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Abstract

Successful derivation of mouse trophoblast stem cells (TSCs) and trophectoderm stem cells (TESCs) greatly facilitates understanding of trophoblast differentiation. However, understanding of early trophectoderm differentiation remains incomplete. Here, we report establishment of a morula-derived trophectoderm stem cell line (MTSCs) from 32-cell embryos that show enhanced and uniformed trophoblast core gene expression. Importantly, distinct from TSCs or TESCs, MTSCs represent a much earlier trophectoderm state (E3.5) than those of TSCs(E5.5-6.5) and TESCs(E4.5-5.5). MTSCs can robustly integrate into all cell lineages of the placenta. Moreover, MTSCs can self-organize to form placenta organoids. When partially differentiated MTSCs aggregated with embryonic stem cells (ESCs), they form blastoids that efficiently implant uteruses. Finally, MSTC medium can efficiently convert ESCs into MTSC-like cells. Thus, MTSCs capture an early blastocyst trophectoderm state and provide a research model for studying trophoblast development.

Materials

	A	B	C
	REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
	Chemicals, peptides, and recombinant proteins		
	RPMI 1640	Gibco	61870036
	DMEM/F-12	Gibco	10565018
	Neurobasal	Gibco	21103049
	GlutaMAXTM	Gibco	35050038
	KnockOut‰Serum Replacement	Gibco	10828-028
	MEM NEAA	Gibco	2281490
	Sodium Pyruvate	Gibco	11360070
	B-27 supplement	Gibco	17504-044
	N-2 supplement	Gibco	17502-048
	Acid Tyrode's Solution	Sigma	T1788
	Hyaluronidase	Sigma	H3506
	Trypsin-EDTA	Gibco	25200056

	A	B	C
	TryPLETM Express	Gibco	12605010
	Gelatin	SARTORIUS	01-944-1B
	OVOILTM	Vitrolife	10029
	2-mercaptoethanol	Gibco	31350010
	L-ascorbic acid 2-phosphate	Sigma	A8960
	Penicillin/streptomycin	Gibco	1114050
	Insulin-Transferrin-Selenium	Gibco	41400-045
	rhFGF-4	R&D system	235-F4
	Heparin	Sigma	H3149
	rhTGF-β1	R&D system	7754-BH-025
	rhBMP4	R&D system	314-BP-010/CF
	Activin A	PeproTech	120-14E
	CHIR99021	Tocris	4423/10
	PD0325901	Tocris	4192/10
	mLIF (ESGRO)	Merck	ESG1107
	XAV939	Sigma	X3004

	A	B	C
	A-83-01	Tocris	2939/10
	Lats-IN-1	TargetMol	T9053
	Y27632	Tocris	1254
	FBS	Vivacell	C04002-500
	FBS	Gibco	16141-079
	BSA	Sigma	A1933
	Mitomycin C	Sigma	M4287
	Sodium selenite	Sigma	S5261
	Transferrin	Sigma	T8158
	8-Br cAMP	BIOLOG	B 007-500
	rmIL-11	PeproTech	220-11
	Sodium Bicarbonate	Sigma	S5761
	Anti-adherence rinsing solution	STEM Technologies	07010
	Gelatin Solution	SARTORIUS	01-944-1B

Derivation of MTSCs Protocol

1 **Preparation of mice**

2 The C57BL/6J and ICR mice were from the Laboratory Animal Center of Yunnan University. All mice were placed in a specific pathogen-free environment, with the temperature controlled (22-24°C) and the light/dark cycle for 12 hours. C57BL/6J mice were used to obtain embryos, and ICR mice were used to prepare pseudopregnant surrogates.

3 **Collection of mouse embryos**

4 At 6-7 PM, 6-8 week-old female mice were given an intraperitoneal injection of 8 IU of PMSG, followed 48 h later by 8 IU of HCG. Subsequently, the female mice were mated 1:1 with the male mice with normal fertility (the female mice were placed in the male cage), and the formation of vaginal plugs was checked and marked at 8-9 am the next day. At 2 p.m., the female mice with vaginal plugs were sacrificed by cervical vertebra removal, and the mice ovary and fallopian tube tissues were obtained from the ultra-clean bench.

5 They were washed twice with PBS buffer, and the enlarged ampulla of the fallopian tube was gently scratched under a stereoscopic microscope with a sterile 1 mL syringe needle to allow the fertilized eggs to overflow naturally. Fertilized eggs were transferred to hyaluronidase solution using a homemade glass Pasteurized pipette for digestion to remove residual cumulus granulosa cells.

6 **In vitro culture of mouse embryos**

7 Fertilized eggs were transferred to KSOM medium and cultured to the 32-cell stage, and the medium was covered with mineral oil. The culture environment was 37 ° C, 5% CO2 and 5% O2, and near saturated humidity.

8 **Preparation of MTSC medium**

9 MTSC medium consisted of RPMI-1640 basal medium supplemented with 20% FBS (VivaCell), 100× Sodium Pyruvate additive, 200× MEM NEAA additive, 25 ng/mL rhFGF4, 1 mg/mL heparin, 0.1 mM The mixture consisted of β-Mercaptoethanol, 1 µM XAV939, 2 µM Lars-IN-1, 10 µM Y27632, 10 ng/mL rhBMP4, 20 ng/mL Activin-A, and 100× penicillin/streptomycin. A 0.22 µm syringe filter was used to filter the medium, and the

prepared medium was stored at 4 ° C. After storage for more than 10 days, the medium was discarded and fresh medium was prepared.

10 **Derivation of MTSC**

11 The zona pellucida of 32-cell stage embryos was removed with acidic Tyrode solution and placed in a 96-well culture plate preseeded with Feeder cells, in which one embryo was placed per well. 150 µL of MTSC medium was added to each well, and the medium was changed every 2 days.

12 After 24 hours, the Outgrowth attached to the feeder layer and began to proliferate outward. After 4-5 days, dark cells in the middle of the derivative were carefully removed with a homemade bent glass needle, the remaining cells were dissociated with TrypLE for 6 min, and the derivative was gently separated into a number of cell clumps using a homemade glass needle, which were passed into new wells for continued culture.

13 Three days later, a single stem cell colony was selected for expansion, and purified MTSC cell lines were obtained.

14 During subculture, when the confluence degree of MTSC cells reached 80-90%, the cells were washed twice with PBS and then digested with appropriate Accutase cell dissociation solution or TrypLE trypsin replacement solution for 8-10 minutes. The cells were recovered by centrifugation and resuspended, and subcultured according to the ratio of 1:4.

15 The feeder layer cells in MTSC need to be removed first when performing embryo chimeric experiments or embryo-like reconstruction experiments. The resuspended cells were seeded in a culture plate precoated with 0.1% gelatin and incubated in an incubator for 30-40 min, at which point most Feeder cells had attached to the bottom of the dish. The plates were gently shaken, the cell suspension was collected and centrifuged, and subsequently MTSC containing almost no Feeder cells was obtained.

16 **Culturing of MTSC organoids**

17 MTSCs were dissociated with 0.05% Trypsin-EDTA (Gibco, 25200056) for 5 min, and resuspended in growth factor reduced matrigel (Corning, 356231) at the liquid state on ice.

18 One 30 µL drop containing 8000-10000 cells was plated per well onto a 24-well culture plate, set at 37°C for 10 min, and overlaid with 500 µL maintenance medium and cultured in a humidified incubator supplemented with 5% CO2 at 37°C. The medium was replaced every 2 days.

19 MTSC organoids were passaged after 7-8 days with 1:8-10 dilution. Organoids were removed from matrigel by cell recovery solution(Corning, 354253) at 37°C, and dissociated by TryPLETM Express(Gibco, 12605010) for 8 min, gently pipetted every 2-3 min.

20 To differentiate MTSC organoids, the organoids were first cultured in a maintenance medium for 4 days and changed to a differentiation medium for another 4 days, and the differentiation medium was changed daily.

21 Maintenance and differentiation media were prepared according to the **reference 1**.

22 **Preparation of ESC medium**

23 ESC medium consisted of N2B27 medium supplemented with 10 ng/mL rhLIF, 1 μM PD0325901, and 3 μM CHIR99021. The N2B27 medium consisted of a 1:1 mixture of DMEM/F-12 basal medium and Neurobasal basal medium. 100× N2 additive (17502-048), 50× B27 additive, 0.1 mM β-Mercaptoethanol, 100× MEM NEAA additive, 100× GlutaMAX additive, and 5% KnockOut Serum were added The mixture consisted of Replacement and 100× penicillin/streptomycin.

24 A 0.22 μm syringe filter was used to filter the medium, and the prepared medium was stored at 4 ° C. After storage for more than 10 days, the medium was discarded and fresh medium was prepared.

25 **ESC to MTSC-Like conversion**

26 When the growth density of Naive ESCs reached about 60% (about 48 hours after passage), the ESC medium was removed by aspiration and replaced with MTSC medium for induction. The induction medium was changed daily.

27 During subculture, when the confluence degree of the cells reached 80-90%, the cells were washed twice with PBS and then digested with appropriate Accutase cell dissociation solution or TrypLE trypsin replacement solution for 8-10 minutes. The cells were recovered by centrifugation and resuspended, and subcultured according to the ratio of 1:4.

28 There is no need to screen the cells in the subculture process, and all the dissociated cells can be subcultured. The induction medium was changed daily.

29 After 3-4 weeks of subculture, almost all of the cells no longer expressed the ESC marker OCT4, but expressed CDX2. ESC-derived MTSC-like cells could be subcultured and maintained similar cell characteristics to MTSC.

Protocol references

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