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At The Cutting Edge – Basic research advances on pituitary stem cell function and regulation.

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Abstract

As a central regulator of major physiological processes, the pituitary gland is a highly dynamic organ, capable of responding to hormonal demand and hypothalamic influence, through adapting secretion as well as remodelling cell numbers among its seven populations of differentiated cells. Stem cells of the pituitary have been shown to actively generate new cells during postnatal development but remain mostly quiescent during adulthood, where they persist as a long-lived population. Despite a significant body of research characterising attributes of anterior pituitary stem cells, the regulation of this population is poorly understood. A better grasp on the signalling mechanisms influencing stem proliferation and cell fate decisions can impact on our future treatments of pituitary gland disorders such as organ failure and pituitary tumours, which can disrupt endocrine homeostasis with life-long consequences. This minireview addresses the current methodologies aiming to understand better the attributes of pituitary stem cells, the normal regulation of this population in the organ and discusses putative future avenues to manipulate pituitary stem cells during disease states or regenerative medicine approaches.

Introduction

Stem cells are characterised by their ability to self-renew and to give rise to differentiated cells, and fulfil an important function in the generation of new cells during growth, homeostasis and repair. Resident stem cell contribution to normal postnatal homeostasis has been demonstrated in the murine

pituitary [1], a step ahead of other endocrine organs of great interest to regenerative medicine, such as the pancreas [2]. Despite the presence of these stem cells, the adult pituitary shows limited regenerative capacity to recover from insult, in either mouse models or human conditions. Endogenous pituitary stem cells display a decline in their potential with age [3], and recent data suggest that their participation in homeostasis during insult is minimal [4]. However certain attributes of endocrine cells allow them to contribute towards enhancing function during insult and enable a degree of resilience to the anterior pituitary. These attributes include: (i) phenotypic plasticity/the possibility to transdifferentiate when a dynamic shift in the population is required e.g. from somatotrophs to lactotrophs as indicated by bihormonal expression during regeneration [5]; (ii) proliferative capacity among the hormone-expressing cell populations; (iii) the ability of reduced numbers of endocrine cells to compensate for hormone production after insult e.g. ablation of somatotrophs in mice, even up to 80%, does not impact on IGF1 levels, however when ablation reaches a threshold of 90% there is a reduction in IGF1 [6]. Combined, these mechanisms may help the organ cope with fluctuations in physiological demand but are clearly not sufficient during insult.

A whole host of markers have been identified for pituitary stem cells, yet the functional relevance of most still remains elusive. Consequently, no solid steps have been made in regulating pituitary stem cell proliferation in a controlled manner, or directing their differentiation *in vivo*. This may be achievable by understanding and manipulating the signalling cues that normally regulate cell activity and fate decisions. Collectively, this information would be applicable to controlling endogenous populations of cells in a broader range of pituitary disorders spanning hypopituitarism, the uncontrolled proliferation of cells during neoplasia, as well as potentially improving hormonal function during ageing.

Recent novel technical advances are enabling us to gain exponential amounts of knowledge on the *in vivo* cellular regulation and function of the pituitary. Genomic, spatial and functional information from basic research efforts will need to be integrated and contextualised in order to better inform translation into the clinic. In this review we present an overview of current basic approaches focusing on understanding the endogenous stem cell population of the pituitary.

Generation of pituitary endocrine cells from pluripotent stem cells

Current regenerative approaches aiming to tackle disorders such as hypopituitarism, rely heavily on understanding the characteristics of normal embryonic development and commitment into pituitary fates. The lack of human pituitary cell lines has been a hindrance to understanding the key steps regulating patterning and lineage restriction. In recent years, pluripotent stem cells have aided in this

process, and through sequential treatment with signalling molecules can be differentiated to eventually specify a pituitary primordium. Pioneering proof-of-principle studies have demonstrated that in this way it is possible to obtain fully functioning hormone-secreting cells *in vitro*, albeit inefficiently, and to even generate these from human ES cells following directed differentiation [7-9]. In previous reports, the generation of anterior pituitary fates from ES cells in three-dimensional culture necessitates an intermediate induction to forebrain fates. Specification of oral ectoderm is achieved through treatment with BMP4, and subsequently SHH agonists to generate definitive anterior pituitary. This occurs at a low efficiency, where the majority of the cells remain neural and only a small fraction on the non-neural (1-7%) express hormones [10]. A higher, but still suboptimal efficiency was obtained in monolayer cultures with human iPSCs. This employed inhibitors of BMP signalling together with inhibitors of TGFβ, Activin and Nodal [9]. The key to this was timed removal of BMP inhibition two days after neural induction, leading to placode induction, followed by activation of the SHH signalling pathway to induce oral ectoderm fates. Earlier exposure to BMP4 can increase the overall placode yield [11]. Recent studies demonstrated derivation of AP hormone-producing cells from human ES or iPS cells, which are able to function in response to stimulus in murine hypopituitarism models [8, 12], with the two-dimensional approach by Zimmer and colleagues generating functional cells at higher efficiency. In order for *in vitro*-generated cells to achieve homeostatic control *in vivo*, these have to be grafted within the pituitary or in immediate contact with the hypothalamus [13-16]. Alternative grafts, such as under the kidney capsule can function without hypothalamic regulation [5, 10] but have the advantage of being less invasive and more easily accessible. It is important to note that regeneration of pituitary cell types would not be a viable strategy to treat hypothalamic hypopituitarism. However, transplantation approaches placing new cells in immediate contact with the hypothalamus may partly circumvent hypopituitarism in the case of pituitary stalk interruption or following traumatic brain injury. Future improvements in these protocols and confirmation of functional hormone producing cells from induced pluripotent stem cells will bring us closer to patient-tailored cell replacement therapies.

Pituitary stem cells are contained within the SOX2+ population

The anterior pituitary is a medium to low turnover organ, where proliferative potential and contribution to all anterior pituitary lineages in the postnatal period of the mouse has been demonstrated by cells expressing SOX2 [1]. This transcription factor serves as a marker of numerous other adult stem cell populations [17], and is also a core factor required for pluripotency of the early embryo, embryonic stem cells and reprogramming. The anterior pituitary forms from Rathke's pouch, a primordium in the oral epithelium, where all cells initially express and require SOX2 [18]. By postnatal stages, the positive cells are located in two niches: the epithelium of the marginal zone and

in scattered throughout the parenchyma, mostly clustered in small groups. These niches persist throughout life and are interconnected by long cellular processes [19, 20]. Our previous work identified that this long-lived population can contribute new hormone-secreting cell types during postnatal life [1], just as they do during organogenesis [21]. Whilst the measure of self-renewal has been limited to *in vitro* experiments [22], the potential to self-renew lies only within a proportion of the SOX2 population, under defined stem cell-promoting conditions [23]. In this context, the proportion of SOX2 cells that adhere, expand to generate more SOX2 cells. This does not mean that other cell types in the pituitary, such as subsets of the committed lineages, do not have self-renewal capabilities and this remains to be tested. It is not known if all SOX2+ cells have the same capacity; it is possible that during *in vitro* isolation, only cells perceiving particular extrinsic or intrinsic signals, or being at a specific phase of the cell cycle are capable of activation and divide under these defined conditions. Intrinsic heterogeneity occurring within this SOX2 population, or influences from their *in vivo* niche, may render them 'active or inactive'. Of relevance, SOX2 is expressed in the human pituitary in a similar pattern to that of rodents [24], and our data indicate that just like in model systems, this putative stem cell population persists until late adult life (Figure 1). Therefore, a thorough characterisation of this long-lived population would be of value to regenerative medicine approaches. Cells with clonogenic potential have been isolated from normal human pituitaries, and these stem-like cells demonstrated generation of differentiated, hormone-secreting cells [25].

Multiple, non-exclusively overlapping markers have been described in *Sox2*-expressing cells, such as *S100b*, *Gfra2*, *Sox9*, *Nestin*, *Cdh1*, *Prop1*, *Prrx1/2*, *Cxadr*, *Grhl2* [1, 21, 22, 26-31], however to date, there are no functional data for a requirement of these in the maintenance and promotion of the postnatal stem cell state. The localisation of PROP1 marks a proportion of SOX2 positive cells that are able to form colonies *in vitro*, however this was shown to be required for enabling transition to the progenitor state and for leaving the epithelium via epithelial-mesenchymal transition [32]. SOX9 positive cells (also expressing SOX2), do generate functional cells of all lineages *in vivo* [21], but the difference in potential between single SOX2 positive only and double SOX2;SOX9 is not yet known, neither is the functional requirement for SOX9.

Addressing the heterogeneity of pituitary stem cells

One thing missing from the current analyses and descriptive characterisations of the stem cell population is the extent of heterogeneity displayed by these cells in their functional attributes. In terms of gene expression, this can be now determined by single cell RNA-Seq approaches [33]. With advances in cell separation and depth of sequencing, several thousand cells can be simultaneously sequenced, however spatial information is lost with these techniques. Methodologies for RNA

sequencing *in situ* are gaining in their cellular resolution and are efficiently analysed, therefore spatial transcriptomic analyses may be the best methodologies for understanding heterogeneity in a niche-dependent context [34-36]. Such approaches are timely and will reveal a wealth of information but hypotheses will need to be functionally tested. It is unlikely that SOX2 positive cells all have the same capacity *in vivo*, especially given their differential *in vitro* potential, heterogeneous marker expression and differential localisation *in vivo*. Therefore, we may be able to elucidate in future if true multipotent SOX2 positive stem cell remain in the adult gland, able to self-renew and give rise to all committed lineages positive for POU1F1 (PIT1), TBX19 (TPIT) and NR5A1 (SF1)/GATA2, or if SOX2 positive cells experience fate restriction, resulting in different uni- or bipotent SOX2-expressing populations. It will also be interesting to determine the localisation of the more active stem cells, to decipher the particulars of the niches promoting or enabling this state.

Simultaneously addressing the *in vivo* behaviour of multiple cell types is not trivial. The most appropriate current genetic tools include multi-colour reporters [37]. The commonly-used Cre recombinase-dependent Confetti reporter for example [38], labels cells driving Cre and their descendants in any of four fluorescent colours in heterozygosity (Fig 2A) (and 16 distinct combinations in homozygosity). These allow the differential labelling of neighbouring cells in a tissue and an independent study of their fates. Caveats of these reporters include a lower recombination efficiency compared to other single-colour ROSA26 reporters, that recombination can sometimes take place as such where no fluorescent protein is expressed (failure to excise the stop cassette), and the fact that whilst Cre is expressed in a cell, the expression of a colour can keep changing until the Cre is no longer active. In our use of $Hesx1^{Cre/+};R26^{Confetti/+}$, by 15.5dpc, each labelled cell has a final colour since *Hesx1* is no longer expressed, revealing a mixed pattern of closely, non-clonal labelled cells. By analysing three months after birth, we find a pattern of frequently segregated colour labelling spanning all fluorophores, indicative of clonal expansion, as well as areas of broad expansion along the epithelium and into the deeper parenchymal layers. Although the use of this reporter in the embryonic model may not be suitable for analyses of the potential of single cells, it is of value to help us understand cell dynamics during growth and to calculate cell turnover in this organ. The Confetti reporter is ideal however, for inducible systems (e.g. CreERT2) where cells become labelled only whilst the administered tamoxifen is still in the system. One issue is that they ought to be optimised for each inducible driver strain. It is advisable to carry out 'nearest neighbour' analyses at short pulses following titred tamoxifen dosage, to define how often two cells next to each other might be labelled with the same colour. When trying to determine if a SOX2 positive pituitary stem cell can be multipotent in adulthood or not, this becomes crucial. An example of its optimised use for clonality in the SOX2+ cells of the anterior lobe during the postnatal growth phase [39, 40], demonstrates asymmetric divisions along the marginal zone epithelium, two weeks post-induction (Figure 2B). Deciphering the extent of pituitary stem cell heterogeneity by combining *in silico* and functional

techniques will help determine the characteristics and possible distinct roles of cell subsets within the stem cell population.

Signalling pathways in the control of proliferative function

We have previously characterised a drop in stem cell potential with age, by determining the *in vitro* capacity of anterior pituitary cells to form clonal colonies [3]. Similarly, the regenerative capacity of the gland following injury has been reported to decline with age [41]. At any stage of life, no more than one twentieth of the SOX2 cells are dividing *in vivo*, or can actively expand *in vitro* [1, 3]. Experiments employing physiological challenge, do suggest that the SOX2 population can be minimally re-activated, for example, estradiol treatment instigates proliferation in the SOX2 compartment, increasing the proportion of dividing cells up to 10% at adulthood [21], a time when divisions are dwindling. Even after challenge, however, stem cell re-activation remains curiously low and is not the main source of new hormone-secreting cells. Therefore, might it be better to focus efforts on more committed cell types that display more proliferative potential? Following adrenalectomy, only up to a fifth of newly generated corticotrophs derive from stem cells [21], and elegant genetic experiments have demonstrated that the majority derive from non-stem cells, either by expansion of committed progenitors or hormone-secreting cells [42]. Lactotrophs can also be stimulated to proliferate, by exogenous administration of estradiol, promoting proliferation both directly and via hypothalamic feedback [43]. These properties may be indicative of a large proportion of hormone-expressing cells that are not actually terminally differentiated, or may not have exited the cell cycle whilst functioning to secrete hormone.

Regenerative potential can be influenced by the enhancement of signalling pathways promoting proliferation. Forced activation of the WNT pathway in SOX2 cells instigates a burst of proliferative activity [1], in-keeping with the major property of this pathway to promote stem cell self-renewal [44]. We have shown that these cells, expressing a stable mutant form of CTNNB1 (β-catenin), in turn up-regulate expression of genes from the *Wnt*, *Fgf*, *Egf*, *Bmp*, *Tgf* and *Hh* families [23], all with the potential to influence proliferation and/or cell fate specification. If normal postnatal SOX2 positive stem cells secrete any of these signals influencing surrounding cells remains to be shown, which would provide an additional function for the relatively sedentary stem cell population in this organ. The outcome of simultaneous enhanced activation of these signalling pathways from mutant SOX2 cells, is the paracrine formation of tumours by a massive proliferative response in non-mutant cells, which happen to lie outside of the *Sox2* lineage [1]. Interestingly, when committed anterior pituitary cells are targeted to express the same non-degradable mutant form of β-catenin, they do not

generate tumours. Recent work reveals that the tumours form when mutant cells enter senescence and robustly activate the senescence-associated secretory pathway (SASP) [45]. The SASP response leads to secretion of a range of mitogens, chemokines and cytokines from the mutated stem cells. In mouse models where the SASP response is low (e.g. through induction of a different mutation elevating the WNT pathway through the *Apc* gene, or mutating *Ctnnb1* in ageing pituitary stem cells), the tumours do not form. This might suggest that even if committed cells did initiate the SASP response, perhaps the levels are not robust enough to induce tumours compared to 'active' SOX2+ stem cells targeted by the oncogenic mutation. This indirect paracrine function of stem cells is of relevance to human pituitary tumour formation, as this mouse model recapitulates the most common pediatric pituitary tumour, adamantinomatous craniopharyngioma, also harbouring mutations in *CTNNB1* activating the WNT signalling pathway. There is evidence of cells isolated from pituitary adenomas with similarities in their *in vitro* capacity and gene expression, to pituitary stem cells [46]. These are also defined by a side population efflux profile upon flow cytometry following Hoechst dye labelling, which can enrich for stem cells in many tissue types, including the pituitary [47]. Definitive evidence for cells with stem-like properties isolated from adenomas has been presented through the propagation of tumour tissue following xenotransplantation [48, 49]. The presence of proliferating SOX2 positive stem cells is prominent in papillary craniopharyngioma tumours, characterised by mutations activating the MAPK pathway [50]. The deregulation of normal developmental signalling pathways may therefore activate proliferative potential, but sustained activation may lead to tumorigenesis, likely facilitated by stem cell involvement.

We recently described the expression of components of the Hippo signalling pathway in the pituitary [51]. This pathway has been well characterised in *Drosophila*, but relatively recently described in mammals [52]. It is comprised of a kinase cascade that negatively regulates key effectors YAP and TAZ, which act to promote proliferation and the stem cell state, as well as to prevent apoptosis. It is therefore unsurprising that deregulation of YAP/TAZ underlies and promotes growth of numerous tumours and cancers [53]. Global deletion of one of the core kinases, LATS1, results in pituitary hyperplasia and hormone deficiency [54]. The impact of LATS1 deletion on YAP/TAZ in the pituitary, as well as the possible link of this pathway to tumour involvement remains to be shown. In the mouse pituitary, localisation of YAP/TAZ is in the nucleus of SOX2 cells and not of the committed lineages (Figure 3A). Since nuclear YAP/TAZ are associated with an active stem cell state, this pathway may be of relevance to the regulation of stem cell potential in the pituitary. Of the genes directly regulated by YAP/TAZ, *Ctgf* is a target in multiple tissues [55]. Surprisingly, mRNA *in situ* hybridisation to detect *Ctgf* expression in the pituitary does not fully recapitulate *Sox2* expression as it is mostly localised in individual cells throughout the parenchyma (Figure 3B). Double *in situ* for *Ctgf* and *Sox2* reveals overlap in some *Ctgf*-expressing cells, however the majority of *Sox2*-expressing cells do not activate this YAP/TAZ target. This could suggest that *Ctgf* may not be a direct target of

YAP/TAZ in the pituitary, however similar analyses with additional direct target *Cyr61* reveal a similar expression pattern (not shown). Alternatively, YAP/TAZ may not be triggering the full transcriptional programme associated with the active stem cell state in all SOX2 cells, if for example this activation is being inhibited. Since there is robust activity of Hippo kinases in these cells, demonstrated by the detection of phosphorylated YAP at serine residue 127, inhibition is indeed taking place [51]. Perhaps down-regulating activity of these kinases may lift the break acting on the proliferative potential of stem cells. Persistent nuclear localisation of YAP/TAZ only in SOX2 cells and detection of *Yap* mRNA in these cells into adulthood, as well as the *Lats1*$^{-/-}$ phenotype are all indicative that the potential to respond might be in place. The vast array of genetic tools available, will allow us to explore the function of this pathway on pituitary stem cells and determine its relevance to applications relating to regenerative medicine and tumours. Since the Hippo/YAP/TAZ pathway is readily druggable, the potential to precisely influence stem cell survival and proliferation through exogenous means may be facilitated [56].

Favoured by recent methodological advances, we are on the cusp of amassing sufficient knowledge of pituitary stem cell attributes and the signalling mechanisms that influence them, to begin manipulating their function and potential *in vivo*. Future research will aim to re-activate, amplify, diminish or differentiate this population in order to facilitate future treatment approaches.

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Figure 1. Immunofluorescence staining against SOX2 in aged human pituitaries. SOX2 is expressed in cells of the parenchyma of two post-mortem pituitaries (Pituitary 1- female, 77 years; Pituitary 2 – male, 89 years). Nuclear localisation is observed in individual parenchymal cells (arrowheads), nuclei are counterstained with DAPI.

Figure 2. Multi-colour labelling of embryonic and postnatal progenitor/stem cells in the mouse pituitary. (A) Genetic strategy employing the R26-Confetti strain, crossed with either a Cre strain driven from the *Hesx1* promoter (embryonic), or CreERT2 strain driven by the *Sox2* promoter (conditional upon tamoxifen administration). Recombination can result in the stable expression of one

of four different fluorophores (nuclear green fluorescent protein (nGFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP), membrane cyan fluorescent protein (mCFP)), which continue to be expressed by descendants. (B) Recombination in the developing pituitary of *Hesx1*$^{Cre/+}$;*R26*$^{Confetti/+}$ demonstrating labelling of numerous scattered cells at 15.5 days post coitum (dpc), followed by analysis at three months postnatal, revealing single-colour clonal expansion of individual embryonic progenitors. A region of expansion (bracket, yellow cells) can be seen spanning the epithelium as well as expanding into the parenchyma. Induction of *Sox2*$^{CreERT2/+}$;*R26*$^{Confetti/+}$ pituitaries at postnatal day 14 (P14) and analysis two weeks later, demonstrates labelling of scattered cells in the intermediate lobe (IL) and anterior lobe (AL) and sporadic duplication of labelled cells along the epithelium (arrowheads).

Figure 3. YAP/TAZ and target expression in the SOX2 positive population of pituitary stem cells. (A) YAP and TAZ localise in the nucleus of SOX2 positive cells, marked by GFP expression in *Sox2*$^{Egfp/+}$ knock-in mice. Adapted from Lodge et al, Front Physiol. 2016 Mar 31;7:114. doi: 10.3389/fphys.2016.00114. (B) RNAscope mRNA *in situ* hybridisation detecting YAP/TAZ target *Ctgf* reveals sporadic robust expression in parenchymal cells (red) and rare expression along the marginal zone epithelium. Cells are counterstained with hematoxylin. Double *in situ* hybridisation detecting *Ctgf* (aqua, arrow) and *Sox2* (red) reveal partial overlap among *Ctgf*-expressing cells (arrowheads), and the majority of *Sox2*-expressing cells do not express *Ctgf*.

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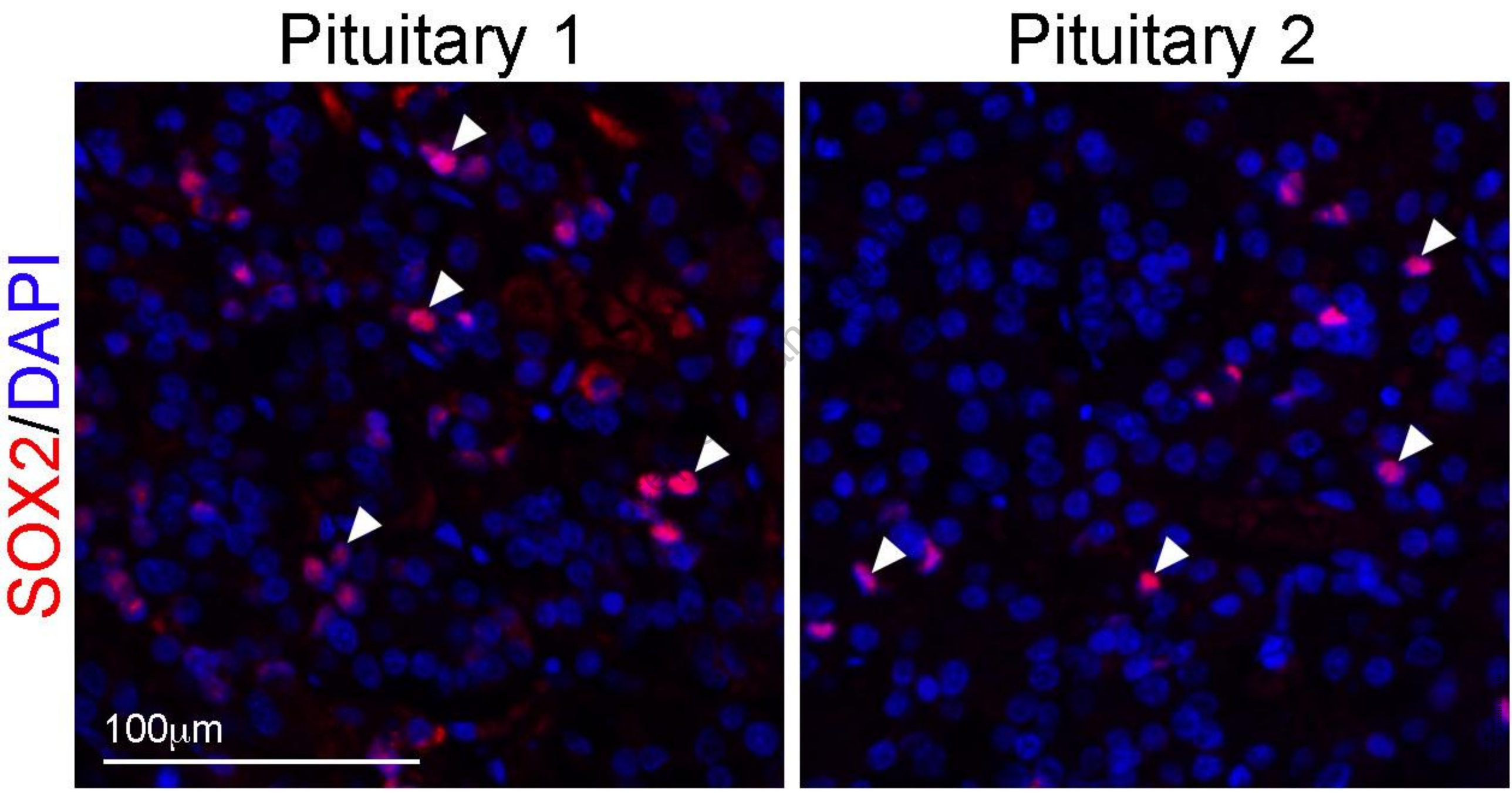
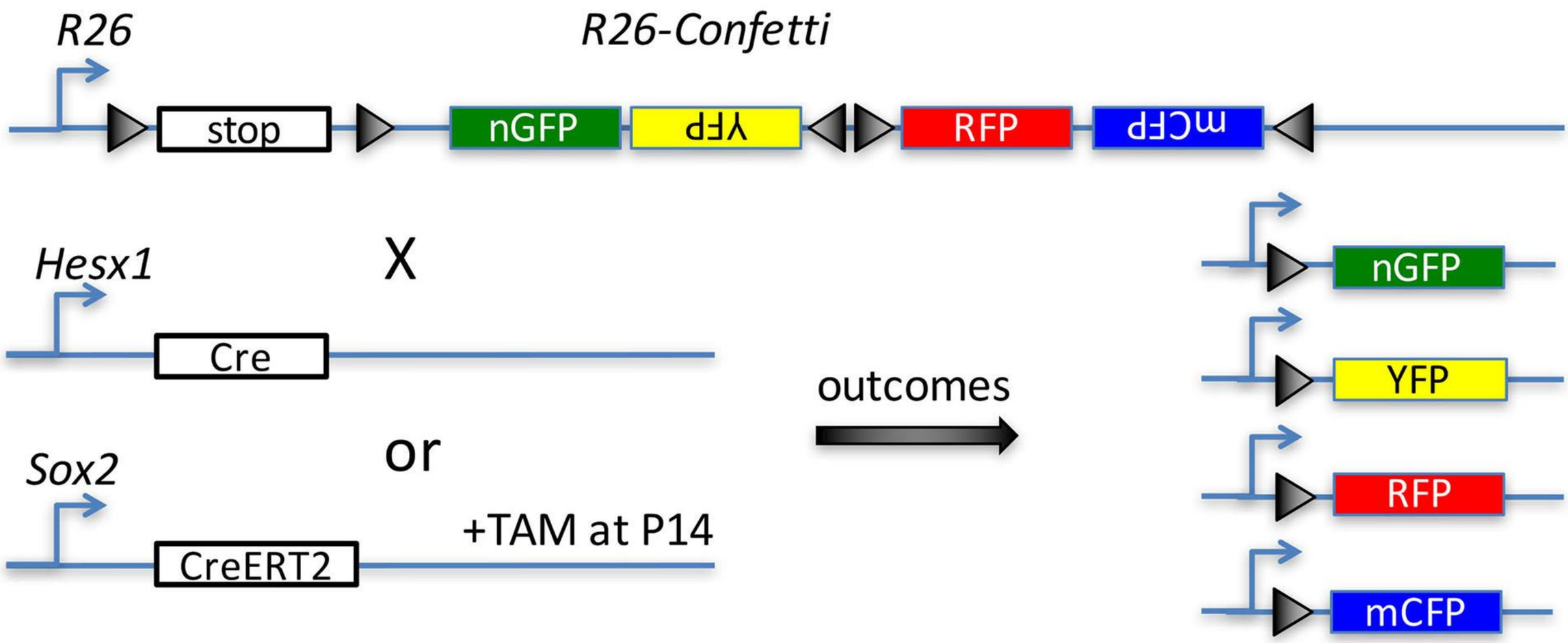
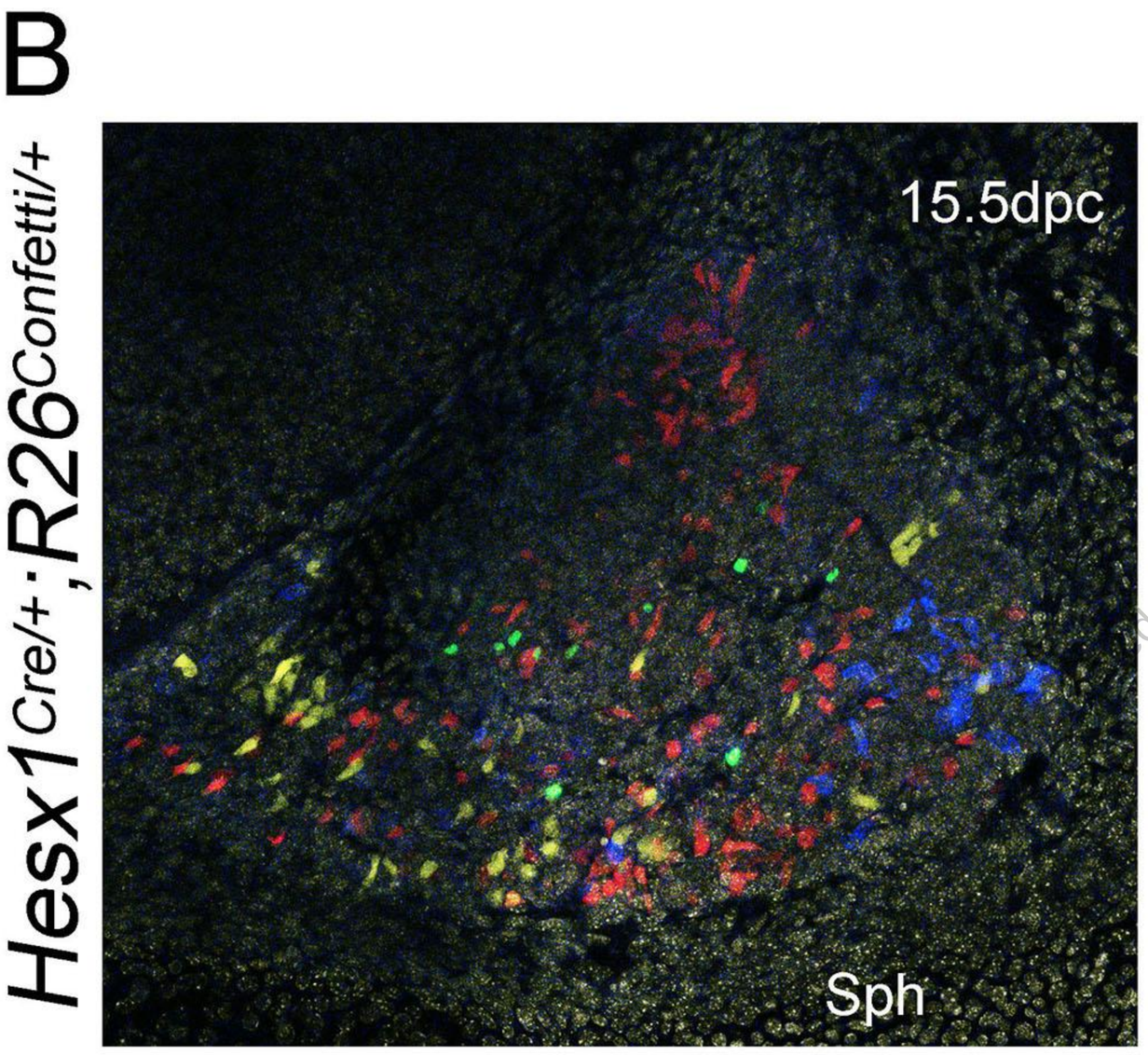
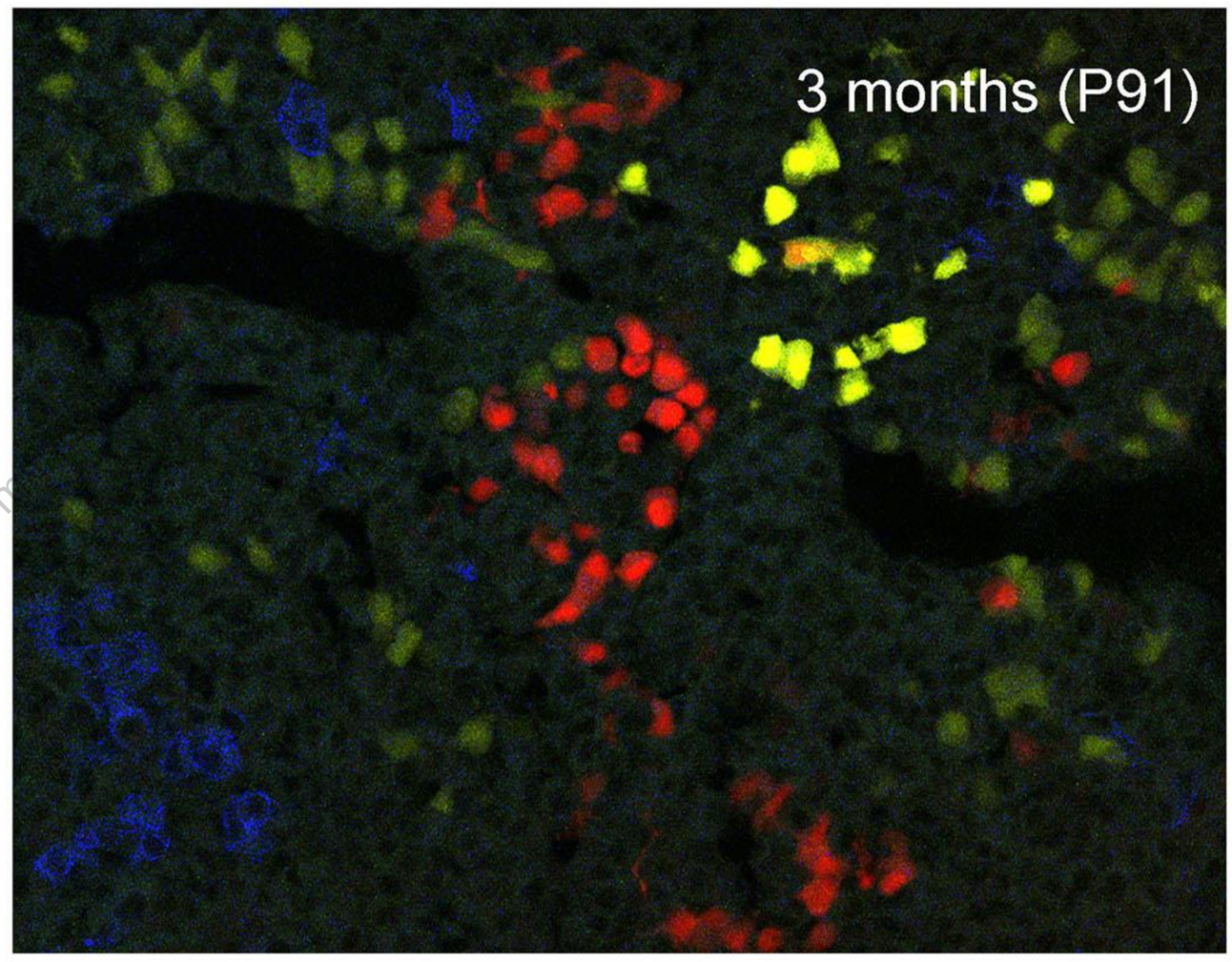

Pituitary 1
Pituitary 2
SOX2/DAPI
100μm

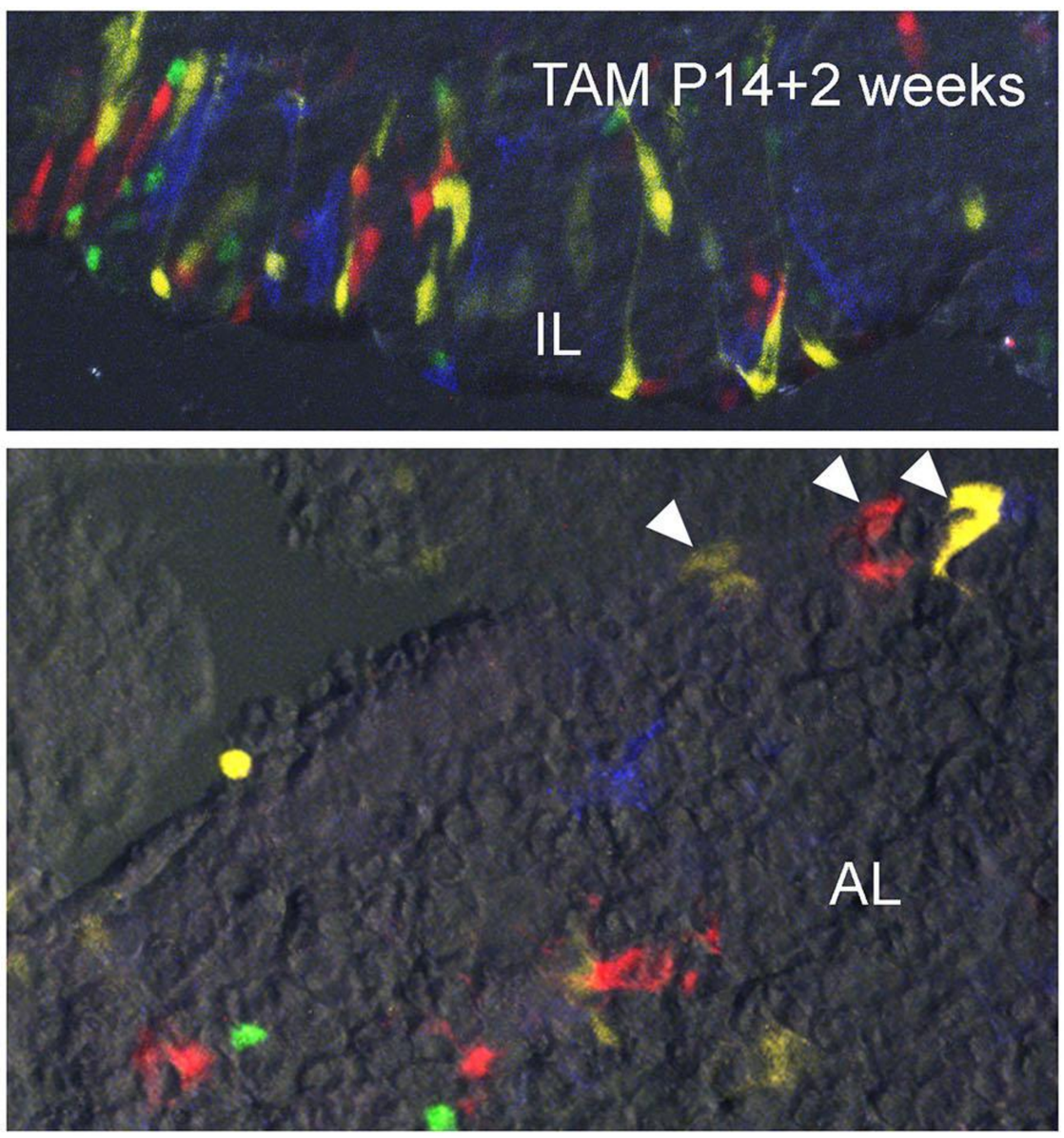
A

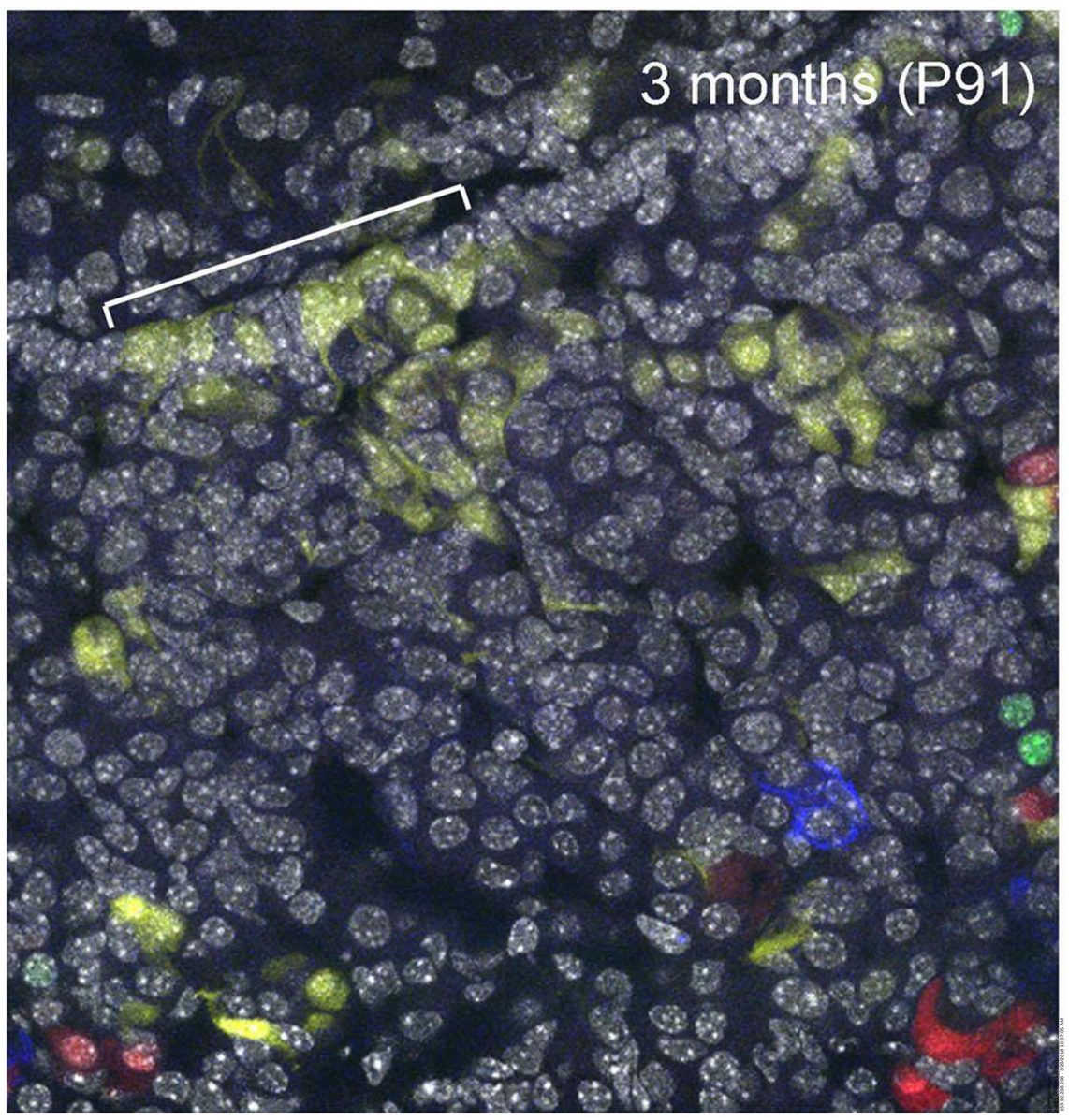

R26
R26-Confetti
stop
nGFP
YFP
RFP
mCFP
Hesx1
X
Cre
Sox2
or
CreERT2
+TAM at P14
outcomes
nGFP
YFP
RFP
mCFP

B


Hesx1Cre/+;R26Confetti/+
15.5dpc
Sph


3 months (P91)


Sox2CreERT2/+;R26Confetti/+
TAM P14+2 weeks
IL
AL


Hesx1Cre/+;R26Confetti/+
3 months (P91)