCT-4, Sox2, Nanog, KLF4, FOXA2, GATA6, and TUJ1 (at a dilution ratio of 1:1000). The cells were then washed, and a fluorescently labeled secondary antibody (dilution ratio of 1:1000) was added for incubation for 1 h. After washing the cells again, DAPI was used to stain nucleic acids and label the cell nucleus. The immunofluorescent stained cells were observed and photographed under a fluorescence microscope.

The relative expression of EPS-specific markers such as NKD1, STOX1, LEF1, UTP14A, WNT8A, and MFSD5 in XF-hEPS cells was detected by qPCR, compared to H9 cells (a traditional embryonic stem cells line). Firstly, total RNA was extracted from the XF-hEPS and H9 cells and transcribed into cDNA using reverse transcriptase. After that, qPCR reactions were performed using specific primers and SYBR Green fluorescent dye. Finally, the relative mRNA expression levels were calculated by comparing the relative expression levels of target genes and internal reference genes in different samples. The primers used were listed in Supplemental Table 1. Data were analyzed using the SPSS22.0 soft package. The differences between the two groups were compared by Student's unpaired t-test. $P < 0.05$ was considered statistically significant.

The multiple differential ability of XF-hEPS cells was detected by EB and teratoma formation For EB, XF-hEPS cells were dissociated into single cells and plated on ultra-low attachment plates in IMDM (Thermo Fisher Scientific,12440-053) supplemented with 15% FBS at a density of 5×10^5 per well for 7 days. Medium change was performed every 2 days. Then, EBs were plated on Matrigel-coated 4-well plates in the same medium for another 7 days. Then, the cells were fixed for immunofluorescence staining of markers presentive for endoderm (FOXA2), mesoderm (GATA6), and ectoderm (TUJ1). To examine the long-term differentiation potential, EB formation and differentiation were performed on XF-hEPS cells in Passages 8 and 20.

For Teratoma formation, XF-hEPS cells were resuspended in PBS supplemented with 30% Matrigel. Kept on ice and drawn into 1 ml syringe immediately before injection. Approximately $2–5 \times 10^6$ cells were injected in the dorsolateral area into the subcutaneous space of NPG mice (Beijing Vitalstar Biotechnology, Beijing 100012, China). Teratomas were recovered 6–8 weeks after grafting, and the animals were euthanized before the tumor size exceeded 1.5 cm in diameter. The teratomas were then embedded in paraffin and processed for hematoxylin and eosin staining of markers presentive for endoderm, mesoderm, and ectoderm.

The chimera capability of XF-hEPS cells was detected by the chimera test We first established XF-hEPS cells with GFP-labeling (hEPSC-Ef1a-GFP-Neo) using the following method. HEPSC-Ef1a-GFP-Neo cell line was established by nucleofection of PBase and PB-Ef1a-GFP-Neo plasmid via Lonza 4D-Nucleofector with P3 Buffer (Lonza, V4XP-9096) into hEPSCs under XF-LCDM medium. Cells were then cultured in chemically defined and xeno-free medium supplemented with 400 μg/mL G418 for 2 passages. The remaining cells were then recovered for 1 passage for further experiment.

For the chimera test, GFP-labeled EPS colonies were picked and disaggregated to a single-cell suspension by pipetting up and down several times in 0.25% trypsin-EDTA. Then trypsin was neutralized, and the cells were centrifuged at 250 g for 3 min at room temperature and resuspended in M2 medium on an ice bath. Approximately 10 to 12 GFP-labeled cells were injected into an 8-cell mouse embryo of ICR background. Chimera embryos were observed every day to detect the presence of fluorescent positive cells by an inverted microscope, and E4.5 chimeral mouse embryos were detected for the immunofluorescence of CDX2 and GFP. E4.5 embryos were first washed in PBS (Corning, 21-040-CVR) droplets 3 times and fixed in 4% paraformaldehyde (DingGuo, AR-0211) at room temperature (RT) for 20 min. Embryos were then blocked with 3% normal donkey serum (Jackson ImmunoResearch, 017-000-121) in PBS plus 0.2% Triton X-100 (Sigma-Aldrich, T8787) at RT for 1 h. Primary antibodies of GFP (1:500, Invitrogen, 2339839) and CDX2 (1:500, BIOGENEX, MU392A) were diluted with a blocking solution and embryos were incubated at 4 °C overnight. The embryos were then washed 3 times in PBS droplets and incubated with secondary antibodies (1:200, Jackson ImmunoResearch) at room temperature for 1 h. After the final washes, embryos were transferred to a confocal dish in PBS droplets covered with paraffin for imaging.

The mice were all purchased from Beijing Vitonlihua Co., Ltd. Mice were bred and housed in an SPF environment.

Morphology, doubling time, and karyotype analysis of XF-hEPS cells

The cell nucleus, cytoplasm, cell membrane of XF-hEPS cells, and the size of XF-hEPS clone were observed and recorded by electron microscopy in the process of derivation.

To detect the proliferation ability of XF-hEPS cells, we calculated the doubling time of the cells. XF-hEPS cells were dissociated into single cells and seeded onto Laminin 521 pre-coated 4-well plates separately at a density of $4*10^4$ cells per well. Cell number was counted using a hemocytometer every 24 h. The doubling time was calculated using the formula: Doubling time = 48×[log2/logNt (number of cells on day 4) -logNo (number of cells on day 2)].

The diploidy of XF-hEPS cells was analyzed using karyotype testing. After cells were treated with colchicine, chromosome slides were prepared through digestion, centrifugation, hypotonic treatment, and fixation. After baking the slides, they were treated with trypsin, and the banding time was adjusted. Then, they were stained with Giemsa, rinsed, and dried for microscopic examination. First, used a 10x lens to find the chromosome karyotype field of view and then switched to an oil immersion lens for observation and photographing. The chromosomes were counted, paired, sorted, and analyzed. Chromosomes with good resolution were carefully selected for karyotype analysis, with 20 cells analyzed per cell line. To examine the long-term genetic stability, karyotype was separately conducted on XF-hEPS cells in Passages 8 and 20.

Genetic testing and bioinformatics analysis

To compare whether the expression profiles of XF-hEPS cells were more similar to hEPS cells on feeder or H9 cells, we sequenced high-throughput expression profiles of all three cell lines.1×10^6 cells were collected for each line and sequenced according to standard procedures. The subsequent library construction and sequencing process was commissioned by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The Illumina NovaSeq Reagent Kit was used for library construction, and bridge PCR amplification was performed on cBot to generate clusters, followed by sequencing on the Illumina NovaSeq 6000 platform. Firstly, the software fastp (https://github.com/OpenGene/fastp) was used to filter the raw sequencing data. After aligning with the reference genome, reads for subsequent transcriptome expression quantification were obtained. The default screening criteria for significantly differentially expressed genes were FDR < 0.05 &|log2FC| ≧ 1. A VENN diagram was created for the differentially expressed genes among various samples, and enrichment analysis was performed. The hEPS cells on feeder and H9 cell line used in this study were generously provided by Professor Hongkui Deng's laboratory (the Key Laboratory of Cell Proliferation and Differentiation of the Ministry of Education, School of Life Sciences, Peking University).

Underlying mechanisms analysis using bulk RNA sequencing

Given that the XF-hEPS condition improved cell line establishment efficiency and cell survival compared to traditional feeder-based hEPS and H9 cultures, we investigated the underlying mechanisms using bulk RNA sequencing. Differential gene expression (DEG) analysis was performed with R package Deseq2. Both differences between XF-hEPS and H9 cells and between XF-hEPS cells and hEPS cells on feeder were detected. Gene Ontology (GO) enrichment analysis with the DAVID database (https://davidbioinformatics.nih.gov) was performed on their shared genes. P value < 0.05 was set as the threshold of significance. Protein-protein interaction analysis with the STRING database (https://cn.string-db.org/) was performed on proliferation-related and anti-apoptotic upregulated genes in XF-hEPS cells. The minimum required interaction score was set as 0.4 (medium confidence) as default. Finally, STRING-GO and STRING-WikiPathways enrichment analyses were also performed with the STRING database to delineate core regulatory networks.

Result

XF-hEPS cell lines were derived successfully and efficiently

As shown in the derivation flow chart (Fig. 1A), the blastocysts without zona pellucida adhered within 24 to 36 h after plating. Outgrowths could be observed after four to five days, and the first passaging could be performed from the sixth to the eighth day. The XF-hEPS cells in passage 1 exhibited typical nest-like growth with large and flat-shaped colonies, a high nucleus-to-cytoplasm ratio, less cytoplasm, and no visible organelles or granules (Fig. 1B), which was similar to that of hEPS cells on feeder. When the cells reach 80-90% confluence, the Tryple Express enzyme is used for passaging. The doubling time of XF-hEPS cells was 45.16 h. XF-hESP cells have a normal karyotype (46, XX or 46, XY) and can be stably passaged for more than 20 generations while maintaining karyotypic stability (Fig. 1C). With chemically defined and xeno-free media, we established 6 XF-hEPS cell lines from 13 discarded blastocytes. Meanwhile, we successfully established hEPS cell lines on feeder-dependent conditions, following the protocol previously reported by Yang et al. [1]. The establishment efficiency reached up

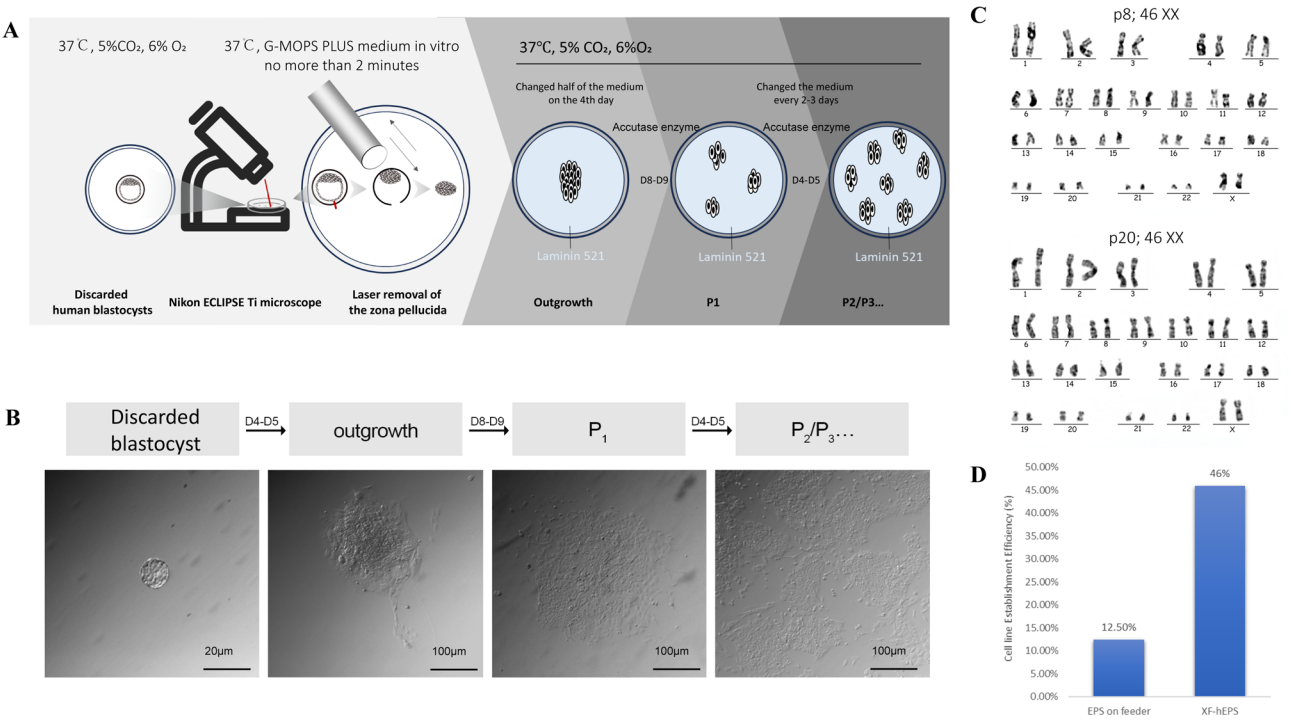

Fig. 1 Derivation of hEPS cell lines from discarded blastocysts under chemically defined and xeno-free condition. **A** Flow chart of the derivation of XF-hEPS cells. **B** Dynamic morphological changes during the establishment of XF-hEPS cells. **C** Karyotype analysis of XF-hEPS cells in passage 8 and 20. **D** hEPS cell lines establishment efficiency under feeder and chemically defined and xeno-free condition

to 46%, which was significantly higher than that of feeder (12.5%) (Fig. 1D).

The pluripotency of XF-hEPS cells

The typical pluripotent markers, such as OCT4, Sox2, Nanog, and KLF4, were all expressed in XF-hEPS cells (Fig. 2A). Furthermore, the EPS-specific markers such as DDX3X, LEF1, NKD1, STOX1, UTP14A, WNT8A, and MFSD5 in XF-hEPS cells were much higher than that in H9 cells (Fig. 2B).

In vitro, XF-hEPS cells could form EB efficiently. After plating and cultured in serum condition, the EB could automatically differentiate into cells from endoderm (FOXA2), mesoderm (GATA6), and ectoderm (TUJ1) (Fig. 3B). When XF-hEPS cells were cultured in vitro to the passage 20, the XF-hEPS cells could also stably differentiate into cells from endoderm (FOXA2), mesoderm (GATA6), and ectoderm (TUJ1) (Fig. 3C).

In vivo, XF-hEPS cells could form teratoma efficiently. The teratoma contained typical gland-like structures of the endoderm, vascular-like structures of the mesoderm, and structures resembling the neural ectoderm (Fig. 3A).

All in all, the XF-hEPS cells were pluripotent and able to differentiate into three germ layers both in vivo and in vitro, akin to hEPS cells on feeder.

Embryonic and extraembryonic differentiation capacity of XF-hEPS cells

The difference between the pluripotency of EPS and classical embryonic stem cells relied on the capability of developing into embryonic and extraembryonic germ cells. By injecting XF-hEPS cells labeled with GFP into mice 8-cell embryos and then observing GFP positive cells' distribution for 3 days in vitro cultivation, we found GFP positive cells in mice embryos both within morula and blastocytes stage (Fig. 4A and B). In some blastocysts, the GFP-positive cells could be observed both in inner cell mass and trophectoderm cells' position (Fig. 4B). By immunofluorescence staining of GFP and CDX2 of E4.5 mouse blastocysts, we further confirmed that GFP labeled XF-hEPS cells presented in the extraembryonic trophoblast layer, which means the embryos presented with GFP+/CDX2+ (Fig. 4C). Taken together, these data suggested that XF-hEPS cells had extraembryonic developmental potential like hEPS cells on feeder.

XF-hEPS cells exhibited expression patterns that are closer to hEPS cells on feeder compared to that of H9 cells

We compared the mRNA expression levels of XF-hEPS cells, hEPS cells on feeder, and H9 ESCs through transcriptome sequencing. The clustering heatmap showed that on the mRNA expression level, XF-hEPS first aggregated with hEPS on feeder and then aggregated with H9. (Fig. 5A). Moreover, PCA analysis also clearly showed

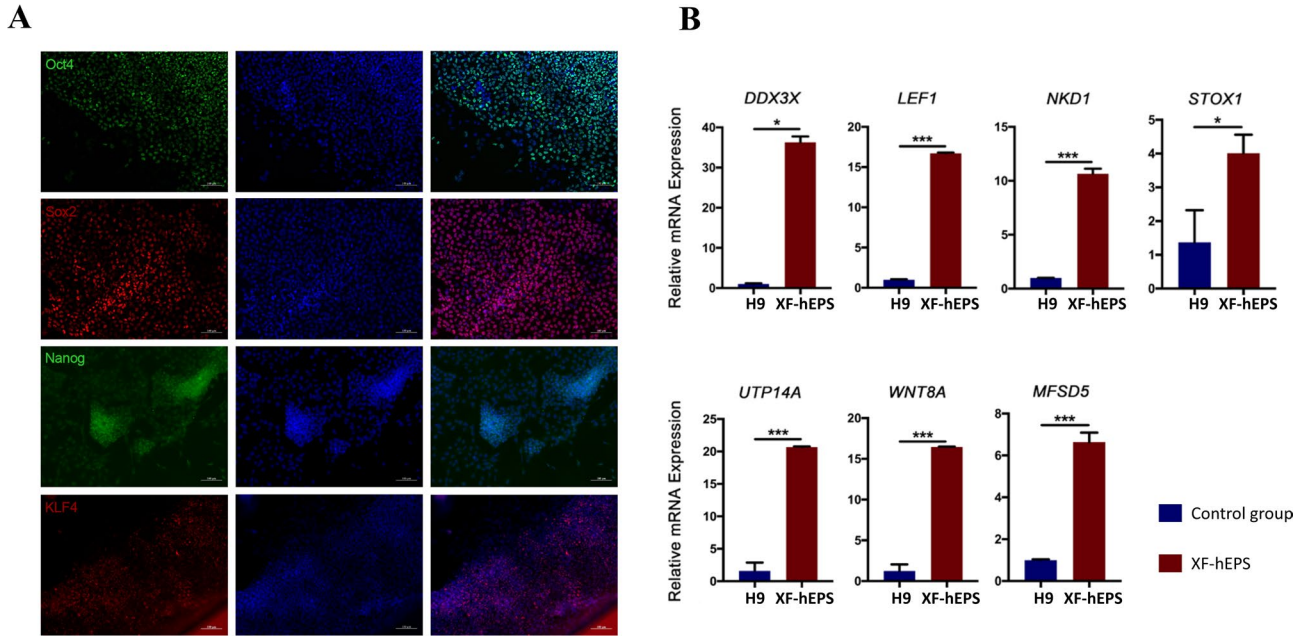

Fig. 2 Characterization of molecular features of XF-hEPS cells. **A** Immunofluorescence staining showing the expression of pluripotency markers OCT4(green), SOX2(red), Nanog(green), KLF4(red) in XF-hEPS cells. Similar images were obtained in three independent experiments. **B** qPCR analysis shows relative mRNA expression levels of EPS-specific markers DDX3X, LEF1, NKD1, STOX1, UTP14A, WNT8A, MFSD5 in H9 ESCs and XF-hEPS cells. Error bars, mean ± SD. All differences between means with $*p < 0.05$, $***p < 0.001$. Statistical significance was analyzed using unpaired t-test

that XF-hEPS are more proximate to hEPS on feeder than H9 spatially (Fig. 5B). Further comparison of the Transcripts Per Million (TPM) value of the gene revealed that there were fewer differentially expressed genes between XF-hEPS cells and hEPS cells on feeder (Fig. 5C). In summary, XF-hEPS cells' expression patterns are closer to hEPS cells on feeder compared with H9 cells on the level of mRNA.

Improved cell line establishment and survival under XF-hEPS conditions are associated with enhanced proliferation and anti-apoptotic gene networks

DEG analysis between XF-hEPS and H9 cells identified 3903 significantly upregulated genes in XF-hEPS cells. A similar comparison between XF-hEPS and hEPS cells on feeder revealed 2586 upregulated genes, with 1410 genes commonly upregulated in both comparisons (Fig. 6A). Gene Ontology (GO) enrichment analysis of these shared genes highlighted terms related to stem cell fate specification, embryonic patterning, cell proliferation (e.g., "positive regulation of cell population proliferation"), and apoptosis suppression (e.g., "negative regulation of apoptotic process") (Fig. 6B), suggesting that XF-hEPS condition enhances cellular fitness.

Next, we focused on 44 proliferation-related (Fig. 6C) and 43 anti-apoptotic genes (Fig. 6D) upregulated in XF-hEPS. Protein-protein interaction (PPI) analysis via STRING, combined with WikiPathways enrichment, revealed that these genes were associated with pluripotency regulation (e.g., embryonic stem cell pluripotency pathways) and key proliferation pathways (e.g., PI3K-Akt and MAPK signaling) (Fig. 6E), further supporting XF-hEPS cells' proliferative advantage.

The STRING-GO analysis demonstrated tight connectivity between proliferation and anti-apoptotic gene sub-networks (Fig. 6F). Strikingly, six FGF family genes (*FGF4*, *FGF8*, *FGF17*, *FGF18*, *FGF19*, *FGF21*) were centrally positioned within these networks (Fig. 6G), suggesting that FGF signaling activation in XF-hEPS may drive the observed improvements in cell line establishment and survival over the traditional condition.

Discussion

Human extended pluripotent stem cells (hEPS) are vital resources in developmental biology and regenerative medicine research, bearing the potential to differentiate into both embryonic and extraembryonic lineages since extended pluripotency represents an intermediate state between primed pluripotency and naïve pluripotency. hEPS cells exhibit a unique transcriptional signature similar to that of pre-implantation embryos, indicating a higher differentiation potency. They can be utilized to imitate early embryonic development in vitro and perform as a promising model for screening new drugs and for research in pharmacology, toxicology, and early human embryonic development.

Currently, no more than 20 reported hEPS cell lines have been established [1, 11, 15]. One of the acquisition

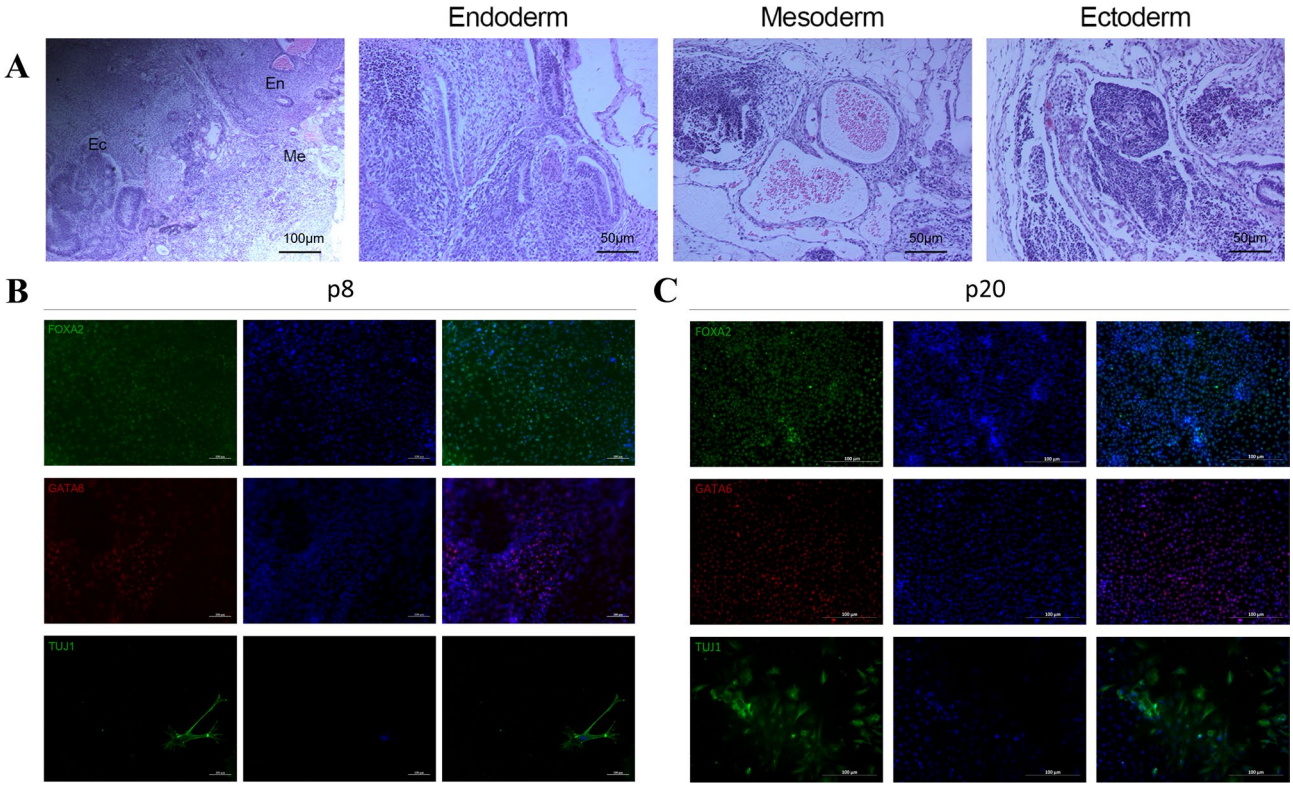

Fig. 3 Characterization of embryonic differentiation capacity of XF-hEPS cells in vivo and in vitro. **A** Teratomas histological analysis of the three germ layers generated from XF-hEPS cells injected into mice. Similar images were obtained in at least three independent experiments. **B** EB immune-staining images showing the lineage markers FOXA2(Endoderm), GATA6(Mesoderm), TUJ1(Ectoderm) formed in the culture of XF-EPS cells in vitro in passage 8. Similar images were obtained in at least three independent experiments. **C** EB immune-staining images showing the lineage markers FOXA2(Endoderm), GATA6(Mesoderm), TUJ1(Ectoderm) formed in the culture of XF-EPS cells in vitro in passage 20. Similar images were obtained in at least three independent experiments

and culture of hEPS rely on traditional culture systems with feeders and animal serum, which makes the establishment of hEPS cell lines from human embryos challenging and with high batch effects. Another approach involves using four transcription factors (Oct4, Sox2, KLF4, and c-Myc) to reprogram somatic cells into induced pluripotent stem cells (iPSCs) in a primed state, then cultured under specific conditions to transform the iPSCs into hEPS cells [1]. However, the induction process of iPSCs involves the introduction of exogenous genes, increasing the risk of harmful gene mutations [16]. In our research, chemical-defined and xeno-free culture conditions utilize recombinant proteins, small molecules, and other components such as substitutes for animal-derived serum proteins instead of feeder cells and animal serum to maintain cell growth [17]. The chemically defined and xeno-free culture condition is simple to operate have well-defined components with controllable concentrations and relatively stable and reliable sources. It avoids the risks of animal-sourced viral infections and xenogeneic gene integration, which helps to enhance the safety and quality stability of hEPS. Such systems are more suitable for clinical cell replacement therapies and for conducting basic research on molecular mechanisms. Meanwhile, we have chosen laminin 521 as the culture substrate to support the culture of hEPS cells, as Miyazaki et al. and Rodin et al. showed that laminin-511/521 are more suitable to support hPSC culture compared with other extracellular matrix proteins [18, 19],. This also helps to reduce the animal-derived components brought by feeder cells.

How to efficiently establish high-quality feeder-free and xeno-free hEPS cell lines from embryos has always been a bottleneck. In this study, we efficiently established hEPS cell lines from early abandoned human embryos using the chemically defined and xeno-free culture method. We believe that the culture system is most closely related to the efficiency of establishing cell lines [20–24]. Previous studies have shown that serum-free culture systems significantly improve the efficiency of establishing cell lines compared to traditional serum-containing feeder systems [25]. Our findings also corroborate this observation. The efficiency of establishing cell lines under feeder-free conditions was 46%, much higher than the 12.5% under feeder conditions. When the number of primary cells is limited, a chemically defined and xeno-free medium is

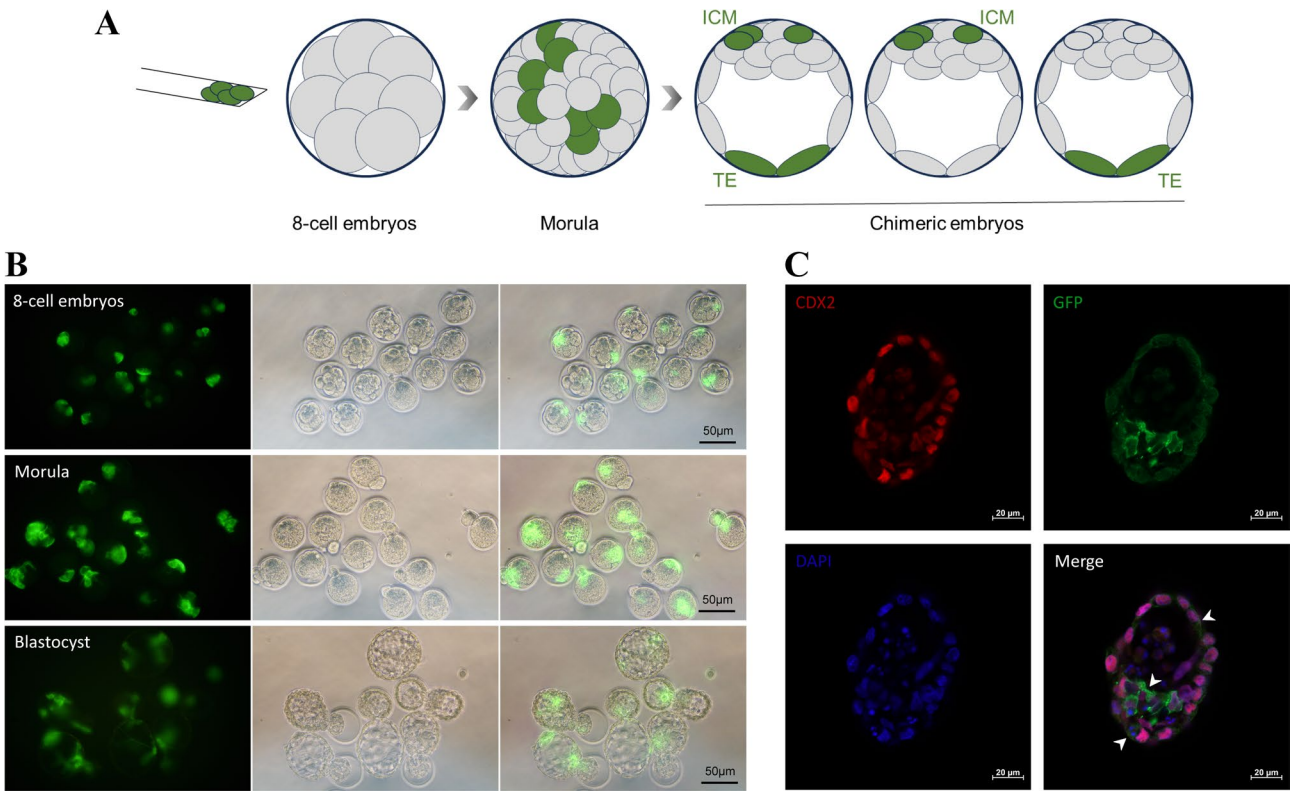

Fig. 4 XF-hEPS cells produce extraembryonic tissues in chimeric mice. **A** Schematic diagram displaying the procedure of generating E4.5 chimeric blastocysts. **B** Representative images of chimeric embryos injected with GFP + XF-hEPS cells (green) at developmental stages of 8-cell, morula and blastocyst. **C** Immunofluorescence analysis of chimeric blastocysts injected with GFP + XF-hEPS cells (green). GFP labeled XF-hEPS cells presented in both inner cell mass (indicated by arrowhead) and the extraembryonic trophoblast layer (indicated by tailed arrow)

more beneficial for the survival and proliferation of EPS cells than a traditional feeder culture. We promote that the timing and manner of subculturing at the P0 stage may also be crucial factors influencing the success rate of cell line establishment. When cells within an outgrowth colony proliferate to a certain extent, the internal cells, derived from the inner cells, cannot directly contact the feeder layer and culture medium due to the tight stacking of the colony, leading to nutrient deprivation and accumulation of metabolic byproducts, leading to deterioration of the cell growth environment and inability to sustain cell growth or even causing cell damage and death. Hence, it is crucial to passage at the proper time. Our study found that the first passage time was on the fourth day under feeder-free and xeno-free conditions. Additionally, mechanical methods are often used to cut and passage the outgrowth in previous reports on establishing cell lines. Although this approach can help maintain a relatively pure stem cell population through passaging, the procedure is complex and the outcomes are closely related to the skill and proficiency of the operator. In the context of this study, we used Accutase for digestion and passaging, which can disperse the dense outgrowth cell colonies into single cells. This digestion fluid is free of animal-derived components, causing less cell damage compared to trypsin [26]. It can be used for a long time while greatly reducing cell damage. It allows for long-term use that substantially minimizes cell injury. Notably, when the cells were enzymatically dissociated, survival efficiency was greatly improved [24, 26]. Compared with previous methods [11–14], the method presented here allowed for EPS cell growth for over 20 generations without apparent changes in phenotype and genetics. The protocol also allows obtaining EPS cells directly from blastocysts without having to isolate ESCs or iPSCs.

The protocol proposed here allows obtaining EPS cells directly from blastocysts without having to isolate ESCs or iPSCs. In fact, the main advantage of hEPS cells derived directly from blastocysts, when cultured under undifferentiated and feeder-free conditions, compared to hEPS cells transformed from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), is the avoidance of genetic risks associated with iPSC reprogramming. Indeed, reprogramming somatic cells into iPSCs (even if later converted into hEPS cells) often requires the introduction of foreign genes (e.g., Oct4, Sox2, KLF4, and c-Myc) [27–29]. This process increases

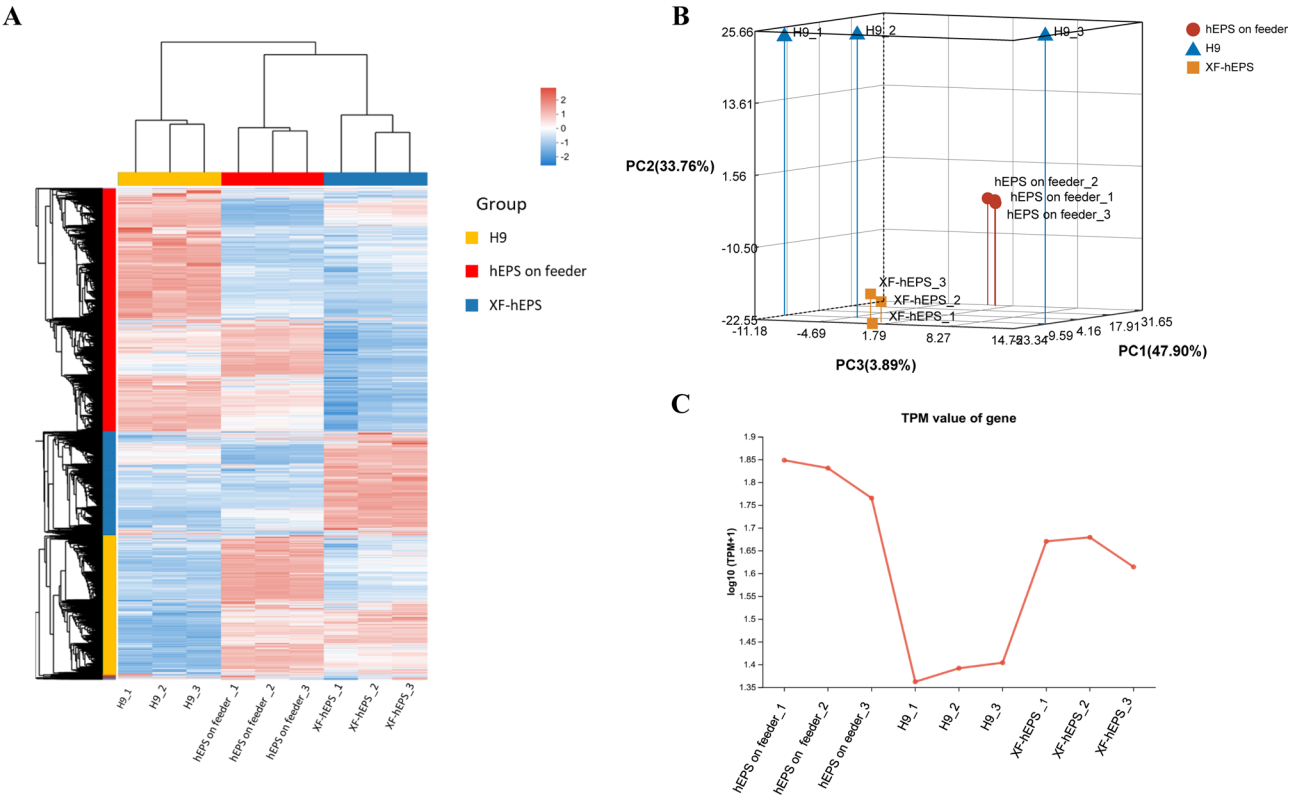

Fig. 5 Transcriptome Sequencing Analysis of multiple pluripotent stem cell lines. **A** Heatmap showing gene expression profiles of H9 ESCs, hEPS on feeder condition and XF-hEPS cells. **B** PCA plot illustrating the expression patterns of H9 ESCs, hEPS cells cultured on feeder condition and XF-hEPS cells spatially. **C** TPM values of gene display each sample of H9 ESCs, hEPS cells cultured on feeder condition and XF-hEPS cells

the risk of genetic instability and developing harmful genetic mutations [30, 31]. Therefore, avoiding reprogramming the cells also avoids the risk of mutation or manipulating the cell genome in a way that could be harmful to the cells, the models using those cells, or for eventual therapeutic purposes. The advantages of using blastocyst derivation include that the hEPS cell lines can be isolated and established directly from waste blastocysts using optimized species, without xenogeneic feeder cells and chemical-free limited media, and bypassing the somatic cell reprogramming step. This approach avoids the risks of transmitting pathogens, exposure to animal antigens that could trigger immune responses, culture contamination by feeder cells [9, 10], and genetic instability or mutations. Therefore, the cells obtained using the methods reported here may have advantages in terms of safety and quality stability, especially for potential clinical applications. Additional studies remain necessary to examine the stability of the cells and the safety of their eventual use for clinical purposes.

The present study had some limitations. While the paper mentions previous methods and improvements, it lacks a direct, detailed comparative analysis between the new method and existing protocols beyond efficiency metrics. Future studies should direct metrics (e.g., cost analysis and practical challenges) among available methods. Second, the study was performed at a single center with a specific set of conditions. Multi-center studies or external validations would be beneficial to ensure the reproducibility and scalability of the findings.

With the continuous advancement of technology, there may be more new methods and approaches to obtain human pluripotent stem cells in the future. These stem cells have broad application prospects in disease modeling, drug screening, regenerative medicine, and other fields, providing new treatment strategies for many currently incurable diseases.

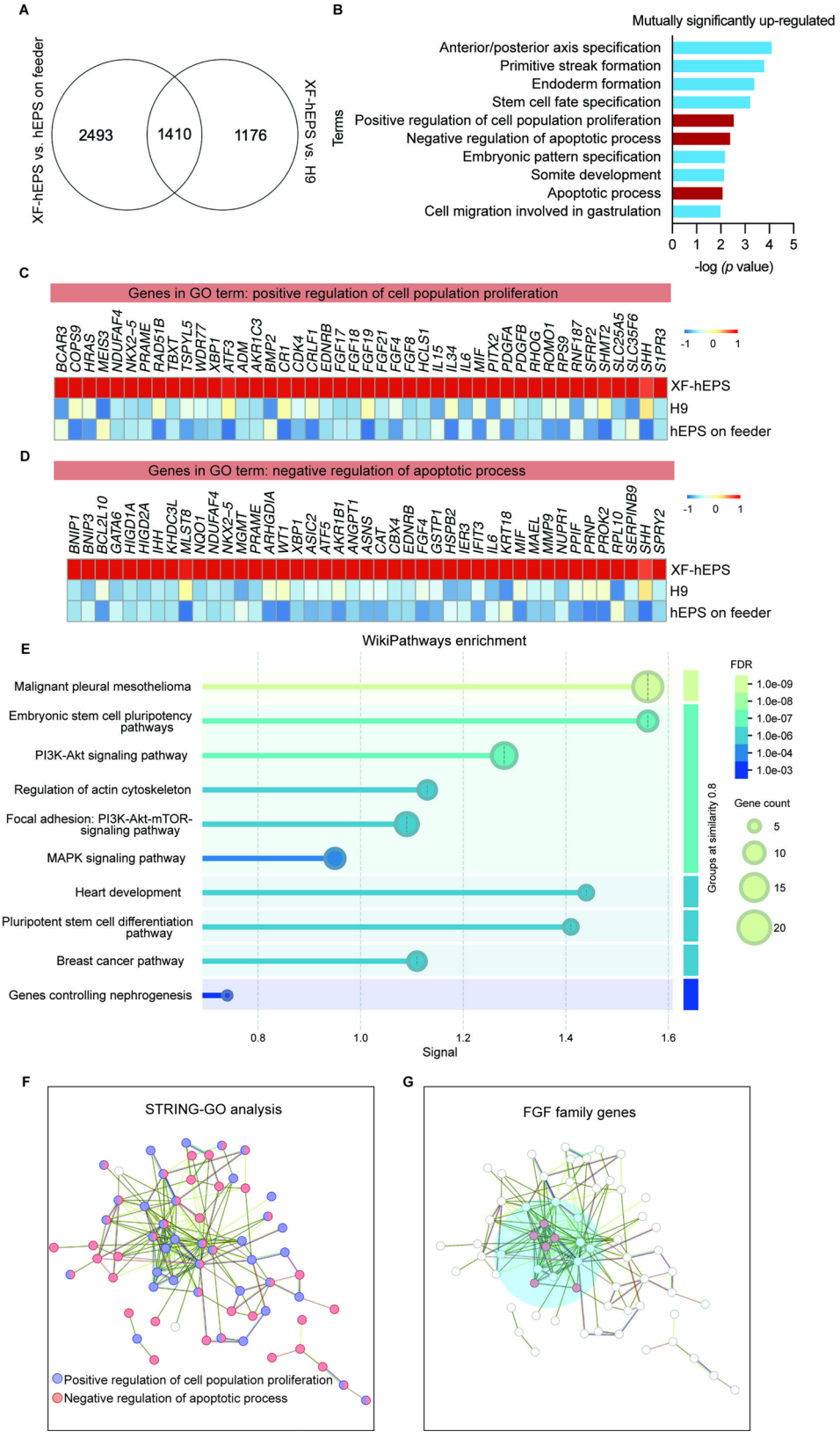

Fig. 6 (See legend on next page.)

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Fig. 6 XF-hEPS cells possess enhanced proliferation and anti-apoptotic gene networks. **A**. Venn diagram presenting the overlap of significantly upregulated genes in XF-hEPS compared with hEPS on feeder and H9. **B**. GO term enrichment analysis of mutually significantly-upregulated genes in XF-hEPS compared with hEPS on feeder and H9. **C**. Heatmap showing genes significantly-upregulated genes in XF-hEPS compared with hEPS on feeder and H9 and were annotated to positive regulation of cell population proliferation. **D**. Heatmap showing genes significantly-upregulated genes in XF-hEPS compared with hEPS on feeder and H9 and were annotated to negative regulation of apoptotic process. **E**. Protein-protein enrichment combined with WikiPathways enrichment of significantly-upregulated genes annotated to positive regulation of cell population proliferation and negative regulation of apoptotic process in XF-hEPS compared with hEPS on feeder and H9. **F**. Protein-protein enrichment combined with GO enrichment analysis of significantly-upregulated genes annotated to positive regulation of cell population proliferation and negative regulation of apoptotic process in XF-hEPS compared with hEPS on feeder and H9. **G**. Protein-protein enrichment analysis for core genes of significantly-upregulated genes annotated to positive regulation of cell population proliferation and negative regulation of apoptotic process in XF-hEPS compared with hEPS on feeder and H9. FGF family genes were highlighted

Supplementary Information

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Supplementary Material 1

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Author contributions
Zhuran Zhao: proposed the concept; designed and performed the study; wrote the initial manuscript. Xi Chen: proposed the concept; designed the study and revised the manuscript. Shan Wang: performed the study. Cheng Shi: proposed the concept; designed and performed the study; wrote the initial manuscript. Jiong Qin: proposed the concept; designed the study; wrote the initial manuscript. Huan Shen: Enrolled patients and collected samples. Min Fu: supervised samples and data collection. Jiayu Li: performed the study. Jun Xu: designed the study and revised the manuscript.

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Data availability
The data supporting this study's findings are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study has been granted approval by the Ethics Committee of Peking University People's Hospital (No. 2022PHB160-001). ALL animal experiments were conducted in accordance with the 3R principles and under the supervision of the Ethics Committee of Peking University People's Hospital (2023PHE104).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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