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Role of YAP/TAZ in cell plasticity

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PUBLICATIONS

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The work presented in this PhD thesis was ideated and coordinated by Prof Stefano Piccolo and it is part of a recent publication of our laboratory (see above). I performed the experiments under the supervision of Dr. Luca Azzolin and Dr. Tito Panciera that are the first authors of the work.

ABSTRACT
(ENGLISH VERSION)

The employment of somatic stem cells (SCs) as therapeutic elements is an important goal in the field of regenerative medicine. However, this is hampered by the fact that tissue stem cells are rare, difficult to purify and maintain in culture. Direct conversion of terminally differentiated cells back into their corresponding stem cells could provide a great effort in this sense. Here we show that ectopic YAP/TAZ expression in primary luminal differentiated cells of the mammary gland epithelium stably converts them into cells that display molecular and functional traits of mammary gland stem cells such as self-renewal, self-organization into structure that resemble the mammary gland *in vitro* and mammary gland reconstitution ability.

ABSTRACT
(ITALIAN VERSION)

L'utilizzo delle cellule staminali somatiche come agenti terapeutici è un importante obiettivo nell'ambito della medicina rigenerativa. Tuttavia, questo è ostacolato dal fatto che le cellule staminale sono rare, difficili da purificare e da mantenere in coltura. La conversione diretta di cellule differenziate nelle corrispondenti cellule staminali potrebbe rappresentare una soluzione a questo problema. In questo lavoro viene mostrato come l'espressione di YAP/TAZ in cellule differerenziate dell'epitelio della ghiandola mammaria conferisca a queste cellule proprietà staminali sia a livello molecolare che a livello funzionale , come la capacità di auto-rinnovamento, l'auto-organizzazione in strutture che ricapitolano la ghiandola mammaria *in vitro* e la capacità di rigenerare un'intera ghiandola mammaria.

EXTENDED ABSTRACT

Unlimited availability of somatic stem cells is a critical aspect for regenerative medicine. Tissue somatic stem cells are rare and difficult to purify hampering the possibility to use them to better understand stem cell biology and to develop regenerative medicine applications. Direct conversion of differentiated cells back into their corresponding stem cells has been proposed as a solution to overcome this problem, as plasticity has been described among tissue populations. Yet, the molecular traits that generate and maintain the stem cell status are poorly understood. YAP and TAZ, the nuclear effectors of the Hippo signaling pathway, are potent inducers of tissue growth during development and they play also a key role in tissue regeneration after damage. Moreover, abnormal YAP/TAZ activity was associated to tissue overgrowth and acquisition of cancer stem cell traits. These evidences suggest a role of YAP/TAZ in the generation and maintenance of the stem cell state when natural or pathological conditions require the expansion of the stem cell compartment.

Here we found that in the mammary gland YAP and TAZ are expressed in the mammary gland stem cells population and that they are the endogenous factors required to sustain the self-renewal ability of the mammary gland stem cells *in vitro*. These evidences prompted us to investigate if YAP/TAZ expression could confer stem cell traits to luminal differentiated cells (LDs) of the mammary gland. We found that expression of YAP/TAZ stably convert LDs in cell displaying molecular and functional characteristics of mammary gland stem cells such as self-renewal capacity and self-organization into tissue like structure that can be expanded as organoid cultures *ex vivo*. Mammary organoids resemble the mammary gland both at structural and functional level. The YAP-induced stem cell state can be transmitted

through cell generations without need of continuos YAP/TAZ expression indicating that a transient expression of YAP/TAZ is sufficient to induce a stable stem cell state and that somatic stem cells can be reprogrammed while preserving their lineage conversion.

INTRODUCTION

Somatic stem cells (SCs) are undifferentiated cells able to self-renew and to generate a differentiated progeny. They are involved in fundamental homeostatic processes such as tissue renewal and tissue regeneration after injury, making them a point of attraction for applications in regenerative medicine. However, the study of the mechanism governing the stem cell status is hampered by the fact that SCs are rare and difficult to purify and expand *ex vivo*. Recently, the development of three-dimensional culture systems allowing stem cells to self-organize into structure called "organoids" represents a revolutionary improvement in this direction. Unfortunately, the generation of organoids requires the isolation of stem cells as starting material.

An attractive alternative to this problem is represented by the direct conversion of differentiated cells back into their corresponding tissue-specific stem cells. Recently, several reports highlighted how somatic cells display an high grade of plasticity, as differentiated cells can return to a stem cell status under particular conditions such as tissue damage (Blanpain and Fuchs, 2014) suggesting that differentiated and stem cell state are interchangeable in response to extrinsic cellular cues.

However, how this plasticity is regulated and the factors involved in the generation and maintenance of the stem cell status are poorly understood.

It has been demonstrated the two transcription co-factors YAP and TAZ are required for the expansion of somatic stem cells during tissue regeneration after damage and for sustaining the aberrant cell growth during the initial phases of oncogenic transformation, suggesting a role of YAP/TAZ as stemness factor (Ramos and Camargo, 2012).

Thus YAP/TAZ could be key regulators of the maintenance or acquisition of stem cell traits whenever generation or expansion of stem cells is required to face natural or pathological conditions.

YAP/TAZ and their regulators

Hippo Signaling Pathway

YAP (Yes-associated protein) and TAZ (Transcriptional co-activator with PDZ-binding domain; also known as WWTR1) are two homologous transcription co-factors that were first discovered as the downstream effectors of the Hippo signalling pathway. The Hippo signalling is at the centre of mechanism that controls tissue growth and the organ size (Pan, 2010).

The components of the intracellular signalling cascade of the Hippo pathway were first discovered in Drosophila; the core cassette is composed by the protein kinase Hippo and its partner Salvador, the protein kinase Warts and its adaptor protein Mob as tumour suppressor (Mats). The investigation of the Hippo pathway revealed that there are several aspects of the pathway that are conserved from flies to mammals. At the core of the mammalian pathway are the two sterile serine/threonine kinase MST1 and MST2 (the orthologs of Hippo in Drosophila): when activated, MST1/2 bind their adaptor protein Salvador1 (SAV1/WW45) leading to the formation of an active enzymatic complex that phosporylate the large tumour suppressor 1 and 2 kinases (LATS1/2), as well as MOB1, allowing the formation of an active LATS1/2-MOB1 complex. The activation of LATS1/2-MOB1 complex lead to the phosphorylation of YAP and TAZ: phosphorylation of YAP at Ser127 and TAZ at Ser89

generate a binding consensus for 14-3-3 proteins leading to the cytoplasmatic retention of YAP and TAZ.

Moreover, LATS1/2-mediated phosporilation also inhibits YAP/TAZ by promoting their degradation through ubiquitination. The phosphorilation of S381 (S311 in TAZ) primes YAP for additional phosporylation by CK1 kinase resulting in the generation of a "phosphodegron" recognized by β-TRCP leading to YAP/TAZ polyubuquitination and degradation. (Liu et al., 2010; Zhao et al., 2010). Despite the presence of the phosphodegron, YAP is a relatively stable protein while TAZ is very protein degradation is the main process controlling TAZ inhibition. Deregulations of the Hippo pathway lead to the accumulation of YAP/TAZ in the nucleus.

YAP and TAZ are transcriptional co-activators lacking DNA binding domain and thus require the association with a DNA-binding partner to activate their transcriptional program. Several reports identified the TEAD family of transcription factors (Scalloped in *Drosophila*) as the most important mediator of YAP and TAZ activity (Zanconato et al, 2015; Zhang et al, 2009; Zhao et al, 2008) (Figure 1A). Moreover YAP and TAZ are involved in the regulation the transcriptional activity of others transcription factors such as SMADs, p73, RUNX, and PPAR-γ (reviewed in Piccolo et al., 2014).

As mentioned above Hippo pathway is involved in the control of the organ size indeed mutations of components of the Hippo pathway or overexpression of YAP/TAZ result in organ overgrowth due to the increase in cell division and a decreased susceptibility to cell death (Piccolo et al., 2014; Zhao et al., 2010). In *Drosophila*, overexpression of *Yorkie* (YAP/TAZ orthologue) leads to a massive growth of the eyes and enlargement of the wing imaginal discs (Figures 1B-C) (Dong et al., 2007; Huang et al., 2005). Since the components of the pathway are conserved from flies to mammals, similar phenotypes were observed in

transgenic mice. For instance, overexpression of YAP in the liver results in a massive liver overgrowth (Figure 1D) (Dong et al., 2007), while conditional knockout of the MST adaptor protein Salvador leads to cardiomegaly (Figure 1E) (Heallen et al., 2011).

Regulation of the Hippo pathway by cell architecture and cell polarity

The core components of the Hippo pathway and the mechanism leading to YAP/TAZ phosphorilation and inactivation are well known but the mechanisms regulating the Hippo pathway are not well understood.

Several reports pointed out a role of cell-cell adhesion and cell polarity (reviewed in Piccolo et al., 2014). Merlin, encoded by NF2 tumour suppressor locus, has been described to be involved in this type of regulation. In confluent monolayer of epithelial cells, Merlin/NF2 is localized at the cell membrane in proximity to cell-cell junctions were it may promote the correct assembly of protein scaffolds that allow the activation of LATS (Yin et al., 2013). Merlin reconstitution in NF2 deficient MDA-MB-231 cell line (breast cancer cells) results in a strong reduction of the YAP/TAZ activity that is totally LATS-dependent (Aragona et al., 2013).

The correct cell architecture and cell polarity have been shown to be relevant regulator of YAP/TAZ activity and this has been linked to the localization of cell polarity determinants, in particular Scribble. At the cell membrane Scribble serves as adaptor for the Hippo kinases promoting the assembly of a MST-LATS-TAZ complex required for LATS activation and TAZ inhibition. The loss of cell polarity and architecture (for instance during the initial phases of the epithelial to mesenchymal transition) leads to Scribble delocalization and activation of TAZ (Cordenonsi et al., 2011).

Recently it has been showed that other polarity determinants are involved in the regulation YAP/TAZ activity. Angiomotin (AMOT) family proteins were shown to interact with YAP/TAZ recruiting them to cell-junctions and inactivating through phosphorilation dependent and independent mechanisms (Chan et al., 2011; Hirate et al., 2014). Moreover it has been showed that α-catenin (the linker between cadherins and the actin cytoskeleton) is an inhibitor of YAP activity in keratinocytes since perturbations in the E-cadherin/α-catenin complex lead to a decrease in YAP phosporilation and promotion of YAP nuclear localization (Kim et al., 2011).

The large variety of connection between Hippo pathway and the cell polarity suggest that the cell architecture and adhesive properties take an important role in the regulation of YAP/TAZ activity.

Role of the Hippo pathway in the early embryonic development

YAP and TAZ have a key role during embryo development as demonstrated by the severe phenotypes associated to YAP/TAZ deletion at the embryo stages. Double knockout mutants die before implantation (Nishioka et al., 2009). YAP null mice die early after gastrulation (E 8.5) and they are characterized by defects in the elongation of the body axis combined with abnormal neural morphogenesis and defects of the extraembryionic tissues. As for YAP knockout, deletion of TAZ is lethal but a fraction of these mice develop to term and die for renal and pulmonary diseases (Makita et al., 2008).

YAP and TAZ play also an important role in cell fate decisions: it has been shown that, at the morula stage, YAP/TAZ are localized in the cytoplasm of inner mass cells while they are enriched in the nucleus of trophoectodermal cells where they promote the expression of Cdx2 and thus the specification of the trophoectodermal lineage (Nishioka et al., 2008).

Regulation of YAP/TAZ by mechanical cues

Hippo-mediated phosphorilation of YAP and TAZ is one the most important mechanisms involved in the regulation of the activity of these two co-transcription factors but it is not the only one. Recent findings highlighted how mechanical signals such as cell-shape, extra-cellular matrix stiffness and cytoskelatal tension are involved in the regulation of YAP and TAZ activity (Dupont et al., 2011; reviewed in Halder et al.,2012).

In cells cultured on stiff extra-cellular matrix (ECM), YAP and TAZ are localized into the nucleus where they can induce the transcription of their target genes; conversely, if cells are cultured on soft ECM, YAP and TAZ are re-localized into the cytoplasm and thus inactivated (Dupont et al., 2011). YAP/TAZ can also respond to changes in cytoskeletal tension: if cells are allowed to spread over the ECM, the cytoskeletal adaptation to the stretch conditions leads to YAP/TAZ nuclear accumulation and activation inducing proliferation and inhibiting differentiation; at the opposite, in cells grown on small adhesive ECM and forced to adopt a round and compact shape, YAP and TAZ are localized in the cytoplasm and cells stop to proliferate and start differentiation. YAP/TAZ appear to be not only sensors of mechanical cues but also crucial mediators of the biological effects of cell shape and ECM stiffness; for instance endothelial cells died when they are forced to be small but increasing YAP/TAZ levels in small cells allow them to proliferate; on the contrary, downregulation of YAP/TAZ in spread cells causes them to die (Dupont et al., 2011). Moreover, ECM stiffness is involved in the differentiation of mesenchymal stem cells (MSC) into different cell types (Engler et al., 2006). High stiffness (elevated YAP/TAZ) prompt cells to differentiate into osteocytes while a softer ECM (low YAP/TAZ) render cells competent toward adipocyte differentiation (Dupont et al., 2011). YAP/TAZ activity could be regulated also by cell density and tissue

architecture. YAP and TAZ inactivation underlies the contact inhibition of proliferation (CIP) (Zhao et al., 2007) that is defined as the arrest of cell division when cells occupy all the space available for their growth. A "two step" model recently explained the role of YAP/TAZ in this phenomenon (Aragona et al., 2013): when cells start to get in contact with each other, YAP/TAZ get phosphorilate by LATS and thus inactivated; this contributes for only 30% of growth inhibition. Successively, as cells continue to proliferate and become crowded, the reduction of the growth area result in an impairment of YAP/TAZ mechanical pathway in a manner not dissimilar from YAP/TAZ inhibition in cells cultured in small adhesive areas.

Crosstalk between YAP and Wnt signaling

YAP and TAZ are not only sensor of the physical environment and tissue architecture but they can also mediate the response to chemical soluble growth factors, indeed it has been recently demonstrated an integration between YAP/TAZ and the Wnt pathway (Azzolin et al., 2014). The core of the Wnt pathway is the regulation of the intracellular transducer β-catenin: in the absence of Wnt, β-catenin is constantly degraded by a cytoplasmic destruction complex consisting of a central scaffold protein Axin, that interacts with other proteins such as adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). In absence of Wnt, the amino-terminal region of β-catenin is sequentially phosphorilated by CK1 and GSK3 resulting β-catenin recognition by β–TrCP and β-catenin ubiquitination and degradation (He et al., 2004). The arrival of Wnt causes functional inactivation of the destruction complex leading to β-catenin accumulation in the nucleus and the formation of nuclear complexes with its DNA-binding partner TCF/Lef (Clevers, 2006). Recently it has been discovered that YAP/TAZ are components of β-catenin destruction complex: they are sequestered in the cytoplasm in the destruction complex and

more, YAP/TAZ association to Axin is required for recruitment of β–TrCP to the complex. Indeed, in "Wnt OFF" conditions, depletion of YAP/TAZ leads to the activation of β-catenin transcriptional program. On the other hand, in "Wnt ON" conditions, the binding between Axin and the Wnt receptor LRP6 leads to the release of YAP/TAZ from the destruction complex. In this situation the destruction complex is "invisible" to β–TrCP, favoring β-catenin accumulation in the nucleus and the activation of Wnt-induced, YAP/TAZ dependent transcriptional program (Azzolin et al, 2014).

YAP/TAZ AND STEM CELLS

Stem cells (SC) display the capacity to self-renew when they divide and to generate a differentiated progeny. This cycling activity is fundamental in maintaining tissue homeostasis and to respond to physiological and environmental stimuli. As mentioned above, Hippo pathway is involved in the control of the organ size and mutation in components of the pathway lead to phenotypes characterized by tissue and organ overgrowth suggesting a role of YAP and TAZ in the regulation of SC and progenitor self-renewal (Ramos and Camargo, 2012; Zhao et al., 2010).

The use of conditional mutants for the Hippo pathway components and inducible transgenic mouse models highlighted a role of YAP/TAZ in the expansion of progenitor cell compartment and in cell fate decision in diverse tissues and organs. The simple overexpression of YAP in the liver of transgenic mice leads to the acquisition of biliary/duct liver progenitor traits by the hepatocyte (Yimlamai et al., 2014). Gain of YAP in the basal layer of the epidermis results in the expansion of the stem cell compartment with increased keratinocytes proliferation, defective stratification and reduced terminal differentiation; vice

versa deletion of YAP from the basal layer in embryos epidermis leads to reduced stem cell's self-renewal resulting in skin loss (Schlegelmilch et al., 2011; Zhang et al., 2011). In the adult intestine YAP expression is restricted to crypt compartment, that is enriched in stem cells, and overexpression of YAP induces the expansion of undifferentiated progenitors (Camargo et al., 2007). YAP/TAZ are also required for the regulation of stem cell expansion after injury, indeed inactivation of YAP severely impairs epithelial proliferation and crypt repopulation in the intestinal epithelium after DSS treatment (dextran sulfate sodium; an inductor of colitis) (Cai et al., 2010).

Some evidences highlighted a role of YAP/TAZ not only in normal stem cells but also in cancer stem cells (CSC). CSCs are the driving force for the growth of solid tumours, sustaining the proliferative potential, the resistance to chemotherapy and the loss of differentiation markers (Visvader, 2012). Recent findings demonstrated that TAZ activity is required to empower CSC characteristics to non-stem cancer cells. In particular, gain of TAZ endows tumor-initiating capacity to non stem-cancer cell populations and loss of TAZ impairs this capacity in CSC (Bartucci et al., 2014; Cordenonsi et al., 2011). Moreover, YAP/TAZ have been associated with the formation of metastasis of lung and breast cancer (Bartucci et al. 2014, Lamar et al., 2012; Lau et al., 2014) and also that loss of TAZ impairs tumorigenic potential and invasiveness in glioblastoma (Bhat et al., 2011).

However, the idea that YAP/TAZ are stemness factors (Ramos and Camargo 2012) is based on distinct and poorly understood observations: the organ overgrowth in Hippo mutants embryos and the preferential localization of YAP/TAZ proteins in the stem cell niche of adult tissues; the role of YAP/TAZ during embryonic development is poorly understood and at the same time the connection between tissue-specific stem cells and their embryonic counterparts is not clear. Moreover conditional inactivation of YAP in adult mammary gland, pancreas,

liver and also double YAP/TAZ inactivation in the intestine showed that YAP/TAZ activity is dispensable for normal tissue homeostasis (Azzolin et al., 2014, Chen et al., 2014; Zhang et al., 2010, Zhang et al., 2014). However YAP and TAZ are essential in conditions in which stem cells need to be expanded for tissue regeneration or after tumour initiation.

ORGANOIDS

The employment of stem cells for use in regenerative medicine and disease modelling *in vitro*, is hampered by the fact that somatic stem cells are rare, difficult to purify and to expand maintaining their multi-lineage differentiation potential *in vitro.*

However, advances in understanding the key role of the stem cell niche and the signal involved in stem cell maintenance lead to the development of 3D culture systems that sustain the stem cell-driven formation of organoids (reviewed in Barker 2016). Organoids can be defined as a cluster of organ-specific cell types developed from stem cells that are able to self-organize in vitro into tissue like structure. They can be generated either from isolated stem/progenitor cells or from isolated fragment of the tissue from the corresponding organ (Koo et al., 2012). In recent years, organoid cultures have been developed from many tissues such as mammary gland (Dontu et a., 2003), small intestine (Sato et al., 2009), liver (Huch et al., 2015), pancreas (Huch et al., 2013), prostate (Gao et al., 2014), stomach (Bartfeld et al., 2015). Organoids resemble the architecture of the organ from which they derive and also recapitulate the stem cell differentiation hierarchy allowing in vitro studying of the mechanism involved in the determination of stem cell fate. Moreover, under appropriated stimuli, they could resemble also specific functions of the tissue from which they derive.

Organoids could be amenable to standard experimental manipulations used for cell lines, such as small interfering RNA and DNA transfections, infection with recombinant viruses and

storage by freezing (Koo et al., 2012). They can also be analysed by standard techniques, such as immunohistochemistry and confocal immunofluorescence, gene expression and mass spectrometry. Importantly, transgenic alleles could be manipulated in organoid culture to follow their effect during time.

The possibility to grow organoids would open new perspectives in the study of stem cells and tissues in various contexts. For instance, adult stem cells can be propagated in organoids for long time (also years) without genomic alterations. Moreover, organoids could have an important role in modelling human disease *in vitro* and also in generating isogenic adult tissues for regenerative procedures and transplantation.

AIM OF THE STUDY

As described in the introduction, tissue cell populations display a certain grade of plasticity leading to the dedifferentiation of differentiated cells back into their corresponding stem cells when natural or environmental conditions require the expansion of the stem cell compartment (Blanpain and Fuchs, 2014).

Disregulation of the Hippo pathway has been recently associated to with cellular dedifferentiation indeed. For instance, in liver the inactivation of the Hippo signaling leads to a massive organ growth due in part to a transient lineage conversion of hapatocytes into biliary cells (Yimlamay et al., 2014). In the context of tumours, it has been shown that the activation of TAZ could confer cancer stem cells traits to non-stem cancer cells (Cordenonsi et al., 2011).

These evidences suggest the possibility that expression of YAP and TAZ in differentiated cell, not only cancer cells but even normal differentiated cells, could confer them characteristics of stem cells.

We tested this hypothesis in one of the most established system to study epithelia's homeostasis: the mammary gland. We first characterized YAP/TAZ expression and role in the subpopulations that compose the mammary gland and then we tested if ectopic YAP/TAZ expression in differentiated cells of the mammary gland could empower them characteristics of stem cells.

RESULTS

Isolation of mouse primary mammary epithelial cells (MECs)

The mammary gland represents one the most important paradigm for the study of epithelia's homeostasis and the mechanisms involved in tissue regeneration and maintenance. Previous works demonstrated that activation of YAP/TAZ in breast cancer cells could confer stem characteristics to non-stem cancer cells (Cordenonsi et al., 2011, Piccolo et al., 2014). Based on these data we thus hypothesized that expression of YAP/TAZ could confer stem characteristics also to normal differentiated mammary gland cells.

To address a possible role of YAP/TAZ in these processes, we started with the isolation of the cell subpopulations that compose the mammary gland epithelium. For this purpose primary mammary epithelial cells were isolated from mammary gland of 8 to 12 weeks old C57BL/6J virgin mice. After mechanical dissection and enzymatic digestion of the glands, we purified by Fluorescence Activated Cell Sorting (FACS) lineage negative (Lin^-) and EpCAM positive epithelial cells using CD49f and CD61 as surface markers (Guo et al., 2012). These procedure allowed distinguishing of three cell populations: a) Mammary gland stem cells enriched fraction (MaSC) ($EpCAM^{low}$, $CD61^+$, $CD49f^{high}$); b) Luminal Progenitors cells (LP) ($EpCAM^{high}$, $CD61^+$, $CD49f^{low}$); c) Luminal Differentiated cells (LD) ($EpCAM^{high}$, $CD61^-$, $CD49f^{low}$) (Figure 2A). These experiments were conducted in collaboration with Professor G. Basso and Dr. C. Frasson (Children's hospital / Città della Speranza, Padova).

Characterization of the mammary gland subpopulations

To validate and further characterize the FACS-purified cell populations we evaluated, by qRT-PCR, the expression of basal and luminal markers. As expected, MaSCs were characterized by the highest expression of basal keratins such as *K14, K5* and a lower expression of luminal keratins such as *K8, K19* when compared to Luminal Differentiated cells and Luminal Progenitors cells. Moreover MaSCs display the highest expression of stem (e.g: *p63, Lgr5, Procr*) and myoepithelial markers (e.g: *Myh11, α-Sma*) (Figure 2B) (Chakrabarti et al., 2014; Wang et al., 2015) that is in line with the finding that the MaSCs enriched population is heterogeneous: it is composed by both basal and myoepithelial cells that have been showed to display stem characteristics. Western blot analysis confirmed the differential expression of basal and luminal keratins among the FACS-purified populations (Figure 2C).

We next functionally characterized our population by measuring their colony forming capacity and self-renewal ability, which are cardinal properties of the stem cells. For this purpose, cells were seeded in colony forming medium containing 5% of Matrigel (see methods) in order to allow the formation of solid outgrowths from single cells. As expected only MaSCs displayed the ability to form colonies that can be passeged through various generation; LPs do form cavitated spherical colonies but these cannot be passaged indicating that these cells are void of self-renewal ability; in contrast LDs remained as single cells (Figure 2D).

Another cardinal property of mammary gland stem cells is the ability to regenerate an entire mammary gland with MaSCs contributing both to the luminal and myoepithelial lineages (Blanpain and Fuchs 2014). As shown in Figure 2E, injection of the FACS-purified MaSCs enriched population into the cleared fat pad of mice triggered regeneration of an entire ductal

tree; in line, LDs were completely void of the capacity to regenerate when injected into the cleared fat pad.

Taken togheter, these findings indicate that our FACS profiling is efficient at identifying the three main populations that compose the mammary gland epithelium.

Western blot analysis showed that YAP/TAZ proteins are expressed in MaSC enriched population and Luminal Progenitors cells while are barely detectable in Luminal Differentiated cells (Figure 2F). Moreover, measuring the expression of two established YAP/TAZ target genes (*Ctgf* and *Axl*) (Zanconato et al., 2015), by RT-qPCR, we found that MaSCs and LP cells display an higher transcriptional YAP/TAZ activity than LDs (Figure 2G).

Based on these data, we next argued if YAP/TAZ could have functional role in the MaSCs. For this purpose I teamed with Tito Panciera and employed mouse conditional knockout alleles of YAP/TAZ ($Yap^{fl/fl}$; $Taz^{fl/fl}$). MaSCs from these mice were FACS-purified as above and single cell suspension of MaSCs was infected with adenoviral vectors bearing the Cre recombinase (Adeno-Cre) or GFP as control (Adeno-GFP). Cells were then plated in colony medium to allow the formation of solid outgrowths from single cells. In these conditions only $Yap^{fl/fl}$; $Taz^{fl/fl}$ MaSCs infected with Adeno-GFP formed solid outgrowths while genetic ablation of YAP/TAZ, by adeno-Cre delivery in $Yap^{fl/fl}$; $Taz^{fl/fl}$ MaSCs, severely impaired the organoid forming ability (Figure 2H) These data indicate that YAP/TAZ not only are expressed and transcriptionally active only in mammary gland stem cells and not in luminal differentiated cells, but also that YAP/TAZ are endogenous factor required for the expansion of MaSCs in vitro.

YAP/TAZ expression revert mammary gland differentiated cells into MaSC-like cells

Given that YAP/TAZ are required for the expansion and self-renewal of mammary gland stem cells and also that the activation of YAP/TAZ in mammary tumors can convert non-stem cancer cells into cancer stem cells (Cordenonsi et al., 2011), we hypothesized that expression of YAP/TAZ may confer stem characteristics also to normal mammary differentiated cells.

To address this, FACS-purified Luminal Differentiated cells, obtained as described above, were plated on collagen-coated dishes and transduced with doxycycline-inducible lentiviral vectors bearing wild-type (YAPwt or TAZwt) or the activated version of YAP and TAZ (YAP5SA or TAZ5SA) lacking the inhibitory LATS phosphorilation sites. As control, cells were transduced with a lentiviral vector encoding for EGFP. Cells were cultured in doxycycline containing medium for 7 days after transduction and then plated at clonogenic density in colony forming medium to allow the formation of solid colonies from single cells (Figure 3A). Interestingly, expression of YAP or TAZ in LDs allowed them to form solid colonies that were indistinguishable from those generated by MaSCs, while LDs transduced with EGFP control vector remained as single cells without ever originating a single colony in 33 experiments (Figure 3B-C). Furthermore, transduction of LDs with an inducible lentiviral vector bearing a transcriptionally inactive form of YAP (YAPS94A; unable to interact with TEAD) had no effect on the colony forming ability (Figure 3B-C), suggesting that this process could be linked to YAP-regulated gene transcription. These data indicate that YAP/TAZ expression in LDs confer them colony forming ability, that is one of the characteristics of stem cells.

We next wanted to evaluate if YAP/TAZ expression in LDs could confer also self-renewal potential, which can be assayed by the ability to serially passage mammary colonies in vitro.

For this purpose I supported a series of experiments carried out by Luca Azzolin, in which we dissociated colonies from natural MaSCs and YAP/TAZ induced MaSCs. Cells were re-seeded then in mammary colony medium without doxycycline. Secondary colonies were counted 2 weeks after seeding, dissociated and re-seeded in the same conditions to allow the formation of tertiary colonies. Colonies from YAP/TAZ-transduced LD cells, similarly to those generated from MaSCs, could form additional generations of colonies after single cell dissociation without changing the colony forming efficiency (Figure 3D-E). Moreover the colony forming ability after passaging was comparable in presence and absence of doxycycline, that is irrespective of ectopic YAP/TAZ expression, suggesting that transient expression of YAP/TAZ is sufficient to confer self-renewal potential to mammary epithelial cells.

To verify if the conversion from LDs to MaSC-like state could be recapitulated also at single cell level, we induced YAP expression in single LDs plated in 96 well-plates (visually verified). Individual transduced LDs cells formed solid colonies with high frequency (18,5% on average in three independent experiments) that could be further passaged as clonal organoids with high efficiency. As expected transduction with EGFP control vector or YAPS94A vector had no effect on the colony forming ability of the single LDs transduced cells (0% either with EGFP and YAPS94A) (Figure 3F). We thus designated these YAP/TAZ induced MaSC-like cells as yMaSCs. Of note, overexpression of YAP in the purified MaSC-enriched population does not increase the colony forming ability indicating that even if rare MaSCs were present in the LDs starting population, these could not be expanded by YAP expression strengthening the notion that yMaSCs originate from YAP induced reprogramming of LDs (Figure 3G).

Lineage tracing of yMaSCs

To further validate the notion that YAP expression converts differentiated cells to a SC fate we carried out genetic lineage-tracing experiments using LD cells purified from *K8-CreERT2; R26-LSL-YFP* mice. For this purpose we FACS-purified LDs from these mice and, after plating, we exposed cells to a tamoxifen pulse leading to the excision of the stop cassette and to the irreversibly YFP labeling exclusively in Luminal Differentiated cells (K8 positive cells) (Van Keymeulen et al., 2011) (Figure 4A). Cells were then transduced with inducible YAP expressing lentiviral vector and plated in Matrigel containing medium in order to allow the formation of colonies; empty vector was used as control (see above). As shown in Figure 4B, colonies that were generated from the reprogramming of LDs were entirely YFP positive attesting their origin from the luminal lineage. To validate that the expression of the CreERT2 was restricted only to LDs (K8 positive), we purified MaSCs from *K8-CreERT2; R26-LSL-YFP*, treated them with tamoxifen and plated in 5% Matrigel containing medium. As shown in Figure 4B, colonies arising from these MaSCs were entirely YFP negative (n=154, 0% YFP +) indicating that the Cre recombinase is active only in LDs and not in MaSCs.

We next performed a complementary experiment taking advantage *of K14-CreERT2; R26-LSL-YFP* mice. In this experimental setup, administration of tamoxifen leads to the YFP labeling exclusively of the MaSC enriched population (K14 positive cells) (Van Keymeulen et al., 2014) (Figure 4A). As above, FACS purified LDs and MaSCs were exposed to tamoxifen pulse in order to label only K14 expressing cells. LDs were then transduced with YAP-encoding vector and plated in 5% Matrigel containing medium to allow the formation of colonies. yMaSC colonies generated from *K14-CreERT2; R26-LSL-YFP* mice were entirely YFP negative (n=122, 0% YFP+) while control colonies generated from endogenous

MaSCs of the same genotype were totally YFP positive (Figure 4B). Taken togheter all these data strengthen the idea that yMaSCs derive from YAP reprogramming of Luminal Differentiated cells and not from the expansion of rare contaminating endogenous MaSCs.

Characterization of the early steps of YAP induced reprogramming

To characterize the initial phase of reprogramming, we carried out immunofluorescence analyses on the earliest yMaSCs emerging as colonies from YAP-expressing LD cells. Interestingly, we found that a large fraction of these colonies consists of cells double positive for K14 and K8 (Figure 5A,C). Moreover colonies were virtually lacking of any staining for the myoepithelial marker α-SMA (Figure 5B,C) suggesting that the early yMaSCs do not represent a transdifferentiation of LDs into myoepithelial cells; rather, the early yMaSCs appeared to represent a progenitor-like state distinct from those prevalently found in the adult mammary gland and reminiscent of embryonic/fetal mammary progenitor cells, that are indeed double K14/K8-positive and α-SMA negative (Wansbury et al., 2011).

To get more insight on the nature of early yMaSCs, I teamed with Tito Panciera and Prof. Michelangelo Cordenonsi and performed RNA-seq analyses comparing the gene expression profiles of early yMaSC colonies, control LDs and MaSC-enriched basal cell populations. As shown in Figure 5D, the LD-to-yMaSC transition triggers downregulation of a group of luminal genes highly expressed in terminally differentiated cells (e.g., *Estrogen Receptor* and *Wap*, blocks A-B) and upregulation of a host of basal markers originally not expressed in LD cells such as *K14, K5, DNp63, LGR4* (block F). In contrast, other markers remain repressed in early yMaSCs, including myoepithelial markers such as *Myh11, Calponin1, Mrtf-a, Myosin-light chain kinase* and *Cd10*, as well as *Procr* (an adult MaSC marker) (Wang et al., 2015) (blocks D-E). This further reinforces the conclusions that YAP does not induce

transdifferentiation of LD into myoepithelial cells. Interestingly, a specific group of genes is exclusively induced in early yMaSCs (block G), and a significant fraction of these (61%) corresponds to genes also expressed in the fetal mammary gland. In line, a significant fraction of the luminal genes (54%) whose expression is retained by early yMaSCs (block C) represents genes also expressed by the fetal mammary gland (Wansbury et al., 2011). These data were validated also by qRT-PCR (Figure 5E). It is important to note that this represents a very transient state, as when yMaSCs were transferred in organoid medium they readily mature into cells displaying a profile matching adult MaSCs that will give rise to fully mature mammary organoids (see below). Also note that 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.

To further validate the notion that YAP/TAZ truly convert LDs to a stem cell fate we take advantage of the *LGR5-GFP* mice (Barker et al., 2012), indeed it has been demonstrated that Wnt signaling is instrumental for the maintenance of somatic stem cells and that several tissue, including the mammary gland contain a Wnt responsive population marked by LGR5 that is enriched in stem cells (Zeng et al., 2010; Clevers et al., 2014). We FACS-purified LDs from these mice and induced to express YAP by doxycycline administration (Figure 5F). LDs from *LGR5-GFP* mice were initially negative for GFP expression but readily turn it on (frequency >20%) upon induction of YAP indicating the activation of the LGR5 promoter and so a switch from a differentiated to a stem cell fate (Figure 5G-H). As control cells transduced with empty vector or YAPS94A (transcriptionally inactive version of YAP) were totally negative for GFP (Figure 5G-H).

The expansion, differentiation and regenerative potential of yMaSCs

To verify if yMaSCs truly represents mammary gland stem cells we tested their ability to self-organize in vitro into mammary tissue-like structures and to differentiate along distinct lineages. Since colonies were not suitable to appreciate and investigate about MaSCs and yMaSCs differentiation potential, we tried to establish a long-term culture system that allows yMaSCs to form mammary-gland like structures in vitro, taking advantage of the "organoids" culture system (Sato et al., 2009). For this purpose MaSC and yMaSC-derived colonies were transferred and embedded into 100% Matrigel, and overlaid with "organoid" medium. To optimize the culture conditions we tested the organoid forming capacity and self-renewal ability of colonies culturing them in different organoid media. We started testing the colony forming medium, in order to preserve the colony identity, but under these culture conditions cells rapidly died without possibility of expansion. Since it was demonstrated that MaSCs are dependent on Wnt protein for their expansion (Zeng e Nusse, 2010) we tried to improve our culture conditions by adding Wnt3a to the organoid medium. In these culture conditions cells maintained their shape and vitality but they were not able to regrow after single cell dissociation.

We next tried to improve the culture conditions by adding EGF, Noggin and R-Spondin (see methods), three factors that were described for the culture of intestinal organoids (Sato et al., 2009) and then maintained for the culture of other organoids type such as prostatic and liver organoids. Under these culture conditions, colonies started to form budding organoids in 2 weeks: 64-75% (depending on the experiment) of yMaSC colonies evolved as organoids that were maintained and passaged without doxycycline. yMaSCs derived organoids were

indistinguishable in growth pattern and size to those generated by endogenous MaSCs (Figure 6A-B).

Histological analysis showed that yMaSCs derived organoids, similar to those generated from endogenous MaSCs, resemble the mammary gland architecture. They were composed by an E-Cadherin positive stratified epithelium (Figure 6C) in which internal cells surrounding a central cavity expressed marker of Luminal Differentiated cells such as K8 and K19 (Figures 6D-E, G-H) while the outer cells expressed markers of basal/stem cells and myoepithelial cells such as K14, p63 and α-SMA (Figures 6D-I). Moreover, histological examination of lineage traced yMaSCs organoids (generated as above from *K8-CreERT2; R26-LSL-YFP* mice) showed that they are composed by YFP positive and K14 positive cells, confirming that they were originated by YAP induced reprogramming of LDs (Figure 6L).

We next moved to characterize yMaSCs at the molecular level. For this, we FACS-purified yMaSCs from organoids and evaluated, by qRT-PCR, the expression of basal and luminal markers, using freshly purified endogenous adult MaSCs and LDs as controls. Gene expression analysis revealed that organoid stage yMaSCs expressed basal markers (including myoepithelial markers such as *α-Sma* and *Myh11*) all to levels comparable to endogenous MaSCs while they are void of expression of luminal markers such as *K8, K18* and *Pgr*. Organoid stage yMaSCs also express genes previously associated to adult MaSCs such as *Lgr5*, *ΔN63* and *Procr* (Figure 7A).

Moreover, unsupervised hierarchical clustering of gene expression profiles showed that organoids from MaSCs and yMaSCs could not be distinguishable (Figure 7B) indicating that MaSCs and yMaSCs are similarly potent.

Mammary gland cells display the ability to produce milk when stimulated by hormones. To test if yMaSC organoids resemble the mammary gland also from at functional level, we

stimulated them by adding to the culture medium the hormone prolactin in presence of insuline and dexamethasone (Wartman et al., 1996) and then we evaluated by qRT-PCR the expression of genes known to be associated with the production of milk. Organoids derived from endogenous MaSCs were used as control. As shown in Figures 7C-D, the addiction of lactogenic stimulus triggered the expression of α *e* β*-casein*, characteristics of milk-producing cells indicating that yMaSCs *in vitro* are able to differentiate in alveolar cells when exposed to the appropriate stimuli.

Taken togheter all these data indicate that yMaSCs, similar to endogenous MaSC, display self-renewal potential and could generate epithelial like organoids that resemble the mammary gland at structural, functional and molecular level.

YAP/TAZ are not only instrumental for the reprogramming of LDs but are also endogenous factors required to preserve the self-renewal potential of yMaSCS derived organoids in vitro. As for endogenous MaSCs, genetic ablation of YAP/TAZ by Adeno-Cre delivery in yMaSCs organoids derived from conditional knockout mice ($Yap^{fl/fl}$; $Taz^{fl/fl}$) bleached their capacity of regrow upon passaging (Figure 7E). Interestingly, induction of exogenous YAP in LDs turned on the expression of endogenous YAP/TAZ that remained expressed in yMaSCs derived organoids after ectopic YAP expression had been turned off (Figure 7F) suggesting that a transient YAP expression is sufficient to endow a loop of YAP/TAZ expression in yMaSCs derived organoids.

Since the ability to trigger tissue regeneration is one of the characteristics of the mammary stem we wanted to test if yMaSCs display regeneration capacity after injection in cleared fat pad of mice. For this purpose, we transduced FACS-purified LD with vectors encoding for

inducible YAPwt and constitutive EGFP in order to trace injected cells. Cells were kept in doxycycline for 7 days and transplanted into the cleared mammary fat pad of NOD-SCID mice. Mice were then kept in doxycycline-free diet (Figure 8A.) Luca Azzolin conducted injection experiments in collaboration with Prof. Antonio Rosato (Istituto Oncologico Veneto IOV-IRCCS). Strikingly, cells acquired the ability to regenerate an entire mammary gland after a transient YAP expression (25%, n=16) (Figure 8B.) Regeneration of an entire mammary gland was observed when as few as 100 yMaSCs were injected in the cleared fat pad (33%, n=6). As control, LD cells transduced only with EGFP did not display any reconstituting activity (0%, n=28) (Figure 8C). By histological examination, reconstituted ductal threes obtained from yMaSCs were indistinguishable from those generated by endogenous MaSCs (Figure 8C); they were composed by an EGFP-positive stratified epithelium (Figure 8D) consist of a basal layer (expressing marker of basal cells such as K14 and α-SMA) overlaid by cells expressing luminal marker such as K8 and K19 (Figure 8E-F). Interestingly, reconstituted mammary glands generated a dense ductal system ending in clusters of milk-secreting alveoli when these mice were impregnated (Figure 8G) indicating that, similar to endogenous MaSCs, yMaSCs retain multilineage differentiation potential *in vivo*.

This collective set of experiments reinforces the notion that transient expression of YAP/TAZ in differentiated cells of the mammary gland is able to convert them into bona fide MaSCs.

DISCUSSION

Dysregulation of the Hippo pathway has been associated to changes in cell fate decision in transgenic liver e mammary cells (Cordenonsi et al., 2011; Skibinski et al., 2014; Yimlamai et al., 2014). However the possibility to *de novo* generate tissue somatic stem cells through YAP/TAZ is fascinating, yet totally unexplored. Here we report that the expression of YAP/TAZ in luminal differentiated cells of the mammary gland epithelium generates cells with molecular and functional characteristics of their corresponding endogenous stem-cells, that can be expanded as self-propagating organoids *ex-vivo*. The stem cell state triggered by YAP/TAZ can be transmitted through several cell generations without continuous expression of exogenous YAP indicating that a transient YAP/TAZ expression is sufficient to stably endow an heritable stem cell state.

YAP turns differentiated cells of the mammary gland into MaSC-like cells

In this work we used several approaches to validate the notion that yMaSC derive from YAP-induced reprogramming of luminal differentiated cells rather than from the amplification of pre-existing stem cells present in the starting population.

A first indication in this sense derives from the characterization of the FACS-purified population. We used a well-established FACS protocol (Guo et al., 2012) and we validated by molecular and functional analysis that it is efficient in identifying the populations that compose the mammary gland epithelium. The experiments invariably provided very clear results: in no case we could detect emergence of colonies in control cultures transduced with

EGFP encoding vector or mutant YAP (YAPS94A) unable to interact with TEAD, indicating that our starting population is completely void of detectable SCs.

A second indication derives from genetic lineage tracing experiments, in which we used established and well characterized Cre driver (*K8-CreERT2*) (Van Keymulen et al., 2011) to label and trace the fate of luminal differentiated cells. Colonies and organoids generated by YAP-induced reprogramming of LDs derived from *K8-CreERT2; R26-LSL-YFP* were entirely YFP positive attesting their origin from differentiated cells. Vice versa, colonies and organoids generated from endogenous MaSCs of the same genotype were invariable void of YFP expression strengthening the notion that Cre driver label exclusively differentiated cells and their progeny. Moreover the possibility that YAP could expand rare stem/progenitors present in the starting populations is inconsistent with the observation that YAP has no effect of the colony-forming capacity of endogenous MaSCs. Finally, the early yMaSCs immediately emerging after YAP expression are very different from normal, adult MaSCs as they resemble fetal mammary stem cells, that do not exist (or would be exceedingly rare) in the adult mammary tissue; the early yMaSCs could only be generated de novo. Collectively these data support the notion that yMaSCs emerge from the YAP-induced reprogramming of luminal-differentiated cells and not from the amplification of contaminant stem cells.

YAP-induced reprogramming generates cells displaying traits of normal SC: yMaSCs can be expanded through multiple generations as self-expanding organoids *ex vivo*; yMaSCs-derived organoids resemble the architecture and the function of the mammary gland indicating that yMaSCs retain multilineage differentiation ability both in *vitro* and also *in vivo*, as injection of yMaSCs in the cleared fat pad leads to the reconstitution of an entire ductal tree. Moreover yMaSCs display remarkable overlaps with endogenous MaSCs also at the transcriptomic level.

Of note, differently from induced pluripotent stem cells (iPSCs), YAP induced stem cells maintain the epigenetic memory of the tissue of origin and are never tumorigenic as we never detected the emergence of tumors after yMaSC injection in the cleared fat pad of mice.

YAP/TAZ and cellular plasticity

Reversion from differentiated to a stem cells state rarely occurs in normal tissues but it occurs in situation such as tissue repair and oncogenic transformation (Blanpain and Fuchs, 2014; Mani et al., 2008; Skibinski et al., 2014; Tetteh et al., 2014; Yimlamai et al., 2014). As described in the introduction, genetic depletion of YAP/TAZ in several adult epithelia has no effect on the normal tissue homeostasis but is fundamental for tissue regeneration, tumor growth (Azzolin et al., 2014; Cai et al. 2010; Zhang et al, 2014) and, as shown in this work, for the in *vitro* expansion of MaSCs. These evidences open a scenario in which YAP/TAZ could be proposed as regulators of cell plasticity and stem cell maintenance, whenever the expansion of endogenous stem cells or *de novo* generation from differentiated cells is required to face physiological or pathological conditions.

Further work is required to understand the means by which YAP overcomes the differentiated state and triggers the generation of a SC state *de novo*. At the molecular level this is clearly linked to the YAP/TAZ transcriptional program, indeed yMaSCs generation is impaired using YAPS94A (unable to bind TEAD transcription factor) for the reprogramming. Gene-expression studies provided some intriguing mechanistic insights, as we found that YAP induces several genes known to be instrumental for SC self-renewal and/or functionally implicated in MaSC biology, such as ΔNp63 (Chakrabarti et al., 2014).

YAP and TAZ are not only instrumental for the reprogramming of luminal differentiated cells but are also the endogenous factors required to maintain the self-renewal ability of MaSCs *ex-vivo*. In line, exogenous YAP can indeed install a self-sustaining loop in differentiated cells leading to a stable re-expression of endogenous YAP/TAZ in ySCs, where they are in turn required to preserve organoid-forming potential. It is thus tempting to propose the existence YAP/TAZ dependent stemness network whose components are still unknown.

We can speculate that, during the reprogramming, YAP/TAZ could act at the level of composite enhancer elements of specific transcription factors to promote the expression of stem-specific genes and repressing the expression of terminally differentiated genes.

YAP/TAZ as a novel paradigm for reprogramming

Immortalized and transformed cell lines are the most important models to study the biology of normal cells and also to investigate pathological aspects of complex disease such as cancer, but their usefulness is still debated. The number of available cell lines is restricted and they are not useful to evaluate some important aspects of cell and tissue biology such as the cell structural organization inside the tissue, the dynamics of tissue growth and also specific functional aspects. Importantly, each cell line is different from genetic and epigenetic point of view as they derived from different patients carrying different genetic alterations. A potential application of the reprogramming procedure presented here is the possibility to generate patient-specific cells starting from tissue specific differentiated cells. The generation of self-organizing tissue specific organoids that recapitulates the functions and the alteration of the tissue of origin would open new perspectives in the field of disease modeling and regenerative medicine.

Further work is required to evaluate if YAP reprogramming could occur also in vivo in order to facilitate tissue repair and regeneration. Direct application of the reprogramming procedure in vivo would provide an alternative to other reprogramming strategies employing either cocktail of transcription factors or the passage through a pluripotent state. In conclusion, the finding that YAP/TAZ can reprogram differentiated cells into their corresponding tissue specific stem cells may have great implications for understanding the mechanisms involved in the regulation of somatic stemness and also for the development of regenerative medicine applications.

EXPERIMENTAL PROCEDURES

Reagents, plasmids and transfections

Doxycycline hyclate, fibronectin, insulin, dexamethasone, dispase, hyaluronidase, NH_4Cl, tamoxifen and 4-OH-tamoxifen were from Sigma. Murine EGF, murine bFGF, human Noggin and murine prolactin were from Peprotech. B27, Collagenase type I, DMEM/F12 and trypsin were from Life Technologies. R-Spondin1 was from Sino Biological. Matrigel was from BD Biosciences (Corning). Rat tail collagen type I was from Cultrex. DNaseI was from Roche.

GFP- and Cre-expressing adenoviruses were from University of Iowa, Gene Transfer Vector Core. For inducible expression of YAP and TAZ, cDNA for siRNA-insensitive Flag-hYAP1 wt, S94A (TEAD-binding mutant (Zhao et al., 2008) and 5SA (LATS-mutant sites (Aragona et al., 2013) and for Flag-mTAZ4SA (Azzolin et al., 2012) were subcloned in FUW-tetO-MCS, obtained by substituting the Oct4 sequence in FUW-tetO-hOct4 (Addgene #20726(Hockemeyer et al., 2008) with a new multiple cloning site (MCS). This generated the FUW-tetO-wtYAP, FUW-tetO-YAPS94A, FUW-tetO-YAP5SA, FUW-tetO-TAZ4SA used throughout this study. FUW-tetO-EGFP plasmid was used as controls, as previously indicated (Cordenonsi et al., 2011). All our constructs are available in Addgene as #.

For stable expression of GFP, we used pRRLSIN.cPPT.PGK-GFP.WPRE (gift of L. Naldini) lentiviral vector.

All constructs were confirmed by sequencing.

DNA transfections were done with TransitLT1 (Mirus Bio) according to manufacturer instructions.

Lentivirus 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 together with packaging vectors pMD2-VSVG and pPAX2 by using TransIT-LT1 (Mirus Bio) according to manufacturer instructions.

Primary mammary epithelial cells (MECs) isolation and induction of yMaSCs

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). Mammary glands were minced and then digested with 6000 U/ml collagenase I and 2000 U/ml hyaluronidase in the DMEM/F12 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 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 for 5 min and 5 mg/ml dispase plus 100 mg/ml DNase I 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.

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).

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).

For induction of yMaSCs, LD cells were transduced for 48 hours with FUW-tetO-YAP, or FUW-tetO-TAZ, in combination with rtTA-encoding lentiviruses. As a (negative) control, LD cells were transduced with either FUW-tetO-EGFP in combination with rtTA-encoding lentiviruses. After infection, adherent cells were washed and treated with doxycycline for 7 days in MG medium for activating tetracycline-inducible gene expression (see scheme in Figure3A) to obtain "yMaSCs". After doxycycline treatment for 7 days in 2D culture, yMaSCs were processed for further assays or analysis. Unless otherwise specificed, yMaSCs were generated from wild-type YAP (FUW-tetO-wtYAP, Addgene #). For the experiment depicted in Figures 4B and 6L we first FACS purified LD cells and MaSC-enriched populations (using CD61 and CD49f as previously described for Figure 2A) from *K8-CreERT2; R26-LSL-YFP/+* or *K14-CreERT2; R26-LSL-YFP/+* virgin female mice. These cells were plated and after attachment they were treated with 4OH-Tamoxifen for 24 hours. Cells were then transduced for 48 hours with FUW-tetO-wtYAP in combination with stable rtTA-encoding lentiviral supernatant. Negative control cells were provided by LD cells transduced with FUW-tetO-MCS (empty vector) in combination with rtTA-encoding

lentiviral supernatants. After infection, cells were washed, treated with doxycycline in MG medium as above.

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, murine EGF, murine bFGF, and heparin) containing doxycycline (2 mg/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, murine EGF, murine bFGF, heparin, Noggin and R-Spondin1). 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.

For the experiment depicted in Figure 3F, cells were plated into 96-well plate as single cells visually verified and their colony forming capacity was monitored. Based on the native fluorescence of EGFP-positive LD cells (negative control cells), we measured that the percentage of infection of our lentiviral-based reprogramming strategy is 50%.

For *a-* and *b-casein* induction (Figures 7C-D), Matrigel-embeded organoids derived from yMaSCs or MaSCs were treated with MG organoid medium supplemented with insulin (10 mg/ml) and dexamethasone (1 mg/ml) in the absence or presence of lactogenic hormone prolactin (5 mg/ml) for 7 days. Organoids were then recovered from Matrigel as before and processed for RNA extraction.

Cleared Mammary Fat Pad Transplantation

For induction of yMaSCs meant for in vivo injection, adherent luminal differentiated cells were transduced for 48 hours with FUW-tetO-wtYAP in combination with stable rtTA- and EGFP-encoding lentiviruses to trace with EGFP fluorescence the generation of transgenic mammary glands from yMaSCs. Negative control LD cells were transduced with FUW-tetO-EGFP, rtTA and pRRL-CMV-GFP. After infection, cells were treated as before (washed, induced with doxycycline for 7 days in MG medium) and then injected in the cleared fat pads (see scheme in Figure 8A). Cell aliquots resuspended in 10 ml PBS/10% Matrigel were injected into the inguinal mammary fat pads of NOD-SCID mice (Charles River), which had been cleared of endogenous mammary epithelium at 3 weeks of age. Animals were then administered doxycycline in the drinking water for 2 weeks and then maintained without doxycycline for additional 8-10 weeks. Transplanted mammary fat pads were examined for

gland reconstitution by whole-mount staining, GFP native fluorescence and immunofluorescence on sections from paraffin-embedded biopsies. Only the presence of GFP-positive branched ductal trees with lobules and/or terminal end buds was scored as positive reconstitution. For whole-mount analysis of mammary glands, freshly-explanted glands were fixed in PFA 4% (2 hours) and ethanol 70% (overnight). Glands were rehydrated, stained overnight with hematoxylin, subsequently dehydrated in graded ethanols, cleared by incubation in benzyl-alcohol/benzyl benzoate (1:2; Sigma) and imaged.

Immunofluorescence, stainings and microscopy

For immunofluorescence on mammary colonies and organoids, outgrowths freshly recovered from Matrigel were embedded in OCT tissue-freezing medium (PolyFreeze, Sigma) and frozen on dry ice. 8 μm cryostat sections for all types of organoids were cut at –20 °C. Sections were mounted on glass slides and dried for at least 30 min. The sections were then fixed with 4% formaldehyde for 10 min. After washing with PBS the sections were permeabilized 10 min at RT with PBS 0.3% Triton X-100, and processed for immunofluorescence using the following conditions: blocking in 10% Goat Serum (GS) in PBS 0.1% Triton X-100 (PBST) for 1 hr followed by incubation with primary antibodies (diluted in 2% GS in PBST) overnight at 4°C, four washes in PBST and incubation with secondary antibodies (1:200 in 2% GS in PBST) for 2 hours at room temperature. Samples were counterstained with ProLong-DAPI (Molecular Probes, Life Technologies) to label cell nuclei. in PBST for 15 minutes, incubated 20 min with DAPI solution and mouted in glycerol.

For immunofluorescence on mammary tissue, biopsies were fixed with PFA, paraffin-embedded and cut in 10 μm-thick sections. Sections were re-hydrated and antigen retrieval

was performed by incubation in citrate buffer 0.01 M pH 6 at 95°C for 20 minutes. Slides were then permeabilized (10 min at RT with PBS 0.3% Triton X-100 for mammary sections and 10 min at RT with PBS 1% Triton X-100 for brain sections) and processed as described above.

Primary antibodies: anti-a-SMA (A2547; 1:400) mouse monoclonal antibody. anti-E-cadherin (610181; 1:1000) was from BD Biosciences. anti-K14 (Ab7800; 1:100) mouse monoclonal antibody, anti-K8 (Ab14053; 1:100) chicken polyclonal antibody were from Abcam. anti-GFP (A6455; 1:100) rabbit serum was from Life Technologies. anti-p63 (H137, sc-8343; 1:50) polyclonal antibody and anti-Vimentin (Vim C-20, sc-7557-R; 1:100) rabbit polyclonal antibody was from Santa Cruz. *K19* was detected using the monoclonal rat anti-*Troma-III* antibody (DSHB; 1:50). Alexa-conjugated secondary antibodies (Life Technologies): Alexa-Fluor-488 donkey anti-mouse IgG (A21202), Alexa Fluor-488 donkey anti-rabbit IgG (A21206); Alexa Fluor-555 goat anti-chicken IgG (A21437). Goat anti-rat Cy3 (112-165-167) was from Jackson Immunoresearch.

Confocal images were obtained with a Leica TCS SP5 equipped with a CCD camera. Bright field and native-GFP (or tdTomato) images were obtained with a Leica DM IRB inverted microscope equipped with a CCD camera (Leica DFC 450C). Live cell imaging was performed with a A1Rsi+ laser scanning confocal microscope (Nikon) equipped with NIS-Elements Advanced Research Software.

Western blot

Western blots were carried out as described in Ref. (Cordenonsi et al., 2011). Anti-YAP/TAZ (63.7; sc-101199) and anti-p63 (4A4; sc-8431) monoclonal antibodies were from Santa Cruz.

anti-GAPDH (MAB347) monoclonal antibody was from Millipore. Anti-K14 (Ab7800) mouse monoclonal antibody and anti-K8 (Ab14053) chicken polyclonal antibody were from Abcam.

Quantitative Real-Time PCR (qRT-PCR)

Cells or tissues were harvested in TriPure (Roche) for total RNA extraction, and contaminant DNA was removed by DNase treatment. qRT-PCR analyses were carried out on retrotranscribed cDNAs with Rotor-Gene Q (Qiagen) thermal cycler and analyzed with Rotor-Gene Analysis6.1 software. Expression levels are always given relative to *Gapdh.*

Microarray experiments

For microarray experiments, Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were used. Total RNA was extracted using TriPure (Roche) from luminal differentiated mammary cells (3 replicates), organoids derived from yMaSCs (3 replicates), and MaSCs (3 replicates).

RNA quality and purity were assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany); RNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). RNA was then treated with DNaseI (Ambion). In vitro transcription, hybridization and biotin labeling were performed according to Affymetrix 3'IVT protocol (Affymetrix). As control of effective gene modulation and of the whole procedure, we monitored the expression levels of tissue-specific markers of differentiated cells or stem/progenitors by qRT-PCR prior to microarray hybridization and in the final microarray data.

All data analyses were performed in R (version 3.1.2) using Bioconductor libraries (BioC 3.0) and R statistical packages. Probe level signals were converted to log2 expression values using robust multi-array average procedure RMA (Irizarry et al., 2003) of Bioconductor *affy* package. Raw data are available at Gene Expression Omnibus under accession number GSE70174.

Global unsupervised clustering was performed using the function *hclust* of R *stats* package with Pearson correlation as distance metric and average agglomeration method. Gene expression heatmaps have been generated using the function *heatmap.2* of R *gplots* package after row-wise standardization of the expression values. Before unsupervised clustering, to reduce the effect of noise from non-varying genes, we removed those probe sets with a coefficient of variation smaller than the 90^{th} percentile of the coefficients of variation in the entire dataset. The filter retained 4511 probe sets that are more variable across the samples .

Differentially expressed genes were identified using Significance Analysis of Microarray algorithm coded in the *samr* R package as in Ref. (Tusher et al., 2001). To identify differentially expressed genes, we selected those probe sets with an FDR $\leq$ 1%.

RNA Sequencing

For RNA-seq, total RNAs was extracted with Trizol from small colonies emerging from YAP-reprogrammed LD cells after 13 days in mammary colony medium and from freshly sorted LD and MaSC-enriched cell populations. RNA-seq libraries were prepared with TruSeq Stranded Total RNA with Ribo-Zero GOLD (Illumina), according to manufacturer's instructions. Sequencing was performed on an Illumina HiSeq 2500 platform. Raw reads were aligned using TopHat (Kim et al., 2013) (version 2.0.5) to build version mm9 of the

mouse genome. Counts for UCSC annotated genes were calculated from the aligned reads using HTSeq (Anders et al., 2015) (version 0.6.0). Normalization and differential analysis were carried out using edgeR package (Robinson et al., 2010) and R (version 3.0.0). Raw counts were normalized according to library size to obtain counts per millions (cpm). Only genes with a cpm greater than 1 in at least 1 sample were retained for differential analysis. Genes were considered differentially expressed with a Benjamini-Hochberg FDR less than or equal to 5%. Luminal genes in Figure 5D were those significantly upregulated in LD cells compared to MaSC-enriched cell populations; Basal genes were genes significantly upregulated in MaSC-enriched cell populations compared to LD cells. Luminal and Basal genes were further divided in three blocks each, corresponding to genes significantly upregulated (blocks C and F), significantly downregulated (blocks A and D), or displaying no significant changes (blocks B and E) in yMaSC colonies compared to MaSC-enriched cell populations (for blocks A, B and C) or LD cells (for blocks D, E or F). Genes specifically upregulated in yMaSC (block G) are defined as genes that were significantly upregulated in yMaSC colonies compared to both LD cells and MaSC-enriched cell populations and displaying no significant difference in expression between LD cells and MaSC-enriched cell populations. The lists of genes of blocks C and G were compared to gene expression signatures derived from (Wansbury et al., 2011), defining groups of genes that were found enriched in the epithelial cells of the fetal mammary bud.

Mice

C57BL/6J mice and NOD-SCID mice were purchased from Charles River. Transgenic lines used in the experiments were gently provided by: Cedric Blanpain (*K8-CreERT2/R26-LSL-*

YFP) (Van Keymulen et al., 2011); Pierre Chambon (*K14-CreERT2*) (Li et al., 2002); Hans Clevers (Lgr5-GFP) (Barker et al., 2007). $Taz^{fl/fl}$ and double $Yap^{fl/fl}$; $Taz^{fl/fl}$ conditional knock-out mice were as described in Ref. (Azzolin et al., 2014). Animals were genotyped with standard procedures (Morsut et al., 2010) and with the recommended set of primers. Animal experiments were performed adhering to our institutional guidelines as approved by OPBA.

TABLES

Table1. Sequences of qPCR oligos

MOUSE GENE	For	Rev
α-Casein	CCCCTTTGGGCTTACTTTCC	CATGAGGTGGATGGAGAATGG
α-Sma	TGCTGTCCCTCTATGCCTCT	GAAGGAATAGCCACGCTCAG
Aldh1L1	CAGGAGGTTTACTGCCAGCTA	CACGTTGAGTTCTGCACCCA
AnkrD1	CTGTGAGGCTGAACCGCTAT	TCTCCTTGAGGCTGTCGAAT
Areg	TGGTGAACGGTGTGGAGAAA	TGTGATAACGATGCCGATGC
Axin2	GAGATGACGCCTGTGGAACC	CCTGCTCAGACCCCTCCTTT
Axl	CGAGGCCAAACTCCCTATCC	GGGCAGAGCCTTCAGTGTGT
β-Casein	CACTCCAGCATCCAGTCACA	GGCATCTGTTTGTGCTTGGG
Bdnf	GGGTCACAGCGGCAGATAAA	GCGAGTTCCAGTGCCTTTTG
Bmp7	AAGGCCACGGAAGTCCATCT	CCAAGGTCTCGGAAGCTGAC
Claudin1	GGGGACAACA TCGTGACCG	AGGAGTCGAAGACTTTGCACT
Ctgf	CTGCCTACCGACTGGAAGAC	CATTGGTAACTCGGGTGGAG
Dll1	TGAAGCCACGGTCAGGGATAC	TGCAGACAGAACATACACCGACT
Erbb3	TGCCAGATACGCACCTCAGA	TACCCCTCCTCTTCCGGTTC
Esr	GGATGCTGAACCGCCCATGA	CAGCCAGGCACACTCGAGAA
Foxa1	ACAGCTACTACGCGGACACG	GCTCGTGGTCATGGTGTTCA
Fzd7	CGGCACCAAGACAGAGAAGC	CAGGGCACAGCGTAGCTCTT
Gapdh	ATCCTGCACCACCAACTGCT	GGGCCATCCACAGTCTTCTG
Gpc3	AACCATGTCTGTGCCCAAGG	GTTCTGCAAGGAAGCGCAGT
Jag1	CCACCGAACACATTTGCAGCG	CTGCAGAGATGGCCACGTGT
Jag2	AGCCTGATCCAGAGCACAGC	CGAGCCACAGCACACTGAAC
Hey2	TGAGAAGACTAGTGCCAACAGC	TGGGCATCAAAGTAGCCTTTA
Igfbp4	GGGTATTCGGTCATCCGACA	GGGCCATTCCTCAGACACAC
K14	AGGACCTGAAGAGCAAGATC	TCCTTGAGGCTCTCAATCTG
K18	ATGACACCAACATCACAAGG	ATCCACTTCCACAGTCAATC
K19	AGGAGCTGAACACCCAGGTC	GGGCTTCAAAACCGCTGAT
K15	TCGCCACTTACCGGAACCT	TTCCATCCACTGATTCCTCCA
K6	ACCACCACCTCCTCCAGCAA	ACACAGCCTCCTCAGTCCCA
K5	TCTCTTCTGGCTACGGAGGA	GAAGCTCATGCCTCCTTGAC
K8	GGACATCGAGATCACCACCT	TGAAGCCAGGGCTAGTGAGT
Kit	AGACTTGCTGGGACGCTGAC	CGAGTTGACCCTCACGGAAT
LGR4	TAACAGCCCCCAAGACCACA	GCGACCAGGAAAATGAACCA
LGR5	TCGTGATCGTCCCACTTCCT	CAGGACCGTTTCTCAACATCG
LGR6	GTGACCCTCATCTCCCGAAC	AAGTGGCTCCCTCTGCCTTC
Ltf	GTCTGGCTGAGAAGGCAGGA	GGTTTGGGGCTATGGCTAGG
Mfge8	GAGGAGCAAGGAAGCAGCAA	ATGCGGTTATGCCAGGACAC
Myh11	GGTGAACGCCCTCAAGAGCA	TCTGAGTCCCGAGCGTCCAT
Nestin	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA
ΔNp63	CCTGGAAAACAATGCCCAGAC	GAGGAGCCGTTCTGAATCTGC
Pgr	TCCGGAACTTACACATTGATGACCA	CCACATGGTAAGGCACAGCGA
Prlr	GGATCATTGTGGCCGTTCTC	CGGAACTGGTGGAAAGATGC
Procr	GGAGAAAGGGCTGGACTGGT	CCCCTCCCACACACACACTT
Sfrp	CTACTGGCCCGAGATGCTCA	TTGTCGCATGGAGGACACAC
Slug	CTCACCTCGGGAGCATACAG	GACTTACACGCCCCAAGGATG
Timp3	GATGCCCCACGTGCAGTACA	CCTTCATACACGCGCCCTGT
Twist	ACGCAGTCGCTGAACGAGGC	GTCAGGGAAGTCGATGTACC
Zeb2	ACACACAGGAAAGAGACCACACC	GCGCTTGCCACATTTGTCACA

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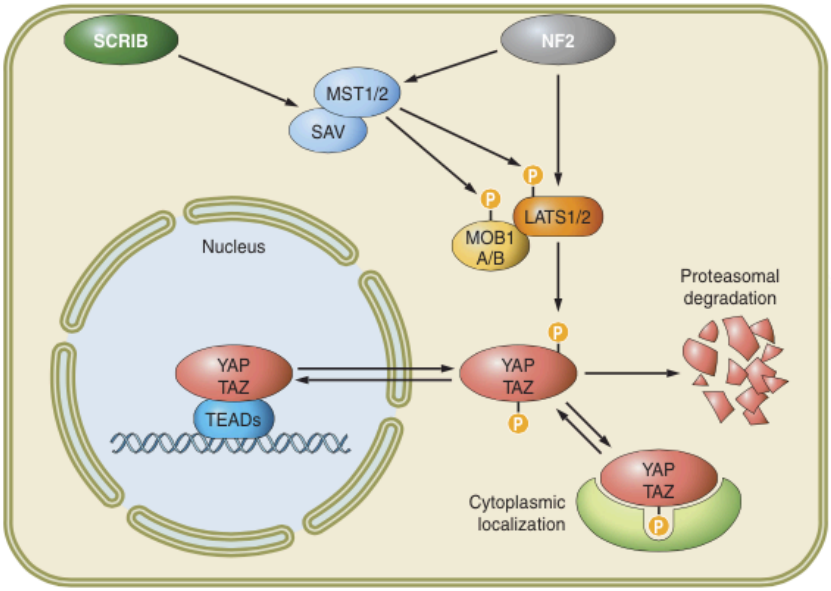
FIGURES

A



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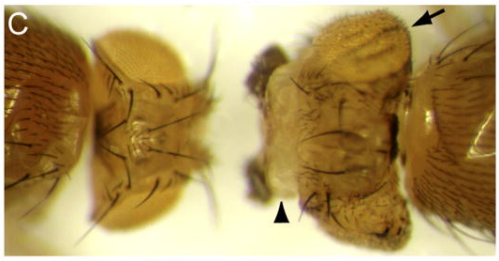

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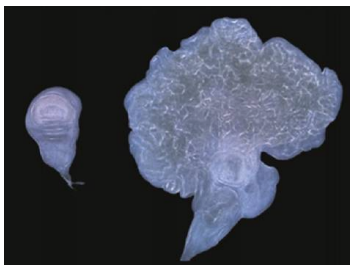

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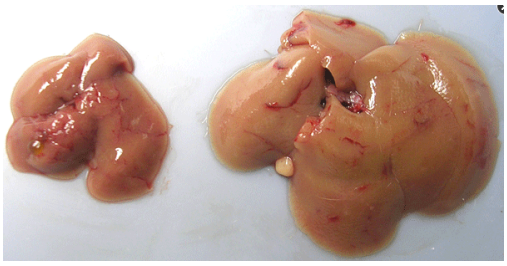


E

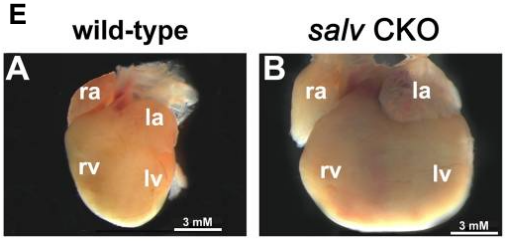


FIGURE 1

Figure 1. The Hippo signaling pathway in organ size control

A, Schematic representation of the mammalian Hippo pathway (Adapted from Piccolo et al., 2014).

B-C, Overexpression of Yorkie (YAP/TAZ orthologue) leads to organ overgrowth in Drosophila. **(B)** Expression of a Hippo-insensitive form of Yorkie in the eye primordium leads to an enlargement of the eye and head cuticle (*right*) compared to control (*left*) (adapted from Dong et al., 2007). (**C**) Yorkie overexpression in *Drosophila* wing imaginal discs results in an increase of the area of the discs (right) compared to control (*left*) (adapted from Huang et al., 2005).

D, YAP overexpression in the liver of transgenic mice leads to organ overgrowth (*right*) compared to wild-type control (*left*) (adapted from Dong et al., 2007).

E, Salvador knockout leads to cardiomegaly in neonatal mice (*right*) compared to control (*left*); ra, right atrium; la, left atrium; rv, right ventricle; lv, left ventricle (adapted from Heallen et al., 2011).

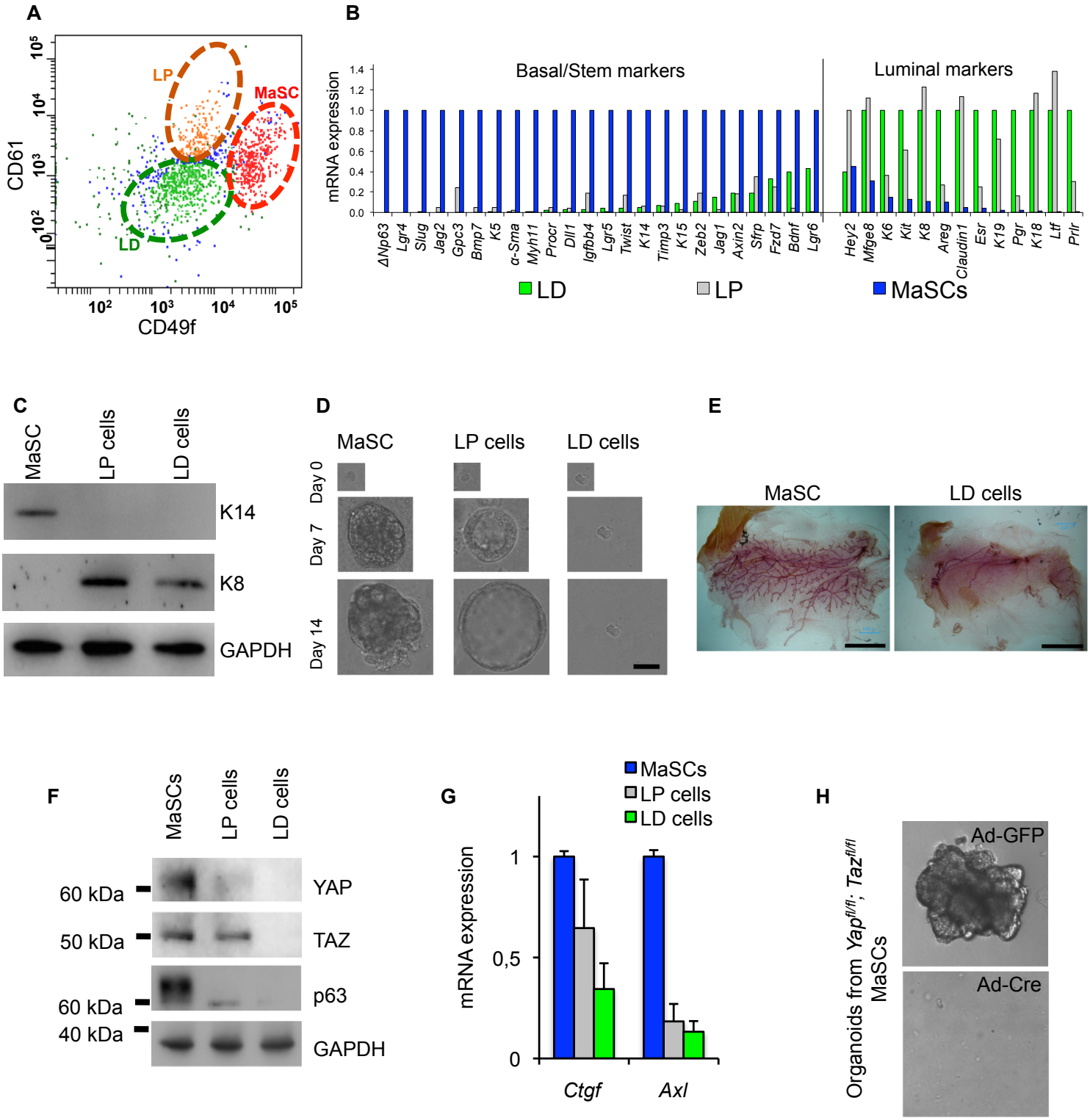

A
LP
MaSC
LD
CD61
CD49f
B
Basal/Stem markers
Luminal markers
mRNA expression
ΔNp63 Lgr4 Slug Jag2 Gpc3 Bmp7 K5 α-Sma Myh11 Procr Dll1 Igfbb4 Lgr5 Twist K14 Timp3 K15 Zeb2 Jag1 Axin2 Sfrp Fzd7 Bdnf Lgr6
Hey2 Mfge8 K6 Kit K8 Areg Claudin1 Esr K19 Pgr K18 Ltf Prlr
LD
LP
MaSCs
C
MaSC
LP cells
LD cells
K14
K8
GAPDH
D
MaSC
LP cells
LD cells
Day 0
Day 7
Day 14
E
MaSC
LD cells
F
MaSCs
LP cells
LD cells
60 kDa
50 kDa
60 kDa
40 kDa
YAP
TAZ
p63
GAPDH
G
MaSCs
LP cells
LD cells
mRNA expression
1
0,5
0
Ctgf
Axl
H
Organoids from Yap^fl/fl; Taz^fl/fl MaSCs
Ad-GFP
Ad-Cre

FIGURE 2

Figure 2. Characterization of the FACS-purified mammary gland cells

A, FACS profile showing the distribution of Lin–/EpCAM+ mammary cells according to their CD49f/CD61 antigenic profile. Three subpopulations were separated: a MaSC-enriched fraction ($EpCAM^{low}CD49f^{high}CD61^{+}$), luminal progenitors (LP, $EpCAM^{high}CD49f^{low}CD61^{+}$), and luminal differentiated cells (LD, $EpCAM^{high}CD49f^{low}CD61^{-}$).

B, Comparison by qRT-PCR for the indicated basal/stem and luminal markers of FACS-purified MaSCs, LP, and LD. Data are normalized to *Gapdh* expression and are referred to MaSC levels for basal genes, to LP levels for *Hey2*, and to LD levels for all the other luminal markers (each set to 1). Results are representative of at least three independent experiments (each using mammary glands from n=20 mice) performed in triplicate.

C, Western blot analysis for the indicated basal and luminal markers in FACS-purified MaSCs, LP and LD cells. GAPDH serves as loading control.

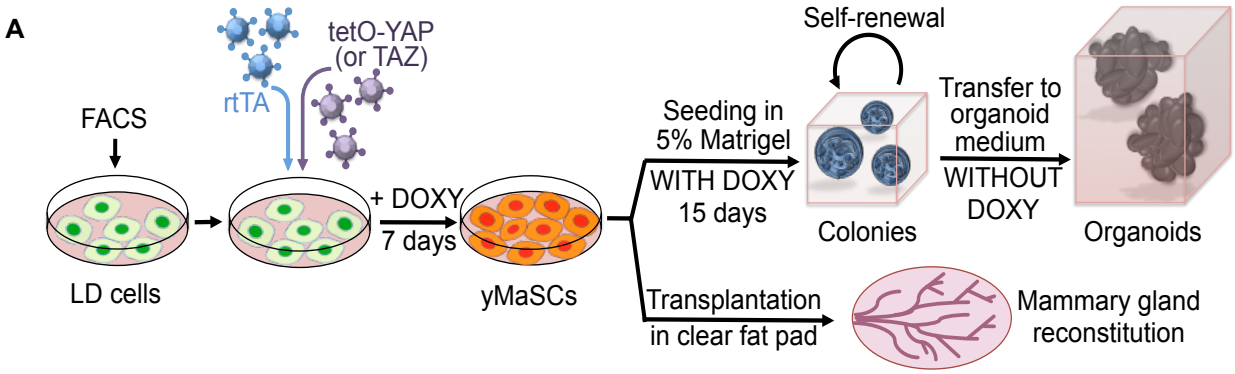
D, Representative images at the indicated time points of mammary colonies formed by the indicated cells in mammary colony medium. Pictures are representative of three independent experiments performed with six technical replicates. Scale bar, 170 μm.

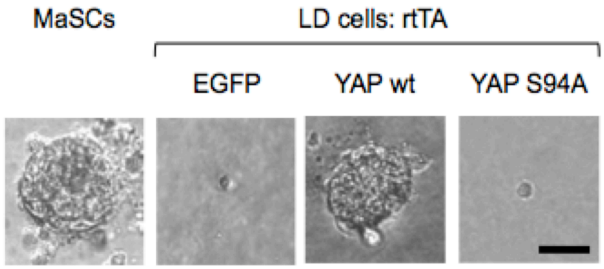
E, Representative images of whole mount hematoxylin staining of cleared fat pads injected with purified MaSCs (leading to outgrowth of a ductal mammary tree) or of LD cells as negative control. Scale bars, 1 cm.

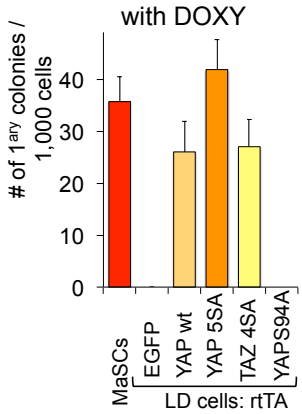
F, Western blots for YAP, TAZ and p63 in the FACS- purified populations; GAPDH serves as loading control.

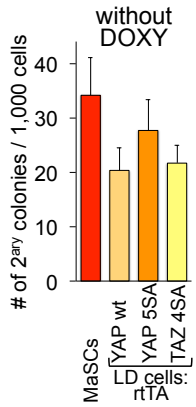
G, qRT-PCRs for *Ctgf* and *Axl* in the indicated cell populations (mean + s.d.). Results are representative of three independent experiments (each using mammary glands from n=20 mice), performed in triplicate.

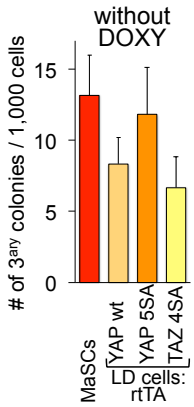
H, Representative images of organoids obtained from *Yap*$^{fl/fl}$*; Taz*$^{fl/fl}$ MaSCs. Organoids were transduced with Ad-Cre or Ad-GFP (as control) during passaging. Panels are representative images of the resulting outgrowths. No organoid ever formed in absence of YAP/TAZ. Scale bar, 250 μm.











F

1 plated cell in 1 single well; visually verified	Experiment			Mean ± SD
	#1	#2	#3	
LD: rtTA/EGFP transduced				
# of single LD cells	n = 56	n = 64	n = 70	
% of single cells that gave rise to a single MaSCs-like colony	0.0%	0.0%	0.0%	0
LD: rtTA/YAP S94A transduced				
# of single LD cells	n = 73	n = 46	n = 50	
% of single cells that gave rise to a single MaSCs-like colony	0.0%	0.0%	0.0%	0
LD: rtTA/YAP wt transduced				
# of single LD cells	n = 71	n = 68	n = 57	
% of single cells that gave rise to a single MaSCs-like colony	19.7%	14.7%	21.1%	18.5 ± 3.4
% of single MaSCs-like colonies that gave rise to an organoid	64.3%	70.0%	75.0%	69.8 ± 5.4

G

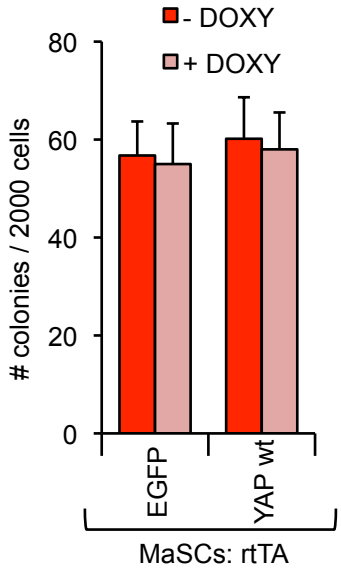


FIGURE 3

Figure 3. Conversion of Luminal Differentiated cells in yMaSCS by YAP/TAZ expression

A, Schematic representation of the experiments performed with LD cells. Doxy stands for doxycycline.

B-C, Representative images (**B**) and quantifications (**C**) of mammary colonies formed by the indicated cells, 15 days after seeding in mammary colony medium. Data in (**C**) are presented as mean + s.d. and are representative of five independent experiments, each with six technical replicates.

D-E, Quantifications of secondary (**D**) and tertiary (**E**) colonies formed by primary mammary colonies after dissociation and re-seeding in mammary colony medium without doxycycline. Data are representative of three independent experiments performed with six technical replicates, and presented as mean + s.d.

F, Quantification of the conversion rates of single LD cells plated in 96-well expressing the indicated constructs plates into a MaSC-like state as determined by % of colony formation. EGFP- and YAP S94A-transduced cells serve as negative control.

G, Quantifications of mammary colonies formed by MaSCs transduced with EGFP or YAP wt encoding vectors, 15 days after seeding in mammary colony medium. Data are presented as mean + s.d.

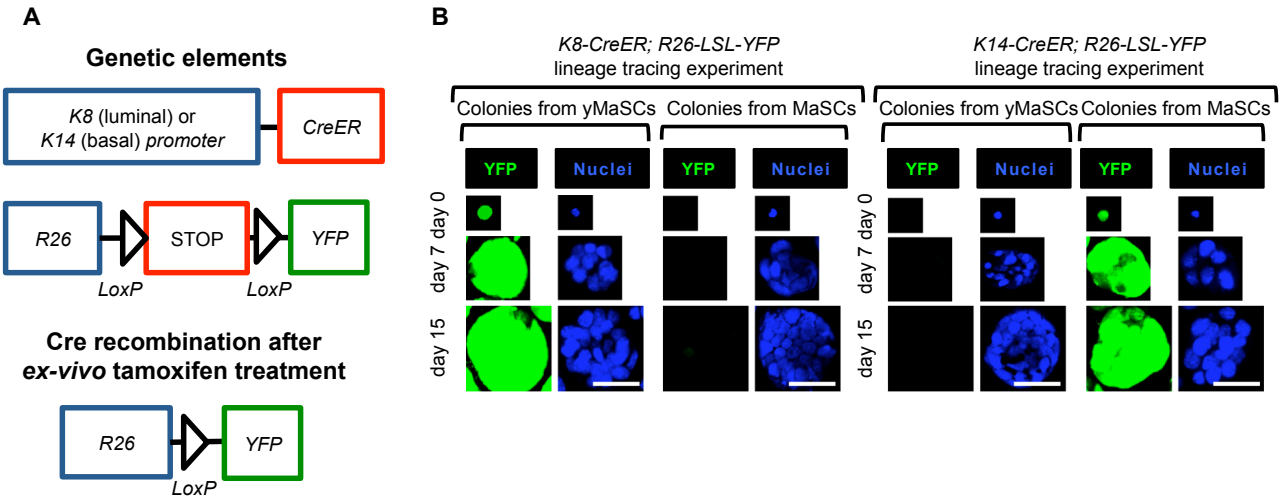

A
Genetic elements
K8 (luminal) or
K14 (basal) promoter
CreER
R26
STOP
YFP
LoxP
LoxP
Cre recombination after
ex-vivo tamoxifen treatment
R26
YFP
LoxP
B
K8-CreER; R26-LSL-YFP
lineage tracing experiment
K14-CreER; R26-LSL-YFP
lineage tracing experiment
Colonies from yMaSCs
Colonies from MaSCs
Colonies from yMaSCs
Colonies from MaSCs
YFP
Nuclei
YFP
Nuclei
YFP
Nuclei
YFP
Nuclei
day 0
day 7
day 15
day 0
day 7
day 15

FIGURE 4

Figure 4. Genetic lineage tracing of yMaSCs

A, Representation of the genetic lineage tracing strategies used to trace mammary cell lineages.

B, Immunofluorescence analysis with anti-YFP of traced cells derived from K8-CreER/R26-YFP or K14-CreER/R26-YFP during colony formation. Days indicate the time in colony medium. Scale bars, 62 μm.

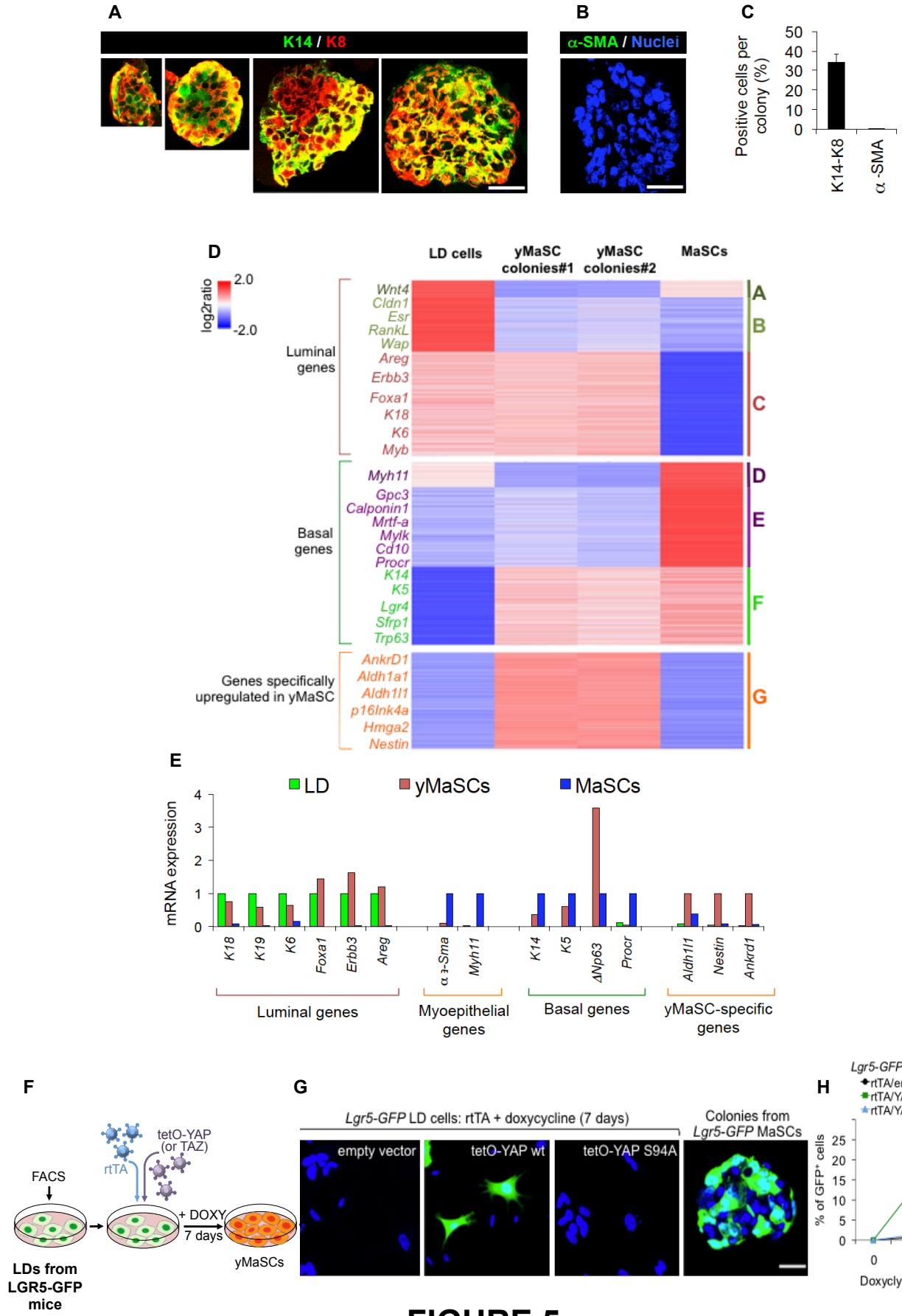

A
K14 / K8
B
α-SMA / Nuclei
C
Positive cells per colony (%)
50
40
30
20
10
0
K14-K8
α -SMA
D
LD cells
yMaSC colonies#1
yMaSC colonies#2
MaSCs
log2ratio
2.0
-2.0
Luminal genes
Wnt4
Cldn1
Esr
RankL
Wap
Areg
Erbb3
Foxa1
K18
K6
Myb
Basal genes
Myh11
Gpc3
Calponin1
Mrtf-a
Mylk
Cd10
Procr
K14
K5
Lgr4
Sfrp1
Trp63
Genes specifically upregulated in yMaSC
AnkrD1
Aldh1a1
Aldh1l1
p16Ink4a
Hmga2
Nestin
A
B
C
D
E
F
G
E
LD
yMaSCs
MaSCs
mRNA expression
4
3
2
1
0
K18
K19
K6
Foxa1
Erbb3
Areg
α -Sma
Myh11
K14
K5
ΔNp63
Procr
Aldh1l1
Nestin
Ankrd1
Luminal genes
Myoepithelial genes
Basal genes
yMaSC-specific genes
F
FACS
rtTA
tetO-YAP (or TAZ)
+ DOXY
7 days
yMaSCs
LDs from LGR5-GFP mice
G
Lgr5-GFP LD cells: rtTA + doxycycline (7 days)
Colonies from Lgr5-GFP MaSCs
empty vector
tetO-YAP wt
tetO-YAP S94A
H
Lgr5-GFP LD cells:
rtTA/empty vector
rtTA/YAP wt
rtTA/YAP S94A
% of GFP+ cells
25
20
15
10
5
0
0
3
7
Doxyclycline (days)

FIGURE 5

Figure 5. Characterization of the early stage of YAP induced reprogramming

A-C, Immunostainings for K14/K8 (**A**, at different stages of the outgrowths) and α-SMA (**B**) in colonies originating from yMaSCs. Scale bars, 36 μm. **C**, quantifications of individual cells, positive for the indicated markers, inside the colonies.

D, Heatmap of genes differentially expressed between LD cells, yMaSCs colonies (two independent preparations) and MaSC-enriched cell population (MaSCs).

E, Comparison by qRT-PCR of FACS-purified MaSC-enriched basal cells (MaSCs) from the mammary gland and yMaSC colonies. Purified LD cells were used as control. Data are normalized to *Gapdh* expression and are referred to LD levels for all the luminal genes, to MaSCs levels for myoepithelial and basal genes, and to yMaSC colonies levels for yMaSC-specific genes (each set to 1).

F, Schematic representation of the experiments performed with LD cells. Doxy stands for doxycycline.

G, Representative images of Lgr5-GFP-positive cells emerging from LDs infected with lentiviruses for rtTA in combination with empty vector or inducible YAP (wt or S94A) after 7 days of treatment with doxycycline. Lgr5-GFP-expressing MaSCs colonies are presented as controls. Scale bar, 31 mm.

I, Quantification of Lgr5-GFP-positive cells emerging from indicated LD transfectants, at different time points during doxycycline treatment. Results are presented as mean + s.d of 10 different fields (each containing 26-50 transfectants), and were repeated twice.

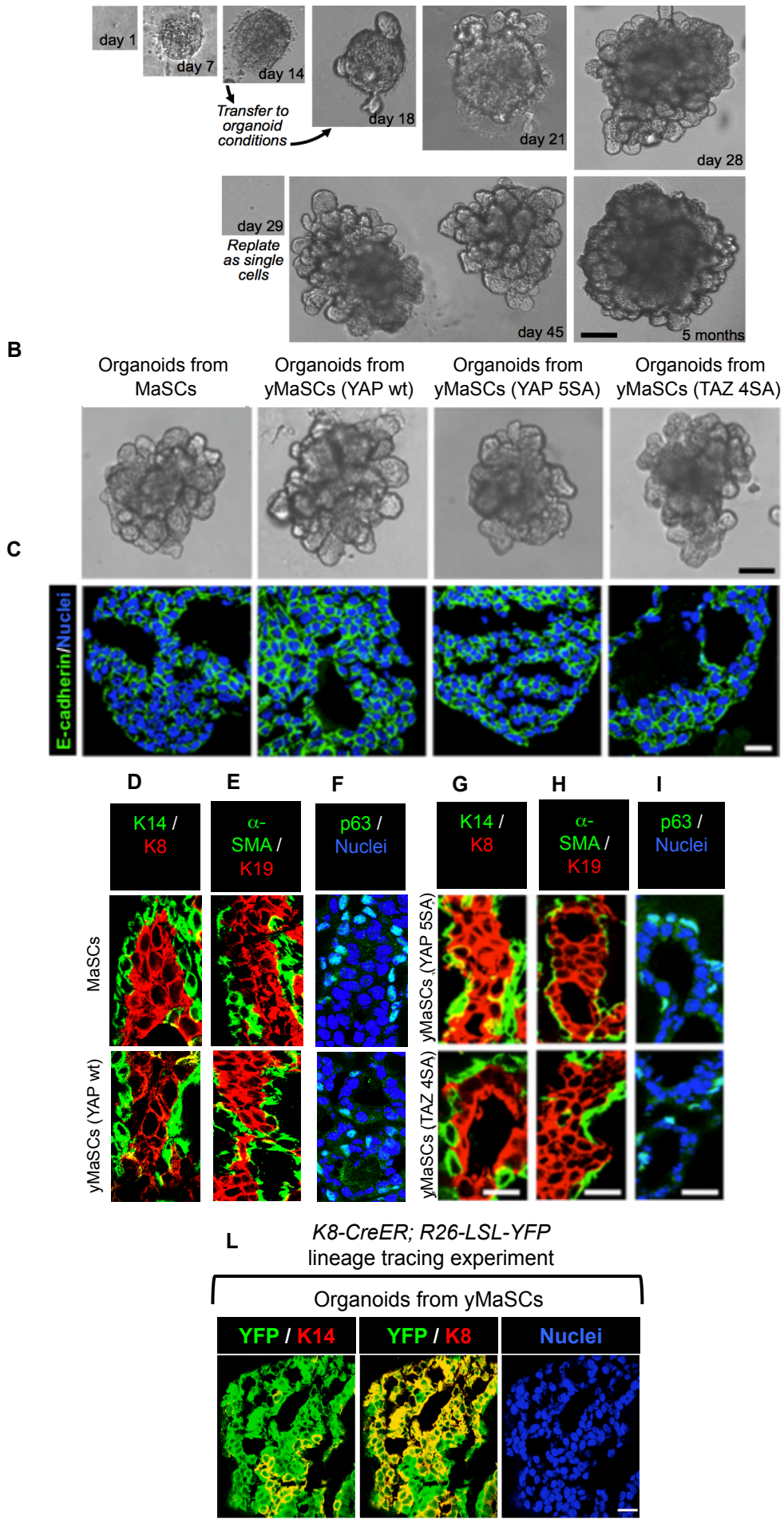

day 1
day 7
day 14
Transfer to organoid conditions
day 18
day 21
day 28
day 29
Replate as single cells
day 45
5 months
B
Organoids from MaSCs
Organoids from yMaSCs (YAP wt)
Organoids from yMaSCs (YAP 5SA)
Organoids from yMaSCs (TAZ 4SA)
C
E-cadherin/Nuclei
D
E
F
G
H
I
K14 / K8
α-SMA / K19
p63 / Nuclei
MaSCs
yMaSCs (YAP wt)
yMaSCs (YAP 5SA)
yMaSCs (TAZ 4SA)
L
K8-CreER; R26-LSL-YFP lineage tracing experiment
Organoids from yMaSCs
YFP / K14
YFP / K8
Nuclei

FIGURE 6

Figure 6. Morphological characterization of yMaSC derived organoids

A, Representative images of yMaSCs outgrowths at the indicated time points. Cultures were in mammary colony medium until day 14 and then transferred to organoid medium in absence of doxycycline. Scale bar 250 mm.

B, Representative images of MaSCs or yMaSCs organoids (derived from reprogramming of LDs with the indicated vectors). Scale bar 250 mm.

C, Immunostaining for E-Cadherin on frozen sections of MaSCs and yMaSCs derived organoiods.

D-I, Confocal immunofluorescence analysis for basal (K14, α-SMA, p63) and luminal markers (K8, K19) on frozen sections of MaSCs and y MaSCs derived organoids. Scale bar, 17 mm.

L, immunostaining with anti-YFP combined with either anti-K14 or anti-K8 antibodies of yMaSCs-derived organoids obtained from K8CreER/R26-YFP-traced LD cells. Scale bar 49 μm.

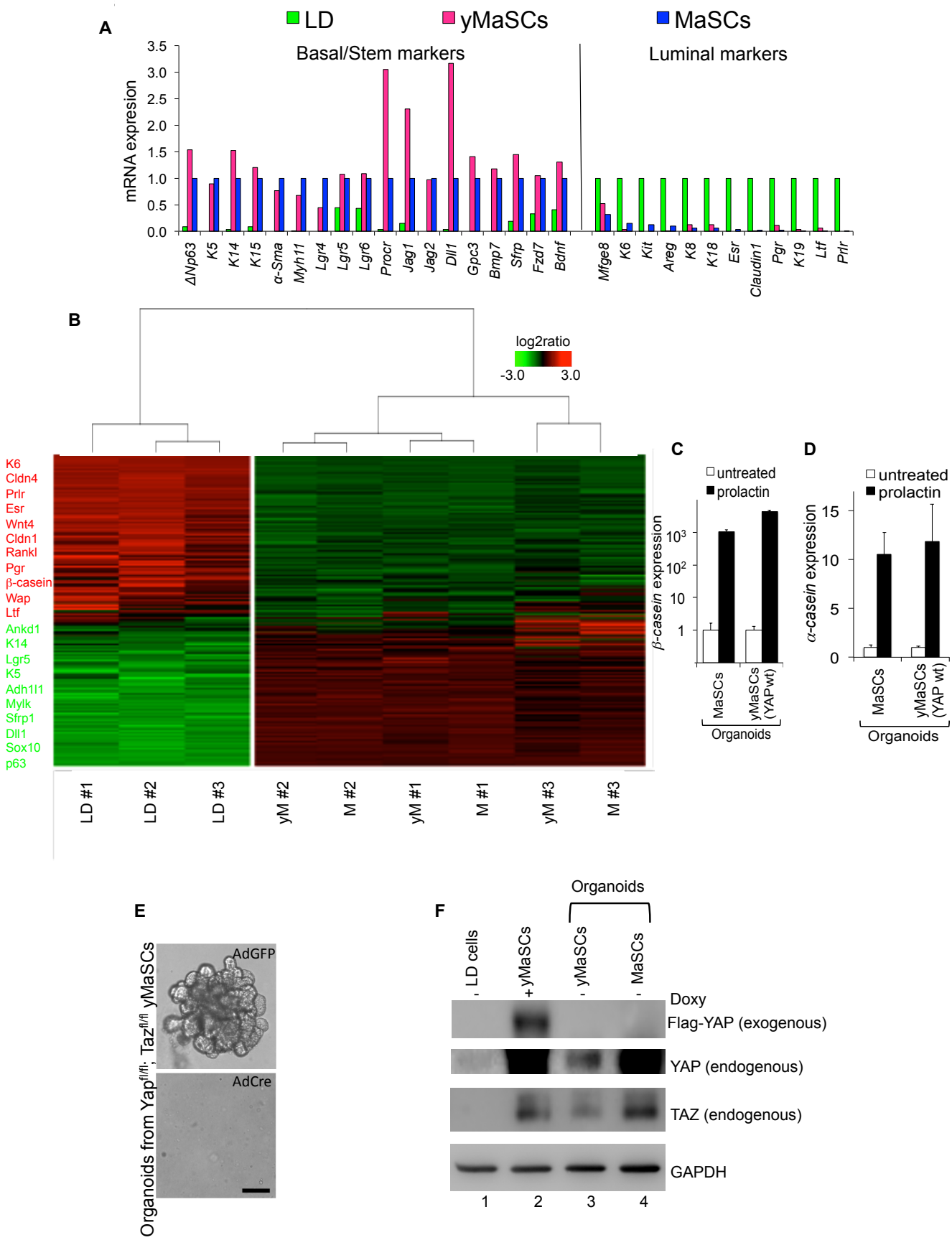

A
LD
yMaSCs
MaSCs
Basal/Stem markers
Luminal markers
mRNA expresion
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ΔNp63
K5
K14
K15
α-Sma
Myh11
Lgr4
Lgr5
Lgr6
Procr
Jag1
Jag2
Dll1
Gpc3
Bmp7
Sfrp
Fzd7
Bdnf
Mfge8
K6
Kit
Areg
K8
K18
Esr
Claudin1
Pgr
K19
Ltf
Prlr
B
log2ratio
-3.0
3.0
K6
Cldn4
Prlr
Esr
Wnt4
Cldn1
Rankl
Pgr
β-casein
Wap
Ltf
Ankd1
K14
Lgr5
K5
Adh1l1
Mylk
Sfrp1
Dll1
Sox10
p63
LD #1
LD #2
LD #3
yM #2
M #2
yM #1
M #1
yM #3
M #3
C
untreated
prolactin
β-casein expression
10³
10²
10
1
MaSCs
yMaSCs (YAPwt)
Organoids
D
untreated
prolactin
α-casein expression
15
10
5
0
MaSCs
yMaSCs (YAP wt)
Organoids
E
Organoids from Yap^fl/fl; Taz^fl/fl yMaSCs
AdGFP
AdCre
F
Organoids
- LD cells
+ yMaSCs
- yMaSCs
- MaSCs
Doxy
Flag-YAP (exogenous)
YAP (endogenous)
TAZ (endogenous)
GAPDH
1
2
3
4

FIGURE 7

Figure 7. Molecular and functional characterization of yMaSC derived organoids

A, FACS-purified MaSC-enriched basal cells (MaSCs) from the mammary gland and FACS-purified yMaSCs from yMaSC-derived organoids were analized for the expression of basal and luminal marker by qRT-PCR. LD cells serves as control. Data are normalized to *Gapdh* expression and are referred to MaSCs levels for basal genes and to LD levels for all the luminal markers (each set to 1).

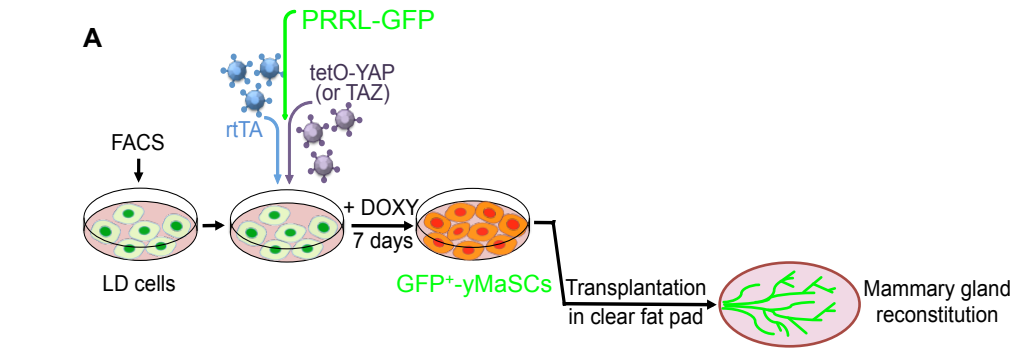
B, Unsupervised hierarchical clustering of gene expression profiles in LD cells, organoids from MaSCs (M) and organoids from yMaSCs (yM). Each column represents one separated biological sample. Genes are ordered according to the decreasing average expression level in LD. Representative genes upregulated in the samples are shown on the left. Red: representative genes upregulated in LDs. Green: representative genes upregulated in MaSC and yMaSC derived organoids.

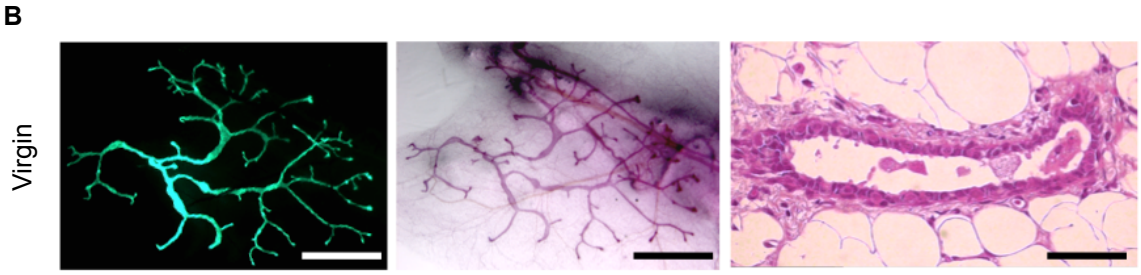
C-D, Treatment with hormon prolactin triggers the expression of *β-casein* **(C)** and α-casein **(D)** in MaSCs and yMaSCs (from YAP wt) derived organoids. qRT-PCR data were normalized to *Gapdh* expression and presented as mean + s.d.; results are representative of two independent experiments performed in triplicate.

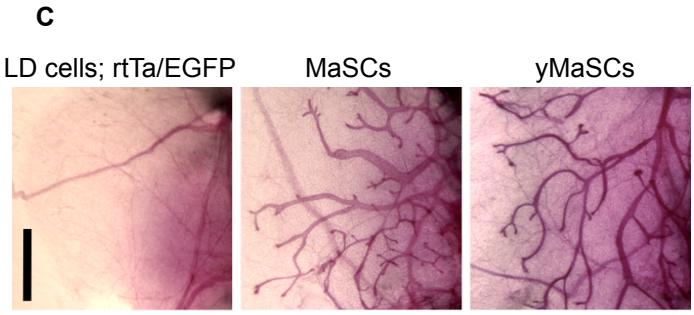
E, Representative images of organoids obtained from $Yap^{fl/fl}$; $Taz^{fl/fl}$ yMaSCs. Organoids were transduced with Ad-Cre or Ad-GFP (as control) during passaging. Panels are representative images of the resulting outgrowths. No organoid ever formed in absence of YAP/TAZ. Scale bar, 250 μm.

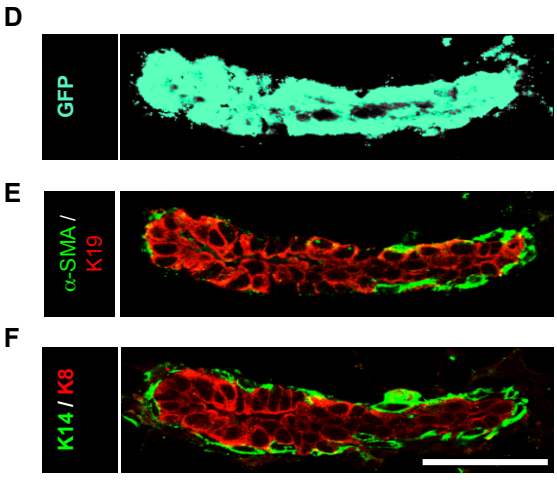
F, Western blots analysis for YAP and TAZ in the indicated cells. Lane 1: FACS-purified LD cells. Lane 2: yMaSCs (wtYAP) after seven days of doxycycline treatment; tagged Flag-hYAP is induced. Lane 3: organoids from yMaSCs cultured in the absence of doxycycline

(Flag-hYAP turned off, but endogenous YAP/TAZ remain expressed). Lane 4: endogenous MaSCs. GAPDH serves as loading control.


A
PRRL-GFP
tetO-YAP
(or TAZ)
rtTA
FACS
LD cells
+ DOXY
7 days
GFP+-yMaSCs
Transplantation
in clear fat pad
Mammary gland
reconstitution


B
Virgin


C
LD cells; rtTa/EGFP
MaSCs
yMaSCs


D
GFP
E
α-SMA /
K19
F
K14 / K8

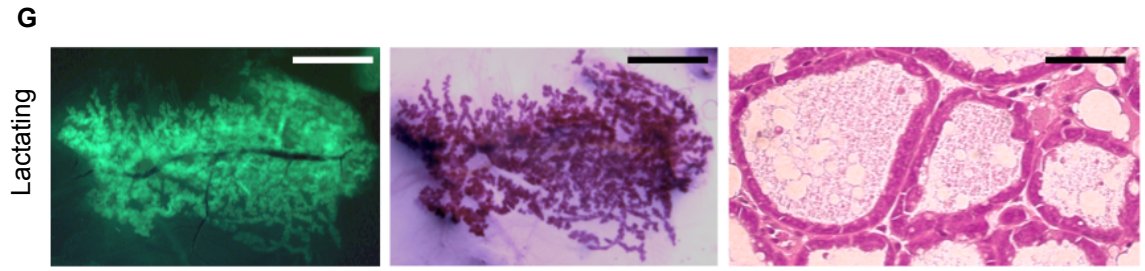

G
Lactating

FIGURE 8

Figure 8. Reconstitution capacity of yMaSC

A, Schematic representation of the injection experiments performed. Constitutive GFP expressing vector (PRRL-GFP) was used to trace the cells. Doxy stands for doxycycline.

B, Mammary gland reconstitution generated by stably GFP-expressing yMaSCs (from YAP wt) in virgin females. Left panel: native GFP fluorescence (scale bar 0,5 cm); Central panel: whole mount hematoxylin staining (scale bar 0,5 cm); Right panel: Hematoxylin staining on histological section (scale bar 21 μm).

C, Hematoxylin staining of cleared fat pad with reconstituted mammary trees from transplanted yMaSCs (from wtYAP), native MaSCs (positive control) and rtTA/EGFP control LD cells (negative control). Scale bar, 0.5 cm.

D-F Immunostaining for GFP **(D)**, and the indicated basal (K14, a-SMA) and luminal (K8, K19) markers **(E-F)** on reconstituted mammary gland sections. Scale bar 21 μm.

G, Mammary gland reconstitution generated by yMaSC in an impregnated female. Left panel: native GFP fluorescence (scale bar 0,5 cm); Central panel: whole mount hematoxylin staining (scale bar 0,5 cm); Right panel: Hematoxylin staining on histological section (scale bar 21 μm). Note that upon gestation and lactation, the mammary gland is constituted by alveoli filled with milk.