LENTIVIRAL PRODUCTION AND INFECTION

(modified from Panciera et al., 2016)

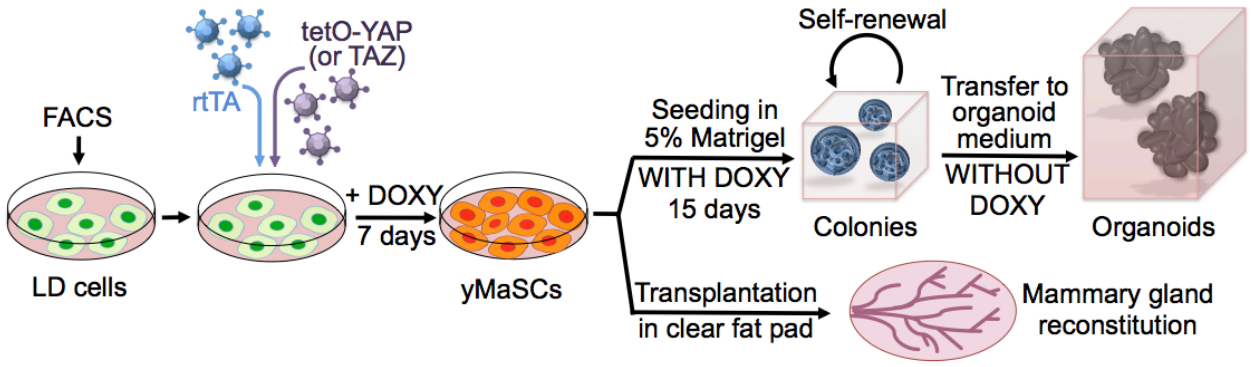
Lentiviral preparation

HEK293T cells (checked routinely for absence of mycoplasma contaminations) were kept in DMEM supplemented with 10% FBS (Life Technologies), Glutamine and Antibiotics (HEK medium). Lentiviral particles were prepared by transiently transfecting HEK293T with lentiviral vectors (10 micrograms/10 cm dishes) together with packaging vectors pMD2-VSVG (2.5 micrograms) and pPAX2 (7.5 micrograms) by using TransIT-LT1 (Mirus Bio) according to manufacturer instructions. Specifically, 60 μl of TransIT-LT1 reagent was diluted in 1.5 ml of Opti-MEM (Life Technologies) for each 10 cm dish, incubated with the vector DNA 15 min at RT and gently distributed over to the cell medium (dish contained about 10 ml of HEK medium). After 8 hr, HEK medium was changed. 48 hr post-transfection supernatant was collected, filtered through 0.45 micrometers and directly stored at -20°C; we did not concentrate viral supernatants.

Lentiviral Infection of primary cells

As example, for a typical infection of a 3 cm dish containing primary cells, we used 500 μl of each unconcentrated viral supernatant diluted to 1x cell-specific medium in 2 ml final volume. For mammary gland cells, we mixed one volume (e.g., 500 μl/3 cm dish) of FUdeltaGW-rtTA supernatant, one volume of the FUW-tetO-YAP (or TAZ) supernatant, and added two volumes of serum-free MG medium with 2X concentrations of supplements. For neurons, for a typical 3.5 cm plate, we mixed 500 μl of FUdeltaGW-rtTA (Addgene # 19780) with 250 μl of FUW-tetO-wtYAP (or negative controls) viral supernatants, 250 μl of HEK medium with 1.5 ml of serum-free Neurobasal medium with 2X B27. We recommend the use of FUW-tetO-wtYAP (Addgene #84009) for reprogramming experiments as detailed in Panciera et al., Cell Stem Cells 2016, as in some primary cell types we experienced growth arrest and premature senescence after infection with the constitutive nuclear/active YAP5SA mutant.

MAMMARY GLAND



Schematic representation of yMaSC induction (from Panciera et al., 2016), distinguishing "colonies" and "organoids" from yMaSCs

Primary mammary epithelial cells (MECs) isolation and culture

Primary MECs were isolated from the mammary glands of 8- to 12-week-old virgin C57BL/6J mice (unless otherwise specified), according to standard procedures (Stingl et al., 2006).

Tissue dissociation: mammary glands were minced and then digested with 6000 U/ml collagenase I (Life Technologies) and 2000 U/ml hyaluronidase (Sigma) in the DMEM/F12 (Life Technologies) at 37°C for 1 hour with vigorous shaking. The digested samples were pipetted, spun down at 1500 rpm for 5 min, and incubated 3 min in 0.64% buffered NH_4Cl (Sigma) in order to eliminate contaminating red blood cells. After washing with DMEM/F12 + 5% FBS, cells were plated for 1 hour at 37°C in DMEM/F12+5% FBS: in this way, the majority of fibroblasts attached to the tissue culture plastic, whereas mammary epithelial populations did not; MEC were thus recovered in the supernatant and pelleted. After washing in PBS/EDTA 0.02%, MECs were further digested with 0.25% trypsin (Life Technologies) for 5 min and 5 mg/ml dispase (Sigma) plus 100 mg/ml DNase I (Roche) for other 10 min. The digested cells were diluted in DMEM/F12+5%FBS and filtered through 40 mm cell strainers to obtain single cell suspensions cells and washed once in the same medium.

FACS protocol: for separating various MEC subpopulations cells were stained for 30 min at 4°C with antibodies against CD49f (PE-Cy5, cat. 551129, BD Biosciences), CD29 (PE-Cy7, cat. 102222, BioLegend), CD61 (PE, cat. 553347, BD Biosciences), EpCAM (FITC, cat. 118208, BioLegend) and lineage markers (APC mouse Lineage Antibody Cocktail, cat. 51-9003632, BD Biosciences) in DMEM/F12. The stained cells were then resuspended in PBS/BSA 0,1% and sorted

on a BD FACS Aria sorter (BD Biosciences) into luminal differentiated (LD) cells, luminal progenitor (LP) cells and mammary stem cells (MaSCs).

Cell culture: primary sorted subpopulations from FACS were plated on collagen I-coated supports and cultured in 2D in mammary (MG) medium (DMEM/F12 supplemented with glutamine, antibiotics, murine EGF, murine bFGF, and heparin with 2% FBS).

Primary mammary epithelial cells infection and induction of yMaSCs

For induction of yMaSCs, LD cells were transduced for 48 hours with FUW-tetO-YAP ~~(Addgene #84009)~~, or FUW-tetO-TAZ, in combination with FUdeltaGW-rtTA lentiviruses. As a (negative) control, LD cells were transduced with either FUW-tetO-EGFP in combination with FUdeltaGW-rtTA (Addgene 19780). After infection, adherent cells were washed and treated with doxycycline for 7 days in MG medium for activating tetracycline-inducible gene expression (see scheme) to obtain "yMaSCs". After doxycycline treatment for 7 days in 2D culture, yMaSCs were processed for further analysis.

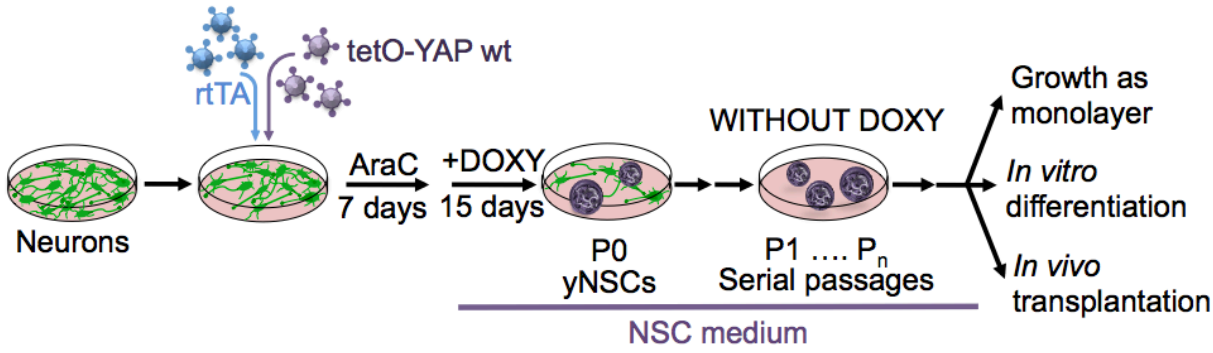
Matrigel culture of mammary colonies and organoids

After infection in 2D cultures and induction with doxycycline for 7 days, mammary cells were detached with trypsin and seeded at a density of 1,000 cells/well in 24-well ultralow attachment plates (Corning) in mammary colony medium (DMEM/F12 containing glutamine, antibiotics, 5% Matrigel, 5% FBS, 10 ng/ml murine EGF, 20 ng/ml murine bFGF, and 4 μg/ml heparin) containing doxycycline (2 μg/ml). Primary colonies were counted 14 days after seeding. To show the self-renewal capacity of yMaSCs independently of exogenous YAP/TAZ supply (i.e, independently of doxycycline administration), primary colonies were recovered from the MG-colony medium by collecting the samples and incubation with an excess volume of ice cold HBSS in order to solubilize Matrigel. After 1 hour, colonies were rinsed 3 times in cold HBSS by centrifugation at 1000 rpm for 5 min and incubated in trypsin 0.05% for 30 min to obtain a single cell suspension. Cells were counted and re-seeded at 1,000 cells/well in 24-well ultralow attachment plates in MG colony medium without doxycycline for further passaging.

For mammary organoid formation, primary colonies were recovered from MG colony medium in cold HBSS and transferred in 100% Matrigel. After Matrigel formed a gel at 37°C, MG organoid medium was added (Advanced DMEM/F12 supplemented with Hepes, GlutaMax, antibiotics, B27 1X, 50 ng/ml murine EGF, 20 ng/ml murine bFGF, 4 μg/ml heparin, 100 ng/ml Noggin and 1 μg/ml R-Spondin1). Note that at this step it is essential to avoid dissociating the primary colonies to single cells but rather to transfer the intact colonies into organoid culture conditions. After few days, colonies started to form

budding organoids. 64-75% (depending on the experiment) of yMaSC colonies evolved as organoids and were maintained and passaged without doxycycline. Also note that, in our hands, direct plating of MaSCs, LD control EGFP-infected, as well as YAP-infected cells, directly into organoid culture conditions did not result in any outgrowth, indicating that the intermediate step in colony culture conditions is required for organoid development. 2 weeks after seeding, organoids were removed from Matrigel, trypsin-dissociated and transferred to fresh Matrigel. Passages were performed in a 1:4-1:8 split ratio every 2 weeks. For analysis, colonies and organoids were recovered from Matrigel as before, and either embedded in OCT medium (PolyFreeze, Sigma) to obtain frozen sections for immunofluorescence or processed for protein or RNA extraction.

NEURONS



Schematic representation of yNSC induction (from Panciera et al., 2016)

Primary neuron isolation and induction of yNSCs

Neurons were prepared from hippocampi or cortices of late (E18-19) embryos or post-natal (P1) pups.

Day 1: Hippocampi and cortices were dissected under the microscope in ice cold HBSS as quickly as possible, incubated with 0.05% trypsin (Life Technologies) 15 min at 37°C and, after trypsin blocking, resuspended in DMEM/10% FBS supplemented with 0.1 mg/ml DNase I (Roche), and mechanically dissociated by extensive pipetting. Cells were then plated on poly-L-lysine-coated wells in DMEM supplemented with 10% FBS, glutamine and antibiotics for hippocampal neurons, or in DMEM/Neurobasal (1:1) supplemented with 5% FBS, B27, glutamine and antibiotics for cortical neurons.

Day 2: the medium of both hippocampal and cortical preparation was changed to fresh DMEM/Neurobasal (1:1) supplemented with 5% FBS, B27, glutamine and antibiotics.

Day 3: For reprogramming experiments neurons were infected with FUW-tetO-wtYAP and FUdeltaGW-rtTA viral supernatants (Addgene 19780). Negative controls were provided by neurons transduced with FUdeltaGW-rtTA alone or in combination with FUW-tetO-EGFP or FUW-tetO-MCS (empty vector). Note that the use of highly concentrated YAP-expressing lentiviruses (such as those obtained by ultracentrifugation) should be avoided. Also, we avoid using YAP5SA as reprogramming reagent, as we observed that infection with of YAP5SA could induce yNSCs rapidly, but in some experiments turned detrimental for long-term self-renewal capacity.

Day 4: the medium was changed and cells were incubated in Neurobasal medium supplemented with 1X B27, glutamine, antibiotics, and 5 μM Ara-C (cytosine β-D-arabinofuranoside; Sigma) for additional 7 days, at the end of which well-differentiated, complex network-forming neurons are visible

Day 11: to induce yNSCs formation, neurons were switched to NSC medium (DMEM/F12

supplemented with 1X N2, 20 ng/ml murine EGF, 20 ng/ml murine bFGF, glutamine, and antibiotics) and doxycycline (2 μg/ml) for activating tetracycline-inducible gene expression. After 7 days, fresh doxycycline was added.

Sphere formation was evident upon YAP induction after 14 days of doxycycline treatment. These "P0" spheres were gently transferred into a 15 ml-plastic tube and let sediment. After discarding the supernatant, spheres were dissociated with TrypLE Express (Life Technologies) and mechanical pipetting at room temperature. TrypLE Express was diluted in NSC medium and cells were resuspended in NSC medium without doxycycline. For the successive passages, spheres were harvested and dissociated and yNSCs were routinely cultured and passaged without doxycycline in NSC medium, as for normal NSCs.

Primary neural stem cells (NSCs) isolation and culture

Neural stem cells (NSCs) were isolated as previously reported (Ray and Gage, 2006) from the telencephalon of C57BL/6J E18 embryos or from mice of the indicated genotype. Telencephalons were minced and digested in trypsin 0.05% for 10 min at 37°C. The cell suspension was treated with DNaseI (Roche) and washed. NSCs were cultured in DMEM/F12 supplemented with 1% N2, 20 ng/ml murine EGF, 20 ng/ml murine bFGF, glutamine and antibiotics. For passages, neurospheres were dissociated into single cells with TrypLE Express (Life Technologies).

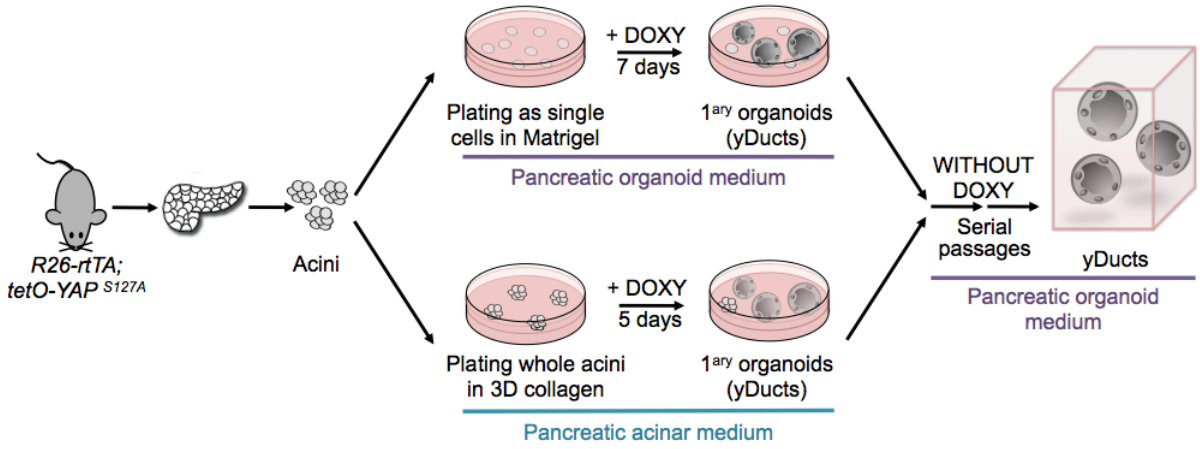
yNSCs differentiation

For neuronal differentiation (Choi et al., 2014), NSCs or yNSCs were cultured over a thin Matrigel layer. Differentiation medium was Neurobasal supplemented with 1X B27, glutamine.

For astrocyte differentiation (Bonaguidi et al., 2005), NSCs or yNSCs were plated on fibronectin coated-plate in NSC medium, to allow a 2D culture. The next day, medium was changed to DMEM (Life Technologies) containing 25 ng/ml LIF, 25 ng/ml BMP4, glutamine, and antibiotics for 2 weeks.

For oligodendrocyte differentiation (Hsieh et al., 2004), NSCs or yNSCs were plated on fibronectin coated-plate in NSC medium, to allow a 2D culture. The next day, medium was changed to Neurobasal (Life Technologies) containing 1x B27, 500 ng/ml IGF, 30 ng/ml T3, glutamine, and antibiotics for 2 weeks.

PANCREAS



Schematic representation of yDuct induction

Pancreatic acinar cells isolation and induction of yDucts

Primary pancreatic acini were isolated from the pancreas of 6- to 9-week-old *R26-rtTA; tetO-YAP^{S127A}* mice, according to standard procedures (Means et al., 2005). Digested tissue was filtered through a 100 μm nylon cell strainer. The quality of isolated acinar tissue was checked under the microscope.

For culture of entire acini, explants were seeded in neutralized rat tail collagen type I (Cultrex)/acinar culture medium (1:1)(Means et al., 2005), overlaid with acinar culture medium (Waymouth's medium (Life Technologies) supplemented with 0.1% FBS (Life Technologies), 0.1% BSA, 0.2 mg/ml SBTI, 1X ITS-X (Life Technologies), 50 μg/ml BPE (Life Technologies), 1 μg/ml dexamethasone (Sigma), and antibiotics) once collagen formed a gel.

For culture of isolated acinar cells, acini were further digested in 0.05% trypsin for 30 min at 37°C to obtain a single cell suspension. Single acinar cells were plated in 100% Matrigel; once Matrigel formed a gel, cells were supplemented with pancreatic organoid medium (Advanced DMEM/F12 supplemented with 1X B27, 1.25 mM N-Acetylcysteine, 10 nM gastrin, 50 ng/ml murine EGF, 100 ng/ml human Noggin, 100 ng/ml human FGF10, 10 mM Nicotinamide, 1 μg/ml R-Spondin1 and antibiotics) supplemented with 0.2 mg/ml SBTI.

For induction of pancreatic organoids, entire acini or single acinar cells were seeded in medium supplemented with 2 μg/ml doxycycline. Negative control cells were cultured in the same conditions in absence of doxycycline. Cells were treated with doxycycline for 7 days and organoid formation was morphologically followed. Organoids were then processed for further analyses.

Matrigel culture of yDucts organoids

To show the self-renewal capacity of pancreatic organoids independently of exogenous YAP supply (i.e, independently of doxycycline administration), organoids were recovered from Matrigel or collagen cultures, trypsinized to obtain a single cell suspension and re-seeded in 100% Matrigel covered with pancreatic organoid medium.

For passaging, organoids were removed from Matrigel by incubation in ice cold HBSS, dissociated with trypsin 0.05% for 30 min to obtain a single cells suspension and reseeded in 100% fresh Matrigel. Organoid cultures were maintained for at least 9 months passaging every 10 days. For analysis, organoids were recovered from Matrigel and processed for immunofluorescence or for protein or RNA extraction.

For normal Ductal organoid preparation and passaging we used the procedure detailed in Huch et al., 2013.