**Universidade de Aveiro** Departamento de Biologia

**2017**

**Tânia Patrícia Dias Fernandes**

# Role of SOX2 on RasV12-mediated transformation

# O papel de SOX2 na transformação mediada por RasV12

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# Role of SOX2 on RasV12-mediated transformation

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor Anxo Figueroa Vidal, Professor Auxiliar do Departamento de Fisiologia da Universidade de Santiago de Compostela e da Professora Doutora Maria de Lourdes Pereira, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro.

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**Keyword** Cell culture, SOX2, RasV12, oncogenic transformation, chemoresistance

**Abstract** The SRY (sex-determining region Y) - box 2 (SOX2) is a master factor in the maintenance of pluripotency and stemness. The transcription factor SOX2 allows the cells to maintain the unique characteristics of the embryonic stem cells (ESCs), such as clonogenicity, pluripotency, self-renewal ability, and conservation of the anti-apoptotic properties of cancer stem cells (CSCs). This factor has an important role in carcinogenesis of several tumors, including gastric, breast, pancreatic, and lung cancers. *SOX2* overexpression can contribute to resistance of cancer cell to drug therapy and has been associated with tumor aggressiveness and worse prognosis. Ras GTPase is a proto-oncogene activated in several types of cancer with low success rate, including carcinomas of the pancreas, colon, lung, thyroid, and myeloid malignancies. This oncogene activates several signalling pathways, which includes the MAPK, PI3K, and RAL. It's signalling is involved in many cellular functions, such as cell proliferation, apoptosis, migration, fate specification, and differentiation. This project aims to investigate the role of SOX2 on RasV12-mediated transformation and the genetic requirements for SOX2 induction mediated by Ras using immortalized mouse fibroblasts and primary mouse embryonic fibroblasts. We also study the effect of SOX2 overexpression on drug therapy using human lung carcinoma and human breast adenocarcinoma cell lines. We have demonstrated that RasV12 overexpression induced the expression of SOX2 and this induction is at level of transcription. We also determined that p53, Rb, and $p19^{ARF}$ factors are not essential for SOX2 induction and that MAPK pathway is required, but not sufficient for SOX2 induction by RasV12. Through a transformation assay we demonstrate that SOX2 overexpression increases the effect of RasV12 in cell transformation and SOX2 silencing mediated by siRNA decreases the transformation capacities of RasV12, so this factor is important in transformation mediated by

RasV12. Several studies show that SOX2 not only influences tumor growth, but it also influences the response of tumor cells to therapeutic drugs. We observed that SOX2 overexpression increased the resistance of human breast adenocarcinoma cells to docetaxel. In conclusion, SOX2 is a key factor in cell transformation mediated by RasV12, as well as has an important role in chemoresistance of cancer cells. Looking at these results, this factor can be a novel target for anti-cancer therapy.

**Palavras-chave** Cultura de células, SOX2, RasV12, transformação oncogénica, quimiorresistência

**Resumo** O fator de transcrição SOX2 (Sex-determining region Y (SRY)-Box2) é um fator importante na manutenção da pluripotência e "stemness". Este fator permite que as células preservem as características únicas das células embrionárias estaminais (ESCs), como a clonogenicidade, pluripotência, capacidade de autorrenovação e a conservação das propriedades anti-apoptóticas de células estaminais cancerígenas (CSCs). O SOX2 tem um papel importante na carcinogénese de vários tumores, como no cancro gástrico, da mama, pancreático e do pulmão. A sobre-expressão de SOX2 pode contribuir para a resistência das células cancerígenas à terapia farmacológica e tem sido associada à agressividade tumoral e mau prognóstico. O proto-oncogene Ras GTPase está ativo em vários cancros com baixa taxa de sucesso, como os carcinomas do pâncreas, colón, pulmão, tiroide e mielomas malignos. Este oncogene ativa múltiplas vias de sinalização, incluindo a MAPK, PI3K e RAL e estas vias estão envolvidas em funções celulares como proliferação celular, apoptose, migração e diferenciação. Este projeto tem como objetivo investigar o papel de SOX2 na transformação mediada por RasV12 e os fatores genéticos importantes na indução de SOX2 usando fibroblastos imortalizados de rato e fibroblastos embrionários primários de rato e o efeito da sobre-expressão de SOX2 na terapia farmacológica usando células do carcinoma do pulmão humano e células do adenocarcinoma da mama humano. Foi possível demostrar que a sobre-expressão de RasV12 induz a expressão de SOX2 ao nível da transcrição. Os fatores p53, Rb e $p19^{ARF}$ não são essenciais na indução de SOX2 por RasV12, no entanto a via de sinalização MAPK é necessária neste processo. Através de ensaios de transformação foi possível demonstrar que a sobre-expressão de SOX2 incrementa o efeito de RasV12 na transformação celular e que o silenciamento de SOX2 usando siRNA diminui a capacidade transformante de RasV12, desta forma este fator é importante para a transformação celular mediada por RasV12.

Vários estudos demonstram que o fator de transcrição SOX2 não só influencia o desenvolvimento tumoral, mas também a resposta das células tumorais à terapia farmacológica. Foi possível verificar que a sobre-expressão de SOX2 aumenta a resistência de células do adenocarcinoma da mama humano ao agente farmacológico docetaxel. Em conclusão, SOX2 é um fator essencial na transformação celular mediada por RasV12, assim como tem um papel importante na resistência das células cancerígenas à terapia. Olhando para estes resultados, este fator pode ser o novo alvo terapêutico na luta contra o cancro.

# List of abbreviatures

| | |
|---|---|
| **AKT** | Protein kinase B |
| **cDNA** | Complementary deoxyribonucleic acid |
| **CSCs** | Cancer stem cells |
| **DMSO** | Dimethylsulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxynucleotide triphosphate |
| **DTT** | Dithiothreitol |
| **EGFR** | Epidermal growth factor receptor |
| **ERK** | Extracellular signal-regulated kinase |
| **ESCs** | Embryonic stem cells |
| **FBS** | Fetal bovine serum |
| **FGFR** | Fibroblast growth factor receptor |
| **GAPs** | GTPase-activating proteins |
| **GDP** | Guanosine diphosphate |
| **GEFs** | Guanine nucleotide exchange factor |
| **GTP** | Guanosine triphosphate |
| **GUSB** | Beta-glucuronidase |
| **HeBS** | HEPES-buffered saline |
| **HMG** | Sex-determining region Y (SRY) – box (SOX) 2 |
| **H-Ras** | Harvey rat sarcoma viral oncogene homolog |
| **HRP** | Horse-raddish peroxidase |
| **Hygro** | Hygromycin resistance |
| **iPSCs** | Induced pluripotent stem cells |
| **KLF4** | Krüppel-like factor 4 |
| **K-Ras** | Kirsten rat sarcoma viral oncogene homolog |
| **LB medium** | Lysogeny broth medium |
| **MAPK** | Mitogen-activated protein kinases |
| **MEFs** | Mouse embryonic fibroblasts |
| **MEK** | Mitogen-activated protein kinase |
| **miRNA** | Small noncoding ribonucleic acid |
| **M-MLV RT** | Moloney murine leukemia virus reverse transcriptase |
| **mRNA** | Messenger ribonucleic acid |

| | |
|---|---|
| **mTOR** | Mechanistic target of rapamycin |
| **MTT** | Thiazolyl blue tetrazolium bromide |
| **Notch** | Neurogenic locus notch homolog protein |
| **N-Ras** | Neuroblastoma rat sarcoma viral oncogene homolog |
| **OCT4** | Octamer-binding transcription |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PI3K** | Phosphatidylinositol 3-kinase |
| **PMSF** | Phenylmethylsulfonyl fluoride |
| **PSCs** | Pluripotent stem cells |
| **Puro** | Puromycin resistance |
| **Ral** | Ras-related protein |
| **Ras** | Rat sarcoma |
| **Rb** | Retinoblastoma protein |
| **RNA** | Ribonucleic acid |
| **RNase Out** | Recombinant ribonuclease |
| **RT qPCR** | Reverse transcription quantitative polymerase chain reaction |
| **SDS** | Sodium dodecyl sulfate |
| **shRNA** | Short hairpin ribonucleic acid |
| **siRNA** | Small interfered ribonucleic acid |
| **SOC medium** | Super optimal broth medium |
| **SOX** | SRY-related HMG box |
| **SOX2** | High mobility group |
| **Sry** | Mammalian testis-determining factor |
| **TEMED** | Tetramethylethylenediamine |
| **TFRC** | Transferrin receptor |
| **TGF-β** | Transforming growth factor |
| **tRNA** | Transfer ribonucleic acid |
| **Wnt** | Wingless-related integration sit |
| **WT** | Wild-type |

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# Introduction

## 1. Cancer

Cancer is known as a disease responsible for uncontrolled growth and spread of abnormal cells. In 2013 1 660 290 new cancer cases were diagnosed, in which about 174 100 cancer deaths were caused by tobacco use. Currently, several forms of cancer can be treated and in 2013 approximated 13.7 million people with cancer history were alive (Asati *et al.,* 2016b).

Carcinogenesis is an event that transforms differentiated tissues into an immature cell population. The progression of this neoplasia in cancer cells lead to accumulation of genetic and epigenetic modifications, by multiple DNA mutations and clonal selection (Atoraih *et al.*, 2016). Therefore, cancer is a disease associated with genomic instability that results, frequently, from oncogene activation (Kotsantis *et al.*, 2016). These alterations make cancer cells return to immature undifferentiated phenotype and acquire novel molecular signatures. Tumor cells share several biological characteristics with normal stem/progenitor cells (Chou *et al.*, 2013), for this the activation of transcription factors of stem cells is an important event in the development of cancer (Atoraih *et al.*, 2016), and the malignant process of the cell involves abnormal cell growth, proliferation, and differentiation (Asati *et al.*, 2016a).

Hanahan and Weinberg suggest that almost all types of human cancers acquired ten capabilities during their development. These hallmarks of cancer are: sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death and deregulation cellular energetic. The development of this multiple hallmarks depends in large part on a succession of changes in the genomes of neoplastic cells (Hanahan and Weinberg, 2011).

The acquisition of gene mutations on driver oncogenes and/or tumor suppressors is one of the main causes of tumor initiation and maintenance, and the cancer cell survival depends on a few oncogenes that sustain the malignancy (Jung *et al.*, 2016).

Replication stress that occur when DNA replication fork progression in S phase slows or stalls is a mechanism proposed to cause genomic instability in cancer. This cause the collapse of forks into DNA double-strand breaks (DSBs) and incomplete sister chromatid separation in the following mitosis. Marks of spontaneous replication stress are found in cancer samples and cells expressing active oncogenes, and replication stress promotes chromosomal instability (Kotsantis *et al.*, 2016).

The overexpression of oncogenes, such as Ras or Myc is sufficient to induce replication stress in culture cells and these oncogenes promoting cell growth and division by acting in the growth factor signalling pathways that stimulate proliferation (Kotsantis *et al.*, 2016).

Several evidences suggest that the genetic and epigenetic alterations of protein-coding genes cannot constitute the entire molecular basis fundamental for the pathogenesis of tumor development. The majority of non-coding RNA (ncRNA) that influence tumorigenesis act to regulate gene expression through a diverse range of molecular mechanisms, and the retrotransposons seems to have a potential role in promoting tumorigenesis (Xue and He, 2014).

The long non-coding RNAs (lncRNAs) regulate individual genes and gene expression through epigenetic regulation or by altering the basal transcriptional machinery. They have a role in, for example, chromosome dosage-compensation, imprinting, epigenetic regulation, cell cycle control, nuclear and cytoplasmic trafficking, transcription, translation, splicing, and cell differentiation. For that, their abnormal expression is associated with numerous diseases including cancer (Jiang *et al.*, 2016).

In cancer cells, several lncRNAs present alterations in the expression levels when compared with healthy tissue of the same origin. Its deregulated are involved in proliferation, apoptosis, and cell-cycle control in the tumorigenesis, and also contribute to the specific process of metastasis (Jiang *et al.*, 2016).

Many microRNA (miRNA) have been identified to promote or suppress oncogenesis in mouse tumor models, cell culture systems, and clinical studies by regulating essential cellular processes during the tumorigenesis. Specific miRNA and components of the miRNA biogenesis machinery have genetic and epigenetic alterations in a diversity of human cancers (Xue and He, 2014).

The initiation and progression of cancers carrying the oncogenic and tumor suppressor mutations that define cancer as a genetic disease (Hanahan and Weinberg, 2011), but the molecular mechanisms that regulate tumor initiation and stemness are still poorly characterized (Boumahdi *et al.*, 2014). One the other hand, genetic profiling of human malignancies have led to a better understanding of cancer biology and a refined molecular classification of cancer types (Schröck *et al.*, 2014).

The regulation of the cell proliferation and apoptotic pathways associated with cell death is an important approach to understand about cancer cells. For that, the identification of cell-cycle regulates and apoptotic stimuli to combat cancer cells is an attractive approach for the discovery and development of potential anti-cancer agents (Asati *et al.,* 2016b).

## 2. The SOX family of transcription factors

The *SOX* (SRY-related HMG-box) gene family was first defined by the discovered of the *Sry* gene, sex-determining region of the Y-chromosome, in humans and mice in the 90s associated with primary male sex determination and, subsequently, testis development. This gene family encodes transcriptional factors with a highly conserved HMG (high-mobility-group) box, which is a 79 amino-acid DNA-binding domain (Zhang and Cui, 2014; She and Yang, 2015). Through the HMG domain, SOX proteins can bind to ATTGTT or related sequence motifs (Kamachi and Kondoh, 2013).

In vertebrates, SOX family is composed of more than 20 *SOX* genes which originated through a process of duplication and divergence (She and Yang, 2015). This family is characterized by three domains: N-terminal, HMG allowing the recognition, and a specific binding to DNA and C-terminal (Marqués-Torrejón *et al.*, 2013; Weina and Utikal, 2014). SOX family are expressed in an extensive variety of tissues and have important role in tissue homeostasis, regulation of organ development (organogenesis), and cell-type specification in many developmental processes from embryonic through to adult stages (Karachaliou *et al.*, 2013; Li *et al.*, 2015; She and Yang, 2015).

The family of SOX transcription factors are divided in eight groups (A-H) based upon HMG box sequence, protein structures, gene organization, and their function (Thu *et al.*, 2014; She and Yang, 2015). Members within a subfamily retain 80% identity in their HMG-domain and other conserved regions and might have overlapping expression patterns, share biochemical properties, and perform synergistic or redundant function. By contrast, members from different subgroups usually develop different functions (Garros-Regulez *et al.*, 2016).

The SRY protein belongs to group A and play an essential role in sex determination. The SOXB group is split into two subgroups, the SOXB1 group proteins composed by SOX1, SOX2, and SOX3 factors that share more than 80% sequence similarity and have an important function for embryonic development and in the central nervous system as transcriptional activators (Marqués-Torrejón *et al.*, 2013; Zhang and Cui, 2014; She and Yang, 2015), and the subgroup SOXB2 (SOX14 and SOX21) act as transcription inhibitors. The proteins SOX4, SOX11, and SOX12 belongs to SOXC group, SOXD group is composed by SOX5, SOX6, and SOX13 proteins, and SOX8, SOX9, and SOX10 protein belongs to SOXE group. Lastly, SOXF group is composed by SOX7, SOX17, and SOX18 proteins, SOX15 and SOX30 proteins belongs to SOXG and SOXH groups, respectively (Kamachi and Kondoh, 2013; She and Yang, 2015).

SOX proteins exhibit their gene regulatory functions only by the formation of complexes with partner transcription factors. A functional SOX-binding site has a binding site for a second

partner protein, which required for SOX-dependent transcriptional regulation. The binding of a single SOX protein alone to a DNA site does not lead to transcriptional activation or repression. In the absence of DNA, SOX proteins are capable of interact with the partner factors, this show that the SOX-partner complexes can form first and then recognize target DNA sites as a complex (Kamachi and Kondoh, 2013).

This protein family do not show their activating or repressive regulatory functions when they bind to DNA on their own, but instead bind specific DNA sequences in combination with partner proteins to exert their effects (Kamachi and Kondoh, 2013).

During the development, the transcriptional activities of SOX proteins are regulated for multiple genetic pathways. The three major aspects of such regulation are: the regulation of the expression levels of SOX proteins in specific cell types and tissues and at precise timings within a series of major developmental stages, regulation of post-translational modifications of SOX proteins, and regulation of recruited partner proteins. In the last case this regulation influence the specification recognition of the binding sites of SOX-partner complexes on the target genes and determine transcription activities and significantly enhance the activation/repression potential (She and Yang, 2015).

## 2.1 SOX2

*SOX2* gene*,* a member of the SRY gene family (Acanda de la Rocha *et al.*, 2016) is in chromosome 3 (3q26.3-q27) of Human genome and encodes for SOX2 protein (Sex-determining region Y (SRY)-box (SOX)2) with 317 amino acids (Weina and Utikal, 2014). The SOX2 protein belongs to SOX transcription factors (Herreros-Villanueva *et al.*, 2013), more precisely to SOXB1 group (Zhang and Cui, 2014) and containing three domains: N-terminal, high mobility group (HMG) DNA-binding domain, and C-terminal domain (Figure 1) (Schröck *et al.*, 2014; Weina and Utikal, 2014).

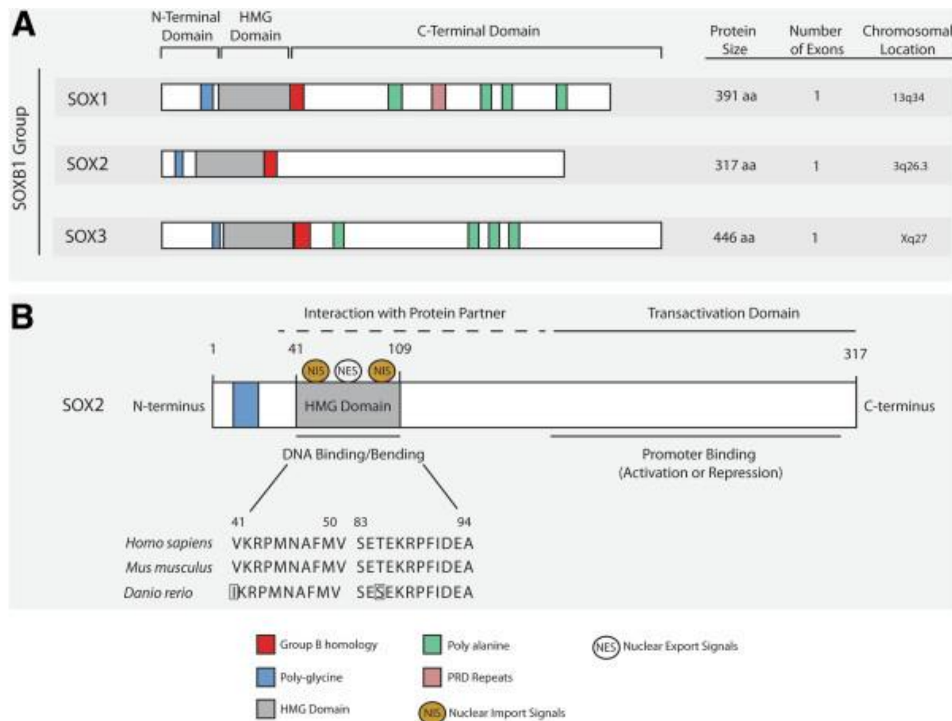


**Figure 1. Homology, structure, and product of *SOX2* gene. (A)** SOX2 belongs to the SOXB1 group of SOX protein. Elements of this group contains three domains: N-terminal, HMG, and C-terminal. **(B)** The HMG domain is highly conserved and serves as potential binding sites for protein partners. Binding of the nuclear import signals (NIS) and nuclear export signals (NES) to the HMG domain leads to regulation of *SOX2* itself. The transactivation domain as the region responsible for promoter binding, which leads to activation or repression of target genes (Weina and Utikal, 2014).

The expression of SOX2 is regulated by intrinsic factors and extrinsic signalling pathways. In the *SOX2* locus there are several regulatory regions (SRR) that are responsible for controlling the expression of this gene, which include SOX2 core promoter and a number of enhancers located upstream and downstream of the gene (Figure 2). These regulatory regions are very conserved across species (Zhang and Cui, 2014).

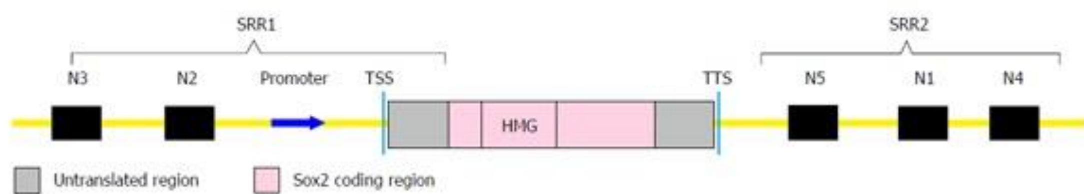


**Figure 2. Transcriptional expression of SOX2 is regulated by multiple enhancers in the locus.** *SOX2* gene locus is represent with SOX2 exon, the location of the N1 to N5 enhancers, the transcription starting (TSS) and termination site (TTS), and regulatory regions 1 (SRR1) and 2 (SRR2) (Zhang and Cui, 2014).

SOX2 protein is found in the cell nucleus and forms a complex with an Oct4 (Octamer-binding transcription factor 4) protein in DNA, regulating the gene expression involved in cell differentiation during embryonic development (Herreros-Villanueva *et al.*, 2013; Weina and Utikal, 2014; Rizzino and Wuebben, 2016). SOX2-Oct4 regulatory complex upregulates a large number of genes (Karachaliou *et al.,* 2013), involved in maintenance of stem cells in adult tissues, sex determination, and development of central nervous system and hematopoietic cells (Thu *et al.*, 2014; Wang *et al.*, 2015).

SOX2 is one of the four pluripotency factors which include Oct4, Myc, and Klf4 (Krüppel-like-factor 4), also called Yamanaka factors. The combination of these factors is sufficient to reprogram differentiated cells in induced pluripotent stem cells (iPSCs), embryonic-like state (Figure 3). SOX2 factor is essential in the last phase of reprogramming process, this is responsible for its own transcription as well as others of pluripotency-associated genes to stabilize the pluripotent state and finally to generate an iPSC. The endogenous *SOX2* locus activation during reprogramming process leads to several transcriptional changes in cells intended to form iPSC (Chanoumidou *et al.*, 2017). The iPS cells exhibit morphological and growth properties of embryonic stem cells (ESCs) and express gene markers of embryonic stem cells (Takahashi and Yamanaka, 2006). The two properties more important of the pluripotent stem cells (PSCs) are indefinite self-renewal in culture and the ability to generate most, if not all, cell types in the body by differentiation into one of the three embryonic germ layers (Zhang and Cui, 2014).

The transcription factor SOX2 allows the cells, through a complex transcriptional regulation network, to maintain the unique characteristics of ESC, such as clonogenicity, pluripotency, self-renewal ability, and conservation of the anti-apoptotic properties of cancer stem cells (CSCs) (Chen *et al.*, 2012; Karachaliou *et al*., 2013). These characteristics enable the initiation of a tumor to maintain its growths and recurrence (Chen *et al.*, 2012), this suggested an oncogenic role of SOX2 (Lundberg *et al.*, 2014).

The physical interaction between Oct4 and SOX2 allow the formation of a heterodimer complex that binds to the *Nanog* promoter along with Klf4 to activate the *Nanog* gene expression, but SOX2 can bind to the *Nanog* gene even without forming the complex with Oct4. The transcription factor SOX2 co-occupies several sites in the genome with Oct4 and Nanog, which acts as master regulates of pluripotency (Zhang and Cui, 2014; Lee *et al.*, 2017). Blocking the expression of SOX2 and Oct4 in pluripotent stem cells and during the initial stages of embryogenesis has terrible developmental consequences (Rizzino and Wuebben, 2016).

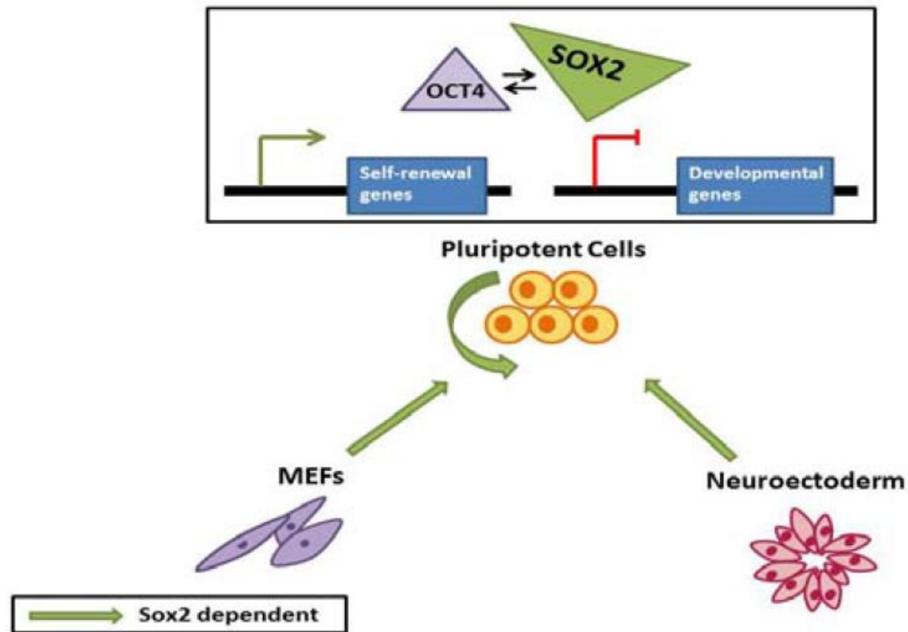


**Figure 3. SOX2 functions in pluripotent stem cells (PSCs).** SOX2 factor participates in the reprogramming of somatic cells to induced PSC (iPSC) and promotes their self-renewal through transcriptional regulation of target genes in conjunction with Oct4 transcription factor (Chanoumidou *et al.*, 2017).

Interaction of SOX2 with several cofactors confers upon this factor greater functional versatility during developmental processes (Zhang and Cui, 2014). Therefore, SOX2 participates in multiple biological process: response to growth factor, tissue regeneration, cell cycle arrest leading to apoptotic process, negative regulation of epithelial cell proliferation, positive regulation of canonical Wnt pathway, regulation of gene expression by regulation of transcription and chromatin organization, positive regulation of cell differentiation, such as somatic stem cell and neuronal stem cell maintenance, glial cell fate commitment, osteoblast differentiation, endodermal cell fate specification and adenohypophysis, pituitary gland, forebrain, and inner ear and eye development (Atoraih *et al.*, 2016).

SOX2 is expressed in adult cells of numerous tissues, such as progenitors of the brain and retina, lens, trachea, tongue epithelium, dermal papilla of the hair follicle, stomach, cervix, anus, testis, and progenitors of pituitary gland. The expression of SOX2 in several adult endodermal and ectodermal stem cell compartments show that this factor is critical for normal tissue regeneration and survival, and the ablation of SOX2$^{+}$ cells in mice results in a disruption of epithelial tissue homeostasis and lethality (Arnold *et al.*, 2011).

Several genes are regulated by SOX2, such as *c-Myc* that promotes cell proliferation, regulates cell growth, apoptosis and self-renewal of stem cells, *Wnt1* involved in oncogenesis

and development processes, *Wnt2* that is essential in carcinogenesis of the esophagus and increases metastatic propensity in pancreatic cancer, *Klf4* involved in stem-like capacity, *EGFR* that modulate DNA synthesis, cell proliferation, cell migration and adhesion, *Oct4* that regulates pluripotency and cell differentiation, and *TGF-β* that is important to control tumor progression and metastasis (Liu *et al.*, 2013).

SOX2 was relevant to several signal pathways directly through its targets or indirectly through its interaction with proteins. The FGFR, IL-4/STAT6, and EGFR/Scr/AKT signal pathways regulated SOX2 expression directly and EGFR/PI3K/AKT pathway also regulated SOX2 expression, but the upstream molecule of SOX2 was TGF-α (Liu *et al.*, 2013). The FGFR pathway regulates SOX2 expression through two signalling cascades, MEK/ERK pathway that regulates SOX2 expression through the final phosphorylation of ERK, which translocated into the nucleus and activates the transcription of SOX2, and PI3K/AKT/mTOR cascade regulates positively the expression of this factor through the activation of the mammalian target of rapamycin complex 1 (mTORC1). The inhibition of mTORC1 in glioma stem cells lead to an inhibition of the SOX2 expression and in self-renewal activity (Garros-Regulez *et al.*, 2016).

SOX2 protein regulates cell growth and tumor invasion through several signalling pathways, such as MAP4K4 (mitogen-activated protein 4 kinases 4), Wnt (Wingless-related integration site)/β-catenin, PI3K (phosphatidylinositol 3-kinase)/AKT (protein kinase B), ERα (estrogen receptor alpha), BMP (bone morphogenetic protein), Notch, JAK/STAT3, and EGFR signalling pathways, forming a complex regulatory network, suggest that SOX2 plays an important role in cell and tumor biology (Atoraih *et al.*, 2016; Chanoumidou *et al.*, 2017). Others signal pathways regulated by SOX2 through its targets or interacting partners are VEGF, p53, ErbB, and cell surface receptor linked signal transduction (Liu *et al.*, 2013).

Vilas and colleagues observed an increase of SOX2 expression in mouse embryonic fibroblasts (MEFs) immortalized by targeting p53 with shRNA (shp53), especially when MEFs deficient in Rb or p130 proteins. In i4F-MEFs (MEFs expressing the four reprogramming factors, Oct4, SOX2, Klf4 and c-Myc) SOX2 levels increased upon knockdown of p53, suggesting that decreasing p53 levels amplifies the already deregulated *SOX2* expression. The increased levels of SOX2 were produced from the endogenous *SOX2* locus, but not from the transgene locus. When p53 is downregulated the low-level *SOX2* expression would be sufficient to activate its own endogenous transcription. This point to a novel function of p53 repressing SOX2 in differentiated cells (Vilas *et al.*, 2015).

Overexpression of SOX2 in WT MEFs cells, but not p53-deficient MEFs lead to a cell growth arrest associated with an increase in the levels of $p16^{Ink4a}$ (p16) and $p19^{Arf}$ (encoded by

alternative reading frames of the *Ink4a/Arf* locus, also known as *Cdkn2a* (Marqués-Torrejón *et al.*, 2013).

In A549 cells the silence of SOX2 by RNA interference could reduce the tumorigenic property of these cells with decreased expression of c-Myc, Wnt1, Wnt2, and Notch (Liu *et al.*, 2013).

SOX2 has an important role *in vivo* and *in vitro* in the survival and proliferation of cancer cells and is a potential candidate biomarker for targets intervention in several cancer types (Ren *et al.*, 2016).

### 2.1.1 SOX2 is a key factor in stemness and embryonic development

SOX2 is a master factor in the maintenance of pluripotency and stemness. This factor is required early in embryonic development to maintain pluripotency and self-renewal in embryonic stem cells (ESCs) and regulates the formation of several epithelia during fetal development (Arnold *et al.*, 2011; Seo *et al.*, 2011). SOX2 expression is initially detected in cells at the morula stage, becoming more specifically expressed in the inner cell mass (ICM) and trophectoderm of the blastocyst during the latter stages (Zhang and Cui, 2014; Zhou *et al.*, 2014; Rizzino and Wuebben, 2016). During the embryonic development the expression of this factor is extinguished in most tissues, but it remains strongly expressed in stem cells of the central nervous system (CNS), retina, and the primordial gut and have an essential role in the maintenance of undifferentiated lineage progenitors such as neural stem cells (Seo *et al.*, 2011; Rizzino and Wuebben, 2016).

SOX2-null embryos die after implantation due to a failure to form a pluripotent epiblast, and blastocyst outgrowths from only trophectoderm-like cells (Zhou *et al.*, 2014). Depletion of SOX2 by gene-knockout or RNA interference compromises the pluripotent stage of mouse and human ESCs as shown by changes in cell morphology, loss of pluripotent marker expression, and differentiation primarily into trophectoderm. In SOX2-null mouse ESCs the overexpression of Oct4 can rescue the pluripotency of these cells, suggesting that SOX2 is important in the maintenance of pluripotent ESCs through promoting and maintaining Oct4 expression (Zhang and Cui, 2014). SOX2 overexpression in embryonic stem cells (ESCs) induce differentiation towards the neuroectodermal lineage, and the expression of this gene is maintained in the developing neuroectodermal (Zhou *et al.*, 2014), but the deletion of this factor in ESCs results in inappropriate differentiation into trophectoderm-like cells (Arnold *et al.*, 2011).

Levels of SOX2 expression need to be tightly regulated to maintain pluripotency of stem cells because increases or decreases in SOX2 expression lead to the loss of pluripotency. Both low and higher levels of SOX2 reduce the promoter/enhancer activity of SOX2-Oct4 target genes. The core transcriptional regulatory formed by SOX2, Oct4, and Nanog is important in the differentiation of ESCs to specific lineages and to maintain the self-renewal of pluripotent stem cells in mouse and human ESCs (Zhang and Cui, 2014; Chanoumidou *et al.,* 2017).

Heterozygous SOX2 mice are phenotypically normal, although the pituitary size, hormone production, and testicular size are reduced. This suggests that SOX2 seems is a central regulator for early Pluripotent Stem Cells (PSCs) formation and embryonic development. Mice deficient SOX2 expression present eye defects, hearing loss, and abnormal development of the hypothalamo-pituitary system (Chanoumidou *et al.*, 2017).

SOX2 is expressed at high levels in ESCs and its expression is downregulated upon differentiation to endoderm or mesoderm while is maintained in the neuroectodermal lineage. This shows that the transcriptional regulation of SOX2 is complex (Zhou *et al.*, 2014). SOX2 is involved in the differentiation and morphogenesis of esophagus, trachea, and lung, whereas its reduction leads to the abnormal development of lung, esophageal atresia, and tracheal-esophageal fistula defects (Chanoumidou *et al.*, 2017).

The conserved expression profile of SOX2 during development and adulthood suggests its involvement at the establishment and proper function of the Central Nervous System (CNS) (Chanoumidou *et al.*, 2017). In neural progenitors cells (NPCs), SOX2 is considered a transcriptional activator as well as a repressor inhibiting the expression of target genes directly or indirectly, such as genes associated with mitosis. In these cells, this factor is upregulated and its expression decreases after differentiation to post-mitotic neuronal and glia cells, thus SOX2 is important to maintain the self-renewal of NPCs *in vitro* and *in vivo*. SOX2 is also essential in the preservation of NSC properties, such as proliferation, self-renewal, and neurogenesis (Zhang and Cui, 2014; Chanoumidou *et al.*, 2017). SOX2 factor is able to reprogram alone somatic cells to induced neural stem cells (NSC) further demonstrates the essential function of SOX2 in these cells (Chanoumidou *et al.*, 2017). It has been reported that the cell cycle regulators E2f3a and E2f3b regulated SOX2 expression and control the proliferation in NPCs, thus affecting adult neurogenesis (Zhang and Cui, 2014). In neural stem cells (NSCs), p21 inhibits the expression of *SOX2* gene by directly binding to the SOX2 regulatory region 2 (SRR2) enhancer downstream this gene. This suggests that the modulation of SOX2 levels by p21 could be a regulatory mechanism to control the proliferation of NSC populations in the adult brain. In p21-null cells increased levels of DNA damage are associated with SOX2 overexpression. To maintain a proper regulation

of cell-cycle transitions in NSCs is essential the repression of SOX2 gene by p21 (Marqués-Torrejón *et al.*, 2013).

SOX2 is also implicated in mesenchymal stem cells (MSC) function, such as positively regulation of cell cycle progression by facilitating G1/S transition through transcriptional activation of cyclinD1 and c-Myc (Chanoumidou *et al.*, 2017).

During the development, SOX2 protein may recruit different partner proteins to realize diverse regulatory functions in specific biologic contexts and targeting several genes involved in development. The complex SOX2-Oct4, in human and mouse, is responsible for targeting genes involved in maintenance of pluripotency in embryonic stem cells such as *SOX2*, *Oct4*, *Nanog*, *Fgf4*, and *Uff1* and is expressed in inner cell mass, embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, pre-implantation embryo, and hindbrain. The function loss of this complex leads to defects in implantation (Zhang and Cui, 2014; She and Yang, 2015).

Binding of SOX2 to Pax6 (paired-box protein 6) is important for initiate lens development by targeting *SOX2*, *Pax6,* and *δ-crystallin* genes. This complex is expressed in retina, optic cup, and lens and function loss of this interaction causes disruptions in eye development. In neural primordial cells, the complexes SOX2-Pou3f2 and SOX2-Brn2 regulates genes, such as *SOX2*, *Nestin*, and *Shh,* and the cooperation between SOX2 and Chd7 are involved in the regulation of neural stem cells. Many other partners are involved in olfactory placode developments and in hair cell formation (Table 1) (Zhang and Cui, 2014; She and Yang, 2015).

SOX2 is very important in both the establishment and maintenance of pluripotent stem cells and its signalling is critical during fetal development for the formation of several endodermal and ectodermal tissues, such as nervous system, lens epithelium, retina, sensory cells of the taste bud, inner ear, and anterior foregut endoderm. Thus, the expression of SOX2 plays important roles at multiple stages of prenatal and postnatal development (Arnold *et al.*, 2011), this suggest that SOX2 seems to be critical for tissue regeneration and survival later in life (Rizzino and Wuebben, 2016).

SOX2 is the only factor that is commonly expressed between ESCs, fetal progenitors, and adult stem cells, and may therefore point toward molecular similarities in the regulation of pluripotent and different adult stem cells (Arnold *et al.*, 2011). SOX2 is a master regulator of stemness and its function is indispensable for the establishment of pluripotent ESC *in vivo* and the generation of iPSC *in vitro* (Chanoumidou *et al.*, 2017).

Several enhancers/regulatory regions of *SOX2* work together to regulate the expression of this gene from early preimplantation embryos to various neural progenitor cells. SOX2 is a key factor in the formation of pluripotent cells in early embryos and finally an essential factor for

embryonic development because this factor regulates distinct target genes involves in these processes (Zhang and Cui, 2014).

The distinct functions of SOX2 depend on many factors, such as expression levels, extracellular signalling, antagonism with tissue specific transcription factors, and partners selection in specific cells and development stages (Chanoumidou *et al.*, 2017). This factor can participate in transcriptional activation or repression depending on the co-factor recruited for the SOX2-partner factor complex. SOX2 knockdown will affect occupancy by the second factor leads to inhibition of development progression if this factor do indeed function as pioneers (Kamachi and Kondoh, 2013).

**Table 1. Example of SOX2 factor partners in the development with target genes and functions** (Kamachi and Kondoh, 2013; Zhang and Cui, 2014; She and Yang, 2015).

| Partner proteins | Target genes | Cell/tissues types | Functions | Loss-of-function phenotype |
|---|---|---|---|---|
| **Oct4** | *SOX2, Oct4, Nanog, Fgf4, Utf1, Fbxo15, Lefty1, Nmyc1, Rex1, Tcf3, DppA4, Trp53, Rest, Zfp42* | Inner cell mass; Embryonic stem (ES) cells; Induced pluripotent stem (iPS) cells; Preimplantation embryo; Hindbrain | Maintain pluripotency | Defects in implantation |
| **PAX6** | *SOX2, Pax6, δ-crystallin, N-cadherin* | Retina; Optic cup; Lens | Initiate lens development | Disruptions in eye development |
| **Pou3f2** | *SOX2, Nestin, Shh* | Neural progenitor; Neural tube | Regulation of neural primordial cells | Septo-optic dysplasia |
| **Eya1, Six1** | *Sostdc1, Atoh1* | Hair cell progenitors; Inner ear; Neurogenic placode | Hair cell fate determination | Defects in hair cell formation; Defects in neuronal progenitor proliferation |

| **Chd7** | *Jag1, Gli3, Mycn* | Neural stem cells | Regulation of neural stem cells | Anophtalmia esophageal-genial syndrome |
|---|---|---|---|---|

### 2.1.2 SOX2 is regulated at translational and post-translational level

SOX2 expression and activity are regulated at translational and post-translational level, and these modifications are important for the several functions of SOX2 (Liu *et al.*, 2013; Zhang and Cui, 2014; Lee *et al.*, 2017). The mediators of SOX2 regulatory network include various transcriptional factors, microRNA, and epigenetic and signalling pathway regulators (Chanoumidou *et al.*, 2017).

MicroRNA (miRNA) are small (approximately 22-nucleotides) noncoding RNAs that regulate the expression of many genes by targeting their mRNAs post-transcriptionally. This binds to the partially complementary targets sites in 3'-untranslated regions of mRNAs, inducing mRNA degradation or translational inhibition. Expression profile of microRNA (miRNA) is different in normal tissues and tumors, and can act as tumor suppressors or oncogenes (Otsubo *et al.*, 2011). MicroRNA could inhibit SOX2 at translational level, for example the miRNA-145 through direct repression of Oct4, SOX2, and Klf4 could inhibit human embryonic stem cells (ESCs) self-renewal and expression of pluripotency genes by targeting the 3'-untranslated region, this promotes lineage-restricted differentiation (Otsubo *et al.*, 2011; Liu *et al.*, 2013). In mouse, the miRNA-134 repress SOX2 expression by targeting its coding region (Otsubo *et al.*, 2011).

In human gastric carcinoma tissues, the expression of SOX2 was frequently downregulated, where 16% it was due to aberrant DNA methylation (Otsubo *et al.*, 2011). Otsubo and colleagues demonstrated that miRNA-126 could inhibit SOX2 expression by targeting two binding sites in the 3' untranslated region of SOX2 mRNA through gain-and loss-function assays (Otsubo *et al.*, 2011).

SOX2 protein can be modified through phosphorylation, acetylation, ubiquitination, methylation, and SUMOylation, these modifications make SOX2 have different activities (Figure 4) (Liu *et al.*, 2013; Zhang and Cui, 2014).

SUMO (small ubiquitin-like modifier) modifications has been recognized as an ubiquitous and critical post-translational modifier observed in organisms of the eukaryotic kingdom. The consequence of this process ranges from the repression of transcriptional factors, to alterations of subcellular localization and activity of targets proteins, to DNA damage repair and signal

transduction (She and Yang, 2015). Through SUMOylation, mouse SOX2 could be modified at lysine247 and the SUMOylation in this lysine or carboxyl terminus impaired the SOX2 binding to the Fgf4 enhancer, therefore, transcriptional activities of SOX2 were negatively regulated through impaired DNA binding (Liu *et al.*, 2013). SUMOylation of SOX2 impairs transcriptional activity and compromises pluripotency (Borkent *et al.*, 2016). Other authors report that lysine47 residue of this protein located in DNA binding domain could be acetylated by a histone acetyltransferase, p300/CBP, and this promoted the nuclear export of SOX2. The increased levels of acetylated SOX2 leads to ubiquitination and proteasomal degradation of this transcriptional factor (Liu *et al.*, 2013).

AKT can phosphorylate SOX2 on threonine118 and this regulates the activity of this transcriptional factor. The phosphorylation of SOX2 increases its protein stability by blocking ubiquitin-mediated proteolysis, but without affecting translocation. This activity of AKT regulates SOX2 function and contributes to self-renewal of embryonic stem cells (ESCs) (Jeong *et al.*, 2010). The phosphorylation of serine249, 250, and 251 in SOX2 protein promotes SUMOylation, inhibiting the binding of the protein to DNA motives (Zhang and Cui, 2014). Interactions between SOX2 and CARM1 (coactivater-associated arginine methyltransferase 1) is required to maintain the identity of ESCs. CARM1, is a protein with arginine methyltransferase activity which facilities the transactivation mediated by SOX2 through methylation on arginine113 of SOX2 (Liu *et al.*, 2013).

SUMOylation of SOX2 protein at lysine247 of its HMG domain interferes with its DNA-binding activity; acetylation of SOX2 at lysine75 leads to nuclear export followed by ubiquitination (Jeong *et al.*, 2010; Lee *et al.*, 2017), because the suppression of SOX2 acetylation at lysine75 residue prevents nuclear translocation of this protein (Lee *et al.*, 2017). SOX2 may also be phosphorylated at serine249, serine250 and serine251 (Jeong *et al.*, 2010).

Upregulation of SOX2 acetylation decreases the expression of SOX2 by ubiquitation and proteasomal degradation, suggesting that acetylation of SOX2 is a key modulator of SOX2 functionality (Lee *et al.*, 2017).

Activities of SOX2 are multiple through translational and post-translational modifications and thereby regulate pluripotency and differentiation (Liu *et al.*, 2013).

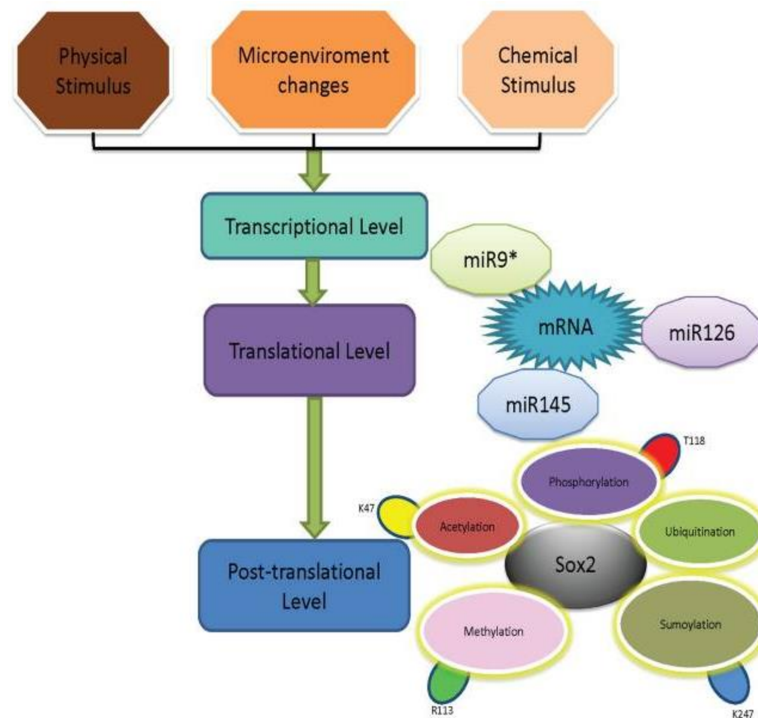


**Figure 4. Multiple modifications in SOX2 for regulate its functions**. SOX2 can be modified at level translational by miRNAs and at level post-translational through phosphorylation, acetylation, ubiquitination, methylation, and SUMOylation. These changes make SOX2 have different activities (Liu *et al.*, 2013).

## 2.2 Role of SOX2 in cancer

SOX2 is one of the key members of the SOX family and play an important role in carcinogenesis of several tumors, including gastric, breast, and pancreatic cancers (Li *et al.*, 2015). This factor is overexpressed in many aggressive tumors and its role as oncogene in malignant proliferation once promote tumor proliferation and growth, maintenance of stemness, cellular migration, invasion, metastasis, tumorigenesis and maintenance of tumorigenicity, apoptotic resistance, and chemoresistance (Liu *et al.*, 2013; Ren *et al.*, 2016).

Overexpression of *Oct4*, *SOX2,* and *Nanog* genes has been detected in breast, head and neck, and prostate cancers. These genes are important for tumorigenicity, metastasis, and radioresistance (Atoraih *et al.*, 2016).

Amplification of *SOX2* gene was detected in 21% of all primary head and neck squamous carcinomas (HNSCC) and, in this carcinoma, SOX2 is associated with resistance to apoptosis-inducing agents including cisplatin though the ability to induced the expression of the antiapoptotic protein BCL-2 (Schröck *et al.*, 2014). Knockdown of SOX2 lead to significant

inhibition of HNSCC with cancer stem cells (CSC) stemness-associated features, including self-renewal capacity, stem cells expression markers, invasion capacity, chemoresistance, and tumorigenesis (Sang Hyuk Lee *et al.*, 2014). Siegle and colleagues identified several novel SOX2 binding partners in human head and neck squamous cell carcinomas (SCCs) that directing SOX2 activity to numerous genomic loci and can function as both a transcriptional activator as well as a repressor (Siegle *et al.*, 2015). In this type of cancer, SOX2 promotes the proliferation via upregulate cyclin B1 expression (Sang Hyuk Lee *et al.*, 2014).

In humans, mutations in *SOX2* gene have associated to anophthalmia and hypopituitarism, and sometimes to learning difficulties, convulsions, motor dysfunctions, and growth problems (Li *et al.*, 2012; Garros-Regulez *et al.*, 2016). However, *SOX2* is upregulated in several types of human cancers, such as medulloblastoma, glioblastoma, breast, gastric and lung cancers, osteosarcomas, and squamous cell carcinomas (Herreros-Villanueva *et al.*, 2013). SOX2 expression is increased in gliomas and glioblastomas in the initial phase and the highest levels are associated with poor outcome. Silencing of *SOX2* gene decreases cell proliferation and might be a novel therapeutic approach to fight against brain tumors (Marqués-Torrejón *et al.*, 2013; Garros-Regulez *et al.*, 2016).

Liu and colleagues discussed that in tumor initiating cells (TICs) derived from adult human glioblastoma SOX2 knockdown lead to inhibition of proliferation and loss of tumorigenicity in immunodeficient mice, indicating that this factor is essential for maintaining the self-renewal capacity of TICs and SOX2 or its downstream molecules might be an ideal target for glioblastoma therapy (Liu *et al.*, 2013).

In pancreatic cancer cells the increased expression of *SOX2* is sufficient for cell proliferation (G1 for S phase transition) by inducing expression of cyclin D3 and repression of $p21^{Cip1}$ and $p27^{Kip1}$. On the other hand, the suppression of *SOX2* maintains the cells in the G0/G1 phase, which is associated with the expression of p21 and p27. Thus, SOX2 protein can facilitate the cell cycle progression in pancreatic cancer cells through regulation of cyclin D3, CDK6 activation, and repression of $p21^{Cip1}$ and $p27^{Kip1}$ (Herreros-Villanueva *et al.*, 2013). In colorectal cancer cells, p21 can directly bind to Cdk1/cyclin B1 and inhibits its activity (Sang Hyuk Lee *et al.*, 2014).

Repression of SOX2 induces cell apoptosis via cleavage of caspase-3 and activation of specific pro-apoptotic factors and inhibit G1/S transition in prostate cancer by regulating cyclin E and regulating cyclin D1 in breast cancer (Ren *et al.*, 2016). In this type of cancer silence SOX2 expression decreased the tumor cell proliferation and colony formation (Liu *et al.*, 2013).

In Ewing's sarcoma, the suppression of SOX2 induce G1/S cell cycle arrest by targeting p21, p27, and cyclin E. The repression of cyclin E and activation of p21 and p27 leads to inhibition of SOX2. SOX2 inhibition promotes the cell apoptosis via the activation of extrinsic and intrinsic

apoptotic pathways through activation of caspases-8 and -9, increased of pro-apoptotic protein Bad and Fas, and decreased of anti-apoptotic protein Bcl-2 and XIAP (Ren *et al.*, 2016).

The overexpression of SOX2 in gastric cancer cells decreased the levels of cyclin D1 (Otsubo *et al.*, 2008), influencing the cell cycle progression (Han *et al.*, 2014), reduced the Rb phosphorylation, and increased the levels of p27 protein. The expression of *SOX2* gene is downregulated and exogenous expression of *SOX2* inhibits cell growth by dragging cell cycle and apoptosis. Thus, *SOX2* gene work as a tumor gene suppressor in gastric cancers (Otsubo *et al.*, 2008). Hütz and colleagues refer that in gastric cancer cells, the inhibition of *SOX2* by cellular transfection decreases cell proliferation and RNA analysis reveals the increased in quantity of p21 (Hütz *et al.*, 2014). However, in human gastric carcinoma, *SOX2* promoter are silenced by DNA methylation (Karachaliou *et al.*, 2013).

SOX2 is overexpressed in 11% of the tumors in colorectal cancer (CRC) and is associated with BRAF$^{V600E}$ mutation, but not to K-Ras mutations in codon 12 and 13. The expression of SOX2 and BRAF$^{V600E}$ mutated are associated with poor patient survival and blocking downstream BRAF (B-Raf) signalling using an MEK-inhibitor resulted in a decreased expression of SOX2, suggesting that SOX2 expression is at least partly regulated by the BRAF/MEK pathway (Lundberg *et al.*, 2014).

In 90% of lung squamous cell carcinoma the SOX2 mRNA expression is increased and associated with poor prognosis, and when SOX2 is silenced the BMP4 factor inhibits cell proliferation (Liu *et al.*, 2013; Fang *et al.*, 2014). SOX2 is also overexpressed in non-small cell lung carcinoma (NSCLC) (Ying *et al.*, 2016), where SOX2 and Oct4 are exclusively expressed in the nuclei of human NSCLC cells and the expression of Oct4 was associated with poor prognosis of lung cancer, we can conclude that this transcription factors are a promising marker in diagnosis and therapy of this cancer (Liu *et al.*, 2013).

About of 27% samples of human small-cell lung cancer (SCLC) have the SOX2 gene amplified and SOX2 knockdown blocked the proliferation of this cell lines and decreases anchorage-independent growth. (Liu *et al.*, 2013; Sang Hyuk Lee *et al.*, 2014). Amplified SOX2 in some types of cancer leads to high levels of this factor (Liu *et al.*, 2013). *SOX2* gene it also amplified in skin squamous cell carcinoma and deletion of this gene in mouse epidermal cells reduce the tumor formation in this tissue (Boumahdi *et al.*, 2014). SOX2 factor is also essential for cutaneous squamous cell carcinomas (SCC) growth in mouse and human, the function of this protein as a key molecule in SCCs because it acts as an integration point of development and oncogenic signalling mechanisms (Siegle *et al.*, 2015).

In glioma stem-like cells (GSCs) SOX2 is overexpressed and promote the stemness, migration, invasion, and maintenance of tumorigenicity, however has not been performed an

exhaustive analysis of SOX2-regulated molecular circuits in GSCs. In GSCs, SOX2 plays prominent roles in driving the growth, treatment resistance, and recurrence of glioblastoma (Acanda de la Rocha *et al.*, 2016). Downregulation of SOX2 by RNA interference (siRNA) decrease proliferation and ability to form tumors *in vivo* and silencing SOX2 leads to reduction of migration and invasion capabilities, increases senescence, and produces an arrest of the cell cycle in G0/G1 (Garros-Regulez *et al.*, 2016).

Silencing of SOX2 in lymph node showed a decreased ability in cell migration and invasion *in vitro* and a reduction of tumor metastasis *in vivo* (Schröck *et al.*, 2014).

Xia and colleagues refer that in lung adenocarcinoma, the overexpression of SOX2 can inhibit K-Ras activation in cancer formation. This type of cancer requires reduced SOX2 expression for your formation (Xu *et al.*, 2014).

Transcription factors Oct4 and SOX2 demonstrate clinical importance in cervical cancer, by the correlation between poor prognosis in patients and overexpressed levels of Oct4 and loss of SOX2. In lung cancer, the high expression of SOX2 is correlated with low patient survival, but in esophageal squamous cancer the low expression of SOX2 is associated with poor prognosis (Lee *et al.*, 2017).

The fact that there are studies showing contradictory effects of SOX2 on cell proliferation suggests that this transcription factor has a different role depending of the cancer type (Weina and Utikal, 2014). So, SOX family can act as an oncogene, tumor suppressor, or both depending on the cellular context (Thu *et al.*, 2014).

In cancer cells, the SOX2 protein is involved in proliferation and cellular growth, cell migration and invasion, stemness and tumorigenicity, resistance to signals of apoptosis and therapy, metastasis and tumorigenesis (Liu *et al.*, 2013; Weina and Utikal, 2014).

SOX2 was identified as a regulatory factor in several key signalling pathways associated with tumor progression, including AKT, Wnt, and MAPK cascades (Ren *et al.*, 2016). In laryngeal cancer cells SOX2 promotes the migration and invasion through the induction of MMP-2 and PI3K/AKT/mTOR pathway (Yang *et al.*, 2014) and in Ewing's sarcoma SOX2 regulated cell-cycle progression and apoptosis via activation of the PI3K/AKT signalling pathway. The signalling pathway play an important role in cell survival and cell growth via direct or indirect regulation of apoptotic factors and cell cycle regulators (Ren *et al.*, 2016).

Li and colleagues refer that *SOX2* overexpression can contribute to chemoresistance to paclitaxel (chemotherapeutic agent) in ovarian cancer through of PI3K/AKT signalling pathway. Chemoresistance cells present a higher expression of SOX2 and the silencing of SOX2 decreased the expression levels of PI3K, AKT, and p-AKT, suggesting that SOX2 directly regulates the PI3K/AKT pathway, and induce apoptosis mediated by paclitaxel. In addition the knockdown of

SOX2 lead to decrease of cell proliferation, for that the transcription factor SOX2 will become an important indicator for ovarian cancer prognosis and a potential target for ovarian cancer therapy (Li *et al.*, 2015).

In breast cancer, SOX2 transcription activity was responsible for the tumorigenicity and cancer stem cell-like phenotypes (Liu *et al.*, 2013), and SOX2 overexpression is associated with resistance to endocrine therapy with tamoxifen in the resistant tumor cells. Downregulating SOX2 expression leads to Wnt signalling decreased and thus re-sensitized the resistant cells to tamoxifen, this suggest that SOX2 is involved in the Wnt signalling in cancer stem cells (Lee *et al.*, 2017).

In human prostate cancer cells, SOX2 overexpression promotes cell proliferation and prevent the cell cycle arrest induced by paclitaxel. The inhibition of the PI3K/AKT signalling pathway attenuated the effects of SOX2 during the treatment with paclitaxel (Li *et al.*, 2014)

During the carcinogenesis, SOX2 can cooperate in the loss of differentiation of the cancer cells and in maintaining the stem cell phenotype in the same (Hussenet *et al.*, 2010). In squamous epithelial cancers, this transcription factor has been implicated as a driver through gene amplification and in prostate cancer is a marker of neuroendocrine differentiation (Mu *et al.*, 2017).

Mu and colleagues show that prostate tumors with intact *TP53* and *RB1* genes are likely to acquire resistance by restoration of androgen receptor (AR) signalling, whereas cancers with deficient *TP53* and *RB1* are more likely to escape therapy by transitioning away from an AR-dependent luminal identity through the reprogramming activity of *SOX2* (Mu *et al.*, 2017).

SOX genes are regulators of cellular programming, which means that any disruption of these genes may lead to several human diseases (Thu *et al.*, 2014). The SOX2 protein has been associated with numerous types of tumours, and in some cases described as an oncogene and controls the physiology of cancer cells while promoting oncogenic signalling and the maintenance of cancer stem cells (Weina and Utikal, 2014). Thus, as SOX2 is expressed in multiple types of cancer is considered an ideal therapeutic target (Karachaliou *et al*., 2013), since the high expression of this factor in primary tumor could profit from more aggressive primary therapy strategies (Schröck *et al.*, 2014).

High levels of SOX2 expression have been associated with tumor aggressiveness and worse prognosis (Garros-Regulez *et al.*, 2016). Wang and colleagues refer that is need more investigation to know the specific role of SOX2 in tumor-associated signalling pathways (Wang *et al.*, 2015).

## 3. The oncogene Ras

Ras superfamily is composed of more than 150 proteins (Fernández-Medarde and Santos, 2011) divided in five families: Ras, Rho, Arf/Sar, Ran, and Rab, and all proteins have a common domain- G-domain when found the GTP binding region (Rojas *et al.*, 2012).

The monomeric Ras proteins are a small guanosine triphosphate hydrolyzing proteins (GTPases) (Asati *et al.,* 2016a; Ilinskaya *et al.*, 2016) with a molecular weight of 21 KDa (Kubuschok *et al.*, 2006) and these proteins are the transcription product of *Ras* gene, a proto-oncogene (Manchado *et al.*, 2016).

The *H-ras* (Harvey rat sarcoma viral oncogene homolog), *N-ras* (Neuroblastoma rat sarcoma viral oncogene homolog), and *K-ras* (Kirsten rat sarcoma viral oncogene homolog) genes are the three human *Ras* genes (Baines *et al.,* 2011).

These small GTPases is regulated by GTP/GDP cycle, Ras is inactive when GDP (guanosine diphosphate) is bound to C-terminal, but when stimulated by GEF (guanine nucleotide exchange factor) released GDP and GTP (guanosine triphosphate) binds to Ras protein, becomes active. However, when GTP is hydrolyzed in GDP by GTPase Ras protein converts to inactive form. If any mutation blocks this cycle by inactivation the GTPase activity, Ras protein remains active (Baines *et al.,* 2011; Rojas *et al.*, 2012; Logsdon and Lu, 2016).

Ras family members are highly conserved across species (Castellano and Downward, 2011) and the signalling of this oncogene can be observed on intracellular membranes, such as cytoplasm, endosomes, endoplasmic reticulum, Golgi apparatus, and mitochondria (Baines, *et al.,* 2011). Since their localization and capacity to regulated different members of the same family and these selective interactions, Ras family are involved in regulation of multiple biological functions (Castellano and Downward, 2011), such as macromolecular biosynthesis, organization of the cytoskeleton, nuclear transport, vesicular trafficking *(*Asati, *et al.*, 2016a), regulation of growth, apoptosis, senescence, differentiation of several types of cells, and anti-cancer effect, by participating in cell proliferation and differentiation via its intrinsic GTPase activity by the transmission of signals initiated in the plasma membrane (Kubuschok *et al.*, 2006; Fernández-Medarde and Santos, 2011; Salzano *et al.*, 2014). Alterations in these pathways can have severe consequences and can result in several abnormalities, including tumorigenesis and immunological disorders (Asati *et al.,* 2016a).

Ras protein is commonly not active until called upon by external cellular signals, but when activated it can influence several cellular functions (Logsdon and Lu, 2016). The functional differences that Ras exhibit are, possibly, associated to with hyper-variable C-terminal region (HVR) which modulates the interaction Ras/membrane to the different locations in organelles

and in signalling cascades (Prior *et al.*, 2012). Ras protein is activated in response to signalling pathways initiated by extracellular stimuli and promote binding to numerous effectors proteins which result in activation of multiple signalling cascades (Asati *et al.,* 2016a).

*H-ras* is an oncogene that promotes proliferation by upregulated general transcription factors to stimulate RNA synthesis. Cells overexpressing H-Ras present an increased expression of the general transcription factor TATA-box binding protein (TBP) that leads to increase RNA synthesis (Kotsantis *et al.*, 2016).

This proto-oncogene becomes an oncogene when mutated, and is associated with several human cancers (Salzano *et al.*, 2014). The oncogenic mutations in Ras affect its GTPase activity, causing accumulation of Ras in active state (with GTP bound) and result on hyperactive signalling that is responsible for initiation and maintenance of tumorigenesis (Manchado *et al.*, 2016).

## 3.1 Signalling pathways activated by oncogene Ras

Ras signalling is involved in many cellular functions, such as cell proliferation, apoptosis, migration, fate specification, and differentiation. This oncogene activates several signalling pathways, which includes the Raf/MEK/ERK mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase-AKT-mTOR (PI3K), and RAL (Castellano and Downward, 2011; Asati *et al.*, 2016b).

Interaction of activated Ras with Raf initiates the Raf/MEK/ERK pathway and the activation of ERK causes phosphorylation of multiple substrates, as kinases that control translation (for example p90) and transcription factors that control genes involved in cell cycle (for example ElK1, Fos, Myc) (Overmeyer and Maltese, 2011; Lee *et al.*, 2017). Therefore, Raf/MEK/ERK cascade is the pathway responsible for transmitting Ras mitogenic signals to the cell cycle machinery (Drosten *et al.*, 2014). The activation of Ras/Raf/MEK/ERK pathway is essential for cell proliferation since this cascade play a critical role in the transmission of signals from growth factor receptor to regulate gene expression, and its inhibition blocks the cell cycle at the G1 phase, which can lead to cellular apoptosis (Drosten *et al.*, 2014; Asati *et al.*, 2016b). However, the retroactivation of the Raf/MEK/ERK cascade promotes the cell proliferation (Drosten *et al.*, 2014). *In vitro* studies show that there is an increased of Ras expression during the G1 and S phase of cell cycle (Morais *et al.*, 2008; Prior *et al.*, 2012).

Drosten and colleagues refer that in normal cells (MEFs), Ras signalling activated the MAPK cascade, which result in inactivation of the p53/p21Cip1/Rb tumor suppressor axis via acetylation of lysine161/162 residues, and consequently allows the cell proliferation. Cells

lacking Ras, the inactivation of Raf/MEK/ERK pathway leads to activation of p53 through acetylation of lysine161/162 residues. The activation of p53 induces p21Cip1 expression that activates Rb and induce the cell cycle arrest. Cells deficient in Ras proteins and with p53/p21Cip1/Rb axis inhibited by absence of p53, leads to retroactivation of the Raf/MEK/ERK cascade and sustained cell proliferation (Drosten *et al.*, 2014).

The PI3K (phosphatidylinositol 3-kinase) – PKB (protein kinase B)/AKT cascade controls cellular processes that are critical for cancer progression, such as cell survival (apoptosis inhibition), transcription, cell cycle progression, translation, metabolism, and motility (Castellano and Downward, 2011; Prior *et al.*, 2012; Asati *et al.*, 2016b). In cells stimulates with growth factors, PI3K has activated when the cells are transiting from G0 to G1 phase and later in G1 phase. This shows that Ras activity is needed throughout all the different phases of cell cycle (Castellano and Downward, 2011).

*In vitro* studies show that H-Ras protein is the best activator of PI3K while K-Ras has a cohesive bound with Raf and Rac (Prior *et al.,* 2012). In human thyroid epithelial cells, PI3K is required for the proliferative response to Ras, so PI3K signalling is necessary to supports tumorigenesis (Castellano and Downward, 2011). Mutations in *K-ras* gene leading to deregulation of signalling pathways such Ras/Raf/ERK and PI3K/AKT (Fernandes *et al.*, 2016). The involvement of various proteins in the Ras signalling pathway becomes more difficult to understand this pathway (Asati *et al.,* 2016a).

Alterations of signal transduction pathways leading to uncontrolled cellular proliferation, survival, invasion, and metastasis are the basis of the carcinogenic process. The abnormal activation of PI3K/AKT cascade is associated with many human cancers, for example in lung cancer (Asati *et al.*, 2016b). The disruption of this pathway promotes oncogenesis because the PI3K activates important mediators of Ras-mediated transformation, such as Rac GTPase, a Rho family protein. In Ras mutant cells, although multiple Ras effectors are essential to initiate tumor formation, the PI3K/AKT signalling is indispensable to maintain tumor growth, PI3K signalling is essential for Ras to drive tumor development; however the inhibition of PI3K alone isn't sufficient to cause regression of tumors once established (Castellano and Downward, 2011). The resistance of cancer cells to doxorubicin is associated with deregulation of the PI3K pathway (Asati *et al.*, 2016b).

Oncogenic Ras and PI3K can form metastasis by promoting the loss of anchorage-dependent growth, which is a key feature in tumor cells. Oncogenic mutations that increase enzymatic activity, constitutively stimulate AKT signalling, induce growth in culture, and cause tumors *in vivo* (Castellano and Downward, 2011).

Deregulation of MAPK cascade is frequently observed in several cancers, such as melanoma, pancreatic, lung, colorectal, and breast cancers because this pathway play a central role in the carcinogenesis. This cascade is involved in the regulation of many cellular processes including proliferation, survival, differentiation, apoptosis, motility, and metabolism (Asati *et al.*, 2016b). In human cells, the interaction between Raf/MEK/ERK and PI3K/AKT pathways is important in Ras-mediated transformation because in these cells the inactivation of the MAPK pathway using different inhibitors decreases AKT phosphorylation (Castellano and Downward, 2011). In the presence of apoptotic signals induced by c-Myc, Ras use specifically the PI3K/AKT pathway to promote the cell survival (Cox and Der, 2003).

In tumorigenesis, different mechanisms activate the Ras signalling pathway, such as mutations in Ras, loss of GTPase-activating proteins (GAPs), and overexpression of receptor tyrosine kinases (RTKs) (EGFR, ERBB2) (Castellano and Downward, 2011). This suggesting that inhibition of components of this pathways may be a potential strategy for cancer therapy (Asati *et al.*, 2016b).

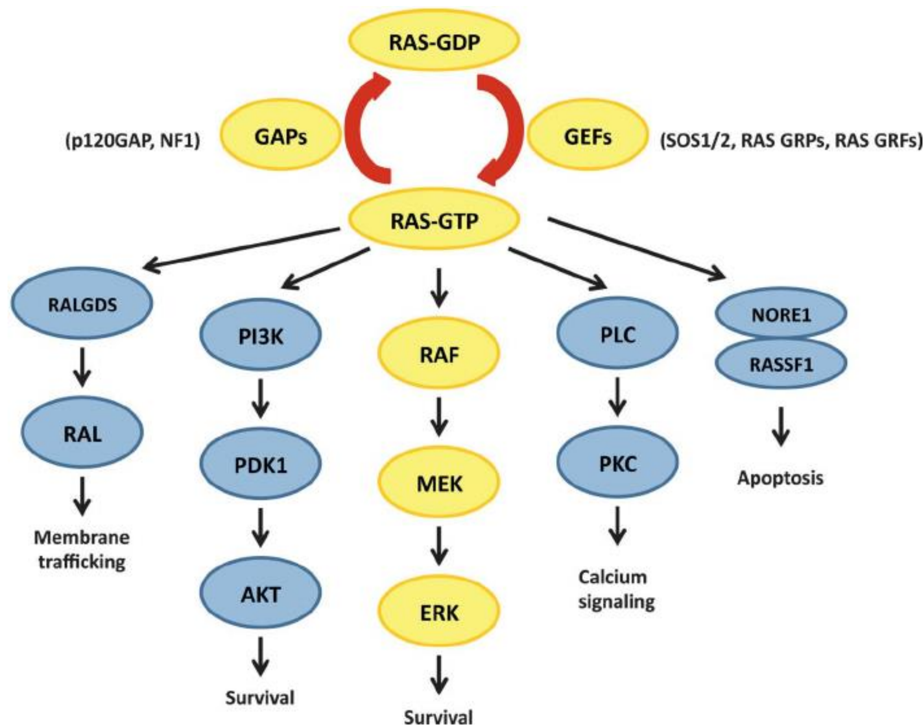


**Figure 5. Ras signalling pathways.** The Ras/RAF/MEK/ERK cascade is the classic RAS/MAPK pathway where RAF binds to the effector region of RAS-GTP, inducing translocation of the protein to the plasma membrane. In the PI3K pathway, PIP3 stimulates the AKT/PKB kinase and several Rac-GEFs, such as Sos1. Ras also activates the RalGDS pathway signalling to enhance cellular transformation. The activation of PLC by Ras leads to break down of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ releases $Ca^{2+}$ from intracellular stores, and DAG activates protein kinase C (PKC), together altering the activity of cellular proteins. The activation of this oncogene

allows the formation of RASSF1-NORE1A complex, activated proapoptotic pathways (Lourenço *et al.*, 2014).

## 3.2 Role of the oncogene Ras in cancer

Oncogenic Ras promotes initiation and maintenance of tumor growth (Castellano and Downward, 2011), and for this is a powerful tool for investigating the cell origin of different cancers (Xu *et al.*, 2014). It is very common to found mutations in one of the three *Ras* genes (*H-ras*, *N-ras* e *K-ras*) and aberrant function of Ras is associated with rapid proliferation diseases and cancers (Fernández-Medarde and Santos, 2011; Prior *et al.,* 2012). The aberrant activation of these proteins facilities all characteristics of the malignant phenotype in cancer cells, including cellular proliferation, transformation, invasion, and metastasis (Ilinskaya *et al.*, 2016).

Studies in the last few years have identified that a single mutation in codon 12 and/or 13 of exon 2 and 61 of exon 3 of *Ras* oncogene are sufficient for tumor formation. These mutations in conserved region promote the binding of GTP and maintains Ras active (Fernández-Medarde and Santos, 2011; Prior *et al.,* 2012).

It is possible found mutations in *Ras* genes in about 33% of all human cancers, but these mutations affect, predominantly, the *K-ras* locus, which is observed in 21,6% of all samples analyzed. In contrast, the rate of occurring mutations in *N-ras* and *H-ras* is very low (8% and 3.3%, respectively) (Baines *et al.,* 2011). Because of the high frequency of *K-ras* mutations and the fact that they appear during the initial stage of tumor progression it can be concluded that K-Ras plays an important role in human tumorigenesis. In a normal mouse, the K-Ras protein is essential for their development unlike the N-Ras and H-Ras proteins are unnecessary (Fernández-Medarde and Santos, 2011). Despite the *H-ras* gene is the isoform less frequently mutated in human cancers it is the most studied, while *K-ras* gene is predominantly mutated in three of the four neoplasms that cause more deaths in US: lung, colon, and pancreatic cancers with a rate of 86% of all Ras mutations (Baine *et al.*, 2011; Asati *et al.*, 2016a).

K-Ras protein is mutated, mainly, in pancreatic ductal adenocarcinomas and lung and colon cancers, H-Ras in bladder cancers and N-Ras in leukaemia, thyroid carcinomas, and malignant melanomas (Fernández-Medarde and Santos, 2011). In 90% of pancreatic cancers *K-ras* gene is mutated (Prior *et al.,* 2012; Logsdon and Lu, 2016).

In primary culture cells, Ras induces premature senescence and apoptosis. The activation of H-Ras and the block of p53 confers mobility to cells of human urothelial cancer and is capable of transforming non-invasive cells in invasive cells (He *et al.*, 2015).

Mutations on *Ras* gene are associated with several types of cancer with low success rate (Drosten *et al.*, 2014), such as carcinomas of the pancreas, colon, lung, thyroid, and myeloid malignancies (Kubuschok *et al.*, 2006; Drosten *et al.*, 2014).

In colorectal cancer 35-45% of all patients that present *K-ras* mutations, this exhibited an early development and progression, so *K-ras* mutation become an important prognostic factor for determination of the colorectal cancer (Asati *et al.,* 2016a). *K-ras* gene is mutated in about 21% to 30% of human lung adenocarcinoma (Mukhopadhyay *et al.*, 2014), activation of K-Ras protein is a potential source of heterogeneity, which hinders their therapy (Xu *et al.*, 2012).

Mutations in Ras pathways that leads to the expression of active Ras proteins, as observed in about 30% of human cancers. The Ras/MAPK/PI3K pathway is activated in human squamous cancers by different mechanisms, this indicates that K-Ras promotes the development of lung cancers (Mukhopadhyay *et al.*, 2014) and the aberrant activation of the Ras/MAPK pathway is correlated with cancer progression and metastatic tumor cell growth (Aksamitiene *et al.,* 2012). In mouse model with K-Ras lung cancers, the inhibition of MEK and PI3K exhibits anti-tumor activity (Shaw *et al.*, 2011).

In pathological conditions, such as inflammation, Ras activity can lead to senescence, cell death or transformation, in absence of tumor suppressors. This condition can initiate a carcinogenesis in pancreatic cells expressing endogenous levels of oncogenic mutant K-Ras (Logsdon and Lu, 2016).

In hepatocellular carcinoma (HCC) Ras protein is not mutated, but their activity is elevated by alteration of regulatory molecules, such as decreased levels of GTPase-activating proteins (GAPs). The decreased of GAPs levels prolonged the Ras signalling that is similarly to the oncogenic mutations in *ras* gene. The overexpression of the oncogenic K-Ras is sufficient to transform adult pancreatic acinar cells without external stimulants. That is, the increased of Ras levels is capable of cellular transformation without oncogenic mutation (Logsdon and Lu, 2016). In pancreatic ductal adenocarcinoma (PDAC), the detection of *K-ras* mutations possibly can be useful prognostic factor for its treatment (Asati *et al.*, 2016a).

The Rasal2, a Ras GTPases-activating protein (Ras-GAP), which can catalyze GTP into GDP and inactivate Ras, acts as a tumor suppressor in bladder cancer (BCa) and modulates the phenotypes of cancer stemness and epithelial-mesenchymal transition (EMT) through MAPK/SOX2 pathway. The suppression of this protein could facilitate BCa cell migration, invasion, stemness, and tumorigenesis and in breast cancer promoted the tumor development as a consequence of activating K- and H-Ras. Rasal2 regulated BCa cells stemness and EMT via MAPK/SOX2 signalling pathway, in which the transcription factor SOX2 acted as an important bridge (Hui *et al.*, 2017).

Involvement of Ras in cancer is accentuated by Ras mutations and deregulation of many regulators or effector pathways, so the study of oncogenic Ras signalling is essential to establish a molecular basis for the pathogenesis of human cancer (Castellano and Downward, 2011), and the use of Ras signalling pathways as therapeutic target is attractive, but in the same time complicated since these pathways are also essentials for the proliferation and survival of the normal cell (Shaw *et al.*, 2011).

## 4. The therapeutic anti-cancer targets

Cancer is considered a cell cycle disease, once only normal cells proliferate in the presence of growth factors and specific mitogenic signals, but the cancer cells proliferated in unregulated way (Diaz-Moralli *et al.*, 2013). Genomic alterations, such as changes in oncogenes, tumor suppressor genes, DNA mismatch repair, and excision repair genes are involved in the most human cancers (Asati *et al.,* 2016a).

The finding of genetic, genomic, and clinical biomarkers redirected the treatment of cancer for a personalized medicine, having consideration a person's susceptibility/progression of disease, the patient's response to therapy and the toxicity of therapeutic for patient. The information from biomarkers permits a rapid disease diagnosis, risk assessment, and better clinical decision. For colorectal cancer (CRC) the microsatellite instability (MSI) and mutations of *K-ras* can be biomarkers of "independent" prognostic value in patients (Asati *et al.*, 2016a).

The increased of CDK (cyclin-dependent kinases) and cyclins expression and loss of CKI (cyclin-dependent kinases inhibitor) and pRB expression are changes associated with cancer caused by chromosomic abnormalities (amplification and translocation of oncogenes and deletion of tumor suppressor genes) or epigenetic inactivation (methylation of suppressors tumor promotors) (Diaz-Moralli *et al.*, 2013). Malumbres refers that the inhibition of oncogenes and suppressor genes such as "checkpoint" kinases affect cancer cells proliferation (Malumbres, 2011).

In cancers induced by changes in *ras* gene, the CDK4 is a good pharmacological target, through its inhibition (Diaz-Moralli *et al.*, 2013) and inhibition of CDK2 to block de cell cycle progression from G1 to S phase (Malumbres, 2011).

The compression about CDK structure permits the development of CDK inhibitors with therapeutic effect against cancer. The molecules developed as CDK inhibitors can be divided into two groups: those that had a broad-spectrum action, such as flavopiridol, olomoucine, R-roscovitine, and kenpaullone, and highly specific, such as fascaplysin, riuvidine, purvalanol A,

and dinaciclib. In some types of cancers, the sensibility for CDK inhibitors depends on the pathogenic-spectrum of mutations. Taxanes and vinca alkaloids are used in breast and ovarian cancer therapy since they interfere with microtubules dynamics (Diaz-Moralli *et al.*, 2013).

The inhibition of an important biological capacity for a cancer, can stop their growth and progression, being necessary more investments in drug for specific targets (Hanahan and Weinberg, 2011).

Cancer cells, in repost to a particular therapy, can reduce the dependence on particular marker and become more dependent of another marker. For example, angiogenesis inhibition in human glioblastomas apparently causes an increase of local invasion and local metastasis. The development of drugs that induce the apoptosis may hyperactivate mitogenic signalling of cancer cells (Figure 6) (Hanahan and Weinberg, 2011).

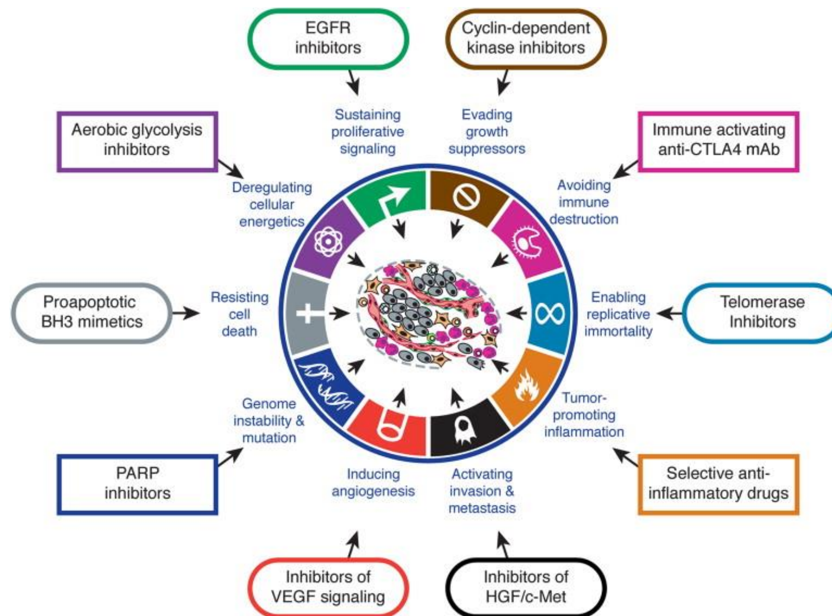


**Figure 6. Therapeutic targeting of the hallmarks of cancer.** Developed drugs that interferes with important capabilities for tumor growth and progression (Hanahan and Weinberg, 2011).

Ras mutated present highly sensitive to MEK inhibition, these inhibitors act on endothelial cell proliferation and tumor cells apoptosis by controlling angiogenesis through increased the level of pro-apoptotic protein BIM. MEK162, sunitinib, and axitinib act as angiogenesis inhibitors (Asati *et al.*, 2016a).

Colorectal cancer patients treated with Cetuximab and Panitumumab as monoclonal antibodies that inhibit the epidermal growth receptor (EGFR) showed significant improvement

in her life, but patient with mutation of codon 12 and 13 in *K-ras* gene exhibited a lack of response to this monoclonal antibodies (Asati *et al.*, 2016a).

The discovery of *K-ras* as a clinical biomarker and a therapeutic target has involved the scientific community to create effective and precise anticancer drugs for cancer therapy (Asati *et al.*, 2016a).

For the lung adenocarcinoma, the K-Ras mutant is a therapeutic target that represents a major goal of clinical oncology. The US Food and Drug Administration (USFDA) approved the trametinib that is an MEK inhibitor in Ras/Raf/MEK/ERK cascade. The components Raf, MEK, and ERK of the MAPK pathway have been used as Ras target, but this strategy has been harm by toxicities associated with their sustained inhibition and/or adaptive resistance mechanisms. USFDA also approved for leukemia treatment the tipifarnib, a Ras inhibitor (Manchado *et al.*, 2016).

Other promising tool for the development of anticancer therapy is the use of several ribonucleases (RNases), for inhibition of Ras signalling though direct binding. These RNases induce selective cytotoxicity in cancer cells by initiated apoptosis (Ilinskaya *et al.*, 2016). The development of small molecules that can restore the GTPase activity of mutant Ras proteins is other strategy for inhibition Ras signalling (Asati *et al.*, 2016a).

Combination treatment of non-small cell lung cancer (NSCLC) with gefitinib and specific inhibitors of the PI3K/AKT and Ras/ERK pathways can be a successful strategy for the development of anti-cancer drugs (Asati *et al.*, 2016a). Recently, a group of investigators suggests that PI3K p110α is a good target for therapeutic intervention in colorectal cancer with *K-ras* mutations (Fernandes *et al.*, 2016).

Cancers with a high prevalence of *K-ras* mutations are difficult to treat and doesn't exist an effective therapy, thus *K-ras* gene is a potential biomarker of pancreatic, colorectal, and lung cancers (Asati *et al.*, 2016a).

The resistance to the antiandrogen drug, such enzalutamide, in prostate cancers can be reversed by inhibiting SOX2, this raises some hope that appropriate clinical interventions could prevent or overcame resistance. Mu and colleagues refer that the direct pharmacologic inhibition of SOX2 is not feasible currently, but can be possible to prevent *SOX2* transcriptional up-regulation following the loss *TP53* and *RB1*. RB1 directly represses SOX2 by recruitment to E2F binding sites in the *SOX2* promoter and TP53 indirectly inhibits SOX2 by up-regulation of its target gene miR-34 that targets *SOX2* mRNA (Mu *et al.*, 2017).

Transplants of glioma stem cells (GSCs) in mice brain using peptide vaccination against SOX2 increase the mice survival. In these cells, miRNA-145 delivery can suppress the tumorigenicity through direct downregulation of SOX2 protein with GSCs becoming more sensitive to

chemotherapeutic agents, such as cisplatin. The use of miRNA might improve current cancer treatments, especially for tumors that have developed a resistance to conventional therapeutic methods. This is a promising therapeutic, but the use of viral vectors for gene delivery may be accompanied by multiple problems, including immune response (Garros-Regulez *et al.*, 2016).

Actuality, approximated 30 different kinases have been identified and multiple inhibitors are developed which reached in Phase I clinical trial (Asati *et al.,* 2016b).

The development of drugs and treatment protocols that cover multiple characteristics/marks of cancer cells will be more effective in cancer therapy (Hanahan and Weinberg, 2011).

# Aims

SOX2 is a stem-cell associated transcription factor that has an important role in maintaining the unique characteristics of embryonic stem cells, such as clonogenicity, pluripotency, and self-renewal (Karachaliou *et al.*, 2013). Its overexpression or gene amplification have been connected with cancer development in several tissues, such as lung and breast (Liu *et al.*, 2013). Thus, SOX2 is a key factor for successful embryonic development whereas its abnormal activity has been associated to cancer development (Chanoumidou *et al.*, 2017).

The present study aimed to investigate the role of SOX2 on RasV12-mediated transformation in order to better understand whether SOX2 increase the oncogenic effect of RasV12 and if this factor influence the response of tumor cells to chemotherapy. Thus, our specifics objectives were:

a) To study the mechanisms by which RasV12 induces SOX2 *in vitro*.
   - Factors involved in induction of SOX2 by RasV12 through several immortalized and primary mouse embryonic fibroblasts cell lines knockdown and knockout for *p53*, *p19*$^{ARF}$, and *Rb* genes.
   - Signalling Ras pathway involved in induction of SOX2 mediated by RasV12 through overactivation and pharmacological inhibition of Ras pathways *in vitro*.

b) To assess the importance of SOX2 in the oncogenic transformation mediated by Ras V12 in immortalized and primary mouse embryonic fibroblasts cell lines *in vitro* through transformation assays.

c) To study the effect of SOX2 overexpression in human lung carcinoma and human breast adenocarcinoma cell lines on drug therapy *in vitro* through MTT assays.

# Materials and Methods

## 1. Cell Culture

### 1.1 Culture conditions

The HEK 293T, W3T3, D3T3, WT MEFs, p53$^{-/-}$ MEFs, ARF$^{-/-}$ MEFs, C39 Tag, A549, and MCF7 parental cell lines (Table 2) were used in this study. Cells were cultured at 37$^{0}$C in a humidified 5% $CO_2$ incubator and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, D5671) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies, 10270), 1% glutamine (Gibco Life Technologies, 25030-024), and 1% penicillin/streptomycin (Gibco Life Technologies, 15140-122) for a confluence of 70 to 80%. Re-plating of cells was accomplished by using trypsin followed by resuspension cells into fresh medium.

Cells infected with vector carrying resistance to puromycin, hygromycin, and G418 were maintained with 2.25µg/ml of puromycin (Sigma, P8833), 250µg/mL of hygromycin B (Invitrogen, 10687-010), and 600 µg/ml of G418 (HyClone, SV30069), respectively. The W3T3 shNTG and W3T3 shp53 cells were maintained in culture with 3µg/mL of puromycin (Sigma, P8833). The A549 EOS GFP Puro, A549 SOX2, MCF7 EOS GFP Puro, and MCF7 Puro cells were maintained with 1µg/mL of puromycin, and the A549 Puro and MCF7 SOX2 cells were selected with 4µg/mL and 200ng/mL of puromycin, respectively.

For cryopreservation, cell pellet was dissolved in 1mL DMEM with 10% of DMSO (dimethylsulfoxide) (Sigma, D2650), a cryoprotectant that prevents the ice crystals formation.

**Table 2. List of parental cell lines.**

| Cell line | Origin of cell line |
|---|---|
| **HEK 293T** | Human embryonic kidney cells transformed with large T antigen |
| **W3T3** | Mouse immortalized fibroblasts cells by 3T3 protocol |
| **D3T3** | Mouse immortalized from Rb$^{-/-}$ embryonic fibroblasts |
| **WT MEFs** | Primary mouse embryonic fibroblasts |
| **p53$^{-/-}$MEFs** | Mouse embryonic fibroblasts p53$^{-/-}$ |
| **ARF$^{-/-}$MEFs** | Mouse embryonic fibroblasts ARF$^{-/-}$ |
| **C39 TAg** | Mouse embryonic fibroblasts immortalized by SV40 TAg |

| | |
|---|---|
| **A549** | Human lung carcinoma cells obtained from Manuel Collado's group, IDIS |
| **MCF7** | Human breast adenocarcinoma cells obtained from Manuel Collado's group, IDIS |

## 1.2 Toxicity curve for antibiotics

### 1.2.1 Hygromycin B toxicity curve for W3T3 cells

Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* it kills prokaryotic and eukaryotic cells by inhibition of translocation of mRNA and tRNA on the ribosome (Borovinskaya *et al.*, 2008). This antibiotic was used for selection of W3T3 cells transfected with pWZL Hygro and pWZL Hygro H-RasV12 plasmids (hygromycin plasmid resistance).

The antibiotic concentration in cell culture depends on cell line and we don't know the toxic concentration of hygromycin B for W3T3 cells we tested the toxic concentration of 0 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL, and 750 µg/mL for this antibiotic in W3T3 cells line. For W3T3 cells with puromycin resistance (W3T3 Puro) we tested the concentration of 0 µg/mL, 50 µg/mL, 100 µg/mL, and 250 µg/mL because we already knew the toxic concentration of hygromycin B for W3T3 cells.

We seeded $1.4x10^4$ W3T3 cells in triplicates in 24 wells culture plates and grown 24 hours before added the respective concentration of hygromycin B. This experiment was maintained eight days in culture, and changed the culture medium every three days.

The optimal antibiotic concentration that kills the control cells in a week after added hygromycin B is 250µg/mL (Figure 7).

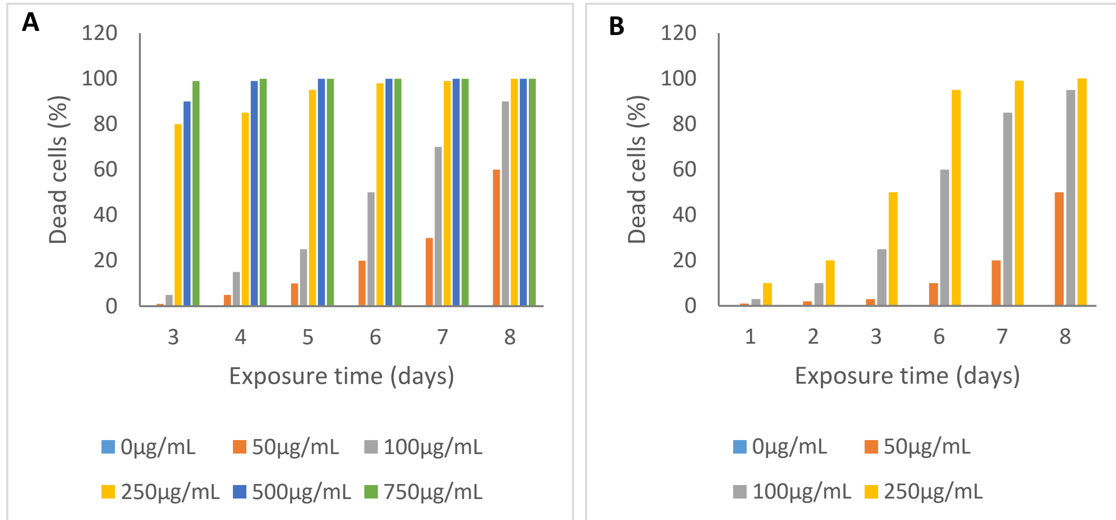


**Figure 7. Optimal concentration of hygromycin B for (A) W3T3 cells and (B) W3T3 cells with puromycin resistance.** The graphs represent the percentage of dead cells for eight days of exposition to different concentrations of antibiotic hygromycin B.

### 1.2.3 G418 toxicity curve for W3T3 shNTG cells

The G418 is an aminoglycoside antibiotic, an analogue of neomycin sulphate that interferes with the function of 80S ribosomes and protein synthesis in eukaryotic cells. We used this antibiotic for selection of W3T3 cells transfected with pInducer RasV12 plasmid.

The toxic concentration of neomycin for W3T3 shNTG cells was unknown, we tested concentrations of 0 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, and 800 µg/mL.

We seeded, $1.4x10^4$ W3T3 cells in triplicates in 24 wells culture plates and growth 24 hours before added the respective concentration of G418. This experiment was maintained eight days in culture, and changed the culture medium every three days.

The optimal antibiotic concentration that kill the control cells in a week after added is 600µg/mL (Figure 8).

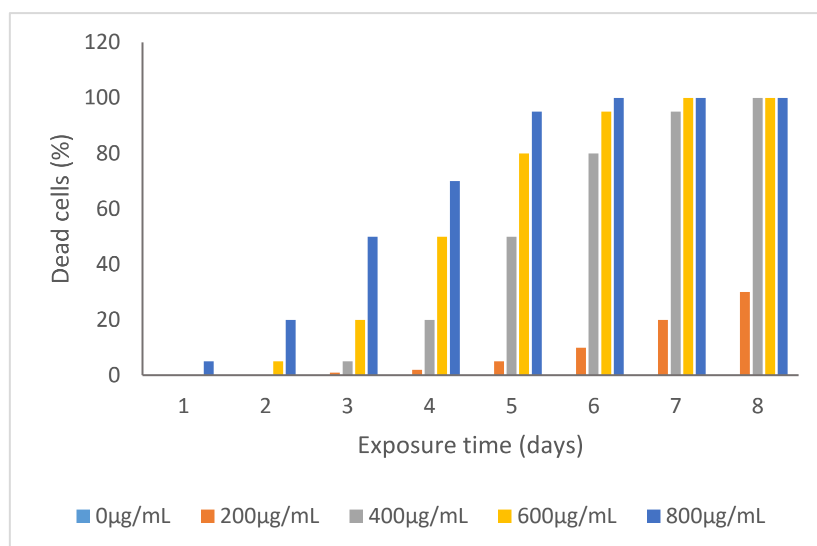


**Figure 8. Optimal concentration of G418 for W3T3 pInducer H-RasV12 cells.** The graph represents the percentage of dead cells for eight days of exposition to different concentrations of antibiotic G418.

## 1.3 Viral infections

### 1.3.1 DNA amplification of retroviral and lentiviral plasmids

In this work, we used $\Psi^2$helper, pBabe Puro, pMXS-SOX2 IP, pBabe Puro H-RasV12, pWZL Hygro, pWZL Hygro H-RasV12, pBabe H-RasV12 C40, pBabe H-RasV12 E38, pBabe H-RasV12 G37, pLp1, pLp2, pLp VSVG, and pInducer 20 RasV12 plasmids. To DNA amplifications we prepared the plasmids at a concentration of 100-200ng/µl and added 2µl of plasmids to 25µl of DH5α competent bacteria, except for lentival plasmid pInducer 20 H-RasV12 that grown in ccdB bacteria. Plasmids were incubated with bacteria 20 minutes in ice, 45 seconds to 42$^0$C, and two minutes in ice again. After, added 1mL of SOC (super optimal broth) medium (Table 3) and incubated for 1.5 hours on a shake to 175rpm and 37$^0$C. We put 100µl of previous solution in a plate of Lysogeny broth (LB) medium + agar with 100µg/mL ampicillin (Sigma, A9518) and incubated 12 to 24 hours to 37$^0$C.

Next, we grow the bacteria in small volume of LB medium (Table 3) with the antibiotic, for that we put a colony of bacteria in 10mL of LB medium with 100µg/mL of ampicillin and incubated for eight hours on a shake to 225rpm and 37$^0$C. After, we growth the bacteria in 250mL of LB medium with 100µg/mL of ampicillin overnight in the same conditions. To increase the number of copies of DNA, the bacteria with pInducer 20 H-RasV12 plasmid were incubated over 12 hours with 170µg/mL of chloramphenicol in the same conditions.

We centrifuged the bacteria in LB medium for 15 minutes to 5000 g at 4ºC and to isolate the plasmid we used a protocol of NucleoBond® Xtra Midi (Macherey-Nagel, 740410-50). The pellet of plasmid was dissolved in TE pH 8.0 (10Mm Tris-Cl pH 8.0 and 1Mm EDTA pH 8.0) and store to -20$^0$C.

To store bacteria colony with the plasmid to -80$^0$C, we added 300µl of glycerol to 700µl of bacteria in LB medium.

**Table 3. Composition of SOC and LB medium.**

| SOC medium | LB medium (1L) |
|---|---|
| 2% tryptone | 10g tryptone |
| 0.5% yeast extract | 5g yeast extract |
| 10mM NaCl | 10g NaCl |
| 2.5mM $KCl_2$ | $H_2O$ |
| 10mM $MgCl_2$ | Adjust the pH 7.5 with NaOH |
| 10mM $MgSO_4$ | |
| 20mM glucose | |

### 1.3.3 Transfection using calcium phosphate-DNA precipitate method

Transfections of the HEK 293T packaging cells, which enables the encapsulation and release of viral particles, with the different plasmids (Table 4), was performed by calcium phosphate method. For retroviral transfections we used 15µg of a plasmid that encodes the structural proteins of the virus (gag and pol) ($\Psi^2$helper) and 15µg of plasmid containing the gene of interest. However, for lentiviral transfections we need 7.5 µg of a lentiviral packaging plasmid containing the HIV-1 gag and pol genes (pLP1), 7.5 µg of a lentiviral packaging plasmid containing the HIV-1 rev gene (pLP2), 7.5 µg of a lentiviral packaging plasmid for expression of the vesicular stomatitis virus G glycoprotein (pLP/VSVG), and 7.5 µg of a lentiviral plasmid containing the gene of interest, in this case pInducer 20 H-RasV12 plasmid (Table 5).

Briefly, 3x$10^6$ HEK 293T cells were plated in 100mm culture plates and incubated for 24 hours. Three hours before the transfection, the culture medium was replaced to 9mL, the cells must be, approximately, with 70% of confluence. In a Falcon tube, we added 500µl of 2x HeBS (1.5 mM $Na_2HPO_4.7H_2O$, 274 mM NaCl, 54.5 mM HEPES) and in an eppendorf tube prepared the

mixture: 50µl of 2.5M $CaCl_2$, 30µg of DNA, and $H_2O$ up to 500µl per culture plate. While bubbling the 2x HEBS was added the mix of $CaCl_2$ and DNA and let sit the mixture 20 minutes at room temperature to allow the formation of calcium phosphate crystals. The solution was placed in each HEK 293T culture plates and 18 hours after incubation changed the medium (washed two times with PBS) to avoid toxic effects on cells. We collected the supernatants of the packaging cells 48 hours after the last medium replaced, centrifuged five minutes to 500 rpm, and filter through a 0.45µm filter. The viral supernatants were frozen at -80$^0$C until used in infections.

**Table 4. Plasmids used in experiments and its function.**

| Plasmids | Function |
|---|---|
| pBabe Puro | Retroviral plasmid control with puromycin resistance |
| pMXS-SOX2 IP | Retroviral plasmid for SOX2 overexpression and puromycin resistance |
| pBabe Puro H-RasV12 | Retroviral plasmid for overexpression of active form of H-Ras and puromycin resistance |
| pWZL Hygro | Retroviral plasmid control with hygromycin resistance |
| pWZL Hygro H-RasV12 | Retroviral plasmid for overexpression of active form of H-Ras and puromycin resistance |
| pBabe H-RasV12 C40 | Retroviral plasmid for overactivation of PI3K pathway and puromycin resistance |
| pBabe H-RasV12 E38 | Retroviral plasmid for overactivation of MAPK pathway and puromycin resistance |
| pBabe H-RasV12 G37 | Retroviral plasmid for overactivation of RalGDS pathway and puromycin resistance |
| pInducer 20 H-RasV12 | Lentiviral plasmid for inducible expression of H-RasV12 (Tet-ON) and neomycin resistance |
| pMIG | Retroviral plasmid with GFP expression |

**Table 5. Amounts of DNA used in transfection with retroviral plasmids and lentiviral plasmids.**

| Transfection with retroviral plasmids | Transfection with lentiviral plasmids |
|---|---|
| 15µg of $\Psi^2$ helper plasmid - encodes the structural proteins of the virus (gag and pol)<br>15µg plasmid containing the gene of interest | 7.5µg pLP1 - lentiviral packaging plasmid containing the HIV-1 gag and pol genes<br>7.5µg pLP2 - lentiviral packaging plasmid containing the HIV-1 rev gene<br>7.5µg pLP/VSVG - lentiviral packaging plasmid for expression of the vesicular stomatitis virus G glycoprotein<br>7.5µg plasmid containing the gene of interest |

### 1.3.4 Viral transduction

#### 1.3.4.1 Infection with viral supernatants

Infections with retroviral supernatants were used to produce different cell lines, through the infection of W3T3, D3T3, WT MEFs, p53$^{-/-}$ MEFs, ARF$^{-/-}$ MEFs, and C39 TAg parental lines with plasmids mentioned in the table 4. We also infected W3T3 shNTG and W3T3 shp53, which were previously generated in the laboratory with pWZL Hygro and pWZL Hygro H-RasV12.

We plated 12 500 cells/cm$^2$ (50-60% of confluence) and incubated for 24 hours. To increase the success rate of this process, we did two rounds of the infection. The viral supernatants were thawed to 37$^0$C and target cells were incubated with 4mL of viral supernatants with 4µg/mL of polybrene (Sigma, H9268) for four hours. The process was repeated one more time and added double volume of fresh medium (8mL) to each plate. After 24 hours since the beginning of viral infection, the medium was replaced and 48 hours after infection started the selection of transformed cells or cells were collected for protein extraction and RNA extraction. Cells were selected with 2.25µg/mL of puromycin for cells infected with plasmid pBabe Puro, pBabe Puro H-RasV12, pBabe Puro H-RasV12 C40, pBabe Puro H-RasV12 E38, and pBabe Puro H-RasV12 G37, 250µg/mL of hygromycin B for cells infected with pWZL Hygro and pWZL Hygro H-RasV12, and 600µg/mL of G418 for cells infected with pInducer 20 H-RasV12 plasmid. Clones were selected until death of cells control.

To originate W3T3 (W3T3 Puro-Hygro), W3T3 Ras (W3T3 Puro-Ras), W3T3 SOX2 (W3T3 SOX2 IP-Hygro), W3T3 SOX2-Ras (W3T3 SOX2 IP-Ras), W3T3 and $p53^{-/-}$ MEFs shEV, W3T3 and $p53^{-/-}$ MEFs shEV Ras, W3T3 and $p53^{-/-}$ MEFs shSOX2, and W3T3 and $p53^{-/-}$ MEFs shSOX2 Ras, the retroviral transductions were divided in two phases. In the first phase W3T3 cells were infected with viral supernatants of Puro and SOX2 IP, and W3T3 and $p53^{-/-}$ MEFs cells were infected with viral supernatants of shEV (short hairpin control RNA) and shSOX2 (short hairpin SOX2 RNA) from Miguel Fidalgo's group, CiMUS. In the second phase cells obtained from the first infections were infected with viral supernatants of Hygro and Hygro-Ras.

Briefly, 12 500 cells/cm$^2$ (50-60% of confluence) W3T3 cells were plated in 100 mm plate culture and were incubated for 24 hours. To increase the success rate of the process, we did two rounds of the infection. The Puro and SOX2 IP viral supernatants were thawed to 37$^0$C and target cells were incubated with 4mL of viral supernatant with 4µg/mL of polybrene for four hours. The process was repeated one more time and added double volume of fresh medium (8mL) to each culture plate. We plated 12 500 cells/cm$^2$ W3T3 and $p53^{-/-}$ MEFs cells (50-60% of confluence) in six wells culture plates and incubated for 24 hours. The shEV and shSOX2 viral supernatants were thawed to room temperature and target cells were incubated with 0.5mL of culture medium and 100µl of viral supernatant with 4µg/mL of polybrene (Sigma, H9268) for four hours, and we added 1.5mL culture medium to each well. The medium of infected cells was replaced 24 hours after starting the viral infections and 48 hours after infection we started the selection of transformed cells with 2.25µg/mL of puromycin for cells transfected and finished when the control cells died.

To the second phase, the cells were seeded at same density in 100mm culture plate and incubated for 24 hours with respective antibiotic. The viral supernatants were thawed to 37$^0$C and target cells were incubated with 4mL of viral supernatant with 4µg/mL of polybrene for four hours. The process was repeated one more time and added double volume of fresh medium (8mL) to each culture plate. After 24 hours since the beginning of viral infection, the medium replaced and 48 hours after infection the cells were selected with 2.25µg/mL of puromycin and 250µg/mL of hygromycin B until the death of the control cells.

The confirmation of the infections was made through western blotting assays to confirm the expression of SOX2 and Ras proteins as mentioned in the point 2.

### 1.4 Cellular response to treatment with doxorubicin

Doxorubicin is a chemotherapeutic agent capable of induce apoptosis by activated the ROS (oxygen-reactive species) and the p53 protein pathway that promote the transcription of p21 protein. In these assays, we intend observe the expression of p53 and p21 through western blotting in samples treated with doxorubicin to check that this pathway still active.

We seeded $8x10^5$ cells per 100mm culture plate of each cell line and grown for 24 hours. Cells were treated with 0.5µg/mL of doxorubicin in DMSO and cells control were treated with same volume of DMSO for 16 hours, depending on the cell line. Following, we proceed to cellular lysis and confirmation of protein expression by western blotting as mentioned in the point 2.

### 1.5 Viability assay

The MTT assay is used to estimate the cell viability, once the viable cells with active metabolism covert MTT (thiazolyl blue tetrazolium bromide) into formazan product, its quantity can be measured by recording changes in absorbance at 570nm using a plate reading spectrophotometer. The formazan accumulates as an insoluble precipitate inside cells as being deposited near the cell surface and in culture medium, and must be solubilized prior with solubilization solution. When cells die lose the ability to convert MTT into formazan, MTT signal is relates with cell number.

For these experiments, we seeded $4x10^3$ of A549 EOS GFP, A549 EOS GFP Puromycin, A549 Puro, A549 SOX2, MCF7 EOS GFP, MCF7 EOS GFP Puromycin, MCF7 Puro, and MCF7 SOX2 cells in 96 wells culture plates and were incubated for 24 hours. The A549 and MCF7 were treated with 0, 0.5, 1, 2.5, 5, 10, 25, and 50µM of doxorubicin, the A549 cells were also treated with 0, 2.5, 5, 10, 25, 50, and 100 nM of docetaxel, and the MCF7 cells were treated with 0, 25, 50, 100, 200, 400, and 800 nM of docetaxel and 0, 25, 50, 100, 200, 500, and 1000 nM of gemcitabine for 48 hours. We added 10µl of MTT solution (5mg/mL) (Alfa Aesa, L11939) per well and incubated for four hours, and added 100µl of solubilization solution (1mL of 1M HCl and 10g of SDS) per well and incubated overnight. The quantity of formazan was measured by recoding changes in absorbance at 570nm.

# 2. Assessment of protein expression by western blotting

## 2.1 Protein extraction and quantification

For protein extractions we plated $8x10^5$ cells in 100mm culture plates and incubated for 24 hours or we collected the cells 48h after viral infection. In ice, we washed the cells two times with PBS $4^0$C and added to the culture plates or cellular pellets (after centrifugation at 1000g for five minutes between washes) 70µl of lysis buffer (Table 6). After, we collected the cells for eppendorf tube, incubated on ice for 20 minutes, and lysis the cells by sonication in three pulses of five seconds with 30% amplitude (keeping the samples in ice to prevent protein degradation). Following centrifuged the samples at 16 000g for 15 minutes at $4^0$C, the protein supernatant was collected and kept at -$80^0$C for later use.

We used the Bradford method to protein quantification. We prepared six BSA standard (1µg/µL) with the concentrations 0, 1, 2, 4, 8, and 10µg/µl and the samples of cell lines. To each sample and standard, we added 1mL of Coomassie (Bradford) protein assay reagent (Thermo Fisher Scientific, 1856209) and the colorimetric reaction could develop for 10 minutes at room temperature. The absorbance (595nm) was measured in a spectrophotometer (Thermo Spectronic, BioMate3), where that standard 0µg/µl was used as blank for the standards lecture and lysis buffer used as blank for the samples.

**Table 6. Composition of RIPA and lysis buffers.**

| RIPA buffer | Lysis buffer |
|---|---|
| 5M NaCl | 1mM $Na_3VO_4$ (sodium orthovanadate) |
| 1M Tris-HCl pH 7.5 | 1M PMSF |
| 0.1% SDS | 1M DTT |
| 1% Triton X-100 | 4mM NaF |
| 1M EDTA pH 8.0 | 1x cocktail of proteinases inhibitors (Thermo |
| 0.5% Sodium deoxycholate | Scientific, 1862209) |
| $H_2O$ | RIPA |

## 2.2 Western blotting

The samples, equal amount, were mixed with a Laemmli charge buffer, a denaturing buffer (50% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.5% bromophenol blue, 0.4M Tris pH 6.8), and

were incubated five minutes to 95$^{0}$C. Thus, we eliminate the protein three-dimensional structure, so proteins are separated on the gel based on their molecular weight.

Proteins from the cell extracts were separated by vertical electrophoresis (25mA and 200V) in denaturing gel of acrylamide: bis-acrylamide of size 8.5x6.5cm with 5% stacking gel and 12% or 15% resolving gel. Two distinct phases divide these two gels: the upper phase (stacking gel) in which the proteins homogenize and the lower phase (resolving gel) that allows the protein separation by molecular weight. The percentage of acrylamide in the resolving gel depends on protein under study.

**Table 7**. **Composition of solution for prepare stracking and resolving gels.**

| | Stracking gel | Resolving gel | |
|---|---|---|---|
| | **5% (4mL)** | **12% (7mL)** | **15% (7mL)** |
| 30% acrylamide mix | 670µl | 2.8mL | 3.5mL |
| 1.5M Tris pH 8.8 | 500µl | 1.75mL | 1.75mL |
| 10% SDS | 40µl | 70µl | 70µl |
| 10% ammonium persulfate | 40µl | 70µl | 70µl |
| TEMED (Sigma, T9281) | 4µl | 2.8µl | 3µl |
| $H_2O$ | 2.7mL | 2.3mL | 1.61mL |

After the electrophoresis, proteins present in the gel were transferred to 45 µm Hybond-PVDF membrane (Merch Millipore, IPVH 00010). The membrane was placed 30 seconds into methanol and submerged in water for least five minutes to activate the membrane, this process is to membrane pass hydrophobic to hydrophilic for that the protein binding to membrane for hydrophobic bond and charge, lastly membrane was transferred to transfer buffer B for least five minutes. We placed six sheets of Whatman in transfer buffer A and C and three sheets of Whatman in transfer buffer B and stack sheets of transfer buffer A, gel, membrane PVDF (polyvinylidene difluoride), and sheets of transfer buffer B and C (Table 8). The stack was positioned onto semi-dry blotter (Sigma) with that gel facing to the positive pole and the membrane for negative pole, once the protein transfer is made from the positive pole for negative pole. Transfers were performed by a current of 0.8mA/cm$^2$ on the membrane for 1.5 hours.

**Table 8. Solutions for semi-dry transference.**

| Transfer Buffer A (1L) | Transfer Buffer B (1L) | Transfer Buffer C (1L) |
|---|---|---|
| 100mL 0.4M e-aminoceproic acid and 0.25M Tris base | 20mL 1.25M tris base | 200mL 1.25M tris base |
| 200mL of isopropanol | 200mL of isopropanol | 200mL of isopropanol |
| 700mL of $H_2O$ | 800mL of $H_2O$ | 600mL of $H_2O$ |

Membranes were incubated, in constant agitation, for least two hours at room temperature in blocking solution (0.05% TNT solution containing 5% milk powder) to block nonspecific protein.

After blocking, membranes were washed, to remove excess buffer, three times for 10 minutes in 0.05% TNT solution. Membranes were incubated with a primary antibody overnight at $4^0$C in constant agitation under the following conditions (Table 9).

**Table 9. Solutions for membrane washing and antibody incubation (TNT) and antibodies used in membrane hybridization.**

| TNT | 0.05% (500mL) | 0.5% (500mL) | 1% (500mL) |
|---|---|---|---|
| 1M Tris HCl pH 7.5 | 12.5mL | 12.5mL | 12.5mL |
| 5M NaCl | 15mL | 15mL | 15mL |
| Tween® 20 (Fisher BioReagents, BP337) | 0.25mL | 2.5mL | 5mL |
| $H_2O$ | 472.25mL | 470mL | 467.5mL |

| Antibody | Applier references | Dilution | Incubation solution |
|---|---|---|---|
| SOX2 | Santa Cruz Biotechnology (Y-17), sc-17320 | 1:500 | 1% TNT |
| pan-Ras | Oncogene (Ab-3), cat#OP40 | 1:200 | 1% TNT |
| α-p53 | Cell signalling (1C12)<br>Santa Cruz Biotechnology (A-1), sc-393031 | 1:1250<br>1:1000 | 1% TNT |

| α-p21 | Santa Cruz Biotechnology (F-5), sc-6246 | 1:200 | 1% TNT |
|---|---|---|---|
| p19$^{ARF}$ | Santa Cruz Biotechnology (5-C3-1), sc-32748 | 1:500 | 1% TNT |
| Phospho-p44/42 MAPK (P-ERK1/2) | Cell signalling (197G2), cat#4377 | 1:1000 | 0.1% TNT + 5% BSA (Sigma, A2153) |
| p44/42 MAPK (ERK1/2) | Cell signalling, cat#9102 | 1:1000 | 0.1% TNT + 5% BSA (Sigma, A2153) |
| α-Tubulin | Sigma (DM1A), cat#T9026 | 1:3000 | 1% TNT |
| α-GAPDH | Millipore (6C5), cat#MAB374 | 1:7500 | 1% TNT |
| *α-Goat* IgG HRP | Santa Cruz Biotechnology, sc-2020 | 1:5000 | 0.5% TNT |
| *α-Mouse* IgG HRP | GE Healthcare, NA931V | 1:5000 | 0.5% TNT |
| *α-Rabbit* IgG HRP | GE Healthcare, NA934V | 1:5000 | 0.5% TNT |
| *α-Rat* IgG HRP | Vector, cat#PI9401 | 1:1000 | 0.5% TNT |

After the incubation, membranes were washed three times for 10 minutes with 1% TNT solution. To allow the detection of antigen-antibody interaction, the membranes were incubated with second antibody associated with HRP (Horse-Raddish Peroxidase) diluted in 0.5% TNT solution for 45 minutes at room temperature. Membranes were washed again three times for 10 minutes in 0.5% TNT solution.

To detect the proteins, membranes were incubated with ECL western blotting substrate 1:1 (Thermo Scientific, 32106) for one minute to room temperature in the dark. The binding of the substrate to peroxidase associated with the antibody (HRP) produces a light reaction that allows its detection by autoradiography. The medical x-ray films were exposed until it was possible to visualize the proteins of interest.

To observe the expression of different proteins in same membrane can be used the stripping and reprobing western blots method, which involves the extraction of membrane-bound antibodies, followed by incubation with the desired antibody. To remove the antibodies, the membrane was incubated at $50^{0}C$ for 30 minutes in stripping buffer (62.5mM Tris pH 6.8; 2% SDS; 100mM ß-mercaptoethanol).

## 3. Cell transformation assay: foci formation assay

The cell foci formation assay allows to test if cells maintain proliferation inhibition by cell-cell contact or they lost this feature. The loss of the competent inhibition by cell-cell contact is a hallmark of cancer cells. In this assay, we mixed the cells to test with W3T3 parental line which maintained contact inhibition, and observed if the interest cell line grows or not over the parental line.

For these experiments we seeded $4x10^5$ W3T3 cells (wild-type) with 400 of interesting cell line in six wells culture plates and were incubated for 11 days. The culture medium was replaced every three days.

Cells were washed two times with PBS $4^0$C, fixed and stained with a staining solution (4% formaldehyde to 37%, 4% glacial acetic acid, 60% methanol, 1% crystal violet) for 30 minutes to room temperature and finally, all excess dye was removed using water. The plates were left to dry for a few hours and the cellular foci counted.

## 4. Inhibition of Ras pathways

### 4.1 RasV12 expression with an inducible system

In these experiments, we used W3T3 cells infected with a Ras Tet-ON plasmid (pInducer 20 H-RasV12) as mentioned in 1.3.4 point. The tetracycline-controlled transcriptional activation is a method of inducible gene expression where transcription is reversibly turned on or off in the presence of the antibiotic tetracycline or doxycycline, a more stable tetracycline derivative. This plasmid has a Tet response element (TRE) that is seven repeats of 19 nucleotide tetracycline operator sequence. In the absence of doxycycline, the gene expression, in this case H-RasV12, is inhibited. In presence of this antibiotic, doxycycline binds to reverse tetracycline-controlled transactivator (rtTA) that binds to TRE and subsequent activated the gene expression.

We must first know if the RasV12 expression is activated in the presence of doxycycline and the time in which we observed increased SOX2 expression after activating the RasV12 expression with doxycycline. For the first point, we seeded $8x10^5$ cells in 100mm culture plates and grow for 24 hours. The cells were treated with 6µg/mL of doxycycline (Sigma, D9891) in PBS while the control cells were treated with same volume of PBS for 24 hours. Following, proceed to cellular lysis and confirmation of Ras protein expression by western blotting as mentioned in the point 2.

For time course experiments, we seeded $3x10^5$ cells in six wells culture plates, incubated 24 hours, and treated 0, 1, 2, 3, 4, 6, 8, 12, and 24 hours with doxycycline. Then, the cells were

collected to RNA extraction and reverse transcription (RT) quantitative PCR analysis, as mentioned in the point 5.

## 4.2 Treatment of cells with Ras pathways inhibitors

To determine the Ras pathway essential for the induction of SOX2 transcription factor by RasV12, we begin by confirming that the inhibitors block the respective Ras pathway. We seeded $8x10^5$ W3T3 pInducer H-RasV12 cells in 100mm culture plates and grow for 24 hours in 10% serum. The medium was replaced by serum deprivation medium (0.2% serum) and the cells were treated with 6µg/mL of doxycycline for six hours and with the respective Ras pathway inhibitor, 10µM PD98059 (Calbiochem, 513000) for six hours, 15µM SB202190 (Calbiochem, 559388) for one hour, 20µM LY294002 for five hours, 2.5µM MK-22062 for four hours, and 1µM GDC-0068 (Ipatasertib) for two hours in DMSO. The controls of these experiments were cells in serum deprivation for six hours, cells in 10% serum for six hours, cells in serum deprivation with doxycycline for six hours, and cells in serum deprivation with doxycycline and DMSO for six hours. The cells were treated in independent culture plate for each Ras pathway inhibitors and controls. Following, proceed to cellular lysis and detection of Ras, p-ERK, and total ERK protein expression by western blotting as mentioned in the point 2.

The next step was to study the mRNA SOX2 expression in the presence of Ras pathway inhibitors, PI3K/PDK1/AKT and MAPK. For this, we seeded $3x10^5$ W3T3 pInducer H-RasV12 cells in six wells culture plates and grow for 24 hours in 10% serum. In the next day, the cells in serum deprivation (0.2% serum) were treated with 6µg/mL of doxycycline for six hours and with the respective Ras pathway inhibitor, 10µM PD98059 for six hours, 20µM LY294002 for five hours, 2.5µM MK-22062 for four hours, and 1µM GDC-0068 for two hours in DMSO. As controls we used, cells in serum deprivation and cells in serum deprivation with doxycycline and DMSO for six hours. The cells were treated in independent culture plate for each Ras pathway inhibitors and controls (Figure 9). Then, the cells were collected to RNA extraction and RT quantitative PCR analysis, as mentioned in the point 5.

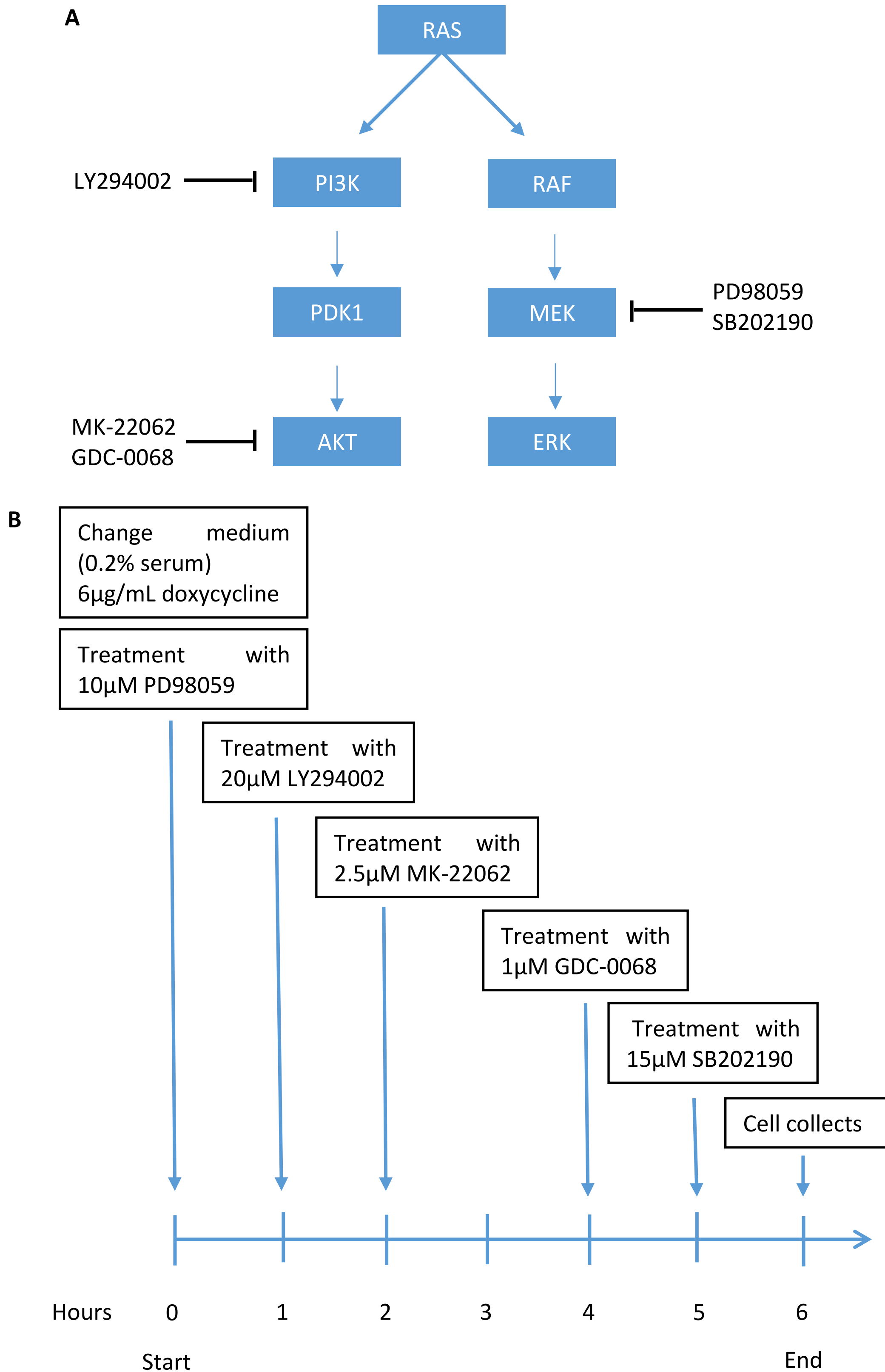


**Figure 9. (A) Inhibitors of the Ras pathways used in W3T3 pInducer RasV12 cells. (B) Experimental timeline for treatment of W3T3 pInducer RasV12 cells with MAPK and PI3K/AKT pathways inhibitors.** Cells were treated with 6µg/mL of doxycycline in serum deprivation (0.2% serum) for six hours and each

culture plate/well were treated with different Ras inhibitors at different times and concentration in the presence of doxycycline

## 5. Gene expression study by RT-quantitative PCR

### 5.1 RNA extraction

To extract RNA from different cell lines, we seeded 12500 cells/$cm^2$ and infected with pBabe Puro and pBabe Puro Ras, as referred to in 1.3.4 point. The cells were washed two times with PBS at 4ºC, 48 hours after the infection, and collected the cells with lysis buffer and β-mercaptoethanol (Sigma, M3148), as referred in the RNA extraction kit. Cells were stored at -21ºC until used.

We seeded $3x10^5$ cells/21$cm^2$ of pInducer H-RasV12 cell line in a six wells culture plates and treated with doxycycline as referred to in point 4. After the treatments, cells were washed two times with PBS at 4ºC, collected, and stored as referred to above.

After thawing the samples, the RNA extractions were performed using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, RTN70) and NucleoSpin® RNA (Macherey-Nagel, 740955) for W3T3 pInducer H-RasV12 cell line. RNA was stored at -80ºC until used to cDNA synthesis.

### 5.2 cDNA synthesis for reverse transcription (RT)

In reverse transcription, double-stranded DNA is synthesized from a RNA strand by a reverse transcriptase enzyme.

For this reaction is necessary a reverse transcriptase (M-MLV RT), dNTPs (to add during the synthesis), random primers (points that serve as a template to initiate the synthesis), and RNase Out (recombinant ribonuclease inhibitor). This method allows to determine, in a relative way, the amount of mRNA expressed in a cell line or tissue. For each sample in study, the greatest amount of cDNA obtained is co-related with highest degree of expression of a particular gene.

The protocol of retrotranscription is based on a series of incubations. We incubated five minutes at 65ºC the mixture of 1µg of sample (RNA), 250ng of random primers (Invitrogen, P/N 58875), and 1.5µl of dNTPs mix (10mM of dATP, dGTP, dCTP e dTTP) (Invitrogen) with a final volume of 19µl. To the last mixture were added 6µl of first strand buffer 5x (Invitrogen, 1820972), 3µl of 0.1M DTT (Invitrogen, P/N y00147), and 1µl of RNase Out™ (Invitrogen, P/N 100000840), and incubated two minutes at 37ºC. Finally, was added 1µl of M-MLV RT

(Invitrogen, 28025-013) and incubated at 25ºC for 10 minutes and 50 minutes at 50ºC. The reaction ended when the solution was incubated at 70ºC for 15 minutes and cDNA is ready to use in the quantitative PCR.

### 5.3 Quantitative PCR

The quantitative PCR allows simultaneous, amplification, and detection of the expression of a gene of interest. This method is based on the use of a cDNA fragment (TaqMan probe) to an intermediate part of the DNA in the sample. This probe carries attached fluorophore in the 5' end and a quencher (fluorescence inhibitory molecule) in the 3' end.

While the primer remains free the fluorescence emitted by the fluorophore is corrected by the inhibitor. However, when the primers bind to the cDNA of the sample the activity of polymerase responsible for the sequence elongation causes the hydrolysis of the inhibitor (quencher). So, the energy emitted by the fluorophore will be detected by a sensor. The amount of fluorescence emitted during each cycle of the PCR will be proportional to the amount of amplified DNA.

These probes used in quantitative PCR assays are designed in such way that the possibility of exogenous DNA amplification is minimal. Thus, for each sample we added a retrotranscription control which didn't include the M-MLV RT enzyme.

During the assays, for the *SOX2* gene, the expression levels of two housekeeping genes were measured, to ensure that the amount of the initial cDNA is equivalent for each sample. Furthermore, the measure of each gene in each sample performed in duplicate or triplicate.

In these experiments, for each sample (1.5µl cDNA + 7.5µl $H_2O$) we added 1µl of 20x TaqMan (Table 10) and 10µl of 2x Master Mix (Applied Biosystems, 94404). The cDNA was amplified (40 cycles) in quantitative PCR instrument (Life technology-applied Biosystems) under the following conditions: 10 minutes at 95ºC, 15 seconds at 95ºC, one minute at 60ºC, and to 4ºC indefinitely.

**Table 10. List of the genes and their TaqMan probes used for RT quantitative PCR analysis.**

| Gene | Probe reference |
|---|---|
| SOX2 | Mm03053810_s1 |
| GUSB | Mm01197698_m1 |
| TFRC | Mm00441941_m1 |

## 6. Statistical analysis

Graphical analysis was performed in Microsoft Excel 2010 software and results were statistically analyzed by T-student test in which the values with $p<0.05$ (* $p <0.05$; ** $p <0.01$; *** $p <0.001$) were considered statistically significant and the graph represent the mean ± SD (standard deviation).

# Results

## RasV12 overexpression induces SOX2 expression

SOX2 has an important role in several biological processes, such as maintenance of pluripotency and stemness (Arnold *et al.*, 2011). In cancer cells, this factor is involved in survival and proliferation both *in vivo* and *in vitro* studies (Ren *et al.*, 2016).

In this project, we used H-RasV12 as an oncogenic model *in vitro*. To determine the effect of SOX2 overexpression in cancer cells we developed cell lines that overexpressed RasV12 (W3T3 Ras), SOX2 (W3T3 SOX2), SOX2 and RasV12 (W3T3 SOX2-Ras), and cell line control (W3T3) by retroviral infection. The advantage of retroviral infections is that each cell contains only one copy of plasmid, so the gene expression isn't related to the number of copies of the plasmid in the cell.

We observed the increased of SOX2 expression in W3T3 SOX2 cells and expression of SOX2 and Ras in W3T3 SOX2-Ras cells. Cells that overexpressed RasV12 (W3T3 Ras) present the levels of Ras increased as well as SOX2 (Figure 10). The cell lines are morphological different from each other, and the cells that overexpressed RasV12 are morphological more elongated than cells expressing basal levels of this oncogene (Figure 11).

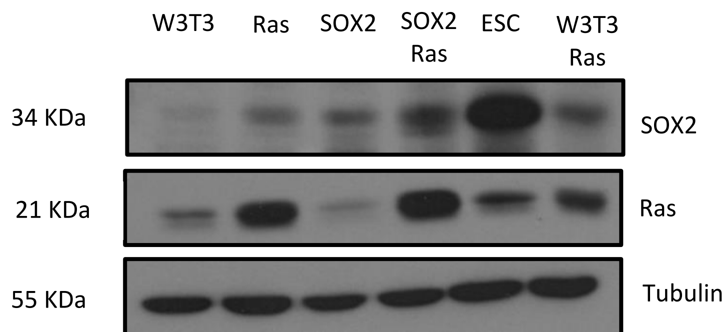


**Figure 10. The expression of SOX2 protein increases in cells expressing RasV12.** The amounts of SOX2 and Ras were determinate by western blotting in W3T3 cells infected with the Ras and SOX2 plasmids after cell selection (A representative result of two independent experiments is shown).

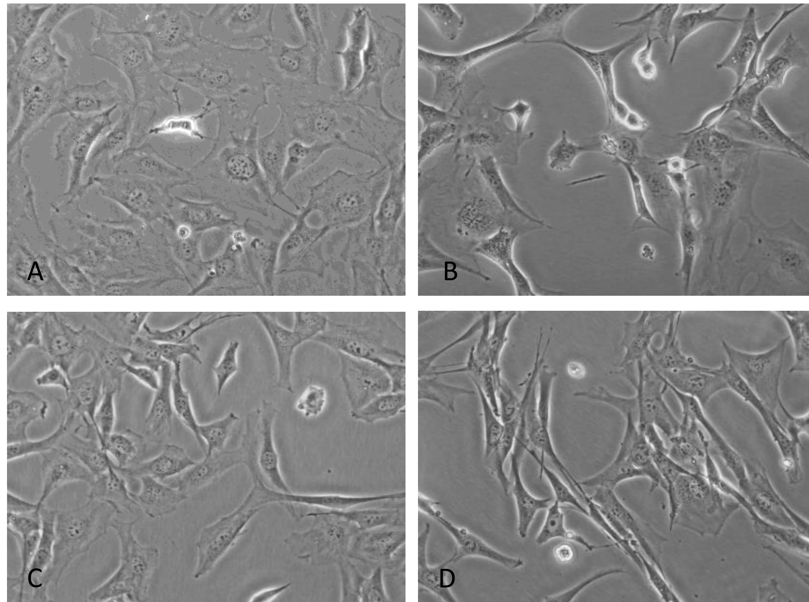


**Figure 11. The (A) W3T3, (B) Ras, (C) SOX2, and (D) SOX2-Ras cell lines are morphological different.** Representative picture of these cells 24 hours after seeded at the density of 13 560 cells/cm$^2$.

## SOX2 cooperates with RasV12 in cell transformation

In normal cells, the cell-cell contact work as an inhibitor of cell proliferation forming monolayer of confluent cells (2D culture). Several types of cancer cells lose the ability to inhibit the proliferation through cell-cell contact (Hanahan and Weinberg, 2011). As already described, cells expressing oncogenic RasV12 lose this ability and form cell foci (Jacobsen *et al.*, 2002).

To determine the effect of SOX2 overexpression in the transformation mediated by RasV12, we plated a foci assay where we use W3T3, to form the monolayer of confluent cells, with the W3T3, W3T3 Ras, W3T3 SOX2, and W3T3 SOX2-Ras cell lines to observe they capacity to form cell foci.

Both the cell lines with RasV12 overexpression, W3T3 Ras and W3T3 SOX2-Ras, formed cell foci and the control cells (W3T3) and cells with SOX2 overexpression (W3T3-SOX2) didn't lose the ability to inhibit the proliferation through cell-cell contact since they weren't able to form cell foci. In cells with SOX2 and RasV12 overexpression (W3T3 SOX2-Ras) the number of foci increased when compared to cells that only overexpressed RasV12 (174.1±4.4 in SOX2 and Ras cells versus Ras cells values of 113.8±4.3), although the cell foci are smaller in size in cells that overexpressed SOX2 and RasV12 (Figure 12). Thus, our results demonstrate that SOX2 increase the effect of RasV12 in cell transformation.

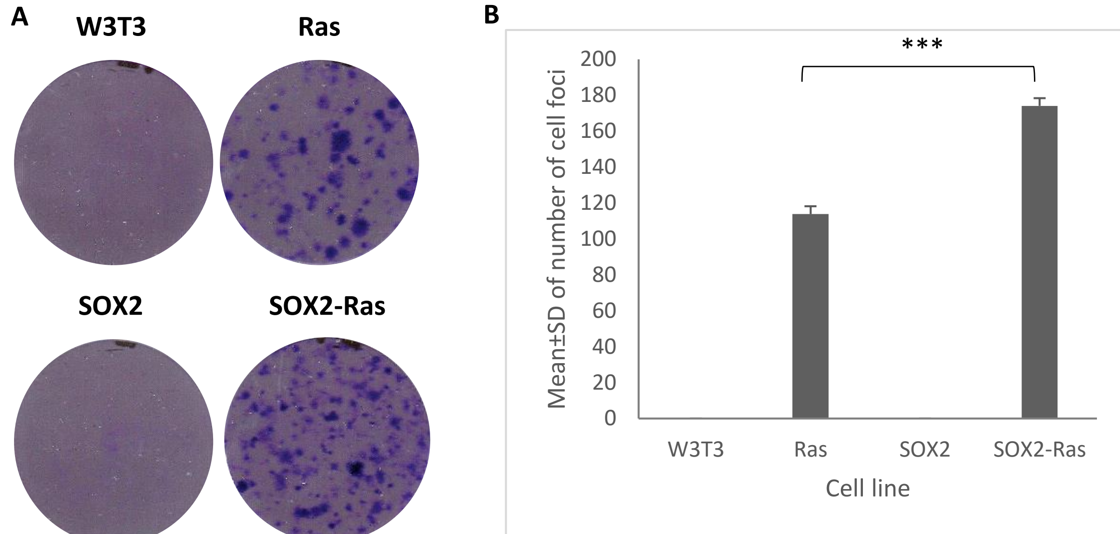


**Figure 12. The transcription factor SOX2 cooperates with RasV12 in cell transformation.** (A) Foci formation by crystal violet staining of W3T3 cells infected with SOX2 and RasV12 plasmids, selected, and 400 of these cells were plated with $4x10^5$ W3T3 cells and grown 11 days prior to staining and counting of foci. (B) Graphic showing the number of cell foci for each cell line, data are shown as mean ± SD of the number of cell foci (***p<0.001). This experiment was repeated thrice for each clone (A representative result of three independent experiments is shown).

## Expression of SOX2 is downregulated by culture time

During the culture time, we observed changes in cell behaviour after about 10 cell passages. When we analyze that SOX2 and Ras expression in the W3T3, W3T3 Ras, W3T3 SOX2, and W3T3 SOX2-Ras we detected the loss of SOX2 expression in W3T3 Ras and W3T3 SOX2 cells (Figure 13). Thus, SOX2 levels decreased during the time culture, approximately 25 days after infection with RasV12 or SOX2 plasmids. So, during the time culture we lose the cells with high levels of SOX2.

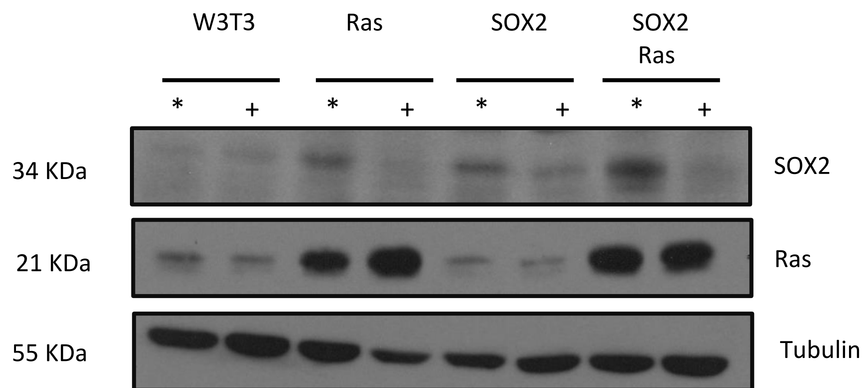


**Figure 13. The expression of transcription factor SOX2 is lost approximately 25 days in culture.** The expression of SOX2 and Ras were determinate by western blotting of cells collected (*) 15 days and (+) 30 days after the infection (A representative result of two independent experiments is shown).

## Acute induction of SOX2 by RasV12 expression

As present in previous results, during the time culture the cells infected with RasV12 loss SOX2 expression. To prevent this loss, we wanted to study SOX2 effects short time after RasV12 expression. So, we tested the degree of infection with a pMIG plasmid (that expresses GFP) and the expression of SOX2 48 hours after the infection.

To analyze the degree of infection, we infected the W3T3 cells with pMIG plasmid and 48 hours after infection observed the amount of EGFP expression in microscopy. We observed that about 90% of the cells are infected with the plasmid (Figure 14A). Using a western blotting method, we detected the increase of SOX2 expression in W3T3 cells 48 hours after the infection with RasV12, without selection (Figure 14B). Thus, we could do the experiments 48 hours after the infection without selection because we have a high degree of infection and can detect SOX2 expression.

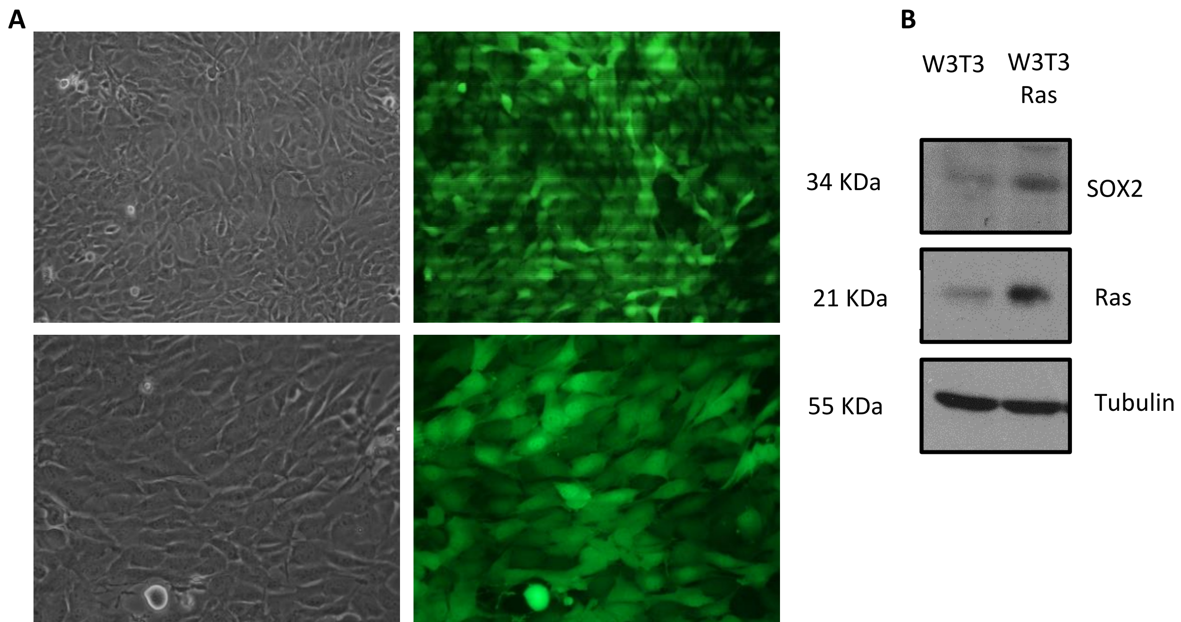


**Figure 14. SOX2 induction can be detected 48 hours after infection in W3T3 cells.** (A) The W3T3 cells were infected with pMIG plasmid and observed the amount of EGFP expression in microscopy 48 hours after the infection. (B) The W3T3 cells were infected with H-RasV12 plasmid, collected 48 hours after the infection, and the expression of SOX2 and Ras were determinate by western blotting.

## Genetic requirements for RasV12-mediated SOX2 induction

Mouse embryonic fibroblasts (MEFs) are a classic model system for studying cell senescence and immortalization. Cells that sustain spontaneous damage in p53/p19$^{ARF}$ pathway (p14 in humans) can overcome this replication block, leading to gradual outgrowth of an immortalized cell population with unlimited growth potential (Odell *et al.*, 2010).

In immortalized MEFs, mutations in *p53* and *p19$^{ARF}$* silencing by biallelic deletion are the two most common routes to spontaneous p53/p19$^{ARF}$ pathway inactivation. The p16/Rb pathway, a senescence master regulator, is also commonly affected during the immortalization. These genetic alterations are also common in human tumors (Odell *et al.*, 2010).

Prevalence of cell lines immortalized by *p19$^{ARF}$* biallelic deletion or *p53* mutations was not unexpected because most 50% of cell lines depending on immortalization protocol, but the surprised is that a significant fraction of cell lines appeared to have retained WT p53 and p19$^{ARF}$ expression (Odell *et al.*, 2010).

The tumor suppressor p53 has an important role in cell cycle, apoptosis, and senescence, inducing factors such as p21 following DNA damage and p16 as a result of premature senescence (Yu *et al.*, 2012; Charni *et al.*, 2016).

Since the immortalization event in W3T3 cells is unknown, we analyze if these cells maintained an active p53 pathway or if these results are due to p53 inactivation. For that, we treated the cells with 0.5µg/mL doxorubicin for 16 hours and analyze the expression of p53 and p21 proteins. Doxorubicin is a chemotherapeutic agent, which can induce apoptosis by the activation of p53 pathway.

Although we didn't observe an increase in p53 protein levels in cells treated with doxorubicin, p21 expression was increased. W3T3 Ras cells seem to have higher basal levels of p21 (something that doesn't happen with the other cell lines; Figure 15). This demonstrates that the p53 pathway remains active in W3T3 cells because we could detect the expression of p21 protein upon p53 inactivation. Thus, p53 inactivation is not the immortalization event in W3T3 cells since these cells behave as p53 positive.

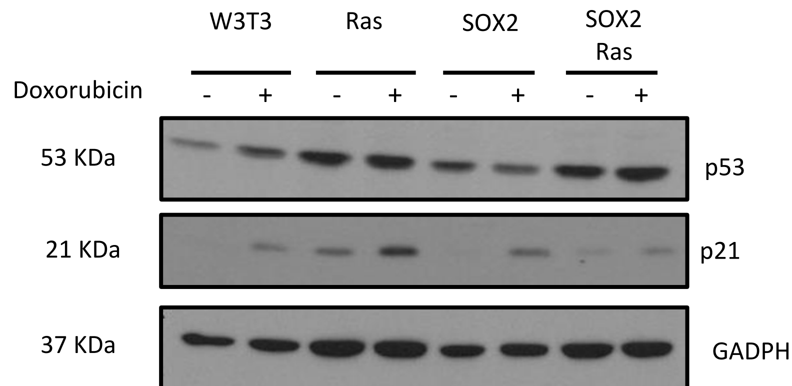


**Figure 15. W3T3 cells infected with SOX2 and H-RasV12 plasmids maintain functionally active p53.** The cells were plated $8x10^5$ cells/100mm dish, grown for 24 hours, and treated with 0.5µg/mL of doxorubicin for 16 hours. The expression of p53 and p21 were determinate by western blotting (A representative result of two independent experiments is shown).

The p53 tumor suppressor is activate and promote the cellular senescence and apoptosis in response to cellular stress, such as the overexpression of Ras (Ramadoss *et al.*, 2016). To determine if p53 is a key factor in increase of SOX2 expression via RasV12, once p53 is functional in W3T3 cells as present in previous results, we infected W3T3 p53 knockdown cells with RasV12 and detect the expression of SOX2 48 hours after infection. We also observed the expression of SOX2 in W3T3 Rb knockout cells (D3T3), where we aren't able to detect the SOX2 expression. We study the expression of other factors as p53 and $p19^{ARF}$, we observed that p53 expression is increased in W3T3 Ras cells and in D3T3 cells the expression of this protein stabilized. The

expression of $p19^{ARF}$ was increased in W3T3 Ras and D3T3 Ras cells (Figure 16). The p53 factor isn't required for SOX2 induction in W3T3 cells upon RasV12 expression.

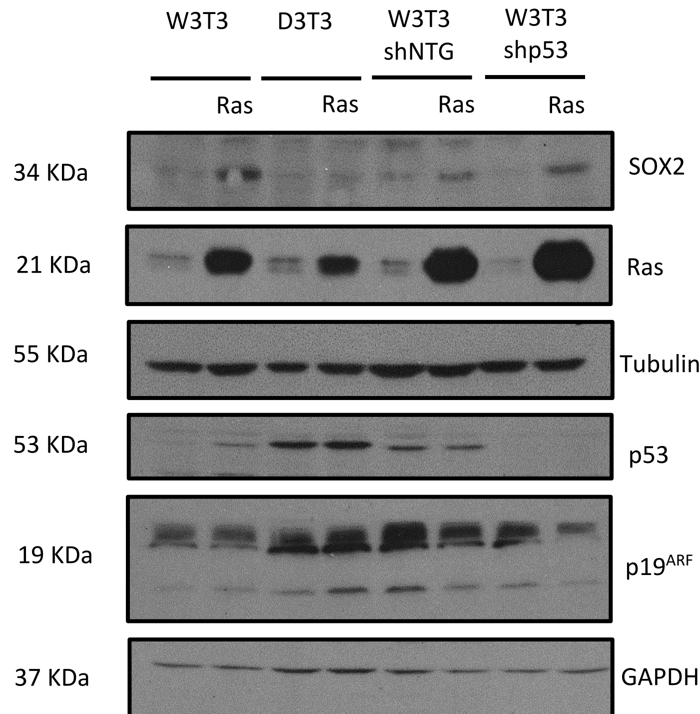


**Figure 16. Expression of SOX2 increases upon RasV12 expression in W3T3, W3T3 shNTG, and W3T3-shp53 cell lines, but not in D3T3 cells.** The W3T3, D3T3, W3T3 shNTG, and W3T3 shp53 cells were infected with H-RasV12 plasmid and collected 48 hours after the infection. The amounts of SOX2, Ras, p53, and $p19^{ARF}$ were assessed by western blotting (A representative result of two independent experiments is shown).

As p53 is stabilized in D3T3 control and Ras cells (figure 15), we wanted to test if p53 remained functional in these cells. Thus, cells were treated with 0.5µg/mL doxorubicin for 16 hours and p53 and p21 expression was measured by western blotting.

We didn't observe the expression of p21 in D3T3 cells treated with doxorubicin, suggesting that p53 is inactive in these cells. So, D3T3 cells are functionally Rb and p53 knockout (Figure 17).

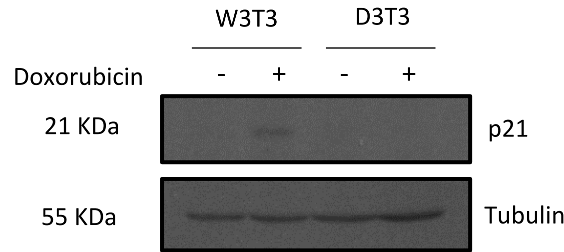


**Figure 17. D3T3 cells don't have active the p53 pathway.** The cells were plated $8x10^5$ cells/100mm dish, grown for 24 hours, and treated with 0.5µg/mL of doxorubicin for 16 hours. The expression of p53 and p21 were determinate by western blotting (A representative result of two independent experiments is shown).

To confirm if the D3T3 cells infected with RasV12 induce SOX2 or not, we selected the cells infected and detected SOX2 expression by western blotting method. After selection, we observed the induction of SOX2 expression by RasV12 in D3T3 cells (Figure 18). Thus, Rb is not required for SOX2 induction by RasV12 expression, although Rb deficiency could delay the induction of SOX2.

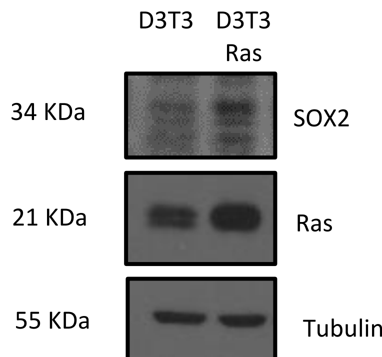


**Figure 18. RasV12-mediated SOX2 induction in D3T3 cells.** The D3T3 cells were infected with H-RasV12 plasmid, selected, and the amounts of SOX2 and Ras determinate by western blotting (A representative result of two independent experiments is shown).

As we don't know the immortalization event of W3T3 cells and, to rule out the influence of other genetic effects, we switch to a genetically defined system as primary mouse embryo fibroblasts (MEFs). We infected WT MEFs, $p53^{-/-}$ MEFs, $ARF^{-/-}$ MEFs, and C39 TAg with RasV12 and detected the expression of SOX2, Ras, p53, and $p19^{ARF}$ 48 hours after the infection. We observed the increase of SOX2 expression in all cell lines that expressed RasV12, except in the $ARF^{-/-}$ MEFs cells where we weren't able to detect the expression of SOX2. In the C39 TAg cells

the increased of SOX2 expression it's not so obvious because the high basal level of SOX2 expression in these cells. The overexpression of RasV12 leads to the increased of SOX2 expression in cells knockout for p53 and Rb, but not in $p19^{ARF}$ knockout cells. Thus, these results confirmed that p53 and Rb are not required for SOX2 induction upon RasV12 expression, but $p19^{ARF}$ could be required in the induction of SOX2.

We can't observe the expression of p53 in WT MEFs and $ARF^{-/-}$ MEFs Ras cells and in C39 TAg cells, TAg inactivates and stabilizes p53. The $p19^{ARF}$ expression is increased in WT MEFs and $p53^{-/-}$ MEFs cells upon RasV12 expression since $p19^{ARF}$ is induced in the presence of oncogenic RasV12 (Figure 19).

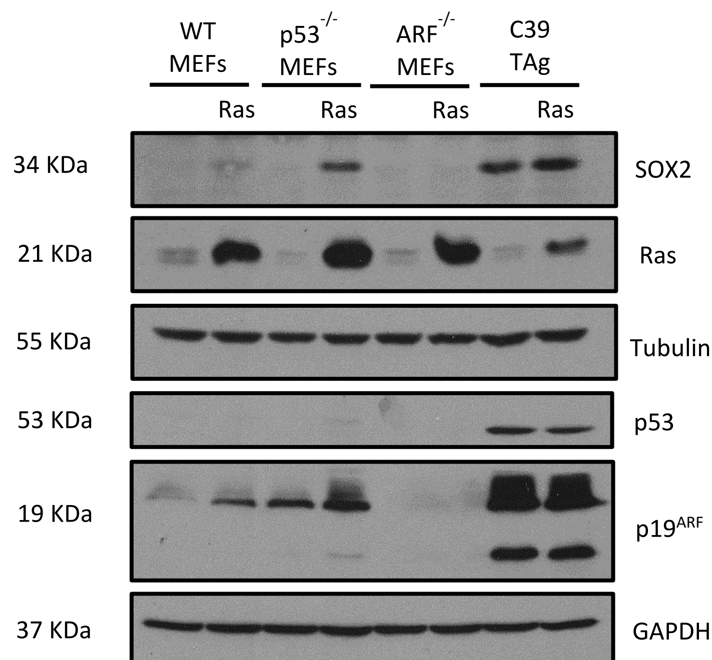


**Figure 19. RasV12 mediated SOX2 induction in WT MEFs, $p53^{-/-}$ MEFs, and C39 TAg cell lines, but not in $ARF^{-/-}$ MEFs cell line.** The MEFs, $p53^{-/-}$ MEFs, $ARF^{-/-}$MEFs, and C39 TAg cells were infected with H-RasV12 plasmid and collected 48 hours after the infection. The amounts of SOX2, Ras, p53, and $p19^{ARF}$ were determinate by western blotting (A representative result of two independent experiments is shown).

SOX2 is induced by oncogenic RasV12 in W3T3, D3T3, W3T3 shNTG, W3T3 shp53, WT MEFs, $p53^{-/-}$ MEFs, and C39 TAg cells.

We wanted to confirm if p53 is functional in these cells because we can't observe the expression of p53 in WT MEFs and $ARF^{-/-}$ MEFs Ras cells, as shown in figure 18. The cells are treated with 0.5µg/mL doxorubicin for 16 hours and detected the p53 and p21 expression by western blotting.

In cells WT MEFs and $ARF^{-/-}$ MEFs cells treated with doxorubicin we detected p21 expression, as well as p53 expression in the last cell line. Thus, the WT MEFs and $ARF^{-/-}$ MEFs cells have the p53 pathway functional (Figure 20).

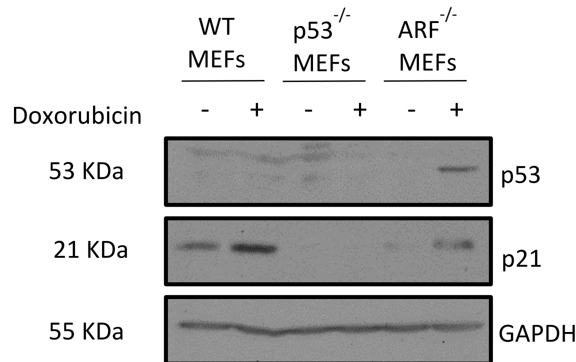


**Figure 20. WT MEFs and $ARF^{-/-}$ MEFs have an active p53 pathway.** The cells were plated $8x10^5$ cells/100mm dish, grown for 24 hours, and treated with 0.5µg/mL of doxorubicin for 16 hours. The expression of p53 and p21 were determinate by western blotting (A representative result of two independent experiments is shown).

## RasV12 induces SOX2 expression at transcriptional level

As observed in previous results RasV12 induced the expression of SOX2 and to determine if this induction is at the level of transcription, we analyze the amount of SOX2 mRNA in cells that overexpressed RasV12. The WT MEFs and ARF$^{-/-}$ MEFs cells were infected with RasV12 and 48 hours after the cells are collected and SOX2 mRNA analysis by RT quantitative PCR.

The SOX2 mRNA mediated by RasV12 is higher in WT MEFs cells than in ARF$^{-/-}$ MEFs cells (5.1±0.75 versus WT MEFs Ras values of 40.7±32). Cells with overexpression of RasV12 have increased levels of SOX2 mRNA, so the oncogenic RasV12 induce the transcription of *SOX2* gene (Figure 21).

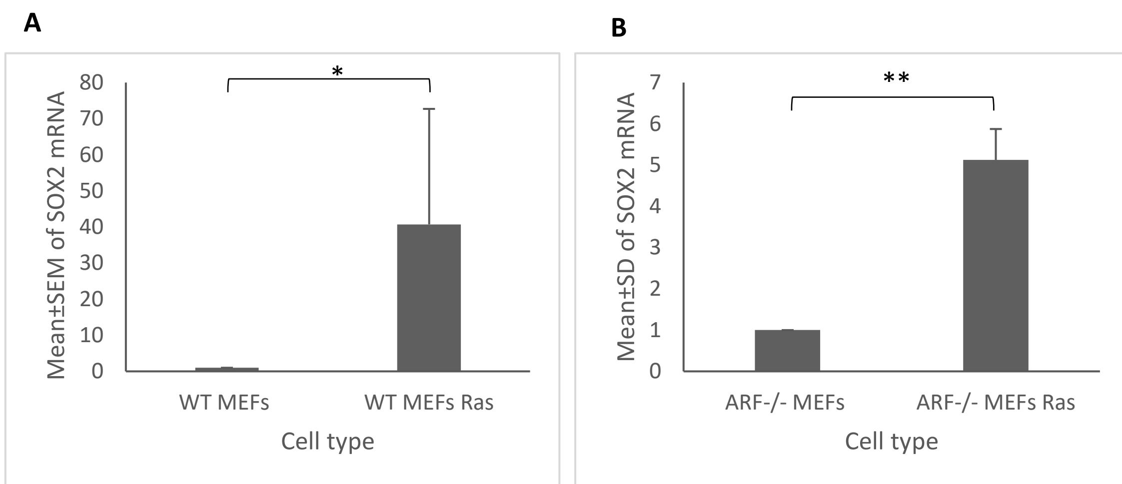


**Figure 21. The transcription factor SOX2 is induced by oncogenic RasV12 at the level of transcription.** The (A) WT MEFs and (B) ARF$^{-/-}$ MEFs cells were infected with H-RasV12 plasmid and collected the RNA 48 hours after the infection. The level of SOX2 mRNA was analyzed by RT quantitative PCR (A representative result of three independent experiments is shown). Graphic showing the mean ± SD of amount of SOX2 mRNA for each cell line (*p<0.05, **p<0.01).

In previous results we observed through protein expression that in MEFs cells KO for ARF (ARF$^{-/-}$MEFs) the oncogenic RasV12 couldn't induce the expression of SOX2 transcription factor 48 hours after the infection with H-RasV12, as we observed in other cell lines (Figure 19). However, through RT quantitative PCR we observed the induction of SOX2 mRNA mediated by RasV12 in the same cells 48 hours after the infection with H-RasV12 plasmid (Figure 21B).

We couldn't detect the protein expression of SOX2 in ARF$^{-/-}$ MEFs cells probably because SOX2 undergoes some post-translational modification, for example SUMOylation.

We conclude that RasV12 induced the expression of SOX2 in ARF$^{-/-}$ MEFs cells, so p19$^{ARF}$ isn't an essential factor for the induction of SOX2 thought the Ras pathway.

## Signalling pathways downstream of Ras that mediate SOX2 induction

Activation of Ras is one of the most common molecular events in human cancer since Ras protein interacts directly with numerous effector proteins, which then induce cellular responses. Mutations in codon 12 of Ras, for example V12, prevent GTP hydrolysis resulting in a constitutively active form of Ras protein (McFarlin *et al.*, 2003).

The activated form of Ras (H-RasV12) induced the expression of the transcription factor SOX2, as shown the previous results, but we don't know which pathway downstream of Ras is responsible for the induction of SOX2. To determine which Ras signalling pathway is involved in SOX2 induction, we selectively activated different Ras pathways using RasV12 mutants for each pathway or, alternatively inhibited the PI3K/AKT and MAPK pathways involved in survival and proliferation using pharmacological inhibitors.

To activate the different Ras pathways, we used three mutants of H-RasV12 C40, E38, and G37. The mutant C40 (H-RasV12C40) have a mutation from tyrosine to cysteine in codon 40 that retains the affinity with PI3K, effector protein of Ras, but greatly reduces the affinity with RalGDS and Raf. Mutations from aspartic acid to glutamic acid in codon 38 (H-RasV12E38) maintain the affinity with Raf, but lacks affinity with RalGDS and PI3K. The mutant H-RasV12G37 have a mutation from glutamic acid to glycine in codon 37 that reduces the affinity with Raf and PI3K, but retains the affinity with RalGDS (McFarlin *et al.*, 2003).

We infected the W3T3 cells with pBabe Puro, pBabe Puro H-RasV12, and the three H-RasV12 mutants, C40, E38, and G37. The cells were selected until death of cells control and observed the expression of SOX2 and Ras in the different mutants by western blotting. Only the infection with the RasV12E38 mutant was able to cause a significant increase in the expression of Ras protein, while in the C40 and G37 mutants we don't detect the expression of Ras. However, when the MAPK pathway is activated by E38 mutant we didn´t observe the increase of SOX2 expression as when all Ras pathways are activated (W3T3 Ras cells). We analyzed also ERK expression to prove if the MAPK pathway is, effectively, activated. We detected the expression of ERK in H-RasV12E38 mutant, so this mutant activated the MAPK pathway, but the activation of this cascade isn't sufficient to induce SOX2 expression as occurs in cells that have all Ras pathways activated. We failed to detect the other RasV12 mutants, so we cannot conclude if these are sufficient or not to induce SOX2 (Figure 22).

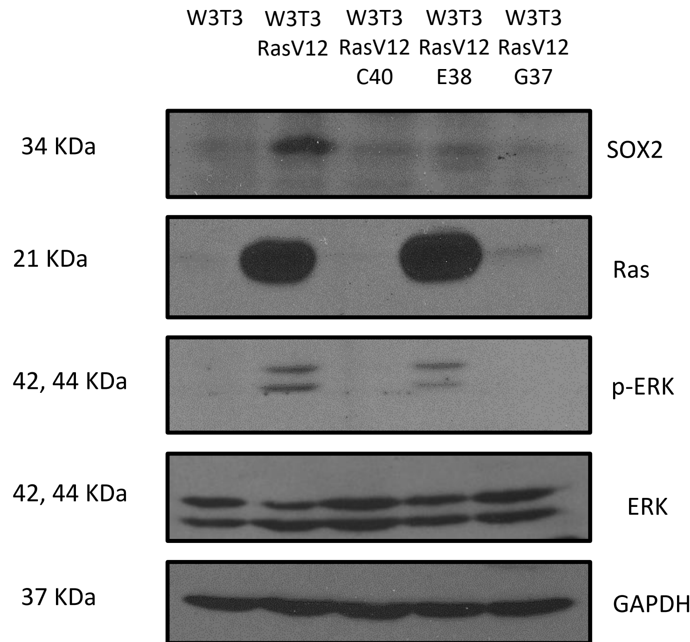


**Figure 22. The overactivation of MAPK pathway by H-RasV12E38 mutant isn't enough for induction of SOX2 expression by RasV12.** The W3T3 cells were infected with H-RasV12 and three different H-RasV12 mutants (C40, E38, and G37) and selected. The expression of SOX2, Ras, phospho-ERK, and total ERK was observed by western blotting (A representative result of three independent experiments is shown).

We next tested which pathway could be required for SOX2 induction through the inhibition of two main Ras pathways, the PI3K/AKT and MAPK cascades. We started by confirming if the W3T3 pInducer H-RasV12 cells induced the expression of Ras in the presence of doxycycline and determining the shortest time in which we could observe the increase in SOX2 mRNA expression. The W3T3 cells were infected with pInducer H-RasV12 plasmid, selected, treated with 6µg/µl doxycycline for 24 hours, and observed the expression of Ras by western blotting. We observed the increased of Ras expression in these cells 24 hours after added doxycycline, so we could use this model for the next steps (Figure 23A).

To determine the shortest time in which it is observed the induction of SOX2 mRNA expression mediated by RasV12, we treated the W3T3 pInducer H-RasV12 with doxycycline during 0, 1, 2, 3, 4, 6, 8, 12, and 24 hours and analysis the amount of SOX2 mRNA by RT quantitative PCR. We observed the induction of SOX2 mRNA expression six hours (2.4 for six hours versus 1.2 for 0 hours) after the activation of RasV12 expression using doxycycline (Figure 23B).

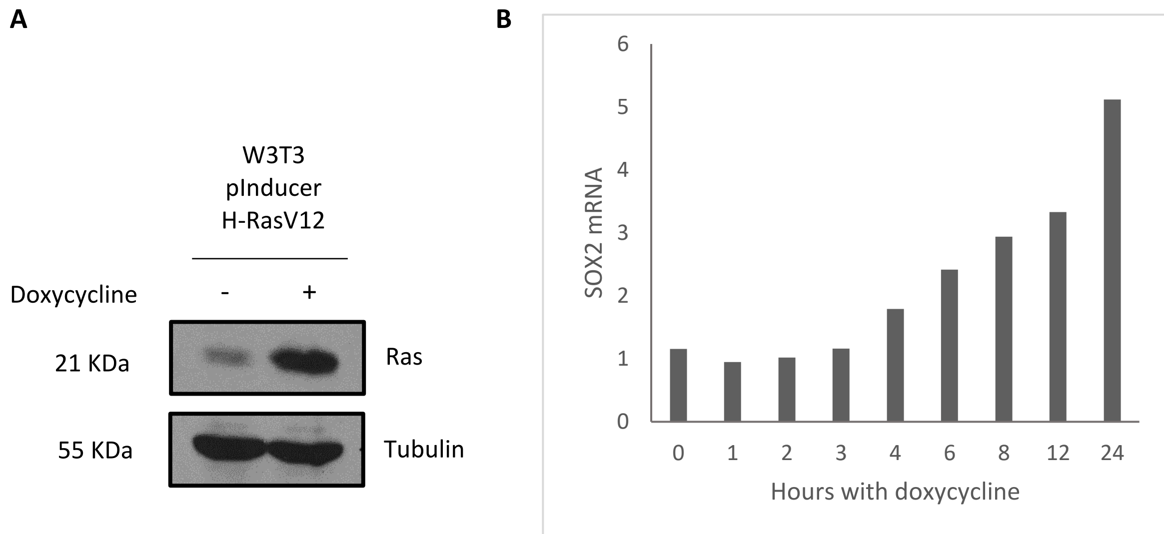


**Figure 23. (A) Doxycycline induced the Ras protein expression in W3T3 pInducer H-RasV12 cells.** Cells were treated with 6µg/µl doxycycline for 24 hours and detected Ras expression by western blotting. **(B) RasV12 induced the SOX2 mRNA expression six hours after the activation of RasV12 with doxycycline in W3T3 pInducer H-RasV12 cells.** Cells were treated with 6µg/µl doxycycline for 0, 1, 2, 3, 4, 6, 8, 12, and 24 hours and the SOX2 mRNA expression analyzed by RT quantitative PCR. Graphic shows the amount of SOX2 mRNA for different times with doxycycline.

To confirm if all inhibitors prevent the activation of the respective Ras pathway, we treated the W3T3 pInducer H-RasV12 cells in serum deprivation with 10µM PD98059 for six hours, 15µM SB202190 for one hour, 20µM LY294002 for five hours, 2.5µM MK-22062 for four hours, and 1µM GDC-0068 for two hours and analyzed the expression of Ras, phospho-ERK, and total ERK by western blotting. We observed that in presence of 6µg/µl doxycycline for six hours the expression of RasV12 is activated, the MAPK inhibitor PD98059 in these conditions inhibit this pathway because we detect the decrease of phospho-ERK expression, but the SB202190 don't inhibit this pathway in under these conditions (Figure 24A).

To analyze the amount of SOX2 mRNA in the presence of Ras inhibitors to know, which is the Ras pathway, PI3K/AKT and MAPK, essential for the induction of SOX2 mediated by RasV12, we treated the W3T3 pInducer H-RasV12 cells in serum deprivation and in presence of 6µg/µl doxycycline with 10µM PD98059 for six hours, 20µM LY294002 for five hours, 2.5µM MK-22062 for four hours, and 1µM GDC-0068 for two hours. After six hours with doxycycline and the time with the respective treatment, we procedure to the RNA extraction and analyzes the amount of SOX2 mRNA by RT quantitative PCR. We observed that when we inhibited the MAPK pathway by PD98059, RasV12 loses the ability to induce the expression of SOX2 (0.53±0.1 versus DMSO control values of 1±0). For the PI3K/AKT inhibitors the values of SOX2 mRNA induction by RasV12

are 1.04±0.36, 1.23±0.22, and 1.27±0.32 for LY294002, MK-22062, and GDC-0068 inhibitors, respectively. This shows that activation of the MAPK pathway is necessary for SOX2 induction mediated by RasV12. We cannot conclude if it's the only essential pathway in the induction of SOX2 expression by RasV12, because we need to confirm if the PI3K/AKT pathway is inhibited with any inhibitor used (Figure 24B).

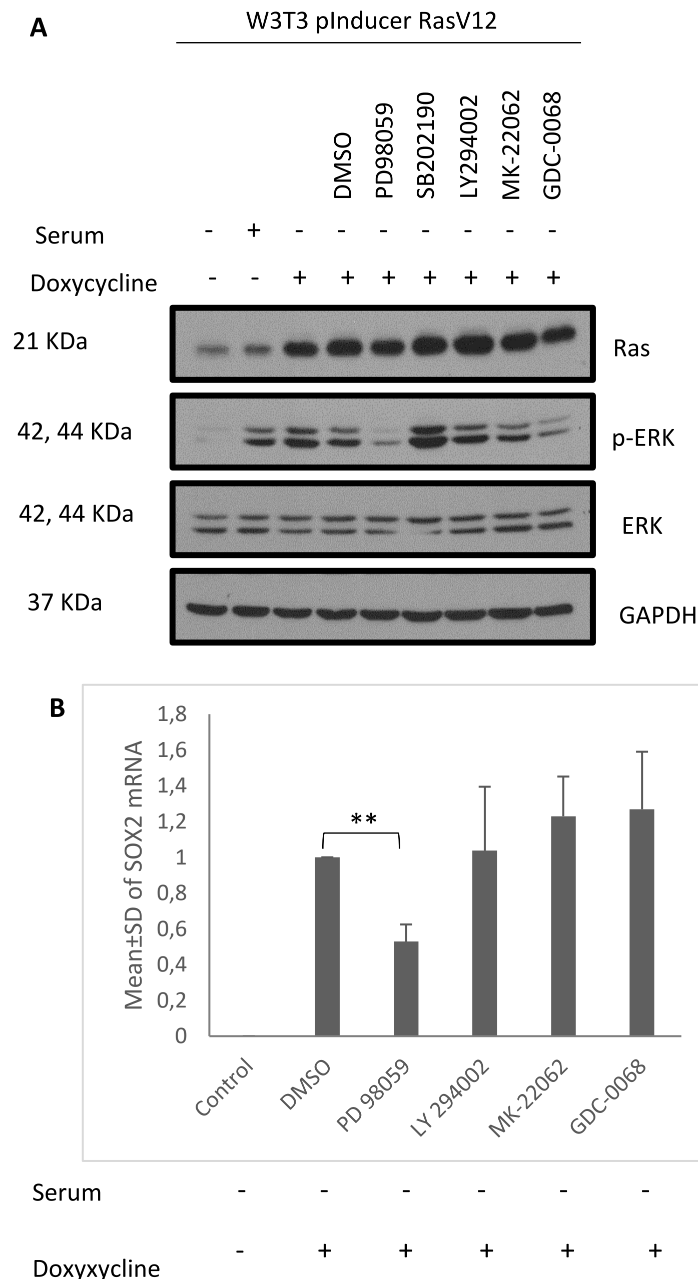


**Figure 24. The MAPK pathway is necessary for SOX2 induction mediated by RasV12.** The W3T3 pInducer H-RasV12 cells were treated in serum deprivation in the presence of 6µg/µl doxycycline with 10µM PD98059 for six hours, 15µM SB202190 for one hour, 20µM LY294002 for five hours, 2.5µM MK-22062

for four hours, and 1µM GDC-0068 for two hours. (A) The expression of Ras, p-ERK, and total ERK proteins were analyzed by western blotting. (B) The amount of SOX2 mRNA was analyzed by RT quantitative PCR (A representative result of three independent experiments is shown). Graphic showing the mean ± SD of amount of SOX2 mRNA for controls and the different treatments (**$p<0.01$).

## SOX2 mediates transformation by RasV12

Previous results showed that the overexpression of SOX2 increase the oncogenic transformation of RasV12 and that RasV12 induces the expression of SOX2.

To determine if SOX2 is an important factor for the oncogenic transformation, we silenced the *SOX2* gene using a shRNA in immortalized W3T3 and in $p53^{-/-}$ MEFs cells. Next, we infected the shRNA cells with H-RasV12, selected the cells, and seeded for a cell foci assay. We checked the effect of the SOX2 knockdown by western blotting 48 hours after the infection with H-RasV12.

We observed that RasV12 was able to induce SOX2 in W3T3 and $p53^{-/-}$ MEFs cells when a control shRNA was used. The shSOX2#1 was not able to prevent SOX2 induction in W3T3. Interestingly, the cells infected with the second shRNA against SOX2 (shSOX2 #2) was able to prevent increase of SOX2 expression upon RasV12, as we intended (Figure 25).

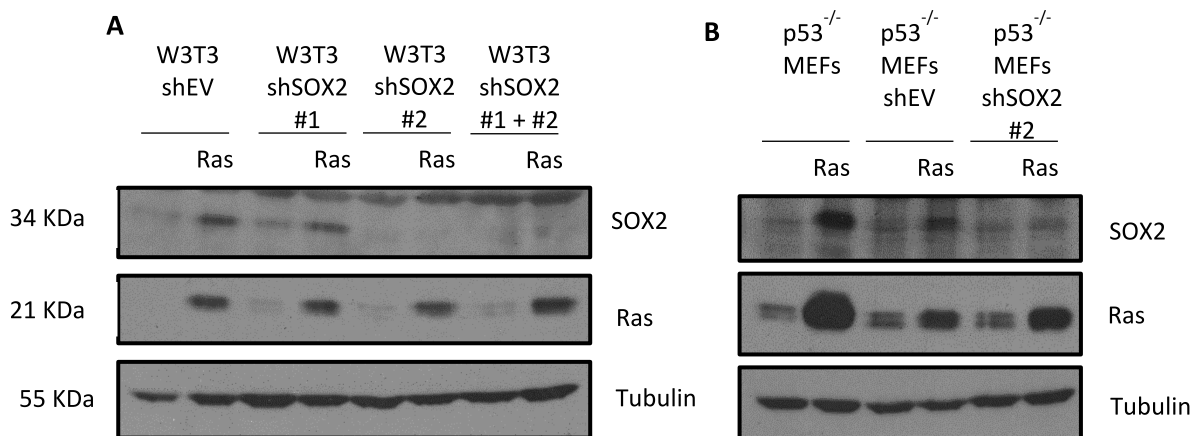


**Figure 25. Efficient knockdown of SOX2 in W3T3 cells and $p53^{-/-}$ MEFs.** (A) The W3T3 cells were infected with empty vector (EV) and two vectors of shSOX2 (#1 and #2) and selected. (B) The $p53^{-/-}$ MEFs cells were infected with empty vector (EV) and shSOX2 #2 vector and selected. The W3T3 and $p53^{-/-}$ MEFs cells shEV and shSOX2 were infected with RasV12 and 48 hours after the infection collected some cells to analyze the SOX2 and Ras protein expression by western blotting (A representative result of three independent experiments is shown for W3T3 cells and a representative result of two independent experiments is shown for $p53^{-/-}$ MEFs cells).

We plated cell foci assays with the control cells (shNTG), and cells with SOX2 down regulated (shSOX2) after the cell selection. We observed that W3T3 shSOX2 RasV12 form fewer cell foci that the control cells (W3T3 shNTG) which express SOX2 (43.8±4.5 for W3T3 shSOX2#2 Ras and 37.1±13.3 for W3T3 shSOX2#1+#2 Ras versus W3T3 shNTG Ras values of 243.0±23.6). In $p53^{-/-}$ MEFs shSOX2 RasV12 we also observed less cell foci and these are smaller than control

cells ($p53^{-/-}$ MEFs shNTG) (58.2±9.8 for $p53^{-/-}$ MEFs shSOX2 Ras versus $p53^{-/-}$ MEFs shNTG Ras values of 83.7±9.0) (Figure 26).

Since, SOX2 knockdown in RasV12 cells leave to formation of fewer foci cell, so RasV12 can't increase the SOX2 expression and its oncogenic transformation capacity decreases.

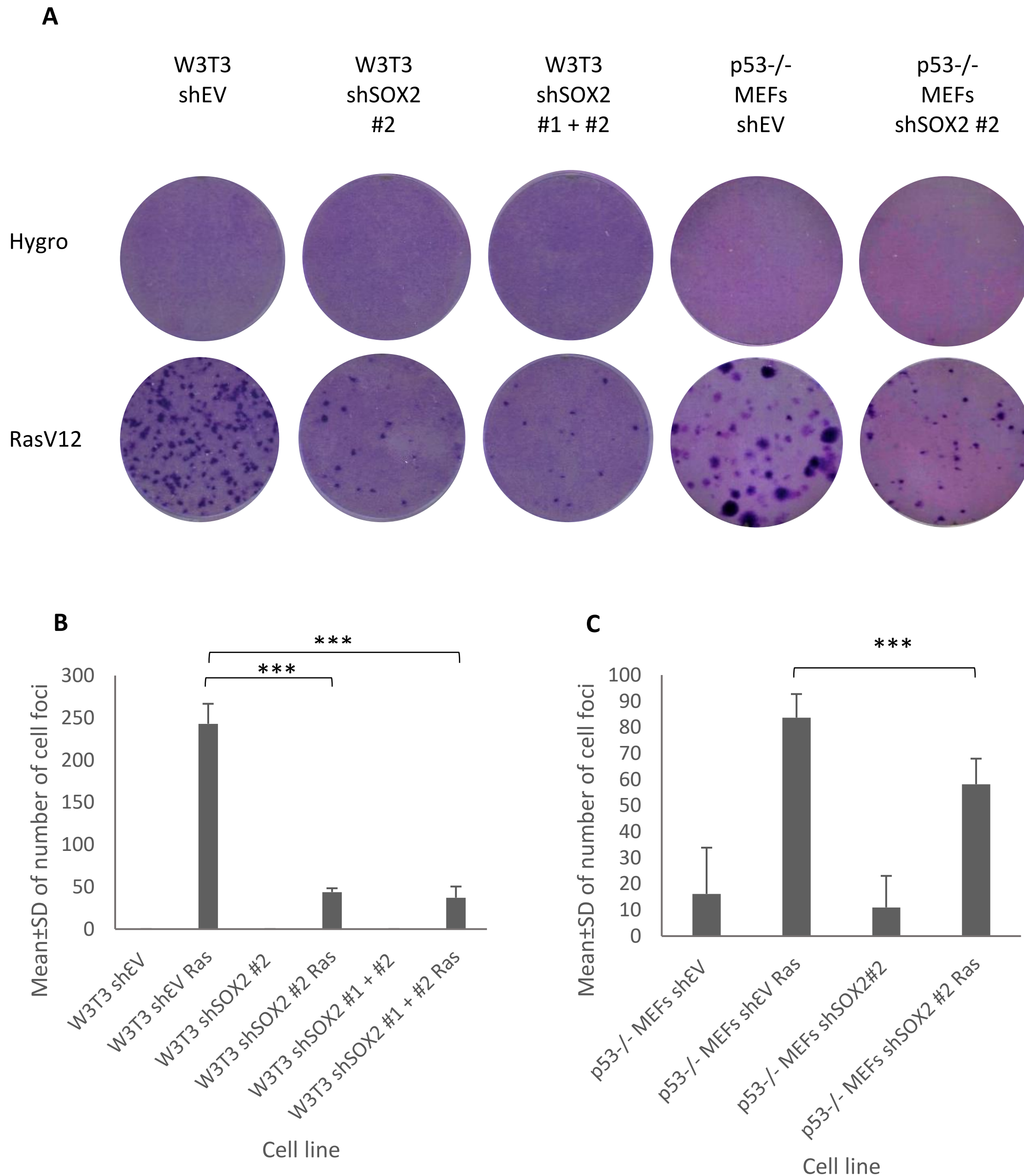


**Figure 26. The transcription factor SOX2 is important for cell transformation mediated by oncogenic RasV12.** (A) Foci formation by crystal violet staining of W3T3 and $p53^{-/-}$ MEFs cells infected with shSOX2 and H-RasV12 plasmids, selected, and 400 of these cells were plated with $4x10^5$ W3T3 cells and grown 11 days prior to staining and counting of foci. Graphics showing the number of cell foci for each (B) W3T3 and (C) $p53^{-/-}$ MEFs cell lines and, data are shown as mean ± SD of the number of cell foci (***p<0.001). This experiment was repeated thrice for each clone (A representative result of three independent

experiments is shown for W3T3 cells and a representative result of two independent experiments is shown for p53$^{-/-}$ MEFs cells).

## SOX2 overexpression increases the resistance of cancer cells to drug therapy

SOX2 not only influences tumor growth, but it also influences the response of tumor cells to drugs used clinically (Wuebben *et al.*, 2016). The development of resistance to therapy is a clinical problem in several cancers, such as breast and lung cancer (Chou *et al.*, 2013; Piva *et al.*, 2014), and the endocrine treatment failure and poor relapse-free survival is correlated with higher levels of SOX2 (Piva *et al.*, 2014).

To study if SOX2 overexpression could cause the development of resistance to drugs used in chemotherapy, we used two tumor cell lines transfected with SOX2 to induce overexpression. We treated control and SOX2 human lung carcinoma (A549) cells with doxorubicin and docetaxel, and human breast adenocarcinoma (MCF7) cells with doxorubicin, docetaxel, and gemcitabine for 48 hours and analyzed the cell viability by MTT assay.

Before starting the treatments, we confirmed the SOX2 expression in these cells by western blotting, and we observed that the A549 and MCF7 cells infected with pMx-SOX2-IP vector expressed higher SOX2 levels when compared to control cells (Figure 27).

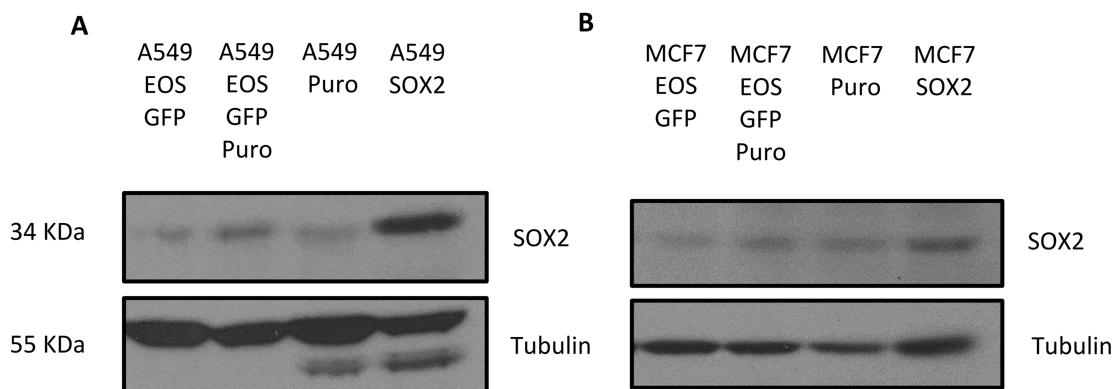


**Figure 27. Increased expression of SOX2 in (A) A549 SOX2 and (B) MCF7 SOX2 cell lines.** The A549 and MCF7 cells were infected with EOS GFP, EOS GFP Puromycin, pBabe Puro, and pMx SOX2 IP vectors from Manuel Collado's group, IDIS. The expression of SOX2 protein were determinate by western blotting.

Our treatment results show that in human lung carcinoma (A549) cells the overexpression of SOX2 factor doesn't increase the resistance of these cells to doxorubicin (Figure 28) and docetaxel (Figure 29), when we compared with the control cells with basal levels of SOX2. However, SOX2 overexpression also doesn't make the A549 cells more sensitive to the treatment. In human breast adenocarcinoma cells (MCF7) the overexpression of SOX2 factor didn't make the cells more resistant or sensitives to doxorubicin (Figure 30) and gemcitabine (Figure 31) drugs. However, MCF7 SOX2 cells treated with docetaxel are more resistance to the therapy in the concentration of 25, 50, 100, and 400nM (0.319±0.03, 0.287±0.04, 0.138±0.02, and 0.189±0.02 for MCF7 SOX2 versus MCF7 Puro values of 0.142±0.03, 0.168±0.02, 0.027±0.03, and 0.088±0.02, respectively) (Figure 32).

SOX2 overexpression makes the human breast adenocarcinoma cells more resistances to docetaxel drug, a paclitaxel derivative used in chemotherapy.

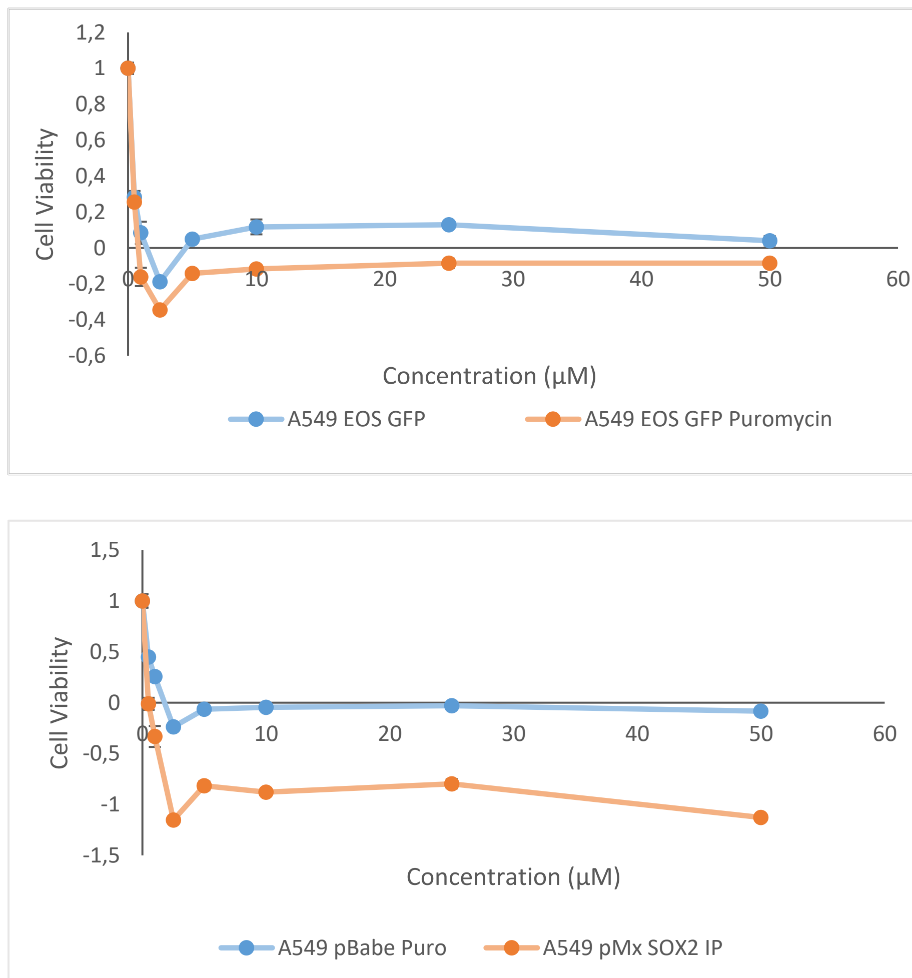


**Figure 28. SOX2 overexpression doesn't increase the resistance of A549 cells to doxorubicin.** Cells were treated with 0, 0.5, 1, 2.5, 5, 10, 25, and 50µM of doxorubicin for 48 hours and the cell viability measure by MTT assay. The graphics represents the cell viability for the different concentration. Data are shown as

mean ± SD of cell viability. This experiment was repeated thrice for each concentration (A representative result of two independent experiments is shown).

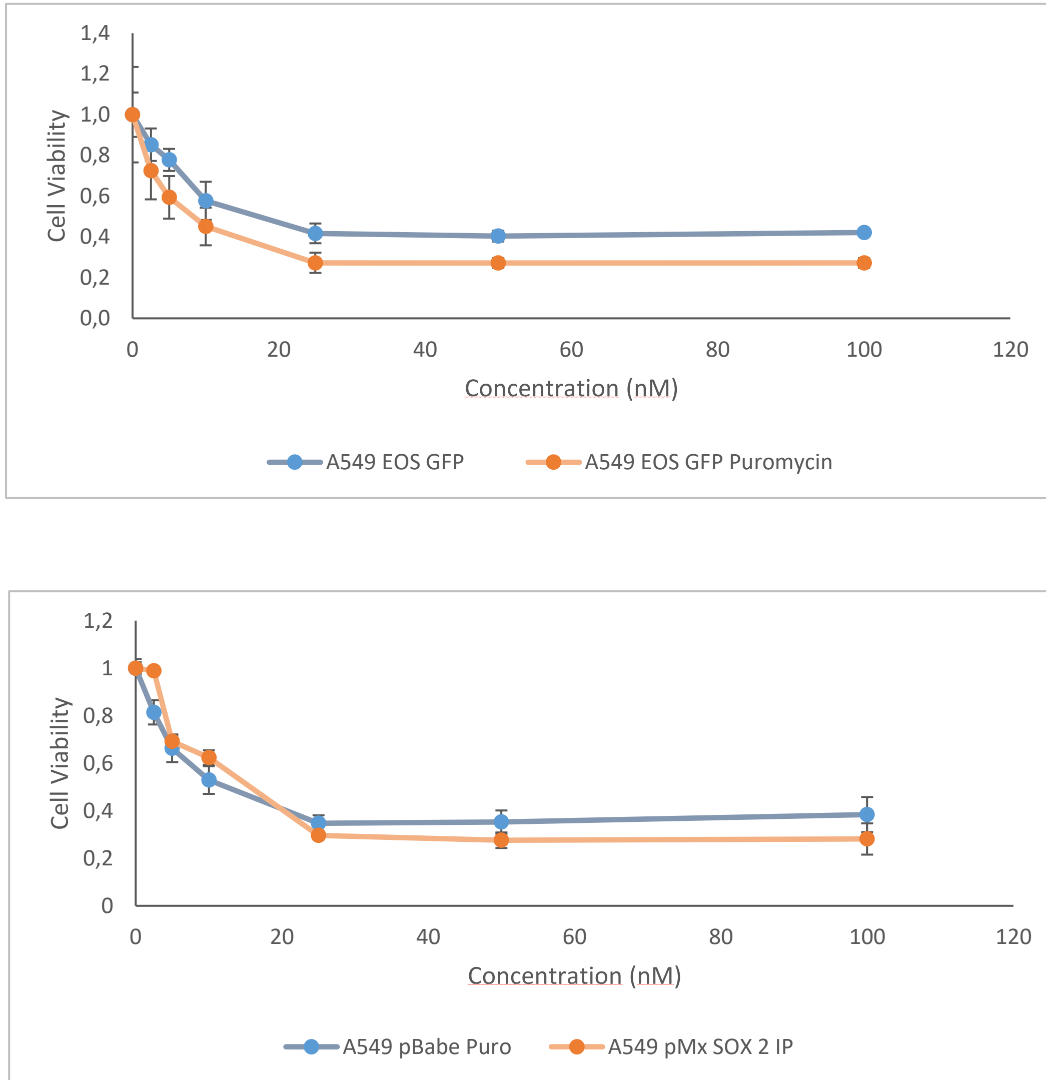


**Figure 29. SOX2 overexpression doesn't increase the resistance of A549 cells to docetaxel.** Cells were treated with 0, 2.5, 5, 10, 25, 50, and 100 nM of docetaxel for 48 hours and the cell viability measure by MTT assay. The graphics represents the cell viability for the different concentration. Data are shown as mean ± SD of cell viability. This experiment was repeated thrice for each concentration (A representative result of two independent experiments is shown).

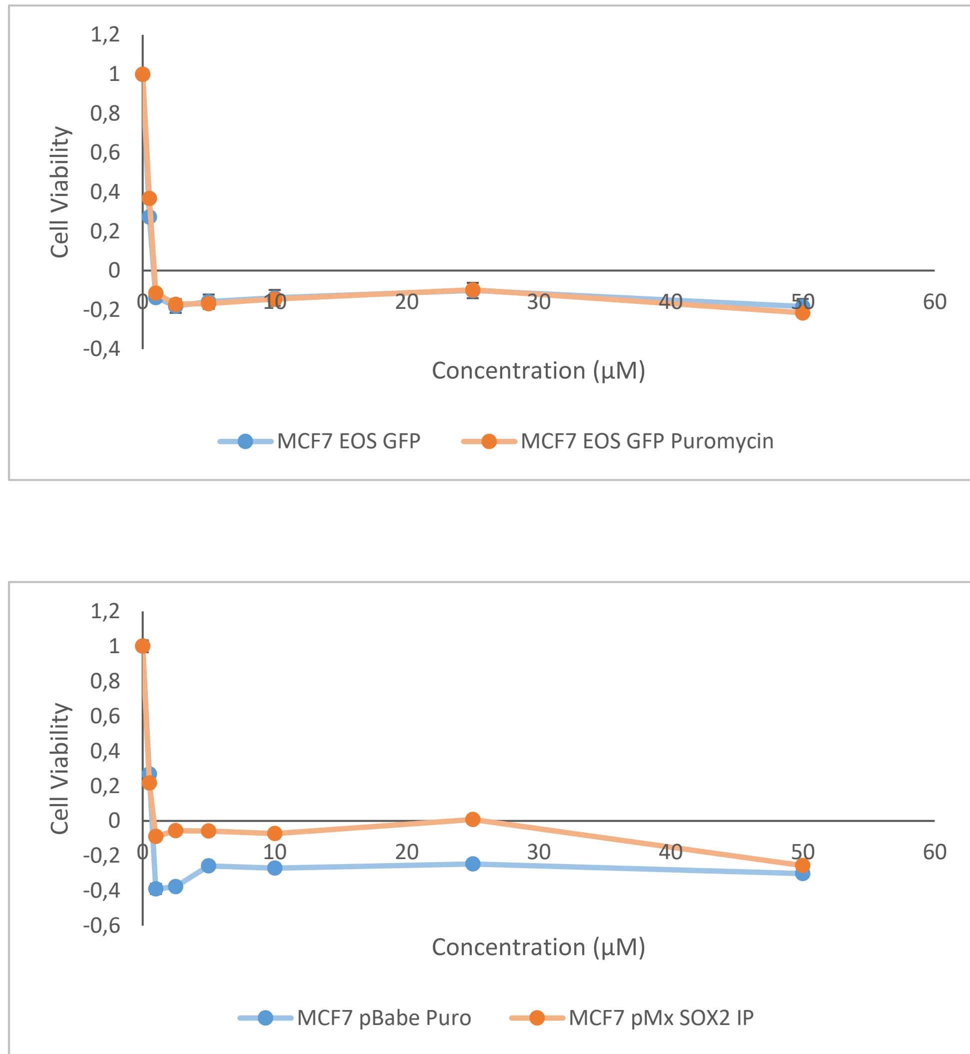


**Figure 30. SOX2 overexpression doesn't increase the resistance of MCF7 cells to doxorubicin.** Cells were treated with 0, 0.5, 1, 2.5, 5, 10, 25, and 50 µM of doxorubicin for 48 hours and the cell viability measure by MTT assay. The graphics represents the cell viability for the different concentration. Data are shown as mean ± SD of cell viability. This experiment was repeated thrice for each concentration (A representative result of four independent experiments is shown).

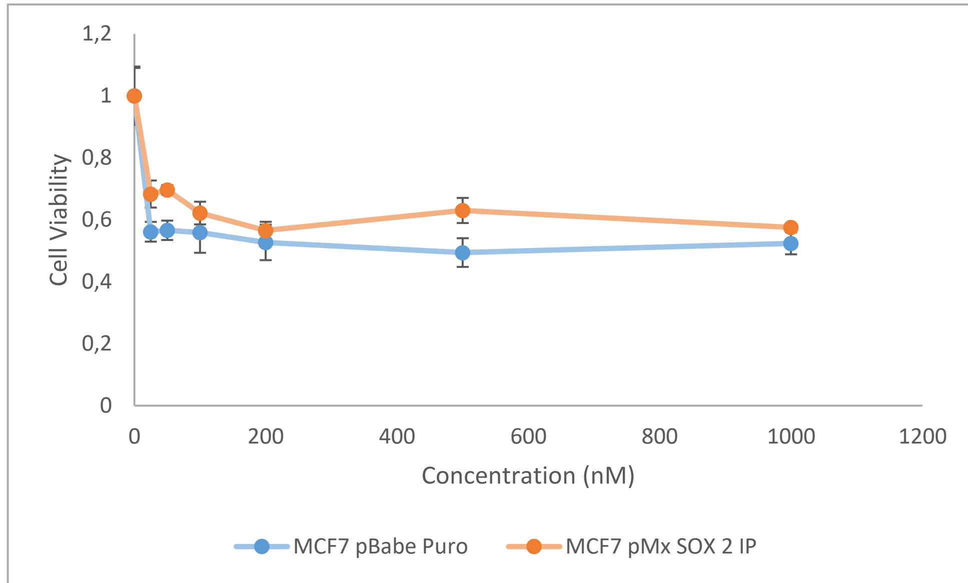


**Figure 31. SOX2 overexpression doesn't increase the resistance of MCF7 cells to gemcitabine.** Cells were treated with 0, 25, 50, 100, 200, 500, and 1000 nM of gemcitabine for 48 hours and the cell viability measure by MTT assay. The graphics represents the cell viability for the different concentration. Data are shown as mean ± SD of cell viability. This experiment was repeated thrice for each concentration (A representative result of four independent experiments is shown).

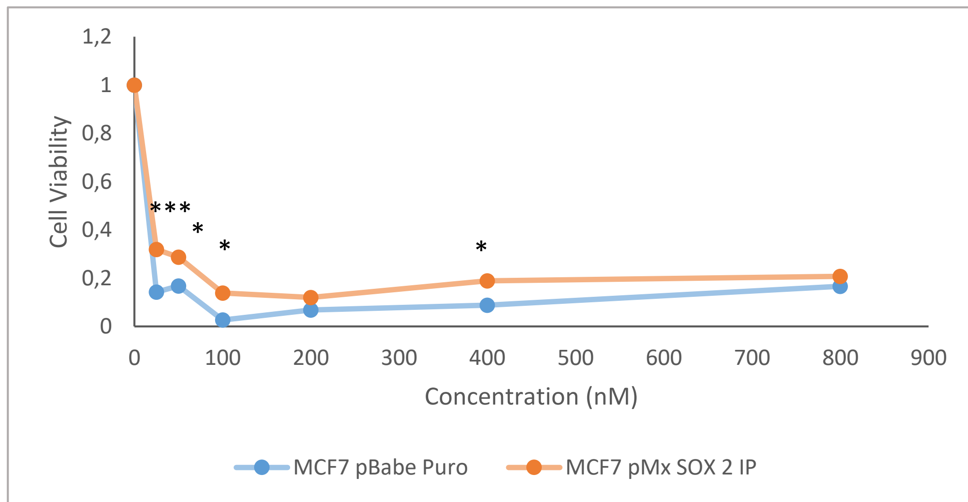


**Figure 32. SOX2 overexpression increases the resistance of MCF7 cells to docetaxel.** Cells were treated with 0, 25, 50, 100, 200, 400, and 800 nM of docetaxel for 48 hours and the cell viability measure by MTT assay. The graphics represents the cell viability for the different concentration. Data are shown as mean ± SD of cell viability (*p<0.05; ***p<0.001). This experiment was repeated thrice for each concentration (A representative result of five independent experiments is shown).

# Discussion

The transcription factor SOX2 is essential for maintaining the pluripotency and self-renewal of embryonic stem cells and play an important role in the reprogramming of differentiated somatic cells into pluripotent stem cells (Bareiss *et al.*, 2013).

Life is not possible without SOX2 expression, in particular during the embryogenesis, but this factor also has a dark side when we talk about tumorigenesis. In the last years, *SOX2* expression has been detected in several tumors, including ovary, lung, skin, brain, breast, and prostate, suggesting that SOX2 also regulates tumorigenesis. SOX2 is a cancer stem cell marker and can induce stem cells properties such as stemness, tumor-initiating capacity, and apoptosis resistance and is also implicated in growth, drug resistance, metastasis, and poor survival. However, only recently the study of the effects of this factor on tumorigenicity, prognosis, and drug resistance in human cancer has begun (Bareiss *et al.*, 2013; Wuebben and Rizzino, 2017).

On the other hand, the implication of Ras proteins in pathological processes, including cancer and physiological processes controlling cellular proliferation, differentiation, and survival has been extensively studied since its discovery 30 years ago. Mutations in *ras* gene are frequent in some of the cancers with the worst prognoses (Fernández-Medarde and Santos, 2011). The Ras activity can lead to senescence, cell death, or in the absence of tumor suppressors, to transformation. This oncogene can be capable of cellular transformation without oncogenic mutation (Logsdon and Lu, 2016).

SOX2 adopts specific roles in individual tumor types because showing divergent expression patterns and function across tumors (Bareiss *et al.*, 2013). Its expression is altered between normal and tumorous tissues, so is important understanding the molecular changes necessary for tumor initiation and progression. Targeting SOX2 expression or its mode of action could be the next step to improve the survival of patients with some of the difficult to treat cancers, but for this is necessary understand mechanistically how SOX2 contributes for the biology of the tumor (Wuebben and Rizzino, 2017).

Despite knowing that SOX2 and Ras are expressed in several types of cancer, the role of SOX2 in oncogenic transformation mediated by Ras isn't much studied. Boumahdi and colleagues refer that SOX2 is essential for growth and maintenance *in vivo* in benign and malignant skin squamous cell carcinoma and has a key role in the tumour initiation. This factor regulates directly and/or indirectly the expression of genes involved in cell proliferation, stemness, chromatin remodelling, and metabolism in primary K-Ras (G12D)- induced skin tumours *in vivo* (Boumahdi *et al.*, 2014). In other hand, Xu and collaborators discuss that in lung

adenocarcinoma the SOX2 overexpression cause Notch inhibition, a direct target of SOX2 transcription factor, and suppresses K-Ras-induced tumors. Thus, SOX2 regulates Notch transcription and the signalling of Notch is important for K-Ras-induced lung adenocarcinoma (Xu *et al.*, 2014).

In the present work, we used different cell lines of immortalized mouse fibroblasts and primary mouse embryonic fibroblasts that overexpressed RasV12 to assess the effect of SOX2 on RasV12-mediated transformation and the genetic requirements for SOX2 induction mediated by RasV12. We also use human lung carcinoma and human breast adenocarcinoma cell lines to study the effect of SOX2 overexpression on drug therapy.

To assess the role of SOX2 in transformation mediated by RasV12 we started by expressing RasV12 and/or SOX2 in immortalized mouse fibroblasts (W3T3) and analyze the transformation capacity of these cells by transformation assay. We showed that cells infected with H-RasV12 plasmid overexpressed Ras, cells infected with SOX2 plasmid overexpressed SOX2, and cells infected with the two plasmids overexpressed SOX2 and Ras, however we also detected overexpression of SOX2 in cells infected with H-RasV12 plasmid. These findings suggest that RasV12 induced the expression of SOX2. In the transformation assay with the same cells we observed that only the cells that overexpressed Ras could form cell foci, however in cells that overexpressed SOX2 and Ras the number of cell foci increased when compared with cells that only overexpressed Ras. These results suggest that SOX2 increases the effect of RasV12 in cell transformation. Castellano and Downward sugest that cooperation between RalGDS and RAF are necessary to induction foci formation in Ras-mediated transformation *in vitro* (Castellano and Downward, 2011). In cell lines derived from many types of cancer the stable overexpression of SOX2 is associated with increase tumor cell growth *in vitro* and *in vivo*. However, SOX2 overexpression inhibits tumor cells growth, as was observed in prostate tumor cell line DU145 and in breast tumor cell lines (Wuebben *et al.*, 2016). The same result was observed by Xu and colleagues wherein SOX2 overexpression inhibits K-Ras induced lung adenocarcinoma formation and leads to adenoma with squamous features (Xu *et al.*, 2014).

In cells that overexpressed SOX2 we observed the loss of its expression after 25 days in culture. Due to this result we tested the acute induction of SOX2 by RasV12 to avoid loss of data. We infected W3T3 cells with H-RasV12 plasmid and analyze the SOX2 expression 48 hours after the infection without selection, we observe that RasV12 induce SOX2 in these conditions.

Gene expression can be regulated by other factors, so to study the genetic requirements for Ras-mediated SOX2 induction we started by observed the p53 and p21 expression in W3T3, W3T3 Ras, W3T3 SOX2, and W3T3 SOX2-Ras cells because mutation in p53 and $p19^{ARF}$ silencing are the two most common immortalization events and we don’t know the immortalized event

in this cell line. The cells were treated with doxorubicin and p53 and p21 expression detected by western blotting. We observed the increased of p53 expression in W3T3 cells treated with doxorubicin and the increased of p21 expression in all the cell lines treated. These findings suggest that p53 inactivation isn't the immortalized event in W3T3 cell line. As p53 is functional we studied whether p53 could be a key factor in the induction of SOX2 mediated by RasV12, we used W3T3 p53 knockdown (shp53) cells and control cells (W3T3 shNTG) infected with H-RasV12 plasmid and detected the expression of SOX2 48 hours after the infection. We observed the increase of SOX2 expression in W3T3 shp53 cells as well as in control cell line, so p53 wasn't requested for SOX2 induction upon RasV12 expression. We also analyzed SOX2 expression in W3T3 Rb knockout cells (D3T3), but we didn't be able to detect the expression of SOX2 48 hours after the infection. However, after cell selection we detect by western blotting, approximately five days after the infection, the increased of SOX2 expression in D3T3 cells. This result suggests that Rb is not required for SOX2 induction by RasV12 expression, although Rb deficiency could delay its induction. We also analyze the expression of p53 and p19$^{ARF}$ in the W3T3, D3T3, W3T3 shNTG, and W3T3 shp53 infected with H-RasV12 plasmid. We observed the increased of p53 and p19$^{ARF}$ in W3T3 RasV12 cells and in D3T3 cells the expression of p53 was stabilized, but the expression of p19$^{ARF}$ was increased when compared to the control conditions. To study the activation of p53 pathway in D3T3 cells we treated the cells with doxorubicin and we didn't observe p21 expression when compared to the control conditions, this suggest that p53 pathway isn't functional in this D3T3 cell line.

To avoid other genetic effects of W3T3 cells because we don't know the immortalization event we used primary mouse embryonic fibroblasts (MEFs) to continue the study of genetic requirements for the induction of SOX2 by RasV12 expression. We infected WT MEFs, p53$^{-/-}$ MEFs, ARF$^{-/-}$ MEFs, and C39 TAg cell lines with H-RasV12 plasmid and detected the expression of SOX2, p53, and p19$^{ARF}$ 48 hours after the infection by western blotting. We showed that RasV12 induced SOX2 expression in WT MEFs, p53$^{-/-}$ MEFs, and C39 TAg cell lines when compared with control cells, although C39 TAg cells have high basal levels of SOX2 as was observed Vilas and collaborators that cells lacking Rb or p130 express higher levels of SOX2 (Vilas *et al.*, 2015). In ARF$^{-/-}$ MEFs cells upon RasV12 expression we don't detect the expression of SOX2. Thus, p53 and Rb is not required for SOX2 induction mediated by RasV12 and p19$^{ARF}$ could be required for SOX2 induction. We only detected p53 expression in C39 TAg cells where its expression was stabilized because the interaction of T antigen (TAg) with p53 block *p53* gene expression and stabilizes p53 as well as inactivated *Rb* expression (Pipas, 2009). Nonetheless, we treated WT MEFs, p53$^{-/-}$ MEFs, and ARF$^{-/-}$ MEFs with doxorubicin to confirm if p53 pathway is activated by detection of p53 and p21 by western blotting. We observed increased of p21 expression in WT MEFs and

ARF$^{-/-}$ MEFs cell lines when compared to the control condition, so in these cells p53 pathway was activated. The p19$^{ARF}$ expression was increased in WT MEFs and p53$^{-/-}$ MEFs upon RasV12 expression. Ferbeyre and collaborators refer that oncogenic *ras* can increase p53 levels and leads mouse embryonic fibroblasts cells to a permanent cell cycle arrest with features of cellular senescence (Ferbeyre *et al.*, 2002) and in neuronal stem cells (NSCs) the increased levels of SOX2 leads to accumulation of p53 protein (Marqués-Torrejón *et al.*, 2013). In WT murine keratinocytes the overexpression of Ras induces cell cycle, which is mediated by increased expression of p19$^{ARF}$ (Meng *et al.*, 2016).

To assess if the induction of SOX2 by RasV12 is at level of transcription we analyze the amount of SOX2 mRNA in WT MEFs and ARF$^{-/-}$ MEFs cells 48 hours after the infection with H-RasV12 plasmid by RT quantitative PCR. We observed the increase of SOX2 mRNA in both cell lines that overexpressed RasV12 when compared to control cell lines. So, RasV12 induce the transcription of *SOX2* gene. We don't observe SOX2 expression in ARF$^{-/-}$ MEFs cells by western blotting. However, we detect SOX2 mRNA by RT quantitative PCR in the same cells 48 hours of the infection with H-RasV12. These findings suggest that p19$^{ARF}$ isn't an essential factor for SOX2 induction in cells upon RasV12 expression. We don't know why we didn't detect SOX2 protein, but probably SOX2 undergoes some post-translational modification, for example SUMOylation. We know through previous studies that p19$^{ARF}$ regulates SUMOylation and SOX2 can be modified at post-translational level by SUMOylation (Tago *et al.*, 2005; Liu *et al.*, 2013). However, we unknown the relationship between p19$^{ARF}$ and SOX2.

MAPK pathway is essential to control the proliferation, differentiation, and survival of eukaryotic cells (Fernández-Medarde and Santos, 2011) and PI3K activation might have an important role during tumor maintenance highlights the importance of this pathway as an anticancer target (Castellano and Downward, 2011). To study which signalling pathways downstream of Ras that mediate SOX2 induction, first we investigated what the Ras pathway is responsible to increase the expression of SOX2. We infected W3T3 cells with mutants of the Ras pathways PI3K/AKT (C40), MAPK (E38), and RalGDS (G37) and after selection detected SOX2 expression by western botting. Our results show that only RasV12E38 mutant activated the MAPK cascade because we observed the overexpression of Ras and phospho-ERK, but we don't detect the expression of SOX2 when compared with control conditions. However, we failed to detect RasV12C40 and G37 mutants because they don't express Ras. This result suggests that RasV12E38 mutant activated the MAPK pathway, but its activation isn't sufficient to induce SOX2 expression as occurs in cells that have all Ras pathways activated. McFarlin show in fibroblasts that activated Ras have higher transformation efficiency in comparison with RasV12 mutants, but Raf of the MAPK pathway appears to be more potent oncogenic effector than PI3K or

RalGDS. Raf is the primary oncogenic effector of Ras and its activation can initiate rat mammary gland carcinogenesis, while affinity with PI3K or RalGDS is sufficient for carcinogenesis after long latency. *In vivo* the Raf pathway may serve as the single most important pathway to target for the prevention and treatment of Ras tumors (McFarlin *et al.*, 2003).

Next, we tested which pathway could be required for SOX2 induction by inhibiting Ras pathways, MAPK and PI3K/AKT, with pharmacological inhibitors. First, we confirmed that doxycycline activates Ras expression in W3T3 pInducer RasV12 (Tet-ON) cells and determine the shortest time in which observed the induction of SOX2 mRNA expression by RasV12. We also confirmed by western blotting that MAPK pathway is inhibited after treated the cells with MAPK inhibitor (PD98059) because we observed decreased expression of phospho-ERK when compared with the control conditions, but we can't confirm the inhibition of PI3K/AKT pathway in cells treated with PI3K inhibitor (LY294002) and AKT inhibitors (MK-220562 and GDC-0068). We analyze the amount of SOX2 mRNA by RT quantitative PCR in cells treated with MAPK and PI3K/AKT pathway inhibitors, we observed that RasV12 loses the ability to induce SOX2 expression when we inhibited the MAPK pathway with PD98059, but RasV12 retains the ability to induce SOX2 expression when we inhibit the PI3K/AKT pathway with LY294002, MK-220562, and GDC-0068 when compared with the control conditions. These findings suggest that MAPK pathway is necessary for SOX2 induction mediated by RasV12, but we don't know if it's the only essential pathway in the induction of SOX2, because we don't observe the increase of SOX2 expression in cells with MAPK pathway active when compared to control conditions. We also need to confirm if the PI3K/AKT pathway is inhibited. Hui and collaborators observed in human non-muscle-invasive bladder cancer (NMIBC) that the transcription factor SOX2 could be regulated by MAPK signalling for maintaining side population or cancer stem cells (CSCs) (Hui *et al.*, 2017). Other author refers that RKIP, a Raf kinase inhibitor protein, inhibits SOX2 via its inhibition of the MAPK pathway, RKIP is normally bound to Raf-1 and cannot be activated by Ras (Lee *et al.*, 2017). These previous results agree with the data observed by us. However, Peltier and collaborators refer that in adult hippocampal neural progenitor cells the expression of SOX2 is regulated by PI3K/AKT signalling pathway, but SOX2 overexpression doesn't increase cell proliferation in contrast with AKT overexpression (Peltier *et al.*, 2011). K-Ras is activated in more than 90% of all pancreatic ductal adenocarcinoma (PDAC) and these tumors are highly dependent on upregulated AKT and MAPK signalling. Overexpression of SOX2 in PDAC cell lines reduced the growth inhibitory effects of trametinib and MK-2206, MEK and AKT inhibitors, respectively. Wuebben and colleagues refer when knocking down SOX2 in PDAC cells and treated with trametinib or MK-2206 they observed reduction in growth of these cells (Wuebben *et al.*, 2016).

To assess the importance of SOX2 expression in transformation by RasV12 we used siRNA-mediated knockdown in W3T3 and MEFs cells and overexpressed RasV12. We confirm the SOX2 knockdown and Ras overexpression by western blotting and analyze the importance of SOX2 in transformation performed a foci assays. In W3T3 and MEFs cells with SOX2 knockdown we observed less cell foci, but in MEFs these cell foci are smaller when we compared with the control condition, cells that expressed SOX2. These results suggest that the induction of SOX2 by RasV12 is essential for oncogenic transformation and SOX2 increase the transformation capacity of RasV12. Several studies report importance of SOX2 expression in cancer cells growth, for example SOX2 promotes esophageal carcinoma growth by regulating the PI3K/AKT signalling pathway (Li *et al.*, 2014). In several cancer cell lines, SOX2 knockdown using short hairpin RNA (shRNA) decreased tumor cell growth *in vitro* and/or *in vivo*, so the expression of SOX2 is required to sustain the growth of cancer cells (Boumahdi *et al.*, 2014). Chou and colleagues refer that in some lung cancer cells SOX2 knockdown caused significant cell death and attenuated tumor growth in xenograft mouse model (Chou *et al.*, 2013). Downregulation of SOX2 expression in pancreatic ductal adenocarcinoma (PDAC) cell lines reduced their growth *in vitro* and *in vivo*, thus SOX2 is required for growth of PDAC (Wuebben *et al.*, 2016). SOX2 is an important oncogene in lung and esophageal squamous cell carcinoma and is universally expressed in neuroglial tumors and together with other pluripotency genes in glioblastoma. Silencing SOX2 in glioblastoma affect proliferation and tumorigenicity (He *et al.*, 2011).

The stem cell signalling is associated with chemoresistance during cancer progression (Chou *et al.*, 2013). SOX2 not only influences tumor growth, but it also influences the response of tumor cells to therapeutic drugs (Wuebben *et al.*, 2016). We study if SOX2 overexpression influences the response of tumor cells to therapeutic drugs. First, we confirmed by western blotting the overexpressed of SOX2 in human lung carcinoma (A549) and in human breast adenocarcinoma (MCF7) cells and treated A549 cells with doxorubicin and docetaxel and MCF7 cells with doxorubicin, docetaxel, and gemcitabine for 48 hours and analyzed the cell viability by MTT assays. We observed that the SOX2 overexpression in A549 don´t increase the resistance or make this cell line more sensitive to doxorubicin and docetaxel, the same result was observed in MCF7 cells treated with doxorubicin and gemcitabine when compared with control conditions. However, MCF7 cells that overexpressed SOX2 are more resistance to 25, 50, 100 and 400 nM of docetaxel therapy. Our results suggest that SOX2 overexpression increase the resistance of human breast adenocarcinoma cells to docetaxel. Several studies show that SOX2 overexpression increased tumorigenicity and chemoresistance, including in gastric and colorectal cancer stem-like cells (Li *et al.*, 2014). Tian and collaborators show that in gastric cancer cells the overexpression of *SOX2* gene increased the insensitivity to the trastuzumab

(antibody) administration and patients exhibit a poor clinical response to this treatment (Tian *et al.*, 2014). In lung cancer cells (A549), SOX2 expression promoted chemoresistance to cisplatin and paclitaxel because this factor increased BCL2L1 expression which protected cancer cells from apoptosis and autophagy. The maintenance of mitochondrial integrity and improves cell survival by SOX2-BCL2L1 signalling leads to increase chemo-resistant in these cells (Chou *et al.*, 2013). In breast cancer cells (MCF-7) the ectopic expression of SOX2 is sufficient to make this cell line more resistance to tamoxifen treatment *in vitro* and *in vivo* (Piva *et al.*, 2014). Ovarian cancer cell lines are resistances to carboplatin, cisplatin, and paclitaxel drugs when expressed SOX2 and are more sensitive when SOX2 is down regulated (Bareiss *et al.*, 2013). In prostate cancer the overexpression of SOX2 is closely correlated with the clinical progress of this cancer (Jia *et al.*, 2011) and is responsible for the paclitaxel drug-resistance and the resistance is mediated by continuous activation of PI3K/AKT pathway (Li *et al.*, 2014). In head and neck squamous cell carcinoma (HNSCC) SOX2 is a mediator of therapy resistance and targeting this factor and related molecular downstream pathways may enhance therapy efficacy in this cancer (Schröck *et al.*, 2014). Lee and colleagues refer that in cells with SOX2 downregulation the cell viability decreases after cisplatin treatment (Lee *et al.*, 2014). In ovarian cancer stem cells, the increased of SOX2 expression enables their selective survival to conventional chemotherapies and promotes tumorigenicity *in vivo*. Bareiss and collaborators suggest that SOX2 expression in cancer stem cells contribute to therapy resistance and disease relapse in patients with ovarian carcinoma. Targeting *SOX2* will improve clinical treatment of ovarian carcinoma by enhancing apoptosis responses to conventional chemotherapies (Bareiss *et al.*, 2013). As SOX2 increases the resistance of the several cancer cells to chemotherapy, this factor may serve as a potential therapeutic target for cancer treatment (Chou *et al.*, 2013).

In conclusion, our results highlight the importance of SOX2 in oncogenic transformation. SOX2 expression is induced by RasV12 at level of transcription and SOX2 cooperates with RasV12 in transformation mediated by this oncogene. We also found that p53, Rb, and $p19^{ARF}$ are not essential factors for RasV12-dependent SOX2 induction. Activation of the MAPK pathway is required, but not sufficient for SOX2 induction mediated by RasV12 expression. SOX2 expression is a clinical problem in the chemotherapy because their overexpression increases the resistance to drug therapy decreasing the survival rate of the patient. Ours results show that SOX2 overexpression increased chemoresistance to docetaxel in breast cell line. In addition, SOX2 is a key factor in oncogenic transformation because their inhibition drastically reduces the ability of RasV12 to cell foci formation. Based on the above results, SOX2 can be the new therapeutic target in the fight against cancer, more precisely in cancer cells with Ras mutations. Future

studies, it is still necessary identified the molecular mechanism and partner factors involved in the induction of SOX2 by RasV12.

# Conclusion and Future Perspectives

The transcription factor SOX2 is an important embryonic stem cells regulator and is aberrantly expressed in multiple types of human tumours (Sang Hyuk Lee *et al.*, 2014). This has an important role in cell cycle regulation, DNA damage repair, and maintaining the totipotency of stem cells (Ying *et al.*, 2016). This factor was amplified and overexpressed in many types of cancer such as breast, pancreatic, gastric, colon, and lung cancers. The high expression of SOX2 in many malignant carcinomas is associated with the invasion and poor prognosis of carcinoma, this suggest the involvement of SOX2 in carcinogenesis (Liu *et al.*, 2013; Lundberg *et al.*, 2014; Ying *et al.*, 2016).

The present study showed that RasV12 induces SOX2 expression and MAPK pathway is required, but not sufficient for SOX2 induction mediated by RasV12 expression. The p53, Rb, and $p19^{ARF}$ factors are not essential for SOX2 induction by RasV12. SOX2 expression increases the ability of RasV12 in oncogenic transformation, as well as SOX2 overexpression increases chemoresistance to docetaxel in breast cell line.

SOX2 binding loci have been extensively analyzed, but need more investigation to elucidate a genome-wide determination of SOX2-interacting partners and a comparative analysis of common target gene loci between SOX2 and synergizing factors (Chanoumidou *et al.*, 2017). In my opinion, SOX2 can be a novel target for anti-cancer therapy as well as can be used in diagnostic, prognostic, and predictive biomarkers. However, know what SOX2 is required in cells transformed by RasV12, what is the mechanism by which RasV12 induced SOX2 expression as well as factors involved in this process, and the discovery of molecules that can block SOX2 expression for therapy in tumor cells that have mutations in *ras* gene may be future lines of investigation.

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