Box 1 Materials for teratoma formation and tissue fixation.

○ hPSC line of interest, e.g. hESC WA01 (H1) (WiCell, lot. no. WA01-DDL-17) or WA19 (WiCell, lot. no. WA19-WB0015). For regular propagation and experimentation, cells are grown in colonies according to the WiCell Feeder Independent Pluripotent Stem Cell Protocol (SOP-SH-002, version D) and maintained on Matrigel-coated plates (Corning, cat. no. 354230; Starlab, cat. no. CC7682-7506) in mTeSR1 (Stemcell Technologies, cat. no. 85850) at 37°C and 5% CO_2 with daily medium change. Colonies are routinely passsaged with Versene (0.02% EDTA) (Lonza, cat. no. BE17-711E) every 4 to 6 days at a ratio of 1:10 to 1:20[60].

CAUTION Make sure to abide by the supplier's terms and conditions regarding the receipt and use of hESC lines, and to follow the institutional and governmental guidelines on human embryonic stem cell research to comply with ethical standards, local laws and policies.

CRITICAL For transplantation, only stable hPSC lines, for which pluripotency *in vitro* is examined on a regular basis, should be used. *In vitro* tests for pluripotency include e.g. assessment of colony morphology, immunofluorescent staining of pluripotency markers Oct-4A, Sox2, Nanog, SSEA4, TRA-1-60 and TRA-1-81, and embryoid body formation[49].

○ Immunodeficient mice, NOD-*scid* IL2Rg^{null} (NSG) (Charles River, Jax stock no. 005557), male, 6-8 weeks old.

CAUTION All animal experiments must be approved by the institutional ethics and animal welfare committee and the national government authorities in accordance to the relevant regulations and laws. This protocol complies with the requirements listed above.

○ Matrigel, growth factor-reduced (GFR) and phenol red-free (Corning, cat. no. 356231)

CRITICAL Standard formulations of Matrigel contain significant amounts of PDGF, IGF-1, TGF-β and other growth-promoting factors. To minimize the impact of exogenously provided growth factors on teratoma formation and especially host cell recruitment, always use Matrigel GFR. This is of particular importance for studies on early events during teratoma development including initiation and primary progression.

○ Dulbecco's phosphate-buffered saline (DPBS) (1X) w/ Ca^{2+} and Mg^{2+} (Lonza, cat. no. 17-513F)

○ 29G insulin syringes, 0.5 ml (Becton Dickinson, cat. no. 324824)

○ Surgical scissors (Fine Science Tools, cat. no. 14090-09, 91401-12 and 91604-09) and forceps (Fine Science Tools, cat. no. 91150-20, 91197-00, 91100-12 and 11003-12)

○ Disposable histology cassettes, 40 x 28 x 6.8 mm (Roth, cat. no. K113.1)

○ Glass beaker, 600 ml, tall form (VWR, cat. no. 213-1174)

○ 4% formaldehyde solution pH 7.0. Phosphate-buffered, methanol-stabilized (<3%) and acid free. Ready-to-use (Roth, cat no. P087)

CAUTION Formaldehyde is classified as hazardous substance. It is extremely flammable, causes skin irritation and eye damage, and is suspected of causing genetic defects and cancer. Wear chemical protection gloves and safety goggles. Dispose of as hazardous waste. Keep container tightly closed and store in a well-ventilated place.

CRITICAL Formalin is the saturated solution of ~37-40% formaldehyde in water. Chemically, 10% formalin, the most commonly used tissue fixative, thus roughly equals 4% formaldehyde. 4%

formaldehyde solutions are usually stabilized by the addition of ~1-3% methanol which suppresses oxidation and polymerization of formaldehyde to paraformaldehyde. Note that commercial grade formalin stock solutions sometimes contain a higher amount of methanol and/or other additives. Since the added methanol can impact fixation, it is recommended to either use the item specified in this protocol, to buy ready-made 10% neutral-buffered formalin or to prepare fresh formalin from paraformaldehyde immediately before use. Do not use formaldehyde solutions at higher concentrations (≥4%). This may cause overfixation of tissue samples.

Box 2 Teratoma formation and tissue fixation procedure, Timing ~8-10 weeks

This short protocol describes the xenografting of hESC into the gastrocnemius muscle of immunodeficient mice to produce teratomas which are immunohistochemically analysed for host cell contribution (**Figs. 5**-**9**; **Supplementary Figs 4** and **5**). The basic procedure can essentially be applied to other cells of interest, e.g. breast cancer cells, to generate xenografts for the analysis of the somatic tumor microenvironment (**Supplementary Fig. 6**).

1 Expand hESC until ~60-70% confluent (mid- to pre-split cycle) and enough for transplantation ($1x10^6$ cells/injection site).

CRITCAL STEP Do not use ≥80% confluent (split-cycle) cultures. Colonies ready to be passaged usually contain a considerable portion of dead and less vital cells which can significantly impact teratoma formation efficiency.

2 Thaw Matrigel stock solution (~10 mg/ml) overnight (~16 h) on ice in a 2°C to 6°C refrigerator or cold room.

CRITICAL STEP Always keep Matrigel on ice as it will start to form a gel at temperatures ≥10°C and rapidly solidify at room temperature. Use chilled tubes and tips. This protocol employs 25 µl Matrigel per injection site. Aliquot remaining Matrigel into working volumes and store at -80°C for later use.

3 Harvest cells with Versene, gently resuspend in an appropriate volume of mTESR1 and determine the number of vital cells.

CRITICAL STEP hESC are prone to dissociation-induced apoptosis. Pipet gently to preserve aggregates. Do not dissociate colonies into single cells by using single cell dissociation reagents or by excessive pipetting using narrow-bore tips as this will decrease teratoma formation efficiency.

4 Pellet cells by centrifugation at 4°C and 200*g* for 5 min and gently resuspend in chilled mTESR1 basal medium (mTESR1 w/o growth factor supplement) or 1X DPBS (w/ Ca^{2+} and Mg^{2+}) at a concentration of $2x10^6$ cells/25 µl. Put the tube on ice.

CRITICAL STEP Since calcium ions are vital co-factors for cadherin-mediated cell adhesion, the use of calcium-containing solutions counteracts single cell dissociation and helps to preserve cell aggregates. Moreover, application of cells at high density as described promotes cell survival and engraftment post injection.

5 Using chilled tips, add an equal volume of Matrigel (25 µl/injection site) to the cell mixture to yield a final concentration of $1x10^6$ cells/50 µl. Mix gently by inverting and put the tube back on ice immediately.

CRITICAL STEP Do not dilute Matrigel below a concentration of ~4 mg/ml This will prevent efficient Matrigel gelling post injection. Information on the lot-specific concentration can be found in the certificate of analysis included with each product shipment.

6 Prepare syringes by drawing 50 µl of the cell/Matrigel mixture. Draw slowly to prevent cell shearing and immediately place on ice. For Matrigel control injections, prepare syringes as described above but omit the cells from the cell diluent/Matrigel mixture.

CRITICAL STEP It is essential to keep samples on ice to preserve cell viability and to prevent Matrigel from gelling. *In vitro* solidifying Matrigel will impair cell injection.

7 Restrain the animal and inject the sample into the gastrocnemius muscle. Expell slowly to prevent cell shearing. Use both hind limbs for injection.

CAUTION Make sure to comply with your institutional regulations on laboratory animal care and animal restraint.

8 Monitor animals by weekly palpation, ~8-10 weeks post injection, teratomas will have formed.

CAUTION Make sure that the size of the generated tumors is within the limits allowed in the ethical guidelines of your institution. Teratomas should be removed once they visibly affect the animals' motility and food and water intake, or if the animals show obvious signs of distress or pain.

9 Sacrifice the mice according to your institutional regulations on experimental endpoints.

CAUTION Obtain your institution's approved protocol as any methods or techniques specified here may become redundant. One commonly used method is to first anaesthetize the mice with a combination of Ketamine and Xylazine, followed by euthanasia via cervical dislocation or carbon dioxide (CO_2).

10 Affix the sacrificied mouse in a supine position by pinning down all four legs and excise the teratoma from the ventral hind limb.

CRITICAL STEP For *in situ* studies of the local teratoma microenvironment, do not dissect and trim the tissue until the teratoma is fully exposed but rather excise the tumor and its adjacent tissue as a whole[7]. Using surgical scissors, make a first incision at the Achilles tendon and cut the skin along the leg. Cut off the fatty connective tissue between the skin and the muscle layer surrounding the tumor. Using forceps, gently grasp the layer and carefully dissect the area immediately surrounding the teratoma. Cut the connecting tissue to release the mass. For Matrigel control-injected mice, excise an equally sized piece of muscle tissue from the injection site.

11 Immediately place the dissected tissues into labeled histology cassettes and transfer them into a container (e.g. glass beaker) with 4% formaldehyde solution. The tissue-to-fixative volume ratio should be at least 1:10. If processing tissues in cassettes as described here, it is sufficient to keep samples submerged in fixative (~10-15 ml/cassette).

12 Fix samples for 24 h at 4°C.

CAUTION Formaldehyde is hazardous. Use leak-proof sealing and label container appropriately.

CRITICAL STEP Fixation time is critical since it affects both, tissue morphology as well as the signal-to-noise ratio after immunostaining. Insufficient fixation of only a few hours fails to adequately preserve tissue morpholgy, thereby impeding or complicating the interpretation of staining results. Next to poorly resolved cell and tissue structures, under-fixation can cause uneven staining due to poor antigen preservation in the less accessible tissue center, leading to a gradual loss of signal. Overfixation (≥48 h, i.e. over the weekend) results in excessive antigen masking with non- or barely retrievable epitopes, causing a general lack of signal and/or high background staining. Do not use old, expired or recycled formaldehyde solutions as these may autofluoresce and increase the general background.

13 After fixation, immediately proceed to tissue dehydration, paraffin-embedding and sectioning following broadly available protocols for standard procedures. Do so by either processing samples manually or by accessing histology core facilities equipped with semi- or fully automated instrumentation and ran by specially trained personnel.

PAUSE POINT After embedding, either directly proceed to tissue sectioning and slide preparation or store tissue blocks at room temperature (20-25°C) in a dust-protective container until ready to be cut. Do not expose to direct sunlight. Paraffin-embedded tissue blocks can generally be stored for years, blocks processed in this study have been stored for up to 18 months without any loss in staining quality. For details on the storage of sectioned tissue, see the step-by-step procedure.

Box 3 Preparation of tissue lysates for immunoblotting, Timing ~1 h

For the preparation of tissue lysates for subsequent SDS-Page and immunoblotting (**Fig. 4b**), snap-frozen teratoma and mouse skeletal muscle (gastrocnemius) tissues (~5-30 mg) are immersed in 70-100 µl chilled Triton X-100 lysis buffer containing 40 mM Hepes pH 7.5 (Sigma-Aldrich, cat. no. H3375), 120 mM NaCl (Sigma-Aldrich, cat. no. S7653), 1 mM EDTA (Sigma-Aldrich, cat. no. E7889), 10 mM β-glycerophosphate (Sigma-Aldrich, cat. no. G9422), 50 mM NaF (Sigma-Aldrich, cat. no. S7920), 1% (v/v) Triton X-100 (Sigma-Aldrich, cat. no. X100), supplemented with 0.5 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich, cat. no. P7626), 2 µg/ml aprotinin (Sigma-Aldrich, cat. no. A1153), 2 µg/ml leupeptin (Sigma-Aldrich, cat. no. L2023), 0.3 µg/ml benzamidin hydrochloride (Sigma-Aldrich, cat. no. B6506), and 10 µg/ml trypsin inhibitor (Sigma-Aldrich, cat. no. T9003), and mechanically homogenized at 5,000 rpm (2x20 s, 5 s break) using a Precellys 24 tissue homogenizer (Bertin, VWR, cat. no. 432-3750) and the CK14/0.5 ml ceramic beads kit (Bertin, VWR, cat. no. 432-0293). This is followed by two freeze&thaw cycles in liquid nitrogen and 20 min incubation on ice. Supernatants containing soluble proteins are collected by centrifugation at 20,000*g* 4°C for 20 min and stored at -80°C.