Transcriptional regulation of Sox2 by the retinoblastoma family of pocket proteins

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ABSTRACT

Cellular reprogramming to iPSCs has uncovered unsuspected links between tumor suppressors and pluripotency factors. Using this system, it was possible to identify tumor suppressor p27 as a repressor of Sox2 during differentiation. This led to the demonstration that defects in the repression of Sox2 can contribute to tumor development. The members of the retinoblastoma family of pocket proteins, pRb, p107 and p130, are negative regulators of the cell cycle with tumor suppressor activity and with roles in differentiation. In this work we studied the relative contribution of the retinoblastoma family members to the regulation of Sox2 expression. We found that deletion of Rb or p130 leads to impaired repression of Sox2, a defect amplified by inactivation of p53. We also identified binding of pRb and p130 to an enhancer with crucial regulatory activity on Sox2 expression. Using cellular reprogramming we tested the impact of the defective repression of Sox2 and confirmed that Rb deficiency allows the generation of iPSCs in the absence of exogenous Sox2. Finally, partial depletion of Sox2 positive cells reduced the pituitary tumor development initiated by Rb loss in vivo. In summary, our results show that Sox2 repression by pRb is a relevant mechanism of tumor suppression.

INTRODUCTION

Cellular reprogramming to induced pluripotency by the combined action of defined transcription factors is a powerful in vitro system to uncover basic mechanisms governing stem cell biology. Given the similarity between cellular reprogramming and oncogenic transformation [1],
others and us addressed the role of tumor suppressor genes opposing this process. In this manner, it was possible to identify crucial barriers impairing the efficient conversion of somatic cells to induced-pluripotent stem cells imposed by well-known tumor suppressor genes such as those encoded by the p53 gene and the Ink4a/Arf locus [2–6].

Further studies on the effect of other tumor suppressor genes in the process of cellular reprogramming allowed us to identify an unprecedented connection between a cell cycle regulator, p27, and a pluripotency factor, Sox2 [7]. A previously unrecognized transcriptional regulatory activity of p27 allows the efficient repression of Sox2 in differentiated cells. Loss of this regulation leads to unscheduled expression of Sox2 in differentiated cells with the drastic phenotypic consequences that characterize p27-null mice: gigantism, pituitary tumor development, and retinal defects. The transcriptional regulation of Sox2 during differentiation is exerted by a partially characterized repressive complex in which, apart from p27, we identified Sin3a, E2F4, and the pocket protein family member p130.

The retinoblastoma family of pocket proteins (composed by pRb, p107 and p130) is a family of negative regulators of the cell cycle with structural homology, and with overlapping and unique functions. The product of the RB gene is considered the member of the family with the most relevant tumor suppressor activity, since it is inactivated in a large number of human cancers [8–12]. In addition, its deletion in mice results in tumors, mainly of the pituitary and the thyroid gland [13]. The other two family members have also been involved in cancer development, although their relevance is secondary compared to pRb [13]. In addition to its role in cancer, pRb controls cellular differentiation during embryonic development and in adult lineages [14], with p107 and p130 also playing regulatory roles in adult cell differentiation [15].

Our previous results investigating the transcriptional repression of Sox2 by p27 in which the pocket protein family member p130 was part of the repressive complex, together with the similarities between the process of reprogramming to pluripotency and oncogenic transformation, prompted us to investigate the role of the pocket protein family members in the transcriptional repression of Sox2. We found that deletion of Rb or p130 leads to impaired repression of Sox2, while loss of p107 has no apparent effect. Furthermore, we identified binding of pRb and p130 to an enhancer downstream of Sox2 important for its regulation, the Sox2-SRR2 enhancer, a region that showed alterations in epigenetic marks upon loss of pocket protein family members. The functional consequences of the defective repression of Sox2 are manifested in the cellular reprogramming of Rb deficient cells to iPSCs in the absence of exogenous Sox2, and in vivo in Rb loss-driven pituitary tumor development.

RESULTS AND DISCUSSION

Cells lacking Rb or p130 express higher levels of Sox2

We previously showed that cell cycle regulator p27 contributes to the transcriptional repression of Sox2 together with pocket protein p130 and consequently, p27 deficient cells or shRNA-mediated knockdown of p130 leads to an increase of Sox2 expression that has measurable phenotypic consequences [7]. To study in more detail the relative potential contribution of the pocket protein family members to the repression of pluripotency genes, and specifically to the regulation of Sox2, we measured by qRT-PCR the mRNA levels of Sox2 and Nanog in early passage primary mouse embryo fibroblasts (MEFs) derived from Rb-, p107-, or p130-null mice. MEFs do not express detectable levels of these pluripotency genes or their levels of expression are negligible. In contrast, the absence of Rb or p130 resulted in moderate but reproducibly increased levels of Sox2 mRNA (Figure 1A, left panel). Surprisingly, cells lacking Rb also showed increased levels of Nanog (Figure 1A, right panel), a pluripotency gene normally not expressed in differentiated cells.

Strikingly, when we analyzed the expression of Sox2 in MEFs from the same genotypes immortalized by an shRNA targeting p53 (shp53) we observed an even higher increase in the expression of Sox2 specifically for MEFs deficient in Rb or p130, but importantly, not in wt or p107-null cells (Figure 1B). In contrast, the increased expression of Nanog remained constant in Rb-deficient MEFs even after immortalization with shp53. Confirming these results, we checked Sox2 levels in MEFs immortalized by the expression of viral oncoprotein large-T (LT) antigen from SV40. LT is known for its ability to block several cellular functions and prominently among them, the tumor suppressor pathways controlled by p53 and the three pocket proteins [16]. In agreement with the above results, MEFs immortalized by LT showed an increase in the expression of Sox2 mRNA by qRT-PCR (Figure 1C, upper panel), with levels high enough to allow clear detection of Sox2 by Western blot (Figure 1C, lower panel). Knockdown of p53 after shp53 expression is not causing an increase of Sox2 in wt cells and this seems to be occurring only in cells deficient in Rb or p130, that already underwent some form of deregulation in the expression of Sox2. To further explore this, we used reprogrammable MEFs derived from a transgenic mouse carrying an inducible cassette for the expression of the four reprogramming factors, Oct4, Sox2, Klf4 and c-Myc (i4F-MEFs) [17]. First we introduced a pluripotency reporter plasmid carrying an active promoter derived from a mouse early transposon (ETn) that is specific for undifferentiated pluripotent stem cells, combined with Oct4- and Sox2-
binding motifs (EOS)[18]. Reprogrammable MEFs carrying this EOS reporter plasmid showed a low level expression of GFP, indicative of a minor leaky expression from the transgenic cassette. When we expressed the shRNA against \( p53 \) in these i4F-MEFs carrying the EOS reporter plasmid a clear increase in the expression of GFP was evident by inspection under a fluorescent microscope. To verify and quantify this observation, we analyzed GFP expression in these cells by flow cytometry (Figure 1D, upper panel). In this manner, we could confirm the visual observation both regarding the alteration of the proportion of GFP positive cells (51.7% in i4F-MEFs versus 71.1% in i4F-MEFs-shp53), and in the intensity of GFP expression (mean fluorescence of 116.38 in i4F-MEFs versus 265.09 in i4F-MEFs-shp53). In agreement with the fluorescence data, these cells showed by qRT-PCR a low level of Sox2 expression that was above the levels observed in wt cells. These low levels of Sox2 expression were sharply increased when shp53 was introduced in i4F-MEFs but not in wt cells suggesting, again, that decreasing the levels of p53 amplifies the already deregulated expression of Sox2 (Figure 1D, lower panel), even when this initial increase of Sox2 expression...
deregulation comes from transgenic expression. The increased levels observed after knockdown of p53 were produced not from the transgene but from the endogenous Sox2 locus, since they were detected using a specific pair of oligonucleotides for the endogenous Sox2. These results imply that a low level expression of Sox2 would be sufficient to activate its own endogenous transcription provided that p53 is downregulated, and point to a novel function for p53 blocking upregulation of Sox2 in differentiated cells.

Taken together, these results show a defective repression of Sox2 transcription in the absence of Rb or p130 that results in low but reproducible levels of this pluripotency gene in primary cells. Lack of p53 leads to higher expression levels of Sox2 only when they are originally deregulated by deficiency in Rb or p130.

**pRb and p130 bind to the Sox2-SRR2 and their absence alters the epigenetic marks on the enhancer**

The main regulatory element of Sox2 in pluripotent stem cells is an enhancer located downstream of the single coding exon of Sox2 gene, called SRR2, bound by Sox2 itself and Oct4 to positively drive its expression during pluripotency [19]. Upon differentiation, Sox2-Oct4 on SRR2 are displaced by a repressive complex formed by, at least, p27, Sin3a, E2F4, and the pocket protein family member p130 that permanently occupies this regulatory element in somatic cells [7]. Based on this, we decided to look for the binding of pRb, p107, and p130 in differentiated cells by chromatin immunoprecipitation (ChIP) using extracts from MEFs. Chromatin precipitated using antibodies against pRb, p107, or p130 produced a clearly distinguishable band when subjected to PCR using oligonucleotides amplifying Sox2-SRR2 (Figure 2A). In contrast, control IgG did not produce an amplification band. This result implies that the three Rb family pocket proteins have the potential to bind to Sox2-SRR2. However, as shown above (Figure 1A), deficiency of p107 does not lead to increased expression of Sox2, probably as a result of compensatory mechanisms by the other members of the family. Functional compensation among pocket proteins is a common feature of the family [13].

Absence of p27 from the repressive complex binding to Sox2-SRR2 leads to an alteration of the active and repressive histone marks present in the enhancer [7]. Given the identification of pRb and p130 bound to Sox2-SRR2 and the derepression of Sox2 observed in these cells, we wondered whether a similar situation might be also occurring in the absence of these pocket proteins. To address this issue we performed ChIP assays with antibodies against the repressive mark H3K27me3 and the active mark H3K4me3 in cell extracts from MEFs deficient for pRB or p130, followed by qRT-PCR to quantify the presence of Sox2-SRR2 in the immunoprecipitates. Using two different sets of oligonucleotides amplifying Sox2-SRR2 we obtained similar results (Figure 2B). The repressive H3K27me3 histone mark seems to be reduced in cells deficient for the Rb and p130 pocket protein family members, while the active H3K4me3 mark appears increased in the absence of p130 with the two sets of primers, and in the absence of Rb at least when using...
one of the two sets of primers. In contrast, ChIP for these same histone marks in the Nanog promoter did not show any alteration (Figure 2B). These changes support the notion that lack of Rb or p130 has a specific impact on the epigenetic marks around the crucial Sox2-SRR2 regulatory element of Sox2.

**Cells lacking Rb can be reprogrammed without ectopic Sox2**

Given the low level expression of Sox2 present in primary MEFs deficient for Rb we wondered whether this deregulated expression could have a phenotypic consequence that could be exposed through the use of the cellular reprogramming system. For this, we performed a cellular reprogramming experiment on wt, Rb, p107, or p130 deficient MEFs, with ectopic expression of Oct4, Klf4 and Sox2 (3F-OKS), or omitting Sox2 (2F-OK). Control reprogramming of wt MEFs with 3F-OKS worked as anticipated giving rise to alkaline-phosphatase-positive (AP+) iPSC colonies after 14 days with the typical morphology and at the expected rate. As predicted, wt cells failed to produce any colonies when only 2F-OK were used in the reprogramming experiments. In contrast, when we used p130 deficient cells we obtained colonies both with 3F-OKS and with 2F-OK, although 2F-OK produced colonies at much lower frequency, as reported [7]. Cells lacking p107 could be reprogrammed at approximately the same rate as wt cells with 3F-OKS while no colonies emerged when using 2F-OK despite repeated efforts. Interestingly, when we used MEFs Rb-null we obtained AP+ iPSC colonies not only with 3F-OKS, as expected, but also when using 2F-OK, the combination omitting Sox2 (Figure 3A). The efficiency and kinetics of colony formation with 2F-OK were very low, similarly to what was previously reported and we confirmed when p130 deficient cells were used. To further prove the pluripotency of the iPSC colonies obtained, we introduced the EOS pluripotency reporter plasmid. EOS lentiviral transduction of MEFs prior to the expression of the reprogramming factors allowed us to observe the emergence of GFP positive iPSC colonies from 2F-OK reprogrammed Rb-null MEFs (Figure 3A). These colonies were picked and expanded in stem cell culture conditions and the mRNA of Oct4, Sox2, Klf4, and Nanog was analyzed by qRT-PCR, confirming the expression of the pluripotency factors (Figure 3B). Similarly, Western blot analysis of protein extracts from these iPSC colonies showed the presence of Oct4, Sox2, and Nanog proteins (Figure 3C). Our Rb-null derived iPSCs with 2F-OK showed similar levels of mRNA or protein for all the pluripotency markers tested than iPSCs obtained from wt or Rb-null MEFs reprogrammed using 3F-OKS, or than control embryonic stem cells (ESCs).

Next we tested the capacity of the 2F-OK iPSCs from Rb-null cells to form embryoid bodies (EBs) in culture. For this, we used the standard hanging drop method to form cell aggregates that were further cultured in non-adherent conditions until they spontaneously showed contractile activity, indicative of cardiomyocyte differentiation. The number and size of the EBs obtained with 3F-OKS in wt or Rb-null, as well as with 2F-OK in Rb-null, was similar (Figure 3D). EBs were collected and RNA was extracted and analyzed by qRT-PCR for markers of differentiation along the three germ layers endoderm, mesoderm and ectoderm, confirming the pluripotent capacity of the iPSCs obtained (Figure 3E). In addition, we analyzed histologically the structures generated within the EBs and observed features of differentiation to the three germ layers (Figure 3F).

These results show that the deregulated expression of Sox2 in Rb deficient cells is sufficient to allow cell reprogramming to iPSCs without the need for exogenous Sox2, demonstrating that this increased expression has relevant functional consequences.

**Partial depletion of Sox2 positive cells alleviates the pituitary tumor phenotype caused by reduced Rb in mice**

Human SOX2 gene locus at 3q26.3 is amplified in several cancer types including glioblastoma, small-cell lung cancer (SCLC) and many forms of squamous cell carcinoma (SCC) [20]. However, the involvement of Sox2 in cancer is not restricted to tumors showing gene amplification, opening up the possibility of other regulatory mechanisms contributing to the potential role of Sox2 in cancer. In this sense, we previously reported the contribution of Sox2 deregulated expression to the development of pituitary tumors in mice deficient for p27 [7]. Our current results were reminiscent of this same situation. Therefore, we wanted to determine if the incomplete repression of Sox2 expression in differentiated cells lacking Rb could contribute to tumor development. Heterozygosity of Rb in the mouse leads to the development of pituitary tumors after stochastic loss of the second Rb allele [21]. Given our previous results, we decided to measure Sox2 mRNA expression in pituitary tissue from wt or Rb-het mice and observed a modest increase in the expression of Sox2 (Figure 4A) consistent with our previous findings in Rb-null MEFs (Figure 1A).

To test if Sox2 positive cells contribute to the emergence of these tumors, we took advantage of a previously reported mouse model in which the thymidine kinase gene (TK) has been inserted into the endogenous Sox2 locus, the Sox2-TK mice [22]. Exposure of Sox2 positive cells to the drug gancyclovir (GCV) induces cell death. Persistent exposure to GCV results in animal death after 1-2 weeks. However, we observed that single intraperitoneal injection of GCV every 2 weeks is well
tolerated by these mice and results in various degrees of Sox2 positive cell depletion. Based on this, we generated compound Rb-het/Sox2-TK mice and subjected them to a repetitive GCV administration protocol in order to test the contribution of Sox2 positive cells to tumors initiated by Rb deficiency (Figure 4B). When the treatments were completed we sacrificed the animals and extracted tissues for histological analysis. Pituitary masses were measured and we observed a tendency to a reduced size of pituitaries from Rb-het/Sox2-TK animals in the GCV-treated group compared to the HBSS-treated control group (Figure 4B). Then, we verified that we produced different degrees of Sox2 positive cell depletion by the GCV administration, as judged by Sox2 immunohistochemical staining in sections from control tissues, esophagus and trachea. Despite the low number of animals analyzed, we observed that when

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**Figure 3: Absence of Rb allows two-factor (Oct4 and Klf4) reprogramming.** a, Representative pictures of iPSC colonies expressing GFP from the EOS pluripotency reporter plasmid (top panels), and stained for alkaline phosphatase (AP, bottom panels). Shown are iPSC colonies obtained in wt MEFs after three-factor expression (Oct4, Klf4, and Sox2; 3F-OKS; left panel), and Rb-null MEFs after three-factor (3F-OKS; middle panel) or two-factor (Oct4, Klf4; 2F-OK; right panel) expression. b, Pluripotency factor (Oct4, Sox2, Klf4, and Nanog) mRNA expression by qRT-PCR in iPSCs obtained from wt primary MEFs reprogrammed by 3F-OKS, or Rb-null with 3F-OKS or 2F-OK. Null expression from MEFs is shown as negative control, and expression in ESCs as positive control. c, Western blot analysis of the expression of pluripotency factors (Oct4, Sox2, and Nanog) in the same set of cells as in (b). d, Representative pictures of embryoid bodies (EBs) obtained after in vitro spontaneous differentiation of iPSCs generated from wt primary MEFs reprogrammed by 3F-OKS, or Rb-null with 3F-OKS or 2F-OK. e, Differentiation factor (Nkx2.5, Dlx3, and Gata4) mRNA expression by qRT-PCR in EBs obtained from iPSCs generated from Rb-null primary MEFs reprogrammed by 2F-OK. f, Representative pictures of H&E stained sections of EBs obtained from iPSCs generated from Rb-null primary MEFs reprogrammed by 2F-OK, and embedded in paraffin. Pictures show examples of ectodermal, endodermal and mesodermal differentiation.
the depletion was efficient, Rb-null pituitaries showed a nearly normal morphology with reduced number of Sox2 positive cells and low cell proliferation, as judged by Ki67 staining (Figure 4C).

Although further studies would be required, these results are in line with our previous observations for reduced pituitary size and tumor development in p27-null mice in the context of lower Sox2 expression using Sox2 heterozygous mice [7]. Interestingly, although Rb is ubiquitously expressed and its inactivation is an extremely frequent event in cancer, Rb deletion in mice is a tumor-initiating event only for some specific cell types. In this sense, it is tempting to speculate that Rb loss might result on pro-tumorigenic expression of Sox2 only for these cell types. Even more, according to our results concomitant inactivation of p53 might have synergistic effects on cancer by enhancing deregulated expression of genes such as Sox2 whose upregulation might be initiated by the loss of Rb.

Taken together, our results unveil an unprecedented mechanism of tumor initiation mediated by loss of the proper strict control exerted by the retinoblastoma family of pocket proteins on the repression of genes, such as Sox2, with crucial functions in regulating the properties of adult stem/progenitor cells.

**Figure 4:** *In vivo* effect of partial depletion of Sox2 positive cells in Rb+/− mice. a, Sox2 mRNA levels in the pituitary of wt (n=4) and Rb-het (n=4) mice. b, Schematic representation of the experimental protocol of gancyclovir (GCV) treatment of compound Rb-het/Sox2-TK mice (upper panel). Intraperitoneal injections were administered at the times indicated (weeks) and mice were sacrificed when they were 42 weeks old. Pituitary mass (n=4 for HBSS; n=3 for GCV) of Rb-het/Sox2-TK mice (lower panel). Means ± s.e.m. are shown. c, Representative pictures of paraffin sections from control HBSS-treated (upper panels) or GCV-treated (lower panels) tissues from Rb-het/Sox2-TK mice. The first three rows of pictures correspond to pituitary sections stained for H&E, proliferative marker Ki67 and Sox2, respectively. The two rows of pictures at the most right part correspond to control tissues, esophagus and trachea, stained for Sox2. Below is shown the quantification of Ki67 and Sox2 positive cells.
MATERIALS AND METHODS

Mice and cells

*Rb*+/− [21], *p107*+/− [23], *p130*+/− [24], Sox2-TK [22], and i4F mice [17] have been previously described. All genotypes were maintained on a C57BL/6 and 129SvJ hybrid background and all comparisons were made among mice derived from the same sets of crosses, and they therefore shared the same genetic background. Animals were kept under SPF conditions and all experiments were carried out under approval of the Santiago de Compostela University Bioethics (protocol number 15005AE/07/01/02/05C/AVF2) in compliance with Principles of Laboratory Animal Care of national laws.

Primary mouse embryo fibroblasts (MEFs, passage 1-2) were obtained from embryos of the indicated genotypes, as described previously [25]. Immortalization was achieved by expression of an shRNA targeting mouse *p53* [26]. Both primary and immortalized fibroblasts were cultured in standard DMEM medium (Sigma) supplemented with 10% FBS (Sigma).

ES cells and iPS cells were cultured on top of feeder layers in DMEM (Sigma) supplemented with serum replacement (KSR, 15%, LifeTechnologies), LIF 1000 u/ml (Millipore), 1x Non-Essential Amino Acids (LifeTechnologies), 1% L-glutamine (Sigma), 1% Streptomycin/Penicillin (Sigma), and 0.1 mM 2-mercaptoethanol (LifeTechnologies).

Protein and RNA expression analysis

For protein expression analysis, cell extracts were prepared using RIPA buffer (150mM NaCl, 10mM Tris-HCl pH 7.5, 0.1% SDS, 1% Triton X100, 5 mM EDTA) for 15 min at room temperature. Crosslinking was stopped by the addition of glycine to a final concentration of 0.125 M. Fixed cells were sonicated in lysis buffer (0.5% NP-40, 1% Triton X-100, 5 mM EDTA, 50 mM Tris-HCl pH 7.5, 150 mM NaCl plus protease inhibitors). For immunoprecipitation, 150 μg of chromatin were diluted in dilution buffer (1% Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA and 10 mM Tris-Hcl, pH 8.0, containing protease inhibitors) and pre-cleared with blocked protein G- sepharose (GE Healthcare). An aliquot of 50 μg of chromatin was reserved as input. The antibodies used for the immunoprecipitation were pRb1 (Santa Cruz, C-15), p107 (Santa Cruz, C-18), p130 (Santa Cruz, C-20), and normal rabbit IgG (Santa Cruz, sc-2027). Immune complexes were precipitated with protein G-sepharose and washed sequentially with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate-Na, 1 mM EDTA, 10 mM Tris-Hcl pH 8.1) and TE buffer. Immunoprecipitated DNA was eluted in elution buffer (1% SDS, 0.1 M NaHCO3) and extracted using the Wizard SV Gel and PCR System (Promega). PCR was carried out using 5 μL of each sample except for the input that was previously diluted.

The primers used for PCR were:

**Sox2-SRR2-F:** 5’-ATTATTCCAGTTCCCCAGTCCAAGC-3’

**Sox2-SRR2-R:** 5’-CCCTCTCCCCCCCCCAGGC-3’

**β-actin-F:**

5’-CAGTTCGCCATGGATGACGATATC-3’

Chromatin immunoprecipitation

To identify the binding of the pocket proteins to the Sox2-SRR2 enhancer, cells were harvested at 90% confluence and fixed by adding 1/10 of fixation solution (0,1M NaCl, 1mM EDTA, 0.5mM EGTA, 50mM Hepes pH8.0 and 11% Formaldehdye) to the culture media for 15 min at room temperature. Crosslinking was performed by the addition of glycine to a final concentration of 0.125 M. Fixed cells were sonicated in lysis buffer (0.5% NP-40, 1% Triton X-100, 5 mM EDTA, 50 mM Tris-HCl pH 7.5, 150 mM NaCl plus protease inhibitors). For immunoprecipitation, 150 μg of chromatin were diluted in dilution buffer (1% Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA and 10 mM Tris-HCl, pH 8.0, containing protease inhibitors) and pre-cleared with blocked protein G- sepharose (GE Healthcare). An aliquot of 50 μg of chromatin was reserved as input. The antibodies used for the immunoprecipitation were pRb1 (Santa Cruz, C-15), p107 (Santa Cruz, C-18), p130 (Santa Cruz, C-20), and normal rabbit IgG (Santa Cruz, sc-2027). Immune complexes were precipitated with protein G-sepharose and washed sequentially with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate-Na, 1 mM EDTA, 10 mM Tris-Hcl pH 8.1) and TE buffer. Immunoprecipitated DNA was eluted in elution buffer (1% SDS, 0.1 M NaHCO3) and extracted using the Wizard SV Gel and PCR System (Promega). PCR was carried out using 5 μL of each sample except for the input that was previously diluted.

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**Sox2-SRR2-R:** 5’-CCCTCTCCCCCCCCCAGGC-3’

**β-actin-F:**

5’-CAGTTCGCCATGGATGACGATATC-3’

**GADPH.**
β-actin-R:
5'-CCGCAGACCGGCCTTGCACATG-3’

The analysis of histone marks was performed essentially as described [27]. Briefly, wt, RB-null, and p130-null MEFs were trypsinized and cross-linked in 1% formaldehyde (Sigma) for 10 min at room temperature (RT). Crosslinking was quenched with 0.125 M glycine for 5 min. Pelleted cells were lysed in 1 ml ChIP buffer and sonicated for 10 min in a Bioruptor (Diagenode). Soluble material was quantified by Bradford assays. 100 μg of chromatin were used to immunoprecipitate histone modifications. The antibodies H3K27me3 (Millipore, 07-449) and H3K4me3 (Diagenode, pAb003-050) were incubated overnight with the chromatin. Immunocomplexes were recovered with 30 μl of protein A or G-agarose bead slurry. Immunoprecipitated material was washed three times with low salt buffer and one time with high salt buffer DNA complexes were decrosslinked at 65°C for 3 h, and DNA was then eluted in 200 μl of water using the PCR purification kit (QIAGEN). Two microliters of DNA was used for each qPCR reaction using SYBR green (Fermentas).

The primers used for Sox2-SRR2 were the ones shown above plus an extra set of primers:

Sox2-SRR2-F:
5’-CGTGGTAATGAGCACAGTCG-3’

Sox2-SRR2-R:
5’-AGGCTGAGTCGGGTCAATTA-3’

The primers used for Nanog promoter were:

Nanog-promoter-F:
5’-CAACTTACTAAGGTAGCCCGAGTCTTAA-3’

Nanog-promoter-R:
5’-CCTCCAAAAGTGCAGGCTTT-3’

Cellular reprogramming to iPSCs

Reprogramming of primary (passage 1-2) MEFs was performed using plasmids pMXs-Oct4, pMXs-Klf4, and pMXs-Sox2 [28] essentially as described [7]. Briefly, retroviral supernatants were produced in HEK-293T cells (3x10^6 cells per 100-mm-diameter dish) transfected by the calcium chloride protocol with the eecotropic packaging plasmid pCL-Eco (5 μg) together with the reprogramming factors (5 μg) in combinations of 3 (Oct4, Klf4, and Sox2) or 2 (Oct4 and Klf4) factors. Retroviral supernatants (10 ml) were collected serially 48 h later and during 36 h, each time adding fresh medium to the cells (10 ml). The recipient MEFs had been seeded the previous day weighing before processing for immunohistochemistry.

To analyze histological differentiation, EBs were cultured over a cell culture dish containing PBS. After 3 days, droplets were collected and transferred to a Petri dish and further cultured in Embryoid Body medium for 15 days before harvested for qRT-PCR and histological analysis.

To analyze histological differentiation, EBs were included in melted agar and the resulting plug was fixed in buffered formalin at 4°C, embedded in paraffin wax, and sectioned at a thickness of 5 μm. Sections were stained with hematoxylin and eosin for pathological examination.

In vitro embryoid body differentiation

In vivo experiments

Starting at 6 weeks of age, compound Rb-het/Sox2-TK mice were intraperitoneally injected with gancyclovir (GCV) at 100 mg/Kg in HBSS (Cymeve, Roche Pharmaceuticals) or vehicle (HBSS) every 2 weeks during the first 20 weeks of life, and then switched to single injections every 4 weeks, to complete a total of 12 injections. Animals were sacrificed when they were 42 weeks old, tissues were removed, and pituitaries were weighted before processing for immunohistochemistry.

For immunohistochemical analysis, tissue was fixed in formalin at 4°C, embedded in paraffin wax, and sectioned at a thickness of 5 μm. Sections were stained with hematoxylin and eosin for pathological examination or processed for immunohistochemical analysis with antibodies against mouse Ki67 (Master Diagnostica, SP6), or Sox2 (CST #3728, C70B1).
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