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PRAS40 and TSC2 regulate apoptosis during early
development of Embryoid Bodies via mTOR

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ABSTRACT

To date the current available treatments are chemotherapy and surgery in cancer and only seldomly treatments or cures for human genetic diseases exist. Therefore scientists are seeking for new methods of therapy and many focus their research on stem cells and possible stem cell therapy as major advances were made in the recent years.

On the one hand embryonic stem cells harbor the potential to differentiate into lineages of the three germ layers and are rapidly proliferating. However, besides the ethical concerns, they have the risk of teratoma formation in SCID mice. On the other hand adult stem cells derived from various tissues, have limited proliferation capacity and differentiation potential. In 2003 the discovery of stem cells in the human amniotic fluid (hAFS cells) smartened up the field of stem cell biology as these cells can differentiate into cells of the three germ layers, are highly proliferative and do not harbor the risk of teratoma formation.

The mTOR pathway plays a critical role in cell growth, cell cycle regulation, translation, apoptosis, energy and nutrient sensing. Furthermore it is deregulated in many cancers and human genetic diseases. One major regulator of the mTOR pathway is TSC2, encoding tuberlin, which forms a complex with TSC1 and mutation in either one gene causes tuberous sclerosis (TSC), a human genetic disease affecting 1:6000 births. In 2003 a novel negative regulator of mTORC1 was identified – the proline rich AKT substrate of 40kDa (PRAS40), which was found to be deregulated in some cancers and has a possible role in diabetes. We proved that hAFS cells are able to form embryoid bodies and that this is dependent on mTOR. Secondly, we showed that these cells were capable of integrating and differentiating into renal structures.

The major aim of this thesis was to study the role of the two major regulators of mTORC1 – TSC2 and PRAS40 – in development using hAFS cells derived embryoid bodies to recapitulate early embryonic development. Knockdown of PRAS40 and TSC2 led to increased apoptosis in embryoid bodies, which could not be rescued by the mTORC1 inhibitor rapamycin; however it was possible to revert this effect by knocking down mTOR. This led us to the conclusion that a) PRAS40 and TSC2 are major anti-apoptotic gatekeepers in early development and b) this regulation depends on rapamycin-insensitive functions of mTOR.

ZUSAMMENFASSUNG

Zurzeit sind chemotherapeutische oder operative Therapie bei Krebs die häufigsten Behandlungsarten und nur selten existieren Therapien für humangenetische Erkrankungen. Deshalb suchen Wissenschaftler nach neuen Methoden und legen dabei ihr Augenmerk auf die Stammzellforschung, welche in den letzten Jahren wesentliche Fortschritte gemacht hat.

Embryonale Stammzellen besitzen einerseits das Potential in alle drei Keimblätter zu differenzieren und haben eine hohe Proliferationsrate. Neben den ethischen Bedenken besteht auch noch das Risiko einer Tumorentstehung. Andererseits existieren adulte Stammzellen aus den unterschiedlichsten Geweben, welche aber nur begrenzte Proliferationsrate und Differenzierungspotential besitzen. Mit der Entdeckung von Stammzellen in humanem Fruchtwasser im Jahre 2003 wurde frischer Wind in das Gebiet rund um die Stammzellforschung gebracht, da diese Zellen in viele Zellarten der drei Keimblätter differenzieren können, hohe Proliferationsraten haben und keine Teratome formen.

Der mTOR Signalweg spielt eine wichtige Rolle bei Zellwachstum, Zellzyklusregulation, Translation, Apoptose, Energie- und Nährstoffhaushalt. Darüber hinaus ist dieser in vielen Krebsarten und genetischen Erkrankungen dereguliert. Ein wesentlicher Regulator des mTOR Signalwegs ist TSC2, das gemeinsam mit TSC1 einen Komplex bildet. Eine Mutation in einem der beiden Gene löst Tuberöse Hirnsklerose aus, eine humangenetische Erkrankung, die 1 von 6000 Geburten betrifft. Im Jahre 2003 wurde ein weiterer Regulator von mTORC1 identifiziert – PRAS40, welcher in manchen Krebserkrankungen dereguliert ist und eine mögliche Rolle bei Diabetes spielt. Wir konnten beweisen, dass es möglich ist, embryoid bodies aus Fruchtwasserstammzellen zu formieren und dass dieser Vorgang mTOR-abhängig ist. Zweitens dazu imstande sind sich in renale Strukturen zu integrieren und dort zu differenzieren.

Das Ziel dieses Projekts war es, die Rolle der zwei wichtigsten Regulatoren von mTORC1 – PRAS40 und TSC2 – in der früher embryonalen Entwicklung, anhand von embryoid bodies aus humanen Fruchtwasserstammzellen, zu studieren. Der Knockdown von PRAS40 und TSC2 löste massive Apoptose aus, welche durch den chemischen mTOR Inhibitor Rapamycin nicht verhindert werden konnte. Allerdings konnte diese aber durch den Knockdown von mTOR revertiert werden. Dies brachte uns zur Erkenntnis dass a) PRAS40 und TSC2 wesentliche anti-apoptotische Schlüsselmoleküle in der frühen embryonalen Entwicklung sind und b) diese Regulation von Rapamycin-insensitiven Funktionen mTORs abhängt.

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1. INTRODUCTION

Medicine and science are definitely one of the fastest growing disciplines in the world and have substantially enriched the quality of life and life span of men. However, heart disease, cancer and a number of severe human genetic diseases still cannot be cured. Therefore scientists all over the world are searching for new ways of therapy and cures. For most cancer patients the only possible therapy is surgery or chemotherapy, which often only help partially. Scientists trade embryonic stem cells as the new potent tool to cure a number of diseases. However, although embryonic stem cells are such a potent source and could be used in many different applications, they do bare some difficulties and problems still to overcome e.g. teratoma formation in SCID mice. The past years, a new source of stem cells was found in the amniotic fluid, the so called amniotic fluid stem cells; and these are a new promising tool, which do not raise ethical issues like embryonic stem cells do.

An important regulator of the insulin signaling pathway is the mTOR complex 1 (mTORC1), which is deregulated in many cancers as some very prominent oncogenes and/or tumor suppressors are upstream of mTORC1 e.g. PTEN, LKB1, AMPK, ERK and its direct upstream regulator TSC2, a tumor suppressor (Rosner et al., 2008a). The tumor suppressor TSC2, encoding tuberin, forms a heterodimer with TSC1, encoding harmatin, and mutations in either gene leads to tuberous sclerosis (TSC) affecting 1:6000 births (Rosner et al., 2008b).

This thesis combines the potential of human amniotic fluid stem cells with the identification of the regulation of the mTORC1 regulators PRAS40 and TSC2 in an *in vitro* embryonic developmental system.

1.1 Stem cells

Since the discovery and isolation of stem cells new revolutionary medical appliances and therapeutics are expected. This new research field has revolutionized developmental genetics and biology, as one can study *in vitro* differentiation of stem cells into all kinds of cells from the three germ layers or *in vitro* development and their future use in regenerative medicine.

1.1.1 Characteristics of stem cells

Stem cells are usually defined to have special characteristics e.g. pluripotency and self renewal. Self-renewal is the ability to go through numerous cell divisions while maintaining an undifferentiated state e.g. asymmetric cell division into an undifferentiated mother cell and a differentiated daughter cell. Pluripotency, on the other hand, is the potential to differentiate into any cell type of the three germ layers. Currently a list of general criteria to evaluate the developmental differentiation potential of cells was provided by Jaenisch and Young in 2008, which include: *in vitro* tri-lineage differentiation like embryoid bodies (EBs), *in vivo* teratoma formation, postnatal chimera, germline contribution, and tetraploid complementation (Jaenisch and Young, 2008).

1.1.2 Embryonic stem cells

As their name suggests, these cells are derived from the inner cell mass (ICM) of the pre-implantation embryo (Odorico et al., 2001). The research field on embryonic stem (ES) cells evolved in the 1950s with the study of teratocarcinoma cells. Then the first mouse embryonic stem cell lines were generated. In 1998 Thomson et al. were the first ones who cultured and expanded human embryonic stem (hES) cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). To maintain their undifferentiated state ES cells express various transcription factors that suppress differentiation and favor pluripotency such as Oct-4, Nanog, Sox-2 (Fong et al., 2008; Mitsui et al., 2003; Pesce and Scholer, 2001). To date most of the attention is drawn to degenerative diseases such as Parkinson's disease, multiple sclerosis or spinal chord injury to be cured through stem cell therapy. On the one hand ES cells raise hope for new and potential therapies to cure severe diseases like cancer or inherited diseases; on the other hand this field is accompanied with ethical concerns, as the generation of ES cells means destroying an embryo.

1.1.3 Induced pluripotent stem cells

This new type of stem cells was first generated in 2006 by Takahashi and Yamanaka and overcomes the ethical concerns of ES cells, as for their generation not a single embryo is needed. Induced pluripotent stem (iPS) cells are pluripotent stem cells developed from a somatic cell. The cell is forced to express certain key genes, namely *Oct 3/4* (POU5F1), the transcription factor *Sox2*, the proto-oncogene *c-Myc* and *Klf4* (Krueppel-like factor 4) to sufficiently reprogram adult cells to cells closely resembling ES cells (Takahashi and Yamanaka, 2006). Modifying the reintroduced sequences by reactivation of *Oct-4* or *Nanog* promoter resulted in even better iPS cells more closely resembling ES cells and

were even able to contribute to the germline (Wernig et al., 2007). However, despite very similar behavior, iPS cells still had a much higher incidence of tumor formation presumably due to the expression of *c-Myc* (Ben-David and Benvenisty, 2011; Maherali et al., 2007). Just recently the Jaenisch group provided an elegant way to generate human iPS cells free from reprogramming factors via Cre-recombinase excisable lentiviruses. The cells maintain a gene expression profile more similar to hES cells and can be differentiated into specific tissue (Soldner et al., 2009). It was assumed until recently that iPS cells behave like their embryonic counterparts regarding tumorigenicity. However, evidence is accumulating that suggests that there are important genomic and epigenetic differences between them influencing their tumorigenicity (Ben-David and Benvenisty, 2011).

1.1.4 Fetal stem cells

1.1.4.1 The function of the amnion and amniotic fluid

The amnion is a membrane surrounding and protecting the embryo during development and pregnancy (Dobreva et al., 2010). Between the fourth and the fifth week of pregnancy the amniotic fluid (liquor amnii) starts to accumulate within the amnion. It increases in volume until approximately the 6th to 7th month of pregnancy, at the end women usually carry about 1 liter of amniotic fluid. The amniotic fluid allows movements of the embryo and functions as a preventing shock absorber that protects against ascending infections, traumas and toxins (Calvin and Oyen, 2007). It contains solids, urea and other extractives from the embryo, protein and sugar. The amniotic membrane has another important task as it suppresses the semiallogenic immune response against the fetus (Bryant-Greenwood, 1998; Calvin and Oyen, 2007).

1.1.4.2 Amniotic fluid cells

Originally, since the 1970s, human amniotic fluid was used to examine the fetus for human genetic diseases through routine prenatal diagnostics. Usually, non invasive techniques such as ultrasound are done routinely in all pregnancies. If these show abnormalities, or if there is a predisposition of genetic diseases in the family or it is a high-risk pregnancy due to the age of the mother, invasive methods are suggested. There are several possibilities: chorionic villus biopsy, which can be performed from the 10th week of gestation, cordocentesis from the 20th week and thirdly amniocentesis, starting around the 15th week. Amniotic fluid as mentioned above slowly starts to build up and reaches its maximum amount in the last trimester of gestation. As soon as the fetus starts

to swallow and subsequently “breathing”, pulmonary, tracheal, nasal and oral particles add up to amniotic fluid (Underwood et al., 2005).

Although culturing of amniotic fluid (AF) cells was broadly established, nothing was really known about these cells and their properties (Prusa and Hengstschlager, 2002). It was only in 2001 that a group tried to engineer tissue constructs from cells found in the amniotic fluid. Immunocytochemical analysis of the AF cells showed a mesenchymal and fibroblast like cell lineage. These cells also proliferated significantly faster than comparable fetal and adult cells (Kaviani et al., 2001). The first time expression of Oct-4, a pluripotency marker for stem cells, was found in some cells of the amniotic fluid by our laboratory (Karlmark et al., 2005; Prusa et al., 2003). The expression of Oct-4 was proven by immunocytochemistry, RT-PCR and Western blotting, and therefore it was suggested that amniotic fluid is a source of pluripotent stem cells. AF cell population is heterogeneous, exhibits various cells in various differentiation stages and from all three germ layers. They either derive from the embryo or the amniotic membrane. Cells derived from earlier gestational stages have a higher expression of endo- and mesodermal markers compared to cells derived from later stages (Perin et al., 2008).

1.1.4.3 Human amniotic fluid stem cells

After it was clear that the human amniotic fluid contains some cells, which express the pluripotency marker Oct-4, several groups started to isolate human amniotic fluid stem cells (hAFS cells) using various protocols (De Coppi et al., 2007; Tsai et al., 2004). All of the isolated cells show overlapping marker expression e.g. Oct-4, CD34, CD44, CD45, CD105 and HLA-ABC and they might all be offspring of the same cell type (Siegel et al., 2008). HAFS cells do belong to the class of fetal stem cells (Figure 1.1.1).

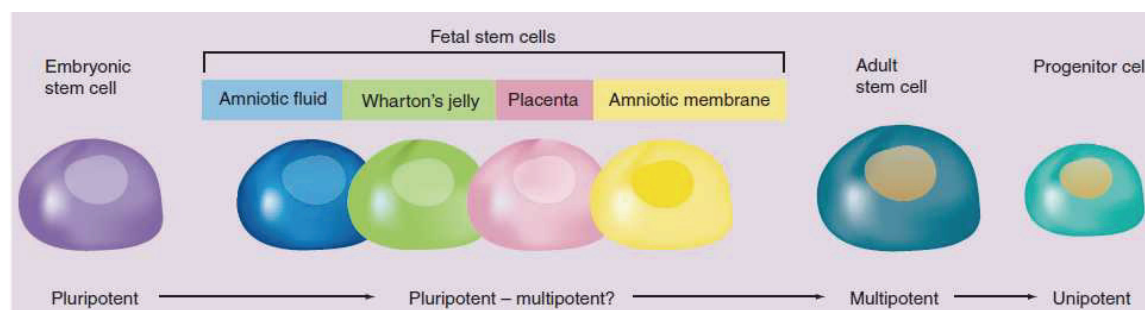


Figure 1.1.1 Model for the differentiation potential of the different stem cell types compared to each other. The fetal stem cells are thought to be a new class of stem cells whose developmental state is somewhere between the embryonic stem cells and the adult stem cells (Pappa and Anagnou, 2009).

It was in 2007 that DeCoppi et al. provided a new isolation protocol for stem cells found in the amniotic fluid. This protocol is based on the fact that 1% of the cells in cultures of human amniotic fluid express the surface marker c-Kit (CD117), the receptor for stem cell factor (De Coppi et al., 2007). C-Kit positive cells were separated from the other cells by magnetic bead cell sorting and established monoclonal cell lines via minimal dilution. Cells do have a typical doubling time of 36h. They exhibit a normal karyotype and retain long telomeres, and after even 250 population doublings they showed a homogeneous and diploid DNA content in the G1 phase of the cell cycle. Another important factor is that these cells do not show teratocarcinoma growth in SCID mice. These hAFS cells could be cultivated and differentiated into lineages of all three germ layers, such as osteogenic, myogenic, adipogenic, endothelial, hepatic and neurogenic lineage (De Coppi et al., 2007; Kolambkar et al., 2007; Perin et al., 2008). These findings strongly support these cells to be pluri- or at least multipotent, which has been accounted solely to ES cells then (Figure 1.1.1). The high potential of hAFS cells was not only shown through differentiation protocols of these cells. Various groups were able to show that hAFS cells could integrate into murine embryonic kidneys (E 12.5 – 18) or murine embryonic kidney rudiments (E 11.5) *in vitro* and differentiate into *de novo* kidney structures e.g. renal vesicle, C- and S-shaped bodies, ureteric bud (Perin et al., 2007; Siegel et al., 2010). Another important finding was that hAFS cells can form EBs, an *in vitro* 3D model system that mimics embryonic development in the very early stages of development and differentiation (Valli et al., 2010) and that EB formation is dependent on mTOR.

1.2 Embryoid bodies (EBs)

The study on EBs represents a unique tool to investigate the very early process of embryonic development. EBs are mimicking stages of pregastrulation development and early gastrulation and therefore are a good tool for scientists to study embryonic development *in vitro* and to search for new cures.

1.2.1 The genesis of embryoid bodies

EBs were first described in the 19th century by the Germans van Waldeyer-Hartz and Pfannenstiel as *in vivo* phenomena of ovarian tumors. The term “embryoid body” was given by the French Albert Peyron, describing them in testicular tumors (Weitzer, 2006). Arnold Levine generated EBs from teratoma cells in 1974 (Levine et al., 1974), being immediately followed by Gail Martin and J.F. Nicolas in 1975, who generated them from teratocarcinoma cells (Evans, 1981; Martin and Evans, 1975; Nicolas et al., 1975; Weitzer, 2006). Finally in 1981 EBs derived from ES cells, isolated from the inner cell mass of murine blastocysts, were generated (Evans and Kaufman, 1981; Martin, 1981). Since then numerous data has been published providing evidence that EBs represent an *in vitro* model for eutherian embryonic development and that the derived somatic cells may be used for cell therapy. It also became evident that EBs represent a good tool to study homozygous null alleles (Robertson et al., 1992) with subtle phenotypes that were either hidden *in vivo* (Bagutti et al., 1996; Milner et al., 1996; Weitzer, 2006) or caused early embryonic lethality (Di Cristofano et al., 1998; Fassler et al., 1995; Rohwedel et al., 1998). However, only after the isolation of hES cells (Thomson et al., 1998), there was a boost in the EB research field. EBs derived from hES cells were shown to have a similar potential to that of murine EBs (Itskovitz-Eldor et al., 2000; Schuldiner et al., 2000).

1.2.2 Embryonic stem cell influence on embryoid bodies

One of the major problems that have to be considered in the ES cells and EB research field is that the ES cells isolated from the inner cell mass of a blastocyst are never from the same origin. Even if isolated at the same time point and from the same mouse strain, ES cells would behave differently. Self-renewal capacity, plasticity and differentiation control, are therefore highly variable among even isogenic ES cell lines generated in different laboratories. EB development is not only influenced by the genetic background but also by the culture conditions. Additional parameters that influence ES cell potential and EB differentiation include the developmental stage of the embryo when ES cells were

generated, time to reach confluency *in vitro*, the source and batch of fetal calf serum and at last the handling of the cells by the investigator (Weitzer, 2006). The initial number of stem cells for example determines the number of ES cells which aggregate to form EB, and the final number of cells in the compact EB strongly influences further development of the cells (Bader et al., 2001; Dang et al., 2004).

1.2.3 Pregastrulation-like development of embryoid bodies

The first cell lineage emerging from the compacted morula is the trophectoderm, which contributes to the placenta (Tanaka et al., 1998). The inner cell mass is then giving rise to the primitive endoderm or the hypoblast (Watson and Barcroft, 2001) and the remaining primitive ectoderm constitutes the epiblast. During implantation, primitive endoderm differentiates into two extraembryonic lineages, the visceral and the parietal endoderm, which both form the yolk sac (Gardner, 1985).

The study of embryonic-like developmental processes in an EB requires size uniformity. This can be achieved through the hanging drop method or suspension culture in 96-Well-Plates. These cultures guarantee the formation of EBs uniform in size and with an equal initial number of ES cells. A critical mass of cells within EBs is essential for development and achieved through a sufficient number of aggregating ES cells. Once an EB has formed, the cell mass fundamentally influences further development of EBs (Bader et al., 2001; Miki, 1999; Nicolas et al., 1975). In hanging drop cultures, mES cells aggregate within 24h and form an early morula-like structure. The initial number of ES cells must be significantly higher than the 16-64 cells of a morula to guarantee the development of all three germ layers in an EB later on (Bader et al., 2001; Dang et al., 2004). Usually, the initial number to form EBs ranges from 300-700 cells. Once ES cells have successfully aggregated and formed small spheres of roughly 50-100µm in diameter, by the 2nd day of *in vitro* development the irregular surface smoothens to morphologically resemble the morula compaction process (Weitzer, 2006). After 3 days EBs can reach a diameter of up to 200µm and the first primitive endoderm cells appear as refractive bubbles on the surface of the EB (Adamson et al., 1985; Grover et al., 1983). The outermost cells of EBs then start to express alphafetoprotein (αFP) (Miki, 1999) and N-acetyl-galactosamine epitopes (Bader et al., 2001). They continue to emerge until the entire surface of the sphere is covered by primitive endoderm by day 4 to 5 (Li et al., 2003; Smyth et al., 1999).

These cells start to secrete components of the extracellular matrix to form a basement membrane (Aumailley et al., 2000; Gao et al., 2004; Murray and Edgar, 2001; Smyth et al., 1999), which effectively isolates the remaining ES cells from at least some environmental influences and consequently generates a new microenvironment for the emerging primitive ectoderm. Some ES cells develop into primitive ectoderm that reorganizes into a columnar epithelium surrounding a central cavity (Ikeda et al., 1999; Smyth et al., 1999). These cysts can either be expanded by apoptosis or the reorganization of the growing EB (Miki, 1999). Summing up EB development perfectly recapitulates the development of the hypoblast by producing primitive endoderm, which in turn gives rise to visceral and parietal endoderm; the latter then undergoes an epithelial to mesenchymal transition to mature mesenchymal parietal endoderm; therefore EBs provide the foundation for consecutive gastrulation-like development (Weitzer, 2006).

1.3 Cell death

Cell death is a fundamental feature of an organism also being essential in embryonic development. It prevents auto-immune response, is essential for tissue homeostasis and DNA damaged cells undergo apoptosis. As true for numerous biological processes there is not only one single mechanism of cell death, there are several different ones ensuring the steady state or which are specialized to work under special conditions or in specific cell types. To date the following mechanisms of cell death are known: apoptosis – the programmed cell death, autophagy and necrosis.

1.3.1 Apoptosis – programmed cell death

Programmed cell death (Lockshin and Williams, 1965) – called apoptosis – is a regulated form of cellular suicide that is tightly controlled by a genetic program (Yuan and Horvitz, 2004). It is associated with certain characteristic morphological and biochemical manifestations such as membrane blebbing, cell shrinkage, chromatin condensation (Figure 1.3.1) and fragmentation of genomic DNA (Bursch et al., 1990; Kerr et al., 1972). After cell blebbing the apoptotic cell breaks into small, membrane-wrapped vesicles, termed “apoptotic bodies”, which are uptaken by macrophages or neighboring cells. Therefore any inflammatory responses are suppressed *in vivo*.

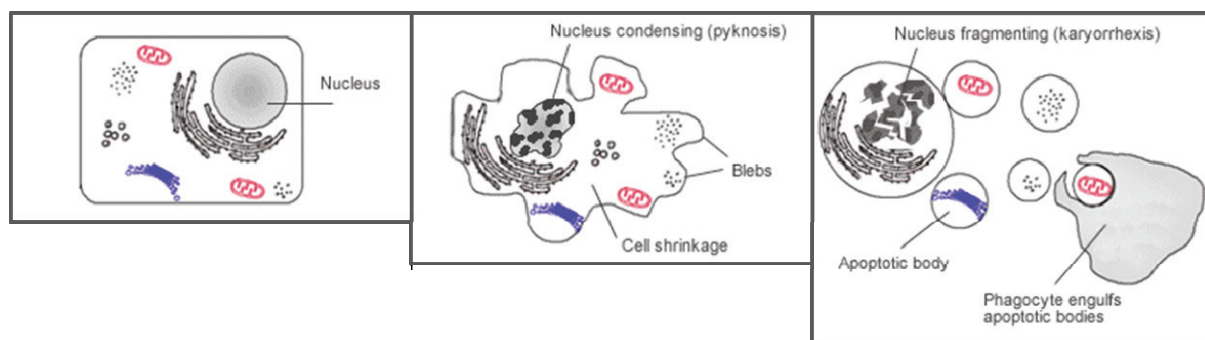
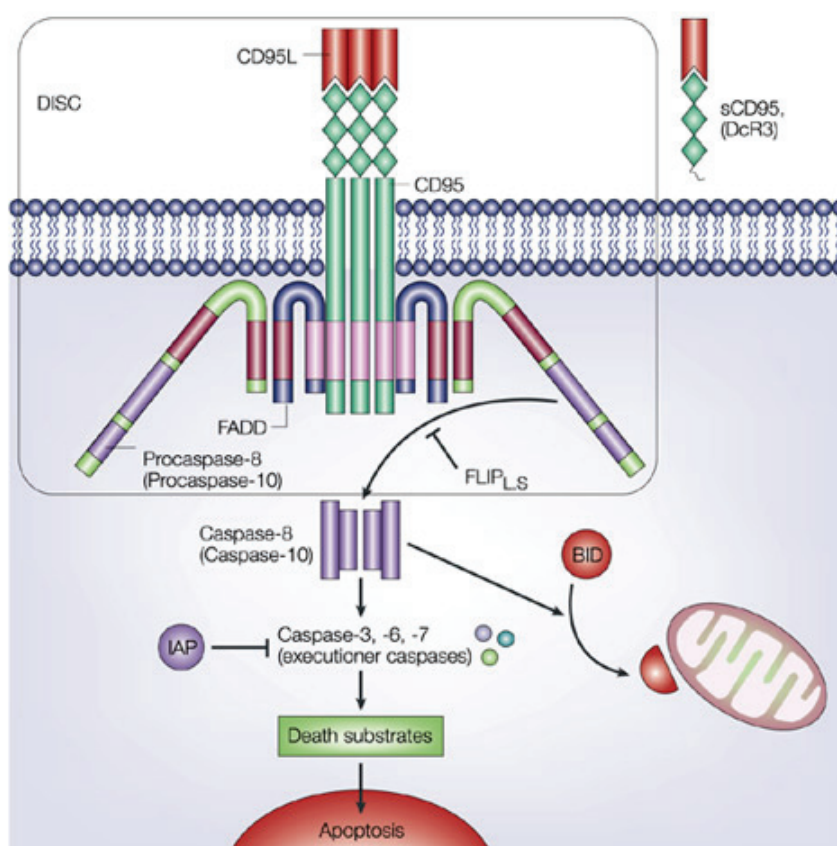


Figure 1.3.1 Scheme of the different stages of apoptosis and its morphological manifestation. Apoptosis is starting first with nuclear condensation, followed by nuclear fragmentation, apoptotic body formation and at last phagocytosis (Figure adapted from <http://en.wikipedia.org/wiki/File:Apoptosis.png>).

Apoptosis can be activated by two different pathways namely the extrinsic and the intrinsic apoptotic pathway. The *extrinsic pathway* is mediated by death receptors on the cell surface (Figure 1.3.2). Death receptors are members of the tumor-necrosis factor (TNF) family and are characterized by an intracellular death domain (Krammer, 1999;

Schmitz et al., 2000). When ligands bind to the respective death receptors, the death domains attract the intracellular adaptor protein FADD (Fas-associated death domain protein), which recruits the inactive forms of the caspase protease family. The initiator caspases, caspase-8 and caspase-10, are then recruited to the death-inducing-signalling complex (DISC). At the DISC complex the caspases are cleaved to activate the executioner/effector caspases e.g. caspase-3 (Danial and Korsmeyer, 2004; Igney and Krammer, 2002) or caspase-8 cleaved BID to turn on the mitochondrial pathway.

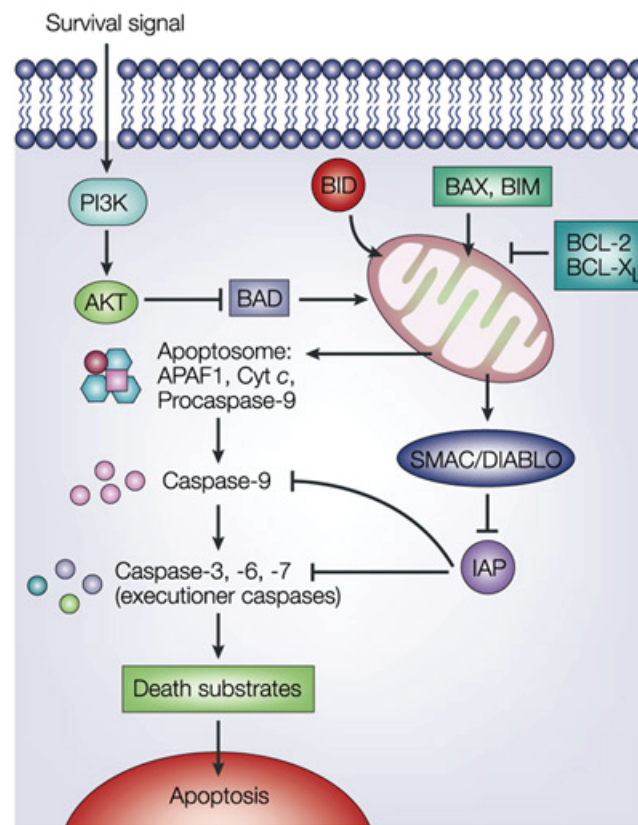


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Figure 1.3.2 Extrinsic or death receptor pathway. Binding to death ligands to their receptor leads to the formation of the DISC. In the DISC, the initiator procaspase-8 is recruited by FADD and is activated by autocatalytic cleavage (Igney and Krammer, 2002).

The *intrinsic or mitochondrial pathway* is initiated via an apoptotic stimulus which affects the mitochondrial membrane. Mitochondria are organelles, existing in most eukaryotic cells, serving as energy suppliers as they produce most of the ATP needed by a cell. They have two very well defined compartments the matrix being surrounded by the inner membrane, and the intermembrane space surrounded by the outer membrane. The

membrane integrity undergoes severe changes during apoptosis concerning both membranes, leading to the dissipation of the inner transmembrane potential ($\Delta\Psi_m$) and the release of cytochrome c and other factors from the intramembraneous space (Liu et al., 1996). The released cytochrome c forms a complex with APAF1 (apoptotic protease activating factor-1), ATP and pro-caspase-9 (Li et al., 1997) in the cytosol (Figure 1.3.3); in this complex, known as the apoptosome, caspase-9 is then activated through proteolysis (Martinou and Green, 2001; Zamzami and Kroemer, 2001), then processes and therefore activates caspase-3, caspase-6 or caspase-7. The active effector caspases cleave each other and start amplifying the proteolytic cascade of caspase activation (Igney and Krammer, 2002; Rathmell and Thompson, 1999).



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Figure 1.3.3 Intrinsic or mitochondrial death pathway. Proapoptotic BCL2 family proteins are important mediators of these signals. Activation of mitochondria leads to the release of cytochrome c into the cytosol, where it binds to APAF1 to form the apoptosome. There initiator caspase-9 is activated. This apoptotic pathway can be inhibited by anti-apoptotic proteins e.g. BCL-2 and BCL-XL and secondly via survival signals from the PI3K pathway (Igney and Krammer, 2002).

Within apoptosis there are two different types the *caspase dependent apoptosis* and *caspase-independent apoptosis*. Caspases do cleave their targets like nuclear lamins or CAD (caspase-activated deoxyribonuclease), which generates 180bp DNA fragments, after aspartic acid residues and catalyze a highly selective pattern of protein degradation. Besides, organelles remain morphologically intact – only subtle changes e.g. membrane permeabilization or protein degradation appear, whereas cell shrinkage and a reduced intracellular potassium level occur (Cohen, 1991; Thompson, 1995). As the remaining material is present in relatively small units, the apoptotic cell is readily phagocytosed by neighboring cells (Huettenbrenner et al., 2003) or engulfed by phagocytes, as they recognize an “eat me” signal, e.g. in mammals phosphatidylserine (PS), on the plasma membrane of a dying cell (Danial and Korsmeyer, 2004; Savill and Fadok, 2000). *Caspase-independent apoptosis* in mammalian cells has been attributed to two mitochondrial proteins endonuclease G and apoptosis-inducing factor (AIF) that translocate to the nucleus upon release (Li et al., 1997; Susin et al., 1999). AIF activates a nuclear DNase (Kroemer and Reed, 2000), which cuts genomic DNA into 50kb fragments giving rise to a distinct nucleo-morphological phenotype of chromatin condensation (Susin et al., 2000). The nuclear translocation of AIF is dependent on poly (ADP-ribose) polymerase-1 (PARP-1). It attaches poly ADP-ribose to nuclear proteins such as histones and its activation leads to apoptosis under several conditions (Yu et al., 2002). Vice versa proapoptotic activity of PARP-1 requires AIF function. Apoptosis during embryoid body cavitation for example is dependent on AIF (Joza et al., 2001).

1.3.2 Necrosis

Necrosis is an accidental form of cell death resulting from various stresses, such as physiochemical injury (temperature, radiation, radicals, toxic trauma), osmotic imbalance, energy deprivation. It is always the outcome of severe acute insults that cause almost instantaneously membrane depolarization and its disruption and cellular swelling (Huettenbrenner et al., 2003; Kerr et al., 1972). The spillage of cellular components induces inflammatory reactions affecting neighboring cells. Studies on excitotoxic neuronal deaths revealed an hyperactivation of NMDA channels and subsequently increases in intracellular Ca^{2+} and Ca^{2+} -dependent pathways through necrosis (Nicotera et al., 1997). Necrosis is an energy independent process, whereas apoptosis is strictly dependent on it; considering this ATP is the switch between apoptosis and necrosis. For

that reason energy depletion leads to necrosis (Eguchi et al., 1997; Grusch et al., 2002; Leist et al., 1997). Table 1.3.1 gives a summarized comparison of apoptosis and necrosis.

Table 1.3.1 Comparison between apoptosis and necrosis

<i>Apoptosis</i>	<i>Necrosis</i>
physiological or pathological	always pathological
single cells	sheets of cells
energy dependent	energy independent
cell shrinkage	cell swelling
membrane integrity maintained	membrane integrity lost
role for mitochondria and cytochrome c	no role for mitochondria
no leak of lysosomal enzymes	leak of lysosomal enzymes
characteristic nuclear changes	nuclei lost
apoptotic bodies form	no apoptotic bodies
DNA cleavage	no DNA cleavage
activation of specific proteases	no protease activation
tightly regulated process	not regulated
evolutionary conserved	not conserved
dead cells ingested by neighbouring cells	dead cells ingested by neutrophils and macrophages

1.3.3 Autophagy

This type of cell death has been implicated for the disposal of intracellular organelles and cytoplasmic material, where lysosomes are involved. These lysosomes assemble and are sequestered within double membrane and are known as autophagosomes. Consumption of these vesicles is divided into four distinct steps: induction and cargo packaging, formation and completion, docking and fusion, and breakdown (Danial and Korsmeyer, 2004). The

autophagosomes further integrate with lysosomes and build up the autophagic vacuoles. Despite a few mitochondria, required for ATP production, the others are degraded. In contrast to apoptosis the cytoskeleton remains intact (Bursch, 2001). Some reports also state that autophagy is a caspase-independent process (Huettenbrenner et al., 2003; Kitanaka and Kuchino, 1999; Quignon et al., 1998). In mammals the autophagy inducing gene is *beclin1* (Liang et al., 1999), a Bcl-2 interacting protein. It links autophagy to the PI3-kinase and mTOR pathways as they can influence shared intermediates, such as p70S6K and mTOR (Danial and Korsmeyer, 2004).

1.3.4 Regulation of apoptosis during development

The development of an embryo is characterized by the means of a high proliferation rate and differentiation of cells. Hand in hand with them, starting at eight-cell stage, the rate of apoptosis increases, as it is known that development and morphogenesis is not only regulated by proliferation and differentiation but also through apoptosis e.g. development of the hand. In the embryo chromosomal abnormalities or suboptimal environmental conditions for example can result in poor embryo development or cell death (Jacobson et al., 1997; Wells et al., 2005; Yuan and Kroemer). These abnormal cells have to be eliminated, due to the fact that the high frequency of cell division could lead to a large number of these cells and would probably lead to an underdeveloped or sick offspring. During late stage apoptosis, when apoptotic bodies have formed, they are either phagocytosed by neighboring cells, dispersed into the blastocyst cavity or perivitelline space in the embryo (Hardy, 1999).

During development of the preimplantation embryo, misplaced, falsely determined, or unneeded cells have to be eliminated. Additionally apoptosis is extremely important for development, such as formation of the amniotic cavity, and control of the cell number of developing organs. Erythrocytes and thrombocytes are also produced by the means of the apoptotic cascade (Jacobson et al., 1997). The Fas system, being one of the key regulators of apoptosis, is expressed from the four-cell stage onward in human embryos (Kawamura et al., 2001). Furthermore, factors which are teratogenic, such as retinoic acid, are able to reduce inner cell mass numbers by inducing apoptosis (Huang et al., 2005; Huang et al., 2003). TNF α is produced by the embryo itself and acts via EGF-R, the survival factor IGF-I is mainly of maternal origin (Hardy and Spanos, 2002). Good key markers for

impaired embryo growth appear to be altered ratios of e.g Bcl-xL:Bak and Bcl2:Bax in favor for Bak and Bax, respectively, leading to apoptotic elimination of altered cells (Jeong et al., 2004; Yuan and Kroemer). A decrease in gene expression between fertilization and the onset of the embryonic genome has also been demonstrated for genes involved in apoptosis, such as the Bcl-2 family (Metcalfé et al., 2004). Other pro-apoptotic genes like RB1 and Bad reach their maximum transcription levels only after compaction, when the first apoptotic signs are observed (Spanos et al., 2002; Wells et al., 2005).

1.4 The mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) pathway is one of the major regulators in protein synthesis and cell growth. The mTOR pathway is activated in response to nutrients (amino acids), growth factors (insulin) and cellular energy (ATP) (Sarbasov et al., 2005a; Wullschleger et al., 2006). The history of mTOR began with the discovery of the anti-fungal macrolide rapamycin in the 1970s, produced by the bacterial strain *Streptomyces hygroscopicus*. The name rapamycin derives from the place it was found, namely on the Easter Island “Rapa Nui”. Initially rapamycin was used as an anti-fungal agent; however, by further investigating this molecule, it was found that it is an effective immunosuppressor used after organ transplantation or chemotherapy. Just lately rapamycin was tested as an anti-cancer therapeutic (Chueh and Kahan, 2005; Di Mario et al., 2004; Vezina et al., 1975; Yang and Guan, 2007). Through rapamycin a protein was found that would bind to it – the target of rapamycin (TOR).

1.4.1 **Structure and genetics of mTOR**

The TOR protein itself is approximately 280kDa, containing several structural and functional domains. In the amino-terminal half a number of HEAT repeats can be found that are important for protein interactions. The FRB domain, located in the middle of the protein, is the binding domain for the FKBP12-rapamycin complex, which inhibits mTOR activity (Brown et al., 1994; Jayaraman and Marks, 1993). The catalytic unit of mTOR is located in the C-terminal region. Its kinase domain places TOR to the family of phosphatidylinositol kinase-related kinases (PIKK). Typical for the PIKK family are so-called FAT and FATC domains (Figure 1.4.1). Overall the TOR protein is very well conserved from yeast to mammals (Hall, 2008).

The mTOR protein is found in two structurally and functionally distinct complexes: mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2). In 1994, the first interacting proteins of this complex were discovered (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994). Much later mTORC2 was found to exist with different proteins associated (Loewith et al., 2002). mTORC1 consists of mTOR, its scaffold protein raptor, mLST8 and is highly sensitive to rapamycin. Newer publications additionally add PRAS40 to mTORC1 and also DEPTOR (Dunlop and Tee, 2009; Ma and Blenis, 2009; Meric-Bernstam and Gonzalez-Angulo, 2009; Peterson et al., 2009). The mTORC2 shares mTOR and mLST8 protein with

mTORC1, but has two other proteins necessary to form that complex – rictor and sin1. Sometimes protor and DEPTOR are also added to the complex. For a long time it was thought that mTORC2 is insensitive to rapamycin, however after a 24h exposure of rapamycin the activity of mTORC2 is diminished (Sarbasov et al., 2006). The dissociation of mTORC2 by rapamycin is achieved through the dephosphorylation of rictor and sin1 (Rosner and Hengstschlager, 2008).

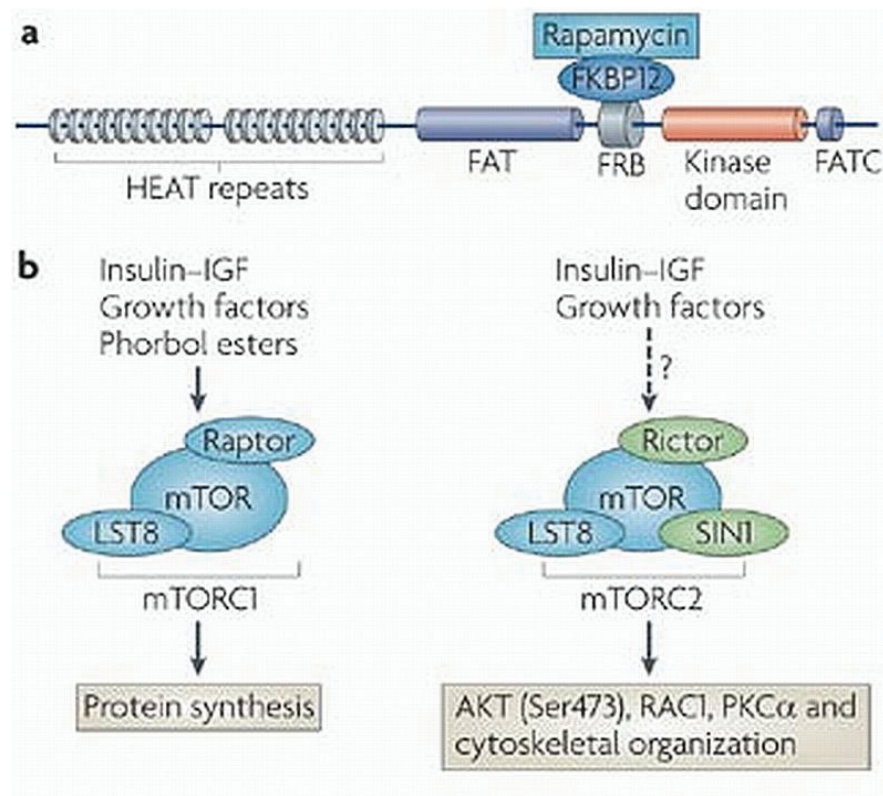


Figure 1.4.1 Structure and complexes of mTOR **a)** Domain structure of mTOR. N-terminal region is composed of tandem HEAT repeats and C-terminal half contains FAT domain, followed by FRB domain, a kinase domain and the FATC domain. **b)** Shows composition of mTORC1 and mTORC2, how these two complexes are stimulated and their downstream effects / function (Ma and Blenis, 2009).

In contrast to mTORC1 the function of mTORC2 seems to be independent of growth factors (Facchinetti et al., 2008) and its function is evolutionary conserved. mTORC2 mainly controls cytoskeleton dynamics, metabolism, proliferation, whole body growth and survival (Cybulski et al., 2009; Jacinto et al., 2004; Loewith et al., 2002; Sarbasov et al., 2004).

The PI3K-AKT-mTOR pathway is activated by growth factors or insulin, which bind to the corresponding tyrosine receptor kinases. This leads to an intracellular phosphorylation event and type Ia phosphoinositide 3-kinases (PI3K) are recruited to the plasma membrane. In the next step PI3K phosphorylates membrane phosphoinositides at the 3' position to generate PI(3,4,5)P3 and related lipids, which lead to recruitment of the serine/threonine kinase AKT to the membrane through its PH domain. To be fully activated AKT is then phosphorylated at S473 by mTOR complex 2 (mTORC2) and other kinases, and at T308 by PDK1 (Alessi et al., 1997; Sarbassov et al., 2005b; Vanhaesebroeck and Alessi, 2000). Active AKT phosphorylates TSC2 on S939 and T1462, as well as PRAS40 on T246, which blocks their inhibitory function towards mTORC1. Besides the PI3K pathway, tuberlin and therefore mTORC1 are also regulated by Rsk, Erk, AMPK and GSK3 β (Dan et al., 2002; Roux et al., 2004; Soulard and Hall, 2007).

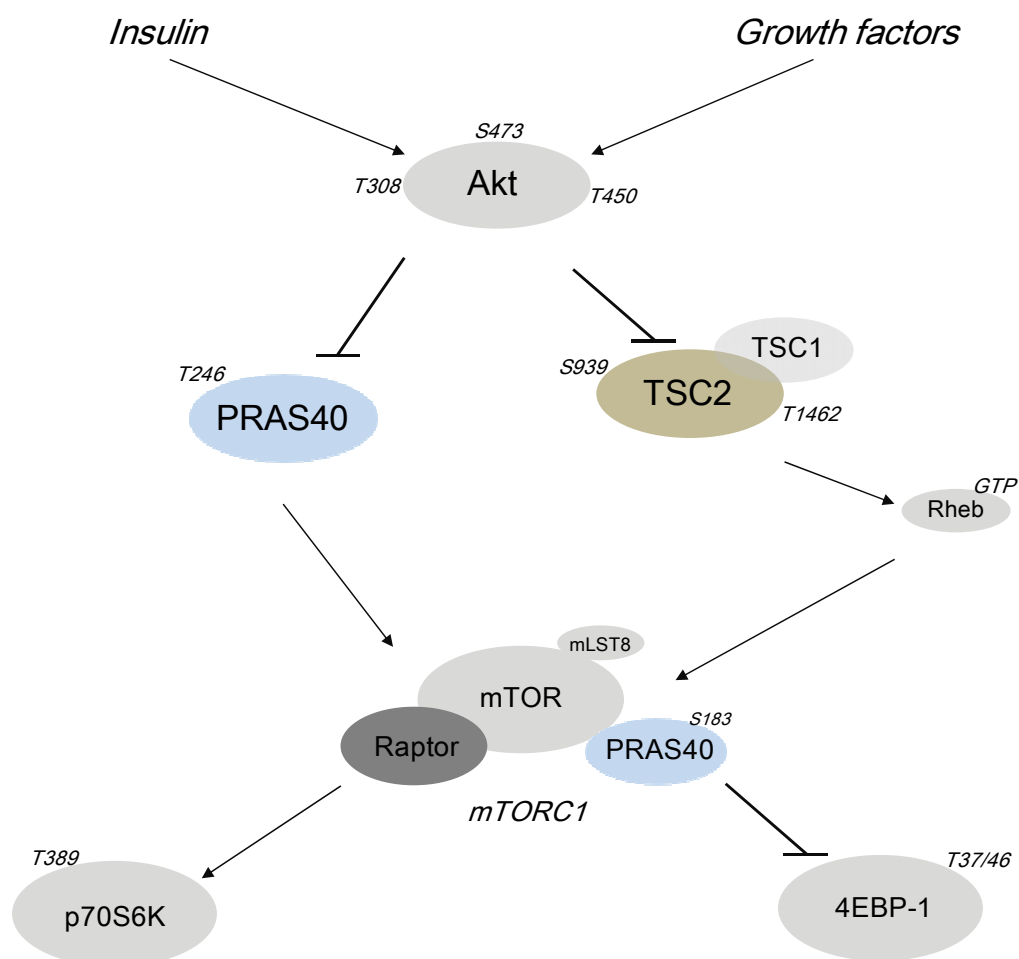


Figure 1.4.2 mTORC1 regulation via AKT-PRAS40 and AKT-TSC.

If tuberlin is phosphorylated by AKT, Rsk or Erk for example it cannot execute its GTPase function towards Rheb. In course of this Rheb, in its active state, subsequently binds to mTORC1 and activates the complex (Avruch et al., 2006; Hall, 2008; Rosner et al., 2006). With the discovery of PRAS40, a second and more direct inhibitor of mTORC1 was found, also being regulated through phosphorylation by AKT, subsequent 14-3-3 binding and removal of the complex (Kovacina et al., 2003; Sancak et al., 2007; Vander Haar et al., 2007), giving AKT two possibilities in regulating mTORC1 (Figure 1.4.2).

mTORC1 has two major downstream targets, p70S6K and 4EBP-1. Active mTORC1 phosphorylates p70S6K on T389 and 4EBP-1 on T37/46. Besides T389 p70S6K has to be phosphorylated on S229/252 by PDK1 to be fully activated (Alessi et al., 1998; Pullen et al., 1998). Fully active p70S6K then activates ribosomal protein S6 via phosphorylation on S240/244. This finally promotes ribosome biogenesis and protein translation. It is obvious that this function of S6 is essential and as a result makes up to 30% of the total protein of a cell (Hengstschlager et al., 2001; Ma and Blenis, 2009; Miloloza et al., 2000; Proud, 2007; Shima et al., 1998; Toker, 2000). While phosphorylation of p70S6K is activating, the phosphorylation of 4EBP-1 by mTORC1 has an inhibitory effect. Phosphorylated 4EBP-1 dissociates from eIF4E, as unphosphorylated 4EBP-1 is bound to it and blocking translation. Free eIF4E is promoting the assembly of eIF4F complex and consequently mRNA translation, especially the cap-dependent translation (Dorrello et al., 2006; Ma and Blenis, 2009; Raught et al., 2004; Shahbazian et al., 2006).

Besides regulating translation and ribosome biogenesis, mTORC1 and mTORC2 are also a major regulators of cell size, growth and cell cycle (Rosner et al., 2009b). Knocking down rictor and raptor led to a severe decrease in cell size and reduced S-phase in cell cycle (Rosner et al., 2009b) and cells that do lack a functional mTORC1 do have smaller cells or the whole organism is smaller (Fingar et al., 2002; Kim et al., 2002). mTORC1 is also regulating autophagy, a special type of cell death, via ATG1 and p70S6K, and angiogenesis via HIF-1 and VEGF, insulin sensitivity, nutrient sensing, ageing and stress responses (Chiang and Abraham, 2007; Dunlop and Tee, 2009; Gwinn et al., 2008; Sabatini, 2006; Selman et al., 2009; Wei et al., 2009).

Far less is known about mTORC2. Besides AKT mTORC2 has some other substrates e.g. PKC α and Rho (Ikenoue et al., 2008; Jacinto et al., 2006). In cells negative for rictor or

mSin1 phosphorylation and protein levels of PKC α are diminished. The phosphorylation of PKC through the components is essential for PKC kinase stability and function (Ikenoue et al., 2008). For a long time mTORC2 was thought to be rapamycin insensitive, in the recent years however several groups proved that long-term treatment with rapamycin leads to dissociation of the mTORC2 complex (Rosner et al., 2009b; Rosner and Hengstschlager, 2008; Sarbassov et al., 2006), and that this is mediated via dephosphorylation of rictor and mSin1 (Dunlop and Tee, 2009; Rosner and Hengstschlager, 2008). The role of the TSC1-TSC2 complex in regulating mTORC1 is known very well. Recent work revealed that cells lacking a functional TSC complex have an impaired activity towards AKT, a mTORC2 substrate. The reduced activity of AKT was shown to be independent of the negative feedback mechanism of p70S6K to IRS (Huang et al., 2008). It was found that TSC complex can physically associate with mTORC2 without involving Rheb, making it clear that there are major differences in the control of mTORC1 and mTORC2 (Huang et al., 2008). It was reported that mTORC2 itself is a potent regulator of mammalian cell size and cell cycle via a mechanism involving the AKT/TSC2/Rheb cascade independently of mTORC1 (Rosner et al., 2009b).

1.5 Tuberous sclerosis

Tuberous sclerosis complex (TSC) is an autosomal dominant, multisystem genetic disease affecting 1 in 6000 births. Mutations in *TSC1*, encoding hamartin, or *TSC2* gene, encoding tuberlin, are causing TSC. It was first described by Bourneville in 1880. Table 1.5.1 gives an overview of various clinical manifestations of tuberous sclerosis, which are important diagnostic criteria (Crino et al., 2006; Roach et al., 1998). For a definite clinical diagnosis of TSC two major features or one major and two minor features are required. For a probable, one major and one minor feature, and possible diagnosis, one major or two or more minor manifestations, are needed.

Table 1.5.1 Diagnostic criteria for Tuberous Sclerosis

Criteria	Age at Onset
<i>Major</i>	
cortical tuber	fetal life
cardiac rhabdomyoma	fetal life
retinal hamartoma	infancy
hypomelanotic macule	infancy to childhood
facial angiofibroma	infancy to adulthood
shagreen patch	childhood
subependymal nodule	childhood to adolescence
subependymal giant-cell tumor	childhood to adolescence
renal angiomyolipomas	childhood to adulthood
ungual fibroma	adolescence to adulthood
lymphangiomyomatosis	adolescence to adulthood
<i>Minor</i>	
multiple pits in dental enamel	
bone cysts	
hamartomatous rectal polyps	
cerebral white-matter radial migration lines	
gingival fibromas	
retinal achromic patch	
"confetti" skin lesions (groups of small pigmented spots)	
multiple renal cysts	

The disease is often (>50%) accompanied by neurological disorders like autism, epilepsy and mental retardation. The neurological defects are also the primary cause of death among children (Kwiatkowski, 2003). The name of the disease, tuberous sclerosis, derives from the cortical tubers found in the brain of patients (Crino, 2004; Crino et al., 2006; DiMario, 2004; Kwiatkowski and Manning, 2005). The pleiotropism of this syndrome is highlighted by the appearance of dysplastic lesions, called hamartomas, in several major organs (Shah and Hunter, 2005). Renal diseases are common in TSC patients, with an 80% chance to develop angiomyolipomas. Although malignant forms and renal cell carcinoma are rare, these lesions can be severe and manifest during early childhood (Bjornsson et al., 1996). In about 60-80% of affected individuals benign cardiac rhabdomyomas are detected perinatally, however they generally do regress during postnatal heart development (Crino et al., 2006; Shah and Hunter, 2005; Smythe et al., 1990). Usually skin abnormalities are involved as well like angiofibromas and fibromatous plaques (Gomez et al., 1999). One clinical feature seems to probably be gender dependent namely pulmonary lymphangiomyomatosis (LAM), which appears more frequently in woman and is very rare in men (Kwiatkowski, 2003). When observing tuberous sclerosis in the perinatal period there is a high frequency of cardiac rhabdomyomas, arrhythmias, cerebral lesions and stillbirth in the fetus, whereas respiratory distress, arrhythmias, murmurs and cardiomegaly are the main signs in the neonate initially. The survival rate of patients diagnosed with TSC in the perinatal period is very low (21%) and as it is associated with a high incidence of morbidity and mortality (Isaacs, 2009).

1.5.1 Genetics of TSC

The tumor suppressor gene *TSC1* is located on chromosome 9q34 and *TSC2* on chromosome 16p13.3. As already stated above one mutation in either gene is sufficient to acquire TSC, and loss of heterozygosity has been documented in a wide variety of tumors. The development of a tumor is assumed to be the outcome of somatic “second hit” mutations according to the Knudson’s tumor suppressor model. Therefore, although TSC is considered an autosomal dominant disease, mutations in the TSC gene are believed to be recessive at the level of the affected cell (Crino et al., 2006; Kwiatkowski, 2003; Rosner et al., 2006). The majority of the disease-causing mutations occur *de novo* in either gene, and only about 1/3 of the TSC mutations are inherited. The distribution of mutations in *TSC1* and *TSC2* is about half in hereditary cases, however in sporadic TSC

mutations *TSC2* stands out. *TSC1* mutations are nearly always described as either nonsense or frameshift mutations leading to truncated hamartin protein. In *TSC2* mutations approximately 20% are missense or nonsense (Astrinidis and Henske, 2005; Rosner et al., 2008b).

Tuberin and hamartin have to physically interact with each other to form a heterodimer (Figure 1.5.1) in order to be a functional complex (Plank et al., 1998; van Slegtenhorst et al., 1998). The two proteins are expressed within multiple organs in the same cell types including the brain, the kidney, lung and pancreas (Plank et al., 1999). So far there exists only one putative functional domain in tuberin, namely a GTPase activating protein (GAP) domain. Rheb, a member of the Ras family, was identified as the specific GTPase downstream of tuberin (Garami et al., 2003; Inoki et al., 2003).

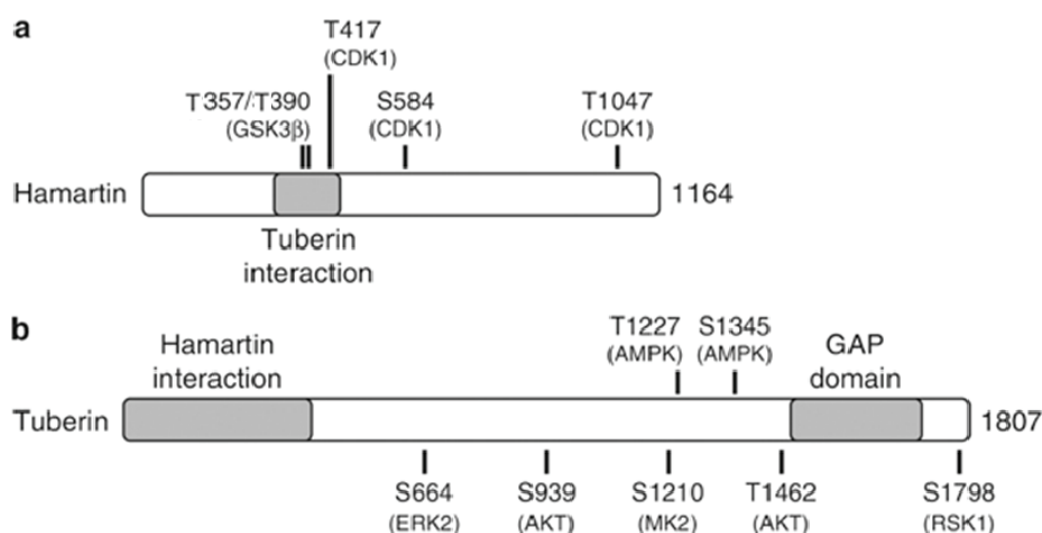


Figure 1.5.1 Domains and phosphorylation sites of hamartin and tuberin. a) Hamartin is a 1164 amino acid long protein with a molecular mass of 130kDa. It interacts with tuberin through amino acids 302-430 (Hodges et al., 2001). It is phosphorylated by CDK1/cyclinB1 at residues T417, S584 and T1047, and by GSK3β at T357 and T390. **b)** Tuberin consists of 1807 amino acids and has a molecular mass of 200kDa. It interacts with hamartin through amino acids 1-418 (Hodges et al., 2001). Besides this domain tuberin has a GTPase activating protein (GAP) domain for Rheb. It is phosphorylated on S939 and T1462 by AKT, at S1210 by MK2, at T1227 and S1345 by AMPK, from RSK2 at S1798 and by ERK at S664 (Astrinidis and Henske, 2005).

1.5.2 Signaling pathway

TSC is incorporated in some major signaling pathways by phosphorylation events that regulate tuberin's GAP activity: PI3K-AKT pathway, the ERK1/2-RSK1 pathway and the LKB1-AMPK pathway (Figure 1.5.2). In brief, as some parts of the pathway were already

explained in previous sections, PI3K is being activated by growth factors and this in turn leads to activation of AKT.

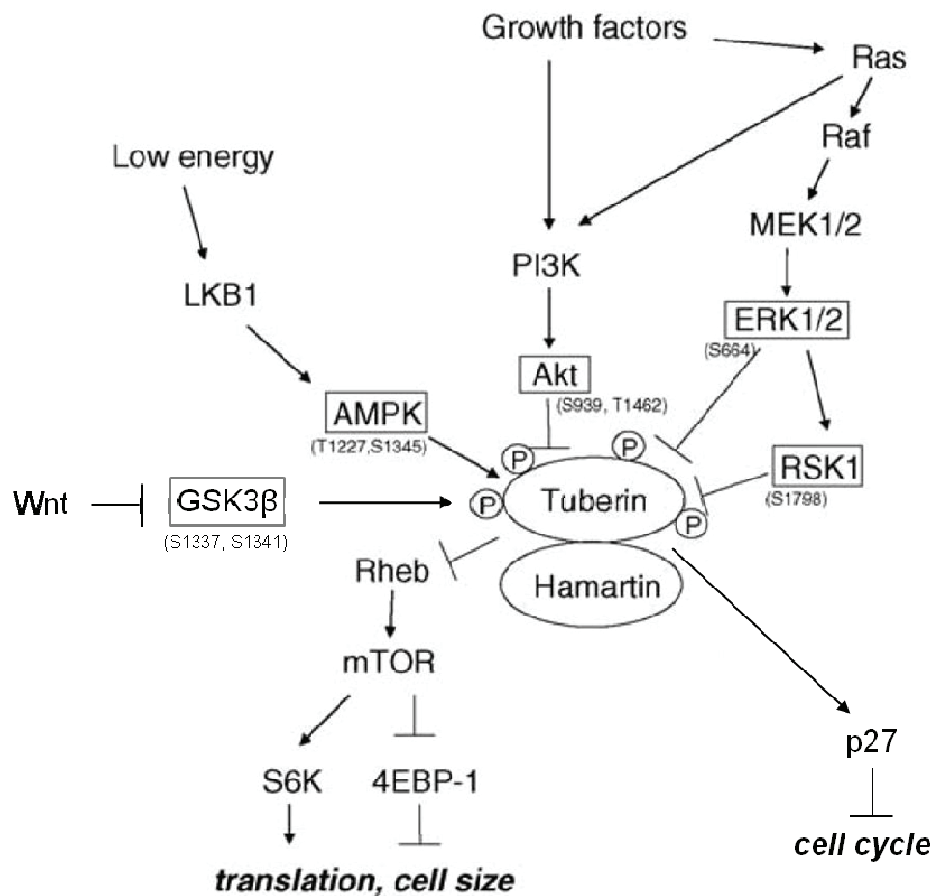


Figure 1.55.2 Upstream and downstream signaling events involving TSC (Rosner et al., 2006).

Akt has numerous downstream targets, to name some: the members of the FOXO family of transcription factors, BAD, GSK3 α and β , members of the cip/kip family (p27 and p21), and last but not least tuberlin (Downward, 2004; Manning and Cantley, 2003). AKT phosphorylation events are inhibitory in general and usually have survival promoting effects. In the absence of growth factors or other stimuli, the TSC1/TSC2 heterodimer has strong inhibitory effects towards Rheb (Ras homology enriched in brain) via its GAP (Saucedo et al., 2003; Stocker et al., 2003; Zhang et al., 2003). Upon induction of the pathway by growth factors, AKT phosphorylates tuberlin on S939, S981, S1130, S1132 and T1462, this in turn inhibits tuberlin's ability to block Rheb via its GAP activity (Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002) and subsequently

Rheb-GTP levels increase (Tee et al., 2003). Elevated Rheb-GTP levels lead to the activation of the mTOR complex 1 (mTORC1), which is positively influencing translation and cell size / cell cycle regulation (Martin and Hall, 2005; Rosner et al., 2009b; Tee and Blenis, 2005). Besides regulating mTOR and therefore indirectly cell size and cell cycle, tuberin can regulate the cell cycle independently of mTOR through the cyclin-dependent kinase inhibitor p27 (Rosner et al., 2006; Rosner and Hengstschlager, 2004). Furthermore it does not only regulate p27, tuberin is influencing its stability and its localization (Rosner et al., 2009a; Rosner et al., 2006; Rosner and Hengstschlager, 2004). In summary, tuberin is a potent regulator of the PI3K-AKT pathway, regulating cell size, cell cycle, translation and apoptosis.

1.6 PRAS40

In 2003 Kovacina et al. identified a novel substrate for AKT called proline-rich AKT substrate of 40kDa (PRAS40) binding 14-3-3 proteins. They showed that PRAS40 was phosphorylated by AKT in vitro at the same site that was phosphorylated in insulin treated cells. Secondly, the activation of an inducible AKT was sufficient to stimulate its phosphorylation. Lastly, the phosphorylation of PRAS40 was diminished in cells lacking AKT1 and AKT2, demonstrating that PRAS40 is indeed a substrate of AKT (Kovacina et al., 2003). Later, other groups recognized that PRAS40 is also an mTORC1 complex component (Oshiro et al., 2007; Sancak et al., 2007; Thedieck et al., 2007; Vander Haar et al., 2007), preferentially binding to raptor but also to mTOR protein itself. However, PRAS40 is not binding to rictor and therefore is not involved in mTORC2 regulation (Oshiro et al., 2007; Sancak et al., 2007; Thedieck et al., 2007; Vander Haar et al., 2007)

1.6.1 **PRAS40 genetics**

PRAS40 gene is located on chromosome 19q13.33 and encodes for three different transcript variants that are all consisting of 256 amino acids but do differ in the 5'-UTR. The highest transcription levels of PRAS40 are found in human liver, heart, and placenta (Kovacina et al., 2003; Nascimento et al., 2006). As the name suggests the protein consists of a proline-rich sequence at the amino terminal domain (Kovacina et al., 2003). This region is followed by two motifs, the TOS (mTOR signaling) and the RAIP motif (Figure 1.6.1), that have been implicated for mTORC1 binding and phosphorylation of its substrates (Nascimento and Ouwens, 2009).

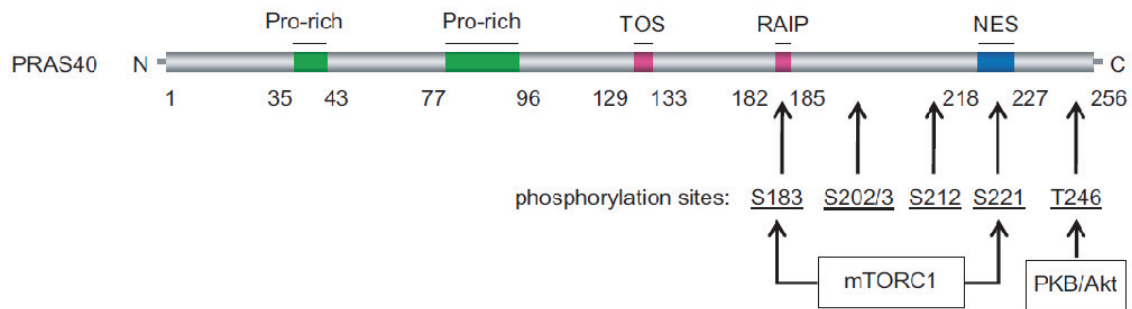


Figure 1.6.1 Primary structure of the PRAS40 protein. PRAS40 consists of two proline-rich stretches followed by a TOS-motif, a RAIP-motif and a NES. Arrows indicate phosphorylation sites of PRAS40 by AKT, mTORC1 and other kinases (Nascimento and Ouwers, 2009).

PRAS40 is a highly conserved protein and the TOS, RAIP and NES-motifs as well as the phosphorylation sites on S183, S221 and T246 share high homology (Vander Haar et al., 2007). The TOS motif is found in many other substrates for mTORC1 and the RAIP motif is also found in 4EBP1 (Beugnet et al., 2003; Tee and Proud, 2002).

1.6.2 Cellular functions of PRAS40

There are different opinions on the role of PRAS40 in the mTOR signaling pathway, ranging from PRAS40 being an inhibitor of mTORC1 (Sancak et al., 2007; Wang et al., 2007), a substrate of mTORC1 (Fonseca et al., 2007; Oshiro et al., 2007; Vander Haar et al., 2007) or both. However, before placing PRAS40 in the mTORC1 signaling pathway, it was found to be a new substrate for AKT, which is phosphorylating it on T246 (Kovacina et al., 2003). The regulation of PRAS40 T246 phosphorylation by AKT seems to be dependent on AKT S473 phosphorylation through mTORC2. Inhibition of mTORC2 activity, by silencing rictor or pharmacologically, reduces T246 phosphorylation of PRAS40 (Thedieck et al., 2007; Toschi et al., 2009). Recently it was shown that the serine/threonine kinase PIM1, playing a role in cell survival, proliferation and differentiation, is also able to phosphorylate PRAS40 on T246. Furthermore PIM1's ability to phosphorylate PRAS40 on that site is independent from AKT and not inhibited by wortmannin. The investigators suggests that PIM1 regulated mTORC1 activity via phosphorylation of PRAS40 (Zhang et al., 2009). As stated above PRAS40 is not only an inhibitor of mTORC1, it is also a substrate for mTORC1 (Figure 1.6.2). Several different phosphorylation sites for mTORC1 on PRAS40 exist: S183, S202/203, S212 and S221 (Oshiro et al., 2007; Wang et al., 2008). The phosphorylation of S183 is promoted by amino acids and insulin and is reduced by the addition of rapamycin strongly supporting

that mTORC1 is the kinase for this site (Oshiro et al., 2007; Wang et al., 2008). From all the other phosphorylation sites, in addition to S183, only S221 is also sensitive to rapamycin (Wang et al., 2008). The kinases that phosphorylate PRAS40 on these other sites yet have to be identified.

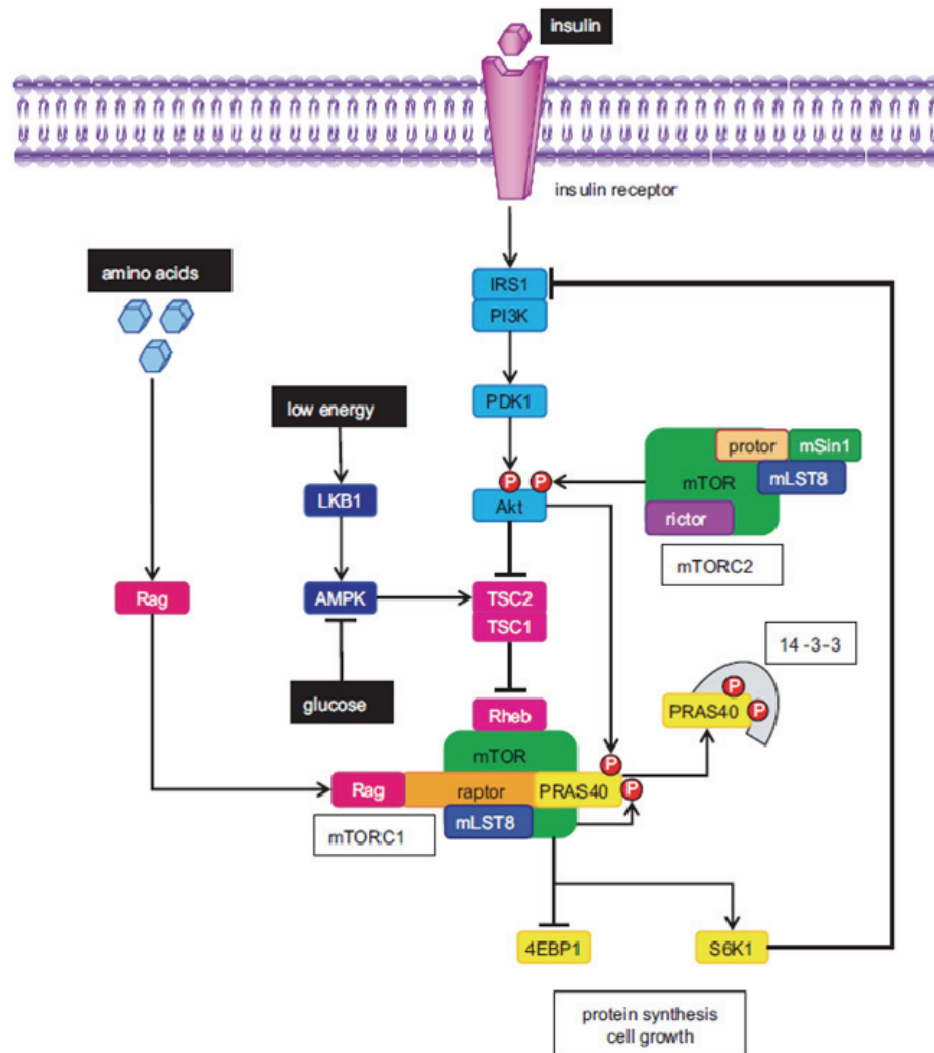


Figure 1.66.2 Regulation of mTORC1 by insulin, amino acids, glucose and energy deprivation. The activation of mTORC1 requires the binding of Rag and Rheb to mTORC1. Full activation of mTORC1 further requires the dissociation of PRAS40 from mTORC1, which requires phosphorylation of PRAS40 by both AKT and mTORC1 and 14-3-3 binding (Nascimento and Ouwens, 2009).

Consistent with a role for PRAS40 as a negative regulator of mTORC1, ectopic expression of PRAS40 led to a significant reduction of cell size (Sancak et al., 2007; Vander Haar et al., 2007). Reciprocally, the silencing of PRAS40 led to an increased cell size in drosophila (Sancak et al., 2007). The inhibitory function of PRAS40 seems to be

partly dependent on the interaction with raptor, supposedly via the TOS motif and S183, the location of the RAIP motif. Based on this it was proposed that PRAS40 functions as an inhibitor of substrate binding on raptor (Oshiro et al., 2007; Wang et al., 2007). There exist contradictory data about the downstream effect of silencing PRAS40; some groups found an upregulation of S6K1 and 4EBP1 phosphorylation upon PRAS40 silencing (Oshiro et al., 2007), however others report that both amino acid and insulin induced phosphorylation of S6K1 and 4EBP1 are reduced in the absence of PRAS40 (Fonseca et al., 2007; Vander Haar et al., 2007; Wang et al., 2007). The requirement of PRAS40 for the phosphorylation of mTORC1 substrates, therefore, also suggests a role for PRAS40 in the assembly or integrity of the mTORC1 complex (Nascimento and Ouwers, 2009).

Another regulatory function of PRAS40 is a role in cell survival and apoptosis. When overexpressed it protects neurons from apoptotic cell death, transient focal cerebral ischemia or spinal cord injury (Saito et al., 2004; Yu et al., 2008). On the other hand, silencing PRAS40 expression has been found to protect against the induction of apoptosis by TNF α or cyclohexamide (Thedieck et al., 2007). It is important to note that rapamycin fails to mimic the proapoptotic effect of PRAS40 silencing and therefore it has been suggested that PRAS40 may regulate apoptosis independently of mTORC1 (Thedieck et al., 2007).

Not that much is known about PRAS40 and diseases. In pre-malignant and malignant breast and lung cancer cell lines both, PRAS40 and phosphorylation on T246 are elevated (Huang and Porter, 2005). Furthermore, PRAS40 T246 levels were elevated in meningiomas and malignant melanomas (Johnson et al., 2009; Madhunapantula et al., 2007). Interestingly, lowering PRAS40 T246 associates with an increased sensitivity of tumor cells to pro-apoptotic stimuli, suggesting that PRAS40 does play a critical role in tumor cell survival (Madhunapantula et al., 2007).

2. MATERIALS AND METHODS

2.1 Cell culture

All cell lines used during this thesis were incubated at 5% CO₂ and 37°C in a Heraeus BBD 6220 incubator (Thermo Scientific, Waltham, MA, USA). All cell lines were obtained from the American Type Culture Collection (ATCC) unless we kindly received them from other colleagues (see in the text).

2.1.1 Cell lines

The human non transformed, non immortalized, primary fetal lung fibroblasts IMR-90 were grown in DMEM-HG medium. The cell lines MCF-7 (mammary gland adenocarcinoma) and Hela (epithelial cervix carcinoma) were also grown in DMEM-HG medium supplemented with 10 % FCS and antibiotics 30 mg/l penicillin, 50 mg/l streptomycin sulphate and 2.5 mM L-glutamine. The Jurkat T cells (human T cells leukemia) are suspension cells and were grown in DMEM-HG medium supplemented with 10 % FCS and 2,5 mM L-glutamine. SK-N-SH cells (human neuroblastoma) were cultured in RPMI medium supplemented with 20 % FCS, antibiotics and 2,5 mM L-glutamine. Cells used for immunofluorescence staining, were seeded and cultured on either glass or permanox slides. Mouse embryonic kidneys (E11.5) were dissected, grown and stained as described (Siegel et al., 2010; Unbekandt and Davies, 2010). Cells were routinely screened for mycoplasma.

2.1.2 hAFS cells

The human AFS cell line Q1 has been established by Anthony Atala via magnetic cell sorting using the CD117 MicroBead Kit (De Coppi et al., 2007). AFS cells were grown in α -MEM minimal essential medium (Gibco-Invitrogen, Carlsbad, CA, USA, 41061), supplemented with 15 % ES-FCS (HyClone, Waltham, MA, USA, 30070.03), 18 % Chang B, 2 % Chang C (Irvine Scientific, CA, USA, C100, C106), 2,5 mM L-glutamine, 50 mg/l streptomycin sulphate and 30 mg/l penicillin. The Q1 hAFS cell line was cytogenetically analyzed according to standard protocols. Chromosome banding was produced by means of a conventional 550-band trypsin-Giemsa analysis. A significant number of metaphases were analyzed for numerical and structural chromosome aberrations (Hengstschlager et al., 2005).

2.1.3 Cell culture methods

2.1.3.1 Thawing of cells

All cells are stored in a liquid nitrogen tank. For thawing cells cryo tubes need to be taken from the tank and rapidly transferred to a 37°C water bath. After thawing the cell suspension is transferred from the cryo tube into a 15ml Falcon tube filled with 1- 5ml of pre-warmed cell culture medium and centrifuged at 1000rpm for 3 to 5 minutes. The supernatant is discarded and the cell pellet is resuspended in the appropriate cell culture medium and seeded into a cell culture dish.

2.1.3.2 Harvesting cells

The culture medium is discarded and then cells are washed once with 1x PBS. The PBS needs to be removed and depending on the size of the cell culture dish an appropriate amount of Trypsin-EDTA is added to the cells and incubated at 37°C for a maximum of 5 minutes. Then the trypsin is inactivated by adding at least two times the amount of medium containing 10% FCS of trypsin used. Cells are transferred to a 15ml falcon tube and centrifuged for 5 minutes at 1000rpm.

2.1.3.3 Passaging of cells

Cells were harvested as described and the resulting pellet is resuspended in an appropriate amount of medium and transferred into new cell culture plates at an appropriate splitting ratio.

2.1.3.4 Freezing of cells

Cells were harvest like in 2.1.3.2 and the obtained pellet is resuspended in 900µl of the appropriate medium. Prior to this cryo tubes need to be prepared containing 100µl (10%) of DMSO. The cell suspension is then added to the 100µl of DMSO and the tube is inverted 3 to 6 times and stored on ice immediately afterwards. After a 20 minutes pre-incubation period on ice the cryo tube is then transferred to -80°C and is stored there for 24 to 72 hours. At last the cryo tube is put into the liquid nitrogen tank for long term storage of the cells.

2.1.4 Embryoid bodies

2.1.4.1 Preparation of methylcellulose

Methylcellulose is a component of the EB medium, which additionally prevents cells from attaching to the bottom of a 96-Well-Plate. 7,5g of methylcellulose (cat# m-0512) are filled into a 500ml bottle containing a magnetic stirrer and then the methylcellulose is autoclaved. The powder is dissolved in 250ml of preheated basal medium (60°C) for 20 minutes. After dissolving the powder another 250ml of basal medium, having room temperature, is added to a final volume of 500ml and is mixed over night at 4°C on a magnetic stirrer. Aliquot the stock solution into 50ml falcons and then clear the methylcellulose by centrifugation at 5000g for 2h at room temperature. Only transfer the clear highly viscous supernatant (90-95% of the stock solution) into 50ml falcon tubes and store aliquots at -20°C.

2.1.4.2 EB formation

EBs were generated using a suspension method with 0,3% (v/v) methylcellulose in 96-well plates with a U shape and non-adhesive cell culture plastic (PAA, Linz, Austria, 34296X) as described (Valli et al., 2010). A single EB is composed of 1000 to 1500 cells in 100 µl medium.

2.1.4.3 Harvesting EB

For protein extraction whole EBs were harvested. For analysis of cell number and cell size / DNA content EBs were harvested, washed 3 times in ice cold 1x PBS and then trypsinized for 15-20 minutes to receive a single cell suspension. Separating the inner and outer cells of EBs was achieved by harvesting EBs, washing them carefully with ice cold 1x PBS, followed by a 2-3 minute incubation with Trypsin-EDTA. Reaction was stopped by adding medium to EBs. EBs and single cells (*outer cells*) were separated by slow centrifugation (300 rpm, 5 minutes, 4°C). Supernatant was collected, transferred into new tubes and centrifuged for 10 minutes, 1000 rpm at 4°C. Cell pellet was then used for further analysis. Remaining EBs were washed with 1x PBS and then trypsinized for 15-20 minutes to receive single cells (*inner cells*). So obtained cell pellets were further processed for analysis.

2.1.5 siRNA transfection of cells

RNA silencing was achieved using human siRNA specific for PRAS40, tuberlin and mTOR (Dharmacon, Lafayette, CO, USA) at a final concentration of 50 nM (Rosner et al., 2010). siRNA was delivered to cells using RNAiMAX reagent (Invitrogen). A pool of four non-targeting siRNAs was used as control for non –sequence-specific effects for each transfection. Analysis and EB formation was performed 72 h after transfection.

2.1.6 DNA transfection of cells

For transfection of cells with plasmid DNA cells need to be grown in antibiotic free medium at a density of 80-90% at the time of transfection and cells need to be seeded 16-24h before transfection. For one 6-Well 1µg of plasmid DNA has to be diluted in 150µl OptiMEM medium and is mixed gently. The second solution consists of 3,75µl Lipofectamine 2000 reagent in 150µl of OptiMEM. Mix both solutions together and mix gently using a vortexer. Incubation time for the DNA-Lipofecatmine solution is at least 20 minutes. Then formed complexes are added to the cells. Cells can be harvested and analyzed 24 to 48 hours after transfection.

Table 2.1.1 Plasmids used for transfections

Gene	Vector backbone	Tag	cataloge #	company
PRAS40	pcDNA3.1(+)	myc		kindly provided by R. A. Roth
PRAS40 S183A	pcDNA3.1(+)	HA	#15674	Addgene
empty	pcDNA3.1(+)		#V790-20	Invitrogen
PRAS40	pRK5	myc	#15476	Addgene
PRAS40 T246A	pRK5	myc	#15479	Addgene
empty	pRK7			kindly provided by J. Blenis

2.1.7 Treatment of cells with chemical compounds

2.1.7.1 Rapamycin treatment

The mTOR specific inhibitor rapamycin (Calbiochem, La Jolla, CA, USA, 553211) is added to the medium at a final concentration of 100nM for 24h, unless otherwise stated, before harvesting the cells. Control cells are treated with the equal amount of DMSO.

2.1.7.2 Wortmannin treatment

Wortmannin is a PI3K inhibitor and therefore blocks phosphorylation of AKT, PRAS40 and Tuberin for example. Before the inhibitor is added, cells are starved for 16 hours or overnight with 0% FCS medium. Then Wortmannin is added to a final concentration of 10 μ M to the cells for 30 minutes. For control cells an equal amount of DMSO is added. After the incubation, serum is added to the cells to reach a final concentration of 10% FCS. Cells are incubated for another 30 minutes. Then cells can be harvested for further investigation.

2.2 Isolation, preparation and analysis

2.2.1 Protein extraction

Total protein was extracted by physical disruption of cell membranes by repeated freeze and thaw cycles. In brief, cells/EBs were washed with PBS and harvested after trypsinization. Cell pellets were lysed in buffer A containing 20 mM Hepes pH 7,9, 0,4 M NaCl, 2,5 % glycerol, 1 mM EDTA, 0,5 mM DTT, 1 mM PMSF, 0,5 mM NaF, 0,5 mM Na₃VO₄ supplemented with 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 0,3 μ g/ml benzamidinchlorid, 10 μ g/ml trypsininhibitor by repeated freeze and thaw cycles. After incubation on ice and centrifugation at 15000 rpm for 20 minutes at 4°C supernatants were collected and protein lysates stored at -80°C. Protein concentrations were determined using Bio-Rad protein assay (Rosner and Hengstschlager, 2008).

2.2.2 Cytoplasmic and nuclear fractionation

For cytoplasmic and nuclear fraction of proteins everything is the same as in 3.2.1 until the second wash step. The pellet gets resuspended in 500 μ l of 1xPBS and transfer 100 μ l of the suspension into a tube for the total protein lysate and the rest into another tube for the cytoplasmic-nuclear preparation. For total protein extraction see section 2.2.1. After the third washing step resuspend the pellet in 5x volume of lysis buffer F1 and incubate 2min at RT, followed by 10 minutes incubation on ice. After incubation NP-40 is added at a final concentration of 1% (v/v) and lysates are homogenized by passing through a 20-gauge needle for three times. Nuclei are pelleted by centrifugation at 2500rpm for 5 minutes at 4°C and supernatant containing cytoplasmic proteins is collected. Remaining nuclei are washed three times with buffer F containing 1% NP-40. The nucleic pellets are

lysed in buffer A by repeated freeze and thaw cycles. Supernatant containing the soluble nuclear proteins is collected by centrifugation at 15000rpm at 4°C for 20 minutes (Rosner et al., 2007b; Rosner and Hengstschlager, 2007).

2.2.3 Flow cytometry and CASY cell counter

EBs were processed as described above to receive single cells. Cells were fixed by rapid submersion in ice-cold 85% ethanol. After fixation over night at -20°C DNA was stained with 0,25 mg/ml propidium iodide, 0,05 mg/ml RNase A, 0,1% Triton X-100 in citrate buffer, pH 7,8 and cell size (FSC) and subG1 fraction was analyzed on a Beckton Dickinson FACSCalibur (Beckton Dickinson, San Jose, CA, USA) (Rosner et al., 2003). For flow cytometric analysis a 96-well plate with EBs was used in triplicate. For cell number determination EBs were trypsinized to obtain single cells and then determined on a Casy Cell Counter (Innovatis). For each measurement 32 EBs were trypsinized to calculate the cell number of a single EB and was performed in triplicate.

2.2.4 Immunocytochemistry

2.2.4.1 Preparation of 4% Paraformaldehyde (PFA)

Dissolve 20g of PFA in 250ml ddH₂O. Add 5ml of 1M NaOH and stir on a magnetic stirrer at 65°C until all PFA has dissolved. Then add 50ml of 10x PBS to the solution and let cool down to room temperature. Adjust the pH to 7,4 with 37% HCl, add another 100ml ddH₂O. Sterile filtrate the solution and make 5-10ml aliquots. These can be stored at -20°C.

2.2.4.2 Immunocytochemistry of cells

Cells on slides were incubated with 4% PFA for 10 minutes at room temperature. Fixed cells were washed with PBS and then first blocked with 0,1% Triton X-100 in PBS for 10 minutes, followed by a second block with 0,5% BSA in PBS/T for 30 minutes at room temperature. After blocking cells were subsequently stained with primary antibody over night at 4°C. Antibodies specific for the following proteins (also see Table 2.2.1) were used: anti- α -fetoprotein (α FP) antibody (R&D Systems MAB4305), anti-laminin antibody (Sigma L9393), anti-nestin antibody (Neuromics MO15012), and anti-WT1 antibody (Dako M3561). After staining with primary antibody cells were washed with PBS and incubated with labeled secondary antibody (1:100 in PBS) for 30 minutes at room temperature: goat TRITC α -rabbit (Sigma) or goat FITC α -mouse (Sigma). Cells were

washed again and DAPI (4,6-diamino-2-phenylindole; 1µg/ml) was added for nuclei staining.

Table 2.2.1 Antibodies used for immunofluorescence stainings

Antibody	Host	Dilution	Company
αFP	mouse	1:100	R&D Systems
Laminin	rabbit	1:50	Sigma-Aldrich
Nestin	mouse	1:200	Neuromics
WT-1	mouse	1:50	Dako
Alexa Fluor 488 mouse	goat	1:400	Molecular Probes, Invitrogen
Alexa Fluor 546 rabbit	donkey	1:400	Molecular Probes, Invitrogen
Mouse-FITC	goat	1:100	Sigma-Aldrich
Rabbit-TRITC	goat	1:100	Sigma-Aldrich

2.2.4.3 Immunocytochemistry of EB

For immunofluorescence stainings of EBs they were incubated with 4% paraformaldehyde for 30 minutes at room temperature and then carefully washed with PBS, followed by washing 15 minutes in TBS/T and subsequently 15 minutes in PBS/T. EBs were blocked for 1 hour in 1 % BSA in PBS/T. EBs were washed and stained with primary antibody over night at 4°C. Antibodies specific for the following proteins were used: αFP (R&D Systems), nestin (Neuromics), and WT1 (Dako). After primary antibody staining EBs were washed and afterwards stained with labeled secondary antibody: Alexa Fluor 488 goat α-mouse (Molecular Probes – Invitrogen A11029) and DAPI over night at 4°C. The next day stained EBs were washed three times with 1x PBS and then transferred onto glass slides. Images were obtained with a Zeiss LSM Exciter confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.2.5 HOPI (Hoechst propidium iodide) staining

Cell culture medium with a final concentration of 5 µg/ml Hoechst dye (HO) and 2 µg/ml propidium iodide (PI) was prepared. Control and treated cells were washed with PBS and treated with TE. The trypsinized cells were harvested in an Eppendorf tube and incubated with the HOPI stain and medium for 1 hour at 37°C. After incubation the cells need to be carefully resuspended to receive a single cell solution. Then 1 drop was put on a glass slide and pictures were taken with the Olympus microscope (up to ten pictures per sample). EBs (Fig. 2A) were directly incubated with the HOPI dye in a 96-well plate and were analyzed on a confocal microscope after 1 h incubation. The analysis of stained cells was done by counting viable, apoptotic and necrotic cells. The Hoechst 33258 dye stains the nuclei of all cells. Nuclear changes, such as chromatin condensation and nuclear fragmentation, are associated with apoptosis. PI uptake indicates loss of membrane integrity being characteristic for necrosis and apoptosis. Necrosis is characterized by nuclear PI uptake into cells without chromatin condensation or nuclear fragmentation (Grusch et al., 2002)

2.2.6 Immunoblotting

Proteins were run on a SDS-Polyacrylamide gel and transferred onto a nitrocellulose membrane. Blots were stained with Ponceau-S to visualize the amount of loaded protein. For immunodetection, antibodies specific for the following proteins were used: tuberin (Santa Cruz Biotechnology, sc-892), phospho-PRAS40 T246 (Cell Signaling, 2997), PRAS40 (Cell Signaling, 2691), mTOR (Cell Signaling, 2972), phospho-p70S6K T389 (Cell Signaling, 9234), p70S6K (Cell Signaling, 9202), α -Tubulin (Calbiochem, CP06), GAPDH (Trevigen, 2275-PC-100). Rabbit polyclonal and monoclonal antibodies were detected using anti rabbit IgG, an HRP-linked heavy and light chain antibody from goat (A120-101P, Bethyl Laboratories); mouse monoclonal antibodies were detected using anti-mouse IgG, an HRP-linked heavy and light chain antibody from goat (A90-116P, Bethyl Laboratories). Signals were detected using the enhanced chemiluminescence method (Pierce).

2.2.7 Microscopy

For phase contrast images EBs were photographed with an Olympus IX51 equipped with a XC50 camera (Olympus, Tokio, Japan). For the measurements of the “inner area” and “whole area” of EBs pictures were randomly taken from three 96-well plates and the area was determined using the microscopic imaging software Cell[^]D (Olympus, Tokyo, Japan). The inner area of the EB is defined as the round shaped inner structure. The cells situated out of the inner EB are integrated in the measurement of the “whole EB” (for a scheme see Figure 3.2.3). All immunostained samples were analyzed on either an Olympus IX51 microscope with a XC50 camera (Olympus, Tokio, Japan) or a Zeiss LSM Exciter confocal microscope (Carl Zeiss, Oberkochen, Germany). The microscopes were not changed within one experiment.

2.2.8 Statistical analysis

Analysis of “inner” and “outer” (whole) area of EBs, flow cytometry data, EB formation and cell number data are all presented as average $\pm$ standard deviation (SD). All comparisons between groups were calculated using Student’s *t*-test (unpaired, two-tailed) with *P*-values $\leq 0,05$ indicating statistical significance.

3. RESULTS

3.1 The localization of PRAS40

Researchers exploring the mTOR signaling pathway are mainly investigating the biochemistry of this complex and its regulation. In the recent years, it got more and more popular to get insights into the localization of the complex proteins, its upstream regulators and downstream substrates (Rosner and Hengstschlager, 2008). Additionally, the localization of proteins can be regulated by other proteins e.g. via phosphorylation (Rosner et al., 2007a). As PRAS40 was found to be a new negative regulator of mTORC1 and additionally a substrate for the same complex, we wanted to investigate PRAS40's localization.

For cytoplasmic and nuclear fractionation logarithmically growing IMR-90 or Hek293 cells were used. The purity of the obtained fractions was investigated by analyzing the expression of α -tubulin (cytoplasmic) and topoisomerase II β (nuclear). It was of importance to use primary, non immortalized, human fetal lung fibroblasts as our cell system of choice, as they resemble the human cell and tissue system in the closest way. In addition, we performed some of the experiments in Hek293 cells, because these cells are widely used by the scientific community.

We found that PRAS40 localization is similar to other mTOR pathway proteins e.g. AKT, mTOR, tuberlin, namely primarily in the cytoplasm (Rosner et al., 2007a; Rosner et al., 2007b; Rosner and Hengstschlager, 2008). Only a very small fraction of the total PRAS40 protein is localized to the nucleus (Figure 3.1.1).

To analyze where active PRAS40 phosphorylated on Thr246 is localized, we had to test the specificity of the phospho-antibody. First we checked if the antibody could recognize the phosphorylation state at all. Phosphorylation of PRAS40 can be inhibited by the well known PI3K inhibitor Wortmannin (100nM), which concomitantly inhibits phosphorylation of AKT on Ser473. We used three different cell lines to test whether the antibody was able to recognize the phosphorylation state of the protein (Figure 3.1.2 A).

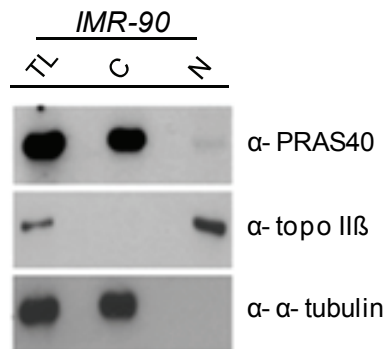


Figure 3.1.1 PRAS40 is predominantly localized to the cytoplasm. Logarithmically growing IMR-90 fibroblasts were lysed and proteins were prepared to obtain either total cell lysates (TL) or cytoplasmic (C) and nuclear (N) fractions. Purity of fractions was demonstrated by using α -tubulin (C) and topoisomerase II β (topo II β) (N) antibodies. Localization of total PRAS40 protein was analyzed.

Secondly we had to prove that the antibody specifically recognizes the phosphorylation site on Thr246. This was done by overexpressing WT and mutated (T246A) PRAS40 in IMR-90 cells and analyzing total and phosphorylated PRAS40 on a Western blot (Figure 3.1.2 B). The results proved that the here used antibody specifically recognizes PRAS40 phosphorylated on Thr246.

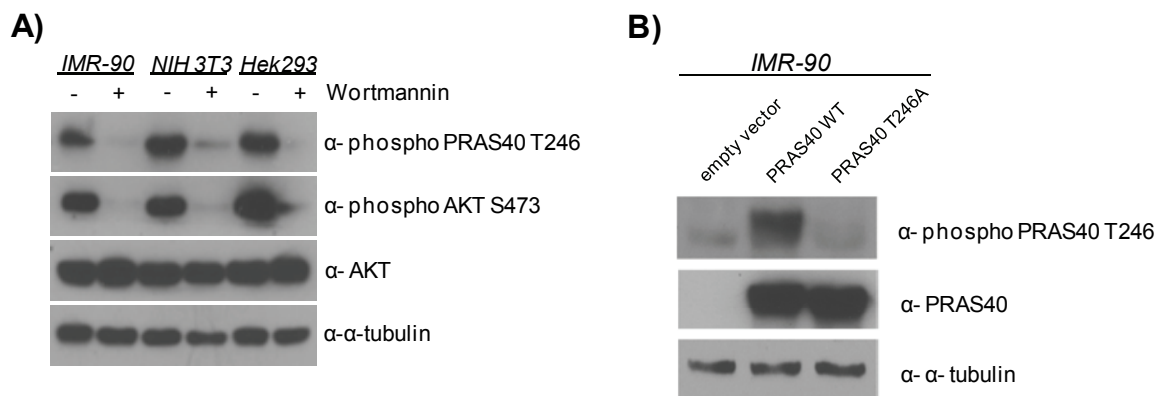


Figure 3.1.2 The phospho-PRAS40 T246 antibody specifically recognizes the phosphorylated protein and is site specific. **A)** Logarithmically growing IMR-90, NIH 3T3 and Hek293 cells were starved of serum over night, treated with or without Wortmannin (100nM) for 30 minutes and were stimulated with serum at a final concentration of 10% (v/v) and directly lysed afterwards. Expression levels of phospho-PRAS40 T246, phospho-AKT S473 and total AKT were analyzed on a Western blot. α -tubulin served as a loading control. **B)** IMR-90 cells were transfected with either wild type PRAS40 (PRAS40 WT) or a mutated form of PRAS40's phosphorylation site in T246 (PRAS40 T246A) as indicated in the Materials and methods section. Whole cell lysates were prepared and protein expression of phospho-PRAS40 T246 and total PRAS40 was analyzed. α -tubulin served as a control of equal loading.

Analyzing phosphorylated PRAS40 on Thr246 on a Western blot we could demonstrate that it is localized the same way as the total protein in logarithmically growing IMR-90 cells (Figure 3.1.3) and also in logarithmically growing Hek293 cells (data not shown).

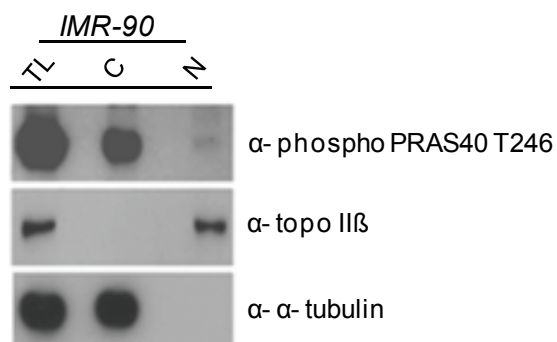


Figure 3.1.3 Phosphorylated PRAS40 is localized mainly to the cytoplasm. Logarithmically growing IMR-90 cells were lysed and proteins were prepared to obtain wither whole cell lysates (TL) or cytoplasmic (C) and nuclear (N) fractions. Purity of fractions was demonstrated by using α- tubulin (C) and topoisomerase IIβ (N) antibodies. Localization of phosphorylated PRAS40 at T246 was analyzed.

The next step was to identify, if the localization of the PRAS40 protein is dependent on its phosphorylation site on Thr246. Therefore we transiently overexpressed wild type and mutant (T246A) PRAS40 in IMR-90 cells for 48h using a lipid based transfection method (Figure 3.1.4). Wild type and mutant (T246A) PRAS40 protein was ectopically expressed to similar levels as can be seen in total lysates of cells analyzed (Figure 3.1.4 left panel). Cytoplasmic and nuclear fractions were obtained and analyzed by Western blot. From the obtained results of these experiments we can say that phosphorylated PRAS40 on Thr246 is localized the same way as wild type PRAS40 ectopically expressed in IMR-90 (Figure 3.1.4).

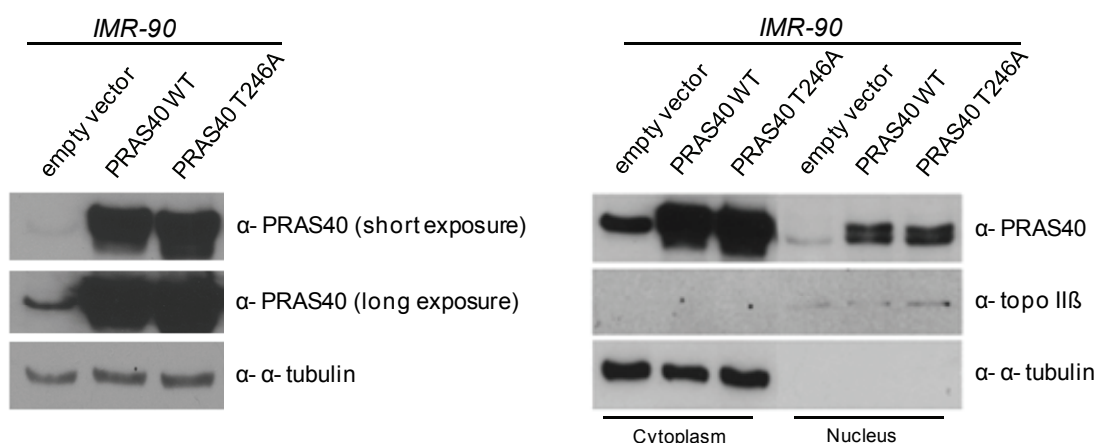


Figure 3.1.4 PRAS40 phosphorylation at T246 is not essential for PRAS40 localization. Total lysates of IMR-90 cells expressing either corresponding empty vector, wild type PRAS40 (PRAS40 WT) or mutant PRAS40 (PRAS40 T246A) were analyzed on a Western blot for expression of PRAS40 protein. α - tubulin served as control for equal loading of proteins (left panel). From the same transfected IMR-90 cells cytoplasmic and nuclear fractions were obtained. α - tubulin (C) and topo II β (N) served as controls for purity of obtained fractions. Localization of total PRAS40 protein was analyzed (right panel).

For another phosphorylation site of PRAS40, namely S183, only the ectopic mutant (PRAS40 S183A) localization experiments could be done do to the lack of a suitable antibody. Secondly the experiments were only conducted in Hek293 cells, as the ectopic expression of the plasmid only yielded satisfying transfection efficiency in this cell line. Western blots of wild type or mutant (S183A) PRAS40 transiently transfected Hek293 cells (Figure 3.1.5) showed that we were able to overexpress PRAS40 protein. However the mutated form of PRAS40 on S183 was expressed at lower levels than ectopic wild type PRAS40. At least from Hek293 cells we can say that phosphorylated PRAS40 on S183 seems to be dispensable for proper or rather normal localization of PRAS40 protein as we could not detect a change in the localization of total PRAS40 protein.

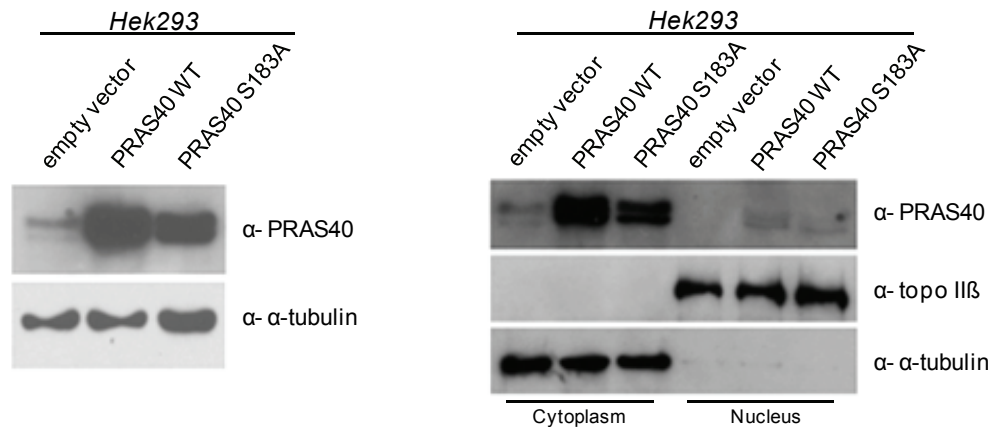


Figure 3.1.5 PRAS40 phosphorylation at S183 is not essential for localization of PRAS40 protein. Total lysates of Hek293 cells expressing either corresponding empty vector, wild type PRAS40 (PRAS40 WT) or mutant PRAS40 (PRAS40 S183A) were analyzed on a Western blot for expression of PRAS40 protein. α - tubulin served as control for equal loading of proteins (left panel). From the same transfected Hek293 cells cytoplasmic and nuclear fractions were obtained. α - tubulin (C) and topo II β (N) served as controls for purity of obtained fractions. Localization of total PRAS40 protein was analyzed (right panel).

To summarize the results we can say that PRAS40 is predominantly localized to the cytoplasm in logarithmically growing IMR-90 fibroblasts and Hek293 cells. Second that PRAS40 total protein localization is not dependent on its phosphorylation sites on Thr246 or Ser183 when ectopically expressed. At last, from the results obtained from the experiments conducted so far, AKT-mediated phosphorylation on T246 does not affect PRAS40 cytoplasmic and nuclear localization.

3.2 PRAS40 and TSC2 regulate apoptosis during early development of embryoid bodies via mTOR

Two major branches are currently investigated in our laboratory, namely the mTOR signaling pathway and hAFS cells and their potential. In the past few years we started to combine those two to study the mTOR pathway and its role in hAFS cells and their differentiation (Rosner et al., 2008a; Siegel et al., 2010; Siegel et al., 2009; Valli et al., 2010). The two major upstream regulators of mTORC1 are tuberlin (TSC2), via regulating Rheb, and proline rich AKT substrate of 40kDA (PRAS40) in a more direct way (Dunlop and Tee, 2009; Inoki et al., 2003; Sancak et al., 2007). One model system to study development and differentiation are so called EBs, which are three dimensional cell aggregates composed of stem cells. The following results will give new insights about the role of PRAS40 and TSC2 in the development of EBs derived from hAFS cells.

The study was done in Q1 cells, which have already been used in previous studies (Rosner et al., 2010; Siegel et al., 2010; Siegel et al., 2009; Valli et al., 2010). They are a monoclonal hAFS cell line, which were kindly provided by Anthony Atala. Handling of cells, EB formation and other methods used for generating these results can be found in the materials and methods section of this thesis. To study the role of PRAS40 and TSC2 in EBs siRNA knockdown technology was chosen, as our laboratory could demonstrate it to be a very efficient tool and knockdowns can be kept up to 15-21 days depending on the cell line (Rosner et al., 2010).

Q1 cells were transfected with non targeting control, human specific PRAS40 or human specific TSC2 siRNAs at a final concentration of 50nM. To prove that the siRNA treatment was successful protein lysates of treated Q1 cells were prepared. Western blot analysis shows efficient knockdown of endogenous TSC2 (tuberlin) and PRAS40 protein. To ensure equal loading of protein samples α -tubulin served as control (Figure 3.2.1). Then EBs consisting of approximately 1000-1500 cells were formed, choosing a suspension culture method with methylcellulose as previously published (Valli et al., 2010). After 24h (day 1) EBs were analyzed morphologically and by measuring EB formation efficiency, area and cell number.

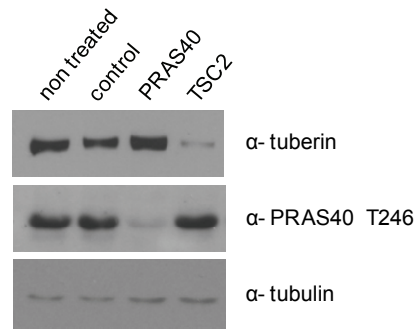


Figure 3.2.1 Monoclonal Q1 human AFS cells were transfected with non-targeting control siRNA, TSC2-specific siRNA or PRAS40-specific siRNA and knockdown of endogenous tuberlin and PRAS40 was confirmed by Western blot analysis. α -tubulin served as a control for equal loading.

One parameter we analyzed was the efficiency of EB formation. One day post start of EB formation the efficiency was approximately the same in all EBs indicating that the formation of EBs per se was not impaired due to knockdown of PRAS40 or TSC2. However 6 days post EB formation the stability / efficiency of EBs dropped to less than 70% in PRAS40 and less than 65% in TSC2 EBs (Figure 3.2.2) being in concordance with morphology observations.

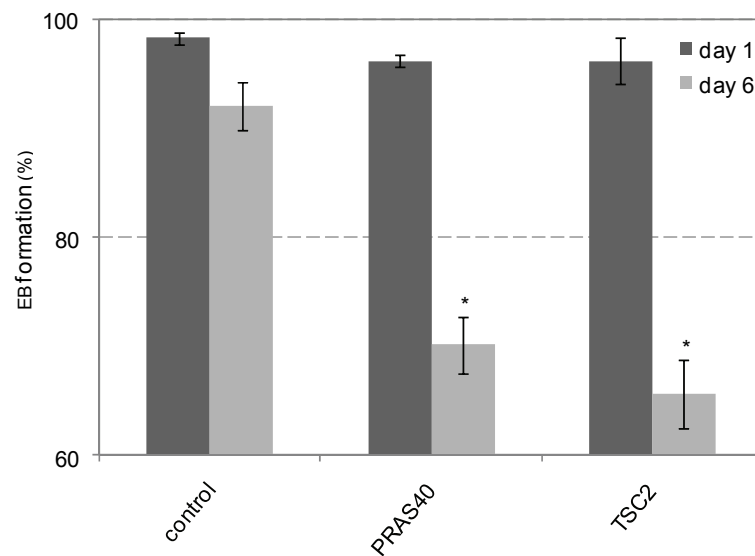


Figure 3.2.2 The number of EBs formed from the so transfected cells was counted on day 1 and day 6 (day 1 control was set to 100%).

For differences in morphology and shape pictures were randomly taken with an Olympus microscope. With a software we were able to analyze the area of the differently siRNA treated EBs (Figure 3.2.4 and 3.2.5). For area analysis ten pictures were taken and area of the “inner area” of EBs and the “outer area” of EBs was measured. The term “inner area” comprises all the cells within the thin outline they are enclosed in. Annexed to this outline a layer of less compacted, brighter round shaped cells appear and are termed as “outer area”. The inner area and the outline of the outer area together are termed whole area of EBs in terms of the subsequently following results (Figure 3.2.3). Alone by observing the morphology of the EBs huge differences could be seen when control EBs were compared to PRAS40 and TSC2 siRNA treated EBs.

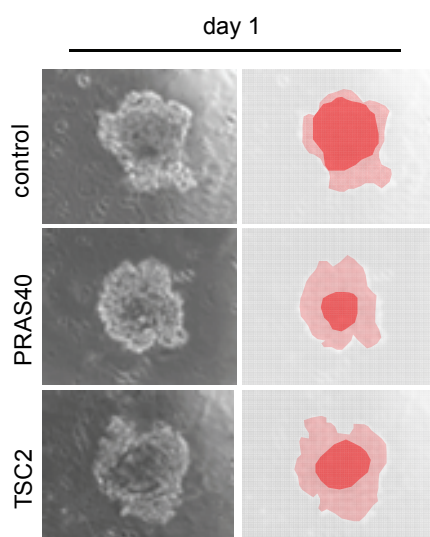


Figure 3.2.3 The role of PRAS40 and tuberlin in EB development of AFS cells. Left panel: representative pictures of day 1 EBs generated from differently transfected Q1 cells (magnification x10). Right panel: schematic presentation of the inner area of the EB (dark red) and the outer area (light red). For detailed explanation see the text.

The outer area was a lot bigger in TSC2 treated EBs and it was even more in PRAS40 EBs compared to control EBs (Figure 3.2.3). Analyzing the whole area of EBs at day 1 there was not much difference within the differently treated EBs (Figure 3.2.4), fitting the morphological impression (Figure 3.2.3).

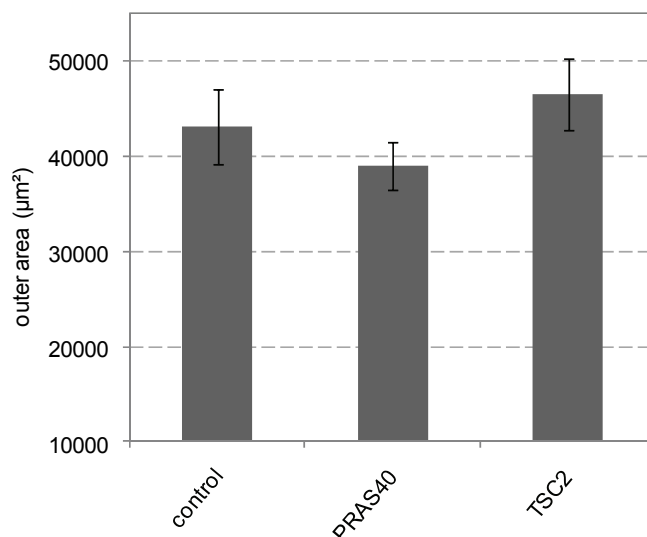


Figure 3.2.4 The whole area (outline of the outer area) of formed EBs was analysed at day 1. For each calculation 10 EBs were photographed, the area was analysed via Cell D image software (Olympus) and the data are presented as average $\pm$ standard deviation.

In agreement with the morphological observation, the inner area of EBs was decreased in TSC2 EBs by approximately 40% and dramatically reduced by 60% in PRAS40 EBs (Figure 3.2.5).

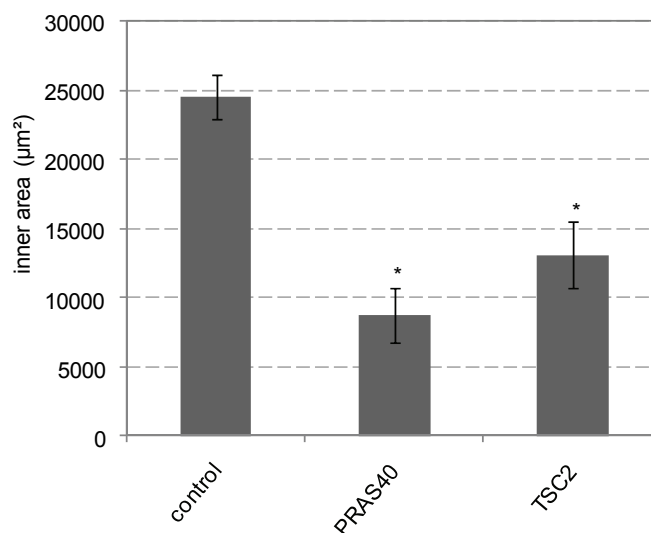


Figure 3.2.5 The inner area (outline of the inner area) of formed EBs was analysed at day 1. For each calculation 10 EBs were photographed, the area was analysed via Cell D image software (Olympus) and the data are presented as average $\pm$ standard deviation. For all statistical analyses Student's t-tests (unpaired, two-tailed) were performed and * indicates statistical significance with a p-value $\leq 0,05$.

To summarize the results obtained from morphological and area analysis we can conclude that the overall EB does not change in size, however due to the strong phenotypic differences we were able to show that PRAS40 and TSC2 EBs have a much smaller inner area of cells compared to control EBs.

To get more insight into EB development we analyzed the cell number of EBs via a CASY cell counter and analyzer. 32 EBs were trypsinized to receive a single cell suspension and then measured with a CASY counter. Results of cell number analysis were quite similar in outcome compared with the area results. A dramatic loss in cell number (Figure 3.2.6) was observed in PRAS40 knockdown EBs (down to ~30%) and in TSC2 knockdown EBs (down to ~60%).

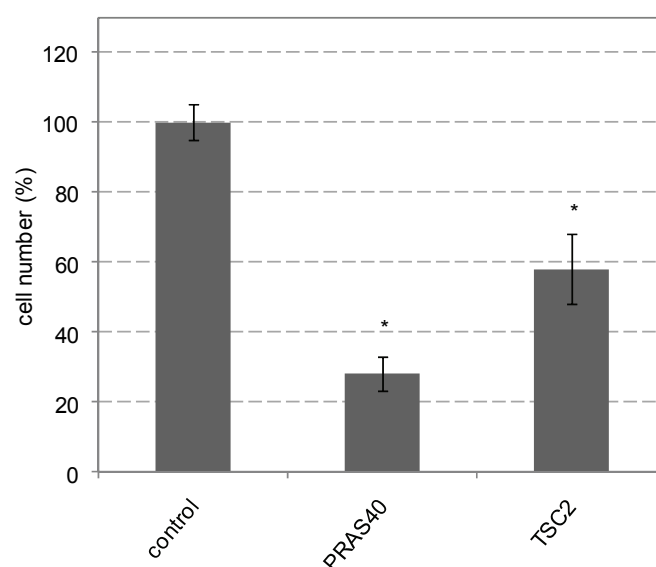


Figure 3.2.6 The cell number of day 1 EBs (32 EBs for each calculation) was determined by a CASY cell counter and analyser and presented relative to the control. For all statistical analyses Student's t-tests (unpaired, two-tailed) were performed and * indicates statistical significance with a p-value $\leq 0,05$.

To further clarify the impact of PRAS40 and TSC2 knockdown in EBs we performed a short time course experiment. 12h and 24h post start of EB formation pictures were taken for morphological differences (Figure 3.2.7). Area analysis of 12h and 24h EBs was conducted.

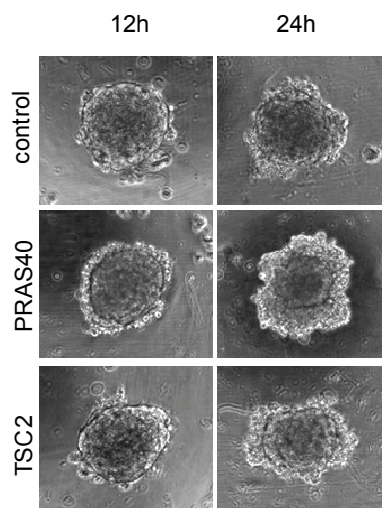


Figure 3.2.7 Representative pictures of 12 hours and 24 hours EBs formed from Q1 cells transfected as indicated (magnification x10).

Already after 12h the area was significantly decreased in PRAS40 knockdown EBs and to a smaller but still significant extent in TSC2 knockdown EBs. 24h later, when analysis was usually performed, the impact on area was even greater, though an overall decrease in area was seen between 12h and 24h post EB formation, presumably resulting from the overall compaction of an EB during that time (Figure 3.2.8).

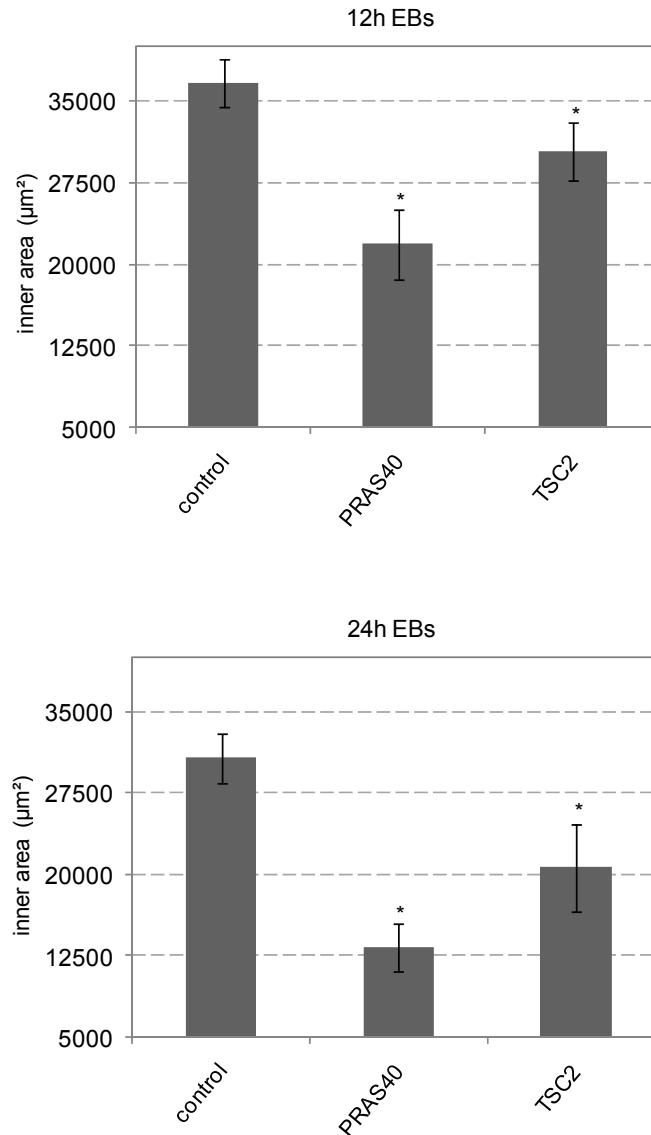


Figure 3.2.8 Area changes of EBs from differently transfected Q1 cells are time course dependent. The inner area of the EBs formed from transfected Q1 cells were analysed at the indicated time points. For each calculation 10 EBs were photographed, the area was analysed via Cell D image software (Olympus) and the data are presented as average $\pm$ standard deviation. For all statistical analyses Student's t-tests (unpaired, two-tailed) were performed and * indicates statistical significance with a p-value $\leq 0,05$.

The decrease of the inner area and the massive drop in cell number needed further clarification. To get more insight into the cellular processes that occur during EB development we stained them with HOPI to check for possible apoptosis.

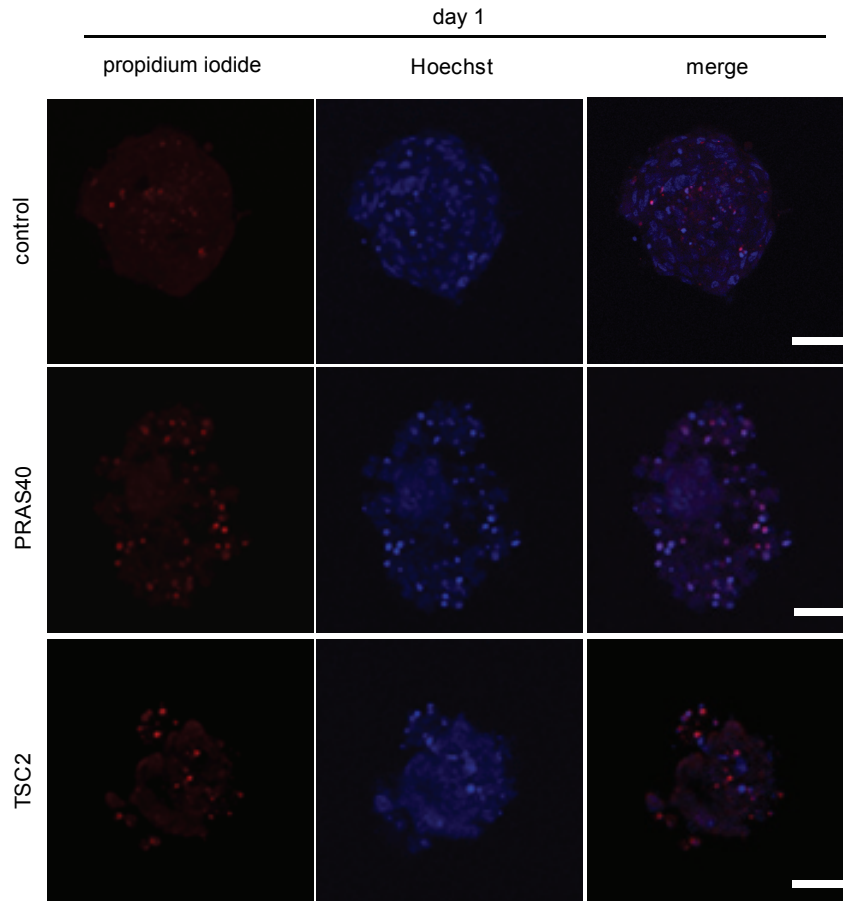


Figure 3.2.9 Knockdown of endogenous tuberin or PRAS40 triggers apoptosis during EB development of AFS cells. Day 1 EBs, from siRNA-treated Q1 cells as described in Fig. 3.2.1, were stained with HOPI for 1h at 37°C and then analysed on a confocal microscope (magnification x25). Scale bars represent 50µm.

Using a confocal microscope we could see that the outgrowth is definitely positive for a HOPI double staining, providing strong evidence that knockdown of PRAS40 and TSC2 in EBs leads to cell death (Figure 3.2.9). To further support the results from HOPI that the outer area was mainly composed of dead cells and, that cells within the original area were viable, whole EBs were trypsinized to receive single cells for flow cytometry.

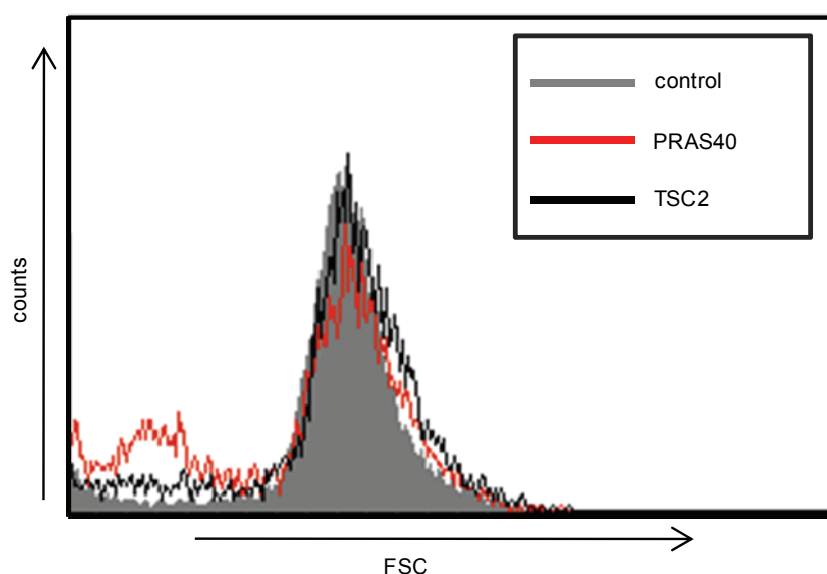


Figure 3.2.10 Whole EBs were trypsinized 24h post start of EB formation and relative cell size was studied via cytofluorometric forward scatter (FSC) analyses.

Cell size (FSC) and subG1 cells were evaluated. Cell size was significantly larger in PRAS40 treated EBs and a slight trend could be observed in TSC2 EBs. The FSC overlay profile showed that cell shrinkage was present in PRAS40 EBs (Figure 3.2.10). PRAS40 EBs had about three times more cells in the subG1 fraction and about 1,5 times more cells in TSC2 were in subG1 compared to control (Figure 3.2.11 A and B).

We performed HOPI staining, which allows discriminating between necrosis, early and late apoptotic cells. For details of HOPI analysis see materials and methods section and publication by Grusch et al., (Grusch et al., 2002). Prior to the staining EBs were photographed for area analysis. In accordance to previous experiments, the area of PRAS40 treated EBs was significantly smaller compared to non treated or control siRNA treated EBs. TSC2 EBs also exhibited a significantly smaller area but to a less extent than PRAS40 EBs. Cells were treated as in previous experiments and 24h post EB formation cells were trypsinized to receive a single cell suspension. Cells were kept in media containing HOPI staining solution and were incubated for 1h at 37°C and 5% CO₂ in an incubator. Afterwards cells were carefully resuspended and several drops of the suspension were put on a glass slide. Pictures of HOPI stained cells were taken (Figure 3.2.12).

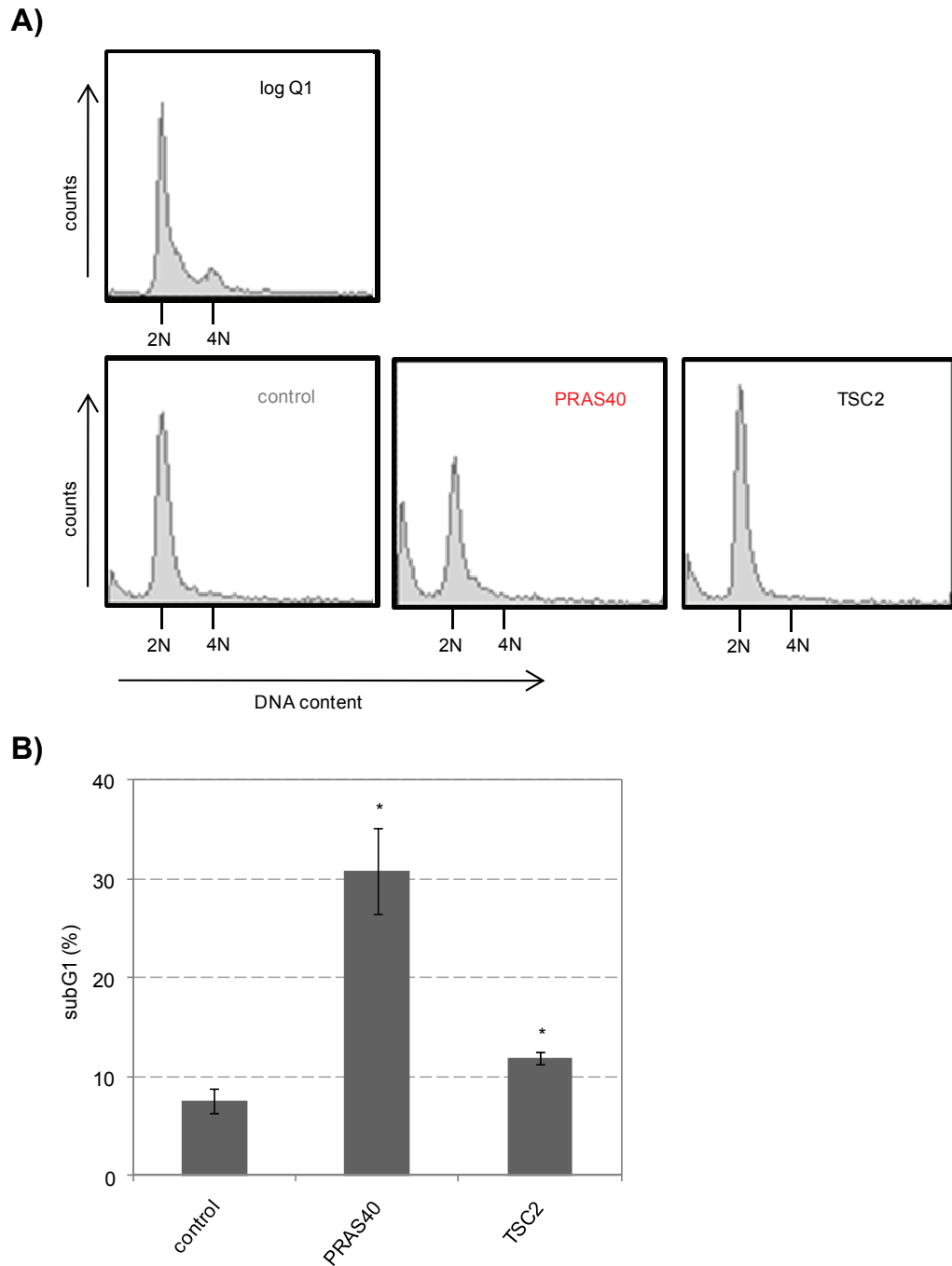


Figure 3.2.11 (A) In the same samples apoptotic cells were cytofluorometrically detected by their sub G1 DNA content. The log Q1 picture represents non-transfected Q1 control cells logarithmically growing in petri dishes without induction of EB formation. (B) Quantitative investigation of the amount of subG1 cells in EBs analysed in (C). Student's t-test was performed and * indicate statistical significance ($p\text{-value} \leq 0,05$; $n=3 \times 96$ well plate).

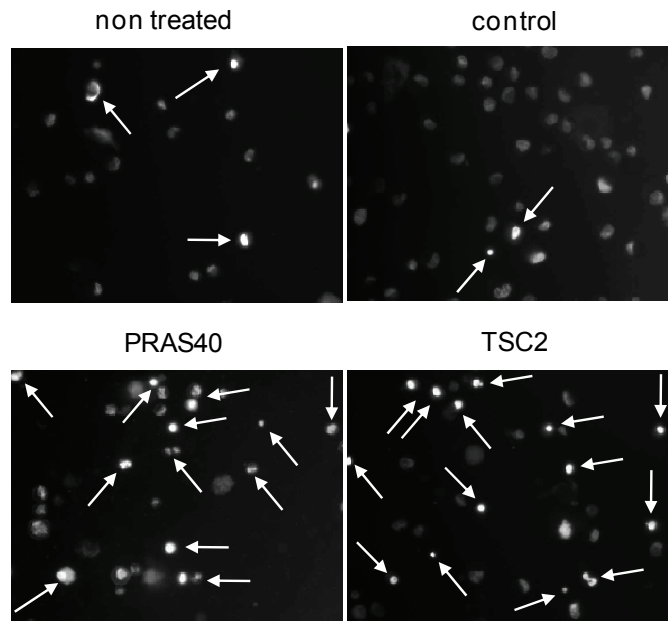


Figure 3.2.12 Downregulation of endogenous tuberlin or PRAS40 triggers apoptosis, but not necrosis, during EB formation of AFS cells. Day 1 EBs formed from Q1 cells, siRNA-transfected as indicated, were trypsinized to receive single cells and stained with HOPI to monitor nuclear chromatin condensation and cell death (arrows) (magnification x20).

For the numerical analysis a minimum of 500 cells per condition were counted. First step of analysis was just to distinguish between living and apoptotic/necrotic cells. In both, non treated and control siRNA treated EBs, the percentage of living cells was above 80%. On the other hand PRAS40 and TSC2 EBs showed a reduction of living cells by approximately 15 to 20% compared to control and portion of apoptotic cells was above 20% (Figure 3.2.13). The second step was to analyze all those cells, which were counted as either apoptotic/necrotic, by counting them as either early apoptotic (only positive for Hoechst dye – bright blue, smaller than living cells, apoptotic bodies), late apoptotic (positive for Hoechst dye and propidium iodide, looking purple, small nuclei and apoptotic bodies) and necrotic cells (positive for both dyes but swollen nuclei, no apoptotic bodies).

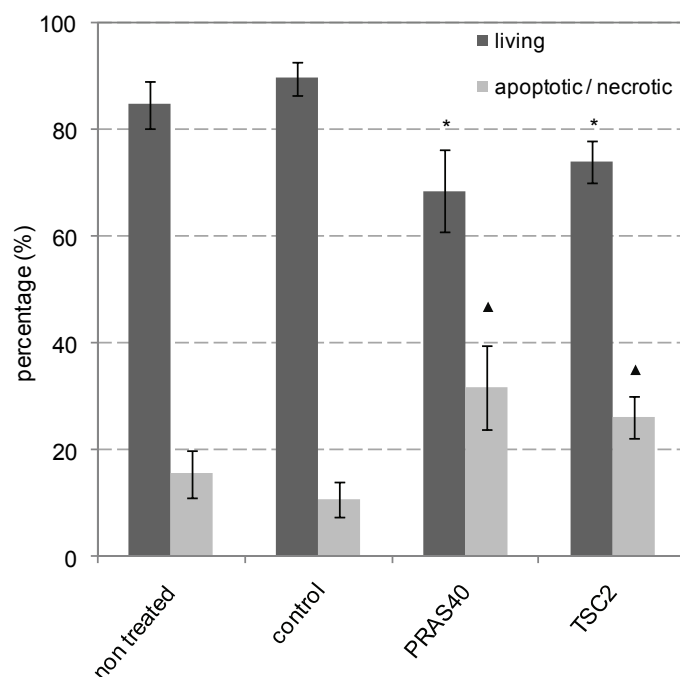


Figure 3.2.13 Living and apoptotic/necrotic cells were counted upon HOPI staining and data are presented as average $\pm$ standard deviation (SD). Statistical analyses was performed via Student's t-tests and * or Δ indicate a p-value $\leq 0,05$ compared with living control cells (*) or dead control cells (Δ) ($n \geq 500$ cells per condition).

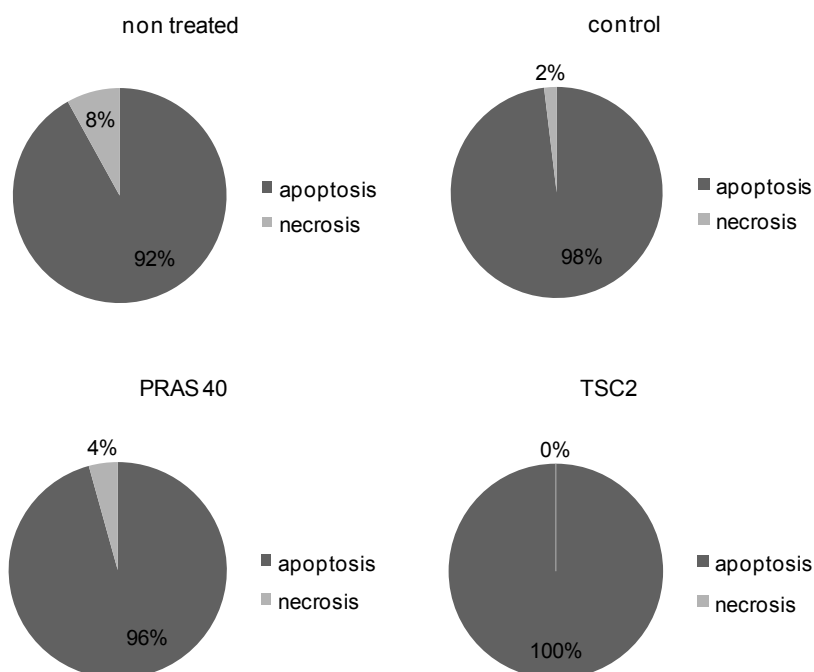
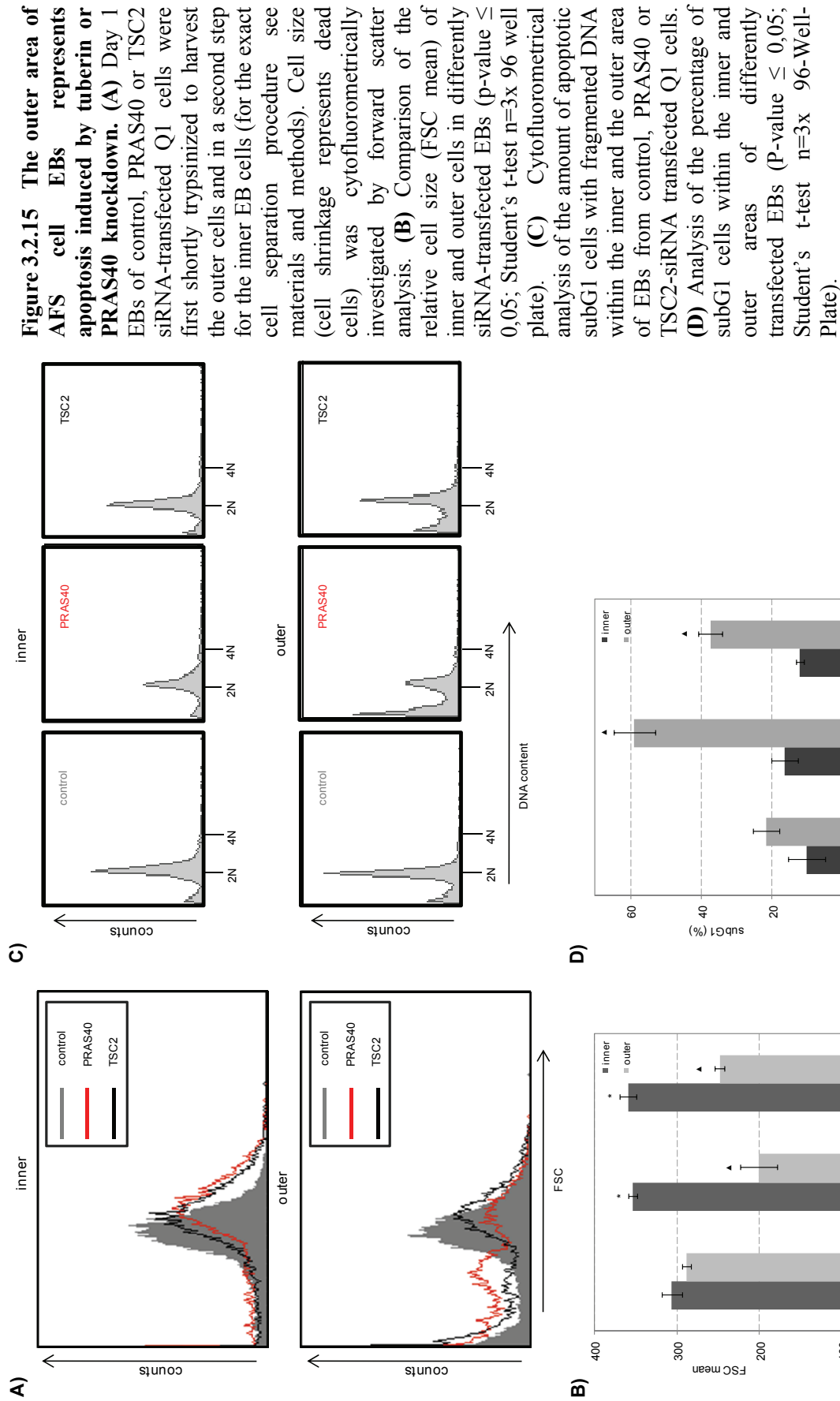


Figure 3.2.14 Apoptotic/necrotic-positive cells were divided into apoptotic and necrotic cells (for details see materials and methods).

Necrosis was never observed in a higher percentage than 8 percent. Therefore we were able to conclude that knockdown of PRAS40 and TSC2 did not trigger necrosis but definitely apoptosis. The differences of early and late apoptosis within one group were not statistically significant. Therefore we decided on just showing the difference between apoptotic and necrotic cell death in EBs to make clear that the phenotype is caused by programmed cell death (Figure 3.2.14).

The conclusion so far is that knockdown of PRAS40 and TSC2 in EBs triggers apoptosis at a high level, leading to reduced area of EBs and a dramatic loss in cell number. This leads to the assumption that PRAS40 and TSC2 play key roles in the early development.

To localize apoptosis we formed differently transfected EBs as previously described. First we shortly trypsinized EBs to get the outer cells of the EB and in a second step of trypsination we received single inner cells (for a more detailed description of separating inner and outer cells of EBs see the materials and methods section). Evaluating cell size of inner and outer cells of the EB via flow cytometric analysis revealed that cells treated with either PRAS40 or TSC2 siRNA within the inner area of an EB were bigger compared to control cells. This is in agreement with other studies on mTOR regulators as mTORC1 and mTORC2 are major regulators of cell size and cell cycle (Rosner et al., 2009b). Vice versa cells out of the inner area of an EB were significantly smaller compared to control cells with PRAS40 siRNA treated cells being the smallest (Figure 3.2.15 A and B). FSC overlays of cells inside (inner) and outside (outer) of the inner area of an EB clearly showed the size differences. Cell shrinkage was seen in PRAS40 and TSC2 siRNA treated EBs, whereas again the PRAS40 phenotype was stronger compared to TSC2 (Figure 3.2.15 A). To answer the question about where apoptosis is residing in the EB subG1 analysis was performed with cells inside (inner) and outside (outer) of the EB. The level and grade of subG1 cells of differently treated EBs could nicely be spotted in DNA profiles depicted (Figure 3.2.15 C). We could demonstrate that the cells inside approximately have the same level of subG1 cells, whereas there were tremendous differences in cells outside. Almost 60% of all the cells of the outer area were in subG1 in PRAS40 EBs and up to 40% of those cells were in subG1 in TSC2 EBs (Figure 3.2.15 D).



We next wanted to assess whether the knockdown of PRAS40 and TSC2 was accompanied with changes in germ layer expression caused by the knockdowns. We choose the following markers for analysis: α FP (alphafetoprotein) as an endodermal marker, nestin as an ectodermal marker, WT1 (Wilms tumor 1) mesodermal marker.

α FP is a glycoprotein, which is usually only expressed in the fetus and expression decreases after birth. It is regulated by p53 and β -catenin and found in elevated levels in adult liver cancer cells (Meier et al., 2006; Peng et al., 2004; Warnes and Smith, 1987). In stem cell biology α FP is used as a marker for endoderm, especially the primitive endoderm. Nestin is a family member of the intermediate filament group and structurally related to the neurofilament proteins. It is highly expressed in the developing brain and other neuronal structures. Nestin is widely used as ectodermal, neuronal and pancreatic progenitor marker of stem cells (Gilyarov, 2008; Messam et al., 2002; Wang et al., 2009). WT-1 is a zinc-finger transcription factor and involved in Wilms' tumor, a renal malignancy in infants and a known regulator of kidney development. It is strongly expressed in mesenchymal derived tissue (Davies et al., 2004; Pritchard-Jones et al., 1990). All of the markers tested were expressed in Q1 cells and the germ layer marker WT-1 was approved as previously published (Siegel et al., 2010). In addition to the stainings performed in Q1, we had to prove specificity of the antibodies. Cell lines positive and negative for the markers were used to do so (Figure 3.2.16).

EBs were generated and fixed in 4% PFA at day 1 and incubated with the following primary antibodies: α FP (endoderm), nestin (ectoderm) and WT1 (mesoderm). EBs were counterstained with DAPI for nuclei staining and embedded onto a glass slide. EBs were analyzed on a confocal microscope. Although we monitored a reduced in size of the inner EB in PRAS40 and TSC2 knockdown EBs, marker specific stainings did not show any difference between control and knockdown EBs (Figure 3.2.17). We conclude that knockdown of PRAS40 and TSC2 in EBs is not deregulating germ layer marker expression.

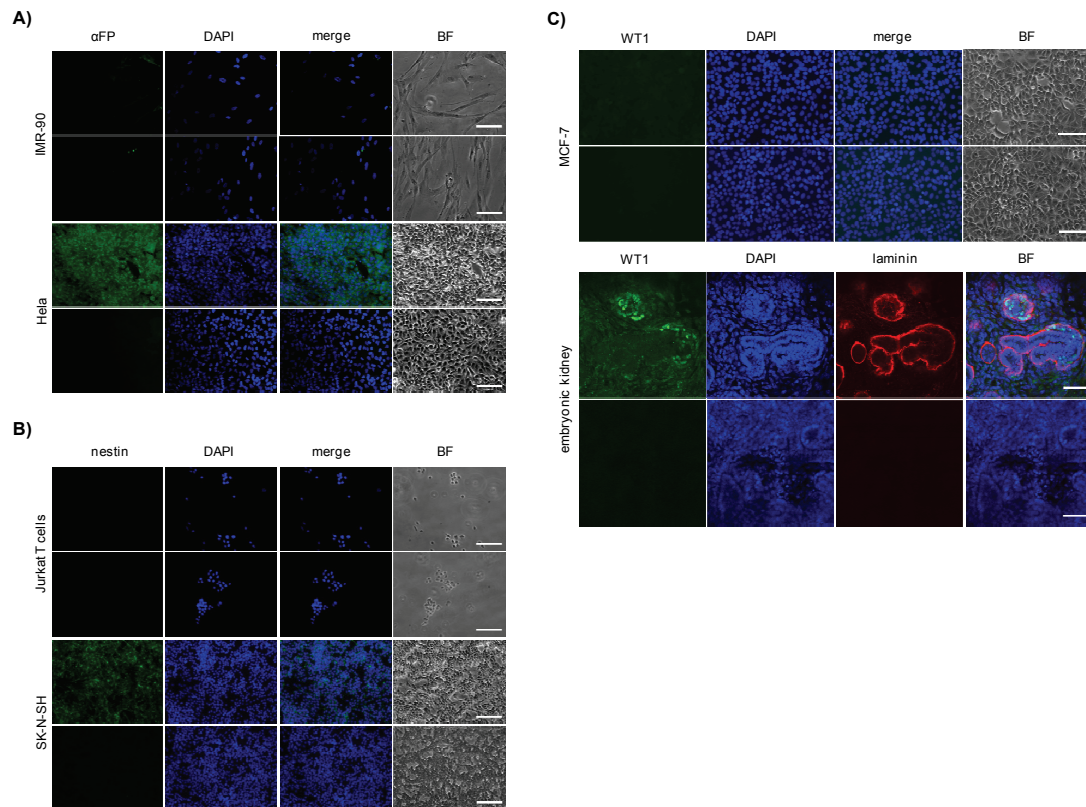


Figure 3.2.16 Analyses of control cells to prove antibody specificity. (A) Staining for α FP with IMR-90 cells serving as negative control and HeLa cells as positive control. Top row represents staining with primary antibody and bottom row represents negative control staining omitting the primary antibody. Scale bars represent 100 μ m. (B) Staining for nestin with Jurkat T cells serving as negative control and SK-N-SH cells as positive control. Top row represents staining with primary antibody and bottom row represents negative control staining omitting the primary antibody. Scale bars represent 100 μ m. (C) Staining for WT-1 with MCF-7 cells serving as negative control and mouse embryonic kidney (E11,5) as positive control. Top row represents staining with primary antibody and bottom row represents negative control staining omitting the primary antibody. Scale bars for MCF-7 represent 100 μ m and for embryonic kidney 50 μ m.

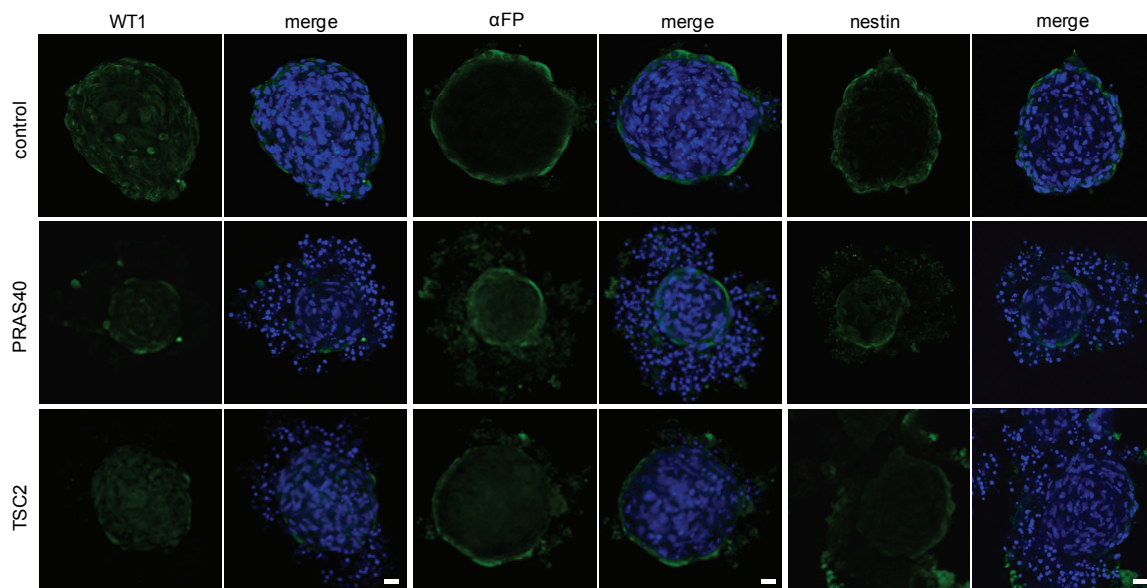


Figure 3.2.17 Knockdown of endogenous tuberlin or PRAS40 is not affecting germ layer specific marker expression. Monoclonal Q1 AFS cells were transfected with non-targeting control, PRAS40- or TSC2-specific siRNA. Day 1 EBs were immunohistochemically analysed for marker expression (WT1-mesoderm; α FP-endoderm; Nestin-ectoderm). Nuclei were counterstained with DAPI in blue (magnification x25). Scale bars represent 20 μ m.

To briefly summarize the results obtained so far we could definitely prove that firstly, PRAS40 and TSC2 are genes essential in early development. Secondly, that these genes do regulate apoptosis in embryoid body development. Thirdly, that knockdown of these genes severely affects area and cell number however not having an impact of the germ layer markers investigated in this study.

The next step was to identify the molecular mechanism by which knockdown of PRAS40 and TSC2 regulate apoptosis. As PRAS40 and TSC2 are both prominent inhibitors of the mTORC1 signaling pathway (Dunlop and Tee, 2009; Inoki et al., 2002; Nascimento and Ouwens, 2009; Rosner et al., 2008a; Sancak et al., 2007; Tee et al., 2003) it was quite self-evident to test whether regulating mTORC1 could revert the phenotype observed in PRAS40 and TSC2 siRNA treated EBs. One of the best ways to efficiently block the mTORC1 signaling cascade is the chemical inhibitor rapamycin. Q1 cells were transfected with control, PRAS40 or TSC2 siRNA, then grown for 48h. Then cells were treated with 100nM rapamycin or DMSO and grown for another 24h (in total 72h as

usual). For EB formation cells were additionally treated with rapamycin or vehicle control (DMSO) and analysis was performed 24h later (Figure 3.2.18).

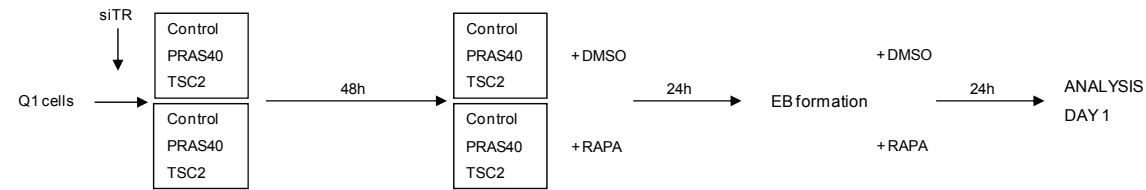


Figure 3.2.18 Scheme of experimental approach of siRNA knockdown and rapamycin treatment of Q1 hAFS cells and EB formation.

For confirmation that rapamycin indeed blocked mTORC1 Western blot analysis was performed from cell lysates of Q1 used for EB formation. Efficient knockdown of PRAS40 and TSC2 was confirmed.

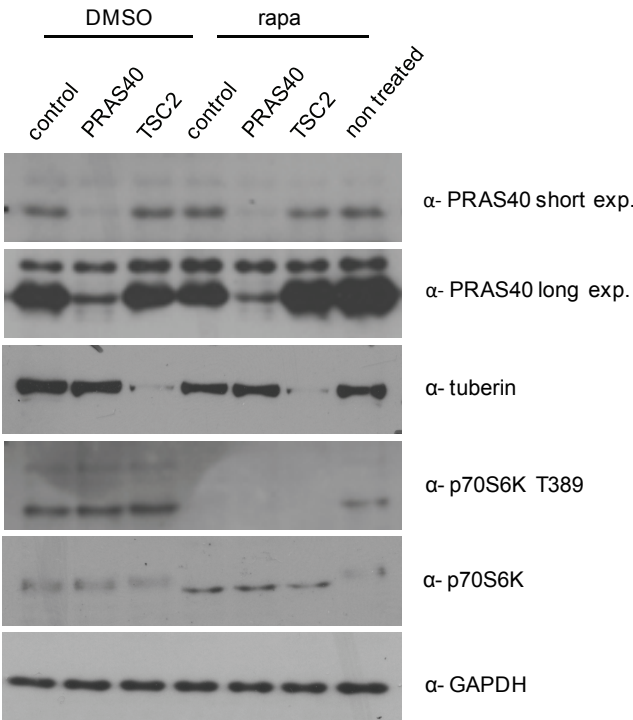


Figure 3.2.19 Q1 AFS cells were transfected with non-targeting control siRNA, PRAS40 siRNA or TSC2 siRNA. 48h after transfection cells were treated with 100nM Rapamycin or vehicle (DMSO). Efficient knockdown of PRAS40 or TSC2 protein was confirmed by Western blot analysis 72 hours after transfection. Additionally, the negative effects of rapamycin on mTORC1 activity were confirmed by analyzing the phosphorylation status of p70S6K T389. GAPDH served as a control for equal loading.

The readouts of mTORC1 activity p70S6K was analyzed. Phosphorylation of p70S6K at T389 was completely blocked by rapamycin and in total p70S6K protein a shift towards the dephosphorylated state was detectable (Figure 3.2.19). Phosphorylation at S240/244 of S6 was quite effectively blocked and in addition to mTORC1 direct read out we could show that phosphorylation of AKT at S473 was slightly induced by rapamycin, triggered via the negative feedback loop of p70S6K towards IRS and then AKT (Dunlop and Tee, 2009; Rosner and Hengstschlager, 2008). GAPDH served as a control for equal loading.

As one of the most prominent features in these EBs was the dramatic phenotype, we analyzed the inner and the whole area of the differently treated EBs. As expected siRNA treated cells together with DMSO did exhibit the usually observed phenotype. Rapamycin could not revert the effects of PRAS40 and TSC2 knockdown EBs it even led to a further decrease of the inner area of siRNA treated EBs compared to control (Figure 3.2.20).

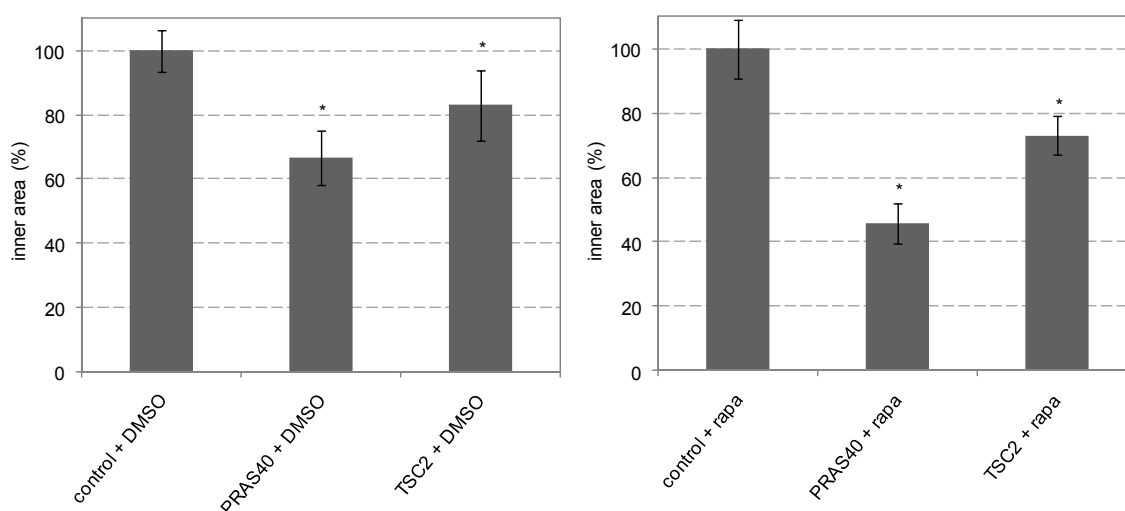


Figure 3.2.20 Knockdown of tuberin or PRAS40 also causes apoptosis in AFS cell EBs treated with the mTOR inhibitor rapamycin. The inner area (outline of the inner area) of formed day 1 EBs was analysed. For each calculation 10 EBs were photographed, the area was analysed via Cell D image software (Olympus) and the data are presented as average $\pm$ standard deviation (control set to 100%).

When evaluating the cell number of differently transfected EBs treated with or without rapamycin we could prove for another time that cell number dramatically dropped by about 50% in PRAS40 treated EBs and item by 20% in TSC2 EBs.

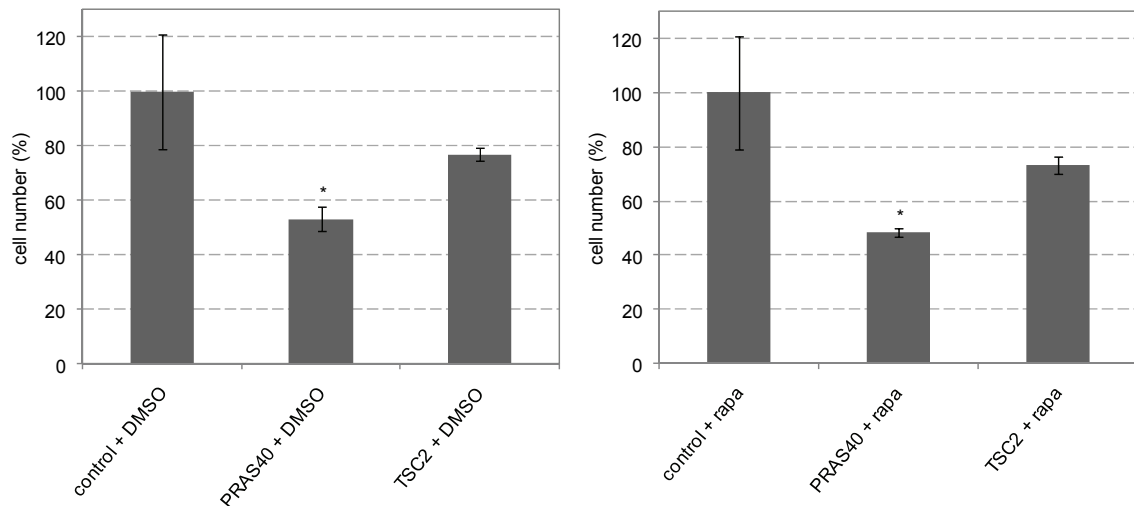


Figure 3.2.21 The cell number of these EBs (32 EBs for each calculation) was determined by a CASY cell counter and analyser. For all statistical analyses in this figure Student's t-tests (unpaired, two-tailed) were performed and * indicates statistical significance with a p-value $\leq 0,05$.

What has already become evident when analyzing the original area of the EBs, moreover at a concentration of 100nM, rapamycin was not capable of rescuing the loss of cells due to apoptosis in EBs treated with PRAS40 or TSC2 siRNA (Figure 3.2.21).

Based on the results obtained from analyzing the EBs and from Western blot analysis we can state that rapamycin was not able to compensate the apoptotic phenotype resulting from PRAS40 and TSC2 knockdown in EBs.

The mTOR protein is present in two different protein complexes, namely mTORC1 and mTORC2, and rapamycin primarily targets mTORC1. In addition there's evidence that TSC2 influences mTORC2 activity (Huang et al., 2008). For that reason and to rule out any doubt about how PRAS40 and TSC2 are regulated in this system (mTOR dependent or independent), we performed double knockdowns with additional mTOR or non-targeting control siRNA in Q1 hAFS cells using 25nM of each siRNA to transfect, resulting in a total of 50nM, and used those cells 72h post siRNA transfection for EB formation.

To confirm that mTOR was indeed knocked down in Q1 cells used for EB formation and that due to that the downstream signaling was affected Western blot analysis was

performed. Although using only half of the amount of siRNA as usual all knockdowns could be verified (PRAS40, TSC2 and mTOR). Deregulating mTOR resulted in a decrease in p70S6K phosphorylation at T389 and consequently a reduced phosphorylation of ribosomal protein S6 at S240/244 (data not shown). Double knockdown of mTOR and PRAS40 or TSC2 could partly revert the effect of the mTOR knockdown (Figure 3.2.22).

More important to us was the difference in the original area of the EBs providing information about cell / EB viability and phenotype to us. EBs treated with control, PRAS40 and TSC2 siRNA and additionally transfected with non targeting control siRNA showed the same phenotype as observed in previous experiments.

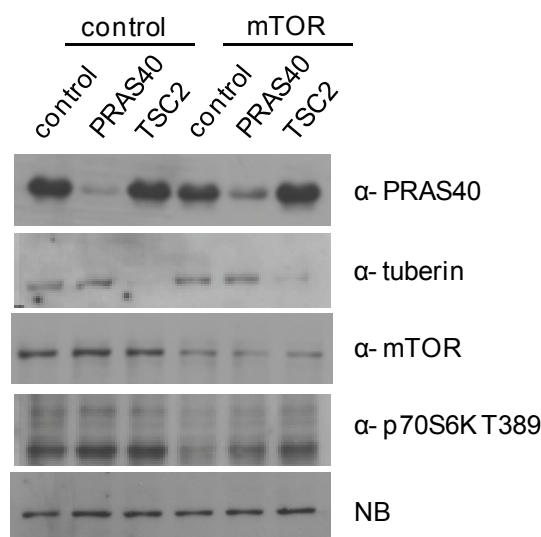


Figure 3.2.22 Q1 AFS cells were transfected with non-targeting control siRNA, PRAS40 siRNA or TSC2 siRNA and co-transfected with either control or mTOR-specific siRNA. Knockdown of endogenous PRAS40, TSC2 or mTOR protein was confirmed by Western blot analysis 72 hours after transfection. Additionally, the mTORC1-dependent phosphorylation status of p70S6K T389 was analysed. A non-specific band served as a control for equal loading.

This goes hand in hand with the area measurements with a significant decrease in TSC2 knockdown EBs and an even higher decrease in area in PRAS40 EBs (Figure 3.2.23). EBs, where mTOR was knocked down, were significantly smaller than control EBs, being consistent with our previously published data, where raptor and rictor knockdown clearly reduced area of EBs (Valli et al., 2010).

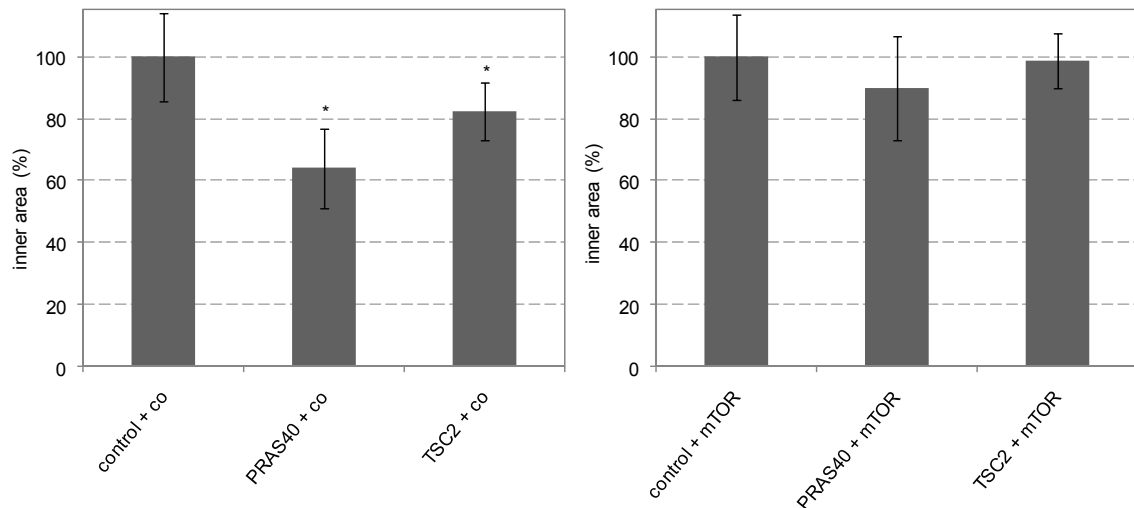


Figure 3.2.23 The apoptotic effects of tuberlin or PRAS40 knockdown depend on mTOR. The inner area (outline of the inner area) of formed day 1 EBs was analysed. For each calculation 10 EBs were photographed, the area was analysed via Cell D image software (Olympus) and the data are presented as average $\pm$ standard deviation (control set to 100%).

From phenotype observation and quantification of the inner area of the EBs we could show that additional knockdown of mTOR was able to compensate the effects of PRAS40 and TSC2 knockdown EBs (Figure 3.2.23).

Analysis of cell number data confirmed again the increased cell death resulting from PRAS40 and TSC2 knockdown in EBs. However when comparing differently transfected EBs additionally treated with mTOR siRNA, we observed a much smaller loss of cells in PRAS40 siRNA treated EBs and TSC2 siRNA treated EBs and moreover decreases were not statistically significant (Figure 3.2.24).

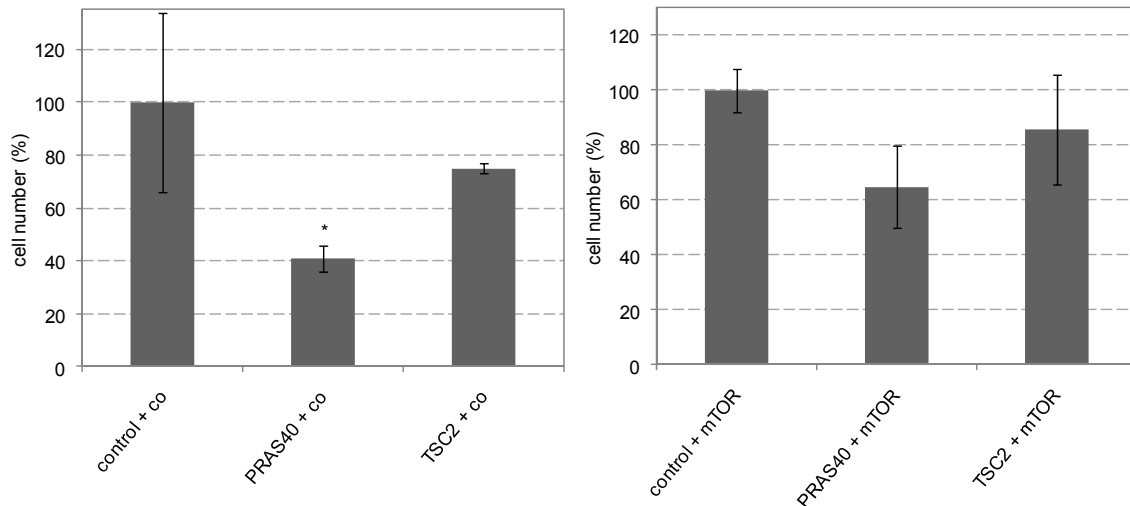


Figure 3.2.24 The cell number of these EBs (32 EBs for each calculation) was determined by a CASY cell counter and analyser. For all statistical analyses in this figure Student's t-tests (unpaired, two-tailed) were performed and * indicates statistical significance with a p-value $\leq 0,05$.

From the results obtained in these experiments we found that mTOR can efficiently compensate the phenotype of PRAS40 and TSC2 knockdown EBs and that PRAS40 and TSC2 regulate EB development via mTOR.

Summing up all the results from this study we conclude that firstly, PRAS40 and TSC2 are major anti-apoptotic gatekeepers in early development and secondly this regulation depends on rapamycin- insensitive functions of mTOR.

4. DISCUSSION

4.1 The localization of PRAS40

PRAS40 was identified as a new substrate for AKT in 2003 (Kovacina et al., 2003), bound by 14-3-3 when phosphorylated on Thr246. Later it was shown that PRAS40 is an inhibitor of mTORC1 until phosphorylated at Thr246 (Sancak et al., 2007; Vander Haar et al., 2007). Furthermore it was found that PRAS40 is part of the mTORC1 complex, acting as a direct inhibitor for substrate binding (Fonseca et al., 2007; Oshiro et al., 2007; Wang et al., 2008; Wang et al., 2007).

Here we show for the first time, that it is primarily a cytoplasmic protein in logarithmically growing human fetal lung fibroblasts IMR-90. We could also show that the localization is the same in Hek293 cells. Our results fit very well to the localization of other AKT substrates e.g. tuberin (Rosner et al., 2007a) and the localization of mTORC1 (Rosner and Hengstschlager, 2008). From the two mTOR complexes, mTOR itself, rictor, sin1 and mLST8 are predominantly cytoplasmic, only raptor can be found equally distributed in the cytoplasm and the nucleus. However the assembly of mTORC1 occurs predominantly in the cytoplasm (Rosner and Hengstschlager, 2008). Therefore it makes sense that PRAS40, as a substrate / component of mTORC1 is primarily found in the cytoplasm to conduct its regulatory function. Multiple other regulators or substrates of mTORC1 signalling e.g. PI3K, AKT and S6K1 are found in both compartments (Furuya et al., 2006; Kim and Chen, 2000; Panasyuk et al., 2006; Saji et al., 2005). PRAS40 possesses an NES (nuclear export sequence) in its C-terminal region suggesting that PRAS40 can shuttle between the nucleus and the cytoplasm (Nascimento and Ouwers, 2009). Some studies reported that PRAS40 is localized to the nucleus. One group reported that in A14 fibroblasts and E2 H9c2 cardiomyocytes gave nuclear phospho-PRAS40 Thr246 staining promoted by insulin (Nascimento et al., 2006). However one has to mention that A14 fibroblasts are NIH3T3 cells overexpressing the human insulin receptor and the same is true for E2 H9c2 cardiomyocytes. Besides as negative controls are missing one has to critically look at the localization of PRAS40 under basal conditions if this staining is really specific. Another group showed that phospho-PRAS40 colocalized with neuron-specific nuclear protein (NeuN) 24 hours after transient focal cerebral ischemia (Saito et al., 2004). Injection of insulin into rats gave nuclear phospho-PRAS40

staining of rat liver and cardiac ventricular tissue sections (Nascimento et al., 2006). One has to keep in mind that most of these experiments were carried out via stimulation or treatment and did not investigate normal basal conditions. Additionally it could be that PRAS40 when strongly phosphorylated by AKT (on Thr246), mTORC1 (on Ser183), PIM-1 (on Thr246) or other currently unknown regulators changes its localization from cytoplasm to the nucleus in its phosphorylated state to conduct e.g. anti-apoptotic (Saito et al., 2006; Saito et al., 2004) or other regulatory functions in the nucleus. Yet this is a subject to clarify.

As PRAS40 is targeted by AKT and phosphorylated by it, we further investigated the role of AKT on PRAS40 localization. In previous studies it has been shown that phosphorylation by AKT can regulate a protein's localization e.g. nuclear and cytoplasmic localization of p27 (Fujita et al., 2002; Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002). As already mentioned above tuberin is mainly localized to the cytoplasm, however its nuclear proportion can be localized to the cytoplasm via active AKT (Rosner et al., 2007a). AKT protein itself is almost exclusively localized to the cytoplasm and to obtain AKT bands of a nuclear fraction on a Western blot requires long exposure times (Rosner et al., 2007b). However from the obtained results, although it seems AKT-mediated phosphorylation of PRAS40 on Thr246 does not affect PRAS40 localization, we still cannot rule out a role for AKT in PRAS40 localization and experiments simply overexpressing constitutive active AKT in IMR-90 and further nuclear and cytoplasmic fractionation should give more insight into PRAS40 localization and its regulation.

4.2 PRAS40 and TSC2 regulate apoptosis during early development of embryoid bodies via mTOR

Amniotic fluid stem cells are an important source for both basic science and regenerative medicine. Besides of being pluripotent (Pappa and Anagnou, 2009) and highly proliferating cells, they do not raise the ethical concerns human ES cells are accompanied with and have a much higher potential compared to adult stem cells. Secondly hAFS cells do not produce teratomas in SCID mice when transplanted, which is a major concern in ES cells (Cananzi et al., 2009; Perin et al., 2008; Siegel et al., 2008; Siegel et al., 2007; Trounson, 2007). Moreover these stem cells can be a useful tool to generate disease specific stem cell lines, which would be optimal biological model systems to recapitulate normal and pathogenic differentiation in vitro (Valli et al., 2010). This has already been demonstrated to be functional using iPS cells from patients with various diseases (Dimos et al., 2008; Maherali and Hochedlinger, 2008; Park et al., 2008). In the past years our group was able to provide new and essential data on hAFS cells, 1) that the mTOR signaling pathway is functional in hAFS cells (Siegel et al., 2009), 2) that hAFS cells have the ability to form EBs and that the mTOR pathway plays a critical role in EB development (Valli et al., 2010) and 3) hAFS cells harbor the potential to integrate and even induce the expression of specific renal markers (Siegel et al., 2010).

The main aim of this thesis was to elucidate the roles of PRAS40 and TSC2, two major regulators of the mTOR signaling pathway, in early embryonic development using hAFS cell derived EBs. EBs served as well recognized in vitro differentiation and early developmental model. We used siRNA to knockdown our genes of interest, as we have reported previously that this could be efficiently achieved in hAFS cells (Rosner et al., 2010; Siegel et al., 2010) and that stability of knockdowns could be maintained for several days, even up to two weeks (Rosner et al., 2010).

The mTOR pathway plays a major role in development demonstrated through knockout mice of e.g. mTOR, raptor, rictor and mLST8, which are components of the two mTOR complexes. mTOR^{-/-} mice were embryonic lethal by E11.5 to E 12.5 (Gangloff et al., 2004; Murakami et al., 2004) proving mTOR to be indispensable during embryonic development. Homozygous embryos showed growth arrest, although they could implant, as they were delayed in development and could not advance beyond E5.5. Furthermore at E5.5 developmental patterning was abnormal resulting in disorganization with no clear

extraembryonic or embryonic boundaries (Gangloff et al., 2004). Another study puts embryonic lethality for mTOR^{-/-} mice already at embryonic stage E6.5 - 7.5 due to impaired cell proliferation of ICM and trophoblasts (Murakami et al., 2004). In addition they also compared the effect of rapamycin with the *mTOR* deletion. Concerning the trophoblast outgrowth rapamycin did mimic the mTOR^{-/-} effect; however rapamycin was unable to impair ICM proliferation (Murakami et al., 2004). Therefore it is suggested that the effect of *mTOR* deletion is stronger than that of rapamycin. Studies on rapamycin by other groups showed that injection of rapamycin from E5.5 to 8.5 resulted in decreased proliferation of the telencephalic primordium, though most of the embryo developed normally (Hentges et al., 2001). In ES cells rapamycin did not block proliferation but rather slowed it down (Jirmanova et al., 2002; Kawasome et al., 1998; Murakami et al., 2004). It was postulated that mTOR is not essential in mouse embryos and ES cells (Jirmanova et al., 2002; Kawasome et al., 1998), however this contradicts the results of Murakami et al. Their explanation is the existence of a rapamycin-insensitive function for mTOR. This is in perfect agreement with the results obtained in this study as rapamycin was not able to revert the apoptotic effects in PRAS40 and TSC2 knockdown EBs, but knockdown of mTOR was.

Later components of both mTOR complexes were knocked out displaying different onsets of abnormal development and embryonic lethality. Raptor^{-/-} mice exhibited defects in ICM and trophoblast cell outgrowth and stopped growth by day 4. By day 7 most of the embryos died and appeared as disorganized apoptotic-like bodies (Guertin et al., 2006). mLST8^{-/-} did not show proliferation defects at the time point mTOR or raptor null embryos did, yet at about midgestation mLST8^{-/-} embryos died at E10.5. Most evident in those null embryos was a failure of development of the cephalic region in comparison to wild type embryos despite no change in apoptosis or cell cycling rates (Guertin et al., 2006). As the mLST8^{-/-} embryos did not exhibit the same severe phenotype as raptor^{-/-} and mTOR^{-/-} it was suggested by Guertin et al., that mLST8 is not required for mTORC1 complex formation and proper function in development. Therefore they studied the phenotype of rictor^{-/-} embryos and showed that those feature very similar characteristics as the mLST8^{-/-} embryo. Both null embryos presumably die from defective vascular development (Guertin et al., 2006). In another publication embryos lacking rictor developed normal until E9.5, followed by growth arrest and death by E11.5 (Shiota et al., 2006). Although no disturbances were seen in gross structure, at the cellular level

hydropic changes occurred, which was evident by nuclear swelling and formation of vacuoles seen in neural epithelial cells, cardiac myocytes, endothelial and mesenchymal cells (Shiota et al., 2006). Shiota et al., suggested that rictor knockout prevents assembly of mTORC2 and that the embryonic lethal phenotype is at least partially due to defects in AKT/PKB signaling.

The negative mTOR regulator TSC2 is also indispensable in early development. TSC2 null embryos died at E9.5 – E12.5 from hepatic hypoplasia (Onda et al., 1999). These embryos were overall smaller and the liver was markedly hypoplastic with dilated sinusoids. Viable embryos at E8.0 – E12.5 were less developed with a delay of approximately 1-2 days. Additionally TSC2^{-/-} embryos were paler and often had pericardial effusions. By E9.0 – E12.5 poor development of other abdominal organs and an abnormal heart size that was shifted inferiorly appeared. The main cause of death in TSC2^{-/-} embryos was due to liver hypoplasia with secondary growth retardation and circulatory failure from anemia (Onda et al., 1999). In another study authors put embryonic lethality of TSC2 null embryos at about E10.5, but some dead or resorbed embryos were already present at E9.0 – E9.5 and some survived even till E12.5 (Kobayashi et al., 1999) indicating a variability in lethal stage. The most prominent phenotypes were the non closure of the neural tube in the head region and an abnormal myocardium with increased cell density (Kobayashi et al., 1999).

For PRAS40, the other regulator of mTORC1 investigated in this study, no knockout mice exist to date and therefore no information about its role in embryonic development. Table 4.2.1 provides a summary of the above described phenotypes of the different knockout mice.

It is more than evident that the mTOR signaling pathway is indispensable in embryonic development; however these data were obtained from mice and so far little data exist for human development. In 2009 a paper was published that revealed that mTOR signaling is needed for proper self-renewal in human ES cells and that impaired mTOR signaling led to differentiation of hES cells and triggered mesoderm and endoderm expression (Zhou et al., 2009). In 2010, for the first time, our group published that hAFS cells are able to form EBs and that the formation of EBs and their differentiation was highly dependent on mTOR (Valli et al., 2010). In this study we show for the first time that two of the major regulators of mTOR, namely PRAS40 and TSC2, are essential in human embryonic

development as they are anti-apoptotic gatekeepers in EB development. At day 1 the EB formation was approximately the same in PRAS40 and TSC2 silenced EBs as in control EBs. Later on, with an onset at about day 3 to day 4, the stability of those EBs was affected by the knockdowns and dramatically dropped to 65 – 70 % in silenced EBs. This provides strong evidence for the necessity of PRAS40 and TSC2 during early development. A major change in morphology of EBs was evident already 12h post start of EB formation as PRAS40 and TSC2 EBs displayed a defined inner and outer area of cells. The analysis of the inner area of EBs revealed a drastic and significant decrease in area in PRAS40 (ranging from ~ 50 – 65 %) and TSC2 (ranging from ~ 60 – 80 %) silenced EBs. Very similar outcome was gained by investigating the cell number of EBs, where PRAS40 and TSC2 knockdown EBs exhibited fewer living cells. Examining the morphology and area of silenced PRAS40 and TSC2 EBs already 12h post start of EB formation, indicated that the PRAS40 phenotype is being displayed earlier than the TSC2 phenotype and additionally seemed to be more severe.

Knockdown of PRAS40 and TSC2 triggered apoptosis in hAFS cell derived EBs. Both proteins are found to be involved in regulating cell survival and apoptosis as they are part of one major cell survival pathway the PI3K/AKT/mTOR signaling pathway. Most studies on PRAS40 and apoptosis were done using different model/tissue systems e.g. spinal cord injury, stroke and cancers. In the area peripheral to the lesion after spinal cord injury, increased expression of phospho-PRAS40 was observed. The increase of phospho-PRAS40 markedly decreased motor neuron death and vice versa downregulation of phospho-PRAS40 increased apoptotic death after spinal cord injury (Yu et al., 2008). PRAS40's neuroprotective role was investigated in transient focal cerebral ischemia (tFCI). Overexpression of phospho-PRAS40 in neurons attenuated apoptotic neuronal cell death after tFCI and furthermore treatment with NGF promoted phospho-PRAS40 expression (Saito et al., 2004). The phosphorylation of PRAS40 and the induced anchoring to 14-3-3 might prevent progression towards apoptosis. Therefore it was suggested that PRAS40 might play a critical role in the survival pathway and should be considered as a therapeutic target for stroke (Saito et al., 2004). PRAS40 also seems to be a regulator of cell survival in cancer as phosphorylation of PRAS40 on T246 was increased in various pre-malignant and malignant lung and breast cancer cell lines. Their conclusion was that the PI3K-AKT pathway is the main pathway PRAS40 is contributing to cell survival (Huang and Porter, 2005). In malignant melanoma PRAS40 deregulates

apoptosis and promotes chemoresistance to apoptosis inducing agents, providing evidence that active PRAS40 gives a selective survival advantage in tumor environment (Madhunapantula et al., 2007).

Conversely to the studies of Yu et al., and Saito et al., downregulation of PRAS40 expression protected against the induction of apoptosis by TNF α or cycloheximide (Thedieck et al., 2007). In their study rapamycin failed to mimic the proapoptotic effect of PRAS40 knockdown and authors suggested that apoptosis was regulated independently of rapamycin-sensitive mTORC1 by PRAS40 (Thedieck et al., 2007). Yet the role of mTORC1 signaling on PRAS40 in apoptosis has not been determined in other studies. Given the observed differences of the impact of PRAS40 silencing on mTORC1 (Fonseca et al., 2007; Oshiro et al., 2007; Vander Haar et al., 2007; Wang et al., 2007; Zhang et al., 2009) further studies are definitely required to elucidate the role of mTORC1 on the regulation of apoptosis via PRAS40 (Nascimento and Ouwens, 2009). This thesis provides new insight into how PRAS40 regulates apoptosis in early development using a human in vitro developmental system. In the here presented study we could show that silencing PRAS40 in EBs derived from hAFS cells triggered massive apoptosis. Using different methods for the measurement e.g. HOPI, subG1 and cell shrinkage via flow cytometry, of cell death we could determine that 1) cell death is increased by approximately 2 to 3 fold in PRAS40 knockdown EBs, 2) cell death is due to apoptosis and not necrosis as determined by HOPI and 3) that the outer area is mainly apoptotic shown through cell shrinkage and subG1 and that there is no difference in subG1 of the inner cells compared to control EBs. TSC2 silenced EBs were also inducing apoptosis in EBs derived from hAFS cells thus to a lesser extent PRAS40 knockdown did. As PRAS40 is linked to apoptosis TSC2 is as well, for example TSC2 overexpressing 3T3 cells were prone to apoptosis (Pasumarthi et al., 2000). In another study high levels of tuberlin triggered apoptosis which was accompanied by p70S6K inactivation, downregulation of phospho-BAD S136 and the upregulation of BAD and BCL-2/BCL-X_L interaction (Freilinger et al., 2006b).

Phenotype	mTOR ^{-/-}	additional information	Rictor ^{-/-}	additional information	Raptor ^{-/-}	additional information	mLST8 ^{-/-}	additional information	TSC2 ^{-/-}	additional information
<i>abnormal developmental patterning</i>	✓	E5.5 disorganization with no clear extraembryonic / embryonic boundaries								
<i>abnormal developmental tissue morphology</i>	✓	trophoblast outgrowth impaired								
<i>inner cell mass degeneration</i>	✓									
<i>abnormal inner cell mass proliferation</i>					✓	inner cell mass explants exhibit reduced expansion				
<i>abnormal extraembryonic tissue morphology</i>	✓									
<i>embryonic growth arrest</i>	✓	E5.5								
<i>embryonic growth retardation</i>	✓	E6.5 - E7.5					✓		✓	
<i>embryonic lethal before somite formation</i>	✓				✓	mice implant but fail to expand and then die				
<i>embryonic lethal during organogenesis</i>	✓	E11.5 - E12.5	✓	showed growth arrest by E9.5 - E10.5; dead by 11.5			✓	mice die at around E10.5	✓	starts at midgestation; homozygotes start dying at ~E9.0-E9.5, with some embryos surviving to E11.5-E12.5
<i>decreased cell proliferation</i>			✓	E9.5						
<i>decreased embryo size</i>			✓	E10.5			✓		✓	by E10.0 smaller than wild type
<i>abnormal placenta labyrinth morphology</i>			✓	trophoblast giant cells number was increased and aberrantly distributed throughout the labyrinth layer in an E16.5 mutant mice			✓	thicker than in wild type mice		
<i>abnormal placental labyrinth vasculature morphology</i>			✓	exhibit fewer maternal-fetal interfaces in most E10.5 mutant placentas						
<i>abnormal trophoblast giant cells</i>					✓	explants exhibit reduced expansion in culture				
<i>liver hyperplasia</i>									✓	
<i>small placenta</i>			✓	placenta was both smaller and thinner than wild-type littermate in an E16.5 mutant mice						
<i>hydrops fetalis</i>			✓							
<i>lethality throughout fetal growth & development</i>			✓	embryonic lethality was observed at later times, and only 1 live embryo was identified among 44 embryos at E16.5						
<i>abnormal vitelline vasculature morphology</i>							✓			

Phenotype	mTOR ^{-/-}	additional information	Rictor ^{-/-}	additional information	Raptor ^{-/-}	additional information	mLST8 ^{-/-}	additional information	TSC2 ^{-/-}	additional information
<i>abnormal heart development</i>							✓	the cardiac wall is slightly thinner than in wild-type mice		
<i>internal hemorrhage</i>							✓	at E9.5, mice occasionally exhibit internal hemorrhage		
<i>abnormal placenta morphology</i>							✓	the interface between the trophoblast giant cell layer and the labyrinth is somewhat disorganized		
<i>abnormal brain development</i>							✓	despite no change in apoptosis or cell cycling rates, cephalic regions fail to develop		
<i>abnormal telencephalon development</i>							✓	telencephalic vesicles are underdeveloped		
<i>abnormal endocardium morphology</i>									✓	at E11.5 and E12.5, mutant endocardia are tightly associated with thickened myocardia
<i>abnormal myocardium layer morphology</i>									✓	at E11.5-E12.5, homozygotes display a thickened myocardium associated with increased cell density
<i>heart hyperplasia</i>									✓	at E11.5-E12.5, homozygotes display a thickened myocardium associated with increased cell density
<i>abnormal heart ventricle morphology</i>									✓	at E11.5 and E12.5, mutant ventricular cavities appear narrowed
<i>open neural tube</i>									✓	at E9.0-E11.5, ~50% display an open neural tube
<i>abnormal gastrulation</i>	✓									
<i>small ectoplacental cone</i>	✓									

Table 4.2.1 Summary of phenotypes of published knockout mice of the mTOR signaling pathway. All knockout mice are embryonic lethal due to abnormal developmental patterning (information summarized in this table was obtained from <http://www.informatics.jax.org/>).

As TSC2 is regulating apoptosis via mTOR and p70S6K mediating BAD phosphorylation, it was suggested that cells lacking functional TSC2 are better protected against apoptotic stimuli and resemble the TSC tumor model (Freilinger et al., 2006b). Figure 4.2.1 represents the signaling pathway TSC2 is involved in to activate the proapoptotic molecule BAD.

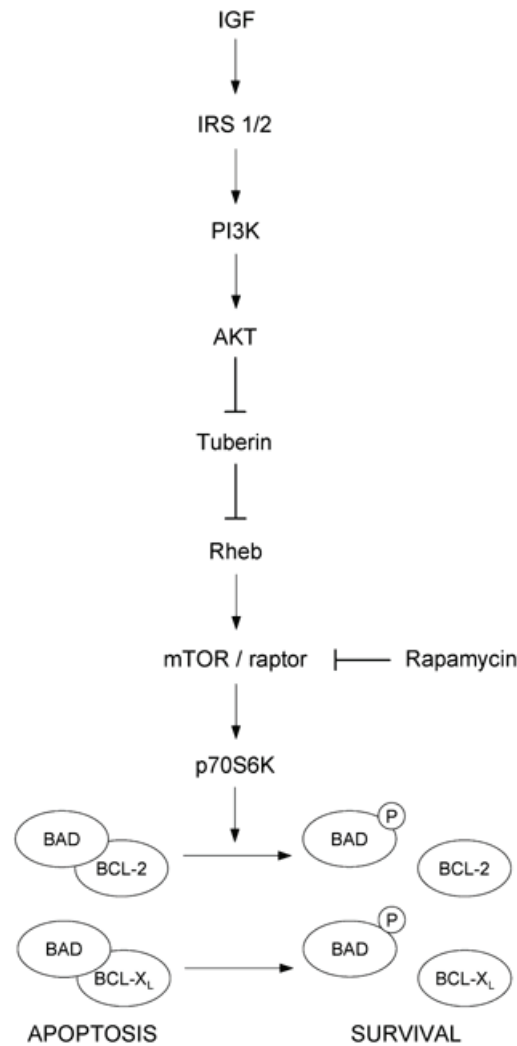


Figure 4.2.1 Tuberin's effects to control p70S6K activity, BAD phosphorylation, BAD/BCL-2 and BAD/BCL-X_L complex formation and apoptosis are negatively regulated by AKT (Freilinger et al., 2006b).

In the same year it was also published that TSC2 negatively affects BCL-2's cell survival function, but still it was suggested that tuberlin might fulfill different functions in apoptosis depending on the exogenous signals (Freilinger et al., 2006a).

Cell survival via regulating tuberlin can also be achieved via Ras signaling pathway, as it negatively regulates tuberlin-induced apoptosis. It was shown that activated Ras was able to downregulate tuberlin's potential to block p70S6K activity (Freilinger et al., 2008). In this report authors mention for the first time a functional connection between oncogenic Ras and the tumor suppressor protein tuberlin. Therefore they suggested that activation of tuberlin might be a useful therapeutic intervention concept in cancers, where Ras is mutated (Freilinger et al., 2008). Figure 4.2.2 views the suggested signaling pathway on how Ras mediates cell survival signals by regulating tuberlin.

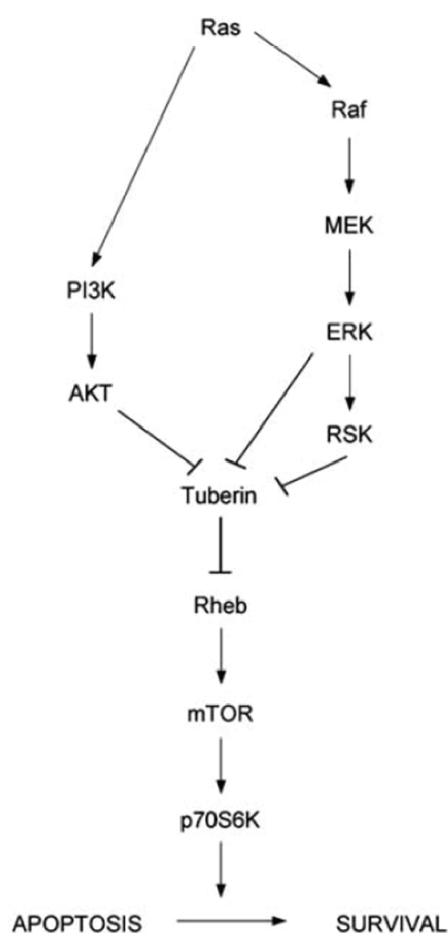


Figure 4.2.2 Suggested pathway for Ras-induced cell survival via regulating tuberlin. Ras-mediated cell survival is accompanied by upregulation of extracellular signal-regulated kinase (ERK) activity, but not of AKT activity.

TSC2 silenced EBs also showed increased susceptibility to apoptosis like PRAS40 silenced EBs but to a much lesser extent, which was already obvious in area and cell number analysis. However, HOPI and flow cytometric analysis also revealed that TSC2 knockdown in EBs is definitely leading to apoptosis and not necrosis, that cell shrinkage

is taking place and that the outer cells of EBs were apoptotic and no visible difference in cell viability was seen in inner cells when compared to control EBs.

Taking together the so far obtained data we can say that PRAS40 and TSC2 are major anti-apoptotic gatekeepers during early embryonic development. Therefore deregulation of any one of the two triggers apoptosis and it is plausible that severe developmental effects would occur *in vivo*.

As the knockdown of PRAS40 and TSC2 in EBs triggered apoptosis and this could have caused deregulation of the germ layers, we stained differently treated EBs with markers for mesoderm (WT1), endoderm (α FP) and ectoderm (nestin). Although the change in the overall EB size was evident through DAPI staining, no significant change in germ layer marker expression was visible in PRAS40 and TSC2 knockdown EBs. Therefore we suggest that PRAS40 and TSC2 are not major regulators of germ layer differentiation and are more important for regulation of cell survival or maybe even homeostasis. Still as we analyzed only three markers it could be possible that those two genes play some role in differentiation of the three germ layers.

PRAS40 and TSC2 are potent negative regulators of mTORC1 (Dunlop and Tee, 2009; Hall, 2008; Rosner et al., 2008a) and mTOR signaling pathway seems to play an essential role during embryogenesis (Guertin et al., 2006; Murakami et al., 2004; Zhou et al., 2009). Using the chemical inhibitor of mTORC1 – rapamycin – we tried to revert the knockdown effects; however we were unable to do so. From Western blot analysis we were able to confirm knockdown of our genes of interest and 100nM rapamycin specifically blocked mTORC1 kinase activity towards p70S6K, which was evident through ablation of T389 phosphorylation of p70S6K. Yet the decrease in area and cell number in PRAS40 and TSC2 knockdown EBs could not be reverted by the addition of rapamycin. Surprisingly, adding rapamycin to controls had no effect on area and cell number, secondly treatment with rapamycin led to further decrease of the area in PRAS40 (from ~ 65% to 45%) and TSC2 (from ~ 83% to 73%) silenced EBs. These effects might be explained as follows: rapamycin preferentially blocks mTORC1 and only later mTORC2 (Rosner and Hengstschlager, 2008). Probably EB development is highly dependent on rapamycin independent functions of mTORC1 or/and mTORC2. This fits well with observations by others as rapamycin did reduce trophoblast outgrowth but was unable to impair ICM proliferation (Murakami et al., 2004). Therefore it was suggested

that rapamycin might not completely block mTOR activity and that the residual activity would be sufficient for embryonic development. Secondly, it was speculated that there exists a rapamycin-insensitive function of mTOR (Murakami et al., 2004). Others even injected rapamycin into developing embryos and, besides some tissue specific growth retardations, the overall embryos developed normally (Hentges et al., 2001).

Our results and previous studies mentioned above rule out a solely mTORC1 dependent pathway. A completely independent role of either mTORC1 or mTORC2 could not be proven with the rapamycin experiment. Taking that into account, we knocked down mTOR in hAFS cell derived EBs targeting both mTOR complexes. Analyzing p70S6K phosphorylation on T389 showed that knocking down mTOR alone did impair phosphorylation. Additional knockdown of PRAS40 slightly increased phosphorylation again and knockdown of TSC2 and mTOR even led to a greater increase of p70S6K T389 phosphorylation. Comparing area of single mTOR knockdown with additional knockdown of PRAS40 and TSC2 we could show that the decrease in area was almost absent in TSC2 (~ 98% compared to 80%) or to a much smaller extent in PRAS40 (~ 89% compared to 64%) and not significant. Analysis of the cell number reflected the results obtained from the area of EBs. Therefore we suggest that PRAS40 and TSC2 triggered apoptosis in EBs is regulated via mTOR. The here investigated mechanisms is dependent on mTORC1 and mTORC2.

To date it is known that there exist rapamycin sensitive and insensitive mechanisms of mTOR signaling. Taking the two main substrates of mTORC1, p70S6K and 4E-BP1, it was shown that rapamycin differentially inhibits their phosphorylation and thus activity (Choo et al., 2008). p70S6K activity is potently inhibited by the addition of rapamycin and starts within minutes of addition (Rosner and Hengstschlager, 2008; Sarbassov et al., 2006). However, after an initial block within the first 6h the phosphorylation of 4E-BP1 recovered and authors suggested that 4E-BP1 phosphorylation is rapamycin resistant still needing mTORC1 activity (Choo et al., 2008). This makes clear that rapamycin differentially regulates mTORC1 substrates (Choo et al., 2008), which could explain our finding that rapamycin could not rescue the apoptotic phenotype in PRAS40 and TSC2 silenced EBs but knockdown of mTOR was. Besides mTORC1, there exists mTORC2 and in the recent years more was learned about this complex and its regulation. PRAS40 has only been found to be associated with mTORC1 and not mTORC2 (Oshiro et al., 2007; Sancak et al., 2007; Thedieck et al., 2007; Vander Haar et al., 2007), yet it was

found that rictor knockdown reduced AKT S473 (Rosner et al., 2009b) and PRAS40 T246 (Thedieck et al., 2007) phosphorylation. This places mTORC2 upstream of mTORC1 and one could speculate that the apoptotic phenotype observed in PRAS40 silenced EBs might be due to deregulated signaling of mTORC2 via PRAS40 and its downstream targets. In addition there exist contrary studies on PRAS40 affecting mTORC1 signaling. In one study silencing PRAS40 induced phosphorylation of p70S6K and 4E-BP1 (Oshiro et al., 2007), whereas in other studies phosphorylation of those two mTORC1 substrates was reduced (Fonseca et al., 2007; Nascimento and Ouwens, 2009; Vander Haar et al., 2007; Wang et al., 2007; Zhang et al., 2009). Therefore it was suggested that PRAS40 might also be playing a role in the assembly and complex integrity of mTORC1 (Fonseca et al., 2007; Nascimento and Ouwens, 2009).

Until 2008 TSC2 has been thought to only regulate mTORC1 but the group of Manning presented results providing evidence that TSC2 complex is also binding and regulating mTORC2 (Huang et al., 2008). They state that an intact TSC complex is required for proper mTORC2 activity and that this regulation is independent of TSC2's GAP activity towards Rheb.

Taking together the data obtained in this study we could show for the first time that PRAS40 and TSC2 are major and essential regulators of early embryonic development. Secondly that those two genes are major anti-apoptotic gatekeepers during development and thirdly that it is regulated via rapamycin-insensitive functions of mTOR. Further investigations have to be done to elucidate the exact mechanism to clarify if those apoptotic effects are regulated via rapamycin-insensitive mTORC1 or mTORC2 or both complexes.

5. LIST OF USED CHEMICALS AND GOODS

5.1 Chemicals and goods

30% acryl amid	BioRad, Hercules, CA, USA
96-Well-Plates, U-Form	Greiner bio-one, Kremsmünster, Austria
Agarose	Sigma-Aldrich, St.Luis, MO, USA
Alpha-MEM minimal essential medium	Gibco-Invitrogen, Carlsbad, CA, USA
Albumin bovine serum fraction V (BSA)	Sigma-Aldrich, St.Luis, MO, USA
Ammonium persulfate (APS)	BioRad, Hercules, CA, USA
Aprotinin	Sigma-Aldrich, St.Luis, MO, USA
Benzamidinchloride	Sigma-Aldrich, St. Louis, MO, USA
Bio Rad - protein assay	BioRad, Hercules, CA, USA
Bromophenol blue	Merck, Darmstadt, Germany
Casycup	Innovatis-Roche, Bielefeld, Germany
Casyton	Innovatis-Roche, Bielefeld, Germany
Chang B + C	Irvine Scientific, Santa Ana, CA, USA
CL-Xposeure films	Thermo Scientific, Waltham, MA, USA
DABCO	Sigma-Aldrich, St. Louis, MO, USA
DAPI	Sigma-Aldrich, St. Louis, MO, USA
D-glucose	Sigma-Aldrich, St. Louis, MO, USA
Dithiotreitol (DTT)	BioRad, Hercules, CA, USA

Dry milk	Maresi, Wien, Austria
DMEM-HG	PAA, Linz, Austria
DMSO	Sigma-Aldrich, St. Louis, MO, USA
EDTA	Sigma-Aldrich, St. Louis, MO, USA
ECL	Thermo Scientific, Waltham, MA, USA
Ethidium Bromide	Sigma-Aldrich, St. Louis, MO, USA
Foetal bovine serum / FCS	PAA, Linz, Austria
GeneRuler 1 kb DNA Ladder	Fermentas, Burlington, Canada
GeneRuler 100 bp DNA Ladder	Fermentas, Burlington, Canada
Glass slides	Lab-Tek, Waltham, MA, USA
Glycerol	Merck, Darmstadt, Germany
Glycin	Sigma-Aldrich, St. Louis, MO, USA
β -Glycerophosphate	Sigma-Aldrich, St. Louis, MO, USA
HEPES	USB, Cleveland, OH, USA
Hoechst33258	Sigma-Aldrich, St. Louis, MO, USA
Isopropanol	Sigma-Aldrich, St. Louis, MO, USA
Lauryl sulphate (SDS)	Sigma-Aldrich, St. Louis, MO, USA
Leupeptin	Sigma-Aldrich, St. Louis, MO, USA
L-glutamin	PAA, Linz, Austria
Lipofectamine 2000	Invitrogen, Carlsbad, CA, USA
Methanol	Sigma-Aldrich, St. Louis, MO, USA

Methylcellulose	Sigma-Aldrich, St.Luis, MO, USA
MgCl	Merck, Darmstadt, Germany
2-Mercaptoethanol min 98%	Sigma-Aldrich, St.Luis, MO, USA
Na ₃ VO ₄	Sigma-Aldrich, St.Luis, MO, USA
NaCl	Sigma-Aldrich, St.Luis, MO, USA
NaF	Sigma-Aldrich, St.Luis, MO, USA
Needles	BD Microlance, Franklin Lakes, NJ, USA
Nitrocellulose membrane	Whatman, Maidstone, UK
Nonidet P40 subsitute	Fluka-Sigma-Aldrich, St.Luis, MO, USA
OptiMEM	Gibco-Invitrogen, Carlsbad, CA, USA
Paraformaldehyde	Sigma-Aldrich, St.Luis, MO, USA
PBS (1x)	PAA, Linz, Austria
Penicillin	Sigma-Aldrich, St.Luis, MO, USA
Pepsin	Sigma-Aldrich, St.Luis, MO, USA
PMSF	Sigma-Aldrich, St.Luis, MO, USA
Ponceau-S	Sigma-Aldrich, St.Luis, MO, USA
Propidium Iodide	Sigma-Aldrich, St.Luis, MO, USA
Reagent Reservoir	Eppendorf, Hamburg, Germany
RNAiMax	Invitrogen, Carlsbad, CA, USA
RNase A	Sigma-Aldrich, St. Louis, MO, USA
RPMI 1640	Gibco-Invitrogen, Carlsbad, CA, USA

Scalpels	Heintel Medizintechnik, Vienna, Austria
Streptomycin sulphate	Sigma-Aldrich, St.Luis, MO, USA
Syringes	BD Plastik, Franklin Lakes, NJ, USA
TEMED Tetramethyl ethylene diamin	BioRad, Hercules, CA, USA
Trizma base (TRIS)	Sigma-Aldrich, St.Luis, MO, USA
Trisodiumcitrate-2H ₂ O	Sigma-Aldrich, St.Luis, MO, USA
Triton X-100	Sigma-Aldrich, St.Luis, MO, USA
Trypsin inhibitor	Sigma-Aldrich, St.Luis, MO, USA
Trypsin/EDTA	Sigma-Aldrich, St.Luis, MO, USA

5.2 siRNAs

human PRAS40 siRNA	Dharmacon, Lafayette, CO, USA
human TSC2 siRNA	Dharmacon, Lafayette, CO, USA
human mTOR siRNA	Dharmacon, Lafayette, CO, USA

5.3 Inhibitors

Rapamycin	Calbiochem, La Jolla, CA, USA
Wortmannin	Cell Signaling, Danvers, MA, USA
LY294002	Calbiochem, La Jolla, CA, USA
Staurosporine	Sigma-Aldrich, St.Luis, MO, USA

5.4 Antibodies

Primary Antibodies

Anti-AKT	#9272	Cell Signaling, Danvers, MA, USA
Anti-AKT S473 (D9E)	#4070	Cell Signaling, Danvers, MA, USA
Anti-cleaved Caspase3 (5A1E)	#9664	Cell Signaling, Danvers, MA, USA
Anti-laminin	#L9393	Sigma-Aldrich, St.Luis, MO, USA
Anti-kip1/p27	#610242	BD Bioscience, Franklin Lakes, NJ, USA
Anti-mTOR	#2972	Cell Signaling, Danvers, MA, USA
Anti-nestin	#MO15012	Neuromics Antibodies, Edina, MN, USA
Anti-GAPDH	#2275-PC-100	Trevigen Inc., Gaithersburg, MD, USA
Anti-p70S6K	#9202	Cell Signaling, Danvers, MA, USA
Anti-p70S6K T389 (108D2)	#9234	Cell Signaling, Danvers, MA, USA
Anti-PRAS40 (D23C7)	#2691	Cell Signaling, Danvers, MA, USA
Anti-PRAS40 T246 (C77D7)	#2997	Cell Signaling, Danvers, MA, USA
Anti- S6 (54D2)	#2317	Cell Signaling, Danvers, MA, USA
Anti- S6 S240/244	#2215	Cell Signaling, Danvers, MA, USA
Anti-tuberin (C-20)	#sc-893	SantaCruz Biotechnology, Santa Cruz, CA, USA
Anti-topoII β	#611492	BD Bioscience, Franklin Lakes, NJ, USA
Anti-WT1 (6F-H2)	#M3561	Dako, Glostrup, Denmark
Anti- α -Fetoprotein	#AF1369	R&D Systems, Minneapolis, MN, USA
Anti- α -Tubulin (DM1A)	#CP06	Calbiochem, La Jolla, CA, USA

Secondary Antibodies

Goat anti-Rabbit	#A120-101P	Bethyl Laboratories, Montgomery, TX, USA
Goat anti-Mouse	#A90-116P	Bethyl Laboratories, Montgomery, TX, USA
Anti-Mouse-Alexaflour488	#A11029	Invitrogen, Carlsbad, CA, USA
Anti-Rabbit-Alexaflour546	#A10040	Invitrogen, Carlsbad, CA, USA
Anti-Mouse-FITC	#F0257	Sigma-Aldrich, St.Luis, MO, USA
Anti-Rabbit-TRITC	#T5268	Sigma-Aldrich, St.Luis, MO, USA

6. BUFFER AND MEDIA

6.1 Buffer and reagents

Buffer A	20 mM HEPES pH7.9 (USB)
	0.4 M NaCl (Sigma)
	25 % glycerol (Merck)
	1 mM EDTA (Sigma)
	0.5 mM NaF (Fluka)
	0.5 mM Na ₃ VO ₄ (Sigma)
	0.5 mM DTT (Sigma)
	dd H ₂ O
Buffer F1	20mM Tris pH 7,6
	50mM β-Mercaptoethanol
	0,1mM EDTA
	2mM MgCl ₂
50xPIM	100 µg/ml leupeptin (Sigma)
	100 µg/ml aprotinin (Sigma)
	15 µg/ml benzamidinchloride (Sigma)
	500 µg/ml trypsin inhibitor (Sigma)

PMSF	0.1 M PMSF (Sigma)
	isopropanol (Sigma)
Buffer B	1.5 M TRIZMA base (Sigma), pH 8.8
	0.4 % SDS (Sigma)
Buffer C	0.5 M TRIZMA base (Sigma), pH 8.8
	0.4 % SDS (Sigma)
4x loading dye	200 mM Tris, PH6.8 (Sigma)
	400 mM DTT (Sigma)
	8 % SDS (Sigma)
	0.4 % bromophenol blue (Merck)
	40 % glycerol (Merck)
Electrophoresis buffer (1 l)	0.25 mol/l TRISMA base (Sigma)
	2 mol/l glycine (Sigma)
	0.035 mol/l SDS (Sigma)
	ddH ₂ O
Harlow buffer (1 l)	0.05 mol/l TRISMA base (Sigma)
	0.39 mol/l glycine (Sigma)
	1 g/l SDS (Sigma)

	20 % methanol (Sigma)
	ddH ₂ O
10x TBS, pH 7.4	1.5 M NaCl (Sigma)
	0.5 M TRISMA base (Sigma)
	ddH ₂ O
1xTBST	10% 10x TBS, pH 7.4
	0.1% Triton X-100 (Sigma)
	ddH ₂ O
FACS-Stain (PI) 100 ml	117,6 mg Trisodiumcitrate-2H ₂ O (Sigma)
	25 mg RNase A (Sigma)
	5 mg PI (Sigma)
	100 µl Triton X-100 (Sigma)
	ddH ₂ O
Mounting medium	2,5% DABCO (Sigma)
	200mM TRIS-HCl, pH8,6 (Sigma)
	90% glycerol (Merck)
	ddH ₂ O
Methylcellulose	7,5g Methylcellulose (autoclaved)
	500ml basal medium

4% Paraformaldehyde	20g Paraformaldehyde
	345ml ddH ₂ O
	5ml 1M NaOH
	50ml 10xPBS

6.2 Cell culture media

DMEM-HG medium	DMEM-HG (PAA)
	10% foetal bovine serum (PAA)
	30 mg/l penicillin (Sigma)
	50 mg/l streptomycin sulphate (Sigma)

RPMI 1640 medium	RPMI 1640 (Gibco)
	10% foetal bovine serum (PAA)
	30 mg/l penicillin (Sigma)
	50 mg/l streptomycin sulphate (Sigma)

Standard medium for hAFS cells	α -MEM minimal essential medium (Gibco)
	15 % FCS (Hyclone)
	2.5 mM L-glutamine (PAA)
	30 mg/l penicillin (Sigma)
	50 mg/l streptomycin sulphate (Sigma)
	18 % Chang B (Irvine Scientific)
	2 % Chang C (Irvine Scientific)

Embryoid body (EB) medium 80% standard medium for hAFS cells
20% Methylcellulose

7. REFERENCES

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8. ABBREVIATIONS

%	percentage
°C	degree Celsius
μ	micro
μg	microgram
μl	microliter
μm	micrometer
μM	micromol
μm ²	square micrometer
4E-BP1	eukaryotic initiation factor 4E (eIF4E) binding protein 1
AF cells	amniotic fluid cells
AFS cells	amniotic fluid stem cells
AKT	v-Akt murine thymoma viral oncogene homolog
AMPK	AMP kinase
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
BAD	Bcl-2-associated death promoter
BSA	bovine serum albumin
CASY	brand name; Casy cell Counter
CD105	endoglin
CD117	c-kit, receptor for stem cell factor
CD34	hematopoietic progenitor cell antigen CD34
CD44	hermes antigen
CD45	protein-tyrosine phosphatase, receptor type C
c-kit	see CD117
c-myc	V-myc myelocytomatosis viral oncogene homolog
DABCO	1,4-Diazabicyclo(2,2,2)octane
DAPI	4',6-diamidin-2'-phenylindol- dihydrochlorid
DMEM	Dulbecco's Modified Eagle Medium
DMEM-HG	Dulbecco's Modified Eagle Medium, high glucose
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
e.g.	exempli gratia (for example)
EB	embryoid body

ECL	enhanced chemilumescence
ECM	extracellular matrix
EDTA	ethylene
eIF4E	eukaryotic translation initiation factor 4E
ERK	extracellular-signal-regulated kinase
ES cells	embryonic stem cells
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FKBP12	FK506-binding protein 12
FOXO	forkhead box protein
FRB domain	FKBP12-rapamycin binding domain
FSC	forward scatter
g	gram
GAP	GTPase activating protein
Gβ1/mLST8	G-protein β-subunit like protein/mammalian LST8
h	human
h	hour
H ₂ O	Aqua dest.
hAFS cell	human amniotic fluid stem cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	hypoxia inducible factor
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G
iPS cell	induced pluripotent stem cell
IRS	insulin receptor substrate
kDa	kilodalton
l	litre
LAM	lymphangioliomyomatosis
LKB1	serine/threonine kinase 11
M	Mol
MAPK	mitogen-activated protein kinase
MC	Methylcellulose
mg	milligram
ml	millilitre
mM	millimol

mRNA	messenger RNA
mSin1	mammalian stress-activated protein kinase interacting protein 1
mTOR	mammalian target of rapamycin
mTORC 1	mammalian target of rapamycin complex 1
mTORC 2	mammalian target of rapamycin complex 2
Oct-4	octamer-binding transcription factor 4
p70 S6K	ribosomal protein S6-kinase, 70 kDa
PBS	phosphate buffered saline
PKK1	3-phosphoinositide dependent protein kinase-1
PFA	paraformaldehyde
pH	potentiometric hydrogen ion concentration
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIKK	phosphoinositide 3-kinase related kinase
PIM	protease inhibitor mix
PIP ₂	phosphatidylinositol 4,5-biphosphate
PIP ₃	phosphatidylinositol (3,4,5)-triphosphate
PMSF	phenylmethylsulfonyl fluoride
PRAS40	proline-rich AKT substrate of 40 kDa
PRR5/protor	proline-rich protein 5/protein observed with rictor
PTEN	phosphatase and tensin homolog
Rag	Ras-related GTP-binding protein
raptor	regulatory associated protein of mTOR
Ras	rat sarcoma
Rheb	ras-homolog enriched in brain
rictor	rapamycin insensitive companion of mTOR
RNA	ribonucleic acid
rpm	rotations per minute
RPMI medium	Roswell Park Memorial Institute medium
RSK1	90kDa ribosomal S6 kinase1
S	serine
S6	ribosomal protein S6
S6K1	ribosomal protein S6 kinase 1
S6K2	ribosomal protein S6 kinase 2
SD	standard deviation

SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
Sox2	SRY (sex determining region Y)-box 2
STK11/LKB1	Serine/threonine kinase 11
T	threonine
TBS	tris buffered saline
TBS-T	tris buffer saline + Triton X-100
TEMED	N, N, N', N'-tetramethylethylenediamine
TOS motif	TOR signalling motif
TSC	tuberous sclerosis complex
TSC1	tuberous sclerosis complex 1 / hamartin
TSC2	tuberous sclerosis complex 2 / tuberin
UK	United Kingdom
USA	United States of America
UTR	untranslated region
v/v	volume/volume
VEGF	vascular endothelial growth factor
α FP	alpha fetoprotein

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11. LIST OF PUBLICATIONS

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