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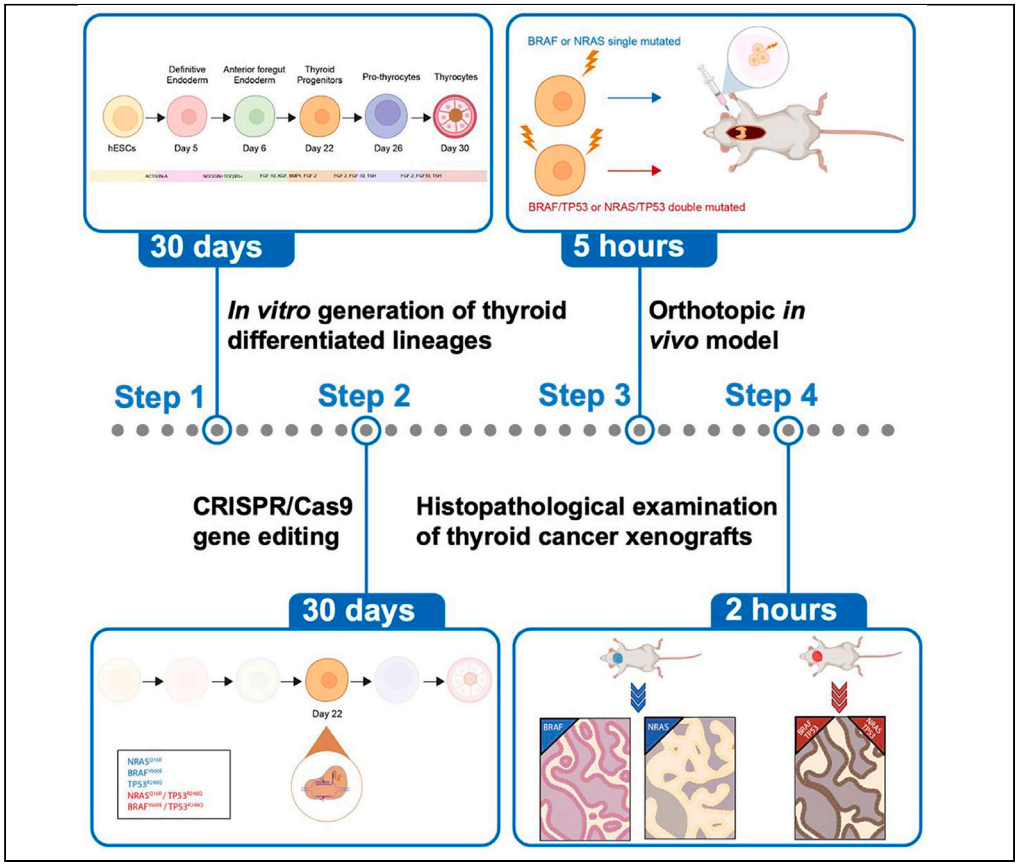
Protocol for generation and engineering of thyroid cell lineages using CRISPR-Cas9 editing to recapitulate thyroid cancer histotype progression



Thyroid carcinoma represents the first malignancy among the endocrine organs. Investigating the cellular hierarchy and the mechanisms underlying the initiation of thyroid carcinoma is crucial in thyroid cancer research. Here, we present a protocol for deriving thyroid cell lineage from human embryonic stem cells. We also describe steps for engineering thyroid progenitor cells utilizing CRISPR-Cas9 technology, which can be used to perform *in vivo* studies, thus facilitating the development of representative thyroid tumorigenesis models.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Differentiation protocol for thyroid cell lineages from human embryonic stem cells

CRISPR-Cas9-mediated cellular engineering for common thyroid cancer genetic alteration

Orthotopic injection of thyroid progenitors to recapitulate thyroid cancer progression

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Protocol for generation and engineering of thyroid cell lineages using CRISPR-Cas9 editing to recapitulate thyroid cancer histotype progression

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SUMMARY

Thyroid carcinoma represents the first malignancy among the endocrine organs. Investigating the cellular hierarchy and the mechanisms underlying the initiation of thyroid carcinoma is crucial in thyroid cancer research. Here, we present a protocol for deriving thyroid cell lineage from human embryonic stem cells. We also describe steps for engineering thyroid progenitor cells utilizing CRISPR-Cas9 technology, which can be used to perform *in vivo* studies, thus facilitating the development of representative thyroid tumorigenesis models.

For complete details on the use and execution of this protocol, please refer to Veschi et al.[1]

BEFORE YOU BEGIN

An innovative *in vitro* differentiation protocol is detailed here to derive thyroid cell lineages from human embryonic stem cells (hESCs) based on Longmire modified differentiation protocol.[2] This protocol was applied to a study addressing the development of thyroid cancer (TC), the third most prevalent malignancy among endocrine organs, by investigating cellular hierarchy and biological mechanisms governing tumor initiation and progression.[1] The procedural steps for engineering thyroid progenitor cells (TPCs) using CRISPR/Cas9 technology are outlined. Importantly, this protocol lays the groundwork for future research endeavors to leverage our experimental model, which involves the engineering of hESC-derived cells. Moreover, this protocol allowed us to obtain thyroid-initiating cancer cells capable of recapitulating *in vivo* the different thyroid tumor histotypes. This model could be suitable for future studies aimed to decipher TC progression or for drug screening purpose.

Institutional permissions

The animal experiments here described received approval from the Italian Ministry of Health (Authorization #25/2020-PR), in line with the national protocols. The animals were

kept in the University of Palermo's animal facility, where they resided in individually ventilated cages (IVC) under specific pathogen-free (SPF) conditions, with unrestricted access to food and water.

1. Aliquot and solution preparation.
   The hESCs (#WA09-RB-001), as well as their differentiated derived progenies, require specific supports and cell culture media supplemented with several components. Prior to starting it is important to ensure that all the necessary components are aliquoted as ready-to-use aliquots, to limit waste of time and avoid freezing and thawing cycles.
   a. Matrigel aliquot preparation.
      i. Thaw the ordered Matrigel overnight on ice and aliquot it in 1 mL ready-to-use aliquots and keep them at −80°C.
      ii. Retrieve aliquots on ice just before use.

   ⚠ CRITICAL: Matrigel thawing needs 6 h.

   ⚠ CRITICAL: Any items that will be in contact with the Matrigel must be chilled keeping them on ice for 5 min.

   b. Freezing solution preparation.
      Achieve a 5% concentration of dimethyl sulfoxide (DMSO) in cell culture medium enriched with 45% of FBS (i.e., 0.5 mL of DMSO + 4.5 mL of FBS + 5 mL of cell culture medium).

   Note: The 500 mL stock fetal bovine serum (FBS) bottle should be heat-inactivated for 30 min at 56°C, preferably with mixing, to inactivate proteins of complement.

   Note: Check the materials and equipment table for precise details regarding the preparation of all aliquots.

2. Media preparation.
   This section outlines the media required for preparing the specific media essential for the differentiation protocol.
   a. Endoderm stem cells (Day 5) into anterior foregut (Day 6) differentiation media.
      Prepare a serum-free medium, composed by 75% IMDM (Thermo Fisher Scientific) and 25% Ham's Modified F12 medium (Euroclone), 1% N2 (Thermo Fisher Scientific), 2% B27 (Thermo Fisher Scientific), BSA (0.05%, US Biological), L-glutamine (200 mM, Euroclone) and ascorbic acid (0.05 mg/mL, Sigma-Aldrich), supplemented with Noggin (100 ng/mL, Novus Biologicals) and the TGF-βR inhibitor SB431542 (10 μM, Sigma-Aldrich).
   b. Anterior foregut (Day 6) into TPCs (Day 22) differentiation media.
      Prepare a serum-free medium containing FGF-10 (10 ng/mL, Novus Biologicals), KGF (10 ng/mL, PeproTech), BMP4 (10 ng/mL, R&D), EGF (20 ng/mL, PeproTech), FGF-2 (500 ng/mL, PeproTech) and heparin sodium salt (100 ng/mL, Sigma-Aldrich).
   c. TPCs (Day 22) to pro-thyrocytes (Day 26) differentiation media.
      Prepare a serum-free medium complemented with FGF-2 (100 ng/mL, PeproTech), FGF-10 (100 ng/mL, Novus Biologicals), heparin sodium salt (100 ng/mL, Sigma-Aldrich) and TSH (1 mU/mL, Novus Biologicals).
   d. Pro-thyrocytes (Day 26) to mature thyrocytes (Day 30) differentiation media.
      Prepare a serum-free medium supplemented with FGF-2 (100 ng/mL, PeproTech), FGF-10 (100 ng/mL, Novus Biologicals), heparin sodium salt (100 ng/mL, Sigma-Aldrich) and TSH (10 mU/mL, Novus Biologicals).

3. WAO9 human embryonic stem cell culture.

This section describes the cell culture support preparation and cell culture protocol for the maintenance of hESCs WA09 (#WA09-RB-001).

a. hESCs WA09 (#WA09-RB-001) support preparation.
   i. Prepare a Matrigel solution with a ratio of 50 μL of Matrigel (Corning hESC-qualified matrix) for 1 mL of hESCs WA09 medium (mTESR1).

⚠ CRITICAL: It is important to keep Matrigel stock solution and Matrigel/mTESR1 mix solution on ice during this step to avoid Matrigel solution solidification, which spontaneously occurs at room temperature.

   ii. Prepare a suitable cell culture vessel, such as a 6-well plate or T25 flask.
   iii. Fill the support with an appropriate volume of Matrigel mix solution, 1 mL for each well in a 6-well plate or 2 mL for T25 flask, to cover the entire area of the support.
   iv. Place the support in the incubator at 37°C for 45 min, allowing the Matrigel layer solidification.
   v. After 45 min, wash twice with an appropriate volume of room temperature 1X PBS (1 mL for 6-well plate or 2 mL for T25 cell plate) to remove the excess of Matrigel mix solution.

Note: Matrigel coated cell support could be stored in this step with an appropriate volume of PBS at 37°C in the incubator for few days.

b. hESCs WA09 (#WA09-RB-001) cell culture passaging.
   i. Thaw hESCs WA09 (#WA09-RB-001) from the liquid nitrogen storage by placing the cryovial at 37°C for 2 min in the laboratory water bath or via ThawSTAR CFT2 Automated Thawing System.
   ii. Carefully dropwise the cells into 5 mL of mTESR1 medium.

Note: Promptly transfer to 5 mL of mTESR1 to rapidly dilute the DMSO present in the freezing solution.

   iii. Centrifuge cells at 300 *g* for 5 min at room temperature.
   iv. Remove the supernatant to eliminate DMSO content.
   v. Count the cells using TRYPAN BLUE and plate the cells using an appropriate volume of mTESR1 medium (2 mL for a 6-well plate and 5 mL for a T25 flask).

Note: Cells confluence is crucial, plate $2 \cdot 10^5$ cells/cm^2.

   vi. Split the cells only when they reach 90% of confluence with a split ratio of 1:2 once a week.
   vii. Change the media every 48 h and freeze $5 \cdot 10^5$ cells for each cryovial using 1 mL of freezing solution.

Note: When detaching cells, employ either Accutase (1 mL/6-well plate) or, as an option, a solution of Trypsin (Euroclone, ECB3052D) diluted with PBS to a concentration of 0.01%, at 37°C. Ensure treatment for a maximum duration of 3 min. Halt enzyme activity by using PBS or FBS-complemented medium at a ratio of 3:1 medium to enzyme.

⚠ CRITICAL: The ROCK inhibitor Y-27632 (10 μM) is added at the cell culture medium after every cell passage to enhance the survival rate of serum/feeder-free single hESCs.[3,4]

4. Designing CRISPR/Cas9 donor and gRNA sequences.
The CRISPR/Cas9 system is a powerful tool for gene editing, consisting of two main components: the Cas9 endonuclease and a noncoding guide RNA (gRNA), and the CRISPR-associated (Cas) protein. The engineered Type II CRISPR/Cas9 system can be leveraged to cleave genomic DNA at a predefined target sequence of interest, usually Cas9. The gRNA is a small RNA molecule that directs the Cas9 protein to a specific target sequence in the DNA, composed of two parts: the CRISPR RNA (crRNA) and the trans-activating CRISPR RNA (tracrRNA). The crRNA recognizes the target DNA sequence through base-pairing, while the tracrRNA assists the binding of the Cas9 protein to the target. The Cas9 protein is a DNA endonuclease that cleaves the target DNA sequence, creating a double-stranded break (DSB) 3 nucleotides upstream from the PAM site. Our CRISPR/Cas9 system uses homology-directed repair (HDR) to repair the DSB thanks to the presence of the donor template, allowing for precise gene editing. The GeneArt CRISPR Nuclease Vector kit offers a simple, ready-to-use, all-in-one expression vector system consisting of both a Cas9 nuclease expression cassette and a guide RNA (gRNA) cloning cassette for rapid and efficient cloning of DNA that encodes target-specific CRISPR RNA (crRNA).
   a. pMA-T donor sequences tailored for the required mutations in the following genes.
      i. BRAF V600E: 5′-ACAGAGAAA-3′
      ii. NRAS Q61R: 5′-GGACGAGAA-3′
      iii. TP53 R248Q: 5′-AACCAGAGG-3′

Note: Precision in designing pMA-T donor sequences is crucial for targeted mutation induction.

   b. Select CRISPR/Cas9 all-in-one OFP vector (Thermo Fisher).
   Choose a CRISPR/Cas9 all-in-one OFP vector (Thermo Fisher) containing.
      i. Cas9 enzyme gene.
      ii. Designed sgRNA sequence.
      iii. Orange Fluorescent Protein (OFP).

⚠ CRITICAL: The selection of the specific sgRNA is pivotal for the efficiency of CRISPR/Cas9 engineering. The sgRNA's specificity directly influences the efficiency of genetic manipulation.

Note: In this protocol, we used the GeneArt CRISPR Nuclease Vector with OFP Reporter Kit, a commercial kit offered by Thermo Fisher Scientific (Thermo Fisher Scientific, A21174). Our CRISPR/Cas9 system employs homology-directed repair (HDR) to accurately repair the Double-Strand Break (DSB) using a guide sequence for homologous recombination. This precise gene editing enables achieving the desired results. Additional information about the commercial kit can be found on the following link (https://www.thermofisher.com/order/catalog/product/A21174?SID=srch-srp-A21174).[5]

Note: The use of GeneArt CRISPR Nuclease Vector with OFP Reporter Kit is not mandatory. Alternative protocols for the introduction of specific mutation through the CRISPR/Cas9 gene editing technology can be used.

The sgRNA sequences selected for the previously detailed mutations are BRAF: TAGCTACAGTGAAATCTCGA; NRAS: CTTCGCCTGTCCTCATGTAT; TP53: TGTAAC AGTTCCTGCATGGG.

Table 1. Potential off-targets candidates

	Site	Mismatches	Sequence	Gene	Sequence Fw	Sequence Rv	Amplicon size
BRAF	WT		TAGCTACAGTGAAATCTCGA				
	OT-1	2	TAGC**C**ACAGTGAAATC**T**TGA	*BRAFP1*	ATG GAT TAC TGA CAC GCC AA	ACA GAA CAA TCC CAA ATG CA	250 bp
	OT-2	3	TAGC**C**ACAGTGAA**G**TCTC**A**A	*THAP4*	TGA CAA GAA GCT AGG GAA CG	TTT CAG TGC ACA CAG ATT GG	279 bp
	OT-3	3	TAGC**C**ACAGTG**C**AATCT**G**GA	*CTNNBL1*	ATG CTG CCA TGA ATT GTG AC	CTC TCA ACA CAC TAC CTC CC	257 bp
NRAS	WT		CTTCGCCTGTCCTCATGTAT				
	OT-1	3	**A**TT**G**GC**A**TGTCCTCATGTAT	*NINL*	GTG GAG AAG GGA GTT TGT CT	AAA GGG AGG AGG GCA TAA TG	296 bp
	OT-2	3	CT**C**CGC**A**TGTCCTC**C**TGTAT	*SEMA5B*	CCT CTC CCA CCT CTC AAA TC	TTT TAC AGA TGG GAA GGC CT	250 bp
	OT-3	4	**T**TTC**A**CCTG**A**CC**A**CATGTAT	*SPATA5*	TCA GTT AAG GCA GGA ACA GG	TGC AGT GTT TGG TTT TCT GT	290 bp
TP53	WT		TGTAACAGTTCCTGCATGGG				
	OT-1	3	TG**A**AACAG**A**TCCT**T**CATGGG	*DCDC2C*	GCT TTC AAT GAC TGT GCC AT	TCA ACT CAG CAA ATC ACA TCC	297 bp
	OT-2	3	TTTAACAGTTC**A**TGC**T**TGGG	*FRAS1*	GAA ATG TAT TAA GCA GCA CAG AA	TCA GTG ATT TGC TTG GTT TTC	300 bp
	OT-3	3	TGTAAC**TTT**G**G**CCTGCATGGG	*SEMA5B*	AAA TGC TTG GCC ATG TTG AG	ATG ATG TGA ACA GGA GGC AG	292 bp

Single guide RNAs (sgRNAs) and the top three predicted potential off-target sites are indicated for each mutated gene. Bold letters correspond to single nucleotide mismatches as compared with the wild-type sequence. The off-target possibilities are indicated as the number of off-target sites found.

c. Perform off-target screen for selected sequences.
The Off-target prediction test was performed using the Synthego off-target detection tool (https://design.synthego.com/#/validate) (Table 1).

⚠ CRITICAL: (i) For optimal outcomes, off-target alignments should exhibit a retention alignment below 95%. (ii) It is strongly recommended to sequence the top three off-target sites to assess potential mutations in these genes after CRISPR/Cas9-mediated editing.

5. Mice management and *in vivo* preparation.
This section describes the common procedures that are required for the completion of the orthotopic injection of engineered hESCs-derived thyroid progenitor cells.
a. Allow the NOD-SCID female mice to undergo a period of acclimatization in a controlled environment in the mice housing facility, spanning a duration of 7 days.

Note: It is noteworthy that, for the *in vivo* thyroid carcinoma experiment, the utilization of either male or female mice is permissible. Our selection of female mice is motivated by the prevalence of thyroid carcinoma in this gender.[6]

b. Ensure to have everything you need for the operating table, ensuring access to all surgery materials.
i. Autoclaved scalpels.
ii. Forceps.
iii. Adaptable lamp above the dissecting microscope.
iv. Dissecting microscope.
v. Isoflurane (anesthetic).
vi. Meloxicam (analgesic).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Oct3/4	BD Biosciences	Cat# 560329
Sox2	BD Biosciences	Cat# 560291
Nanog	BD Biosciences	Cat# 560873
CXCR4	R&D Systems	Cat# FAB170P-100
c-kit	BD Biosciences	Cat# 561682
Fox A2	BD Biosciences	Cat# 561589
PAX8	Novus Biologicals	Cat# NBP3-08274PE
TTF1	Miltenyi Biotec	Cat# 130-118-307
TSH-R	Santa Cruz Biotechnology	Cat# sc-32262
TPO	Abcam	Cat# 203340
Thyroglobulin	Novus Biologicals	Cat# NBP-34748PE
NIS	Abcam	Cat# 17795
CD133	BD Biosciences	Cat# 567033
ABCG2	BD Biosciences	Cat# 561451
Nestin	BD Biosciences	Cat# 561231
HNF-4α	Santa Cruz Biotechnology	Cat# sc-374229
Sox17	BD Biosciences	Cat# 561591
Chemicals, peptides, and recombinant proteins		
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat# D5879
B-27 supplement (50X)	Thermo Fisher Scientific	Cat# 17504044
BSA	US Biological	Cat# 9048-46-8
N-2 supplement (100X)	Thermo Fisher Scientific	Cat# 17502048
L-glutamine 100 × 200mM	Euroclone S.p.A.	Cat# ECB3000D
Nicotinamide	Sigma-Aldrich	Cat# N0636
N-Acetyl-L-cysteine	Sigma-Aldrich	Cat# A9165
Ascorbic acid	Sigma-Aldrich	Cat# A4403
TSH	Novus Biologicals	Cat# 4610-TH
FGF-2	PeproTech	Cat# 100-18B
TGF-β-III protein	Sigma-Aldrich	Cat# GF176
Noggin	Novus Biologicals	Cat# 6057-NG
TGF-βR Kinase inhibitor VI, SB431542	Sigma-Aldrich	Cat# 616461
FGF-10	Novus Biologicals	Cat# 345-FG
KGF	PeproTech	Cat# 100-19
BMP4	R&D Systems	Cat# 314-BP
EGF	PeproTech	Cat# AF-100-15
Heparin sodium salt	Sigma-Aldrich	Cat# 9041-08-1
Meloxicam	LEYCAM	Cat# AB008
Isoflurane	Sigma-Aldrich	Cat# 792632
X-tremeGENE HP DNA transfection reagent	Roche	Cat# 6366236001
Betadine	Viatris	Cat# 037114016
HEPES	Fisher Scientific	Cat# 15630106
Accutase	Thermo Fisher Scientific	Cat# A1110501
Penicillin-Streptomycin	Euroclone S.p.A.	Cat# ECB3001
7-AAD	BD Biosciences	Cat# 559925
Antibiotic-Antimycotic 100X	Euroclone S.p.A.	Cat# ECM0010D
Critical commercial assays		
StemDiff definitive endoderm kit	STEMCELL Technologies	Cat# 05110
High-capacity c DNA Reverse Transcription Kit	Applied Biosystems	Cat# 43-688-14
DNeasy Blood & Tissue Kit	QIAGEN	Cat# 69582
Hotstartaq Plus Master Mix Kit	QIAGEN	Cat# 203645
MinElute PCR Purification Kit	QIAGEN	Cat# 28004

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BigDye Terminator v3.1 Cycle Sequencing Kit and BigDye X-Terminator Purification Kit	Applied Biosystems	Cat# 4337455
Ion AmpliSeq Library Kit Plus	Thermo Fisher Scientific	Cat# 4488990
Ion Xpress Barcode Adapter Kit	Thermo Fisher Scientific	Cat# 4471250
the Ion GeneStudio S5 Plus System using the Ion 510 & Ion 520 & Ion 530 Kit	Thermo Fisher Scientific	Cat# A34019
Experimental models: Cell lines		
WA09	WiCell	Cat# WA09-RB-001
Experimental models: Organisms/strains		
NOD.Cg-Prkdc^{scid}/J (NOD SCID), 4–6 weeks old, female	Charles River Laboratories	Strain# 001303
Oligonucleotides		
FOXE1 Forward: 5′-CACACTCAACGACT GCTTCCTC-3′; Reverse: 5′-CAGGAAGCTGCCG CTCTCGAA-3′	Integrated DNA Technologies	N/A
HHXE Forward: 5′-CCAGGTGAGATTC TCCAACGAC-3′; Reverse: 5′-CTCCATTTAGCGC GTCGATTCTG-3′	Integrated DNA Technologies	N/A
18S Forward: 5′-GCAGAATCCACGCC AGTACAAG-3′; Reverse: 5′-GCTTGTTGTCCAGA CCATTGGC-3′.	Integrated DNA Technologies	N/A
Recombinant DNA		
OFP vector	Thermo Fisher Scientific	Cat# A21174
pMA-T customized donor DNA NRAS Q61R: 5′-GGACGAGAA-3′	Thermo Fisher Scientific	N/A
pMA-T customized donor DNA BRAF V600E: 5′-ACAGAGAAA-3′	Thermo Fisher Scientific	N/A
pMA-T customized donor DNA TP53 R248Q: 5′-AACCAGAGG-3′	Thermo Fisher Scientific	N/A
CRISPR-Cas9 all-in-one OFP vector	Thermo Fisher Scientific	Cat# A21174
Software and algorithms		
Synthego off-target detection tool	Synthego	https://design.synthego.com/#/validate
FlowJo Software	BD Biosciences	https://www.flowjo.com/solutions/flowjo
4Peaks Software	Griekspoor and Tom Groothuis	https://nucleobytes.com/
Graphpad Prism 8	GraphPad Software	http://www.graphpad.com/
Other		
Matrigel	Corning	Cat# 354277
Matrigel	BD Biosciences	Cat# 354230
FBS	Corning	Cat# 35-079-CV
mTESR1 media	STEMCELL Technologies	Cat# 85850
IMDM	Thermo Fisher Scientific	Cat# 12440053
Opti-MEM	Thermo Fisher Scientific	Cat# 11058021
Advanced DMEM/F12	Thermo Fisher Scientific	Cat# 12634010
Ham's F12	Euroclone S.p.A.	Cat# ECB7502L
PBS	Euroclone S.p.A.	Cat# ECB4004L
Trypsin	Euroclone S.p.A.	Cat# ECB3052D
Trypan blue	Sigma-Aldrich	Cat# T8154

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
25 cm^2 Rectangular canted neck cell culture flask with vent cap Corning	Corning	Cat# 430639
75 cm^2 U-shaped canted neck cell culture flask with vent cap Corning	Corning	Cat# 430641U
Costar 6-well clear TC-treated multiple well plate, individually wrapped, sterile	Corning	Cat# 3516
Nunc 15 mL conical sterile polypropylene centrifuge tubes	Thermo Fisher Scientific	Cat# 339650
Nunc 50 mL conical sterile polypropylene centrifuge tubes	Thermo Fisher Scientific	Cat# 339652
1.7 mL microcentrifuge tubes	Corning	Cat# 3620
27-gauge needle Hamilton syringe	Hamilton Company	Cat# 7803-01
ABI PRISM 3130 Genetic analyzer	Thermo Fisher Scientific	Cat# 627-0040R
ThawSTAR CFT2 automated thawing system	STEMCELL Technologies	Cat# 100-0650
FACSLyric	BD Biosciences	Cat# 651165
Ion GeneStudio S5 Plus System	Thermo Fisher Scientific	Cat# A38195
FACSMelody	BD Biosciences	Cat# 661768

MATERIALS AND EQUIPMENT

WA09 coating

Reagent	Final concentration	Amount
Matrigel BD Biosciences Cat# 354277	50 μL/mL	50 μL (6-well plate; 9.6 cm^2)
Advanced DMEM/F12 medium	N/A	1 mL
Total	**N/A**	**1.05 mL**

Store at 4°C for up to 1 month.

WA09 cell culture media

Reagent	Final concentration	Amount
mTESR1 media	N/A	2 mL (6-well plate; 9.6 cm^2)
Total	**N/A**	**2 mL**

Store at 4°C for up to 1 month.

Day 5/6/22/26/30 coating

Reagent	Final concentration	Amount
Matrigel BD Biosciences Cat# 354230	50 μL/mL	50 μL (6-well plate; 9.6 cm^2)
Advanced DMEM/F12 medium	N/A	1 mL
Total	**N/A**	**1.05 mL**

Store at 4°C for up to 1 month.

From day 0 to day 5

Reagent	Final concentration	Amount
StemDiff definitive endoderm kit (STEMCELL Technologies)	N/A	2 mL (6-well plate; 9.6 cm^2)
Total	**N/A**	**2 mL**

Store at 4°C for up to 1 month.

Day 5 cell culture media

Reagent	Final concentration	Amount
IMDM	75%	360 mL
Ham's Modified F12 medium	25%	120 mL

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Continued

Reagent	Final concentration	Amount
N2	1X	5 mL
B27	1X	10 mL
BSA	0.05%	0.25 mL
L-glutamine	1X	5 mL
ascorbic acid	0.05 mg/mL	250 μL
Noggin	100 ng/mL	200 μL
TGF-βR inhibitor SB431542	10 μM	50 μL
Total	**N/A**	**500.75 mL**

Store at 4°C for up to 1 month.

From Day 6 to Day 22 cell culture media

Reagent	Final concentration	Amount
Advanced DMEM/F12 medium	N/A	450 mL
N2	1X	5 mL
B27	1X	10 mL
BSA	0.05%	0.25 mL
L-glutamine	1X	5 mL
Penicillin-Streptomycin	1X	5 mL
HEPES Buffer Solution 1 M	10 mM	5 mL
Nicotinamide	10 mM	10 mL
N-Acetyl-L-cysteine	1 mM	1 mL
EGF	20 ng/mL	200 μL
FGF-2	500 ng/mL	500 μL
Heparin sodium salt	100 ng/mL	25 μL
FGF-10	10 ng/mL	200 μL
KGF	10 ng/mL	50 μL
BMP4	10 ng/mL	5 μL
Total	**N/A**	**492.01 mL**

Store at 4°C for up to 1 month.

Day 22 cell culture media

Reagent	Final concentration	Amount
Advanced DMEM/F12 medium	N/A	458 mL
N2	1X	5 mL
B27	1X	10 mL
BSA	0.05%	0.25 mL
L-glutamine	1X	5 mL
Penicillin-Streptomycin	1X	5 mL
HEPES Buffer Solution 1 M	10 mM	5 mL
Nicotinamide	10 mM	10 mL
N-Acetyl-L-cysteine	1 mM	1 mL
EGF	20 ng/mL	200 μL
FGF-2	100 ng/mL	100 μL
Total	**N/A**	**499.55 mL**

Store at 4°C for up to 1 month.

Day 26 cell culture media

Reagent	Final concentration	Amount
Advanced DMEM/F12 medium	N/A	454 mL
N2	1X	5 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
B27	1X	10 mL
L-glutamine	1X	5 mL
Penicillin-Streptomycin	1X	5 mL
HEPES Buffer Solution 1 M	10 mM	5 mL
Nicotinamide	10 mM	10 mL
N-Acetyl-L-cysteine	1 mM	1 mL
EGF	20 ng/mL	200 μL
FGF-2	100 ng/mL	200 μL
FGF-10	100 μg/mL	20 μL
heparin sodium salt	100 ng/mL	2.5 μL
TSH	1 mU/mL	31.2 μL
Total	**N/A**	**495.35 mL**
Store at 4°C for up to 1 month.		

Day 30 cell culture media		
Reagent	Final concentration	Amount
Advanced DMEM/F12 medium	N/A	454 mL
N2	1X	5 mL
B27	1X	10 mL
L-glutamine	1X	5 mL
Penicillin-Streptomycin	1X	5 mL
HEPES Buffer Solution 1 M	10 mM	5 mL
Nicotinamide	10 mM	10 mL
N-Acetyl-L-cysteine	1 mM	1 mL
EGF	20 ng/mL	200 μL
FGF-2	100 ng/mL	100 μL
FGF-10	100 μg/mL	20 μL
heparin sodium salt	100 ng/mL	2.5 μL
TSH	10 mU/mL	312 μL
Total	**N/A**	**495.63 mL**
Store at 4°C for up to 1 month.		

CRISPR/Cas9 gene editing		
Reagent	Final concentration	Amount
X-tremeGENE HP DNA Transfection Reagent	N/A	12 μL
Opti-Mem	N/A	500 μL
OFP vector (Thermo Fisher, A21174)	N/A	1 μg
pMA-T customized donor DNA plasmid	N/A	5.5 μg
Total	**N/A**	**variable**
Prepare a ready-to-use mix.		

STEP-BY-STEP METHOD DETAILS

Timing: 30 days (for step 1)

Timing: 5 days (for step 1a)

Timing: 1 day (for step 1b)

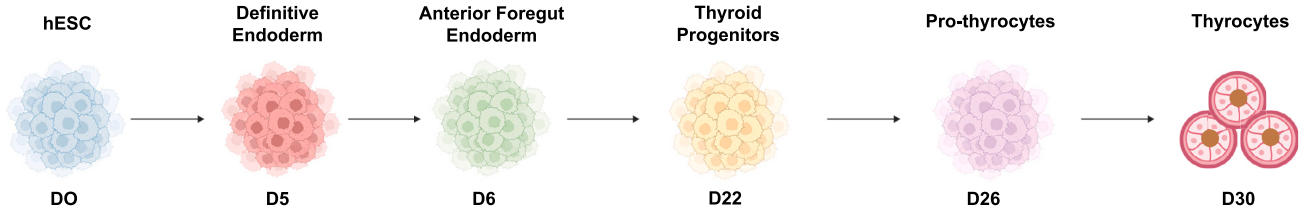

Figure 1. Differentiation model of human embryonic stem cells (hESC) in a complete thyroid lineage

Timing: 16 days (for step 1c)

Timing: 4 days (for step 1d)

Timing: 4 days (for step 1e)

Timing: 30 days (for step 2)

Timing: 5 h (for steps 2a)

Timing: 10 min (for step 2c)

Timing: 2 h (for step 2d)

Timing: 5 h (for steps 2e)

Timing: 1 day (for step 2g)

Timing: 30 days (for step 3)

Timing: 5 h (for step 3a)

Timing: 2 h (for step 3b)

1. Differentiation protocol for establishing thyroid cell lineage from human embryonic stem cells WA09 (#WA09-RB-001).
   This section delineates the technical specifications required to successfully execute the differentiation process aimed at achieving distinct stages of the thyroid cell lineage from human embryonic stem cells (hESCs) WA09 (#WA09-RB-001) (Figure 1).
   Day 0: Human Embryonic Stem Cells.
   Day 5: Definitive Endoderm.
   Day 6: Anterior Foregut Endoderm.
   Day 22: Thyroid Progenitors Cells.
   Day 26: Pro-Thyrocytes.
   Day 30: Mature Thyrocytes.

   Note: It is recommended to use Matrigel-coated flasks throughout the thyroid differentiation process. Prepare a mix combining the Matrigel with an appropriate volume of DMEM/F12 media for each step of the differentiation process. Spread the mixture carefully to ensure complete coverage inside the flask and incubate for 20 min to allow it to solidify. Wash off any excess mixture using 1 mL of PBS for 6-well plate, 5 mL of PBS for a T25 flask, and 10 mL for a T75 flask, preparing the flask for subsequent cell seeding. Use new flasks and a fresh Matrigel coating mixture for every *in vitro* cell passage, to avoid the reduction of Matrigel

thickness, which may impact the differentiation process. Using new flasks and freshly prepared Matrigel ensures optimal conditions for cell growth and differentiation.

a. Differentiation of hESC (Day 0) to definitive endoderm (Day 5).
   i. In the morning, count cells using Trypan blue and seed $2 \cdot 10^6$ WA09 cells per well of a 6-well plate coated with the Corning hESC-qualified matrix (Cat# 354277) using the StemDiff definitive endoderm kit (STEMCELLL Technologies), as indicated in the preparation step and materials and equipment, to induce definitive endoderm formation.

⚠ CRITICAL: The number of cells and the confluency is pivotal in this step, it is important to plate a high number of cells, because during this passage you should expect approximately an 80% cell death.

   ii. Add ROCK inhibitor Y-27632 (final concentration 10 μM) to cell culture medium.
   iii. Approximately 6 h later, check the cells to ensure their attachment.
   iv. Perform medium refreshments every 48 h.

WA09 cell culture media		
Reagent	Final concentration	Amount
mTESR1 media	N/A	2 mL (6-well plate; 9.6 cm^2)
Total	**N/A**	**2 mL**
Store at 4°C for up to 1 month.		

Note: Please be aware that the media contains Activin A, which plays a pivotal role in specifying endoderm from human embryonic stem cells.

Pause point: Cells at Day 5 can be stored in liquid nitrogen, to be used as backup cell culture.

b. Differentiation of definitive endoderm stem cells (Day 5) into anterior foregut (Day 6).
   i. Prepare a Day 6 differentiation medium, for anterior foregut differentiation as indicated in materials equipment.
   ii. Detach the cells using Accutase (see before you begin, step 3b).
   iii. Centrifuge cells at 300 *g* for 5 min.
   iv. Discard the supernatant and resuspend the pellet in an appropriate volume of StemDiff definitive endoderm kit media.
   v. Count the cells using TRYPAN BLUE and seed $2 \cdot 10^6$ WA09 cells per well of a 6-well plate coated with the Corning hESC-qualified matrix (Cat# 354277), using the above-described serum-free medium for 24 h, to induce anterior foregut endoderm differentiation.

Day 5 cell culture media		
Reagent	Final concentration	Amount
IMDM	75%	360 mL
Ham's Modified F12 medium	25%	120 mL
N2	1X	5 mL
B27	1X	10 mL
BSA	0.05%	0.25 mL
L-glutamine	1X	5 mL
ascorbic acid	0.05 mg/mL	250 μL
Noggin	100 ng/mL	200 μL
TGF-βR inhibitor SB431542	10 μM	50 μL
Total	**N/A**	**500.75 mL**
Store at 4°C for up to 1 month.		

Note: This is the last differentiation step wherein cells are seeded in 6-well plate coated with Corning hESC-qualified matrix that is specific for the early stage of differentiation. From the next step of differentiation, the Matrigel solution to be used is BD, cat. 354230.

From Day 6 to Day 22 cell culture media		
Reagent	Final concentration	Amount
Advanced DMEM/F12 medium	N/A	450 mL
N2	1X	5 mL
B27	1X	10 mL
BSA	0.05%	0.25 mL
L-glutamine	1X	5 mL
Penicillin-Streptomycin	1X	5 mL
HEPES Buffer Solution 1 M	10 mM	5 mL
Nicotinamide	10 mM	10 mL
N-Acetyl-L-cysteine	1 mM	1 mL
EGF	20 ng/mL	200 µL
FGF-2	500 ng/mL	500 µL
Heparin sodium salt	100 ng/mL	25 µL
FGF-10	10 ng/mL	200 µL
KGF	10 ng/mL	50 µL
BMP4	10 ng/mL	5 µL
Total	**N/A**	**492.01 mL**
Store at 4°C for up to 1 month.		

Pause point: Cells in this step can be stored in liquid nitrogen prior moving on the next differentiation stage.

c. Differentiation of anterior foregut (Day 6) into TPCs (Day 22).
   i. Prepare fresh Day 22 TPCs differentiation medium as indicated in materials and equipment.
   ii. Prepare 6-well plate support coated with Matrigel (Cat# 354230), using a ratio of 50 µL of Matrigel per mL of medium.
   iii. Count and seed $5 \cdot 10^5$ Day 6 cells per well into coated 6-well plate by re-suspending the cells in 2 mL of the Day 22 TPCs differentiation medium.
   iv. Refresh the media twice a week by adding 2 mL of fresh medium. Upon reaching confluency, detach the cells and perform a 1:2 split.

⚠ CRITICAL: It is crucial to sustain a minimum cell confluence of around 70%.

Note: By this step up to day 30, use Matrigel (Cat# 354230) coated flask.

Note: This cell population, which represents the TPCs, can be used to perform CRISPR/Cas9 gene editing.

Pause point: Cells in this step can be stored in liquid nitrogen prior moving on the next differentiation stage.

d. Differentiation of TPCs (Day 22) into pro-thyrocytes (Day 26).
   i. Prepare a Day 26 pro-thyrocytes differentiation medium, as indicated in materials and equipment.
   ii. Prepare a T25 adhesion flask coated with Matrigel (Cat# 354230).
   iii. Count and plate $5 \cdot 10^5$ Day 5 cells for each T25 adhesion-coated flask.

iv. Change the media twice a week (i.e., Monday-Thursday).

Day 26 cell culture media		
Reagent	Final concentration	Amount
Advanced DMEM/F12 medium	N/A	454 mL
N2	1X	5 mL
B27	1X	10 mL
L-glutamine	1X	5 mL
Penicillin-Streptomycin	1X	5 mL
HEPES Buffer Solution 1 M	10 mM	5 mL
Nicotinamide	10 mM	10 mL
N-Acetyl-L-cysteine	1 mM	1 mL
EGF	20 ng/mL	200 µL
FGF-2	100 ng/mL	200 µL
FGF-10	100 µg/mL	20 µL
heparin sodium salt	100 ng/mL	2.5 µL
TSH	1 mU/mL	31.2 µL
Total	**N/A**	**495.35 mL**
Store at 4°C for up to 1 month		

Pause point: Cells in this step can be stored in liquid nitrogen prior moving on the next differentiation stage.

Note: at this step it is possible to proceed with CRISP/Cas9 gene editing in order to generate mutated TPCs (see "Engineering of D22 TPCs using CRISPR/Cas9 technology" step 2).

e. Differentiation of pro-thyrocytes (Day 26) to mature thyrocytes (Day 30).
   i. Prepare fresh Day 30 mature thyrocytes differentiation medium, as indicated in materials and equipment.
   ii. Prepare a T25 adhesion flask coated with Matrigel (Cat# 354230).
   iii. Count and plate $5 \cdot 10^5$ Day 5 cells for each T25 adhesion-coated flask.
   iv. Change the media every 48 h.

Day 30 cell culture media		
Reagent	Final concentration	Amount
Advanced DMEM/F12 medium	N/A	454 mL
N2	1X	5 mL
B27	1X	10 mL
L-glutamine	1X	5 mL
Penicillin-Streptomycin	1X	5 mL
HEPES Buffer Solution 1 M	10 mM	5 mL
Nicotinamide	10 mM	10 mL
N-Acetyl-L-cysteine	1 mM	1 mL
EGF	20 ng/mL	200 µL
FGF-2	100 ng/mL	100 µL
FGF-10	100 µg/mL	20 µL
heparin sodium salt	100 ng/mL	2.5 µL
TSH	10 mU/mL	312 µL
Total	**N/A**	**495.63 mL**
Store at 4°C for up to 1 month.		

f. Flow cytometry-based cell positivity analysis.
   i. Use Accutase to harvest cells and transfer them into 15 mL tubes.
   ii. Centrifuge cells at 300 *g* for 5 min.

iii. Remove supernatants and resuspend cells in the specific growth medium using within in 5 mL polystyrene tubes (Corning).
iv. Count viable cells using trypan blue.
v. Employ pipetting techniques to disperse cell clumps effectively.
vi. Use a 70 μm cell strainer to filter single cells and prevent further clump formation.
vii. Resuspend $1 \cdot 10^5$ cells in a volume of 200 μL of 2% paraformaldehyde for 20 min at 25°C to fix them.
viii. Centrifuge cells at 300 *g* for 5 min.
ix. Remove supernatants and resuspend cells in 100 μL of PBS 0.1% Triton X-100 for 15 min at 25°C, to permeabilize them.
x. Centrifuge cells at 300 *g* for 5 min.
xi. Remove supernatants and proceed with the step 8.
xii. Resuspend $1 \cdot 10^5$ cells in a volume of 100 μL were exposed for 1 h at 4°C to specific markers : Oct3/4 (40/Oct-3 Alexa-Fluor 647, mouse IgG1K, BD Biosciences, 20 μL/sample), Sox2 (245610 PE, mouse IgG2a BD Biosciences, 20 μL/sample), Nanog (N31-355 PE, mouse IgG1, BD Biosciences, 20 μL/sample), CXCR4 (FAB170P PE, mouse IgG2a, R&D system, 1 μL/sample), c-kit (YB5.B8 PE, mouse IgG1 BD Biosciences, 20 μL/sample), Sox17 (P7-969 PE, mouse IgG1k, BD Biosciences, 5 μL/sample), Fox A2 (N17-280 PE, mouse IgG1, BD Biosciences 5 μL/sample), PAX8 (PAX8/1492 APC, mouse IGg2a, Novus 5 μL/sample), TTF1 (REA1090 FITC, mouse IgG1, MACS Miltenyi Biotec, 16 μL/sample), TSH-R (4C1 FITC, IgG2a Santa Cruz, 2 μL/sample), TPO (203340, Anti-rabbit IgG H + L Alexa-488, Abcam, 2 μL/sample), Thyroglobulin (SPM221 PE, mouse IgG1, Novus, 2 μL/sample), NIS (SPM186, goat anti-mouse IgG (H + L) Alexa-488, Abcam, 5 μL/sample), CD133 (W6B3C1 FITC, mouse IgG1, BD Bioscience, 20 μL/sample), ABCG2 (5D3/CD338 APC, mouse IgG2b, BD Bioscience, 10 μL/sample), Nestin (25/Nestin PerCP 5.5, mouse IgG1, BD Bioscience, 5 μL/sample), HNF-4α (H-1, goat anti-mouse IgG (H + L) Alexa-488, Santa Cruz, 4 μL/sample), or corresponding isotype matched controls (IMC).
xiii. Analyze the cell positivity for those markers using the FACS Lyric cytometer (BD Biosciences).

g. Real-time PCR-based gene expression analysis.
i. Extract RNA from a cellular pellet using TRIZOL reagent (Thermo Fisher Scientific).
ii. Retro-transcribe 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).
iii. Analyze mRNA expression levels of FOXE1 (F: 5′-CACACTCAACGACTGCTTCCTC-3′; R: 5′-CAGGAAGCTGCCGCTCTCGAA-3′), and HHXE (F: 5′-CCAGGTGAGATTCTCCAACGAC-3′; R: 5′-CTCCATTTAGCGCGTCGATTCTG-3′) thyroid transcription factors. Endogenous gene control used to calculate the ΔΔCt is the 18S (F: 5′-GCAGAATCCACGCCAGTACAAG-3′; R: 5′-GCTTGTTGTCCAGACCATTGGC-3′).

Note: The more precise way to check cell heterogeneity is represented by single-cell RNA-seq technique.

Note: During the differentiation protocol of hESCs into thyroid committed lineages it is possible to observe morphological changes, which ultimate with the generation of thyroid follicle-like phenotype, characterized by formation of budding structures. The efficiency of the differentiation protocol can also be monitored by studying specific markers of hESCs (Oct3/4, Sox2, Nanog, CXCR4), endoderm cells (C-kit, Sox17, FoxA2), thyroid progenitor cells (HHEX and FOXE1), and thyroid differentiated cells (PAX8, TTF1, TSH-R, TPO, TG, NIS) (Figure 2).

2. Engineering of D22 TPCs using CRISPR/Cas9 technology.

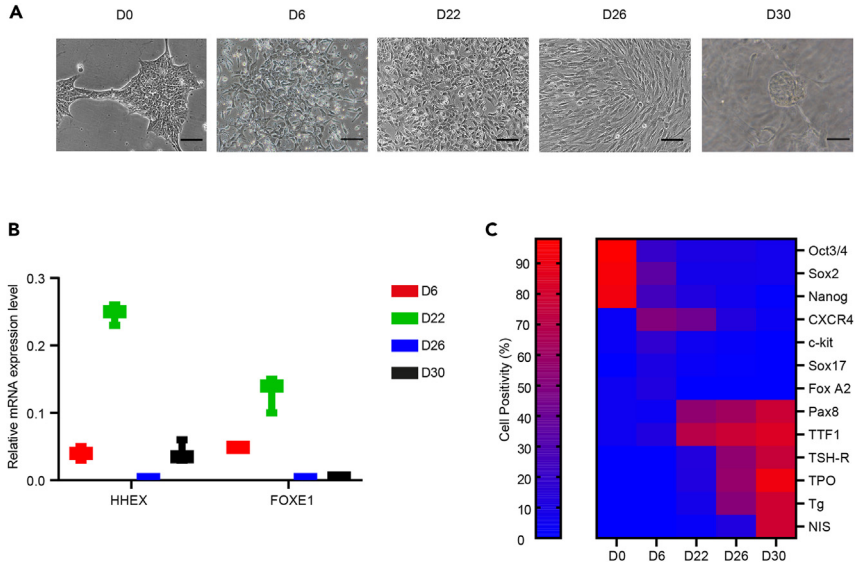

Figure 2. Validation of hESC-derived differentiated progenies

(A) Phase contrast microscopy of hESCs-derived stages of thyroid differentiation. Scale bars measure 20 μm.

(B) mRNA levels of HHEX and FOXE1 in hESCs-derived specific stages of thyroid differentiation. Data represent mean ± SD of three independent experiments.

(C) Heatmap representing flow cytometry analysis of specific markers in hESCs-derived various stages of thyroid differentiation lineages (D0, D6, D22, D30). Data are presented as mean ± SD from three separate experiments.

This section outlines the technical procedures involved in engineering TPCs through CRISPR/Cas9 technology to achieve single or double mutations, in $BRAF^{V600E}$ or $NRAS^{Q61R}$ and $TP53^{R248Q}$ (Figure 3).

a. day 0-*BRAF* or *NRAS* editing via CRISPR/Cas9.

   i. Acclimatize X-tremeGENE for 20 min at room temperature before use. Then, vortex the solution for 5 s.

⚠ CRITICAL: It is crucial to ensure to thaw enough volume of X-tremeGENE before to start.

   ii. Keep Opti-Mem medium at room temperature at least 30 min before to start the transfection protocol.

Note: It is important to use the Opti-Mem medium for the transfection protocol, its composition is optimized to increase transfection efficiency and improve cell viability post-transfection in serum free condition.

   iii. Prepare one Matrigel (Cat# 354277)-coated T25 flask for each CRISPR/Cas9 transfection adding 50 μL of Matrigel for 1 mL of media.

   iv. Allow the resulting solution to solidify for 45 min at 37°C.

   v. Add 100 μL of Opti-Mem medium to a 1 mL tube.

   vi. Add 8 μg of total DNA: 2.5 μg of specific *BRAF* or *NRAS* CRISPR/Cas9 All-in-One OFP Vector, and 5.5 μg of $BRAF^{V600E}$ *or* $NRAS^{Q61R}$ pMA-T donor, to the 1 mL tube previously prepared.

   vii. Add 12 μL of X-tremeGENE maintaining a ratio of 3:2 with the DNA amount to the same tube.

   viii. Gently shake the DNA-Opti-Mem-X-tremeGENE mix solution for 15 s.

   ix. Keep the DNA-medium- X-tremeGENE mix at room temperature for a minimum of 40 min. In the meantime, start D22 TPCs cell count and then follow the next steps.

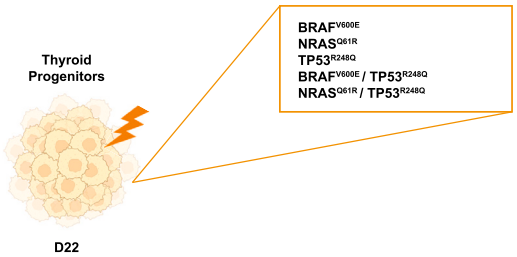

Figure 3. Engineering of D22 TPCs via CRISPR/Cas9 technology

⚠ CRITICAL: Avoid the use of tubes made of polystyrene for X-tremeGENE HP Transfection Reagent: DNA complex preparation. If polystyrene materials cannot be avoided, ensure you pipette the transfection reagent directly into the serum-free medium (e.g., Opti-Mem).

x. Count D22 TPCs and prepare $5 \cdot 10^5$ cells for each CRISPR/Cas9 mediated cellular transfection, resuspending them in 500 μL Opti-mem medium.
xi. After 40 min of incubation, add the D22 TPCs to the DNA-Opti-Mem-X-tremeGENE mix solution.
xii. Vortex Cells-DNA- X-tremeGENE mix for 30 s. Maintain the mix at room temperature for the subsequent 4 h. Gently mix every 30 min for 20 s.
xiii. Centrifuge the cells at 300 *g* for 5 min.
xiv. Resuspend the cells in 1 mL of TPC differentiation media and transfer them to 15 mL tubes.
xv. Wash the tubes with 10 mL of TPC differentiation media and centrifuge the cells at 300 g for 5 min.
xvi. Resuspend the resulting pellet in 5 mL of TPC differentiation media and transfer the cells to the Matrigel (Cat# 354230)-coated T25 flasks, to be stored at 37°C.

b. day 1- Cell maintenance.
Refresh the cell culture medium 24 h following cell transfection, to avoid X-tremeGENE toxicity.

c. day 2-Transfection efficiency evaluation.
Post-Transfection Evaluation.
48 h after transfection, replace the TPC differentiation medium. Starting from this time-point it is possible to assess the expression of the reporter gene.

Note: The OFP fluorescent signal arises from the OFP reporter gene within the CRISPR/Cas9 All-in-One OFP Vector, which includes the gRNA. CRISPR/Cas9 All-in-One OFP Vector is transiently transfected, therefore the cell fluorescence can only be evaluated during the first month (*i.e.*, by fluorescent microscopy analysis, or flow cytometry analysis; Ex λ: 548, Em λ: 573), subsequent to the transfection.

Note: 48 h post-transfection fluorescence signal should be present, ideally within a range of 10%–30% of plated cells.

⏸ Pause point: If you intend to work with single mutated BRAF or NRAS engineered TPCs, you may proceed directly to point e.

d. day 3- Enrichment of OFP positive cells.
i. Use Accutase to harvest cells and transfer them into 15 mL tubes.
ii. Centrifuge cells at 300 *g* for 5 min.
iii. Remove supernatant, count cells, and resuspend them at $1 \cdot 10^6$ cells/mL density, in growth medium in 5 mL polystyrene tubes (Corning).

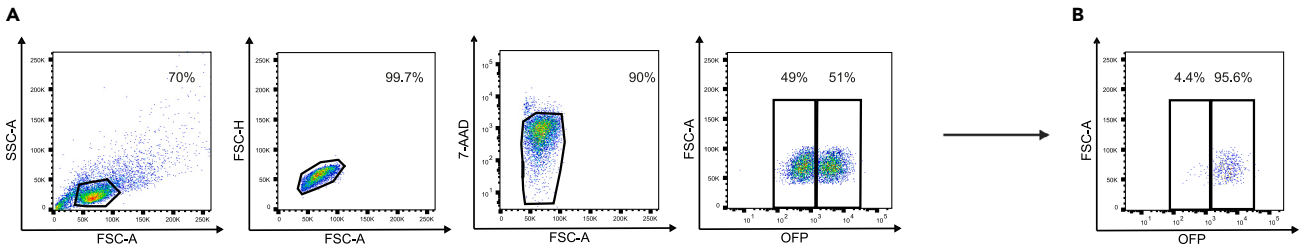

Figure 4. Schematic diagram of gene-edited OFP-positive cell sorting

(A) Gating strategy for the sorting of OFP^+ transduced TPC.

(B) Post sorting evaluation of OFP^+ TPC cell population.

iv. Employ pipetting techniques to disperse cell clumps effectively.

v. Use a 70 μm cell strainer to filter cells and prevent further clump formation.

vi. Add 5 μL of 7-AAD (7-Aminoactinomycin D; 0.25 μg/test) directly to the cell medium, to mark $1 \cdot 10^6$ cells.

vii. AAD marks dead cells allowing indirectly the selection of live cells.

Note: Avoid prolonged sample storage; prepare each sample slightly ahead of the sorting schedule.

viii. Initiate the sorter following the instructions of the manufacturer.

ix. Load a pre-saved experiment layout for OFP-positive cells or create a new one using a negative control.

x. Load tube containing negative control with OFP negative D22 TPCs and establish a gating strategy for OFP-positive cells (SCC-A versus FSC-A: cells; FSC-H versus FSC-A: single cells; 7AAD vs. FSC-A; FSC-A versus OFP: OFP positive cells and OFP negative cells) (Figure 4). Analyze data by FlowJo Software.

Note: Ensure the gating strategy is not overly restrictive, especially in instances of low positivity, to prevent inadvertent exclusion of identical cells.

xi. Start analyzing the D22 TPCs CRISPR/Cas9 engineered cell population.

Note: Monitor the stability of the cell cloud during sorting; fluctuations in laser intensity or fluidic changes may impact stability.

xii. Begin the sorting process and document events.

Note: The cell sorter calculates the number of sorted events, but it may not correspond to the effective number of alive sorted cells; it is recommended to count cells before plating.

xiii. Collect OFP-positive cells into FACS tubes containing 500 μL of D22 cell culture medium.

xiv. Centrifuge at 300 *g* for 5 min.

xv. Remove supernatants and resuspend the cells in 2 mL of medium.

xvi. Plate the cells in another Matrigel-coated support to reach 80% of confluence.

⚠ CRITICAL: To avoid cell death and maximize the efficiency of the flow-cytometry fluorescence-based sorting, keep the sample on ice and work in dark conditions.

e. day 4-TP53 editing via CRISPR/Cas9

i. X-tremeGENE (HP, Roche) thawing.
Acclimatize X-tremeGENE for 20 min at room temperature before use. Then, vortex the solution for 5 s.

⚠ CRITICAL: It is crucial to ensure to thaw enough volume of X-tremeGENE before to start.

ii. Opti-Mem medium Thawing.
Keep Opti-Mem medium at room temperature at least 30 min before to start the transfection protocol.

Note: It is important to use the Opti-Mem medium for the transfection protocol, its composition is optimized to increase transfection efficiency and improve cell viability post- transfection in serum free condition.

iii. Preparation of Matrigel (Cat# 354277)-Coated T25 Flasks.
Prepare one Matrigel (Cat# 354277)-coated flask adding 50 μL of Matrigel for 1 mL of media, then allow the resulting solution to solidify for 45 min at 37°C.
iv. Add 100 μL of Opti-Mem medium to a 1 mL tube.
v. Add 8 μg of total DNA: 2.5 μg of *TP53* CRISPR/Cas9 All-in-One OFP Vector, and 5.5 μg of $TP53^{R248Q}$ pMA-T donor to the 1 mL tube previously prepared.
vi. Add 12 μL of X-tremeGENE maintaining a ratio of 3:2 with the DNA amount to the same tube.
vii. Gently shake the DNA-Opti-Mem-X-tremeGENE mix solution for 15 s.
viii. Keep the DNA-medium- X-tremeGENE mix at room temperature for a minimum of 40 min. In the meantime, start D22 NRAS or BRAF mutated TPCs cell count and then follow the next steps.

⚠ CRITICAL: As suggested by the manufacturer, avoid the use of tubes made of polystyrene for X-tremeGENE HP Transfection Reagent: DNA complex preparation. If polystyrene materials cannot be avoided, ensure you pipette the transfection reagent directly into the serum-free medium (e.g., Opti-Mem).

ix. Count D22 NRAS or BRAF mutated TPCs and prepare 5·105 cells for CRISPR/Cas9 mediated cellular transfection, resuspending them in 500 μL Opti-Mem medium.
x. After 40 min of incubation, add the D22 NRAS or BRAF mutated TPCs to the DNA-Opti-Mem-X-tremeGENE mix solution.
xi. Vortex Cells-DNA- X-tremeGENE mix for 30 s. Maintain the mix at room temperature for the subsequent 4 h. Vortex the mix every 30 min for 20 s.
xii. Centrifuge the cells at 300 *g* for 5 min.
xiii. Resuspend the cells in 1 mL of TPC differentiation media and transfer them to 15 mL tubes.
xiv. Wash the tubes with 10 mL of TPC differentiation media and centrifuge the cells at 300 *g* for 5 min.
xv. Resuspend the resulting pellet in 5 mL of TPC differentiation media and transfer the cells to the Matrigel (Cat# 354230)-coated T25 flasks, to be stored at 37°C.

f. day 5- Cell maintenance.
i. Refresh the cell culture medium 24 h following cell transfection, to avoid X-tremeGENE toxicity.
ii. Split the cells only when they reach 90% of confluence with a split ratio of 1:2 once a week.
iii. For the first 4 passages following transfection, it is highly recommended to freeze 50% of the cells, thus generating 4 backup vials.
iv. One month after cell transfection (to avoid the sequencing of the donor plasmids used for gene editing), these cells can be used for DNA extraction and gene editing analysis.

Note: In our protocol, we use the commercial kit from ThermoFisher Scientific (catalog no. A21174) to confirm the introduction of specific mutations ($BRAF^{V600E}$, $NRAS^{Q61R}$, and $TP53^{R248Q}$). However, this kit contains only the OFP reporter gene, which limits our ability to verify and discriminate, via fluorescence microscopy or FACS analysis, the subsequent introduction of a second mutation into our double mutated cells (BRAF/TP53 and NRAS/TP53). To address this limitation, we recommend introducing the second mutation using a kit containing another reporter gene (i.e., GFP or YFP). By using two distinct reporter genes for each mutation, we can effectively validate, based on their fluorescence, the introduction of two different mutations into our experimental system without resorting to DNA sequencing.

g. Genome editing evaluation.
   1-month post-transfection, during which the cells have been maintained in D22 TPCs cell culture medium, verify the CRISPR/Cas9 genome editing via SANGER and/or NGS techniques. The results from both Sanger sequencing and NGS analyses are shown in Table 2. Details about the used primers, the gene locus, and the amplicon size for Sanger and NGS sequencing are included in Table 3 and Table 4, respectively.

Note: CRISPR/Cas9 All-in-One OFP Vector is transiently transfected. A notable fluorescence at least of 30% should be visible after 7 days until 10 days.

Note: Following the protocol, you will obtain a heterogeneous population of TPCs enriched in single or mutated NRAS or BRAF/TP53 cells. To note, freeze 3 cell vials in liquid nitrogen after 7–10 days post-transfection.

Note: TP53 single mutation is not individually explored due to its limited ability to contribute to tumor initiation when inactivated alone. During the *in vitro* experiments, utilizing CRISPR/Cas9 technology for obtaining double mutated TPCs, TP53 inactivation remains consistent, whether performed during the first or second round of engineering.

Note: To evaluate the CRISPR/Cas9 technology mutational rate, perform a sequencing analysis with specific primers directed against the inserted mutations. We suggest performing both the SANGER analysis checking the presence of the mutations, followed by the NGS analysis to identify low mutational rate.

For Sanger sequencing.

i. DNA from CRISPR/Cas9 engineered cells was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Cat# 69582) and amplified with the HotStarTaq Plus Master Mix Kit (Qiagen, Cat# 203645).
ii. The amplified products were purified with the MinElute PCR Purification Kit (Qiagen, Cat# 28004).
iii. The BigDye Terminator v3.1 Cycle Sequencing Kit and BigDye X-Terminator Purification Kit (Applied Biosystems, Cat# 4337455) were used for sequencing and purification, respectively.
iv. Sanger sequencing was performed to detect specific mutations BRAFV600E, NRASQ61R, and TP53R248Q using capillary electrophoresis on an ABI PRISM 3130 Genetic Analyzer.
v. Capillary electrophoresis was carried out on the ABI PRISM 3130 Genetic Analyzer or by BMR Genomics service (Padua, Italy). The resulting electropherograms were visually analyzed using 4Peaks Software (Griekspoor and Tom Groothuis, nucleobytes.com).
vi. The electropherograms were analyzed using 4Peaks Software.
   For NGS sequencing.

Table 2. Analysis of CRISPR/Cas9 gene editing performed by Sanger and NGS, showing wild type (WT) and edited sequences (MUT), as well as indel mutation percentage (INDEL), in engineered hESC at different stages of differentiation (Day 6, Day 22, Day 26, and Day 30)

		Percentage (%)																	
		$BRAF^{V600E}$						$NRAS^{Q61R}$						$TP53^{R248Q}$					
Differentiation stage	Mutation	MUT	WT	Indel	MUT	WT	Indel	MUT	WT	Indel	MUT	WT	Indel	MUT	WT	Indel	MUT	WT	Indel
Day 6	NTC	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00
	BRAF	100.00	0.00	0.00	99.85	0.15	0.00												
	NRAS							100.00	0.00	0.00	99.83	0.17	0.00						
	TP53													100.00	0.00	0.00	99.45	0.55	0.00
	BRAF/TP53	100.00	0.00	0.00	99.80	0.20	0.00							100.00	0.00	0.00	99.30	0.70	0.00
	NRAS/TP53							100.00	0.00	0.00	99.25	0.75	0.00	100.00	0.00	0.00	98.90	1.10	0.00
Day 22	NTC	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00
	BRAF	67.30	32.70	0.00	52.33	47.67	0.00												
	NRAS							76.60	23.40	0.00	99.80	0.20	0.00						
	TP53													15.00	85.00	0.00	14.90	85.10	0.00
	BRAF/TP53	95.60	4.40	0.00	86.16	13.84	0.00							93.00	7.00	0.00	90.44	9.56	0.00
	NRAS/TP53							100.00	0.00	0.00	93.37	6.63	0.00	22.90	77.10	0.00	33.71	66.29	0.00
Day 26	NTC	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00
	BRAF	74.60	25.40	0.00	57.63	42.37	0.00												
	NRAS							100.00	0.00	0.00	100.00	0.00	0.00						
	TP53													100.00	0.00	0.00	94.85	5.15	0.00
	BRAF/TP53	44.10	55.90	0.00	14.27	85.73	0.00							31.87	68.13	0.00	26.85	73.15	0.00
	NRAS/TP53							35.50	64.50	0.00	99.82	0.18	0.00	11.20	88.80	0.00	11.50	88.50	0.00
Day 30	NTC	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00
	BRAF	95.00	5.00	0.00	85.37	14.63	0.00												
	NRAS							73.90	26.10	0.00	99.90	0.10	0.00						
	TP53													84.40	15.60	0.00	82.49	17.51	0.00
	BRAF/TP53	77.20	22.80	0.00	58.90	41.10	0.00							4.00	96.00	0.00	7.41	92.59	0.00
	NRAS/TP53							16.90	83.10	0.00	99.51	0.49	0.00	27.10	72.90	0.00	31.10	68.90	0.00
		Sanger			NGS			Sanger			NGS			Sanger			NGS		

Table 3. Sanger sequencing details, including gene names, chromosome loci, amplicon region and size, and forward (Fw) and revers (Rv) primer sequences

Genes	Locus	Amplicon	Size of the amplicon	Sequence Fw	Sequence Rv
BRAF BRAF	chr7:140453128 chr7:140453136	BRAF (AMPLP223465859), hg19,chr7,140453078, 140453103,140453221, 140453249	118	CCA CAA AAT GGA TCC AGA CAA CTG TT	GCT TGC TCT GAT AGG AAA ATG AGA TCT AC
TP53 TP53 TP53 TP53 TP53	chr17:7577538 chr17:7577549 chr17:7577555 chr17:7577564 chr17:7577559	TP53 (AMPLP261746128), hg19,chr17,7577489,7577509, 7577613,7577636	104	GGC TCC TGA CCT GGA GTC TTC	CTC ATC TTG GGC CTG TGT TAT CTC
NRAS NRAS	chr1:115256504 chr1:115256529	NRAS (AMPL7156573949-glio), hg19,chr1,115256410,115256439, 115256557,115256584	118	GCT CCT AGT ACC TGT AGA GGT TAA TAT CCG	CAA GTG GTT ATA GAT GGT GAA ACC TGT T

vii. NGS libraries were created using the Ion AmpliSeq Library Kit Plus (https://www.ampliseq.com/login/login.action) from Thermo Fisher Scientific. These libraries were generated from a customized panel targeting 25 genes, including BRAF, TP53, and NRAS. The panel contained 1525 amplicons, with 10 ng of genomic DNA used for each primer pool.

viii. The libraries were barcoded with the Ion Xpress Barcode Adapter Kit (Thermo Fisher Scientific, Cat# 4471250) and quantified by qPCR using the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific, Cat# 4468802). Automated template preparation and chip loading were conducted on the Ion Chef System.

ix. Sequencing was performed on the Ion GeneStudio S5 Plus System using the Ion 510 & Ion 520 & Ion 530 Kit (ThermoFisher Scientific, Cat# A34019).

x. Initial data analysis was conducted with Torrent Suite v.5.10.1, including alignment to the hg19 reference genome. Single nucleotide variant (SNV) annotations were performed with Ion Reporter v5.18.2.0.

xi. Variants were filtered based on a variant allele frequency (VAF) of 3%, read depth greater than 100, a Phred quality score above 40, and a p-value below 0.

Note: Gene editing has to be validated by Sanger, or NGS sequencing, to confirm the engineering protocol and to evaluate its efficiency.

3. Generation of an orthotropic *in vivo* model.

This section provides a detailed description of all the steps required for the generation and histopathological examination of thyroid orthotopic tumors.

a. Orthotopic injection of engineered TPCs.

i. Count $3 \cdot 10^5$ D22 TPCs, single or double transfected, using trypan blue.

Note: Use cells early passage

ii. Resuspend the cells in 50 μL of Matrigel-mix solution composed by 80% Matrigel BD and 20% Sterile PBS.

iii. Maintain the resuspended cells on ice.

iv. Anesthetize each NOD-SCID 4/6 old weeks female mouse using Meloxicam (drinkable solution, 0.3 mg/kg/day) and Isoflurane (inhalation, 5% induction and 2% maintenance).

⚠ CRITICAL: Anesthesia will keep the mice Anesthetized for a maximum of 40 min.

v. Shave and sterilize the neck area with Betadine.

Table 4. NGS sequencing information, including gene names, chromosome number, chromosome start/end region point, number and length of generated amplicons, number of covered and missed bases, and overall coverage

Name	Chromosome	Chr_Start	Chr_End	Num_Amplicons	Total_Bases	Covered_Bases	Missed_Bases	Overall_Coverage
NRAS (AMPLP239816250)	chr1	115251059	115251184	1	125	125	0	1
NRAS (AMPLP239853329)	chr1	115251184	115251310	1	126	126	0	1
NRAS (AMPLP237210508)	chr1	115252156	115252257	1	101	101	0	1
NRAS (AMPLP237243672)	chr1	115252257	115252374	1	117	117	0	1
NRAS (AMPLP242632724)	chr1	115256402	115256523	1	121	121	0	1
NRAS (AMPLP242658097)	chr1	115256523	115256653	1	130	130	0	1
NRAS (AMPLP237217291)	chr1	115258627	115258704	1	77	77	0	1
NRAS (AMPLP237248971)	chr1	115258704	115258781	1	77	77	0	1
TP53 (AMPLP239769572)	chr17	7572830	7572936	1	106	106	0	1
TP53 (AMPLP239807548)	chr17	7572936	7573040	1	104	104	0	1
TP53 (AMPLP326843856)	chr17	7573886	7574019	2	133	133	0	1
TP53 (AMPLP343115170)	chr17	7573997	7574069	2	72	72	0	1
TP53 (AMPLP239556324)	chr17	7576606	7576680	1	74	74	0	1
TP53 (AMPLP241066932)	chr17	7576806	7576927	1	121	121	0	1
TP53 (AMPLP242292875)	chr17	7577006	7577127	2	121	121	0	1
TP53 (AMPLP261669586)	chr17	7577088	7577196	2	108	108	0	1
TP53 (AMPLP261728092)	chr17	7577392	7577509	2	117	117	0	1
TP53 (AMPLP261746128)	chr17	7577508	7577613	2	105	105	0	1
TP53 (AMPLP262327837)	chr17	7578141	7578219	1	78	78	0	1
TP53 (AMPLP262347540)	chr17	7578219	7578298	1	79	79	0	1
TP53 (AMPLP262578967)	chr17	7578304	7578428	1	124	124	0	1
TP53 (AMPLP262610068)	chr17	7578428	7578557	1	129	129	0	1
TP53 (AMPLP263757129)	chr17	7579278	7579385	1	107	107	0	1
TP53 (AMPLP263769320)	chr17	7579385	7579512	2	127	127	0	1
TP53 (AMPLP263779137)	chr17	7579511	7579601	2	90	90	0	1
TP53 (AMPLP263209938)	chr17	7579609	7579728	1	119	119	0	1
TP53 (AMPLP242494828)	chr17	7579834	7579949	1	115	115	0	1
BRAF (AMPLP222629880)	chr7	140434389	140434494	2	105	105	0	1
BRAF (AMPLP222635553)	chr7	140434444	140434549	2	105	105	0	1
BRAF (AMPLP222636793)	chr7	140434559	140434661	1	102	102	0	1
BRAF (AMPLP223519358)	chr7	140439604	140439721	1	117	117	0	1
BRAF (AMPLP223175118)	chr7	140449078	140449190	1	112	112	0	1
BRAF (AMPLP223177865)	chr7	140449190	140449274	1	84	84	0	1
BRAF (AMPLP223460541)	chr7	140453023	140453102	1	79	79	0	1
BRAF (AMPLP223465859)	chr7	140453102	140453221	1	119	119	0	1
BRAF (AMPLP223719489)	chr7	140453947	140454053	1	106	106	0	1
BRAF (AMPLP223960162)	chr7	140476646	140476763	2	117	117	0	1
BRAF (AMPLP223967627)	chr7	140476752	140476865	2	113	113	0	1
BRAF (AMPLP223971374)	chr7	140476865	140476944	1	79	79	0	1

(*Continued on next page*)

Table 4. ***Continued***

Name	Chromosome	Chr_Start	Chr_End	Num_Amplicons	Total_Bases	Covered_Bases	Missed_Bases	Overall_Coverage
BRAF (AMPLP225222024)	chr7	140477788	140477876	1	88	88	0	1
BRAF (AMPLP326889377)	chr7	140481297	140481391	1	94	94	0	1
BRAF (AMPLP326913823)	chr7	140481391	140481511	1	120	120	0	1
BRAF (AMPLP222680486)	chr7	140482785	140482885	1	100	100	0	1
BRAF (AMPLP222684242)	chr7	140482885	140482992	1	107	107	0	1
BRAF (AMPLP223588249)	chr7	140487295	140487412	1	117	117	0	1
BRAF (AMPLP223739184)	chr7	140494111	140494215	2	104	104	0	1
BRAF (AMPLP223741661)	chr7	140494198	140494286	2	88	88	0	1
BRAF (AMPLP223700378)	chr7	140500127	140500214	1	87	87	0	1
BRAF (AMPLP223708886)	chr7	140500214	140500294	1	80	80	0	1
BRAF (AMPLP223944056)	chr7	140501130	140501252	1	122	122	0	1
BRAF (AMPLP223950351)	chr7	140501252	140501327	1	75	75	0	1
BRAF (AMPLP222258987)	chr7	140507664	140507784	1	120	120	0	1
BRAF (AMPLP222259284)	chr7	140507784	140507865	1	81	81	0	1
BRAF (AMPLP224404546)	chr7	140508688	140508770	1	82	82	0	1
BRAF (AMPLP224410546)	chr7	140508770	140508888	1	118	118	0	1
BRAF (AMPLP222895407)	chr7	140534301	140534421	1	120	120	0	1
BRAF (AMPLP222903910)	chr7	140534421	140534524	1	103	103	0	1
BRAF (AMPLP222922922)	chr7	140534524	140534620	1	96	96	0	1
BRAF (AMPLP222935598)	chr7	140534620	140534698	1	78	78	0	1
BRAF (AMPLP223103332)	chr7	140549819	140549943	1	124	124	0	1
BRAF (AMPLP223104608)	chr7	140549951	140550074	1	123	123	0	1
BRAF (AMPLP224119138)	chr7	140624307	140624441	2	134	134	0	1
BRAF (AMPLP224119999)	chr7	140624378	140624505	2	127	127	0	1

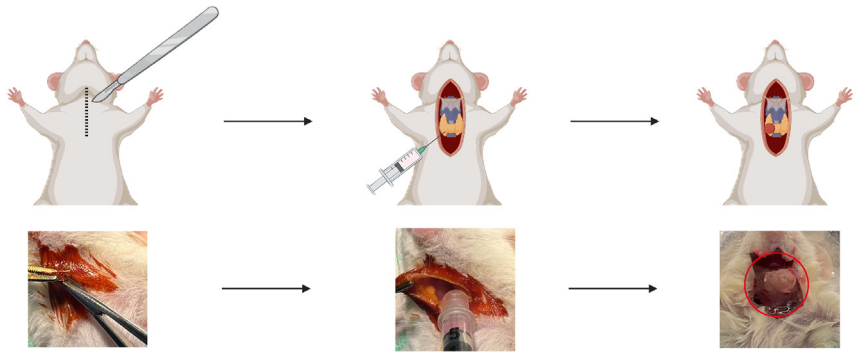

Figure 5. Orthotopic injection of engineered thyroid progenitor cells recapitulates thyroid cancer *in vivo*

vi. Create a vertical incision in the neck using scissors and clamps.
vii. Incise the underlying neck central infrahyoid muscles and move them laterally to create a central space between the mussels.
viii. The trachea will be visible beyond the incised muscles as tubular structure composed of white cartilaginous rings. Identify the right thyroid lobe in the upper-right corner flanking the trachea as a little translucent gland.
ix. Perform orthotopic injection of Day 22 engineered cells using a 27-gauge needle Hamilton syringe (Figure 5).

Note: Ensure that the cell mix solution stay inside the thyroid. The correct performance of orthotopic cell injection is proven by observation of thyroids welling.

Note: Prevent the formation of bubbles while aspirating the cell-Matrigel solution into the syringe.

Note: Timing is contingent upon the number of mice scheduled to be included in the experiment. 5 h were calculated on 6 mice.

⚠ CRITICAL: Keep the cell-Matrigel mix solution on ice throughout the experiment until the use.

x. Reposition the infrahyoid muscles.
xi. Close the incision using surgical nylon suture.
xii. Reapply Betadine to the operated neck area.
xiii. Maintain the mice under a warm temperature lamp until they regain consciousness.
xiv. Monitor mice weekly to evaluate surgical recovery and tumor growth.

b. Mice sacrifice and tumors harvesting.
This section outlines the endpoint of the orthotopic *in vivo* experiment. Follow Directive 2010/63/EU guidelines (D.lgs 26/2016).
i. Euthanize mice by inducing isoflurane-induced loss of consciousness, followed by cervical dislocation.
ii. Surgically remove tumors and organs and store them in sterile PBS for subsequent analysis.

Note: Timing is contingent upon the number of mice scheduled to be included in the experiment. 2 h were calculated on 6 mice.

Note: Single Mutations: $BRAF^{V600E}$ TPCs generate tumors that recapitulate characteristics of papillary thyroid carcinoma. $NRAS^{Q61R}$ TPCs generate tumors that recapitulate features

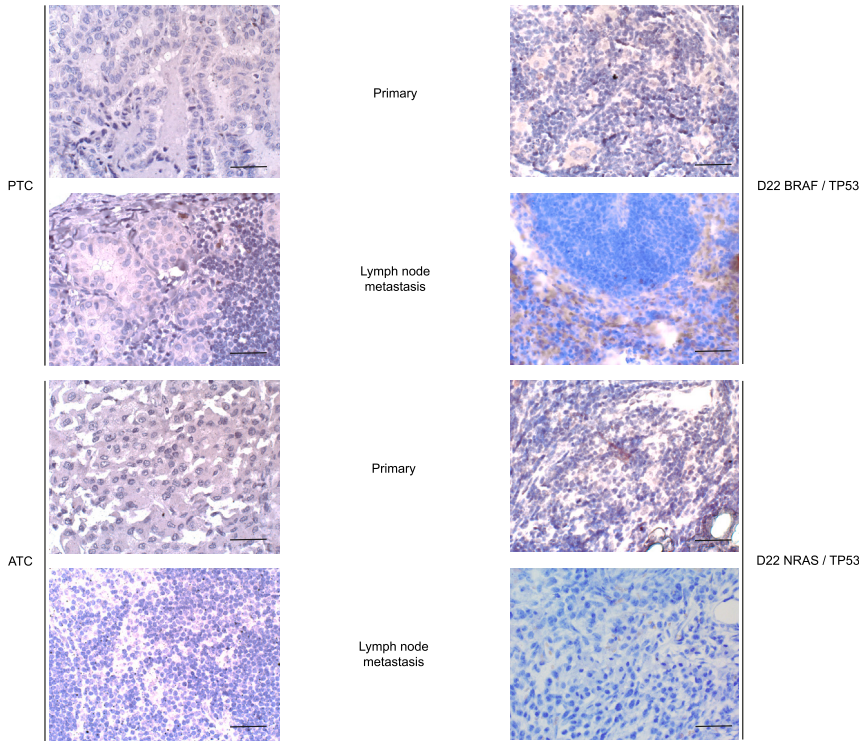

Figure 6. Histopathological comparison of TPCs-derived xenografts and patient-derived thyroid tumors demonstrates that the orthotopic injection of engineered thyroid progenitor cells recapitulates thyroid cancer *in vivo*

Scale bars represent 100 μm.

resembling follicular histotype. Double Mutations: $BRAF^{V600E}$ or $NRAS^{Q61R}$ and $TP53^{R248Q}$ TPCs generate tumors that recapitulate characteristics associated with anaplastic thyroid carcinoma. Single mutated TPCs generate orthotopic xenografts after approximately 30 days, while double mutated ones are able to give rise orthotopic tumors after about 15 days.

Note: Following orthotopic injection of mutated TPCs, it is crucial to check the morphology of generated xenografts, to demonstrate the recapitulation of PTC/ATC thyroid tumors (Figure 6).

Remember to strictly adhere to ethical and safety guidelines throughout the entire procedure.

EXPECTED OUTCOMES

The presented protocol endeavors to mirror thyroid differentiation lineage *in vitro*, delineating a comprehensive timeline through a replicable methodology enabling the generation of cells at various thyroid differentiation stages. This protocol allows to obtain human thyroid differentiated follicular cells from hESCs. This approach facilitates an enhanced exploration of individual cell roles during embryogenesis and thyrogenesis. Aiding in the identification and characterization of tumor-originating cells within TC. Employing the CRISPR/Cas9 technology ensures stable genetic editing of cells, thereby paving the way for investigating prevalent genetic alterations associated with TC evolution. With this protocol we have highlighted an innovative genetic and temporal tumorigenesis model for TC, highlighting the role either of the genetic mutation and the timing in which they occur during the carcinogenesis. Moreover, the potential application of this model extends to the investigation of tumorigenesis mechanisms in various organs derived from the definitive endoderm

throughout their lineage differentiation. This protocol explains how to pinpoint the thyroid gland's location while operating on a live mouse using an orthotopic *in vivo* model and provides step-by-step instructions for injecting thyroid-engineered cells directly into the thyroid gland. This model is especially impactful because it closely recapitulates how follicular derived thyroid tumors actually grow in the tumor microenvironment, making it more translational if compared to other *in vivo* models. Therefore, the orthotopic model allows for a more detailed study of how cancer spreads during the metastatic process.

LIMITATIONS

Although the differentiation protocol effectively induces the creation of a complete thyroid lineage, underlying the differences between the several differentiation stages, as demonstrated in our work,[1] that protocol doesn't involve completely the cellular bulk population. Accordingly, the resulting heterogeneous population may be enriched in several differentiating stages during the protocol. **Adherence to the technical procedures outlined in this protocol is crucial to maximize the differentiation effect on the cell population**. CRISPR/Cas9 technology-mediated cellular engineering generates a heterogeneous population enriched in mutated cells. The orthotopic *in vivo* model, specifically in our thyroid model, faces technical limitations. The thyroid gland, situated in the right corner of the trachea, appears notably small and, for this reason, difficult to identify. Caution is advised during syringe penetration to prevent inadvertent damage to surrounding structures (such as vessels). Of particular importance is to avoid any leakage of the cellular-Matrigel mix from the thyroid gland during the injection process, as this precaution significantly enhances the efficacy of the tumor growth experiment.

TROUBLESHOOTING

Problem 1

Heterogeneity after the differentiation protocol.

Possible solution

The differentiation protocol may result in a heterogeneous cell population enriched in various differentiated stages. To enhance differentiation efficacy, strict adherence to the aforementioned guidelines is crucial, particularly in terms of media preparation and achieving optimal cellular confluence (recommended 60% of confluency at the seeding).

Problem 2

CRISPR/CAS9 low efficacy.

Possible solution

The CRISPR/CAS9 technology exhibits low efficacy, leading to the generation of a heterogeneous cell population, even in terms of mutations. To enhance editing efficacy, the initial consideration should involve the design and testing of multiple RNA guides. This approach not only improves genome editing efficacy but also reduces the potential for off-target effects, contributing to an overall enhancement of CRISPR/CAS9 editing efficiency.[7]

To address the low efficacy of CRISPR/Cas9, our protocol suggests enriching the cell population with effectively mutated cells through FACS-mediated OFP+ cell sorting. Although this method yields a highly effective mutation rate, it still results in a heterogeneous population, enriched with mutated cells. For a more thorough resolution, a more complicated and time consuming clonogenic selection method may be employed, isolating individual cells that have incorporated the mutations.

Problem 3

Cellular damage after cell sorting.

Possible solution

The engineered cells will be sorted via FACS to enrich the gene edited OFP^+ population. During the analysis cells integrity could be damaged by FACS sorting system. We recommend to plate sorted gene edited OFP^+ cells at higher confluence comparing the described above (80%–90%) to maintain them closed to limit their high mortality after the FACS analysis. Of note, the day after the plating you could find cellular debris because of the cellular damage and bacteria contamination. In the latter we recommend adding rock inhibitor and antibiotics in the medium.

Problem 4

Challenges encountered during cells injection in the thyroid.

Possible solution

The challenge lies in maintaining the syringe accurately within the small size of the thyroid gland, making it difficult to ensure proper positioning. Additionally, the correct alignment of the syringe determines the success of the injection, preventing any leakage outside the gland. An effective solution to enhance result efficacy involves having another person assist during the orthotopic injection. This assistant can aid the operator in maneuvering the syringe from the outside, facilitating a more precise and controlled injection.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Giorgio Stassi (giorgio.stassi@unipa.it).

Technical contact

For inquiries regarding the technical details of executing the protocol, please reach out to Dr. Simone Di Franco (simone.difranco@unipa.it), the designated technical contact who will provide the necessary answers.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze data sets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization and visualization, V.D.P., F.V., S.D.F., and G.S.; methodology, V.D.P., F.V., A.T., V.V., C.M., M.L.I., M.G., S.D.B., and M.T.; writing – original draft, V.D.P. and F.V.; writing – review and editing, V.D.P., F.V., S.D.F., and G.S.; supervision and funding acquisition, S.D.F. and G.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Veschi, V., Turdo, A., Modica, C., Verona, F., Di Franco, S., Gaggianesi, M., Tirrò, E., Di Bella, S., Iacono, M.L., Pantina, V.D., et al. (2023). Recapitulating thyroid cancer histotypes through engineering embryonic stem cells. Nat. Commun. *14*, 1351. https://doi.org/10.1038/s41467-023-36922-1.

2. Longmire, T.A., Ikonomou, L., Hawkins, F., Christodoulou, C., Cao, Y., Jean, J.C., Kwok, L.W., Mou, H., Rajagopal, J., Shen, S.S., et al. (2012). Efficient derivation of purified lung and thyroid progenitors from embryonic stem cells. Cell Stem Cell *10*, 398–411. https://doi.org/10.1016/j.stem.2012.01.019.

3. Li, X., Meng, G., Krawetz, R., Liu, S., and Rancourt, D.E. (2008). The ROCK inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation. Stem Cells Dev. *17*, 1079–1085. https://doi.org/10.1089/scd.2007.0247.

4. Li, X., Krawetz, R., Liu, S., Meng, G., and Rancourt, D.E. (2009). ROCK inhibitor improves survival of cryopreserved serum/feeder-free single human embryonic stem cells. Hum. Reprod. *24*, 580–589. https://doi.org/10.1093/humrep/den404.

5. Liang, X., Potter, J., Kumar, S., Zou, Y., Quintanilla, R., Sridharan, M., Carte, J., Chen, W., Roark, N., Ranganathan, S., et al. (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. J. Biotechnol. *208*, 44–53. https://doi.org/10.1016/j.jbiotec.2015.04.024.

6. Siegel, R.L., Giaquinto, A.N., and Jemal, A. (2024). Cancer statistics, 2024. CA. Cancer J. Clin. *74*, 12–49. https://doi.org/10.3322/caac.21820.

7. Feng, S., Wang, Z., Li, A., Xie, X., Liu, J., Li, S., Li, Y., Wang, B., Hu, L., Yang, L., and Guo, T. (2021). Strategies for High-Efficiency Mutation Using the CRISPR/Cas System. Front. Cell Dev. Biol. *9*, 803252. https://doi.org/10.3389/fcell.2021.803252.