The role of *LKB1* in the pathogenesis of Osteosarcoma

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By

Laure Alice Estelle Duhamel

Genetics and Cell Biology of Sarcoma group
Cancer Institute

I, Laure Alice Estelle Duhamel, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Osteosarcoma (OS) is a rare malignant mesenchymal neoplasm, but the third most common cancer in adolescents. Understanding the molecular pathogenesis of this disease is essential to design new, effective therapeutic strategies to improve patient survival. The recent report that *LKB1*-deficient mice develop bone-forming tumours begs the question whether loss of this tumour suppressor gene plays a role in human OS pathogenesis.

We found that LKB1 protein expression was reduced in 20 of 26 (77%) human OS cases by Western blot and 153 of 259 (59%) by immunohistochemistry. Downstream, the mTOR pathway was activated in 137 of 158 cases (87%) and this activation was correlated to LKB1 loss, providing new insights into potential treatments.

No copy number loss of the *LKB1* region was identified in 93 OS cases by interphase fluorescent *in situ* hybridization. Four of 12 informative cases had concomitant loss of one parental allele at the locus of one single nucleotide polymorphism and reduced protein expression; one of them possessed only one *LKB1* copy by qPCR. Direct sequencing of 21 cases failed to detect *LKB1* mutations and all these cases expressed similarly high levels of LKB1 mRNA by qRT-PCR, irrespective of their protein expression. It suggested that *LKB1* is regulated post-transcriptionally in OS.

In silico analysis of our OS microRNA data showed that this cannot be accounted for by microRNA directly targeting LKB1 mRNA and a preliminary study could not exclude the involvement of SIRT1.

The knock-down of *LKB1* by shRNA in the osteoblast cell line HOB had only subtle effects on cell proliferation and survival. Its role in OS pathogenesis was confirmed by knock-in in LKB1-deficient OS cell lines, which induced reduced cell proliferation.

To conclude, LKB1 protein expression is reduced in a subset of OS by a post-transcriptional mechanism, leading to increased cell proliferation in the tumour.

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PUBLICATIONS

Research papers (in preparation)

- *MDM2* and *MDM4* amplification are common events in parosteal osteosarcoma but rare in other subtypes
  Laure Duhamel, Hongtao Ye, Dina Halai, Bernadine Idowu, Nadège Presneau, Roberto Tirabosco, Adrienne M Flanagan
- mTOR pathway activation in human osteosarcoma
  Laure Duhamel, Asem Shalaby, Dina Halai, Bernadine Idowu, Nadège Presneau, Adrienne M Flanagan
- Reduced expression of the tumour suppressor gene *LKB1* in a subset of osteosarcoma
  Laure Duhamel, Stephen Damato, Dina Halai, Asem Shalaby, Hongtao Ye, Bernadine Idowu, Malihe Eskandarpour, Nadege Presneau, Adrienne M Flanagan

Conference abstracts

- Reduced expression of the tumour suppressor gene *LKB1* in a subset of osteosarcoma
  Laure Duhamel, Stephen Damato, Dina Halai, Asem Shalaby, Hongtao Ye, Bernadine Idowu, Malihe Eskandarpour, Nadege Presneau, Adrienne M Flanagan
  Pathological society Summer meeting, Saint-Andrews, UK, 29 June – 1 July 2010, plenary oral presentation
- Amplification of *MDM2* and *MDM4* in osteosarcoma
  Laure Duhamel, Hongtao Ye, Dina Halai, Bernadine Idowu, Nadege Presneau, Adrienne M Flanagan
  Pathological society Summer meeting, Saint-Andrews, UK, 29 June – 1 July 2010, oral presentation

Posters

- Reduced expression of the tumour suppressor gene *LKB1* in a subset of osteosarcoma
  Laure Duhamel, Stephen Damato, Dina Halai, Asem Shalaby, Hongtao Ye, Bernadine Idowu, Malihe Eskandarpour, Nadege Presneau, Adrienne M Flanagan
  Eurobonet network annual meeting, Paris, France, 11&12 January 2011
- Amplification of *MDM2* and *MDM4* in osteosarcoma
  Laure Duhamel, Hongtao Ye, Dina Halai, Bernadine Idowu, Nadege Presneau, Adrienne M Flanagan
  Eurobonet network annual meeting, Paris, France, 11&12 January 2011
- Role of LKB1 and the mTOR pathway in osteosarcoma pathogenesis
  L Duhamel, D Halai, A Shalaby, H Ye, B Idowu, N Presneau, AM Flanagan
  Pathological society Winter meeting, Imperial College London, UK, 7&8 January 2011, winner of the Sir Alistair Currie poster price
  Eurobonet network annual meeting, Münster, Germany, 18-20 January 2010
- Biomarkers for osteosarcoma
  Laure Duhamel, Asem Shalaby, HongTao Ye, Dina Halai, Bernadine Idowu, Nadège Presneau, Adrienne M Flanagan
  Bone Cancer Research Trust, Saint-Albans, UK, 27 June 2009

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µl	Microliter
03	Clone V3LHS_639003
04	Clone V3LHS_639004
143B	Human osteosarcoma cell line (derived from HOS)
21	Clone V3LHS_645421
293T	Human embryonic kidney 293T cell line
35	Clone V2LHS_232235
4EPB1	Eukaryotic translation initiation factor 4E-binding protein 1
AA	Amino acid
A549	Human lung adenocarcinoma epithelial cell line
Akt	v-akt murine thymoma viral oncogene homolog 1
AMPK	5' adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
bp	Base pair
BSA	Bovine serum albumin
Cal72	Human osteosarcoma cell line
cDNA	Complementary deoxyribonucleic acid
CGH	Comparative genomic hybridisation
chr	Chromosome
CRUK	Cancer research UK
Ct	Threshold cycle
CT	Computerised tomography
Cys	Amino acid cysteine
d	Day
DAB	Diaminobenzidine
DAPI	4’,6’-dianidino-2-phenylindole
ΔCt	Delta (difference) of threshold cycles
Δ ΔCt	Comparative Ct method
DMEM	Dulbecco’s modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediamine tetraacetic acid
eIF-4E	Eukaryotic initiation factor E4
EV	Empty vector
FACS	Fluorescent activated cell sorting

FBS	Fetal bovine serum
FISH	Fluorescent *in situ* hybridisation
G292	Human osteosarcoma cell line
GAPDH	Glyceraldhyde 3-phosphate dehydrogenase
GEM	Gene expression microarray
GR	Good response to chemotherapy
h	Hour
H&E	Haematoxylin and eosin staining
HAL	Human osteosarcoma cell line
HF1	Human immortalised fibroblastic cell line
HOB	Human immortalised osteoblast cell line
HOS	Human osteosarcoma cell line
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
Jurkat	Human immortalised T lymphocyte cell line, derived from leukemia patient
KD	Kinase dead construct
KPD	Human osteosarcoma cell line
LB	Lysogeny broth (Lennox)
LKB1	Serine-threonine kinase 11
LOH	Loss of heterozygosity
Lys	Amino acid lysine
MG63	Human osteosarcoma cell line
MHM	Human osteosarcoma cell line
min	Minute
miRNA	Micro ribonucleic acid
ml	Mililiter
mM	milimolar
MNNG	Human osteosarcoma cell line (derived from HOB by treatment with N-methyl-N'-nitro-N-nitroguanidine)
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
mTOR	Mammalian target of Rapamycin
mTORC1	Mammalian target of Rapamycin complex 1 (Raptor)
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NA	Not available
ng	Nanogram
nmol	Nanomolar

NS	Non-silencing vector
o/n	Overnight
OB	Osteoblastoma
OHS	Human osteosarcoma cell line
OS	Osteosarcoma
OSA	Human osteosarcoma cell line
OST	Human osteosarcoma cell line
P	Parental
p-	Phospho
p53	Tumour protein 53
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline solution
PC	Pressure cooking
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PI3K	Phosphoinositide-3-kinase
PITA	Probability of interaction by target accessibility
PJS	Peutz-Jeghers syndrome
PR	Poor response to chemotherapy
PTEN	phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Reverse-transcriptase quantitative polymerase chain reaction
RB	Retinoblastoma protein 1
Rheb	Ras homolog enriched in brain
RNU48	small nucleolar RNA, C/D box 48
RPMI	Roswell Park Memorial Institute medium 1640
RPS18	ribosomal protein S18
RPS6	Ribosomal protein S6 kinase
s	Second
S6K	p70 S6 Kinase
SaOS2	Human osteosarcoma cell line
SDS	Sodium dodecyl sulfate
Ser	Amino acid serine
shRNA	Short hairpin ribonucleic acid
SNP	Single nucleotide polymorphism
SOC	Super Optimal broth with Catabolite repression
TBS	Tris buffered saline solution
TBST	Tris buffered saline solution with tween 20
Thr	Amino acid threonine
TKR	Tyrosine kinase receptor
TMA	Tissue microarray

TSC	Tuberous sclerosis complex
U2OS	Human osteosarcoma cell line
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WB	Western blot
ZK58	Human osteosarcoma cell line

Chapter 1: **INTRODUCTION**

1.1 Osteosarcoma

Osteosarcoma (OS) is a rare malignant mesenchymal neoplasm that is characterized by the production of osteoid matrix by the tumour cells. The term osteosarcoma was introduced as early as 1805 by the French surgeon Alexis Boyer (Peltier 1993). Although the cancer has been extensively studied since the start of the 20th century, it has a high mortality rate and its genetics are poorly understood.

1.1.1 Disease characteristics

1.1.1.1 Incidence

The incidence of OS is low, affecting between 2 to 4.8 persons per million per year (Bielack et al. 2005, Ries et al. 1999). It represents less than 0.2% of all cancers (American Society of Cancer)(Whelan et al. 2010), but it is the most common bone cancer and the third most common cancer among adolescents (Kansara & Thomas 2007). It affects about 150 persons per annum in the UK (Cancer Research UK (CRUK) and Tan et al. 2006) and 450 to 600 in the US (Ries et al. 1999). Its incidence is slightly higher in men compared to women – with a ratio of 1.5 to 1 (Bullough 2004) – which may be due to differences in growth observed between the sexes. It is also slightly higher in black populations.

1.1.1.2 Clinical presentation

Patients with OS present symptoms of pain and swelling. The pain often increases with physical activity and persists at night. Pathological fracture occurs in 5% of the cases. The symptoms have usually appeared several months before diagnosis. The neoplastic tissue is positive for alkaline phosphatase and some patients present with increased levels of serum lactate dehydrogenase (Bacci et al. 1994) and alkaline phosphatase (Bacci et al. 1987). The lesions, which may be osteolytic or sclerotic (or both), can be detected radiographically. Computerized tomography (CT) or magnetic resonance imaging (MRI) demonstrates the extent of the tumour and is useful for

planning the biopsy approach and the treatment (Gibbs et al. 2001). The diagnosis should be confirmed at the histological level on a needle core biopsy prior to treatment. Due to the high occurrence of metastatic disease in the lungs (section 1.1.3), a CT of the chest is also routinely performed at the time of diagnosis.

1.1.1.3 Age group

Two thirds of patients with OS present between 10 and 25 years old (Bullough 2004), the age of maximum bone growth. In one third of the cases, patients are affected later in life, after 50 years of age, in which case it usually occurs on the background of another condition (section 1.1.2).

1.1.1.4 Sites

OS can develop in any bone, but it primarily affects long bones in the vicinity of the growth plate: predominantly the distal femur, the proximal tibia and the humerus (Figure 1.1).

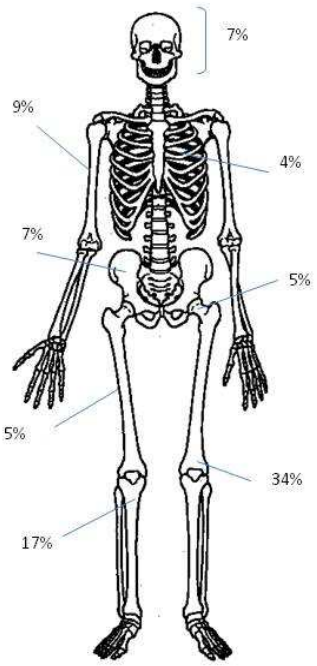


Figure 1.1: OS sites and frequency.
Possible location and occurrence (% of total) of central high-grade OS (Bullough 2004).

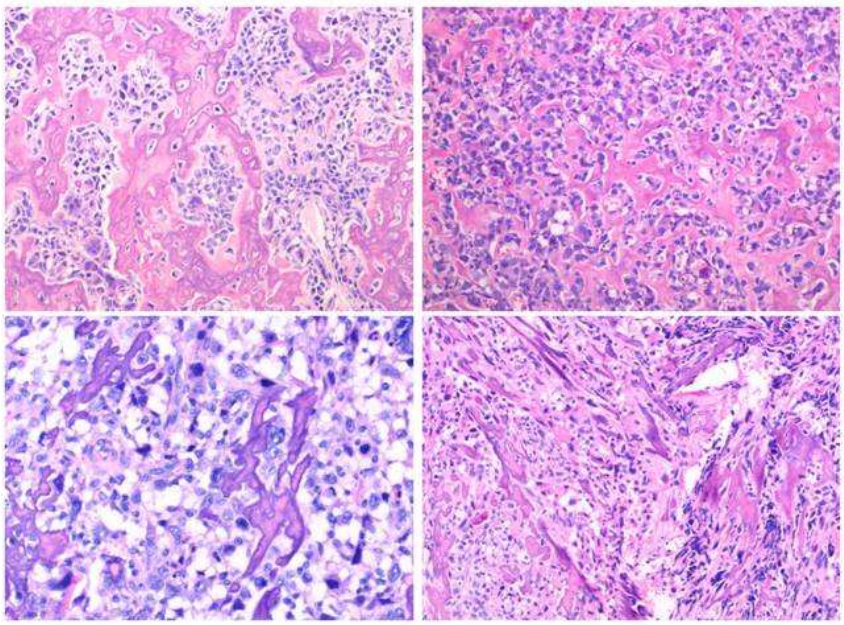
1.1.1.5 Subtypes and classification

OS can be separated into two groups according to the World Health Organisation (WHO) classification: central OS and surface OS.

Over 75% of cases represent central OS; they are sited in the medullary canal and are characterised by their histological subtypes. The most common is osteoblastic, accounting for about half of the cases, followed by fibroblastic and chondroblastic, each accounting for about a quarter of the cases (Cleton-Jansen et al. 2005). The remaining cases are represented by rare variants including telangiectatic, giant cell rich, osteoblastoma-like and small cell OS (Figure 1.2 and Figure 1.3). Different histological subtypes are frequently found in different areas of a tumour, making a strict classification difficult.

Surface lesions are less frequently observed and include two main subtypes: parosteal or periosteal, depending on their localisation on the bone. Unlike central OS, parosteal OS occurs in young adults (age peak is about 30 years) and is more common in women – ratio of 1.5 to 1 (Bullough 2004). According to the WHO classification, periosteal OS is more frequent in adolescents.

A. Osteoblastic subtype



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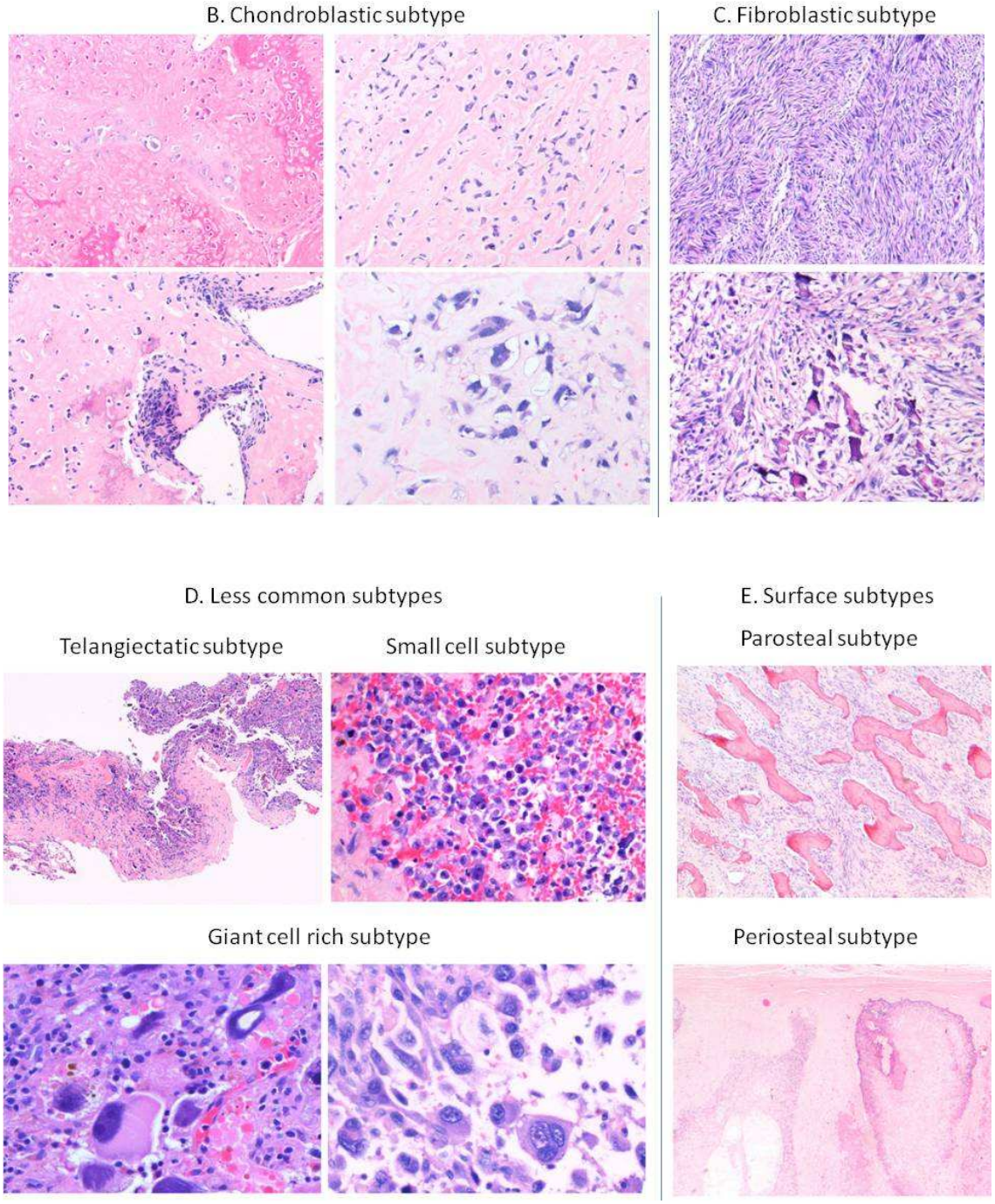


Figure 1.2: H&E sections illustrating the different histopathological subtypes of central (A to D) and surface (E) OS.

1.1.1.6 Grading

Central OS is generally a high-grade neoplasm. Parosteal subtype is a low-grade neoplasm and usually responds poorly to chemotherapy, making treatment difficult despite its low-grade status. It can also recur and dedifferentiate, hereby becoming a high-grade neoplasm. Periosteal OS is classified an intermediate-grade tumour.

1.1.1.7 Aggressive Osteoblastoma versus Osteosarcoma

Osteoblastoma (OB) and osteoid osteoma are benign bone lesions but can behave locally in an aggressive manner. The lesion is excised and patients are not treated with chemotherapy. A small proportion of OB can be aggressive, and on occasions, the differential diagnosis between osteoblastoma-like OS (Figure 1.3) and an aggressive OB is difficult at the microscopic level. Since they are treated differently, it would be valuable to have molecular markers to distinguish them.

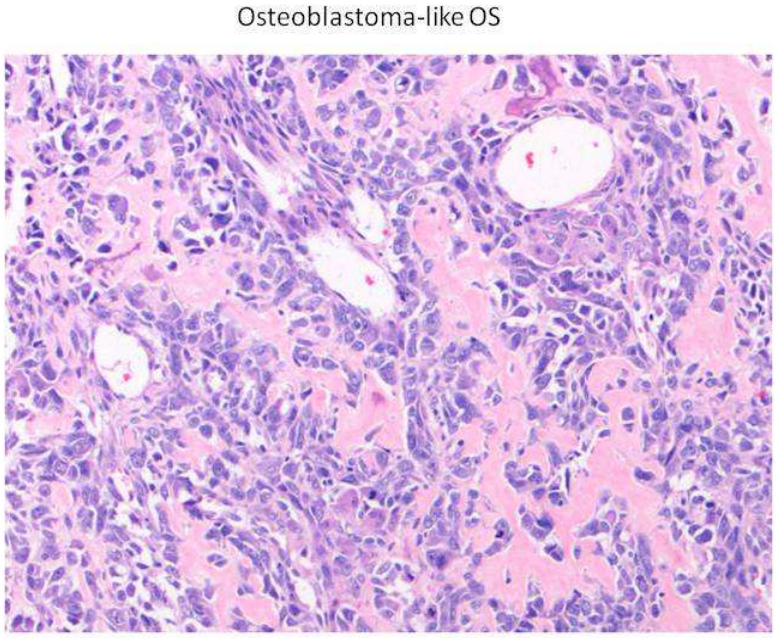


Figure 1.3: H&E section of an osteoblastoma-like OS.

1.1.2 Causes and risk factors

Environmental and genetic factors have been identified to play a role in OS. Several familial syndromes have been associated with this disease and are presented in this section. The exact role of the genes implicated in those syndromes is detailed further in section 1.1.5.1.

1.1.2.1 Rapid bone growth

Rapid bone growth has been noticed to coincide with age peak of OS; it is thought to be related to OS development. The incidence of OS in girls peaks at an earlier age than in boys, as is their growth spurt. Young patients with OS are relatively taller than the average young for their age. The disease is also predominantly seen in metaphyseal portions of the bones undergoing the most rapid growth such as the distal femur or proximal tibia (Figure 1.1 and section 1.1.1.4). However, OS neither occurs exclusively during the patient bone growth nor near the growth plate of bones.

1.1.2.2 Radiation-induced Osteosarcoma

Exposure to radiation and radiotherapy gives an increased risk of developing OS in the long term, within 4 to 40 years (median of 12 to 16 years). The use of diagnostic radioconstrast and alkylating agents could also increase the risk of OS development.

1.1.2.3 Paget's disease of bone

Patients with abnormal bone turnover, such as Paget's disease of bone, have an increased risk of developing OS; in these cases, patients develop the disease generally over 40 years of age. Paget's disease of bone was discovered in 1877 by Sir James Paget and has been associated with defects in the genes *PDB2* (18q), in the IL-1/TNF signalling pathway, or *SQSTM1* (e.g. *PBD3* or *p60,* 5q35.3) and *MAPK8* (10q11.22) in the RANK signalling pathway (Kansara et al. 2007). It is mostly a localised and clinically silent condition, often diagnosed upon autopsy or complications (including fracture, arthritis, heart failure or sarcoma). It is associated with a high level of osteoclastic resorption of bone. Increased bone resorption results in increased osteoblast proliferation and activity (Bullough 2004). The theory is that this contributes to the onset of OS.

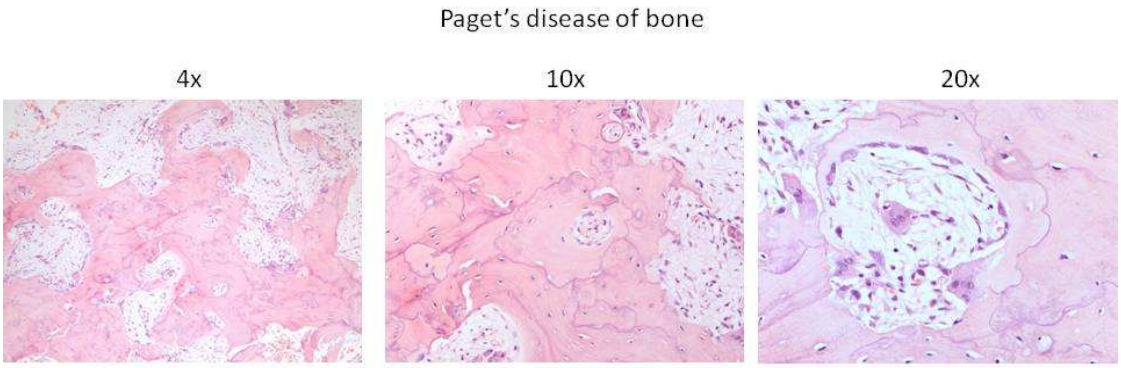


Figure 1.4: H&E sections at 3 different magnifications illustrating the appearance of Paget's disease of bone.
The mosaic pattern acquired by the bone and the presence of numerous osteoclats are typical features of Paget's disease.

1.1.2.4 Retinoblastoma

The strongest association between OS and a genetic disease is with hereditary retinoblastoma. This condition is caused by a germline mutation in the tumour suppressor gene retinoblastoma gene 1 (*RB*) on chromosome (chr) 13q14.2 (section 1.1.5.1). This condition leads to the development of malignant tumours in the retina at an early age, usually before 5 years old. The incidence of OS in patients affected with this condition is increased by 2000 fold in the skull in cases having radiation treatment and 500 fold in the extremities (Hansen et al. 1987, Wong et al. 1997).

1.1.2.5 Li-Fraumeni syndrome

Patients with Li-Fraumeni syndrome, caused by a germline mutation of *p53* (17p13.1), have a 15 fold increased incidence of OS.

1.1.2.6 Other pathologies and syndromes related to Osteosarcoma

Bone dysplasia such as fibrous dysplasia, enchondromatosis (Ollier's disease) or hereditary multiple exostoses, and other bone conditions such as chronic osteomyelitis are associated with an increased risk of developing OS.

Other syndromes are associated with this cancer, including Rothmund-Thompson (caused by a mutation in *RecQL4*, located on 18q24.3), Bloom (*BLM,* e.g. *RecQL3,* 15q26.1*)* and Werner (*WRN,* e.g. *RecQL2,* 8p12-p11.2) syndromes. They all affect the DNA helicase family, *RecQL* (section 1.1.5.1).

1.1.3 Current treatments

OS is currently treated with neoadjuvant and adjuvant chemotherapy, and surgery. Radiotherapy, when delivered at standard doses, is ineffective on OS tumours, so it is usually not prescribed as a treatment (Picci 2007), although recent clinical studies have demonstrated its potential in combination with chemotherapy (Mahajan et al. 2008).

1.1.3.1 Surgery

Surgery was the exclusive treatment for OS until the 1970s when chemotherapy was introduced. The procedure consists of the surgical resection of all malignant tissue with wide margins. Complete removal of the tumour is essential as only 10% of patients are cured with chemotherapy alone (Jaffe et al. 2002). Thanks to the progress in orthopaedic surgery, amputation of the whole limb involved by the tumour can be avoided in most cases (Kim et al. 2010): limb-sparing (or limb salvage) procedures are now used in 90% of the cases and do not affect patient survival when radical margins are used. As OS mainly affects young children and adolescents, this surgical technique limits the long-term burden of the surgery and greatly improves the patient's quality of life.

1.1.3.2 Chemotherapy

The introduction of chemotherapy as a treatment for OS resulted in a dramatic improvement in overall patient survival, from 20% with surgery only to 60-70% on average (Greene 2002). No significant change of this survival has been observed since then. It is hypothesised that the pre-operative chemotherapy targets the undetectable micrometastases present in most patients (Bruland et al. 2005). This would explain its efficiency. It also helps to shrink the primary tumour, facilitating its resection and the use of limb-sparing surgery (Gibbs et al. 2001, Kim et al. 2010). Patients of older age do not tolerate chemotherapy as well as young patients and are sometimes treated with surgery only.

The chemotherapeutic agents used for the treatment of OS are doxorubicin (Cortes et al. 1974, Pratt et al. 1974b), cisplatin (Baum et al. 1981, Gasparini et al. 1985, Ochs et al. 1978), methotrexate (Jaffe et al. 1974, Pratt et al. 1974a, Pratt et al. 1980), and more recently ifosfamide in combination with etoposide (Marti et al. 1985, Miser et al. 1987, Saleh et al. 1990).

The patient's response to the pre-operative chemotherapy is assessed on the resected tumour by a pathologist. In tumours which show a good response to chemotherapy, over 90% of the tumour is necrotic. Tumours which have less than 90% necrosis are classified as showing a poor response to chemotherapy (Salzer-Kuntschik et al. 1983, Winkler et al. 1988). The five year survival of patients with good response to chemotherapy is around 75 to 80%, compared to around 45 to 55% for poor responders (Bielack et al. 2002, Whelan et al. 2000).

1.1.4 Disease progression

1.1.4.1 Recurrence and metastasis

OS frequently recurs or metastasises, either early (e.g. metastasis is present at the time of diagnosis) or late (e.g. metastasis appears several years after treatment). The most common site of metastasis is the lung but metastases to bone or soft tissue are also reported. About 20% of patients have detectable distant metastasis at diagnosis (Cleton-Jansen et al. 2005). Patients with metastatic disease have a worse prognosis and their five year survival rate is far lower. It drops to 10% when the metastases are present at diagnosis compared to 55% with localised disease (Bielack et al. 2002, Whelan et al. 2000) and 90% for patients with a OS tumour classified as low-grade (CRUK).

1.1.4.2 Prognostic markers

Clinical factors

At present there are few prognostic factors used in the clinic for OS. The patient response to chemotherapy has been the most widely employed one, along with either the presence or the absence of metastatic disease. Patients with poor response or metastatic disease have little additional treatment options at present and a worse prognosis. Hence they would benefit from the identification of new therapeutic targets. No molecular marker has so far been confirmed as a predictor of the patient's response to chemotherapy or of risk of developing metastatic disease. There is a great need for such biomarkers as they would ensure a better tailoring of the patient's treatment.

Different histological subtypes show different responses to the chemotherapy and can therefore be used as prognostic factors. For example the chondroblastic subtype usually responds poorly to chemotherapy (Cleton-Jansen et al. 2005). This may be due to the low cellularity of the tumour and the presence of a high quantity of chondroid matrix, which could hamper the delivery of the drug to the cancer cells.

Incomplete surgery is a negative prognostic factor and the classification of the surgical margin can be used to predict the patient's risk of relapse. Radical surgical margins are recommended to prevent local recurrence (Picci et al. 1994). The excision margins are routinely assessed upon

resection, to ensure no tumour remains after surgery. Axial location and tumour size, when it can be evaluated, can also give an indication of the prognosis of the patient (Bielack et al. 2002, Bieling et al. 1996).

Finally, according to CRUK, patients who are young have a higher chance of recovery. Elevated serum alkaline phosphatase as well as lactate dehydrogenase has also been shown as an indicator of poor prognosis (Bacci et al. 1987, Bacci et al. 1994).

Possible molecular markers under review

P-glycoprotein expression is the most promising prognostic marker identified to date. *P-glycoprotein*, (e.g. *ABCB1* or *MDR1,* 7q21.12) is a member of the ABC-transporter family and part of the multi-drug resistance (MDR) subfamily. This molecule enables cells to expel chemotherapy drugs, which could explain its association with poor prognosis in 94 OS cases (Serra et al. 2006). Its use to stratify patients for chemotherapy has been suggested by the authors but the screening of a larger cohort is required. The use of other multidrug resistance proteins as biomarkers, such as MRP and BCRP1 also needs to be investigated further.

Several biomarkers are reported in the literature to be associated with patients' prognosis and metastasis (Yamamoto et al. 2010), including tyrosine kinase receptors like *EGFR* and *Erbb2* (Kersting et al. 2007, Onda et al. 1996)(section 1.1.6.1), *ezrin* (section 1.1.5.2 and 1.2.3.1), *S100A6* (Luo et al. 2008), *annexin 2* (Mintz et al. 2005), matrix metalloproteinases (MMP) like *MMP-9* (Peng et al. 2002) or *SDF-1* (Kansara et al. 2007, Tang et al. 2008). They have been identified by prospective whole genome analysis of clinical samples or by the profiling of *in vivo* and *in vitro* models. Yet, none of them is currently assessed as a routine in the clinic and the screening of large cohorts of human patients is warranted prior to their use. Once implemented, these markers may unveil new therapeutic targets.

1.1.5 Molecular pathology: uncovering the pathogenesis of Osteosarcoma

Although OS has been studied for decades, very little is understood of the molecular mechanisms causing the disease. This can be explained both by the wide heterogeneity of the disease, despite its rarity, and by the complex genetic profile of the tumours. Several approaches have been undertaken to explain OS pathogenesis: the study of the genetic abnormalities of the disease, gene expression or protein expression profiling of the tumours, use of *in vivo* models and study of the cell of origin of the tumour were the most fruitful ones. They are detailed in this section.

1.1.5.1 Genetics of Osteosarcoma

OS cases are not characterised by a consistent chromosomal aberration. Unlike in Ewing's and other sarcomas, no translocation has been identified as a hallmark of this cancer. Abnormal karyotypes with frequent aneuploidy and complex chromosome abnormalities have been detected but without a clear pattern between different cases. It has been suggested that a defect in chromosomal stability or DNA repair may be an early event in the pathogenesis of OS, leading to these inconsistent abnormalities (Khanna 2008). The most commonly observed genetic abnormalities occurring in familial syndromes or *in vivo* models are described below.

Role of *RB* and its pathway

Among the genes implicated in OS pathogenesis, *RB* is the best known one. *RB* plays a major role in the transition from G1 to S phase, by regulating the function of the transcription factors E2F1, 2 and 3 (Figure 1.5). Therefore deregulation of the protein can affect the control of cell proliferation. In addition to patients developing OS after suffering from retinoblastoma (section 1.1.2.4), loss of *RB* expression has been reported frequently in sporadic OS cases (Kansara et al. 2007), via loss of heterozygosity (LOH) in up to 70% of cases (Tang et al. 2008), structural rearrangement or point mutations (Miller et al. 1996b).

Other genes in the same pathway as *RB* are also frequently deregulated in OS (Figure 1.5). *CDK4* (on chr 12q14), which can negatively regulate RB by phosphorylation, has been found amplified in some OS cases. They were mostly of the parosteal subtype, with up to 65% of cases of this subtype having this abnormality (Tang et al. 2008, Yoshida et al. 2010), rather than high-grade central

tumours (Nielsen et al. 1998). High levels of *cyclin D1* (chr 11q13.3), whose protein forms a complex with CDK4, have also been reported in some cases (Tang et al. 2008). Alterations of the CDK inhibitor genes which can regulate CDK4, *CDKN2A* – e.g. p16 protein - and *CDKN2B* - e.g. p15 protein - but not on *CDKN2C* - e.g. p18 protein - have also been reported in a subset of OS cases and cell lines (Miller et al. 1996a). Alterations of *CDKN2A* were more frequent in absence of *RB* alterations (Nielsen et al. 1998).

Role of the *p53* pathway

The fact that patients with the Li-Fraumeni syndrome (section 1.1.2.5) develop OS suggests the *p53* pathway plays a role in this cancer. *p53* is involved in DNA repair, cell cycle arrest and apoptosis by regulating downstream genes including *p21* and *Bax* (Figure 1.5). Loss of *p53* function in sporadic cases has also been found in 40 to 60% of the high-grade cases (Tsuchiya et al. 2000), by allelic loss (75 to 80% of cases), point mutations (20-30%) and gene rearrangements (10-20%) (Miller et al. 1996b, Tang et al. 2008).

In addition to *p53* direct inactivation, several genes in its pathway are de-regulated in OS. *Mouse double minute 2 gene* (*MDM2,* 12q14.3-q15), an inhibitor of p53, is amplified in OS (Ladanyi et al. 1993). This genetic event seems more frequent in parosteal OS (Gamberi et al. 2000, Ragazzini et al. 1999) and can be detected at the protein level (Yoshida et al. 2010). It was observed independently of *p53* alterations (Miller et al. 1996b). This genetic abnormality was confirmed by a study by fluorescent *in-situ* hybridisation (FISH) in our group, in which 179 OS cases, including 24 parosteal were screened (Duhamel et al. 2010).

CDKN2A (chr 9p21.3), which encodes for both p16 and p14 proteins (and can hence regulate both *p53* and *RB*) has been reported as mutated, lost or methylated in OS cases (Lopez-Guerrero et al. 2004, Miller et al. 1996a, Tsuchiya et al. 2000), although it was suggested that small or large deletions of this gene are more frequently observed than methylation (Mohseny et al. 2010).

Mutations in *CHK2* (chr 22q12.1), which regulates p53 stability, has been reported in a small number of cases (Miller et al. 2002) and is also responsible for the Li-Fraumeni syndrome in the absence of p53 mutations (Bell et al. 1999).

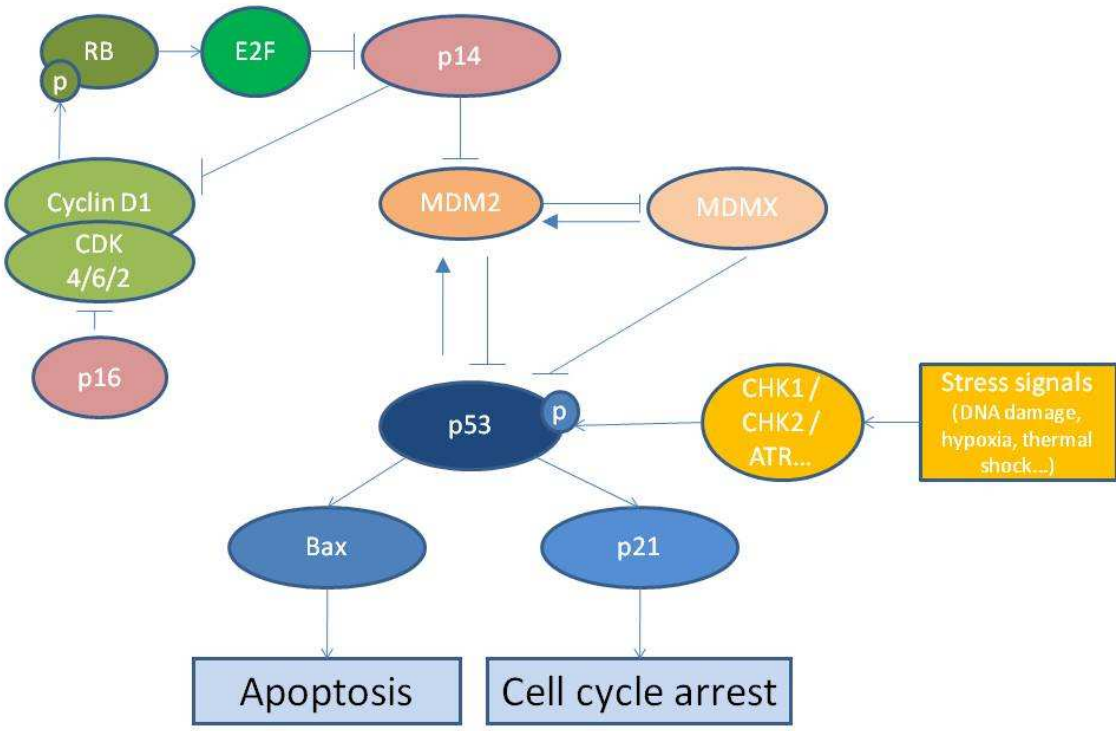


Figure 1.5: Scheme of p53 and RB pathway.

Role of *RecQL* family

RecQL helicases (involved in Werners, Bloom and Rothmund Thomson syndromes, section 1.1.2.6) participate in the maintenance of genomic stability and DNA repair. The exact function of these proteins in OS is unknown. In sporadic OS, no mutations in these genes were identified in one study (Nishijo et al. 2004) but the proteins may be overexpressed compared to their level in osteoblasts (Maire et al. 2009).

Role of the oncogene *MYC*

The oncogene *MYC*, or *v-myc* (avian) myelocytomatosis viral oncogene homolog (chr 8q24.21), is amplified at the genetic level in 7 to 12% of cases and also at the protein level in a small cohorts of patient (Gamberi et al. 1998, Tang et al. 2008). *MYC* is an oncogene that has been implicated in

several hematopoietic tumours and is a transcription factor that regulates processes like cell cycle, apoptosis and cellular transformation.

Role of *FOS*

The oncogene *FOS* (chr 14q24.3) has also been associated with OS as it is overexpressed in 61% of cases in one study at the protein level (Wu et al. 1990). This has been confirmed by a study on a small cohort of patients, with overexpression of FOS at the mRNA and protein level, and was associated with metastasis (Gamberi et al. 1998). FOS is a transcription factor that dimerises with proteins of the JUN family and is involved in cell growth, differentiation, transformation and bone metabolism.

1.1.5.2 Whole genome and epigenome analysis

Several groups have used genomic and epigenomic analysis to uncover the pathogenesis of OS, using a wide range of techniques and heterogeneous cohorts of samples.

Copy number changes in Osteosarcoma

As mentioned earlier, OS displays a great range of genetic instability; cytogenetic, comparative genomic hybridisation (CGH) or single nucleotide polymorphism (SNP) studies have identified a wide range of copy number variation. Although the results are not always consistent, gain of 6p, 8q and 17p (containing *p53* and *COPS3*, which regulates *p53*) are the most frequently observed abnormalities (Cleton-Jansen et al. 2005). Also, gain of chromosome 1, and loss of chromosome 9, 10, 13 and 17 are commonly seen (Bridge et al. 1997). Overall, gains are more frequent than losses. Although informative, those studies have not really enabled to characterise further OS genetic profile as many genes loci they identified were from already known candidates (section 1.1.5.1).

Gene expression profiling

Several studies have focused on the establishment of a gene expression profile of human OS using gene expression microarrays (GEM). Their results are difficult to interpret due to the heterogeneity of the samples and the controls selected. Nevertheless, they led to the identification of interesting markers like *ezrin* (Khanna et al. 2001, Leonard et al. 2003)(section 1.2.3.1), *EBF2* (Patino-Garcia et al. 2009), which is part of the RANK pathway (section 1.1.6.1), and of the role of the Wnt-β-catenin pathway in OS pathogenesis (Cleton-Jansen et al. 2009, Kubista et al. 2010)(see discussion in section 1.1.5.3). Three studies compared the genomic profile of good responders and poor responders (Man et al. 2005, Mintz et al. 2005, Ochi et al. 2004), but unfortunately their results did not overlap.

Other studies have focused on *in vitro* and *in vivo* models to identify putative genes responsible for resistance to chemotherapy or metastasis, by comparing a cell line with enhanced chemo-resistance or metastatic potential to their parental counterpart (Bruheim et al. 2009, Nakano et al. 2003, Rajkumar et al. 2008, Yamamoto et al. 2010). They confirmed the potential role of markers like ezrin, matrix metalloproteinases or VEGF, introduced in previous paragraphs. However, these findings still need to be confirmed on large cohorts of human tumours.

Epigenomic studies

At present, only one group has published analysis of whole genome methylation in OS cases (Sadikovic et al. 2009) and cell lines (Sadikovic et al. 2008) and integrated their results with their GEM and CGH arrays data. They identified a differential expression of *Runx2*, which controls osteoblast differentiation (section 1.1.5.4) and confirmed the role of known genes and pathways like *MYC* or the *p53* pathway. The methylation profile of OS cell lines has also been screened by Kansara et al, who found that *Wifi1*, an inhibitor of the Wnt family, was silenced by hypermethylation (see discussion in section 1.1.5.3).

The discovery of small non coding mRNA, or microRNA (miRNA), in recent years has opened a new insight on the post-transcriptional regulation of protein expression. miRNA can bind to mRNA and either prevent their transcription or induce their degradation (Flynt et al. 2008). However, there are very few reports on miRNA profiles of OS published to date. One group has published the miRNA signature of 200 tumour tissues from 22 different sarcomas, including 15 OS cases (Sarver

et al. 2010). However, there are no published articles with an analysis of the data obtained for these cases and no details on the pathology and clinical characteristics of the tumours chosen. Three studies identified miRNA implicated in chemotherapy response using *in vitro* (Song et al. 2010, Song et al. 2009) or *in vivo* (Gougelet et al. 2010) models: miRNA-140, miRNA-215, miRNA-92a, miRNA-99b and miRNA-132. Both studies found different targets, which may be due to the fact that they use either different models or chemotherapy drugs. Additionally, unpublished data from our group failed to identify miRNA that could be used as biomarkers for chemotherapy response by comparing the miRNA signature of 11 human OS biopsies showing a poor response to chemotherapy in the subsequent tumour resection and 13 showing a good response. Hence these results need to be confirmed further on a large cohort of patients. miRNA-143 and miRNA-21 were the focus of two studies and related them to decreased cell apoptosis (Zhang et al. 2010a) and cell invasion and migration (Ziyan et al. 2010) respectively. However, these two studies focused the expression of only one miRNA, selected due to its role in other cancer, so these results need to be confirmed by whole genome analysis in larger cohorts of patients. The effort of the Eurobonet network, which currently studies the miRNA and gene expression profiles of OS cases and cell lines (unpublished data) will help to understand better the role of miRNA in OS.

Deep sequencing: the Cancer Genome Project

The Sanger Institute is currently analysing the mutation status of 246 human OS samples by deep sequencing (Forbes et al. 2008). Mutations have been reported in *p53* in 43 of 165 cases analysed (26%), in *CDKN2A* in 23 of 246 (9%) cases with 3 of 10 (30%) in the p14 transcript and in *RB* in 12 of 86 (14%) cases, confirming the results previously published on this disease (section 1.1.5.1). Interestingly, novel mutations were also found, with 4 of 43 cases (9%) mutated in *BRAF,* 2 of 75 (3%) in *EGFR*, 1 of 164 (>1%) in *FBWX7* and 1 of 10 (10%) in *TSC1*. As further samples and further genes are analysed, novel markers are likely to be uncovered.

Conclusion

Overall, the prospective analysis of the genomic and epigenomic features of OS using high resolution technology has not yet given a clear picture of the disease. The complexity of this cancer is such that the profiling of the tumours has yielded little transferable data, with hundreds of potential targets identified and yet few confirmed in subsequent publications. Hence most studies conducted so far have failed to distinguish the passenger genetic events from the driver ones. Only a handful of recent studies have lead to novel findings and a better understanding of the disease, with the identification of the role of *ezrin* and of the Wnt-β-catenin pathway.

The integration of the results of gene expression and copy number studies is hampered by the selection of small cohorts of tumours with heterogeneous profiles, mixing different subtypes, different grades and different biological material (cell lines, xenografts, biopsies, resection pre or post-chemotherapy). The limited availability of tumour tissue, due to the rarity of the disease, explains these designs. *In vitro* and *in vivo* models have therefore been widely employed to overcome this problem but the significance of the results has rarely been confirmed on large cohorts of patients. Most of the targets identified from these studies are yet to be translated into biomarkers or treatment.

The use of appropriate controls is essential in the analysis of the genomic and epigenomic data. As the cell of origin of OS has not been clearly identified (section 1.1.5.4), there is no consensus on the matching normal tissue to use. OB does not transform into OS and no other benign disease progressing into OS has been reported, limiting the choice for a matching control. Different groups have chosen different approaches; MSC and osteoblasts are the most common controls employed. Other studies have simply compared tumours or cell lines between them, according to their chemoresistant or metastatic properties, without normal reference. This absence of standardisation in the methodology is contributing to heterogeneity of the results observed.

Deep sequencing of OS as performed at the Sanger Institute may help the understanding of the genetics of this disease and to identify the driver genetic events. Studies integrating results of GEM, CGH (or SNP), methylation and miRNA arrays may also help to better decipher the mechanisms of OS pathogenesis, compared to the use of one method alone. Such an effort is currently being undertaken within the Eurobonet network (unpublished data). However, the use of high numbers of cases along with their matching normal tissue and a strict clinico-pathological

classification of the cases has to be achieved to overcome the difficulties encountered with previous studies.

1.1.5.3 Proteomic analyses and additional pathways implicated in the disease

Proteomic results

The recent publication of proteosome analysis of OS cell lines compared to osteoblast controls has enabled the identification of potential markers such as Prohibitin (Liu et al. 2009), which plays a role in the G1-S phase transition in the cell cycle, AHA1 (Guo et al. 2007) which can bind to Hsp90, a potential therapeutic target in OS (section 1.1.6.1) or CD151 (Zhang et al. 2010b). Two studies performed on human tumour samples confirmed the deregulation of ezrin (Folio et al. 2009, Li et al. 2010) among other targets. However, as in genomic studies, the protein identified by this method needs to be validated further in large cohorts of patients.

Wnt-β-catenin pathway

As detailed in the previous paragraph, genomic and epigenomic analyses of OS have identified that the Wnt pathway plays a role in OS pathogenesis (section 1.1.5.2). The Wnt (or wingless-type MMTV integration site) family, can act through a non-canonical pathway or a canonical pathway, though β-catenin. This latter pathway has been shown to play a role in bone development (Hartmann 2006). However, the mechanism of its action is controversial in OS, with studies suggesting it is activated and that β-catenin or Wnt ligands are present (Haydon et al. 2002a, Iwaya et al. 2003, Kansara et al. 2009) and other studies suggesting it is downregulated in OS (Cai et al. 2010, Cleton-Jansen et al. 2009). To be active, β-catenin needs to be transported to the nucleus, where it can act as a transcription factor; Cai et al show then that in 90% of 52 OS cases, no nuclear β-catenin staining was observed by immunohistochemistry and argue that previous publications showed accumulation of cytoplasmic β-catenin rather than nuclear. The mechanism of transport of this transcription factor to the nucleus when it accumulates in the cytoplasm is not well understood; further studies are warranted to determine the exact role of the Wnt pathway in OS.

mTOR and Ezrin

The PI3K-Akt-mTOR pathway has been suggested to participate to the pathogenesis of OS in several studies, mostly in connection to *ezrin*, which may act upstream of this pathway. This will be developed in section 1.2.3.

1.1.5.4 *In vivo* models used to understand disease progression

Transgenic mice

Several transgenic mouse models have been reported to develop OS tumours, confirming the genetic findings of the disease (detailed in 1.1.5.1). Mice lacking both alleles of *Rb* die *in utero*, but surprisingly, mice lacking one allele failed to develop OS or even retinoblastoma (Jacks et al. 1992). On the contrary, *p53* null mice were viable and developed OS among other cancers (Jacks et al. 1994), as did mice lacking both *Bax* and *p14* (Eischen et al. 2002). Mice lacking one allele of *p53* also developed OS but at a later age (Donehower et al. 1992, Jacks et al. 1994, Purdie et al. 1994). $Nf2^{+/-}$ mice have been reported to develop highly metastatic OS with long latency. Merlin, encoded by *NF2*, can destabilise p53 by inhibiting MDM2, which may account for this result. However, no mutation was detected in *NF2* in 22 OS cases (Stemmer-Rachamimov et al. 1998). Mice overexpressing *Fos* develop OS, and the exogenous gene was found to be specifically overexpressed in osteoblasts (Grigoriadis et al. 1993, Ruther et al. 1989).

Conditional knock-outs in the osteoblast lineage

The role of osteoblasts in OS pathogenesis suggested by the previous report on *Fos* has been confirmed further by the recent use of conditional knock-out of *p53* and *Rb* in transgenic mice in the osteoblast lineage.

One group restricted the loss of one or both alleles of *p53* by using a *Col1A1*-Cre construct, reported to express the Cre-recombinase specifically in osteoblasts (Liu et al. 2004). However, Col1A1 (collagen type 1 alpha 1) can be found in other tissues such as retina; this gene is not implicated in the differentiation process of the osteoblast lineage and its expression is not restricted to one progenitor. Both homozygous and heterozygous *p53* deletion lead to tumours

development, with a shorter latency for the homozygous deletion; 60% of them were OS (Lengner et al. 2006).

A more specific conditional knock-out strain was then established by two different groups by using an alternative o*sterix*-Cre construct (Berman et al. 2008, Walkley et al. 2008). O*sterix*, or *SP7*, is a transcription factor regulated by *Runx2* that is expressed in the transition from osteoblast progenitors to preosteoblasts; these express alkaline phosphatase and Col1A1, so the knock-out happens in a less differentiated cell than in the model of Liu et al (Figure 1.6). In this model, mice lacking one allele of *Rb* did not develop OS, and only one animal lacking both alleles of *Rb* developed OS in the two studies. The $p53^{+/-}$ and $p53^{-/-}$ strains developed OS; loss of *Rb* in addition to the loss of *p53* gave shorter latency and higher metastatic potential to the tumours.

The tumours recapitulated the features of human OS as confirmed by cytogenetic region enrichment analysis (Walkley et al. 2008); they were not exclusively osteoblastic as areas of chondrogenic differentiation were present. Furthermore, cell lines isolated from the tumours spontaneously arising from the preosteoblasts double mutants $p53^{-/-}$ and $Rb^{-/-}$ were able to dedifferentiate into an adipogenic lineage, and expressed the mouse cancer stem cell marker Sca-1 (Berman et al. 2008). This suggests that an oncogenic event in a committed progenitor was sufficient to induce a de-differentiation of the cells back to a multipotent MSC-like phenotype. Further research is needed to establish whether more mature progenitors, such as mature osteoblasts, can generate similar tumours.

Xenograft models

For rare tumours like OS, it is essential to generate xenografts to increase the availability of tissue, to study the onset of metastatic disease and to run pre-clinical studies. Immunocompromised mice injected either orthotopically, subcutaneously or intravenously with human or mouse cell lines and primary tissue gave rise to a large number of *in vivo* models which have helped to understand the onset of OS. Many were used subsequently for genomic analysis. However obtaining such xenografts is technically challenging and yields a low success rate, as confirmed by unpublished data from our group.

Only a small subset of human OS cell lines have been reported to grow *in vivo*, with sometimes conflicting reports, which may be explained by the use of different strains of mice, and are: U2OS

(Manara et al. 2000), SaOS2 (Dass et al. 2006), OST, MNNG (Chen et al. 1997) and 143B (Kim et al. 2002b). Additionally, many of those xenografts do not recapitulate the features of a classical OS *in vivo*, as they do not produce osteoid and/or alkaline phosphatase (as we observed with xenografts of the MNNG cell line) and rarely spontaneously metastase to the lung (Ek et al. 2006). By selection of metastatic clones after *in vivo* injections, metastatic cell lines were generated by different groups, such as the SaOS2-LM6 cell line (Jia et al. 1999) or the K7M2 cell line (Khanna et al. 2001). These models have facilitated the understanding of metastasis in OS, with for example the identification of the role of ezrin.

1.1.5.5 Cancer initiating cells and Osteosarcoma

The genetics of Osteosarcoma and *in vivo* models point toward an osteoblast origin

The cancer initiating cells in OS has not been definitely identified. The large range of histological subtypes observed between cases or even within the same tumour points toward undifferentiated mesenchymal stem cells (MSC). However, as the tumour produces osteoid and is positive for osteoblasts markers like alkaline phosphatase, it may arise from an osteoblast progenitor instead. This is strongly supported by the study of transgenic mice models (section 1.1.5.4) and additional evidence points to this hypothesis.

The role of *RB* in OS (section 1.1.5.1) also suggests an osteoblast origin; *RB* can regulate *Runx2* (Figure 1.6), required for the differentiation of preosteoblasts into committed progenitors and finally osteocytes (Tang et al. 2008). Several whole genome and whole epigenome analyses of OS cases and cell lines have identified genes responsible for osteoblast differentiation, like *Runx2* (Sadikovic et al. 2009) to be de-regulated.

Moreover, the use of differentiating agents on OS cells lines induces both apoptosis and alkaline phosphatase production (Haydon et al. 2002b, Haydon et al. 2007); this suggests that OS is generated from a cell of the osteoblastic lineage having blocked its ability to differentiate into osteocytes. Similarly, in some human and mouse OS cell lines that did not express *osterix* (Figure 1.6), transfection with this transcription factor lead to a decreased tumourigenicity and metastatic potential *in vitro* and *in vivo* (Cao et al. 2005). Hence, re-expression of *osterix* may have overcome the mechanism that blocked differentiation of the cell lines. However, this data needs to be validated by further studies.

Identification of the cancer initiating cell in Osteosarcoma: the cancer stem cell model

Several groups have recently focused on the identification and characterisation of cancer stem cells in human tumours and cell lines to understand tumour pathogenesis. The cancer stem cell (CSC) model postulates that a subset of cells within the tumour have a stem cell phenotype and hence are the tumour initiating cells (Cho et al. 2008, Reya et al. 2001). Thanks to this feature, they are thought to be able to resist chemotherapy treatment as they possess drug pumps (a characteristic of stem cells) and to drive recurrence and metastasis of the tumour. Since up to 40% of patients with OS die within five years as their tumours recur and metastasise (section 1.1.3), the design of new therapeutic treatment targeting the cells resistant to chemotherapy is essential.

Several markers have been proposed to identify CSC in various cancers, such as CD44 and CD24 (Al Hajj et al. 2003) or ALDH1 (Ginestier et al. 2007) in breast cancer, CD133 in prostate cancer (Collins et al. 2005) and glioblastoma (Singh et al. 2004), Oct3/4 and Nanog in lung cancer (Chen et al. 2008), among others (Yang et al. 2008). Yet, the field has proven difficult, with poorly reproducible results and high technical challenges (Check 2007). CD133, which has been used extensively in the CSC field, was proven a controversial maker (Bidlingmaier et al. 2008) and gave poorly reproducible results, especially in colon carcinoma (Labarge et al. 2008, Shmelkov et al. 2008). Furthermore, Quintana et al reported that the new mouse strain NOD-SCID gamma grew xenografts from single melanoma tumour cells, irrespective to their expression of known markers like CD133 and in contradiction to previously published data using less immunocompromised mouse models (Quintana et al. 2008).

Finally, the lack of a single, well-characterised and specific surface marker to identify MSC or osteoblasts, the putative cancer initiating cells in OS (see previous paragraph) make this approach even more challenging.

Nevertheless, some attempts have enabled characterisation of some features of the CSC in OS. Side population assays in OS tumours and cell lines, which use the ability of stem cells to efflux the dye Hoechst 33342 to identify them by FACS analysis (Goodell et al. 1996), lead to the isolation a sub-population of cells with CSC features (Murase et al. 2009, Wu et al. 2007). A high proportion was associated with poorer prognosis. Generation of sarcospheres (3D spherical and clonal colonies growing from CSCs) from human, rat or canine OS tissue or cell lines showed that they expressed ALDH1, Stat3, the ES markers Oct3/4, Nanog and Bmi-1, and the MSC markers Stro-1, CD105 and CD44 (Fujii et al. 2007, Gibbs et al. 2005, Honoki et al. 2010, Wang et al. 2009, Wilson

et al. 2007, Zhou et al. 2010). Other studies corroborated the findings by investigating the role of some of these markers like Oct3/4, ALDH1 and Stro-1 in drug resistance and tumourigenecity (Adhikari et al. 2010, Di et al. 2009, Levings et al. 2009, Wang et al. 2010a). CD116, nestin and CD133 were also suggested as CSC markers in OS (Adhikari et al. 2010, Tirino et al. 2008, Veselska et al. 2008).

Although these reports confirmed the validity of the CSC model in OS, unpublished data from our group failed either to detect or to associate with patient prognosis the expression of the markers Oct3/4, Nanog, ALDH1, and CD44 detected by IHC in 162 patients. Hence, further studies are needed to translate these findings into usable prognostic and therapeutic candidates.

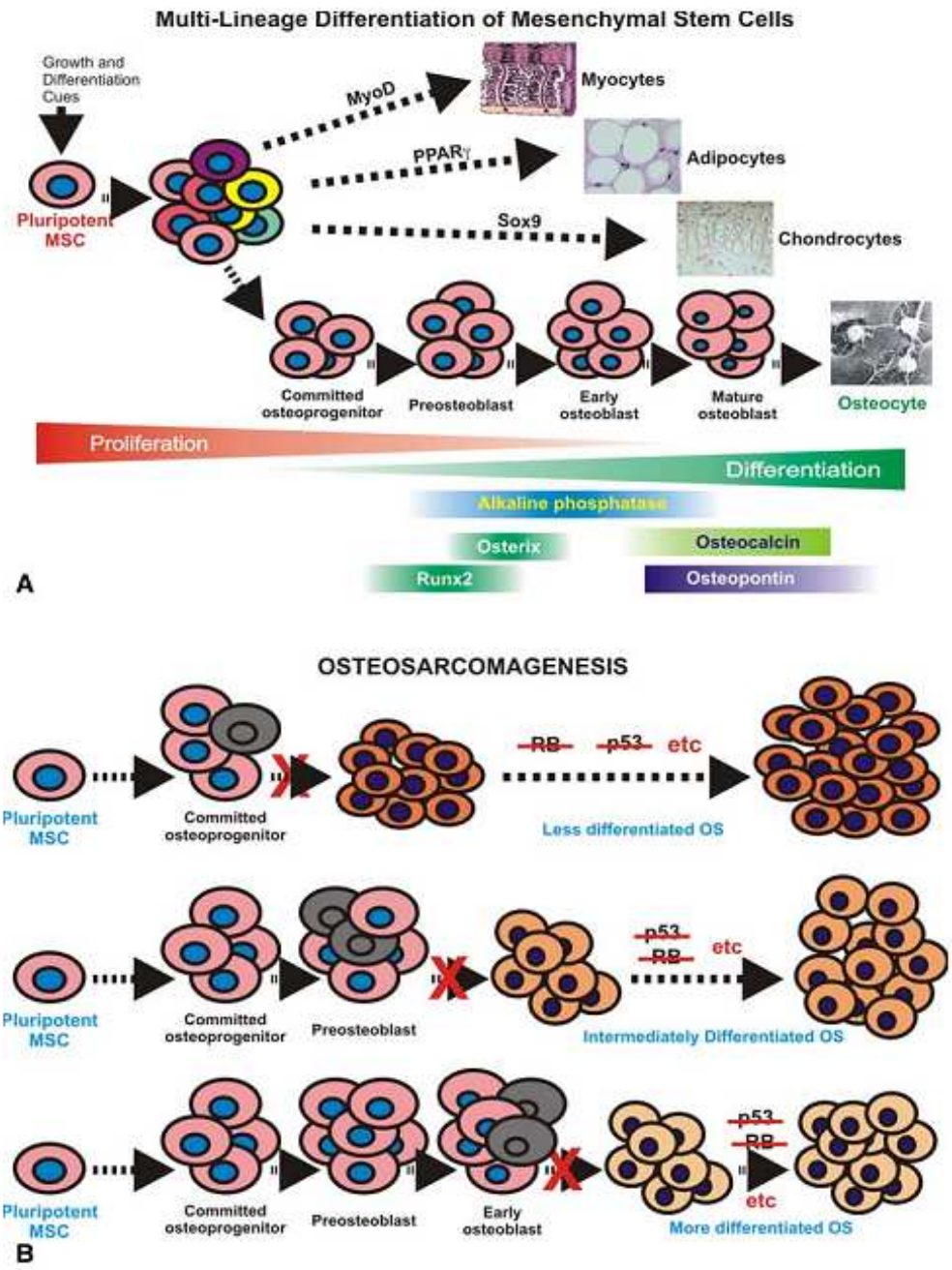


Figure 1.6: Schemes from Tang et al presenting the differentiation process in mesenchymal cells and possible mechanisms leading to OS initiation (Tang et al. 2008).
The cells in black in B. indicate the cancer initiating cells.

1.1.6 Potential treatments under investigation

1.1.6.1 New therapeutic targets

Although the genetics of OS have not been fully deciphered (section 1.1.5.1), several potential therapeutic targets have been identified from observation of the gene and protein expression of OS tissue, or from *in vitro* and *in vivo* systems.

Overcoming Osteosarcoma resistance to chemotherapy

To improve the response of patient to chemotherapy, different approaches have been tested. First, alternative routes of delivery of the chemotherapy treatment have been tried, such as an intra-arterial method; but it was not shown to improve patient's response (Hughes 2009). Delivering higher doses of chemotherapeutic agents has also been attempted; high dose methotrexate does not give better survival and has increased acute and long term side effects (Blay 2007). The management of the chemotherapy regimen, in terms of combination of the drugs used and the timing of the drug delivery, has also been extensively studied in different trials. However current treatment with a combination of the five drugs presented in the paragraph 1.1.3 still gives the best results (Ferrari et al. 2007). As patients receive a very intensive treatment, the use of protectors to reduce the side effects of the drugs is envisaged (Smeland et al. 2004) and the long term side effects need to be monitored and minimised.

Another approach would be to target specifically the cells resisting chemotherapy; this is the basis of the CSC model (section 1.1.5.4). It is at present too early to translate any of the results in this field into the clinic.

Finally, the use of prognostic markers predicting the response of patients to chemotherapy, such as P-glycoprotein (section 1.1.4.2), could lead to better management of the treatment given to the patient. The set up of therapeutic strategies to target the cells expressing these markers is still under review. As such, P-glycoprotein inhibitors are under development and could be used as therapeutic targets.

Many clinicians have expressed concerns in recent publications that the main focus of OS treatment in recent years has been for chemotherapy (Bielack 2010). The strategy chosen has been to optimise the drugs given to the patient, reaching very high doses delivered to the tumour.

However no significant progress has been achieved on the patient's survival and the use of new targeted therapy is warranted.

Targeting metastases

At present, patients presenting with metastases have no treatment available except surgery and further chemotherapy, yielding a very low success rate. To improve the management of metastatic disease, delivery of the chemotherapeutic agents directly to the lungs by intra-pleural or inhalation routes could ease the targeting of the metastatic sites. Preclinical studies in animals and phase I trials have shown that inhalation therapy with gemcitabine is a feasible option in OS (Gagnadoux et al. 2006, Verschraegen et al. 2004).

Additionally, the treatment of bone metastasis could be achieved by exploiting the affinity of bone to phosphates and phosphanates. The principle is to direct radiotherapy treatment toward the metastatic site, thus overcoming the inefficiency of this treatment under standard conditions (Picci 2007). By administrating a radioactive agent like Samarium conjugated to phosphate or phosphanates during the radiotherapy treatment, and using radio-sensitizing agents such as gemcitabine at the end of this radiotherapy treatment, some bone metastasis may be effectively treated. This treatment was tested in both an animal and a pre-clinical study (Anderson et al. 2005, Lattimer et al. 1990), but further assessments are necessary.

MDM2 as a therapeutic target

MDM2, which inhibits p53, is amplified in a majority of parosteal OS (section 1.1.5.1). A new inhibitor is now available against this protein, Nutlin3-A. It has proven its therapeutic potential on OS cell lines *in vitro* as a single agent, inducing apoptosis in the *MDM2*-amplified OSA and MHM cell line and cell cycle arrest in the *p53* wild type non *MDM2*-amplified U2OS cell line (Tovar et al. 2006). As a combined agent with TRAIL, it induced apoptosis in the HOS cell line (Hori et al. 2009). This drug has also proven efficient *in vivo* as it reduced the tumour volume of xenografts from the MHM and OSA cell lines (Tovar et al. 2006). *MDM4* could then compensate for the activity of *MDM2* in repressing p53, making Nutlin treatment inactive (Hu et al. 2006). Recent data from our group showed that concomitant amplification of *MDM4* in *MDM2*-amplified cases is rare (Duhamel et al. 2010); thus most patients with *MDM2* amplification would benefit from Nutlin

treatment. Phase II and III clinical trials are needed to assess the role of this inhibitor in the treatment of parosteal OS. However, the rarity of this subtype presents an obstacle to such studies.

Targeting osteoclasts

Bone maintenance is insured by the balance between bone resorption by the osteoclasts and bone formation by the osteoblasts. Defects in the bone resorption mechanism, as in Paget's disease (section 1.1.2.3), can lead to the development of OS. Although OS is thought to arise from an osteoblast progenitor (section 1.1.5.5), the cross-talk between osteoblasts and osteoclasts may play a role in the pathogenesis of the disease. Osteoclast-targeted therapy aims to decrease the activity of osteoclasts in order to control both the bone destruction and the increased osteoblast activity in the tumour (Akiyama et al. 2008). Three strategies can achieve this aim, all acting at different levels on the RANK pathway, which controls osteoclasts proliferation, differentiation and survival.

First, the inhibition of tumour-induced osteoclastogenesis can be achieved by targeting RANKL, with inhibitors like OPG and RANK-Fc. OPG has been shown to have a therapeutic effect in OS in a murine model (Lamoureux et al. 2007). Biphosphanates and aminobiphosphanates, such as zolendronate or zolendronic acid, can suppress bone resorption, offering a second option. Zolendronic acid has an effect in human cell lines *in vitro* (Ory et al. 2007, Ory et al. 2008) and in OS progression and metastatic potential *in vivo,* with or without chemotherapy (Heymann et al. 2005, Ory et al. 2005). Finally osteoclast apoptosis could be induced by the use of Src inhibitors, such as dasatinib. Src is activated in the OS cell lines U2OS and SaOS2, and treatment of those with dasatinib induced apoptosis and reduced invasion and cell motility (Shor et al. 2007). Another Src inhibitor, SI-83, had similar effects on the SaOS2 cell line (Spreafico et al. 2008b). However, dasatinib had no effect *in vivo* in one study (Hingorani et al. 2009).

Phase III clinical trials are warranted to assess further the effect of osteoclast-targeted therapy in humans as it could impair skeletal development, a major drawback in a disease affecting young children and adolescents (section 1.1.1.3).

Angiogenesis

Targeting angiogenesis to reduce tumour growth and progression is widely accepted as a promising treatment option in cancer. OS tissues have been shown to express high levels of vascular endothelial growth factor (VEGF) and of its receptor (VEGFR) and their expression correlates to poor prognosis. Pre-clinical studies have shown the effect of the inhibitor AZD2171 against OS xenografts (Maris et al. 2008) and phase III clinical trials are underway (section 1.1.6.2).

PI3K-Akt-mTOR pathway and treatment

It has been suggested that the PI3K-Akt-mTOR pathway is playing a role in OS (section 1.2.3.2 and Figure 1.11). Inhibitors against mTOR like Rapamycin or its analogues, or against Akt and PI3K, could be used for the treatment of OS and this will be developed further in section 1.2.3.2.

TKR as therapeutic targets

Tyrosine kinase receptors (TKR) are surface proteins that can channel to different pathways the molecular signals from the cell's environment (e.g. growth factors, cytokines and hormones), to control cell processes. Due to their function in signal transduction, they have been extensively studied in other cancers and have offered successful therapeutic targets, such as Herceptin targeting ErBB2 in breast cancer. Several growth factors and their receptors have been shown to have a role in OS progression. c-MET, the TKR for the hepatocyte growth factor (HGF) has been associated to disease progression (MacEwen et al. 2003, Scotlandi et al. 1996). The insulin-like growth factor 1, IGF-1, and its receptor IGFR have been shown to play a role in bone development (Kasukawa et al. 2004). However, the blockage of IGF-1 release from the liver in a pre-clinical study on dog failed to show an effect on OS tumour growth (Khanna et al. 2002), even though it had an effect on the MNNG cell line *in vivo* (Pinski et al. 1995). This could be explained by the fact that the molecule used failed to alter the hormone level in the tumour microenvironment, although IGF-1 serum levels decreased in the animal. *ErBB2* has been associated with poor prognosis in OS (Onda et al. 1996) whereas *EGFR* amplification is frequent (Freeman et al. 2008, Wen et al. 2007) and associated with good prognosis in one study (Kersting et al. 2007)(section 1.1.4.2). Nevertheless, reports of *ErBB2* overexpression are not always consistent so this receptor may not play a major role in OS pathogenesis (Anninga et al. 2004); further studies are required to validate this target.

LOH in *FGFR* has been detected in OS but no mutation was identified (Mendoza et al. 2005) so the role of this TKR is unclear.

Overall, these reports suggest that TKR inhibitors such as trastuzumab or gefitinib could provide potential treatment in OS. Imatinib mesylate, which can inhibit both c-kit, the platelet derived growth factor receptor (PDGFR), and the drug resistant pump BCRP1 (from the MDR family, section 1.1.4.2) could also offer a promising therapy as it induces apoptosis in OS cells *in vitro* but not *in vivo* (McGary et al. 2002). It could help to overcome OS resistance to chemotherapy (Houghton et al. 2004).

Hsp90 as a therapeutic target

Heat shock protein 90 (Hsp90) is a molecular chaperone that can regulate the expression of certain proteins, including LKB1 (Boudeau et al. 2003b, Nony et al. 2003), p53 (Nagata et al. 1999, Sasaki et al. 2007), IGF-1, Akt and cMET. Hsp90 may play a role in OS as its expression was associated with a better response to treatment in 20 patients (Trieb et al. 2000), or mostly because of the role of its targets such as p53 (section 1.1.5.1). It could be used for therapy as suggested by the effect of 17AAG on OS tumours and cell lines in preliminary reports (Bagatell et al. 2007, Gazitt et al. 2009) . Other inhibitors against Hsp90 exist, like geldanamycin or STA-1474 and they are both reported to target Hsp90 and its downstream proteins in preclinical models of OS (Bagatell et al. 2005, McCleese et al. 2009). One limitation of these inhibitors is that Hsp90 could also target tumour suppressor genes, and not only oncogenes (section 1.2.1.5). Further clinical trials should be set up to demonstrate the effect of those inhibitors in the treatment of OS.

Osteosarcoma treatment using interferons and stimulation of the immune system

Interferons α, β and γ are cytokines that help to regulate the immune system. They have been shown to have an effect on malignant tumour cells: they are able to induce growth inhibition and the release of tumour necrosis factors, to increase cell differentiation (leading to either apoptosis or maturation of cancer cells), to have anti-angiogenic effects, and to stimulate the host's immune system (Whelan et al. 2010). The effect of interferon on OS progression has been demonstrated both *in vitro* and in the clinic. Interferon α action was shown *in vitro* in cell lines as early as 1977 (Strander et al. 1977) and *in vivo* in a mouse xenograft model the following year (Crane, Jr. et al. 1978). This data was confirmed later in other *in vivo* models (Brosjo et al. 1985, Masuda et al. 1983). Two clinical trials in Scandinavia, conducted on 89 patients between 1971 and 1990, showed that interferon α led to an increase in relapse-free survival (Muller et al. 2005). Another study that included 158 patients assessed the role of treatments with low doses of interferon β; it found no difference in disease free survival after 30 months (Winker 1984). The EURAMOS 1 trial is now partly assessing their role and will be presented in the next paragraph.

1.1.6.2 Current phase III trials

Several trials, from either phase I, II or III, are recruiting patients with OS, mainly in the US. Their aim is mainly to confirm the activity of the targets discussed in the previous paragraph. Some early phase trials are recruiting OS as part of a trial on sarcomas in general; the results of such trials are difficult to interpret, as other sarcomas usually greatly differ from OS. Here are detailed the main phase III trials for OS now running.

EURAMOS 1

The main clinical trial in the UK for OS at present is the intergroup trial called the European and American Osteosarcoma Study or EURAMOS 1. It is running across centres in North America, with the North American Children Oncology Group (COG), and in Europe with the German-Austrian-Swiss Cooperative Osteosarcoma Study Group (COSS), the European Osteosarcoma Intergroup (EOI) and the Scandinavian Sarcoma Group (SSG). It is a phase III, open label, randomised clinical trial. Its objective is to optimise patient therapy to improve the outcome of both good and poor

responders. This is achieved through the use of additional post-operative agents, such as interferon-α.

Eligible patients have a central high-grade OS that is resectable, and are under 40 years old. The target is to recruit 1,400 patients in 4 years; it has already included 901 patients since its start in February 2008 (Marina et al. 2010). The design of the trial is presented in Figure 1.7 (http://www.ctu.mrc.ac.uk/euramos). In both good and poor responders, patients are randomised before being distributed to each branch of treatment.

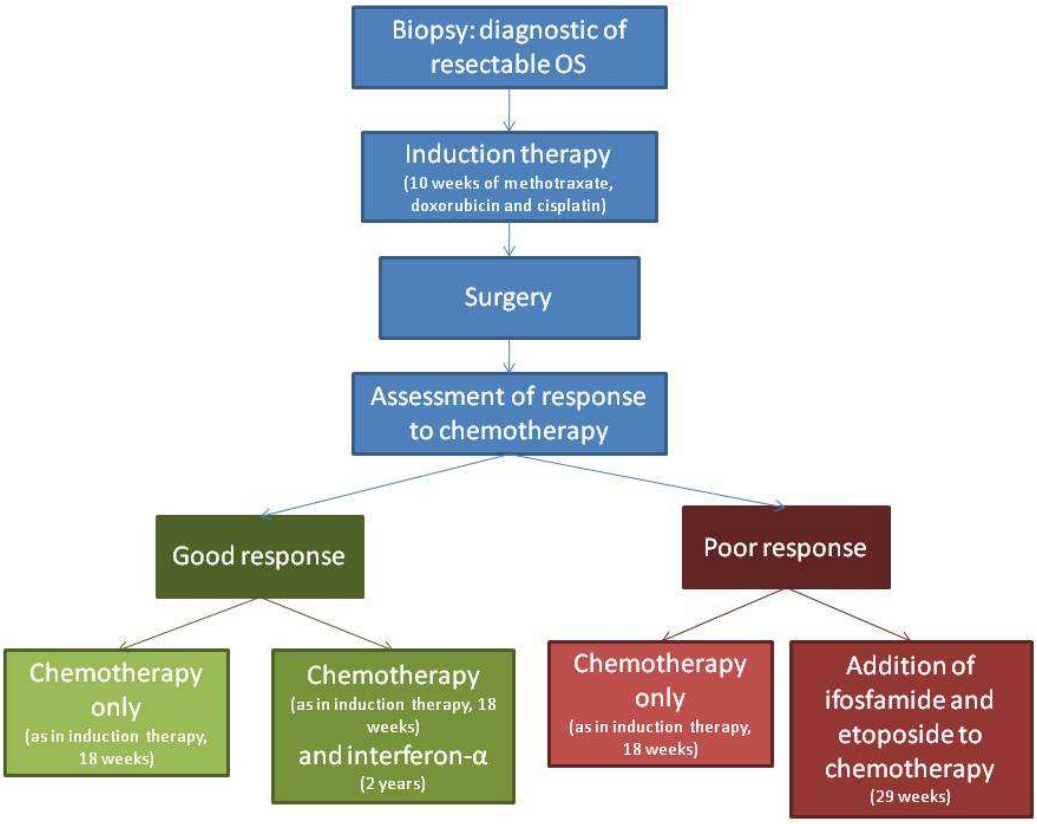


Figure 1.7: Diagram of EURAMOS trial design.

Others trials

Among the other phase III trials including OS presently running, two of them are assessing the potential role of Zolendronic acid in the treatment of this cancer either as a single agent (in India) or combined to chemotherapy (in France). One trial is assessing the role of an anti-angiogenic factor, bevacizumab, which is a humanized monoclonal antibody against the VEGF, in addition to chemotherapy treatment in high-grade patient with OS under 30 years of age. Phase I, II and III trials are also underway in different centres around the world to improve the management of the chemotherapy given to the patients, in term of dosage, drug combination, time and method of drug delivery.

1.1.7 Conclusion

OS is an aggressive cancer that is not well understood. The understanding of its pathogenesis is hampered by its rarity and its complexity. Concerning its molecular characterisation, the *RB* and *p53* pathways have been shown as essential for its progression; this links OS pathogenesis mainly with pathways regulating cell cycle progression from G1 to S phase and apoptosis. Other pathways such as Wnt and Akt-mTOR pathway may also play a role in OS pathogenesis, but further studies are needed to confirm these hypotheses. A line of evidence suggests that the tumours arise from osteoblasts, which have yielded innovative ideas for treatment design, such as osteoclast-targeted therapy. International efforts in the implementation of multi-centred clinical trials, for example EURAMOS, and in the integration of research data, as in the Eurobonet network, are essential to tackle the challenges of this disease.

1.2 LKB1 and the mTOR pathway: potential role in Osteosarcoma

1.2.1 LKB1

1.2.1.1 LKB1 and its complex

LKB1 is the protein encoded by the serine/threonine kinase 11 gene or *STK11* (referred to as *LKB1* in this work), located on chr 19p13.3 at 190 kb from the microsatellite marker D19S886. It belongs to the same protein kinase family as Akt (EC 2.7.11.1.247). The gene is well conserved across species, with single orthologs identified in many mammals. *LKB1* was discovered by linkage analysis in Peutz-Jeghers patients in 1998 (Jenne et al. 1998), suggesting its role in this syndrome (section 1.2.1.3). Following this association, *LKB1* has been identified as a tumour suppressor gene (section 1.2.1.4). Its main role is to regulate cell polarity and metabolism through its interaction with several downstream kinases (section 1.2.1.2).

Splice variants

LKB1 codes for two splice variants, $LKB1_l$ and $LKB1_s$, the latter being recently discovered. Both isoforms contain the same eight first coding exons but they differ in the ninth and last one: $LKB1_s$ has a shorter C-terminal with 39 instead of 63 residues, and lacks the serine 428 (Ser^{428}) phosphorylation site and the cysteine 430 (Cys^{430}) farnesylation site (Denison et al. 2009). However the significance of these differences is not well understood as both proteins have similar localisation and as these residues are not essential for the kinase activity (Alessi et al. 2006). Both isoforms are ubiquitously expressed, but $LKB1_s$ is only the dominant isoform in testis and spleen. It seems to be involved in spermatogenesis and male knockout mice for this isoform are sterile (Denison et al. 2009, Towler et al. 2008). Therefore $LKB1_l$ will be solely studied in this work and will be subsequently referred to as LKB1.

Protein structure

LKB1 mRNA transcript contains 3276 nucleotides with 10 exons, the last one being non-coding, and it is translated in a protein of 433 amino acids for a molecular weight of 48.6kDa (reference Q15831 in Uniprot). The protein is localised either in the nucleus or in the cytoplasm of the cell (see next paragraph). Its structure includes a serine/threonine kinase domain from amino acid (AA) 49 to 309 (Figure 1.8). The C- and N-terminals do not have homologies with other proteins. The C-terminal is thought to be the regulatory domain and the N-terminal to contain a nuclear localisation signal, whose role needs to be assessed further.

LKB1 contains at least four auto-phosphorylation sites on Thr^{185}, Thr^{189}, Thr^{336} and Thr^{402}, and five phosphorylation sites, Ser^{31}, Ser^{307}, Ser^{325}, Ser^{428} and Thr^{363} (which also may be auto-phosphorylated as discussed in the next paragraph), one farnesylation site, Cys^{430}, and one acetylation site on Lys^{48} (Figure 1.8). The role of these sites is discussed further in paragraph 1.2.1.5.

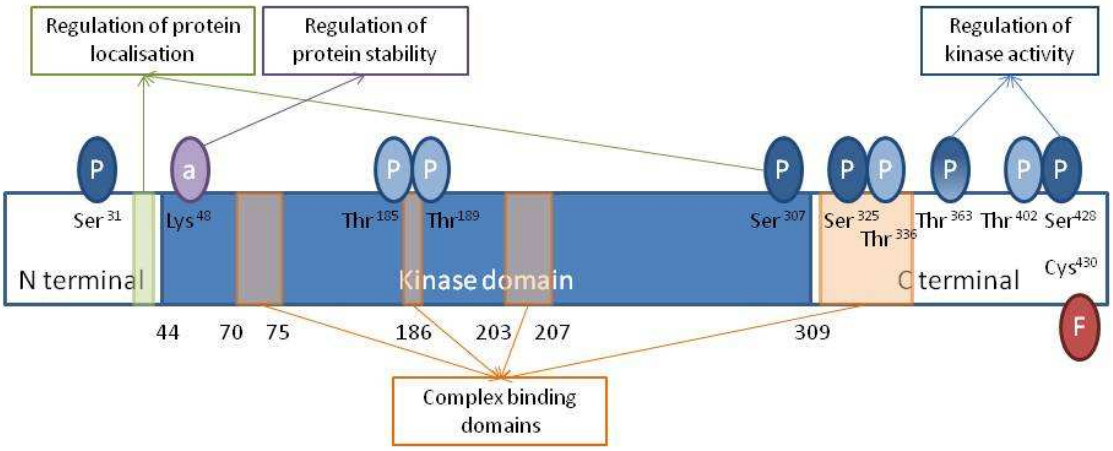


Figure 1.8: LKB1 protein domains.
Farnesylation site indicated by F, acetylation by a, phosphorylation by P (dark blue for phosphorylation by other kinases and pale blue for autophosphorylation sites).

LKB1 complex

LKB1 protein is part of a heterotrimeric complex with either of the isoforms α or β of Strad (e.g. STRADA or LYK5 for α, and STRADB for β, STE20-related kinase adaptor alpha/beta on chr 17q23.3 and 2q33.1 respectively) and MO25 (e.g. CAB39 α or β, calcium binding protein 39 on chr 2q37.1). Both isoforms of both proteins interact with LKB1. LKB1 is barely active as a monomer, as demonstrated by mutating *LKB1* in the Strad-binding domain or use of siRNA against Strad (Baas et al. 2003). Upon binding to Strad, LKB1 activity is enhanced by 100-fold by conformational change (Hawley et al. 2003).

The structure of the complex was discovered recently by crystallography (PDB reference 2WTK). It showed that once in the complex, LKB1 stably acquires a canonical closed conformation with a re-order of its activation loop, which is typical of activated kinases (Zeqiraj et al. 2009a). To achieve this, Strad acquires a closed conformation and binds to LKB1 kinase domain. Once bound, the two proteins are phosphorylated by LKB1, on Thr^{329} and Thr^{419} for Strad, and Thr^{336} and Thr^{363} for itself. Mutations on those 4 residues do not affect the complex formation and the activity of LKB1 kinase; Stradβ does not even have those sites and still interacts with LKB1. Strad itself is a pseudokinase and lacks any activity; however, once in the complex, its closed conformation with an ordered activation loop is typical of active kinases. MO25 stabilises the complex by binding to both Strad and LKB1 (Boudeau et al. 2003a), as a scaffold protein; more precisely, it interacts with Strad regulatory helix and with LKB1 activation loop in the C-terminus (Zeqiraj et al. 2009a). The interaction between Strad and MO25 facilitate the allosteric change of Strad into its closed conformation and its binding to ATP. Both these are required to fully activate LKB1 (Zeqiraj et al. 2009b) and may participate in keeping Strad in the appropriate conformation.

Once synthesised, LKB1 protein is imported to the nucleus by importins α and β. Stradα and MO25 diffuse passively to the nucleus. By binding to its complex, LKB1 is re-localised from the nucleus to the cytoplasm, where it mainly plays its role (Baas et al. 2003). Stradα was identified as the facilitator of this transport, and not Stradβ or MO25 (Dorfman et al. 2008): it promotes the export of the complex from the nucleus thanks to its binding to CRM1 and exportin-7. Stradα also competes with the importins binding site on LKB1 to prevent its re-localisation to the nucleus. Stradβ lacks the binding sites to exportin-7 and CRM1 and was shown to inefficiently transport LKB1 to the cytoplasm.

In conclusion, the formation of LKB1 complex (and not phosphorylation like for most kinases) is the essential step in the kinase regulation: it promotes its activity both directly, by an allosteric mechanism, and indirectly, by the control of its localisation.

1.2.1.2 Function of LKB1

The main function of LKB1 is to activate by phosphorylation of their kinase domain the AMPK subfamily of kinases (or AMPK-related kinases). This subfamily contains 14 identified members: AMPK 1 and 2, SIK 1 and 2, NUAK 1 and 2, MARK 1, 2, 3, and 4, BRSK 1 and 2, QSK and SNRK (Jaleel et al. 2005, Lizcano et al. 2004). They have been associated with pathways implicated in cell metabolism, cell polarity and apoptosis as will be detailed below. They have been identified by sequence homology in their kinase domain. Upon phosphorylation by LKB1, their activity is enhanced by 50-fold (Alessi et al. 2006). However, they have different length and different functional domains. Their role is redundant and their expression may be cell-type dependent. LKB1 has also been reported to interact with other pathways regulating cell differentiation, via an unknown mechanism. This multi-functional role is illustrated in Figure 1.9.

Regulation of cell metabolism: the central role of AMPK

The first identified and best known effector of LKB1 is AMPK, or 5'-adenosine monophosphate activated protein kinase. It is a heterotrimeric complex composed of the following subunits: α, the catalytic one, and β and γ, the regulatory ones. There are two isomers of the α-subunit, named 1 and 2. AMPK is first activated by the binding to AMP to its γ subunit (Carling et al. 2008). This enforces an allosteric mechanism and eventually inhibits the dephosphorylation of AMPK Thr^{172} site on the catalytic subunit, required for complete activation. This phosphorylation can be performed either by LKB1 as shown by biochemical assays (Hawley et al. 2003, Shaw et al. 2004) or by the Ca^{2+}/calmodulin-dependent protein kinase β (CaMKKβ). Both proteins are integrating cell metabolic status: LKB1-mediated activation takes place in response to AMP/ATP increased ratio, whereas CaMKKβ-mediated activation happens in the presence of increased Ca^{2+} levels in the cytoplasm (Carling et al. 2008, Jansen et al. 2009). AMPK can phosphorylate LKB1 on Ser^{31}, maybe as a feedback after its activation; however the exact role of this phosphorylation is unknown.

AMPK is an energy-sensor; its role is to switch off cell metabolism in response to the scarcity of ATP and subsequent increase of AMP. This prevents cell growth and proliferation in nutrient deficient conditions. It is accomplished through the regulation of enzymes such as acetyl-CoA carboxylase (ACC), through the inhibition of CREB transcription by phosphorylation of its co-activator CRTC as shown in hepatocytes (Koo et al. 2005), or through its inhibition of protein translation via the mTOR pathway (Inoki et al. 2003) as described in paragraph 1.2.2.1.

SIK1 and 2 (salt-inducible kinase 1, on chr 21q22.3 and salt-inducible kinase 2 or QIK, on chr 11q23.1) have also been reported to play a role in cell metabolism by phosphorylation of CRTC, like AMPK. SIK1 was shown to be active in adrenocortical cells (Katoh et al. 2004), and SIK2 in adipose tissue (Horike et al. 2003) and pancreatic islet cells (Screaton et al. 2004), and both were reported to act in the liver (Shaw et al. 2005). The SIK kinases can be inactivated by hormones (Katoh et al. 2004). This could explain how the action of AMPK and SIK kinases on the same targets is coordinated: one is responding to cellular energy levels, and one to external hormonal stimuli.

Apoptosis and anoikis regulation

In addition to its part in cell metabolism, a novel role of SIK1 has been discovered recently, linking it to p53 and anoikis (Cheng et al. 2009). SIK1 was identified as a regulator of p53 by phosphorylation in anchorage independent conditions by siRNA screening in a soft agarose assay. Loss of SIK1 enabled cells to survive in those conditions and promoted metastasis *in vivo* (section 1.2.1.4).

Similarly, AMPK has been reported to control a p53-mediated apoptosis response but in conditions of energy stress (Jones et al. 2005, Shaw et al. 2004).

Finally, NUAK 1 (NUAK family SNF1-like kinase 1 or ARK5, on chr 12q23.3) and NUAK 2 (NUAK family SNF1-like kinase 2 or SNARK, on chr 1q32.1) have also been associated with resistance to apoptosis, one in nutrient starvation (Suzuki et al. 2003) and the other in glucose starvation (Suzuki et al. 2003) or in tumour cell lines (Legembre et al. 2004).

LKB1 and cell polarity: AMPK, MARK and BRSK

The role of *LKB1* in cell polarity has been first discovered by genetic screening of its orthologs, *Par4* in the *Caenorhabditis elegans* model and *dLKB1* in the *Drosophila* model (Alessi et al. 2006). The Par family can define different membrane domains, hereby polarising the cell; Par1, (MARK and BRSK in humans) for the lateral domain, and Par3 and 6 for the apical domain, (Jansen et al. 2009). The protein Par5 (14-3-3 protein) and Par4 (LKB1) can respectively inhibit or activate by phosphorylation the effectors, Par1, 3 or 6. They regulate this polarisation mechanism and control asymmetric division, which is an important process in several epithelial tissues. Even though these mechanisms are quite different in invertebrates and vertebrates, these studies suggest that LKB1 plays a similar role in humans.

This was demonstrated in intestinal epithelial cells as they became polarised following LKB1 expression (Baas et al. 2004). Mammalian epithelial cells mutants in the C-terminal of LKB1 also failed to become polarised, confirming further this link (Forcet et al. 2005). AMPK was shown to be one of the LKB1 effectors implicated in cell polarisation (Zhang et al. 2006, Zheng et al. 2007), by potentially targeting myosin regulatory light chains. It is of note that NUAK1 may play a role in cell adhesion by interacting with myosin phosphatases (Zagorska et al. 2010).

As mentioned above, MARK 1 to 4 (MAP/microtubule affinity-regulating kinase 1 to 4, respectively on chr 1q41, 11q13.1, 14q32.32 and 19q13.32) and BRSK 1 and 2 (BR serine/threonine kinase 1 and 2 or SAD-A and B, respectively on chr 19q13.42 and 11p15.5) share homology with the Par1 proteins. There is little evidence of the role of MARK proteins other than by homology. MARK proteins were reported to phosphorylate Tau proteins, which are best known for their major role in the onset of Alzheimer's disease. This may lead to the destabilisation of microtubules and, overall, cell polarity (Katajisto et al. 2007). They are also thought to phosphorylate the microtubule-associated proteins, and can then re-shape microtubules skeleton, centrosome positioning and cell polarisation (Drewes et al. 1998, Kojima et al. 2007). BRSK proteins have been detected mainly in the brain. Mice lacking those two isoforms cannot form axons and dendrites due to a deficiency in their neuron polarisation (Kishi et al. 2005).

Other downstream kinases

The roles of other kinases activated by LKB1 are not well defined. QSK (or SIK family kinase 3 on chr 11q23.3) has been identified to play a role in cell cycle progression by a knock-down experiment in drosophila (Bettencourt-Dias et al. 2004). SNRK has been reported to be only present in the testis (Jaleel et al. 2005), where the alternative splice variant of LKB1 is present. This may indicate that $LKB1_s$ and SNRK cooperate to regulate spermatogenesis. Further studies will possibly uncover novel functions of LKB1.

LKB1 and action on other pathways

Beyond its role in the activation of its downstream kinases, LKB1 has been shown to regulate other key signalling pathways by mechanisms that are not fully understood and which may be indirect effects.

In addition to its role via the interaction of its downstream kinase AMPK with TSC2 (section 1.2.2.1), LKB1 is thought to regulate the mTOR pathway by directly phosphorylating PTEN (Mehenni et al. 2005b)(Figure 1.11 for pathway scheme).

Similarly, LKB1 role on migration has not been restricted to its action on cell polarity via MARK and BRSK. It has also been demonstrated to inhibit cell motility (Deguchi et al. 2010) via the phosphorylation of PAK1 (p21 protein (Cdc42/Rac)-activated kinase 1 on chr 11q14.1). The interaction of LKB1 with PAK1 and Cdc42, which activates PAK1, has been mentioned before (Zhang et al. 2008) and the protein has been shown to co-localise at the leading edge of migrating cells. Furthermore, an integrated genetic and proteomic screen have shown the association of LKB1 loss with invasion and metastasis in lung cancer, via the activation of EMT and focal adhesion markers (Carretero et al. 2010). The authors established that LKB1 and Src were cooperating in an antagonistic fashion for the regulation of both cell growth via the mTOR pathway, and migration via the PAK1 pathway.

More evidence of LKB1 role in the p53-pathway has been uncovered with its interaction with p21, downstream target of p53, and with its binding to p53 in the nucleus, to stabilise it by phosphorylation (Zeng et al. 2006). LKB1 is recruited by p53 to activate the transcription of p21 by binding to the gene's promoter region. This study suggests that LKB1 complex could have a function in the nucleus and not only in the cytoplasm. LKB1 and p21 low expression were

correlated in another study on pancreatic cancer. This confirmed the potential interaction between LKB1 and the p53 pathways to mediate cell cycle arrest (Morton et al. 2010).

In xenopus, inhibition of the ortholog of LKB1, XEEK1, led to developmental abnormalities characteristic of defective Wnt signalling, and XEEK1 was shown to phosphorylate GSK3β, thereby activating the Wnt-β-catenin pathway (Ossipova et al. 2003). It was previously shown that Wnt and the mTOR pathway were co-regulated in human due to the involvement of GSK3β and AMPK (Inoki et al. 2006); the discovery of Ossipova et al imply that LKB1 could be participating to this mechanism. Two recent reports (Chaiyapan et al. 2010, Ma et al. 2010) underline that β-catenin protein is present in Peutz-Jeghers patients, who lack LKB1 expression (section 1.2.1.3 below); however, the localisation of β-catenin reported in those publication is cytoplasmic, not nuclear, so the protein may not be active (section 1.1.5.3 for discussion on Wnt pathway). On the contrary, LKB1 was shown to inhibit the Wnt pathway as its expression *de novo* induced a de-phosphorylation of GSK3β (Lin-Marq et al. 2005). As one model is in xenopus and one in human, it may account for these opposite results. It is also possible that LKB1 action on Wnt may be context dependent (depend on energy level in the cell), or different in developmental and in adult settings. Overall, the interaction between the Wnt pathway and LKB1 should be studied further.

Finally, LKB1 has been associated in the cross-talk between cell types via the TGF-β pathway, which regulates cell growth and death. LKB1 expression in endothelial cells promotes vascular smooth muscle recruitment via TGF-β, linking LKB1 and angiogenesis (Londesborough et al. 2008). Similarly, LKB1 loss was shown to disrupt the cross-talk between the mesenchymal and epithelial compartment mediated in the intestines by TGF-β (Katajisto et al. 2008), as will be developed in 1.2.1.3. The exact mechanism leading to the deregulation of TGF-β signalling by LKB1 has to be elucidated.

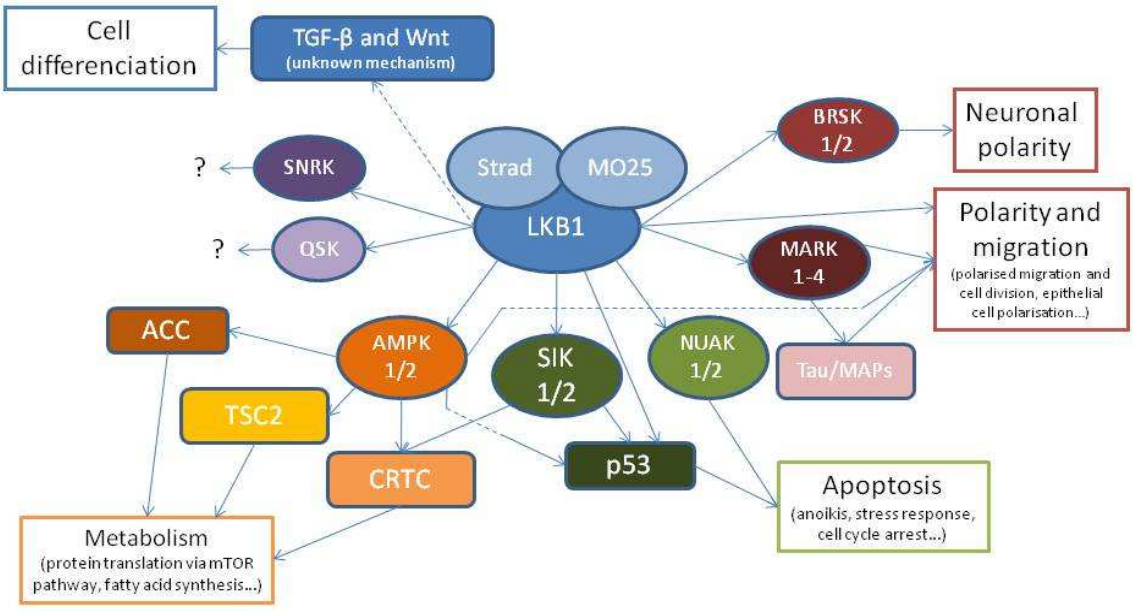


Figure 1.9: Scheme illustrating the downstream kinases of the LKB1 complex and their known function.

Conclusion

LKB1 can be seen as a master pathway regulator due to its high number of downstream targets and its involvement in many major cell regulatory functions. However, as developed in paragraph 1.2.1.1, once expressed and bound in complex with Strad and MO25, LKB1 is constitutively activated. How LKB1 is directed among its many function is not fully understood, but it seems to be cell type dependent and context dependent. The study of LKB1 function suggests it integrates environmental signals to activate its proper downstream effector. Other regulation mechanisms will be developed in paragraph 1.2.1.5.

1.2.1.3 LKB1 and Peutz-Jeghers syndrome

Peutz-Jeghers syndrome (PJS) is an autosomal dominant disease described first by Peutz in 1921 and Jeghers in 1949 (Jeghers et al. 1949, Peutz 1921). *LKB1* has been associated with PJS first by CGH analysis then by linkage analysis (Hemminki et al. 1997). Mutations in *LKB1* were then discovered in patients with PJS (Jenne et al. 1998). Screening for mutation and LOH in proteins interacting with LKB1, including Strad and MO25, has failed to reveal any other abnormalities to this date (Alhopuro et al. 2005, de Leng et al. 2005). The use of recent techniques like multiplex ligation dependent probe amplification has confirmed that PJS is mainly caused by genetic inactivation of *LKB1* in around 80% of patients (Aretz et al. 2005). The syndrome is not only a familial disease as 10 to 20% of patients acquire PJS by spontaneous germline mutation of *LKB1*.

The disease is characterised by the presence of mucocutaneous melanin pigmentation appearing in early childhood and the development of hamartomatous polyposis in the patients' gastrointestinal tract. The condition is rare, affecting 1 in 50,000 to 1 in 200,000 persons in the world (Jansen et al. 2009). The symptoms are abdominal pain, bleeding, occlusions and intussusceptions and appear between 20 to 30 years of age. They are due to the development of large polyps, potentially from the small hyperplastic lesions observed throughout the digestive tract of patients with PJS. These larger polyps can occur at any location in the gut, but are mostly seen in the small intestine (Vasen 2000). The main treatment for patients with PJS is surgery, to remove the polyps before the development of any complications.

Although the polyps are benign, patients with PJS have an increased risk for developing a variety of cancers: a 22 to 130-fold increased risk for pancreas cancer, 15-fold for gastro-oesophageal, small bowel or colorectal cancer, 8.5-fold for gynaecological and testicular cancer, 6-fold for breast cancer, 4 to 9-fold for lung cancer. Female patients with PJS also have an increased risk for developing sex cord tumours with annular tubules; they are benign, bilateral and multifocal, whereas they are usually malignant and unilateral in sporadic cases (Jansen et al. 2009). Approximately 93% of patients with PJS suffer from cancer by the age of 43 (Boudeau et al. 2004). Although LKB1 expression is ubiquitous, the tissues expressing LKB1 at higher level in normal adult conditions also seem to be the ones where patients with PJS develop tumours most frequently (Sanchez-Cespedes 2007).

Patients with PJS have usually lost only one copy of LKB1, mostly by germline mutation, although some cases also lose the remaining allele. They can display mutations throughout the gene, and over 100 different ones have been reported; but interestingly, they target the kinase activity domain of LKB1, the C-terminal domain but not the N terminal. In the C-terminal, one frequently observed mutation is a stop codon to suppress the last 20 amino acids, which are associated with LKB1 localisation. A set of mutations in the C-terminal of LKB1 that disrupt the kinase ability to control intestinal epithelial polarity have also been uncovered (Forcet et al. 2005). Single AA substitutions that prevent the binding of LKB1 with Strad have also been found in patients with PJS (Zeqiraj et al. 2009a).

Mouse models have been developed to understand the role of LKB1 in PJS. Lkb1 null mice do not survive beyond embryonic day 10.5. This shows that LKB1 is essential for embryonic development, probably due to the interaction between LKB1 and Wnt signalling (Ossipova et al. 2003), and its role in axon development (Kishi et al. 2005). However, mice presenting only a monoallelic loss of Lkb1 develop polyps that mirror the human conditions (Jansen et al. 2009). This confirms the observation made in patients with PJS: loss of only one allele of LKB1 is pathogenic.

The mechanism of polyps formation has been recently discovered using a conditional knock-down *in vivo* model (Katajisto et al. 2008). Loss of one or two alleles of *Lkb1* restricted to the mesenchymal compartment in the intestines is sufficient to induce polyp formation in mice by disrupting the mesenchymal-epithelial interaction. The authors suggested that the reduced expression of LKB1 at the protein level in the mesenchymal cells resulted in a reduced TGF-β signalling to the epithelial cells. It hereby created an increased proliferation of the gut epithelium, and led to the formation of polyps. This hypothesis was confirmed by IHC in polyps from patients with PJS.

1.2.1.4 *LKB1*, a tumour suppressor gene

Functionally, *LKB1* regulates processes that are targeted in cancer cells, such as: cell polarity, cell proliferation in response to environmental stimuli, anoikis and apoptosis. Cell migration is a polarised phenomenon that demands cytoskeletal reorganisation; loss of *LKB1* was demonstrated to promote cell migration and metastasis (Carretero et al. 2010, Taliaferro-Smith et al. 2009). The disruption of cell polarity also leads to abnormal and chaotic tissue structure and can also impair asymmetric division, which is important in the regulation of stem cell differentiation or renewal during its cell division. *LKB1* loss may promote cell de-differentiation (linking it to the cancer stem cell model). Moreover, the de-connection of cell growth and environmental conditions (e.g. sustained growth in nutrient and energy deprived condition) is a hallmark of cancer cells; LKB1 regulates key proteins implicated in this mechanism, such as AMPK. LKB1 has also been linked to angiogenesis (Londesborough et al. 2008), essential for tumour maintenance. It interacts with pathways that have been associated with cancer development, for example the p53, Wnt-β-catenin or Akt-mTOR pathways (section 1.2.1.2). Taken together, this underlines that *LKB1* is relevant to cancer biology.

As patients with PJS have a significantly increased risk of developing cancer, *LKB1* was described as a tumour suppressor gene. The *Lkb1*$^{+/-}$ mouse model, developed to study PJS, was also reported to spontaneously develop tumours, mainly hepatocellular carcinoma late in age (Nakau et al. 2002), confirming this status.

Tumour suppressor gene versus haploinsufficiency

According to Knudson's "two-hits" hypothesis (Knudson, Jr. 1971), identified from the study of *RB*, some genes are negative regulators of cell growth; tumour formation is promoted upon their loss. They are defined as tumour suppressor genes. To inactivate these genes, two genetic (or epigenetic) abnormalities are necessary; only the loss of function of both of the alleles can achieve a tumourigenic effect. Classically, these abnormalities are a mutation (germline or somatic), a loss of a chromosomal region (microdeletions as well as whole loci), a mitotic recombination of the genomic region containing the lost or mutated allele or gene silencing by methylation (Presneau et al. 2003). However this notion is now questioned by the scientific community, as haploinsufficiency or loss of only one of the two alleles of certain tumour suppressor genes has

appeared to be sufficient to promote tumours (Payne et al. 2005, Shannon et al. 2008, Smilenov 2006). Change in gene dosage (and therefore protein dosage) may be sufficient to mediate an effect.

LKB1, a haploinsufficient gene?

Although LKB1 has been considered a tumour suppressor gene, its haploinsufficient effect is now under investigation. The loss of one allele of *LKB1* is sufficient to be pathogenic: Peutz-Jeghers patients can have only one allele mutated and mice with only one allele deleted develop polyps. In some cases, biallelic loss of *LKB1*, with LOH reported in addition to the mutation characteristic of the syndrome, was noted; it occurred in a minority of human polyps (typically in 25 to 40%) (Katajisto et al. 2007). Other studies in *Lkb1*$^{+/-}$ mice (Jishage et al. 2002, Miyoshi et al. 2002, Rossi et al. 2002), and in humans (Hernan et al. 2004) detected a functional *LKB1* allele in the polyps.

Concerning the development of cancer from the benign PJS polyps, it has been suggested by different studies that the loss of the second allele of *LKB1* in patients was a necessary step for cancer development. Several studies demonstrated that tumours arising on the background of PJS displayed a biallelic loss of *LKB1* in humans (Connolly et al. 2000, Entius et al. 2001, Sato et al. 2001, Wang et al. 1999b), in accordance with the "two-hits" hypothesis. The hepatocellular carcinomas that develop spontaneously in *Lkb1*$^{+/-}$ mice also display a loss of both alleles of *Lkb1* (Nakau et al. 2002).

However, this last finding was challenged by the recent results obtained by the same group showing that heterozygous loss of *Lkb1* coupled with activation of β-catenin led to tumour development in mice (Miyoshi et al. 2009). Monoallelic loss of *Lkb1*, when in cooperation with *Kras* mutation, was sufficient to induce spontaneous lung cancer and even metastasis in transgenic mice (Ji et al. 2007). The same genetic background mediated a tumourigenic effect in an *in vivo* transgenic model of pancreatic cancer (Morton et al. 2010). Overall, heterozygous loss of *LKB1* appears sufficient to promote tumourigenesis, at least when combined with another event; this begs the question of whether *LKB1* has a haploinsufficient role in other cancers. In any case, LKB1 plays a role in cancer and especially lung cancer, as will be developed in the next paragraphs.

Role in lung cancer

LKB1 function in lung cancer has been well studied after the discovery of its involvement in this disease by the group of Pr Sanchez-Cespedes. Loss of the chromosomal arm 19p, and in particular the 19p13 region, where *LKB1* locus is, was frequently reported in lung cancer; about 80% of non small cell lung carcinoma (NSCLC) and 30% of small cell lung carcinoma have this abnormality (Rodriguez-Nieto et al. 2009). *LKB1* was then associated with this locus (Sanchez-Cespedes et al. 2002) and mutation screening confirmed that the gene was biallelically lost. The mutations were mostly observed in NSCLC, but to a lesser extent than the chromosomal loss. Absence of LKB1 was confirmed at the protein level by immunohistochemistry in 159 patients (Conde et al. 2007). Biallelic loss of *LKB1* was also noted in human lung cancer cell lines. Therefore, in human sporadic lung cancer, the "two-hits" hypothesis is validated. However, there is no in depth report on the significance of the loss of only one allele of *LKB1,* without mutation. A haploinsufficient role of *LKB1* in those tumours cannot be excluded.

The mutations observed in lung carcinoma were very varied: intronic deletions, mutations in splicing sites, and exonic mutations in exons 1 to 8, leading to the generation of a non functional (mutation in the kinase domain) or truncated protein, were all observed. Most of the mutations observed in PJS were also detected in lung carcinoma (Sanchez-Cespedes 2007). Promoter methylation was rarely observed (Sanchez-Cespedes et al. 2002). Interestingly, *LKB1* loss was associated to the NSCLC type, and especially to adenocarcinoma (Fernandez et al. 2004), smoking, absence of *EGFR* mutations(Rodriguez-Nieto et al. 2009), and non-Asian background (Gao et al. 2010). Finally, the loss of LKB1 seems to be an early genetic event as shown by the screening of lung adenomatous hyperplasia compared to lung adenocarcinoma (Ghaffar et al. 2003).

Further studies have associated LKB1 with higher metastatic potential in this cancer, especially when cooperating with *Kras* mutations (Carretero et al. 2010). Other studies confirmed this by showing its role in epithelial to mesenchymal transition in lung cancer (Roy et al. 2010, Upadhyay et al. 2006). Finally, targeting of the mTOR pathway in patients lacking *LKB1* have been suggested as a potential treatment (Mahoney et al. 2009).

Role in other cancers

Surprisingly, LKB1 was reported to be lost in a low number of sporadic tumours in the past. Even though patients with PJS develop pancreatic, intestinal and breast cancer, there are few reports of somatic mutations in these cancers (Sanchez-Cespedes 2007). This may be accounted for by the technique used for mutation screening; large deletions have been reported in Peutz-Jeghers and lung cancer patients, including intronic deletions, which may not be detected by simple exonic mutation screening. They may also be masked by contamination from normal tissue. Another explanation is that *LKB1* can have a haploinsufficient function in other tumours, and additional findings may have been overlooked if only one "hit" had been detected. In the latest release of COSMIC, from the Cancer Genome Project, 229 samples are reported to carry mutations in *LKB1* out of 4209 (5.4%) samples. The tumours mutated included not only lung, but also cervix, intestine, prostate and skin cancers. In other studies of sporadic cancer, some cases with mutations of *LKB1* were reported in head and neck cancer (Kenanli et al. 2010, Qiu et al. 2006), cervical cancer (Wingo et al. 2009) hepatocellular carcinoma (Kim et al. 2004) and gastric tumours (Park et al. 1998), with promoter methylation in colorectal cancer (Trojan et al. 2000) and with loss of *LKB1* chromosomal region or LOH in colorectal (Forster et al. 2000, Trojan et al. 2000), ovarian (Wang et al. 1999a) sex cord-stromal (Kato et al. 2004), breast (Forster et al. 2000, Yang et al. 2004) and brain cancers (Sobottka et al. 2000). In these cases, *LKB1* loss of function was not necessarily a homozygous event, and the effect of this loss was not always investigated. LOH in the 19p13.3 locus is the most commonly reported event and usually affects a high proportion of the cases scrutinised. Loss at the protein level was found in a subset of pancreatic and biliary tumours (Sahin et al. 2003). This suggests that the role of LKB1 in other cancers was underestimated as its potential haploinsufficient effect was unknown.

The development of conditional mouse models has helped to understand the role of *LKB1* in tumourigenesis, by mimicking the onset of disease observed in patients with PJS in a specific tissue. There are very few reports on natural cancer occurrence in *Lkb1*$^{+/-}$ mice, which is unexpected when compared to the data obtained with conditional models. Patients with PJS develop cancer late in life, whereas the polyps appear in adolescence. It is possible that the *Lkb1*$^{+/-}$ mice studied for polyp formation were not kept long enough or died of complications from the polyps before the onset of these cancers.

As such, a mouse strain with *Lkb1* loss restricted to the mammary gland developed grade 2 ductal or solid papillary breast carcinoma that recapitulated the features of PJS breast cancers (McCarthy et al. 2009). Only a few mice with monoallelic loss of *Lkb1* developed tumours after 120 weeks, but nearly 30% with homozygous loss of *Lkb1* died of the disease within 80 weeks. Also, the cancer developed late in the lifetime of the mice, after 10 months of age. Overall, this demonstrated a role for *LKB1* in breast cancer. This finding was confirmed in an immunohistochemical study of sporadic human breast carcinoma (Fenton et al. 2006).

Similar findings were reported in gynaecological cancer, with female *Lkb1*$^{+/-}$ mice that survived past 40 weeks of age spontaneously developing endometrial carcinomas (Contreras et al. 2008). This confirmed the hypothesis that the *Lkb1*$^{+/-}$ mice develop cancer late in life, making it difficult to study. It was not reported whether a second allele of *LKB1* was spontaneously lost in these tumours or if another genetic event occurred, but a conditional mouse model lacking both *LKB1* alleles in the uterus was established to confirm the results. Loss of *LKB1* protein was also detected in a cohort of 190 human cases and associated with increased invasiveness, in common with the findings in lung cancer (see paragraph above).

To conclude, in light of the possible haploinsufficient effect of *LKB1* in tumourigenesis, there is evidence that this gene plays a role in gynaecological, pancreatic, intestinal, breast and liver cancers, in addition to lung cancer.

1.2.1.5 Regulation of LKB1

The regulation of LKB1 activity is a complex process. Although essential, it is not restricted to its binding to Strad and MO25. The study of LKB1 function revealed that its effect was cell type dependant and controlled by environmental stimuli (section 1.2.1.2). As little is known on the downstream kinases of LKB1, the regulation of LKB1 has been mainly investigated by the study of its interaction with other molecules in its environment. A scheme summarising the main results found is presented in Figure 1.10.

Activation of LKB1: role of the complex

Mutations on *LKB1* in complex binding sites in cancer (Boudeau et al. 2004) or PJS (Zeqiraj et al. 2009a) underline the importance of the complex formation in LKB1 regulation. Yet little is known on how the formation of the complex is regulated.

One hypothesis involves the chaperones Hsp90 and Cdc37 (cell division cycle 37 homolog, on chr 19p13.2). As the LKB1 complex does not spontaneously arise when mixing the three proteins (Boudeau et al. 2003a), the two chaperones may be required to facilitate its assembly. Moreover, Hsp90 can stabilise LKB1: use of Hsp90 inhibitors like geldanamycin (section 1.1.6.1) induced degradation of LKB1 by the proteosome (Boudeau et al. 2003b). Mutations in *LKB1* in its binding domain to Hsp90 and Cdc37 were reported in testicular cancer, underlying the importance of these chaperones in *LKB1* regulation (Nony et al. 2003). This also uncovers a major unwanted effect of treatment of cancer and patients with OS with geldanamycin (section 1.1.6.1), as it may promote tumour growth by downregulation of *LKB1*.

No other protein interacting with the complex has been identified, and complex formation is not dependent on phosphorylation of any of the three kinases, as discussed in paragraph 1.2.1.1. The regulation of LKB1 complex activity could also be achieved by regulation of Strad and MO25 protein expression.

Report on miRNA 451 regulating MO25

There has not been any report of regulation of LKB1 expression by miRNA. However, a recent report has shown that miR451 can repress MO25α and hence regulate the LKB1-AMPK pathway and glioma cells response to metabolic stress, by shifting the equilibrium between cell proliferation and cell migration (Godlewski et al. 2010b, Godlewski et al. 2010a). miR-451 overexpression reduces the availability of the active LKB1 complex but does not influence the level of expression of LKB1 itself. This finding uncovers another possible mechanism of regulation of LKB1 activity, through the regulation of the expression of the proteins of the complex by miRNA. More studies are needed to establish whether this mechanism is present in other cell types and other diseases.

Regulation of LKB1 through the regulation of its localisation

Although playing its role in the cytoplasm, LKB1 is only present there when bound to its complex, as developed in section 1.2.1.1. But the regulation of this transport has not been fully deciphered. For example, the role of Stradβ that cannot transport the LKB1 to the cytoplasm but still activates it is unknown. The mechanism needs to be better understood to fully comprehend the regulation of LKB1. It may offer the cell another way to control the action of LKB1 complex, even though it is constitutively activated.

One hypothesis is that LKB1 re-localisation to the cytoplasm is controlled by its phosphorylation by PKC-ζ (or protein kinase C zeta, PRKCZ, on chr 1q36.33) on Ser^{307}, a novel phosphorylation site recently discovered (Xie et al. 2009). As AMPK stimulates PKC-ζ activity, this mechanism uncovers a feedback between LKB1 and its activating kinases. Ser^{428} could also potentially regulate cellular localisation after its phosphorylation by PKC-ζ, PKA (cAMP-dependent protein kinase) or RSK (90kDa ribosomal protein S6 kinase)(Song 2008). However, the isoform $LKB1_s$ which lacks this site, is also localised in the cytoplasm (Denison et al. 2009, Towler et al. 2008). Therefore these mechanisms could be used as a tuning of LKB1 availability but they are not essential for its function.

STK11IP (serine/threonine kinase 11 interacting protein or LIP1 on chr 2q35) is reported to play a role in LKB1 localisation, but neither its function nor its mechanism of interaction with the LKB1-Stra-MO25 complex are understood (Smith et al. 2001). Co-expression of the two proteins

increased the amount of LKB1 in the cytoplasm, and the two proteins may play a role in TGF-β signalling via binding of both STK11IP and LKB1 to SMAD4. Another study has suggested that SKT11IP was mainly localised in the cytoplasm, due to a localisation signal in its N-terminal domains, and that it could block NF-kappa B activation (Liu et al. 2003).

The control of LKB1 activity by regulation of its localisation is not restricted to the mechanism of transport of the complex to the cytoplasm. LKB1 is also reported to co-localise with E-cadherins at adherens junctions, where it can mediate its action on cell polarisation (Sebbagh et al. 2009). LKB1 kinase activity was shown not to be dependent on E-cadherins; this suggests instead that E-cadherins control LKB1 function by enabling its recruitment to the cell membrane. There may be other proteins promoting the co-localisation of LKB1 with its downstream kinases to regulate the action of LKB1. Similarly, the Cys^{430} AA on LKB1 is thought to enable its integration to the plasma membrane, and could hereby regulate LKB1 function in cell polarity, but its role in mammals has not been investigated (Alessi et al. 2006).

Role of phosphorylation: another layer in LKB1 regulation

Although phosphorylation usually promotes kinase activity by allosteric change, LKB1 phosphorylation sites could regulate it at a different level. First they may play a role in the transport of LKB1 complex to the cytoplasm as mentioned above. Mutation of Ser^{428} enabled cell growth for the G361 cell line in soft agarose so the site may participate to the control of cell growth in response to environmental stimuli, such as anchorage-free conditions (Alessi et al. 2006). The phosphorylation the same site by brain derived neurotrophic factor (e.g. BDNF) has also been shown in one study, and promoted axon formation (Winckler 2007), linking phosphorylation and control of cell polarisation. Additionally, ATM (ataxia telangiectasia mutated on chr 11q22.3) can bind to LKB1 after DNA damage (Fernandes et al. 2005) and phosphorylate it on Thr^{363}, although the function of this mechanism is not fully investigated (Alessi et al. 2006). LKB1 could also be regulated by phosphorylation by ERK, enabling interaction between the mTOR and RAF-MEK-ERK pathways; therefore B-RAF activation by the mutation V600E plays its oncogenic role partly by inhibiting LKB1 action on AMPK and the mTOR pathway (Esteve-Puig et al. 2009, Zheng et al. 2009). To conclude, the various phosphorylation sites of LKB1 may present an opportunity for the cell to tune the protein action and direct it towards specific downstream kinases, enabling the integration of environmental stimuli.

The ability of the complex to autophosphorylate has also been noted, but its role is not fully explained. Since the sites are phosphorylated upon LKB1 binding to Strad, it indicates a potential feedback function in the regulation of complex formation or in cellular localisation. However mutating these residues has no direct effect on complex formation or re-localisation.

SIRT protein family

Recently, a new mechanism of regulation of LKB1 expression by SIRT1 (sirtuin 1 or silent mating type information regulation 2 homolog 1, on chr 10q21.3) has been reported. SIRT1 is a class III NAD-dependent deacetylase enzyme. It regulates histone H3 or other protein acetylation status in correlation with the cells NAD^+/NADH ratio, e.g. with the cells metabolic status. SIRT1 is therefore a metabolic sensor, as is AMPK whose activity depends on the AMP/ATP ratio. Interaction between SIRT1 and LKB1, which is upstream of AMPK, is therefore not surprising.

One study showed that the activation of SIRT1 using polyphenols lead to the increase of LKB1 phosphorylation at Ser^{428}, whose role is mentioned in the paragraphs above, and AMPK activity (Hou et al. 2008). The authors infer that SIRT1 plays a role in LKB1/AMPK pathway and that it can regulate hepatocyte lipid metabolism. The actual mechanism of this regulation was uncovered in another study which showed that LKB1 was acetylated on various lysine residues, Lys^{48} seemingly being the most important one as assessed by point mutation (Lan et al. 2008). Overexpression of SIRT1 in the 293T cell line induced LKB1 deacetylation, and conversely SIRT1 downregulation by shRNA promoted LKB1 acetylation. The deacetylation of LKB1 increased its binding to Strad, its transport to the nucleus and its activation of downstream kinases such as AMPK.

However, this interpretation is not shared by other groups. On the contrary, Zu et al have shown that knocking down SIRT1 in endothelial cells promoted LKB1 expression and phosphorylation on Ser^{428}, and AMPK activity. SIRT1 overexpression led to the deacetylation of LKB1 in primary porcine aortic endothelial cells, as was also shown in Lan et al. But in this case, it led to its ubiquitination and degradation by the proteosome. SIRT1 was shown to be a negative regulator of AMPK activation in this study. Zu et al studied a model of senescence using porcine endothelial cells whereas Lan et al and Hou et al used a human embryonic kidney and a human hepatocyte cell line in normal growth conditions. Downregulation of LKB1 did not induce senescence in those two cell

lines as it did in the endothelial model (Zu et al. 2010). LKB1 regulation mechanism via SIRT1 may be cell type and context dependent.

Additionally, there seems to be either a feedback loop or an interaction between AMPK and SIRT1: AMPK activation could lead to SIRT1 activation. It was demonstrated that treatment with an AMPK activator in glucose deprivation conditions inhibited myoblast differentiation and changed the NAD^+/NADH ratio (Fulco et al. 2008). This effect was not observed in *Sirt1*$^{+/-}$ mouse myoblasts or human myoblasts with *SIRT1* knock-down by shRNA. Hence, AMPK can also regulate SIRT1 activity. Further proof was given as the activation of AMPK in myocytes and mouse fibroblasts disturbed the NAD^+/NADH ratio leading to SIRT1 activation, which then deacetylated its target PGC1-α (Canto et al. 2009). A recent report argues that activation of SIRT1 and AMPK could be promoted by FGF21 activation in adipocytes (Chau et al. 2010). This suggests that regulation of the SIRT1-LKB1-AMPK pathway may also be mediated by TKR and not only by changes in AMP/ATP or NAD^+/NADH ratios.

SIRT1 is part of a family of seven members. Hence other proteins of this family could also change LKB1 acetylation status. SIRT3, and not SIRT1, has been reported to activate LKB1 by deacetylation in a mouse model of cardiomyocytes, in hypertrophic conditions (Pillai et al. 2010). Another study has shown that treatment of glomerular epithelial cells with resveratrol prevented LKB1 acetylation, thereby restoring its activity; this mechanism was independent of SIRT1 (Lee et al. 2010b). The same treatment applied to hepatocytes (Shin et al. 2009) and neurons (Dasgupta et al. 2007), also found to be independent from SIRT1 activation. This confirms that LKB1 acetylation may not depend solely on SIRT1.

In conclusion, SIRT1 can regulate LKB1 acetylation status and this will influence protein stability and function. However this may lead to both an increased or decreased protein activity, in different models. This is probably part of a more complex mechanism which coordinates AMPK and SIRT1 activation in response to the energy status of the cells. Therefore there may be another player whose role is to be discovered and whose action may depend on cell type or environmental factors. Cancer cells often develop the ability to disconnect their growth from energy sensors. The alteration of the cooperation between SIRT1 and AMPK in cancer cells has not been investigated. As SIRT1 function is related to differentiation (Fulco et al. 2008), a process also altered in cancer cells, the role of SIRT1 may differ in cancer cells compared to normal differentiated cells. This is emphasized by the finding that SIRT1 inhibition in stem cells can alter both AMPK and telomerase

activity, which is also disrupted in cancer (Narala et al. 2008). Further studies on the role of SIRT1 in LKB1 regulation in the context of cancer cells are required as the evidence to date is not fully conclusive.

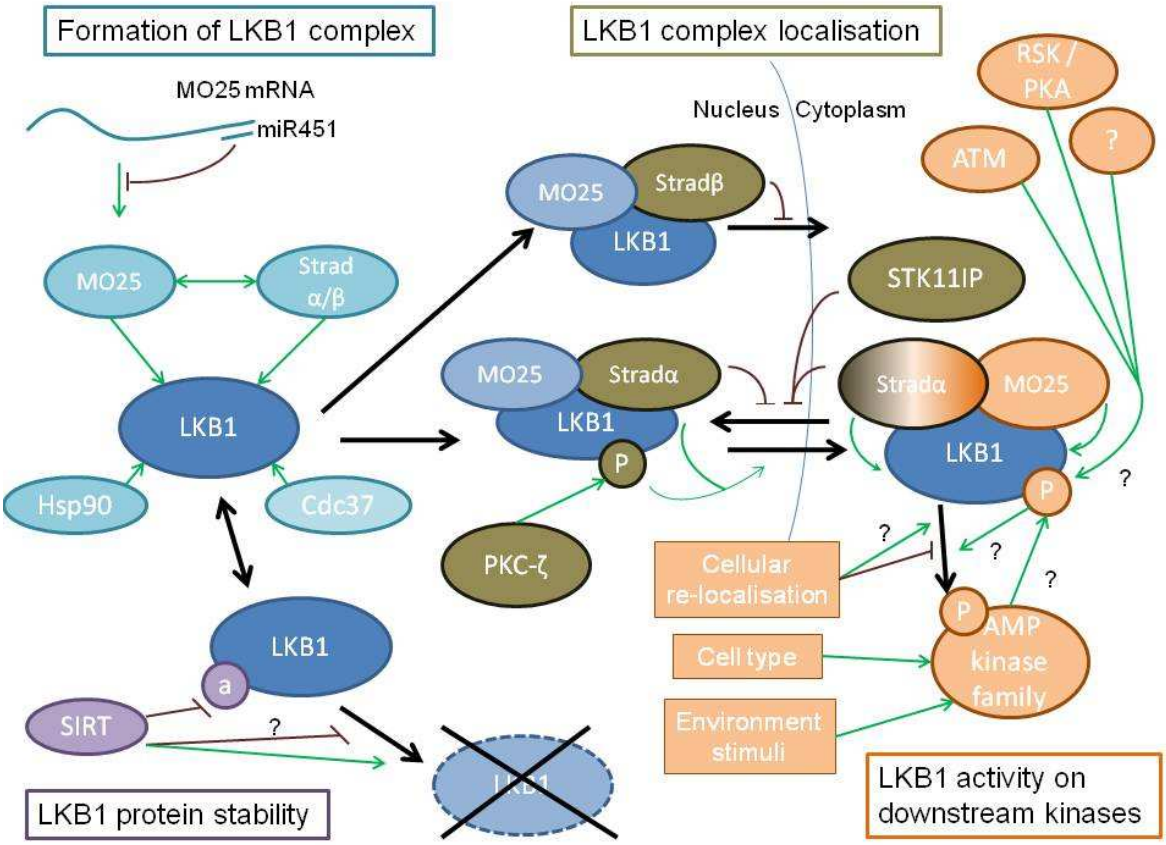


Figure 1.10: Scheme of LKB1 regulation mechanisms.
Plain black arrows represent the different equilibriums of LKB1. Green arrows show a positive regulation and red straight connectors a negative regulation or inhibition. Each molecule's colour illustrates the mechanism in which they participate. Question marks illustrate processes that are not fully understood. 'P' represents phosphorylation, and 'a' acetylation of the target protein.

1.2.1.6 Conclusion

LKB1 is an essential and yet not fully characterised serine/threonine kinase involved in many key cellular pathways, related to cell metabolism, polarity, migration, apoptosis and differentiation. Most of the knowledge on this kinase is very recent, with some aspects, like the role of some of its downstream effectors and their regulation, still undiscovered. LKB1 is constitutively activated once in complex with Strad and MO25, and may be regulated further by localisation, phosphorylation, acetylation and protein interactions. This has uncovered novel regulation mechanisms for kinases, beyond a simple activation by phosphorylation as observed for most of them. Understanding this mechanism better will help to comprehend the role of LKB1 with regards to its multiple downstream targets. LKB1 function is cell type and context dependent, its action being coordinated by environmental stimuli. Its role in sporadic cancer and especially lung cancer has only been identified recently, although its tumour suppressor gene status has been long known thanks to its association with PJS. Whether *LKB1* can have a haploinsufficient effect needs to be investigated further. It would therefore be interesting to assess its role in sarcomas and OS in particular.

1.2.2 The mTOR pathway

One of the main pathways downstream of LKB1 is the mTOR pathway (Figure 1.9), whose role in cancer has been intensively studied as it is a promising target for specific drug therapy.

1.2.2.1 Pathway activation

The mTOR pathway is involved in cell growth and proliferation via the regulation of mRNA translation initiation. It is regulated both by the PI3K/Akt and LKB1 pathways. Their regulation and activation in normal conditions is described here and a schematic diagram is shown in Figure 1.11.

Activation of the cascade by TKR

The PI3K/Akt/mTOR pathway activity is initiated by the binding of a growth factor to its TKR, such as IGF to IGFR; IGFR becomes hereby active and autophosphorylates. It then phosphorylates the insulin receptor substrate (IRS), which activates PI3K, or phosphatidylinositol 3-kinase. Other TKR and their corresponding adaptors can play the same role and their expression is cell type dependent.

PI3K and PTEN

PI3K is a heterodimeric protein composed of a regulatory subunit, p85, and a catalytic subunit, p110. IRS can recruit and interact with p85 to ensure that it phosphorylates phosphatidylinositol-3,4-bis-phosphate (e.g. PIP2) into phosphatidylinositol-3,4,5-bis-phosphate (PIP3) (Franke et al. 1997). The equilibrium between PIP2 and PIP3 is regulated further by PTEN (phosphatase and tensin homolog on chr 10q23.31) which can dephosphorylate PIP3 back into PIP2 to counter PI3K signalling (Radu et al. 2003).

Akt

PIP3 then recruits Akt (v-akt murine thymoma viral oncogene homolog 1, also referred to as protein kinase B, PKB, on chr 14q32.33) and PDK1 (3-phosphoinositide-dependent protein kinase 1) to the plasma membrane in order for PDK1 to partially activate Akt by phosphorylation of Thr^{308} (Stephens et al. 1998). For complete activation, Akt needs to be phosphorylated on Ser^{473} (Toker et al. 2000), which is accomplished by the complex mTORC2 (Sarbassov et al. 2004, Sarbassov et al. 2005)(see below). The main role of Akt is the activation of the mTOR pathway, but it is not restricted to this. Akt can phosphorylate BAD (BCL2-associated agonist of cell death, on chr 11q13.1) for apoptosis regulation, GSK3β (glycogen synthase kinase 3 beta on chr 3q13.33) for cell metabolism or the transcription factor family FOXO for cell proliferation (Downward 1998, Tran et al. 2003).

TSC complex

Upon its activation, Akt can regulate the mTOR pathway via the TSC complex (Inoki et al. 2002). Tuberous sclerosis complex 1 (TSC1), or hamartin, and tuberous sclerosis complex 2 (TSC2), or tuberin, respectively on chr 9q34 and 16p13.3, are bound in a heterodimer complex and normally inhibit the activity of Ras homologue enriched in brain (Rheb, on chr 7q36.1), a small GTPase. TSC1 stabilises the protein complex whereas TSC2 is its active kinase part. The TSC complex converts Rheb from its GTP form, which is necessary for mTOR activation, to its GDP form, which is unable to activate mTOR (Tee et al. 2003). Upon phosphorylation by Akt on Thr^{1462}, TSC2 activity is inhibited and this results in activation of the mTOR pathway (Tee et al. 2002). TSC2 phosphorylation may accelerate its ubiquitination and hence its degradation (Plas et al. 2003). However, nutrient starvation leads to the phosphorylation of AMPK by LKB1, which in turn can activate the TSC complex via the phosphorylation of Thr^{1227} and Ser^{1345} (Inoki et al. 2003). This enables the cell to inhibit the mTOR signalling pathway and hence cell proliferation in stress conditions. The TSC complex is therefore a key regulator of the pathway activity as it can either be activated by LKB1 signalling or inhibited by Akt signalling, depending on environmental conditions.

mTOR complex

mTOR, the mechanistic target of Rapamycin (chr 1p36.22), also called FRAP, is a 289 kDa serine/threonine kinase part of either the mTORC1 or mTORC2 complexes. It is the active part of the complexes, which are composed of mLST8 (MTOR associated protein, LST8 homolog or G protein beta subunit-like, on chr 16p13.3) and either raptor (regulatory associated protein of MTOR complex 1, on chr 17q25.3) for complex 1 (Kim et al. 2002a) or rictor (RPTOR independent companion of MTOR, complex 2 on chr 5p13.1) for complex 2 (Sarbassov et al. 2004). Both complexes have different functions; mTORC1 is Rapamycin sensitive and promotes cell growth when activated by Rheb, whereas mTORC2 (also called PDK2) is not Rapamycin sensitive and can regulate the signalling pathway through a feedback loop that activates Akt. However, the role of mTORC2 beyond this is still poorly understood and more research on this complex is needed. mTORC1 and 2 are thought to be in equilibrium, hence regulating the whole pathway. AMPK has also been reported to be able to inactivate mTORC1 directly by phosphorylation of Raptor. When active, mTORC1 can be phosphorylated on Ser^{2448} by S6K as a feedback regulation (Chiang et al. 2005).

mTOR main effectors: 4EBP1 and S6K

mTORC1, when active, phosphorylates S6K (ribosomal protein S6 kinase, 70kDa, polypeptide 1 or RPS6KB1 on chr 17q23.1) on multiple sites, the most important being Thr^{289}. When activated, p-S6K phosphorylates multiple targets to stimulate mRNA translation, but mainly RPS6 (40S ribosomal protein S6 on chr 9p22.1). The activation of RPS6 by phosphorylation on several sites, including Ser^{235} and Ser^{236} (Ferrari et al. 1991), leads to its recruitment; it enhances top-dependent mRNA translation, coding for ribosomal proteins, elongation factors or growth factors such as IGF (Wan et al. 2007). mTORC1 also phosphorylates 4EBP1 on several sites including Thr^{70}. 4EBP1 is a small molecule that binds to the eukaryotic translation initiation factor eIF-4E; its phosphorylation induces release of eIF-4E which then drives the translation of cap-dependent mRNA, such as cyclins, MYC or VEGF (Holz et al. 2005).

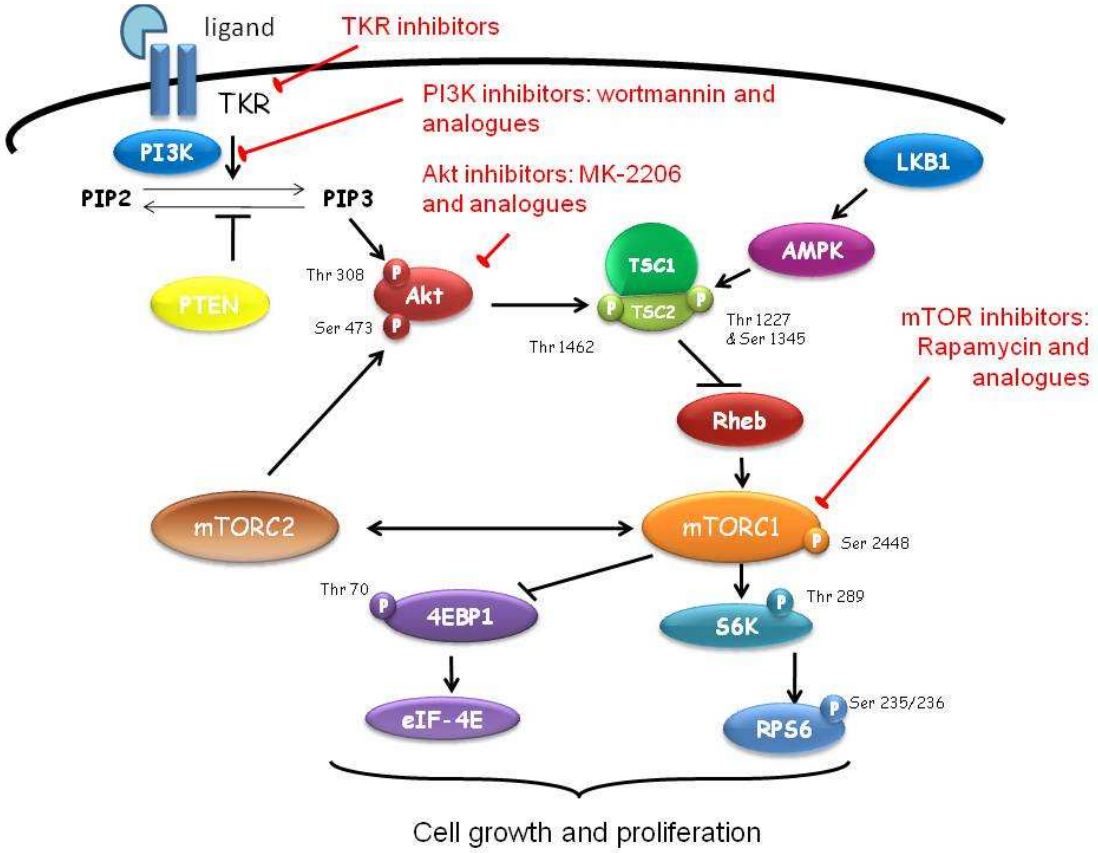


Figure 1.11: Scheme of the mTOR pathway.
The arrows indicate a positive and the straight link a negative regulation. Known inhibitors that could be used for therapeutic treatments are indicated in red.

1.2.2.2 Role of the mTOR pathway in cancer

The mTOR pathway as a key regulator of cancer cell proliferation

The mTOR pathway is a key regulator of cell growth and is controlled by several feedback loops and interaction between pathways; for instance, *LKB1* ensures limitation of pathway activation (and hence cell growth) in nutrient deprivation conditions. Cancer cells have the ability to disconnect their proliferation signals from environmental stimuli; the deregulation of the mTOR pathway in tumours is then not surprising. It leads to uncontrolled cell proliferation and survival of the tumour cells. Additionally, as 4EBP1 controls the translation of the mRNA of targets such as VEGF, cyclins and MYC; then, activation of this pathway indirectly promotes angiogenesis and deregulation of cell cycle entry. Finally, as mTORC2 activates Akt, which can - beyond TSC2 - phosphorylate a wide range of targets (some being associated with control of apoptosis), constitutive activation of mTOR may lead to resistance to apoptosis of tumour cells.

mTOR constitutive activation in cancer

The mTOR pathway is often constitutively activated in cancer following genetic abnormalities, such as the constitutive activation of TKR by mutation, amplification of those TKR, loss of PTEN or mutations in the TSC complex. All these events are frequently reported in cancer or are characteristic of syndromes associated with an increased cancer risk, such as Cowden's syndrome for PTEN, and tuberous sclerosis for TSC1 and 2 (Wan et al. 2007). Genetic events and deregulation of pathways that indirectly regulate the mTOR pathway, such as LKB1 loss (Shaw 2009), Ras activating mutations (Roux et al. 2004) or Wnt pathway deregulation (Menon et al. 2008) can also lead to constitutive activation of this signalling cascade. In that case, no obvious genetic event would be detected within molecules of the pathway.

The mTOR pathway in different cancers

Among others, the mTOR pathway has been reported to play a role in ovarian cancer (Altomare et al. 2004), lung adenocarcinoma, breast cancer (Menon et al. 2008), glioblastoma (Riemenschneider et al. 2006), and sarcomas, including peripheral nerve sheath tumour, schwannoma, rhabdomyosarcoma and synovial sarcoma (Dobashi et al. 2009). Our group reported

that about 65% of a cohort of 50 chordomas had the PI3K/Akt/mTOR pathway activated and would therefore be responsive to inhibitors of this pathway (Presneau et al. 2009). Its role in OS will be discussed in paragraph 1.2.3.2.

1.2.2.3 The mTOR pathway as a target for therapy

mTORC1 complex is sensitive to the antifungal agent Rapamycin, as mentioned in paragraph 1.2.2.1. Rapamycin in mammalian cells binds to the FK506 binding protein 1A 12kDa (FKB1A). This leads to an increased ability of the complex formed to inhibit mTORC1, but not mTORC2 (Mita et al. 2003). The complex is thought to function as an allosteric inhibitor of the mTOC1 kinase activity and once attached, the interaction of mTORC1 and Rapamycin appears irreversible (Chiang et al. 2007). This blocks the phosphorylation of mTORC1 effectors, S6K and 4EBP1. Several analogues have been developed by pharmaceutical companies to enhance its solubility and its stability: CCI-779 or tensirolimus by Wyeth, AP23573 or ridaforolimus by Ariad and RAD001 or everolimus by Novartis. Novartis have also developed another compound, NVP-BEZ235, which is active on both PI3K and mTORC1. The inhibitors have been tested in preclinical studies and in phase I /II trials on a wide range of cancers, including renal, breast, haematological and sarcomas. They are well tolerated and induced a stable disease or a partial response in a subset of cases (MacKenzie et al. 2007, Wan et al. 2007). The relevance of these inhibitors to OS is detailed in paragraph 1.2.3.2.

Another approach to target the mTOR pathway would be to use TKR inhibitors, PI3K inhibitors such as wortmannin or LY294002, and Akt inhibitors such as MK-2206. However, those inhibitors act upstream in the signalling cascade, and they would affect many other pathways. Their effect would therefore be less specific than an mTOR inhibitor. Also, due to the interaction of other pathways with mTOR signalling, tumour cells could bypass the repression of the pathway by these inhibitors.

1.2.2.4 Conclusion

As the mTOR pathway provides relevant targets for cancer therapy, its role in OS has been investigated, and this is described in the next paragraph.

1.2.3 LKB1 and the mTOR pathway in Osteosarcoma

1.2.3.1 Ezrin and osteosarcoma

Ezrin (cytovillin or villin2 on chr 6q25.3) is a linker protein between the plasma membrane and the actin cytoskeleton of the ERM protein family (ezrin/radixin/moesin) and it plays a role in cell surface functions. It is present in an inactive closed conformation in the cytoplasm; upon its phosphorylation, the molecule becomes active and changes to an open conformation. It relocates to the cell membrane and links itself to the actin cytoskeleton. This physical connection may influence membrane extension, motility, invasion and adherence processes. It also facilitates signal transduction from growth factor receptors and adhesion molecules, thereby integrating signals from the extracellular matrix or from cell to cell contacts to promote growth. Hence it plays a role in the signalling of MEK/ERK, Akt and MAPK pathways (Hunter 2004). To perform its function, ezrin can form a complex with moesin, Merlin, the protein coded by *NF2*, associated with OS (section 1.1.5.4), and CD44, which is overexpressed in dedifferentiated OS and promotes invasion in OS cell lines (Peng et al. 2002, Peng et al. 2005). This associates ezrin with tumourigenesis, cell migration (Sainio et al. 1997) and metastatis in several cancers (Hunter 2004, Weng et al. 2005).

Ezrin in sarcomas

Confirming its role in cancer and sarcomas in particular, ezrin has been shown to be highly expressed by IHC in Ewing sarcoma, leiomyosarcoma, rhabdomyosarcoma, peripheral nerve sheath tumours, schwannoma and synovial sarcoma compared to normal tissue (Bruce et al. 2007). Ezrin was therefore suggested as a diagnostic tool to differentiate conventional chondrosarcoma from chondroblastic OS and a therapeutic target in dedifferentiated chondrosarcoma (Salas et al. 2009), but a more recent study did not confirm this finding (Machado et al. 2010).

Ezrin detection in human Osteosarcoma and evidence correlating it to metastasis

High expression of ezrin in OS has been mentioned earlier in section 1.1.5. Ezrin has been detected by high throughput screening of human OS, such as in GEM (Leonard et al. 2003) and proteomic analysis (Folio et al. 2009, Li et al. 2010). It was among the top deregulated genes in a GEM comparing metastatic and non metastatic mouse OS cell lines (Khanna et al. 2001). It was found to be necessary for metastasis using this same model *in vivo*, and to act through the Akt and MAPK pathways (Khanna et al. 2004).

The expression of ezrin and its association with metastasis was confirmed in several studies at the protein level by IHC (Boldrini et al. 2010, Kim et al. 2009, Kim et al. 2007, Park et al. 2006, Park et al. 2009, Salas et al. 2007) and at the mRNA level by qRT-PCR (Ogino et al. 2007, Salas et al. 2007, Wang et al. 2010b, Xu-Dong et al. 2009). It is of note that exclusive cytoplasmic expression was associated with better disease free survival chances compared with both cytoplasmic and membranous expression (Ferrari et al. 2008). Nucleic expression of phosphorylated ezrin was reported in OS in one study (Di et al. 2010), uncovering an unknown role of ezrin in OS.

These results imply that ezrin could be a therapeutic target in OS. Its inhibitor, sorafenib, was suggested for therapy in OS as it showed anti-tumour activity *in vitro* and *in vivo* in a pre-clinical study (Pignochino et al. 2009).

Little is known on how ezrin is regulated. One recent study has shown that ezrin was dynamically phosphorylated during metastasis progression, possibly by the protein kinase C family (Ren et al. 2009). Furthermore, ezrin has also been reported recently to regulate metastasis in one OS cell line through the binding of integrin β4, e.g. CD104 (Wan et al. 2009), partially elucidating its role. The mechanism by which it can activate signalling pathways such as mTOR, ERK and MAPK still needs to be deciphered.

Conclusion

Further study on the biological function and regulation of ezrin will help to understand better its role in OS. The screening of large cohorts of patients would enable assessment of its role as a therapeutic target and prognostic factor, as the result of early studies is encouraging.

1.2.3.2 mTOR and Osteosarcoma

Animal models and links to Ezrin

The group of Pr Khanna, which has discovered the importance of ezrin expression in OS, has also demonstrated how this protein may play its role, by linking it to the mTOR pathway. They found that the metastatic *in vivo* murine model used previously to identify ezrin by GEM (Khanna et al. 2001) was sensitive to Rapamycin treatment (Wan et al. 2005). Furthermore, the mTOR pathway was activated in this model, and suppression of ezrin led to a decrease in the phosphorylation of mTOR pathway effectors, S6K and 4E-BP1. Ezrin plays an important role in the signal transduction of TKR, which activates the Akt/mTOR pathway, but the exact mechanism of this link needs to be deciphered further. Furthermore, the role of the mTOR pathway in OS was confirmed in another animal model, as mTOR and S6K were activated in canine OS cell lines (Gordon et al. 2008).

Evidence of constitutive mTOR pathway activation in human Osteosarcoma

Further studies have shown that the mTOR pathway is potentially activated in human OS, as suggested by the animal models. First, several TKR appear to play a role in OS development and are even considered as targets for therapy (section 1.1.6.1); their action can be channeled through the mTOR pathway. Moreover, genetic events have been reported in this pathway, leading to its constitutive activation in tumours; PTEN was reported as lost in OS tumours by SNP array and FISH, with a biallelic deletion in 4 of 27 cases and monoallelic in 9 of 27 (Freeman et al. 2008). In a recent release of COSMIC, 1 of 10 screened OS samples were mutated for TSC1. OS cells have also been suspected to increase the activation of the mTOR pathway in their environment, possibly via growth factors. The use conditioned media from mouse OS cells on osteoblastic cell lines promoted their growth of (Mori et al. 2008). This may increase bone proliferation in the tumour environment, and may also have an endogenous effect on the tumour cells. Finally, a recent publication showed that mTOR and S6K were expressed in some OS cases and correlated this expression with prognosis in 65 patients (Zhou et al. 2009). However, the study failed to assess whether the pathway was activated as the phosphorylated status of the mTOR pathway effectors was not recorded.

Preclinical studies on Osteosarcoma

As many drugs have been designed to target the mTOR pathway (Figure 1.11 and section 1.2.2.3), most publications have focused on the activity of these inhibitors on OS rather than deciphering the mechanism of activation of the pathway in OS. Several preclinical studies have shown the effect of Rapamycin and its analogues on human OS. Rapamycin has been shown to be a potent inhibitor of cell growth in OS cell lines, and reduced the activity of S6K, mTOR and Akt (Gazitt et al. 2009). However, it did not induce apoptosis. A preclinical study in canine OS demonstrated that Rapamycin was well tolerated and induced reduced activity of RPS6 in the tumour and PBMC fraction, an effect that was dose dependent (Paoloni et al. 2010). Screening of Rapamycin action *in vitro* and *in vivo* on a panel of paediatric sarcoma cell lines revealed that 1 of 6 OS cell lines responded to Rapamycin (Houghton et al. 2008). Several preclinical studies on different sarcomas, including OS, have reported the potential of these inhibitors of this pathway in OS treatment. A phase II clinical trial of ARIAD pharmaceutics on sarcoma displayed clinical benefit after treatment with a Rapamycin analogue, AP23573 or ridaforolimus in 15 of 50 bone tumours, including 3 OS (Staddon et al. 2006). The trial showed evidence that patients with bone sarcoma, leiomyosarcoma and liposarcoma had an overall improved survival from 9 to 17 months. A phase III trial is under way to study metastatic sarcomas, including OS, and its results are being analysed. In a subset of the patients recruited in this trial, phosphorylation of RPS6 was a predictive marker of the tumour response to therapy (Iwenofu et al. 2008). The design of this trial can be improved by the optimisation of biomarkers to classify which patients would benefit from the treatment. The use of another inhibitor, NVP-BEZ235 from Novartis, has been assessed *in vivo* and *in vitro* on OS cell lines in combination and in the absence of chemotherapy drugs (Manara et al. 2010). As with other mTOR targets, it inhibited cell cycle progression, migration and adhesion but did not induce apoptosis. Its combination with doxorubicin or IGFR inhibitors yielded better responses.

To conclude, the first studies of the role of mTOR inhibitors are promising but treatment design can be improved with biomarkers and combination therapy.

Conclusion

There is evidence that the mTOR pathway plays a role in OS pathogenesis and, potentially downstream of Ezrin, in the development of metastases. Little is known of the mechanism of activation of the mTOR signalling pathway in OS. Further studies of the genetic events leading to its constitutive activation in OS are warranted. Moreover, the role of the mTOR pathway has not been yet confirmed on a large cohort of human patients. This would enable assessment of whether the therapeutic targets would potentially benefit selected patients.

Preclinical studies have shown the ability of Rapamycin or its analogues to stop cell growth in OS tumours, but not to induce apoptosis. These compounds also only have cytostatic effect in other cancers, leading to G1-cell cycle arrest (Menon et al. 2008). Their effect is enhanced in combination with other traditional agents, including chemotherapy drugs like doxorubicin, or new targets like TKR inhibitors. Using inhibitors that could target both mTORC1 and mTORC2 could also enhance patient response to the drug. Phase III clinical trials, as the one set up for ridaforolimus, are required to assess fully the potential of these inhibitors. Such trials may also identify biomarkers which may stratify patient for treatment.

1.2.3.3 *LKB1* role in Osteosarcoma pathogenesis

In vivo model and LKB1 in osteoblast proliferation

LKB1 heterozygous loss has been associated with osteogenic tumours in a recent publication using an *in vivo* model. Robinson et al have observed that about 2% (6/300) of the cohort of $Lkb1^{+/-}$ mice became paralysed at about 300 days of age (Robinson et al. 2008) due to the development of an osteoblastic tumour in the spinal column (Figure 1.12). Moreover, study of 12 asymptotic mice revealed that abnormal osteoblastic proliferation was also present. The malignancy of the lesion was difficult to assess, but was reported as representing osteoblastosis rather than osteosarcoma. Hence LKB1 may play a role in osteoblastoma or OS. $Lkb1^{+/-}$ mice and $p53^{+/-}$ mice develop OS (Wei et al. 2005), but as $p53^{+/-}$ mice also develop OS (section 1.1.5.4), it was difficult to assess the effect of LKB1 in this process. The abnormalities are observed late in life in the mice so it is possible that they remain undiagnosed in patients. However, one case of osseous metaplasia in 3 of 15 hamartomatous polyps in a patient with PJS was reported in 1995 (Narita et al. 1995), suggesting further that LKB1 is relevant to bone biology.

As described in paragraph 1.1.5.5, OS may arise from osteoblasts that have lacked their differentiation potential. AMPK have been associated with cell differentiation in myoblasts (Fulco et al. 2008)(section 1.2.1.5). A recent article has shown that osteoblast differentiation is associated with a decreased AMPK activity (Kasai et al. 2009) in a mouse osteoblast cell line. In reduced glucose conditions, AMPK phosphorylation was increased and osteoblast differentiation inhibited. The use of an AMPK activator recapitulated this phenotype. Expression of a constitutively active AMPK also inhibited differentiation. Conversely, AMPK phosphorylation decreased during osteoblast differentiation. This supports the hypothesis that LKB1 loss in osteoblasts may induce a deregulation of their differentiation potential and hence promote tumourigenicity.

Further evidence of the role of LKB1 in OS has been demonstrated as expression of LKB1 in OS cell lines induced a TRAIL mediated-apoptosis (Takeda et al. 2007).

Overall, this points toward a pathogenic role of LKB1 in osteoblast proliferation and possibly an involvement in OS.

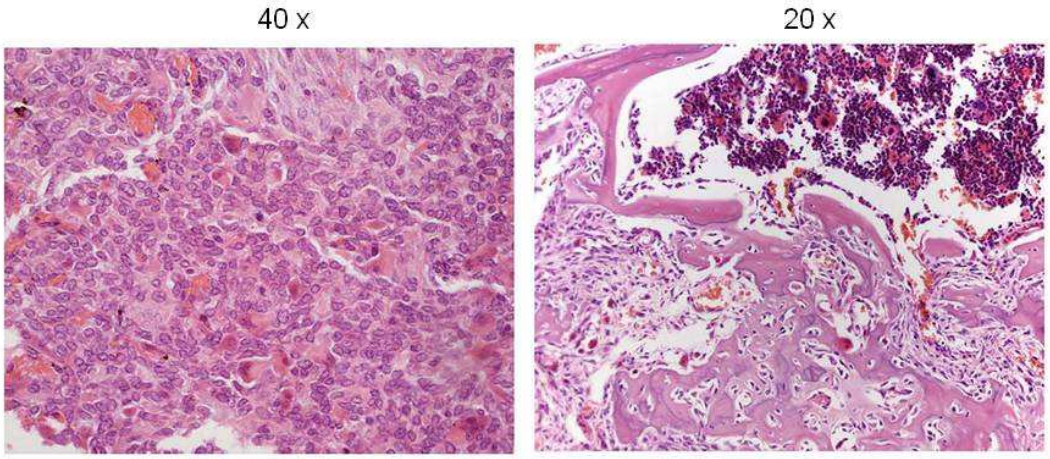


Figure 1.12: H&E sections of the osteogenic tumour detected in *Lkb1*$^{+/-}$ mice (Robinson et al. 2008).

Genetic alteration of *LKB1* in Osteosarcoma

The study of genetic abnormalities, and in particular of chromosomal loss in OS, has not given a clear picture of OS genetic drivers (section 1.1.5.2). It is of note however that loss of the *LKB1* locus, 19p13.3, part or even the whole of chromosome 19, have already been reported in some cases (Bayani et al. 2003, Ozaki et al. 2002, Squire et al. 2003, Stock et al. 2000, Tarkkanen et al. 1999, Tarkkanen et al. 1995). Breakpoints were reported in over 10 cases by cytogenetics at 19p13 (Bridge et al. 1997). This suggests that *LKB1* may be lost in OS, like has been found in lung cancer. Gains of chromosome 19 have also been reported by three groups (Bayani et al. 2003, Ozaki et al. 2002, Zielenska et al. 2004, Zielenska et al. 2001), but this occurred less often than loss of the chromosome. Moreover, aneusomy is a very common event in OS and this does not exclude a role of LKB1 in OS pathogenesis. *LKB1* has not been identified as deregulated in published GEM or proteomic studies, and no mutation of the gene has been recorded to this date. More investigation is needed to fully decipher the genetic status of *LKB1* in OS and of its contribution to bone tumourigenicity.

LKB1 is at the crossroad of key signalling pathways playing a major role in Osteosarcoma

LKB1 function (paragraph 1.2.1.2), has been associated with several signalling pathways that play a role in OS.

First, LKB1 major signalling route is by activation of AMPK, which can then inhibit the activation of the mTOR pathway, mainly by phosphorylation of TSC2. Loss of LKB1 in OS would then prevent the shutdown of the mTOR pathway in energy-deprived cells. As the mTOR pathway has been reported to play a role in OS, mainly via ezrin (section 1.2.3.1 and 1.2.3.2), it raised the question whether LKB1 participates in OS pathogenesis. This would also provide a new light on the genetic mechanism of mTOR pathway activation in OS.

Moreover, LKB1 has been shown to cooperate with Src to regulate the activation of the mTOR pathway (Carretero et al. 2010) and also cell migration and metastasis, as discussed in paragraph 1.2.1.2. This cooperation could explain why dasatinib treatment, which targets Src, failed in OS *in vivo* (section 1.1.6.1). Src was also associated with metastatic potential in OS in a cell line model (Azuma et al. 2005), as it was in a metastatic *Lkb1* deficient lung cancer model in mice (Carretero

et al. 2010). Similarly, anoikis resistance in SaOS2 cell line was shown to be dependent on Src activation (Diaz-Montero et al. 2006). A better understanding of LKB1 role in OS could help to refine the treatment design with Src inhibitors in OS.

Similarly, the Wnt-β-catenin pathway has been suggested to be inactive in OS, although this is controversial (section 1.1.5.3). LKB1 has been suggested to regulate the interaction between the mTOR and the Wnt-β-catenin pathway (section 1.2.1.2), although its exact role is not well understood. Assessing the role of LKB1 in OS could help to resolve those questions in the future.

LKB1 has been shown to interact with TGF-β signalling and this function was even suggested to play an essential role in polyp formation in patients with PJS (section 1.2.1.2)(Katajisto et al. 2008) by disrupting the interaction between epithelial and mesenchymal cells in the intestine. In bone biology, TGF-β can stimulate the proliferation of osteoblast progenitors, participate to the control of their differentiation in different lineages and decrease bone resorption by inducing osteoclast apoptosis. Disruption of bone remodelling has been associated with OS, with Paget disease of bone giving an increased risk factor for developing this cancer. The targeting of osteoclasts is even considered as a potential treatment for OS (section 1.1.6.1) Therefore, disruption of the TGF-β signalling and the interaction between osteoclasts and osteoblasts via the disruption of LKB1 could hypothetically participate in OS pathogenesis.

Finally, the major genetic event associated with OS is the alteration of the p53 pathway (section 1.1.5.1). LKB1 has been shown to interact with this pathway either via its downstream kinases AMPK, SIK and NUAK, or more directly by interaction with p53 and/or p21 (section 1.2.1.2). It would then be very interesting to assess the role of LKB1 in OS.

1.2.4 Conclusion

Overall, in addition to the work of Robinson et al, underlining the link between *LKB1* and bone biology, *LKB1* was found to play a role in key signalling pathways, implicated in OS, including the mTOR pathway, and there is some evidence of loss of its locus in sporadic cases. A better understanding of the role of *LKB1* in OS could help to decipher the regulation of those pathways in OS and to design innovative therapeutic strategies targeting them.

1.3 Aims

The importance of the mTOR pathway in OS was demonstrated by pre-clinical studies; LKB1 is a key playor in this pathway, and has been linked to OS either directly with a mouse model, or indirectly as a player in major pathways deregulated in OS, as described in 1.2.3.3. Therefore, the overall aim of this thesis was to assess the role of LKB1 in the pathogenesis of osteosarcoma.

As the most studied effector of LKB1 is AMPK, which regulate the activation of the mTOR pathway depending on nutrient availability, the first focus of this work was to assess the activation of this mTOR pathway in a large cohort of human cases (Chapter 3).

To achieve our main aim, the level of expression of *LKB1*, a tumour suppressor gene, was evaluated in human OS clinical samples and in human OS-derived cell lines at the protein level and correlated to the activation of the mTOR pathway (Chapter 4).

Having identified the loss of LKB1 protein in OS, the next objective was to investigate the molecular mechanism explaining this loss, by assessing the presence of genetic abnormalities of *LKB1*, such as mutation or gene deletion (Chapter 5), the mRNA expression level of *LKB1* (Chapter 5) and investigating *LKB1* post-transcriptional regulation (Chapter 6).

Finally, the functional role of LKB1 loss in OS pathogenesis was studied using two models: a knock-down of *LKB1* in an osteoblast cell line (Chapter 7) and its knock-in in LKB1-negative OS cell line (Chapter 8).

Chapter 2: MATERIALS AND METHODS

2.1 Clinical samples

The tissue samples and the clinical data were obtained from the archives of the Department of Pathology, Royal National Orthopaedic Hospital (RNOH) in Stanmore, UK. Paraffin embedded and snap-frozen tissue samples were used, dating from 1997 to 2010. The study complied with the standards of the Central Office for Research Ethics Committees (COREC) (REC: 07/Q0506/8). The material was handled as recommended by the Human Tissue Act (HTA licensing number 12325 and 12055). The histology of each sample was reviewed by a pathologist to confirm the diagnosis before being used in this work. For paraffin embedded tissue, the blocks with necrotic tissue were excluded; whenever possible, blocks with minimal decalcification were chosen. For frozen tissue, the histology was reviewed using an H&E every 10 sections cut.

2.2 Cell culture

The cell lines used in this study are detailed in the table below (Table 2.1). Except the Jurkat cell line which was grown in suspension, the cells were maintained as monolayers at 37°C with 5% CO2 and high humidity in the appropriate medium (GIBCO®, Invitrogen, Paisley, UK) as detailed in Table 2.1, supplemented with 10% FBS (GIBCO) and with 100 U/ml penicillin G, and 100µg/ml streptomycin (Invitrogen). When the cells reached 80 to 90% confluence, they were split using trypsin-EDTA (GIBCO) 1:3 to 1:10, depending of their growth rate, two to three times a week. Cells were stored frozen in FBS supplemented with 10% DMSO at a density of 1 to 3 million per ml. They were counted using a Neubauer haemocytometer (Weber Scientific International Ltd., Middlesex, UK) diluted 1:2 in trypan blue (Sigma Aldrich, Dorset, UK) for dead cell identification before freezing or prior to any assay.

Cell line	Description	Medium	Origin
293T	Human embryonic kidney cell line HEK2931T	DMEM	ATCC biobank
A549	Human lung adenocarcinoma epithelial cell line	DMEM	
Jurkat	Human immortalised T lymphocyte cell line, derived from leukaemia patient	DMEM	
143B	Human OS cell line derived from HOS	DMEM	
MG63	Human OS cell line	DMEM	
G292	Human OS cell line	DMEM	
HOS	Human OS cell line	DMEM	
U2OS	Human OS cell line	DMEM	
SaOS2	Human OS cell line	DMEM	
HOB	Human OS cell line	DMEM	Kindly provided by Pr Boschoff, Viral Oncology, Cancer Institute, UCL, UK
HF1	Immortalised human fibroblast	DMEM	Kindly provided by Dr Funes, Viral Oncology
Cal72	Human OS cell line	DMEM	DSMZ biobank
OST	Human OS cell line	RPMI	Kindly provided by Dr Strauss, Cancer Institute
MHM	Human OS cell line	DMEM	Eurobonet biobank
OSA	Human OS cell line	DMEM	
MNNG	Human OS cell line derived from HOS	DMEM	
ZK58	Human OS cell line	DMEM	
KPD	Human OS cell line	DMEM	
OHS	Human OS cell line	DMEM	
HAL	Human OS cell line	DMEM	

Table 2.1: List of the cell lines used.

2.3 *In vivo* work

Xenografts were generated with the OS cell lines MNNG, U2OS and SaOS2 in immunocompromised mice. The study complied with the regulations of the Animal (Scientific Procedures) Act 1986 (project license reference PPL70/6666 and personal license reference PIL70/21069). The cell lines were cultured as described in the previous paragraph (section 2.2), harvested and counted and two millions of cells were resuspended in 60µl of PBS. The cells were then mixed in 60µl of cold matrigel (BD Bioscience, Oxford, UK) and kept on ice. They were injected subcutaneously in two flanks of two NOD.Cg-*Prkdc*^{scid} *Il2rg*t^{m1Wjl}/SzJ mice, also referred to as NOD-Scid gamma (Jackson Laboratories, Maine, USA). The mice, highly immunosuppressed, were kept in a controlled environment as described in the project license: the drinking water and the food were autoclaved and the cages were kept in a depressurised category 2 laboratory. The xenografts were left to grow for up to 20 weeks, or until they reached the limits stated in the project license, e.g. 14mm wide. At that point, the animal was sacrificed and the xenografts were harvested and snap-frozen.

2.4 Molecular pathology

2.4.1 TMAs

Seven tissue microarrays (TMA) with 0.6mm cores were constructed for this project using a manual tissue arrayer (Beecher Instruments Inc, USA).

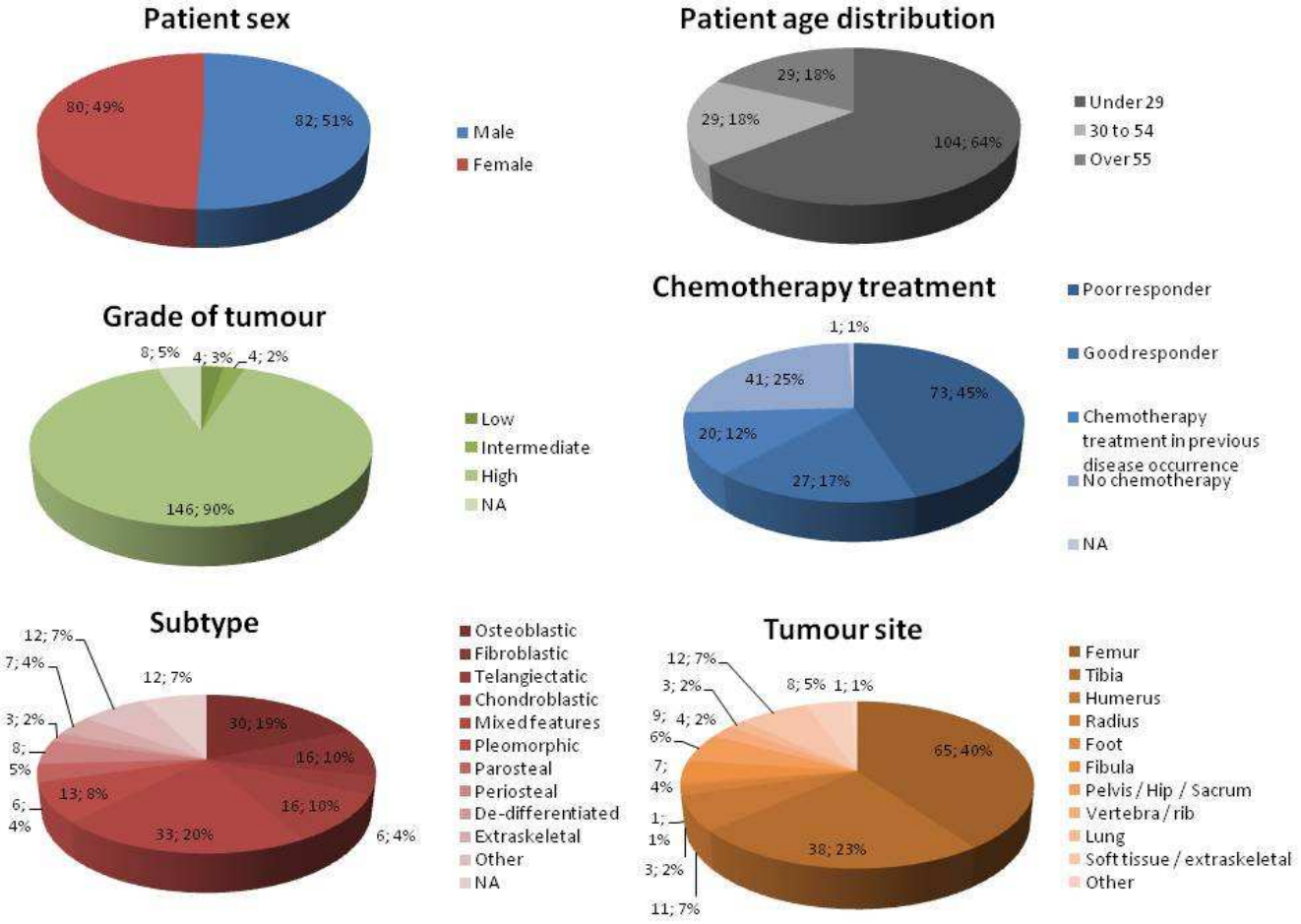
The first two TMAs were used for the screening of the mTOR pathway and contained 162 resected OS cases in duplicate to quintuplicate including 69 cases from poor responders to chemotherapy (PR), 27 from good responders (GR) and 63 cases without chemotherapy before the resection. All subtypes and sites available were included. They were collected between 2000 and 2007. The mean age of the patients was 30.0 years old, ranging from 4 to 85 years old. There were 134 primary tumours, 11 metastasis and 17 recurrences, and 53 cases presented a progressive disease, with recurrence or metastasis reported at diagnosis or in subsequent follow-ups. More details on the clinical data are provided in the Figure 2.1.

Five other TMAs including the same and more recent cases were designed for the study of the expression of LKB1 and p-TSC2 in OS. They totalled 268 resected OS cases in duplicate to triplicate, with 109 PR, 70 GR and 87 without chemotherapy. All subtypes and sites available were included. The mean age was 28.7 year old and was in the same range as the first set. The cases were collected between 2000 and 2009. There were 225 primary, 28 recurrent and 14 metastatic cases; 75 cases presented with progressive disease. More details on the clinical data are provided in the Figure 2.2.

Additionally, a TMA with 51 OB cases collected between 1997 and 2008 was constructed. The mean age of the patients was 19.3 years old, ranging from 3 to 54 years old. More details on the clinical data are provided in Figure 2.3.

Normal and cancer tissues were used as controls and for orientation in the TMAs.

All cases were reviewed by a pathologist, as mentioned in section 2.1, to select an area representing the characteristics of the tumour histology, where the punches were taken.



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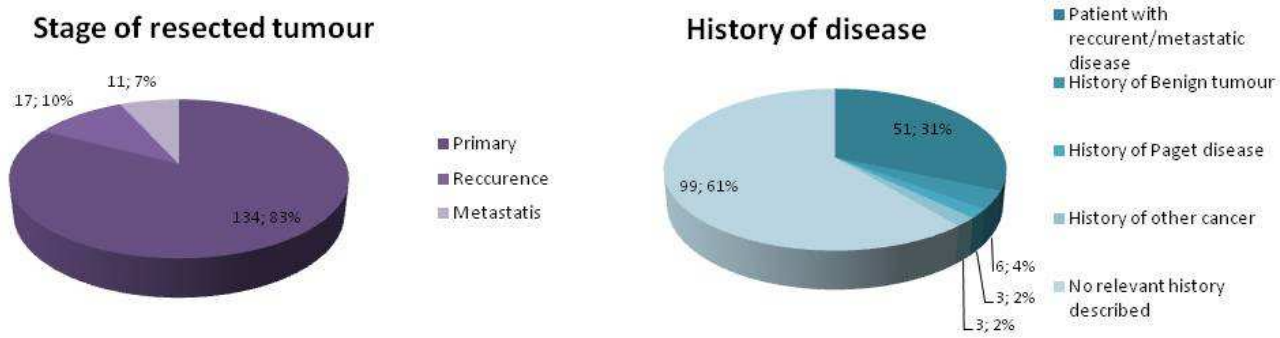
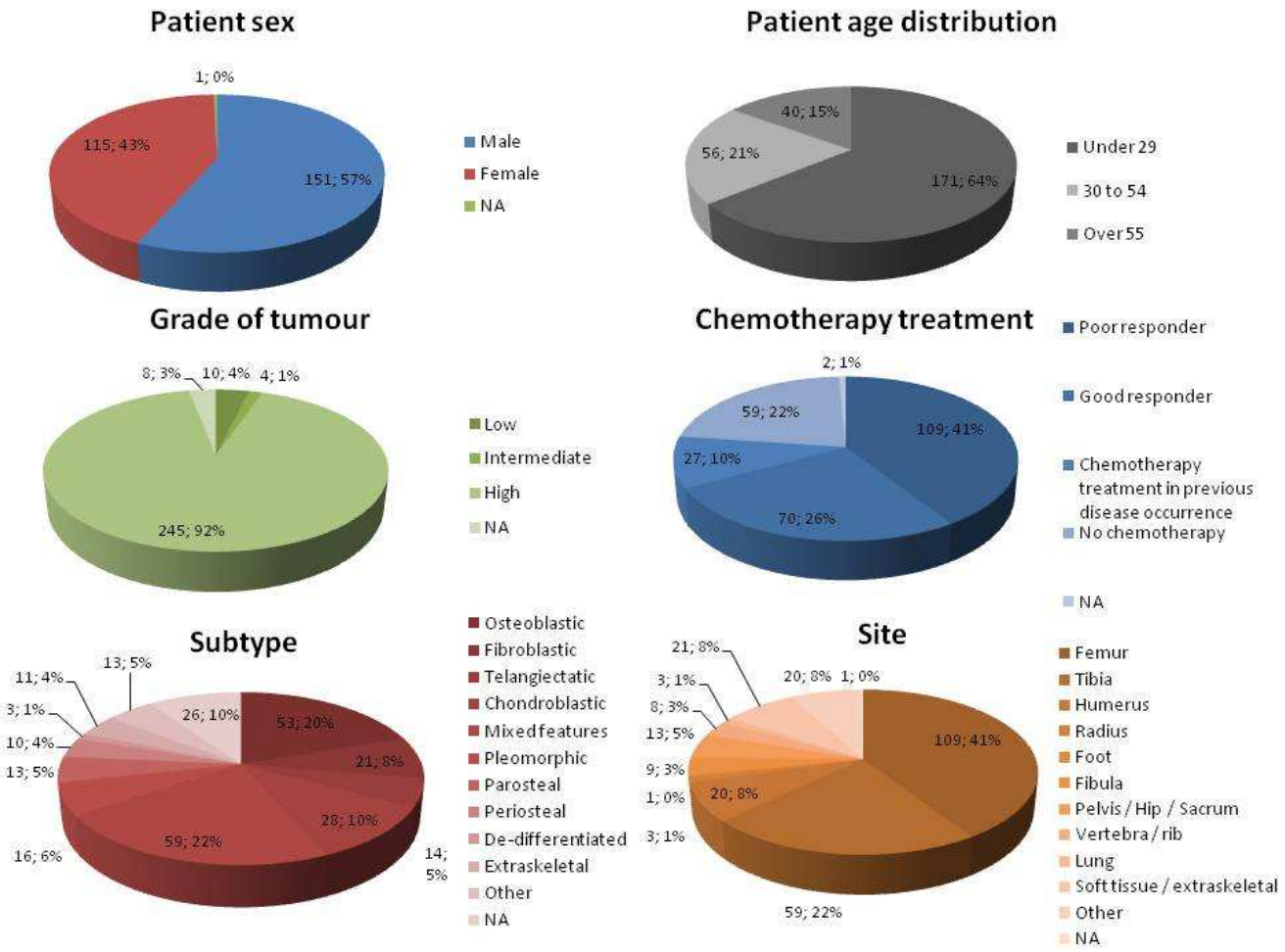


Figure 2.1: Clinical characteristics of the cases included in the first set of OS TMAs.
The number of cases concerned and percentage they represent among all cases are indicated on the charts for each category.



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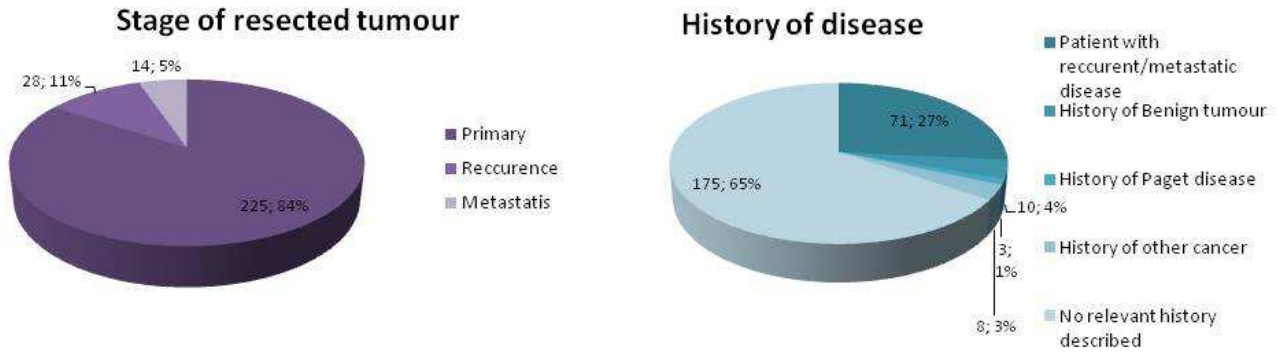


Figure 2.2: Clinical characteristics of the cases included in the second set of OS TMA.
The number of cases concerned and percentage they represent among all cases are indicated on the charts for each category.

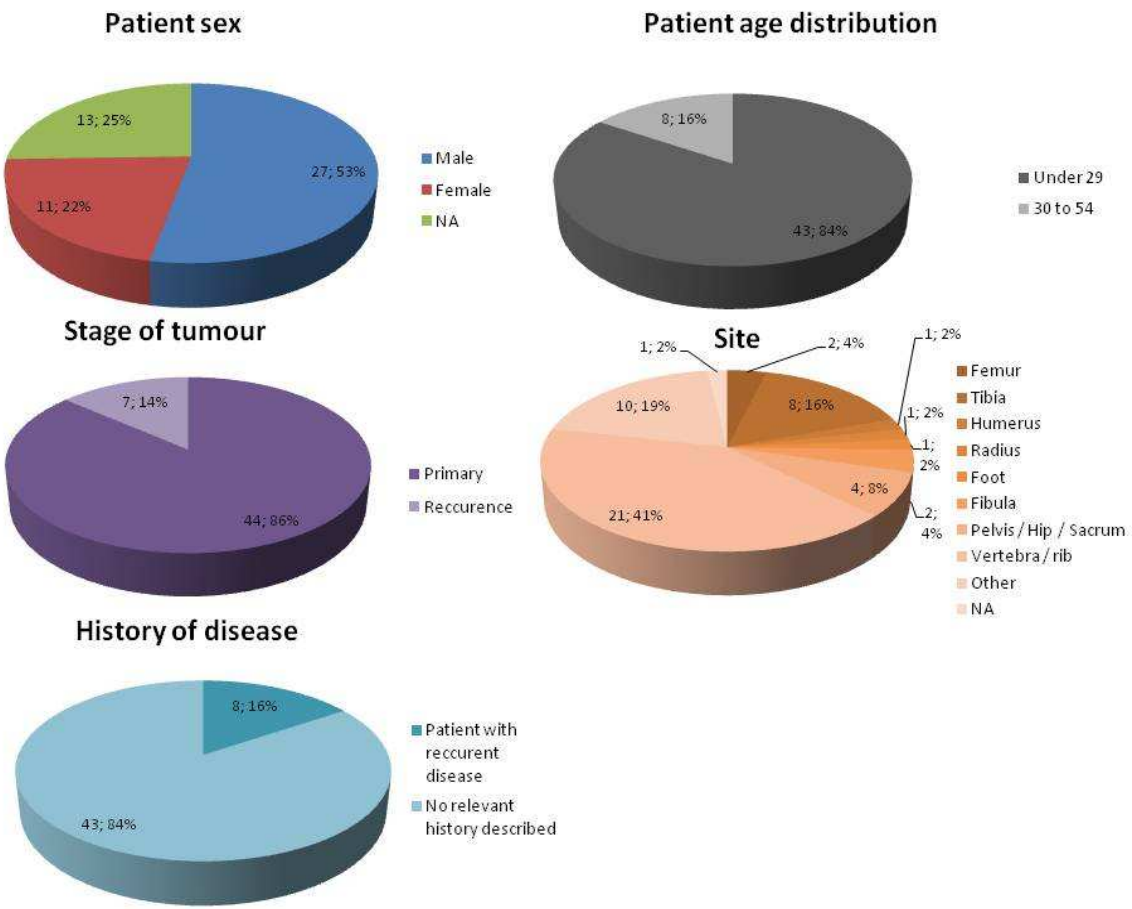


Figure 2.3: Clinical characteristics of the cases included in the osteoblastoma TMA.
The number of cases concerned and percentage they represent among all cases are indicated on the charts for each category.

2.4.2 Immunohistochemistry

2.4.2.1 Slide staining

Three µm thick paraffin-embedded sections were de-waxed by successive baths of xylene, ethanol and water, pre-treated and incubated with the primary antibody as described in Table 2.2. Pre-treated slides were either stained manually with the Ventana iVIEW DAB kit (Ventana Medical Systems, Strasbourg, France) or automatically using the Ventana NexES Autostainer (Ventana Medical Systems), following the manufacturer's instructions. The slides were counterstained with haematoxylin. A positive, an isotype and a negative control (e.g. no primary antibody added) were performed on tissue controls. An H&E was performed for each slide to review its histology. The slides were kindly scanned by Dr Dahmane Oukriff (Department of Pathology, UCL) using the Nanozoomer HT system (Hamamastu, Welwyn Garden City, Herts, UK) and analysed using the Nanozoomer Digital Pathology software.

2.4.2.2 Immunohistochemistry scoring

Both the intensity of the staining and the number of cells stained were scored for each core on the slides scanned with the Nanozoomer, as described above. For the number of cells stained, a score of 0 was given when no or sparse tumour cells (less than 1%) were stained, 1 for less than 10%, 2 for between 10 and 60% and 3 for above 60%. For the staining intensity, a score of 0 corresponded to an absence of staining, 1 to a weak staining intensity (less intense than the positive control), 2 to a medium staining intensity (comparable to the positive control) and 3 to a high staining intensity (above the positive control). The score of each of the duplicated cores for the same case were then averaged, to give a final score for the staining intensity and for the extent of the staining. Unequivocally immunoreactive cases, with a score for intensity equal or above 1 and a score for the % of cells stained strictly over 1, were considered as expressing the protein. Cases not immunoreactive, with a score equal or below 1 for the extent of the immunoreactivity, were considered as negative for protein expression. Difficult cases, with a low intensity and/or a low % of cells stained, were reviewed further by a pathologist to confirm the scoring (Dr Hongtao Ye, Dr David Delaney, Dr Asem Shalaby or Pr Adrienne Flanagan) and were finally considered either positive or negative. The staining of non-neoplastic cells as internal positive and negative controls was monitored in each

case to validate the scoring. Equivocal cases, with only few cells remaining on the slide or a too weak staining, or with no internal controls, were excluded. The scoring of the IHC for LKB1 was assessed by Dr Stephen Damato following the same method.

Antibody (p-site), clone, species	Source	Antigen retrieval step	Incubation of Primary	Dilution	Method	Positive control
p-Akt (Ser^{473}), clone 736E11, rabbit polyclonal	Cell Signalling Technology, MA, USA, ref 37875	6min PC	Overnight at 4°C	1:50	Manual	Breast cancer
PTEN clone 28H6, mouse monoclonal	Abcam, Cambridge, UK, ref ab10945	2min PC	30min at 37°C	1:100	Automated	Breast cancer
p-mTOR (Ser^{2448}), rabbit polyclonal	Cell Signalling Technology, ref 2976S	3min PC	30min at 37°C	1:50	Automated	Breast cancer
p-S6K (Thr^{389}), rabbit polyclonal	Cell Signalling Technology, ref 92055	4min PC	Overnight at 4°C	1:50	Manual	Breast cancer
Total S6K, rabbit polyclonal	Abcam, ref ab59246	6min PC	Overnight at 4°C	1:50	Manual	Breast cancer
p-RPS6 (Ser$^{235/236}$), rabbit polyclonal	Cell Signalling Technology, ref 4857	3min PC	30min at 37°C	1:100	Automated	Breast cancer
Total RPS6, mouse monoclonal	Cell Signalling Technology, ref 2317	2min PC	30min at 37°C	1:100	Automated	Breast cancer
TSC1, rabbit polyclonal	Cell Signalling Technology, ref 4906	2min PC	30min at 37°C	1:25	Automated	Breast cancer
p-TSC2 (Thr^{1462}), rabbit polyclonal	Abcam, ref ab59274	4min PC	Overnight at 4°C	1:25	Manual	Breast cancer
Total TSC2, clone C20, rabbit polyclonal	Santa Cruz, CA, USA, ref SC893	2min PC	30min at 37°C	1:100	Automated	Breast cancer
p-4EBP1 (Thr^{70}), rabbit polyclonal	Cell Signalling Technology, ref 9455	2min PC	Overnight at 4°C	1:50	Manual	Breast cancer

The Table continues on the next page

Antibody (p-site), clone, species	Source	Antigen retrieval step	Incubation of Primary	Dilution	Method	Positive control
eIF4E, rabbit polyclonal	Cell Signalling Technology, ref 9742	2min PC	30min at 37°C	1:100	Automated	Breast cancer
LKB1, clone Ley 37DG6, mouse monoclonal	Abcam, ref ab15095	2min PC	Overnight at 4°C	1:600	Manual	Normal lung and skeletal muscle
Rabbit IgG isotype control	Dako, Cambridge, UK, ref X 0903-028	Same protocol as tested antibody				
Mouse IgG1 isotype control	BD Pharmingen, Oxford, UK, ref 555746	Same protocol as tested antibody				

Table 2.2: List of antibodies used for IHC and staining method used.

2.4.3 Fluorescence in situ hybridisation

2.4.3.1 Probe preparation

BAC probes were selected from the RP11 library of the Sanger Institute: RP11-81M8 for *LKB1* and RP11-91H11 for the telomeric control on chromosome 19 (BACPAC Resource centre, Oakland, CA, USA). The BAC clones were spread on LB agar overnight and a single clone was amplified in LB overnight. The DNA was extracted by a crude miniprep protocol, amplified using GenomiPhi DNA Amplification v2 kit (GE healthcare, Amersham, Bucks, UK) as instructed by the manufacturer, and labelled by nick translation in either Spectrum-Orange (LKB1 probe) or Spectrum-Green (telomeric probe) with reagents from Vysis (Abbot molecular, Maidenhead, Berks, UK) as instructed by the manufacturer. Both probes were tested on a metaphase spread of normal cells to ensure that they hybridised to the correct chromosomal area.

2.4.3.2 *In-situ* hybridisation

After optimisation of the protocol on tonsil and OS resections, interphase fluorescence *in situ* hybridisation (FISH) was performed on the OS and OB TMAs. The slides were de-waxed, pressure-cooked for 3min in distilled water and digested in 0.006% pepsin in 1mM HCl for 20min. They were then washed twice in distilled water, dehydrated and air-dried. The probes were hybridised on the slides for up to 48h at 45°C in a humidity chamber, after 5min denaturation at 73°C on a hot plate. The slides were then washed in 50% formamide (Sigma) in 2x SSC (Sigma) at 42°C three times for 5min, then in 2x SSC at 42°C three times for 5min, in 1% Igepal (Sigma) in PBS once for 5min at room temperature, and three times briefly in distilled water. They were counterstained with DAPI and observed under a fluorescence microscope (Olympus, Southall, Middlesex, UK) using AnalySIS software (Olympus).

2.4.3.3 Scoring method for fluorescence *in-situ* hybridisation

Fifty non-overlapping nuclei were counted for each case. Four normal controls were used to determine the cut-off values for disomy, copy number loss and gain of *LKB1* and polysomy. The cut-off was calculated using the mean plus two standard deviations of the scoring obtained for the normal controls. Therefore, disomy was defined as the presence of two copies of *LKB1* and the telomere in over 90% of the cells. Copy number loss of *LKB1* was defined by a ratio of *LKB1* over telomere strictly less than 1 in at least 20% of the cells and copy number gain by a ratio strictly over 1 in at least 10% of the cells. Polysomy was defined as the presence of at least 3 copies per cell of both *LKB1* and the telomere in over 10% of the cells. Additionally, the scoring was performed independently by a pathologist, Dr Hongtao Ye. The results of both scores were then examined and divergent cases were reviewed to reach a final decision on the status of the case.

2.5 Sample processing

2.5.1 Genomic DNA extraction from tissue and cell lines

One to three 80% confluent 75cm^2 flasks of the cell lines were harvested in trypsin-EDTA, washed in PBS twice and stored as a pellet at -20°C until the genomic DNA was extracted. Twenty frozen sections from both tumour and normal tissue were cut for each of the 21 OS and 1 OB cases used in this study. For one case, no snap-frozen normal tissue was available and blood was used instead. The DNAeasy kit (Qiagen, Crawley, West Sussex, UK) was used for the DNA extraction, as instructed by the manufacturer.

Briefly, the pellet or the cut sections were resuspended in 180µL of ATL buffer. Twenty µL of proteinase K was added and the samples were digested at 56°C in a water-bath for 10min for the cell lines, and 1 to 2h for the tissue samples. Two hundred µL of AL buffer was added, mixed for 15s with the sample and then incubated for 10min at 70°C to digest any mRNA remaining. Two hundred µL of 100% ethanol was added and the sample was mixed. The solution was loaded on a column and the DNA bound to it by centrifuging for 1min at 8,000 rpm. The samples were then washed once with 500µL of AW1 buffer and once with 500µL of AW2 buffer. They were eluted in 100 to 200µL of AE buffer, depending of the initial amount of frozen tissue or cells in the sample, after 5min incubation with the elution buffer on the column. The samples were stored at -20°C. The concentration and quality of DNA obtained was monitored using a NanoDrop UV spectrophotometer. Tumour cases yielding less than 100ng/µL were amplified using the GenomiPhi DNA Amplification v2 kit (GE healthcare) according to the manufacturer's instructions.

2.5.2 miRNA extraction from tissue and cell lines

The mRNA and miRNA of 21 OS resections, 24 OS biopsies and 1 OB case, as well as of 19 cell lines was extracted using the miRNeasy extraction kit (Qiagen), following the manufacturer's instructions. Briefly, for the patient samples, 30 frozen sections were resuspended in 700µL of Qiazol lysis reagent and homogenized by vortexing. For the cell lines, the cells were plated in one 10cm dish, and when reaching 70 to 80% confluence, they were washed twice in PBS and removed from the dish using 700µL of Qiazol. The Qiazol resuspended samples were stored at -80°C between 1h to a few weeks, to increase the final yield. The homogenate was then thawed, vortexed and incubated for 5min at

room temperature. Chloroform was added (140µl for 700µl of Qiazol) and the tube was shaken vigorously for 15s. The sample was incubated for 2-3min at room temperature and centrifuged for 15min at 13,000rpm at 4°C. The upper aqueous phase was collected and 1.5 volumes of 100% ethanol added. After mixing thoroughly by vortexing, the sample was transferred into a miRNeasy mini spin column and centrifuged at 10,000rpm for 15s at room temperature. The RNA was washed with 350µL of RWT buffer. The remaining DNA was digested on the column by adding 80µl of a mix of 10µL of DNase I (Qiagen) and 70µL of buffer RDD and incubated at room temperature for 15min. The sample was then washed once with 350µL of buffer RWT, and twice with 500µL of buffer RPE. Finally, the sample was eluted in 30 to 50µL of RNase free water and stored at -80°C. The concentration and quality of the mRNA and miRNA obtained was monitored using a NanoDrop UV spectrophotometer.

2.5.3 Protein extraction from tissue and cell lines

For the cell lines, the cells from one to three 80% confluent 10 cm dishes or 75cm^2 flasks were harvested using trypsin-EDTA, washed twice in PBS, pelleted and stored at -20°C until the protein was extracted. When the cells were cultured in smaller dishes (6-well plates or 6cm dishes), the lysis buffer was directly added on the cells for 5min on ice after two washes with PBS. The cells were then scraped out of the plate and incubated further in the lysis buffer as described below. For the tissue samples, 30 frozen sections of the 27 OS cases and 1 OB case were cut and directly used for the extraction.

For each sample, a lysis buffer mix containing 500µL of radio immunoprecipitation assay (RIPA) buffer (50mM Tris at pH8, 100mM NaCl , 5mM EDTA and 1% Triton X100), 5µl of protease inhibitor (Sigma), 5µl of phosphatase inhibitor (Sigma) and 5µl of 1% SDS (Sigma) was prepared. The sample was resuspended in 300 to 500µL of the lysis buffer and incubated for 30min on ice. The sample was occasionally pipetted up and down during this incubation. The lysate was then centrifuge for 5min at 10,000rpm to get rid of the cell debris and the supernatant was collected.

The concentration of the protein lysate was assessed using a BCA protein assay kit (Fisher Scientific, Loughborough, Leicestershire, UK), and compared to a BSA standard of 1:2 dilutions from 2mg/ml to 0.0625mg/ml.

2.6 Molecular biology

2.6.1 Polymerase chain reaction

LKB1 was amplified by polymerase chain reaction (PCR) to study the occurrence of any abnormalities in the gene in OS cases. The genomic or amplified DNA extracted from the tumour and normal tissue of 21 OS and 1 OB cases and two control cell lines (A549 and Jurkat) were used for each reaction. The primers were designed to hybridise to the intronic sequences flanking each exon, to enable the amplification of the entire exon and to include the single nucleotide polymorphisms (SNP) or possible somatic mutations of interest for this study (section 2.6.2 and 2.6.3). These primers (Table 2.3) were either previously described (Kato et al. 2004, Sanchez-Cespedes et al. 2002) or designed using the online software Primer3 version 0.4.0 (Rozen et al. 2000) or by hand. Their properties (CG content, molecular weight, melting temperature and self-complementarity) were monitored using Primer3 or Oligo Calculator version 3.26 (Kibbe 2007) and blast against the human genome using Ensembl BlastView and NCBI blast tools.

For each reaction, 2.5µl of 10X Hot start buffer (Qiagen), 1µl of a 10pM dilution of each forward and reverse primers, 1µl of DNA (50 to 300ng), 0.2µl of a mix of 10nM dNTP and 0.1µl of 1U Hotstart polymerase enzyme (Qiagen) were mixed in a total volume of 25µl. In some exons that showed unspecific amplification with the positive control, 5µl of Q solution was added to enhance the specificity of the amplification reaction (Table 2.3).

A touchdown PCR protocol was employed with annealing temperature reduced by 1°C per cycle from 65°C to 62-58°C (Table 2.3), followed by 35 further cycles at 62-58°C . Each cycle was performed as follows: 94°C for 1min, annealing temperature for 1min, 72°C for 1min. The polymerase was activated at by an initial step at 95°C for 15min before cycling and a final elongation step at 72°C was performed for 10min.

2.6.2 Mutation screening: sequencing

The amplified DNA was cleaned using the Qiaquick PCR purification kit (Qiagen) following the manufacturer's instructions and sequenced with the same primers used for the PCR amplification by UCL Scientific Support Service. Sequencing reactions were run using GenomeLab™ DTCS Quick Start chemistry (Beckman Coulter UK Ltd, High Wycombe, Bucks, UK) and were analysed on a CEQ™ 8000 Genetic Analysis System (Beckman Coulter). The sequences were reviewed using the software Sequence Scanner v1.0 (Applied Biosystems, CA, USA).

Exon	Direction	Sequence (5' to 3')	Product length	Addition of Q solution	Final annealing temperature
1	Forward	AGGAAGGACCGCTCACCCG	410 bp	Yes	58°C
	Reverse	AACCATCAGCACCGTGACTG			
2	Forward	AGTGTCCTAACTGTGTCCTC	405 bp	No	58°C
	Reverse	GGAAGAGGAGCAGGCCT			
3	Forward	GCCTTTTCAGAGGGGTGGC	341 bp	Yes	58°C
	Reverse	TATCAGGACAAGCAGTGTGG			
4-5	Forward	CAGCTGCAAAGGGGACC	456 bp	Yes	60°C
	Reverse	AGTGTGCGTGTGGTGAGTGC			
6	Forward	GTCAACCACCTTGACTGACC	252 bp	No	58°C
	Reverse	ACACCCCCAACCCTACATTT			
7	Forward	CCTGACAACAGAGGCTGGG	546 bp	Yes	60°C
	Reverse	GTCCGCTGCTCTGTCTTCC			
8	Forward	CCTGAGTGTGTGGCAGGTAC	375 bp	Yes	62°C
	Reverse	TGTTGCAGACAGGCAGGCAC			

Table 2.3: List of primers used to amplify *LKB1.*

2.6.3 SNP study: restriction digestion

To confirm the data obtained by FISH, 21 OS and 1 OB cases were investigated at the locus of several SNPs along the gene. The status of five SNPs was assessed by a restriction digestion method, as previously described (Sobottka et al. 2000). Briefly, using the primers listed in the previous paragraph (Table 2.3), exons 1, 2, 3, 7 and 8 (and the portion of surrounding intron containing the SNPs) were amplified by PCR from the extracted DNA from both the tumour and normal tissue of 22 cases and cleaned using the Qiaquick PCR purification kit as previously described.

The five restriction enzymes used were BamHI-HF, BsrBI, BstNI, BstUI and EarI, (New England Biolabs, Hitchin, Herts, UK). Eight µl of the clean PCR product were then digested with 1µl of the appropriate enzyme and 1µl of buffer; the buffer, temperature and incubation times were used as recommended by the manufacturer (Table 2.5). Half of the digestion product was run on a 2% low melt agarose gel, with the tumour and the normal for each case in adjacent wells.

According to the results observed, the cases were classified as:

- Presenting a parental allelic loss if the tumour had a homozygous profile and the normal a heterozygous profile (see possible nucleotide changes in Table 2.4)
- Not presenting a parental allelic loss when both the tumour and the normal had a heterozygous profile
- Not informative when the normal is homozygous.

The cases presenting an allelic loss had both their normal and tumour tissue sequenced to confirm the results obtained.

The remaining SNPs in exons 2, 3 and 7, detailed in the Table 2.4 and for which no restriction enzyme could be used, were studied by sequencing of both the tumour and the normal samples, in cases with loss of one parental allele detected by restriction digestion. The SNPs on exons 1 and 6, without restriction digestion enzyme usable as well, were only studied by sequencing of the tumour, so the cases were classified as either not informative (tumour homozygous) or as presenting no allelic loss (tumour heterozygous).

SNP ID	Location in LKB1	Nucleotide change	Reported Frequency	Restriction enzyme used	Exon sequenced for detection
rs56354945	Exon 1	A/C	NA	BamHI	Exon 1
rs3764640	Intron 1/2	G/T	G&T 41%	None	Exon 1
rs650599	Intron 1/2	C/T	C60%, T40%	None	Exon 2
rs57281474	Intron 1/2	C/T	NA	None	Exon 2
rs11552325	Exon 2	T/C	NA	None	Exon 2
rs2075604	Intron 2/3	G/T	G&T 21%	BstUI	Exon 2
rs34928889	Intron 2/3	A/G	NA	BstNI	Exon 3
rs12608721	Intron 3/4	G/C	NA	None	Exon 3
rs9282859	Exon 6	C/T	C100% in EU	None	Exon 6
rs2075607	Intron 7/8	G/C	C&G 33%	BsrBI	Exon 7
rs2075608	Intron 7/8	C/T	C&T 35%	None	Exon 7
rs59912467	Exon 8	C/G	NA	EarI	Exon 8

Table 2.4: List of SNPs studied and restriction enzymes used.

SNP ID	Exon amplified	Restriction enzyme	Incubation temperature	Incubation time	Buffer	Observed band size
rs56354945	Exon 1	BamHI	37°C	10min	4	C allele: 323 + 87 bp A allele: 410bp
rs2075604	Exon 2	BstUI	60°C	10min	4	T allele : 313 + 92bp G allele : 250 + 63 + 92bp
rs34928889	Exon 3	BstNI	60°C	10min	2 and BSA	A allele: 201 + 47 + 37 + 32 + 24bp (only 201bp is detected) G allele: 131 + 70 + 47 + 37 + 32 + 24 (only 131 and 70bp were detected)
rs2075607	Exon 7	BsrBI	37°C	1h	4	C allele : 215 + 331bp G allele: 152 + 63 + 331bp
rs59912467	Exon 8	EarI	37°C	1h	4	C allele : 246 + 129bp G allele: 375bp

Table 2.5: Reaction conditions of the digestion with restriction enzymes used for the SNP study.

2.6.4 Semi quantitative real time RT- PCR

2.6.4.1 cDNA synthesis

cDNA was synthesised from mRNA extracted from the tissue or cell lines (section 2.5.2) using the superscript III first strand DNA kit (Invitrogen). For each reaction, 100 to 200ng of total RNA were mixed with 1µl of random primer, 1µl of 10mM dNTP and made up to 10µl of RNAse free water. The solution was heated to 65°C for 5min and then incubated on ice for at least 1min. Then 2µl of 10X buffer, 2µl of 0.1M DTT, 4µl of 25mM $MgCl_2$, 1µl of RNAseOUT and 1µl of the enzyme superscript III (200U/µl) were added as a mastermix to add up to 20µl to each tube. The mix was incubated for 10min at 25°C, then for 50min at 50°C. The reaction was inactivated at 85°C for 5min. The cDNA obtained was stored at -20°C until use.

2.6.4.2 Real time RT-PCR

The amplification step was performed in a Microamp optical 96-well plate (Applied Biosystems, Birchwood, Warrington, UK). Each well contained in a total reaction volume of 25µl diluted in RNAse free water: 1µl of cDNA, prepared as described above, 0.75µl of forward and 0.75µl of reverse primer at 10pmol (specific to the gene to be amplified, see sequences detailed in Table 2.6), 12.5µl of SYBR® green PCR Master Mix (Applied Biosystems). The primers were designed using the online software Universal ProbeLibrary Assay Design Center (Roche, Burgess Hill, UK). Each reaction was performed in duplicate. The amplification was performed on an Realplex4 Mastercycler (Eppendorf, Cambridge, UK), with a first step at 95°C for 10min and then 40 cycles with 95°C for 15s, 60°C for 1min with a fluorescent reading at the end of this step (with a 520nM filter, SYBR dye). The relative expression of the gene of interest was quantified using either the ΔCt or the comparative Ct method (ΔΔCt), relative to the appropriate housekeeping gene, as explained below.

The forward and reverse primer concentration was optimised for amplification efficiency and primer dimer formation monitored by a ramping step (melting curve). Ribosomal protein 18s (RPS18) was used as a housekeeping gene for SIRT1 and β-actin was used for LKB1, to ensure that the ΔΔCt method was applicable. This was optimised by testing up to three housekeeping gene and using a 1 in 5 serial dilution of the U2OS cell line, from neat cDNA to a 1 in 3125 dilution.

Gene	Direction	Sequence (5' to 3')	Product length
LKB1	Forward	GTACGAACCGGCCAAGAG	76 bp
	Reverse	AGCCGGAGGATGTTTCTTC	
β-actin	Forward	ATCACCATTGGCAATGAGCG	98bp
	Reverse	TTGAAGGTAGTTTCGTGGAT	
SIRT1	Forward	AAATGCTGGCCTAATAGAGTGG	78 bp
	Reverse	TGGTGGCAAAAACAGATACTGA	
RPS18	Forward	CGCCGCTAGAGGTGAAATTC	62 bp
	Reverse	TTGGCAAATGCTTTCGCTC	

Table 2.6: List of qRT-PCR primers used.

2.6.5 Western Blot

2.6.5.1 Protein migration and transfer

The detection of LKB1 protein from fresh-frozen tumour samples or from cell lines was performed by Western Blot (WB). The protein was extracted as described in paragraph 2.5.3 and 20 to 30µg of lysate were denatured at 100°C for 5min with 7.5µl of loading buffer in a total volume of 30µl. A lysate of the Jurkat cell line was used as a positive control and of the A549 cell line – which carries a homozygous mutation in *LKB1*, Q37*, confirmed by sequencing and FISH (Sanchez-Cespedes et al. 2002) – as a negative control. One lane on each blot was loaded with the ColorPlus prestained protein marker broad range (NEB), to estimate the molecular weight of the transferred protein. The lysates were resolved on a 12% SDS-polyacrylamide gel at 90V for 2h and the proteins were transferred onto immobilion-P transfer membrane (Millipore Corporation, Bedford, MA, USA) by semi-dry electro-transfer method at 20V for 55min. The membrane was washed in 0.1% tween 20 in Tris buffered saline solution (TBST) and either blotted immediately or stored at -20°C.

2.6.5.2 Membrane blotting

The membrane was blocked for 1h in 5% bovine milk diluted in TBST, and incubated with the primary antibody in 5% milk in TBST, at the dilution described below (Table 2.7) with shaking. The membrane was then washed three times for 15min in TBST, and incubated for 1h at room temperature with shaking in a dilution of 1:5000 of the secondary antibody in 5% milk in TBST. The membrane was washed again three times for 15min in TBST.

2.6.5.3 Development

The blotted membrane was incubated for 1min with a mix of solution A and B of ECL™ Western Blotting Detection Reagents (Amersham, GE healthcare) to visualize the bound antibody. The chemiluminescent signal was then detected using Hyperfilm (Amersham, GE healthcare) after 1s to 15min exposure. The blots were then rinsed in TBST and either stored at -20°C or stripped using the Restore Western Blot stripping buffer (Thermo Scientific, Cramlington, Northumberland, UK) and used with another antibody.

2.6.5.4 Quantification by densitometry analysis

The blots were then scanned and the images analysed using the software ImageJ (version 1.42, National Institutes of Health, USA) by recording the absolute band intensity (the integrated density) on inversed 8-bit images. The blots chosen for the quantification had an exposition time selected so as to ensure that the positive and loading control were not overexposed. To quantify the expression of LKB1 protein in the samples and compare it to the controls, the integrated density of the bands for LKB1 and for the loading control GAPDH was recorded in all experiments (n=26) for Jurkat and A549, to give the mean of the ratios of the relative LKB1 protein expression compared to GAPDH. The 95% confidence interval of this relative ratio for Jurkat and A549 was then calculated as the mean of the ratios plus or minus 1.96 times the standard error of all the measurements. Cut-off values were then set to define a score of high protein expression (score of 2) when the figure for the cell line was within the 95% confidence interval obtained for Jurkat, of very low or no protein expression (0) when the densitometry was within the 95% confidence interval for A549 and of low protein expression (1) in-between.

Antibody	Source	Primary / Secondary	Dilution	Incubation method
LKB1 clone Ley 37DG6, mouse monoclonal	Abcam, ref ab15095	Primary	1:5,000 to 1:10,000	Overnight at 4°C
GAPDH clone 6C5, mouse monoclonal	Advanced Immunochemical Inc, Long beach, CA, USA	Primary	1:5,000 to 1:10,000	1h at room temperature
Anti mouse – HRP, goat polyclonal	Dako, ref P0447	Secondary	1:5,000	1h at room temperature

Table 2.7: List of antibodies used for WB.

2.6.6 Semi quantitative PCR

The amount of the alleles A and G in the SNP rs34928889 was quantified by Taqman® SNP assay (Applied Biosystem) by semi quantitative PCR, using the assay C2830637_10, as instructed by the manufacturer. Briefly, the genomic DNA was diluted to a concentration of 5ng/µl. The amplification step was performed in a Microamp optical 96-well plate (Applied Biosystem). Each well contained 0.5µl of the primers from the SNP assay, 10µl of the Taqman® Universal PCR master mix No AmpErase UNG, 5.5µl of water and 4µl of the diluted genomic DNA (e.g. 20ng). Each reaction was performed in quadruplicate and the experiment was performed twice. The amplification was performed on an Realplex4 Mastercycler (Eppendorf), with a first step at 95°C for 10min and then 40 cycles with 95°C for 15s, 60°C for 1 min with a fluorescent reading at the end of this step (FAM dye, 520nM filter and VIC dye, 550nM). The relative expression of the gene of interest was quantified using the comparative Ct method (ΔΔCt), relative to the housekeeping RNAse P (VIC dye, 550nM, reference 4316844).

2.7 miRNA microarray

2.7.1 Sample preparation

miRNA was extracted from 20 frozen sections of 24 OS biopsy samples using the miRNeasy extraction kit as described in paragraph 2.5.2 above. The samples were selected by a pathologist and the quality of the tissue cut for the extraction was reviewed on H&E sections as described in paragraph 2.1. The quality of the miRNA fraction in the extracted RNA was assessed using the Agilent Small RNA kit (Agilent, Wokingham, Berks, UK). Briefly, the RNA was diluted to a concentration between 25 and 100ng/µl. The chip was loaded with gel-dye matrix, conditioning medium and marker. One µl of each of the samples and the ladder were pipetted into the appropriate wells. The chip was vortexed for 1min at 2400rpm and analysed using the Agilent Bioanalyser 2100 (Agilent) straight away.

After the quality assurance step, the samples were diluted to a concentration of 25ng/µl in RNAse free water and processed for hybridization on the array (see below).

2.7.2 Microarray processing

The human miRNA microarray system v2 from Agilent was used, with a kit containing three high definition 15K glass slides of 8 samples each (designed from Sanger miRBase database release 10.1, with 723 human and 76 human viral miRNAs). The samples were prepared and hybridized on the chip as instructed by the manufacturer, using the protocol version 1.6 from March 2009.

Briefly, the RNA was dephosphorylated using calf intestine phosphatase (TaKaRa, Saint-Germain-en-Laye, France) for 30min at 37°C. The miRNA was then denaturated using DMSO at 100°C for 5 to 10min. The samples were cooled on ice and labelled by ligation of a Cyanine 3-Cp on the 3' end of the miRNA by ligation using a T4 RNA ligase (Ambion, Applied Biosystems) with a 2h incubation at 16°C. The samples were desalted with a MicroBioSpin 6 Column (BioRad, Hemel Hempstead, Herts, UK). The purified samples were dried at 45°C in a vacuum concentrator for 1h and resuspended in 18µl of RNAse/DNAse free water, 4.5µl of 10X GE Healthcare blocking agent, and 22.5µl of 2X Hi-RPM hybridization buffer. The samples were incubated for 5min at 100°C, cooled and loaded on the glass slide arrays. After 20h of hybridization at 55°C in an Agilent microarray

hybridization chamber, the glass slides were washed for 5min at room temperature in Wash buffer 1 and then 5min at 37°C in Wash buffer 2. The slides were scanned in an Agilent microarray scanner G2 505B (Agilent). Microarray images were automatically analysed using Feature extractionTM software (Agilent).

2.7.3 Data analysis

The raw data were processed, normalised and analysed by the bioinformatician Dr Stephen Henderson. The analysis was performed using Bioconductor and the limma packages for the R statistical programming language. The median and background signals were extracted, and the set of probes were corrected for the background and normalised.

2.7.4 Target predictions method

Target predictions for the genes implicated in *LKB1* regulation and for the miRNA identified by the data analysis were obtained using Targetscan algorithm (Lewis et al. 2005) and/or PITA algorithm, established in the Weizmann Institute (Kertesz et al. 2007).

2.8 *LKB1* silencing by shRNA knock-down

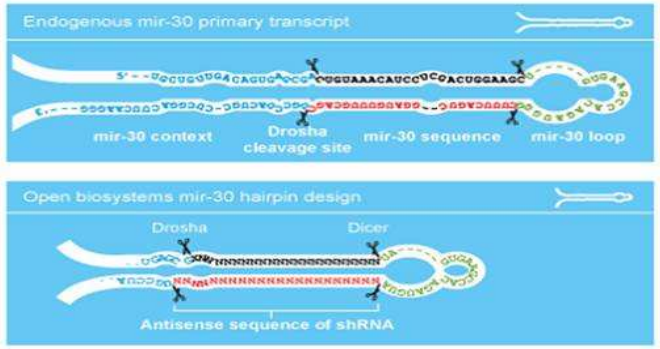
2.8.1 shRNA clones

The knock down of *LKB1* was performed using four clones of the Open Biosystems pGIPZ human lentiviral shRNAmir library (Figure 2.4 Table B. for sequences and reference) obtained from the Scientific Support Team in UCL; they will be abbreviated as clone 35, 21, 03 and 04, using the last two digits of their full ID number. The shRNA constructs induce a permanent knock down in the recipient cells. The shRNA sequence is inserted into a miR-30 construct, inside a pGIPZ lentivirus plasmid directed by a CMV promoter, as illustrated below. The plasmid also includes a GFP tag to monitor transfection efficiency and the genes for antibiotic resistance against Ampicillin, Zeomycin and Puromycin for selection (Figure 2.4). An empty vector and a non-silencing construct were used as controls. The lentiviral VSVg-envelop expressor plasmid pMDG and gag-pol expressor p8.91 were also provided by the Scientific Support Team.

2.8.2 Preparation of plasmid DNA

The GIPZ hairpin clones or controls and the packaging vectors were received as an LB agar stab. The bacteria was streaked on to a LB agar plate supplemented with 100µg/ml of Ampicillin and grown overnight at 37°C. A single colony was picked for each vector and cultured overnight at 37°C under agitation in 5ml of LB low salt media (20g/l of LB, 10g/l of peptone and 5g/l of yeast extract) as recommended by Open Biosystems. Half of this culture was used to seed a large scale culture of 200ml of LB low salt media, supplemented with Ampicillin. The other half was used to create a stock and to extract the plasmid DNA using the Qiaprep Spin Miniprep kit (Qiagen), as instructed by the manufacturer. Briefly, the culture was centrifuged 3min at 8,000rpm at room temperature, and the pellet was resuspended in 250µl of buffer P1. Then 250µl of the lysis buffer P2 was added and the tube was inverted several times to mix; 350µl of the neutralization buffer N3 was added and the tube was inverted again. The solution was centrifuged for 10min at 13,000rpm to get rid of the debris and the solution was loaded onto a spin column and centrifuged for 1min at 13,000 rpm. The column was washed with 0.5ml of PB buffer and 0.75ml of PE buffer. The plasmid DNA was eluted in 50µl of EB buffer by 1min centrifugation at 13,000rpm, after 1min incubation on the column.

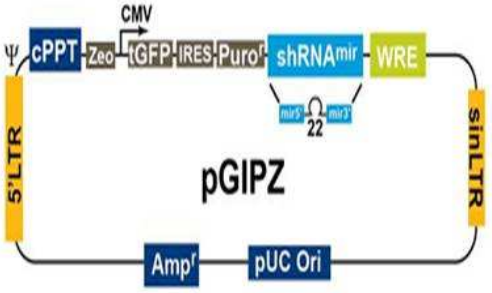
A.



B.

Clone	Mature shRNA sequence	Binding region in LKB1
V2LHS_232235	ATTTATTGCCAAATTTGGG	3′ UTR
V3LHS_639004	TTTTGTGCCGTAACCTCCT	Exon 2
V3LHS_639003	TTCTGCTTCTCTTCGTTGT	Exon 2
V3LHS_645421	AAGAAAATGGAACCAACCA	3′ UTR
Non silencing	ATCTCGCTTGGGCGAGAGTAAG	None

C.



Vector Element	Utility
CMV Promoter	RNA Polymerase II promoter
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
TurboGFP	Marker to track shRNAmir expression
IRES-puro resistance	Mammalian selectable marker
Amp resistance	Ampicillin (carbenicillin) bacterial selectable marker
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance of plasmid in *E. coli*
SIN-LTR	3' Self inactivating long terminal repeat (Shimada, et al. 1995)
RRE	Rev response element
Zeo resistance	Bacterial selectable marker

Figure 2.4: shRNA lentiviral constructs.
A. miR30 primary transcripts as used in Open Biosystems library. B. Sequence of clones and binding region. C. Map of the vector pGIPZ from Open Biosystems shRNA library.

The plasmid DNA concentration and quality was assessed using the NanoDrop. The GIPZ clones were digested using the restriction enzymes XhoI and MluI (Promega, Southampton, UK) in buffer D and BSA for 1h at 37°C to ensure the presence of the hairpin construct in the plasmid amplified by culture.

The larger scale culture was incubated overnight at 37°C under agitation. The plasmid DNA was then collected using the Qiagen Plasmid Maxi kit as instructed by the manufacturer. Briefly, the culture was decanted into 50ml tubes and centrifuged at 4,000rpm for 15min at 4°C. The pellets were then pooled and resuspended in 5ml of buffer P1, and lysed with 10ml of buffer P2. The tubes were inverted for mixing and incubated for 5min at room temperature. Ten ml of pre-chilled buffer P3 were added to neutralise the reaction and the solution was incubated for 20min on ice before centrifugation at 3,500rpm for 30min at 4°C. Meanwhile, the QIAGEN tip columns were equilibrated with 10ml of QBT buffer. The plasmid in the supernatant obtained was bound to the column, washed twice with 30ml of QC buffer and eluted in 15ml of QF buffer. The DNA was then precipitated using 10.5ml of isopropanol. After 30min centrifugation at 4,000rpm, the pellet was washed in 5ml 70% ethanol, centrifuged again for 10min at 4,000rpm and air-dried. The DNA was dissolved in 100 to 500µl of EB buffer. The plasmid of the shRNA clones was sequenced as described in paragraph 2.6.2 to confirm that the hairpin had the expected sequence. The primer used was: 5' - GCATTAAAGCAGCGTATC - 3'. The concentration and quality of the plasmid DNA was evaluated using the NanoDrop as described previously.

2.8.3 Lentivirus production

The 293T cell line was maintained as described in paragraph 2.2, splitted by 1:4 to 1:6 three times a week and never grown over 80% confluence. The day before the transfection, the cells were seeded in a 10cm dish at a density of 3.5 million cells in 8ml of complete medium. On the day of transfection, the plasmid DNA mix was prepared using 12µg of DNA of the p8.91 vector, 4µg of the pMDG vector and 16µg of the GIPZ plasmid. The mix was then added to 5ml of Optimem and filtered through a 0.22µm filter. Five ml of Optimem medium was added as well as 1µl of a stock solution of 10mM PEI. The mix was left to incubate at room temperature for 20min. The cells were gently washed twice with warm Optimem in the mean time. The DNA mix was then added to the cells. After 4h incubation at 37°C and 5% CO_2, the medium was replaced by 25ml of warm complete

medium without antibiotics and incubated overnight. The following morning, the medium was replaced with 8ml of fresh complete medium without antibiotics. The supernatant containing the virus was then collected 48h and 72h after transfection. It was filtered using a 0.45µm filter and aliquoted into 1ml fractions that were stored at -80°C.

2.8.4 Infection and virus titration

The infection of the recipient cells, i.e. the HOB cell line, was performed as follows: 80% confluent cells were harvested, counted and 10^5 cells were plated in one well of a 6-well plate in 500µl of complete medium without antibiotics and 0.5µl of polybrene (Sigma). The cells were incubated for 30min at 37°C in 5% CO_2. Meanwhile, the virus was thawed and diluted appropriately in complete medium without antibiotics in a final volume of 1ml. One µl of polybrene was added to the virus, and incubated for 15min on ice. The virus mix was then added to the cells, using one well per clone; one well without virus was kept as a control. The cells were incubated for 5h to overnight with the virus. The medium was then changed and the cells left to grow in culture for 2 more days. Three days after infection, the presence of green fluorescence in the cells was monitored by direct microscopic observation.

For the titration, the volume of virus used was 1ml (no dilution), 0.3ml, 0.2ml, 0.1ml, and 50µl. Three days after infection, the cells were harvested, washed in PBS and analysed by flow cytometry (FACS) to assess the fraction of GFP positive cells using the CyAn ADP High-Performance Flow Cytometer and the Summit v4.3 software (Beckman Coulter, High Wycombe, UK). The dilution that yielded around 20% of GFP positive cells was selected. This ensured that around one copy of the plasmid was present in every infected cell.

Using the virus dilution optimized by the titration, the HOB cell lines were infected with each of four clones with knocked-down LKB1 and two controls – empty vector (EV) and non-silencing shRNA (NS). The cells were then passaged and selected three days after infection using Puromycin at a concentration of 1µg/ml. This concentration was optimized beforehand by performing a Puromycin kill curve. A control well was seeded to ensure that 100% of the non infected cells were dead after 3 days. The cells were kept on selection for 7 days (i.e. two passages), and their fluorescence after selection was monitored by microscopic observation.

RNA and protein was harvested from the cell lines infected with each of the four clones and the two controls as described in paragraphs 2.5.2 and 2.5.3. The efficiency of the knock-down was then assessed by qRT-PCR and WB to detect whether LKB1 expression was reduced at the mRNA and protein level compared to the controls.

2.9 *LKB1* overexpression by knock-in

2.9.1 Knock-in plasmid

A pBABE plasmid containing the FLAG-tagged human cDNA sequence of either *LKB1* (referred to as LKB1 clone) or of a kinase dead mutant of *LKB1* (referred to as KD clone) under an SV40 promoter (plasmid 8592, Addgene, Cambridge, MA, USA) was used as described previously (Shaw et al. 2004):

http://www.addgene.org/pgvec1?f=c&identifier=8592&atqx=lkb1&cmd=findpl

The plasmid also contained genes for Ampicillin and Puromycin resistance. Its map is given below (Figure 2.5). The protein obtained was the main isoform of LKB1 (Uniprot reference Q15831), as discussed in the Introduction (section 1.2.1.1). The kinase dead mutant contained a single amino acid switch, K78I in exon 1, due to two single nucleotide mutations: A233T and G234C. Its kinase inactivity has already been described (Shaw et al. 2004). The plasmid was transformed in the strain DH5a. The kinase dead mutant was used as a control in the functional assays. An empty vector construct was also prepared but was not used in the functional assays. A retroviral VSVg-envelop expressor plasmid and gag-pol expressor were obtained from Dr Funes (Viral Oncology, Cancer Institute).

2.9.2 Empty vector cloning

The empty vector (referred to as EV clone) was constructed by digesting the KD construct to take out the insert, purifying the open vector, and ligating it back into an empty vector.

Two µg of KD plasmid was digested in triplicate with 1µl of SalI and 1µl of EcoRI in 5µl of buffer 4 (NEB) for a final volume of 50µl for 20min at 37°C. The digested plasmid was run on a 0.8% agarose gel for 2h; the linear vector without the insert was cut out and the DNA extracted with the Qiaquick gel extraction kit as instructed by the manufacturer. Briefly, 750µl of QG solution was added to the agarose gel and incubated for 30min at 50°C. Ten µl of NaAc and 250µl of isopropanol were added and the liquid was loaded onto a Qiaquick column. The DNA was washed with 500µl of QG buffer and then 750µl of PE buffer. The DNA from the triplicates was pooled and eluted in 30µl of EB buffer after 1min incubation on the column.

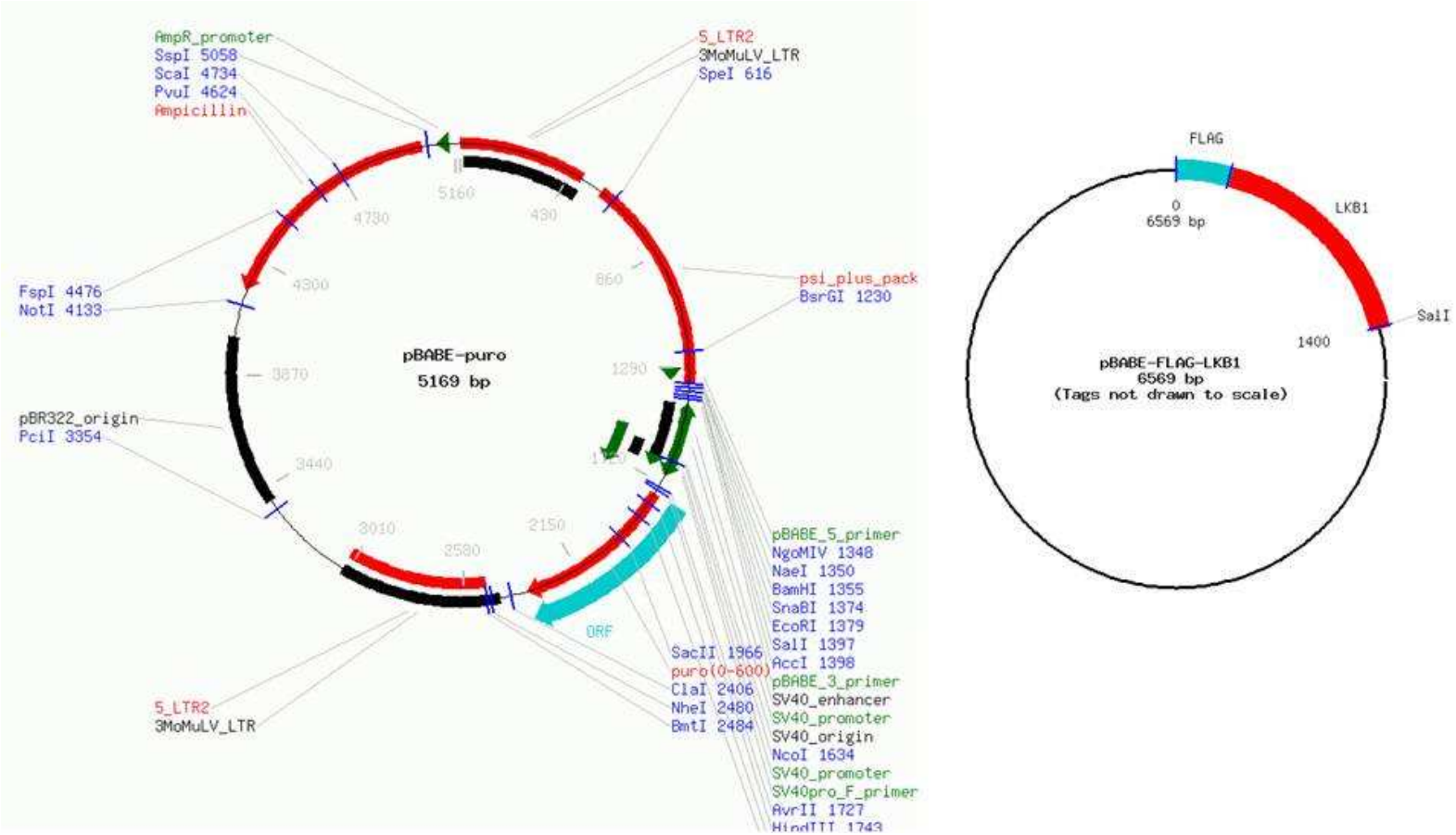


Figure 2.5: Map of the pBABE plasmid and LKB1-pBABE construct used.
Restriction digestion sites on the plasmid map are in blue, primers in green and specific coding regions (such as antibiotic resistance) in red.

The ends of the open plasmid were then blunt-ended as the restriction enzymes were not compatible. One hundred ng of plasmid was digested in a final volume of 20µl in T4 ligase buffer with 1µl of 10µmol of dNTP and 1µl of Klenow enzyme. The mix was incubated for 1h at 37°C and the enzyme was heat-inactivated for 20min at 75°C. The vector was then self-ligated by adding 1µl of T4 ligase to the mix and incubated at 16°C overnight. The ligation product was transformed as described in the next paragraph.

2.9.3 Plasmid production

The LKB1 and KD clones were streaked from the bacterial stab on LB agar supplemented with 100µg/ml of Ampicillin and grown overnight at 37°C. The VsVg and gag-pol plasmid and the EV construct were transformed in One Shot TOP10 chemically competent *E. coli* (Invitrogen) as instructed by the manufacturer. Briefly, 25µl of cells were thawed on ice. One µg of retroviral packaging plasmid or 5µl of ligation product for the EV construct were added to the cells and the tube was gently flicked. The cells were left for 30min on ice, and then heat-shocked at 42°C in a water-bath for 45 sec. They were returned to ice for 5min, added to 900µl of SOC medium and incubated for 1h at 37°C with shaking. The SOC medium was then plated on LB agar at different concentrations and grown overnight at 37°C.

Single colonies were then picked and cultured overnight at 37°C under agitation in 5ml of LB. Half of this culture was used to seed a large scale culture of 200ml of LB supplemented with Ampicillin. The other half was used to create a stock and extract the plasmid DNA using the Qiaprep Spin Miniprep kit (Qiagen), as instructed by the manufacturer (section 2.8.2). The plasmid DNA concentration and quality was assessed by analysis on a NanoDrop. The LKB1 and KD clones were digested using the restriction enzymes SalI and EcoRI (NEB) in buffer 4 for 20min at 37°C to ensure the presence of the insert in the plasmid amplified by culture. The EV clone was digested in buffer 4 and incubated for 10min at 37°C using either BamHI or NotI (NEB), to check the vector had the appropriate size, or SalI and EcoRI (NEB), to check for the absence of the insert.

The larger scale culture was incubated overnight at 37°C under agitation. The plasmid DNA was then collected using the Qiagen Plasmid Maxi kit as instructed by the manufacturer (section 2.8.2). The concentration and quality of the plasmid DNA was evaluated using the NanoDrop and the

sequence of the LKB1 and KD clones were checked by sequencing in both directions using the primers described in Table 2.8.

Primer	Direction	Sequence
pBABE 5'	Forward	CTTTATCCAGCCCTCAC
pBABE 3'	Reverse	ACCCTAACTGACACACATTCC

Table 2.8: Primers used for sequencing the LKB1 insert in the the pBABE plasmid.

2.9.4 Retrovirus production

Retrovirus production was performed similarly to the lentiviral production described in paragraph 2.8.3. The 293T cell line was seeded in a 10cm dish at a density of 3.5 million cells in 8ml of complete medium the day before transfection. On the day of transfection, the plasmid DNA mix was prepared using 4.8µg of the gag-pol plasmid, 1.6µg of the VsVg plasmid and 8µg of the pBABE plasmid (LKB1, KD or EV clones). The mix was then added to 800µl of Optimem and 64µl of a diluted stock of PEI. The mix was left to incubate at room temperature for 20min. The cells were gently washed twice with warm Optimem. The DNA mix was then resuspended in 8ml of warm Optimem and added to the cells. After 5h incubation at 37°C and 5% CO_2 the media was replaced by 8ml of warm complete medium without antibiotics. The supernatant containing the virus was then collected 48h and 72h after transfection. It was filtered using a 0.45µm filter and aliquoted in 1ml fractions that were stored at -80°C.

2.9.5 Infection

The OS cell lines SaOS2, OSA and OST were used as recipient cells for the infection. When the cells reached 80% confluence, they were harvested, counted and $2.5x10^5$ cells for SaOS2 and OSA, $5x10^5$ cells for OST were plated in one well of a 6-well plate for each clone in 1ml of complete medium without antibiotics and 1µl of polybrene. While the cells were incubating for 30min at 37°C in 5% CO_2, the virus was thawed, 1µl of polybrene was added to 1ml of undiluted virus, and it was incubated for 15min on ice. The virus was then added to the cells, with one well per clone

(LKB1, KD or EV) and one control well without virus. The cells were incubated for 5h to overnight with the virus. The medium was changed and the cells were left in culture for two more days. They were then passaged and put on selection in Puromycin at 5µg/ml for OSA and OST, and 7.5µg/ml for SaOS2. No green fluorescent marker was present in the vector, and therefore a titration was performed to ensure that about 20% of the cells survived after 3 days of selection. The cells were cultured until they reached 80% confluence and kept in selection medium for one more passage.

The efficiency of the infection was assessed by qRT-PCR and WB on harvested RNA and protein from the cell lines for each of the clones as described in paragraphs 2.5.2 and 2.5.3.

2.10 Functional assays

To assess the effect of *LKB1* knock-down or knock-in, several functional assays were set up. The assays were performed in normal glucose conditions (4.5mg/ml) – either in DMEM supplemented with 10% FBS or in RPMI for the OSA cell line – in low glucose conditions (1mg/ml) – in DMEM supplemented with 10% FBS – or without glucose and pyruvate – in DMEM supplemented with 10% FBS. The transwell and soft agarose assays were only performed in normal conditions. Cell densities were adapted to the cell line size to have about 80% confluency at the time of the experiment.

2.10.1 Soft agarose assay

To test the ability of cells to form colonies, a soft agarose assay was performed. Sterile 6% low melting point agarose (Sigma) was prepared in distilled water. From this stock solution, 2% agarose was prepared in complete DMEM with glucose. This solution was diluted to coat the bottom of the wells of 6-well plates with 0.6% agarose. The plate was placed at 4°C for 30min to settle and then placed in a 37°C incubator. Cells were harvested and 10^4 cells were seeded in 0.3% agarose as an upper layer. Each condition was performed in triplicate. The plate was left at 4°C for 30min to solidify the agarose, and then placed back in a 37°C and 5% CO_2 incubator. Complete medium with glucose was added the next day in each well and changed regularly.

The plates were monitored several times a week to detect the formation of colonies and kept for up to one month. Upon the detection of colonies, the plates were photographed at two time points with the imaging system G:Box (Syngene, Synopics, Cambridge, UK) and the number of colonies was quantified with the companion software GeneSnap (v7.09.11, Syngene).

2.10.2 Proliferation assays

2.10.2.1 MTS assay

Cells were plated in triplicate in 100µl of medium in a 96-well plate at three increasing densities: 10^3, $2x10^3$ and $3x10^3$ for the HOB cell line, 10^3, $2x10^3$ and $4x10^3$ for the SaOS2 cell line and $2x10^3$, $4x10^3$ and $8x10^3$ for the OST cell line. One triplicate without cells was used for background subtraction for the absorbance reading. The cells were grown in the usual conditions and their proliferation was assessed every 24h from day 1 to day 4 using the MTS assay CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) as instructed by the manufacturer. Briefly, 20µl of MTS solution was added to each well and they were incubated at 37°C and 5% CO_2 for 1h. The reaction was then blocked by adding 25µl of 10% SDS to the cells, and the absorbance at 490nm was measured using a Varioskan Flash plate-reader and its companion software SkanIt (Thermo Scientific).

2.10.2.2 Growth curve

The growth curve of the different cell lines was recorded using a non invasive live cell imaging system within the incubator, the Incucyte FLR (Essen BioScience, Welwyn Garden City, Herts, UK) and its companion software version 2010A (Essen BioScience). A kinetic quantification of the growth of the cells was obtained by recording the average well confluency from at least six points per well. The cells were plated at a density of 10^4 cells per well for the HOB, $2x10^4$ for SaOS2 and OSA, and $4x10^4$ for OST in a 24-well plate, in duplicate for each condition. After 1h, the cells were placed in the Incucyte and the growth was recorded for up to a week with measurements at least every two hours.

2.10.2.3 Cell cycle analysis

A profile of the cell cycle was obtained using propidium iodide (PI) staining. The cells were plated in the appropriate condition in two wells of a 6-well plate with 10^5 cells for HOB, $2x10^5$ cells for SaOS2 or $4x10^5$ for OST. After 2 to 3 days, the cells were harvested by pooling the wells for each condition, washed in PBS and resuspended in 0.5ml of PBS. They were then fixed in 3ml of cold 70% ethanol by adding the PBS solution drop by drop to the ethanol while vortexing. They were stored at -20°C at least overnight. The cells were then centrifuged for 8min at 1800rpm and the ethanol discarded. They were washed in 1ml PBS, resuspended in 500µl of a PI master mix containing 50µg/ml PI (Sigma) and 100µg/ml RNAse A (Sigma) diluted in PBS and incubated for 30min at 37°C. They were then centrifuged again at 1800rpm for 8min and resuspended in PBS. The cell cycle profile was analysed by FACS as described in paragraph 2.8.4. The gating was performed manually. The gates for the G0/G1, S and G2/M phases were set manually in the histogram for the PI area, to ensure that the peak of the G0/G1 region was detected at half of the value of the peak for the G2/M phase. Hence the exact distribution of the population for each phase was not deconvolutionized with a software specialized for cell cycle analysis. The histograms obtained for the different conditions to compare were overlaid before adjusting the gates for the different phases, to ensure that they were all analysed with the same gating.

2.10.2.4 BrdU proliferation assay

Proliferation was recorded using a BrdU proliferation assay, with the FITC BrdU flow kit from BD Pharmingen, as instructed by the manufacturer. Briefly, the OST cell line was plated in three wells at a density of $2x10^5$ cells in 2ml of complete media. After 36h, the cells were pulsed with 20µl of 1mM diluted stock of BrdU (BD Pharmingen) for 1h. The cells were then harvested by pooling the three wells of the same condition, washed in PBS and fixed and permeabilised by 30min incubation on ice in 100µl of BD Cytofix/Cytoperm buffer. They were washed with 1ml of 1X BD Perm/Wash buffer and stored overnight in 1ml PBS. The following day, they were permeabilised by 10min incubation on ice in 100µl of BD Cytoperm Plus buffer, washed with 1ml of Perm/Wash buffer and fixed again by 5min incubation in BD Cytofix/Cytoperm buffer on ice. They were then resuspended in 100µl of a diluted DNAse solution (BD Pharmingen) at a 300µg/ml final concentration and incubated for 1h at 37°C to expose the incorporated BrdU. After a wash in 1ml

of Perm/Wash buffer, the cells were stained for BrdU using the anti-BrdU FITC-labelled antibody from BD, diluted 1:50 in 50µl of Perm/Wash buffer. After 20min incubation at room temperature, they were washed with Perm/Wash buffer and resuspended in 20µl of 7-AAD (BD Pharmingen) and 700µl of PBS. The amount of incorporated BrdU was assessed by recording the fluorescence of the FITC fluorophore by FACS as described in paragraph 2.8.4.

2.10.3 Migration assays

2.10.3.1 Scratch assay

The scratch assay was performed as previously described using the Incucyte to get images of the scratch non-invasively and to record the confluence in the well in the area of the scratch (Liang et al. 2007). Cells were plated in a 24-well plate at near confluence and allowed to grow overnight in complete medium with glucose. Each condition was performed in duplicate on the plate. At confluence, a scratch was performed in each well using Essen BioScience Wound-Maker for 24-well plates. The wells were then washed twice with PBS to remove the floating cells and the appropriate medium for the studied condition was added to the well. The growth in the scratch area was recorded using the Incucyte live-cell imaging system as described in paragraph 2.10.2.2, with images taken at least every two hours.

2.10.3.2 Transwell assay

Cell migration was assessed further using a 24-well transwell assay (Appeleton Wodds, Birmingham, UK) with 8µm transwells. Cells were plated at a density of $8x10^3$ cells for SaOS2 and $1.6x10^4$ for OST in 100µl of complete DMEM without glucose inside each transwell. The bottom of the well contained 1ml of complete DMEM with normal glucose levels. This stimulated the migration of the cells. After an overnight incubation at 37°C and 5% CO_2, the wells were rinsed with PBS and the cells were fixed with 4% paraformaldehyde for 10min at room temperature. The transwells were washed with PBS and the cells were then permeabilised with 0.5% triton-X in PBS for 3min. They were then washed with PBS and stained with Crystal violet for 5min. After 3 to 4 washes, the transwells were mounted under a coverslip and the migrating cells were observed

under an inverse-light microscope. The number of migrating cells was then counted in four different areas for each well.

2.10.4 Anoikis and apoptosis assay

To assess the effect of LKB1 on cell death, anoikis and apoptosis assays were performed. Cells were plated at the same density than for the cell cycle assay (section 2.10.2.3), either as adherent cells (apoptosis assay) or in suspension (anoikis assay) in a 6-well plate with two wells per condition. The suspension culture was obtained by coating the bottom of the well with 2% sterile agarose. After 2 to 3 days, the cells were harvested by pooling both wells of each condition and washed in PBS and stained with Annexin V antibody conjugated with APC (BD Pharmingen) as instructed by the manufacturer. The cells were resuspended in 100µl of 1x binding buffer (BD Pharmingen) and 5µl of Annexin V antibody, and incubated for 15min in the dark at room temperature. Then 200µl of binding buffer and 2µl of a 2mg/ml stock solution of PI were added to the cells and they were analysed within the next hour by FACS as described in paragraph 2.8.4.

2.11 Statistics

All experiments were repeated as independent replicates. Error bars represent the standard deviation from the mean, and mean and standard deviation were calculated using descriptive statistics. Pearson Chi square test (for unpaired categorical data in large cohorts) or Fisher exact test (for unpaired categorical and binomial data in smaller cohorts), Student's t-test (for unpaired continuous and normally distributed data), Mann-Whitney U test (for unpaired continuous and skewed data comparing two groups), one-way ANOVA (for normal unpaired continuous data comparing more than two groups), Kruskall-Wallis test (for skewed unpaired continuous data comparing more than two groups) and Wilcoxon Signed-Rank test (for paired categorical data) were used where appropriate, and differences were considered statistically significant if $p<0.05$. The Shapiro-Wilk test was used to assess the normality of the distribution of the data. The Spearman rho test was used for regression analysis to correlate two not normally distributed continuous sets of data and the Pearson correlation test for normally distributed continuous sets of data. The performance of the markers tested for clinical diagnostic was assessed with the specificity and the sensitivity of the binary classification obtained using the result of the protein expression of the marker, compared to a defined clinical characteristic. The sensitivity was calculated as the ratio of the proportion of people testing positive and with the clinical characteristic compared to the proportion of people testing positive. It represents the probability to have a positive test result given that the patient has the characteristic. The specificity was calculated as the ratio of the proportion of people testing negative and without the clinical characteristic compared to the proportion of people testing negative. It represents the probability to have a negative test result given that the patient does not have the characteristic. The software SPSS version 18.0 (SPSS Inc, Chicago, US) was used for statistical calculations.

Chapter 3: THE ACTIVATION OF THE MTOR PATHWAY IN OSTEOSARCOMA

3.1 Introduction and aim

3.1.1 mTOR pathway and relevance to Osteosarcoma

There is strong evidence suggesting aberrant activation of the mTOR pathway in human OS and therefore Rapamycin and its analogs could be used as therapeutic targets (section 1.2.3.2). However the activation of this pathway has not been confirmed in large cohorts of patients. Moreover, the molecular mechanism responsible for this activation remains unclear, beyond the possible role for ezrin (section 1.2.3.1). The exact mechanism by which ezrin could activate the pathway has not been deciphered, although it is recognised that it can help to integrate extracellular signalling to activate downstream cellular signalling.

There is a lack of markers that can predict response of OS to chemotherapy or the progression of the disease (section 1.1.4.2). Ezrin is one of the most promising markers for association with metastasis, and has been detected in many screening studies both at the mRNA (Leonard et al. 2003) and protein level (Folio et al. 2009). As ezrin action is mainly mediated through the mTOR pathway (Khanna et al. 2004), there may be other molecular markers within this pathway that could be used as prognostic markers or help in the design of targeted therapy.

3.1.2 Aim and objectives

The aim in this chapter was to assess mTOR pathway activation and to evaluate the usefulness of mTOR inhibitors in OS therapy. To do so, the presence and/or the phosphorylation of the key molecules of the pathway were investigated. The objectives were:

1. Assess the activation status of a variety of molecules in the pathway
2. Decipher possible mechanisms of constitutive activation of the pathway
3. Assess whether these results could be used to identify an informative prognostic biomarker

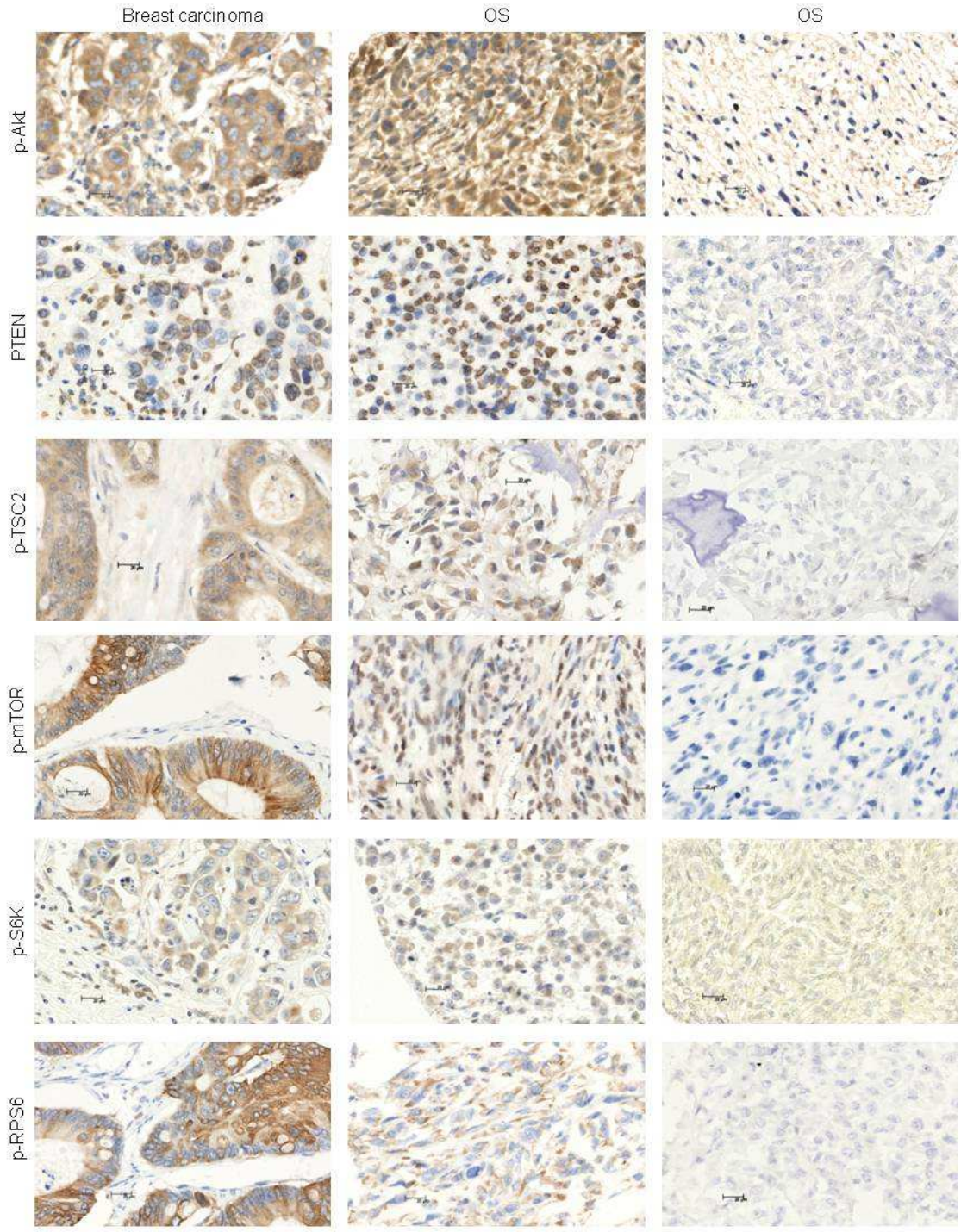
3.2 mTOR pathway activation in Osteosarcoma

3.2.1 IHC results and protein activation

The presence and/or phosphorylation of key molecules of this pathway were studied by IHC as described in section 2.4.2 and as previously published (Presneau et al. 2009). The role of the molecules studied and of their eventual phosphorylation sites (p-Akt (Thr^{308}), PTEN, TSC1, TSC2 and p-TSC2 (Thr^{1462}), mTOR and p-mTOR (Ser^{2448}), S6K and p-S6K (Thr^{389}), RPS6 and p-RPS6 ($Ser^{235/236}$), p-4EBP1 (Thr^{70}) and eIF4E) have been detailed in paragraph 1.2.2.1. Two TMAs generated from 162 resections, with 69 PR and 27 GR were used (section 2.4.1).

3.2.1.1 Summary of the IHC results

All the proteins screened showed a strong immunoreactivity in the positive control: the immunoreactivity of all of the antibodies was mainly cytoplasmic, except for the one to PTEN, which showed nuclear reactivity. Representative positive and negative cases by IHC are presented in Figure 3.1 below. Most cases showed activation of Akt (154 of 159, 97%). PTEN was absent in 46 of 140 (33%) cases, and this could account for constitutive activation of the pathway in these cases, (section 3.4.4.1). Disruption of the TSC1/2 complex was found in more than 60% of the cases as shown by phosphorylation of Thr^{1462} in 91 of 136 (67%) cases and by the absence of the TSC1 protein in 63 of 139 (45%) cases. mTOR activation by phosphorylation of Ser^{2448} was detected in 47 of 146 (32%) cases and its downstream targets S6K and 4EBP1 were activated in 108 of 156 (69%) and 130 of 133 (98%) cases respectively. S6K's target RPS6 was activated in 106 of 148 (72%) cases and eIF4E was detected in 127 of 141 (90%) cases. The IHC results are summarised in Table 3.1 below.



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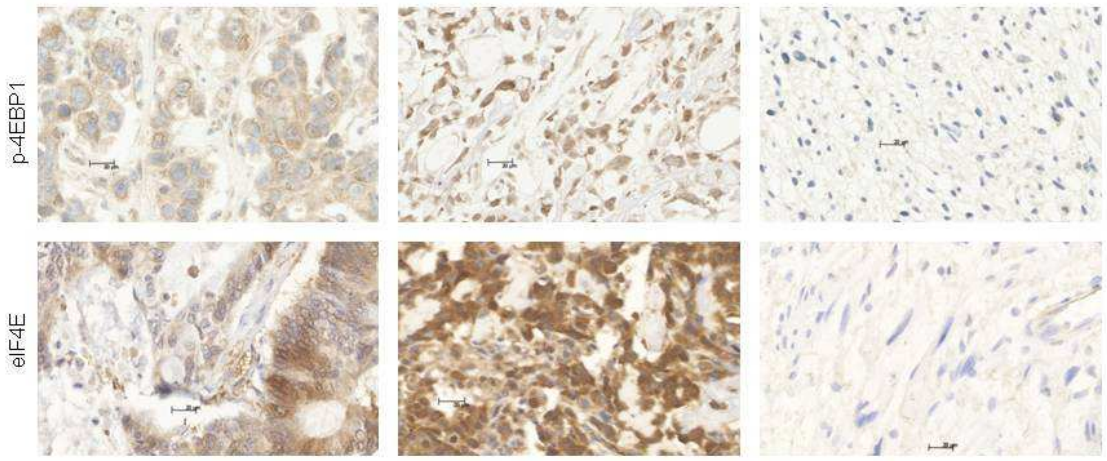


Figure 3.1: Transmitted light photomicrographs of the IHC for the key molecules used to assess the activation of the mTOR pathway.
The images are from the scanned TMAs. The antibody is indicated on the left. The first column represents the positive controls (breast carcinoma). The center panel shows representative OS cases with positive reactivity and the right hand panel representative OS negative for immunoreactivity. The bar corresponds to 20µm (magnification x40).

Protein	Ratio of positive cases for protein phosphorylation (%)	Ratio of positive cases for total protein expression (%)
PTEN	NA	94 / 140 (67%)
Akt	154 / 159 (97%)	NA
TSC2	91 / 136 (67%)	139 / 144 (97%)
TSC1	76 / 139 (55%)	NA
mTOR	47 / 146 (32%)	127 / 144 (88%)
S6K	108 / 156 (69%)	143 / 144 (99%)
RPS6	106 / 148 (72%)	121 / 145 (83%)
4EBP1	130 / 133 (98%)	NA
eIF4E	NA	127 / 141 (90%)
Pathway activation	**137 / 158 (87%)**	

Table 3.1: Summary of the IHC scoring results obtained for all cases and all antibodies tested.
A case is considered to have activation of the pathway if p-S6K, p-RPS6 or p-mTOR are detected.

3.2.1.2 Relationship between 4EBP1 activation and eIF4E presence

The activation of 4EBP1 was detected in a large majority of the cases. eIF4E is released following phosphorylation of 4EBP1, as detailed in the Introduction (section 1.2.2.1). Both p-4EBP1 and eIF4E were expressed in 105 of 119 (88%) cases and at least one of them was expressed in 152 of 155 (98%). This arm of the mTOR pathway was activated in virtually all OS cases screened.

3.2.1.3 Relationship between S6K and RPS6 activation

Activation of S6K and RPS6, the biomarkers mostly monitored to assess the activation of the mTOR pathway in the literature (MacKenzie et al. 2007, Presneau et al. 2009), was found in approximately two third of the cases. Activation of both molecules was detected in 81 of 146 (55%) cases and of either in 133 of 158 (84%). Similarly, a wide majority of the cases showed activation of this arm of the pathway, but to a lesser extent than the 4EBP1 one.

3.2.1.4 Relationship between mTOR, S6K and RPS6 activation

The activation of mTOR was detected in 32% of the cases. Concomitant mTOR and S6K activation was present in 44 of 146 (32%) cases. The phosphorylation of either mTOR or S6K was observed in 121 of 158 (77%) cases and only 4 cases with mTOR activation showed no immunoreactivity for p-RPS6 or p-S6K. Overall, 137 of 158 (87%) cases expressed p-mTOR, p-S6K or p-RPS6, and expression of one of these three markers was used to define the pathway as activated.

3.2.1.5 Relationship between p-TSC2 expression and pathway activation

Since virtually all cases were immunoreactive for p-Akt, only the detection of p-TSC2 could indicate whether the activation of the pathway was mediated by Akt, via the inhibition of the TSC complex. p-TSC2 immunoreactivity was detected in 88 of 120 (73%) informative cases showing pathway activation. Hence, in three out of four cases, activation of the pathway could be directly attributed to Akt. The relationship between p-TSC2 expression and pathway

activation was highly statistically significant ($p=9.49x10^{-7}$ with the Wilcoxon signed-rank test). Cases with p-TSC2 expression were 1.36 times more likely to present with activation of the pathway (95% confidence interval [1.17; 1.48]).

3.2.1.6 Conclusion

The mTOR pathway is activated in a vast majority of OS cases, as shown by the phosphorylation of mTOR, S6K or RPS6 in 87% of our cohort (Figure 3.2).

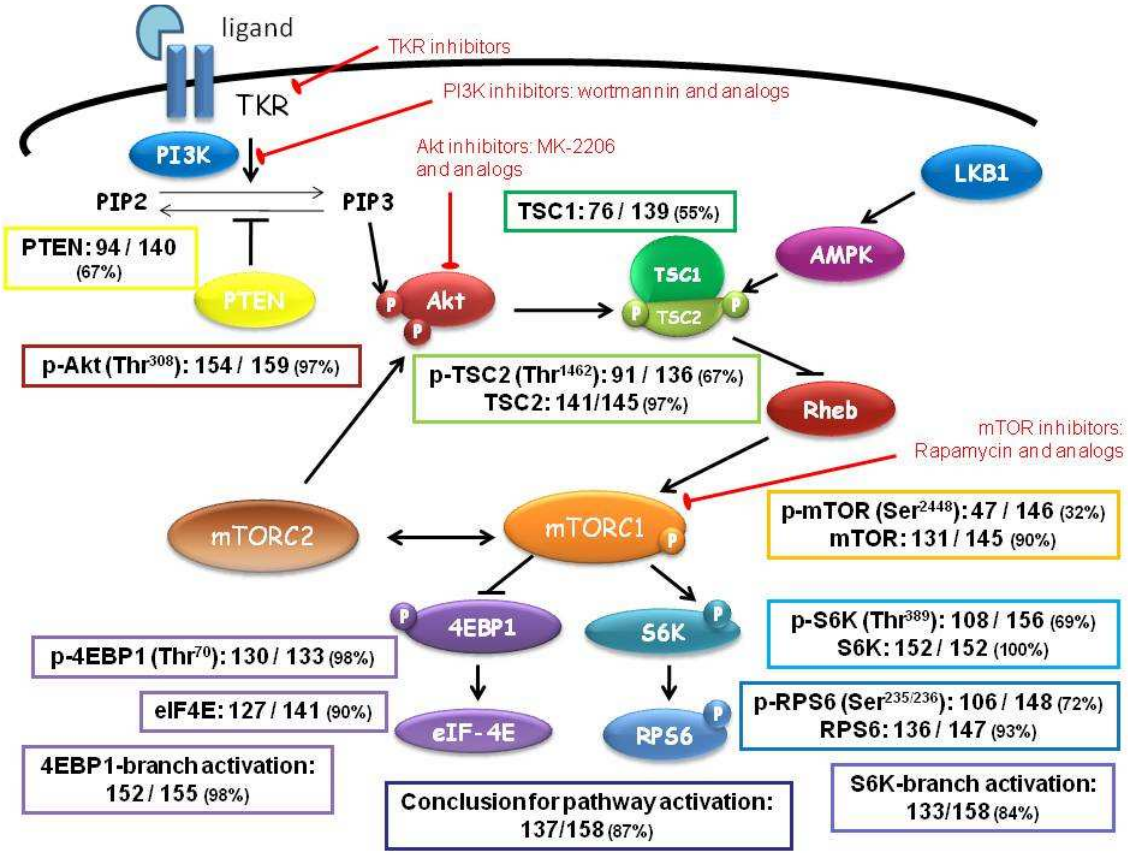


Figure 3.2: Scheme of the mTOR pathway including the IHC results obtained in 158 OS cases.
Results are indicated as a ratio of positive immunoreactivity compared to the total number of informative cases screened (and the equivalent percentage), either for the phosphorylated (p-) or the total protein. Note: the ratio of total protein was corrected to take into account false negative cases.

3.2.2 Constitutive activation of the mTOR pathway

3.2.2.1 Phosphorylated versus total protein expression

To investigate the reason for the absence of phosphorylation of TSC2, mTOR, RPS6 and S6K in a subset of cases, the total protein expression for these molecules was assessed on the same TMAs. Total TSC2, mTOR, S6K and RPS6 was detected in 139 of 144 (97%), 127 of 144 (88%), 143 of 144 (99%) and 121 of 145 (83%) cases respectively (Table 3.1). However, when these numbers were corrected to take into account the false negative cases (cases that were either negative or uninformative for the total protein but positive for the phosphorylated protein), the total protein was considered to be present in 141 of 145 (97%) cases for TSC2, 131 of 145 (90%) for mTOR, 152 of 152 (100%) for S6K and 136 of 147 (93%) for RPS6 (Figure 3.3). Therefore, the total protein was detected in most cases and eventual genetic abnormalities were not studied further.

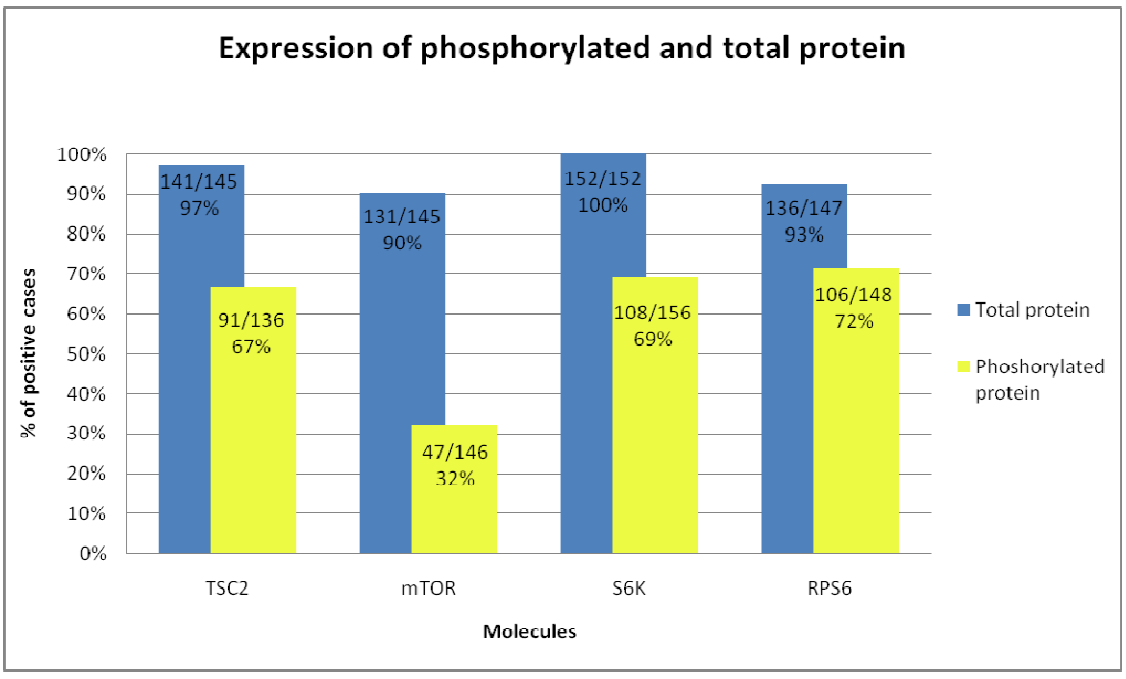


Figure 3.3: Graph illustrating the expression of phosphorylated protein compared to their total protein expression in OS cases.
The ratio of positive cases over all informative ones and the equivalent percentages are given in the graph. The number of cases expressing the total protein was corrected to take into account the false negative cases.

3.2.2.2 Relationship between TSC1 and TSC2 expression: potential loss of the TSC complex

For mTOR to be activated, the TSC complex needs to be repressed, as detailed in paragraph 1.2.2.1. This can be achieved by phosphorylation of TSC2 by Akt; constitutive activation of Akt would then lead to a constitutive activation of mTOR. However, absence of the TSC2 or TSC1 protein have the same effect, by preventing the formation of the TSC complex. Although TSC2 was present in virtually all cases, TSC1 was not detected in 63 of 139 (45%) cases. Out of the 38 cases showing no p-TSC2 immunoreactivity but expression of total TSC2 protein, 19 lacked TSC1 expression. However, p-TSC2 was detected in the absence of TSC1 immunoreactivity in 37 of 127 (30%) cases.

3.2.2.3 RPS6 protein expression

The locus for *RPS6* (9p22.1) is in close vicinity to the locus of *CKDN2A* (9p21.3), coding for the p16 protein. The latter has been reported as lost in OS (Kansara et al. 2007) as detailed in the Introduction (section 1.1.5.1). We therefore screened for p16 by IHC for the 11 cases negative for total RPS6. This was performed by UCL Advanced Diagnostics, Department of Pathology, UCL. Nine of the 11 cases were informative; 3 lacked p16 protein expression, and 6 expressed the protein. Hence a small subset of OS tumours may have lost both the *CDKN2A* and *RPS6* gene region, accounting for the lack of immunoreactivity observed. As only a very small subset of the cases presented this abnormality, it was not studied further.

3.3 Biomarkers for prognosis

3.3.1 Identification of a prognostic marker

The best prognostic marker to date is the patient's response to chemotherapy (section 1.1.4.2). Predicting this response before treatment would be very useful. Therefore, the expression of candidate biomarkers was studied in the tumours which showed a good (GR) and poor (PR) response to chemotherapy.

3.3.1.1 p-TSC2 is a candidate prognostic marker

The IHC results for the total and phosphorylated proteins that were screened in PR compared to GR is illustrated in Figure 3.4. Statistically significant differences between the two groups were found for p-TSC2 (p= 0.0005 with the one-tailed Fisher exact test) and total RPS6 (p= 0.048). However, the difference in total RPS6 immunoreactivity between PR and GR was only barely statistically significant, with a p-value was very close to 0.05. Additionally, when taking into account the false negative cases (cases positive for p-RPS6 and either uninformative or negative for RPS6) the total protein expression was 63 of 69 (91%) for the PR and 23 of 23 (100%) for the GR. This difference was not significant. Hence, the previous result for total RPS6 immunoreactivity was considered to be an artifact. Only the use of p-TSC2 as a prognostic marker was investigated further.

3.3.1.2 Validation of p-TSC2 in a larger cohort of patients

To confirm the results obtained with p-TSC2 as a predictor of patient response to chemotherapy, a larger cohort of patient was screened using the additional TMAs prepared, with a total of 267 cases including 109 PR and 70 GR. Their clinical data are presented in Figure 2.2 in the Materials and Methods chapter. There were 30 of 267 (11%) non-informative cases (11%); p-TSC2 was positive in 135 of 237 (60%) informative cases.

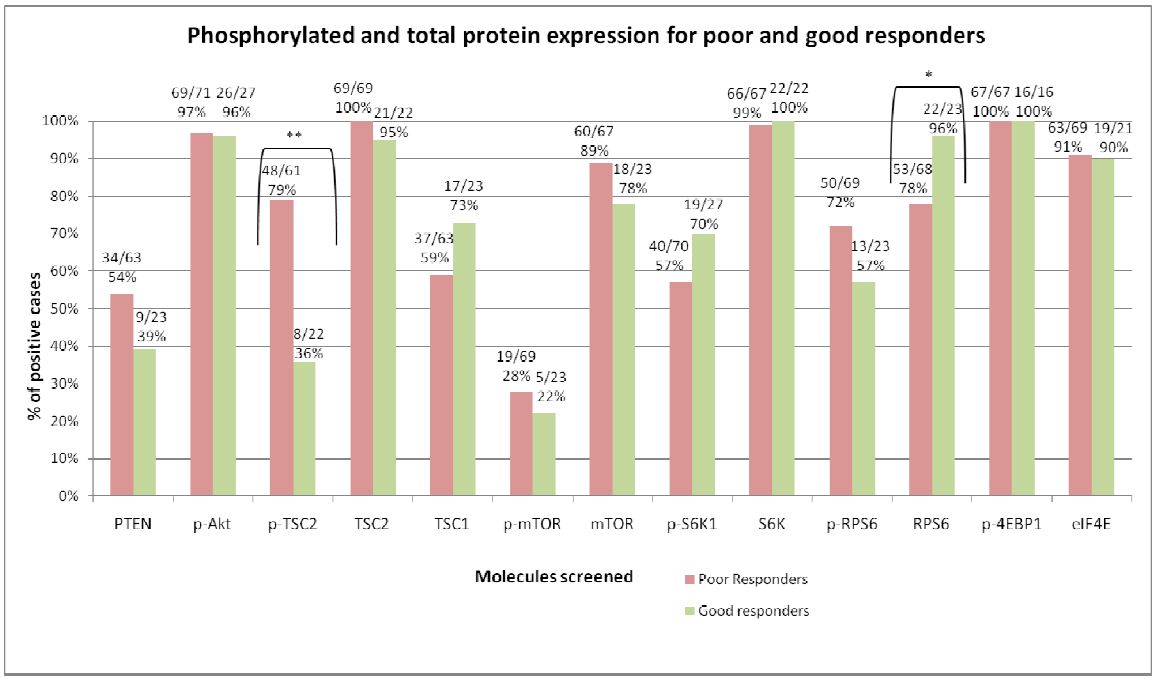


Figure 3.4: Graph showing the immunoreactivity of the phosphorylated and total proteins screened for good and poor responders.
* for p<0.05 and ** for p<0.01 with the one-tailed Fisher exact test.

There was a significant association between p-TSC2 expression and the chemotherapy status of the patient (p=0.009 with the Chi Square test). This included the following categories: PR, GR, no chemotherapy treatment or previous chemotherapy treatment received in earlier disease presentation. When restricted to the PR and GR categories, 57 of 97 (59%) PR expressed p-TSC2 compared to 25 of 63 (40%) GR, and there was a significant difference between those two groups (p=0.018 with the Chi square test), as illustrated in Figure 3.5. In other words, the relative risk of responding poorly to chemotherapy is 1.35 times higher if p-TSC2 is detected (95% confidence interval [1.05; 1.74]). Using p-TSC2 to predict the response to chemotherapy would give a test's sensitivity of 58.8% (95% confidence interval [52.3%; 64.6%]) and a specificity of 60.3% (95% confidence interval [50.7%; 65.3%]). This was consistent with the results found in the smaller cohort of patients.

3.3.1.3 Relationship between p-TSC2 and other clinical data

No association with p-TSC2 was found for patient age and sex, or tumour site, histopathological subtype or grade. Patients presenting with recurrent or metastatic disease were then compared to patients without disease progression reported (Figure 3.5): 46 of 66 (70%) tumours in patients with progressive disease expressed p-TSC2 compared to 89 of 172 (52%) without (p=0.012 with the Chi Square test). Hence, the relative risk of disease progression was found to be 1.76 times higher when p-TSC2 was detected (95% confidence interval [1.13; 2.79]). Using p-TSC2 as a predictive marker for disease progression would give a test's sensitivity of 69.7% (95% confidence interval [59.6%; 78.5%]) and a specificity of 48.3% (95% confidence interval [44.4%; 51.6%]). To conclude, expression of p-TSC2 was associated with a worse prognosis.

3.3.2 Pathway activation and clinical setting

The relationship between the activation of the mTOR pathway (p-mTOR, p-S6K or p-RPS6 immunoreactivity) in the 162 cases screened and their clinical characteristics as presented in the Materials and Methods chapter (Figure 2.1) was investigated. No relationship was found with most of the clinical features (patient sex and age, chemotherapy treatment, tumour grade, subtype and site) as assessed using the Chi square or one-tailed Fisher exact tests. No difference was found either between patients with a poor and a good response to chemotherapy: 58 of 69 (84%) PR had pathway activation, compared to 22 of 27 (81%) GR. However, a relationship was found between the pathway activation and disease progression: patients with reported recurrence or metastasis had activation of the pathway in 48 of 51 (94%) cases, whereas 89 of 107 (83%) cases had pathway activation in the absence of disease progression at the time of the study (Figure 3.5). Although small, this difference is statistically significant (p=0.045 with the one-tailed Fisher exact test). The relative risk of presenting with progressive disease is 2.45 times higher if the pathway is activated (95% confidence interval [0.98; 7.21]). Using the pathway activation as prognostic test for disease progression would give a sensitivity of 94.4% (95% confidence interval [86.4%; 97.9%]) and a specificity of 16.8% (95% confidence interval [13.2%; 18.6%]).

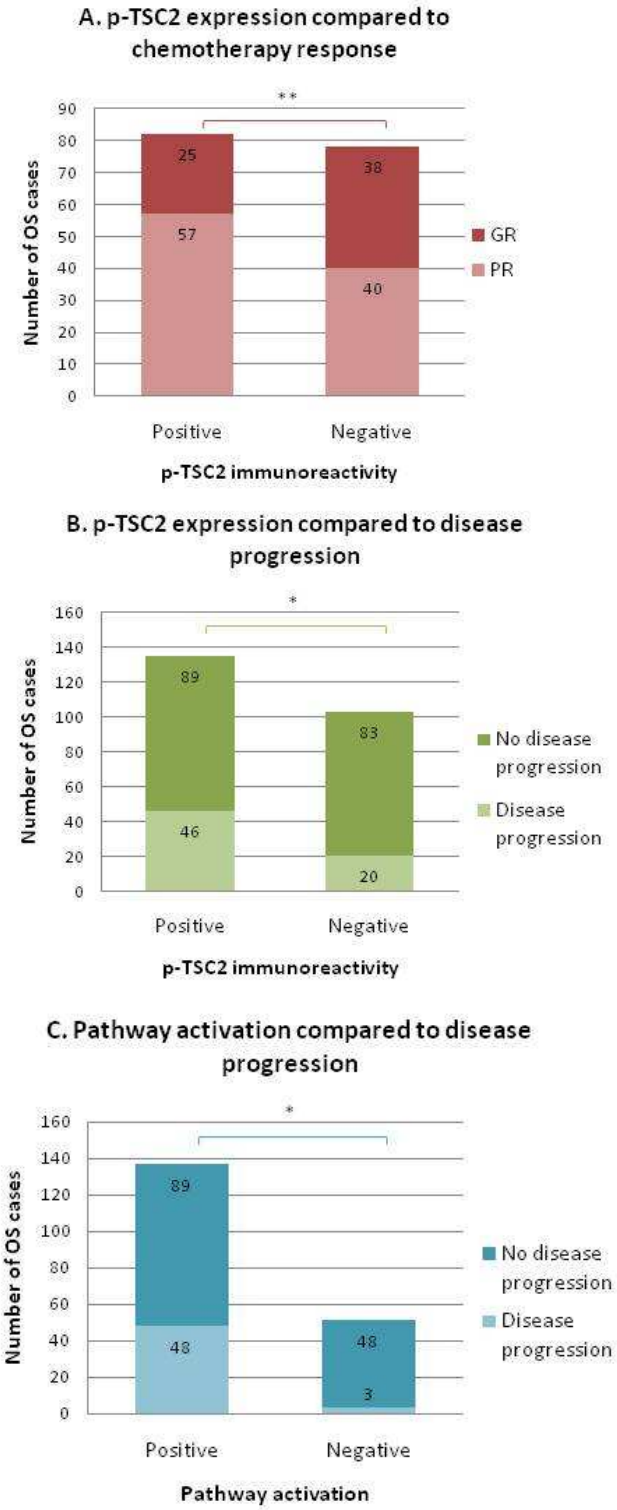


Figure 3.5: Bar chart illustrating the relationship between p-TSC2 expression (A. and B.) and mTOR pathway (C.) activation with the clinical data.
* for $p<0.05$ and ** for $p<0.01$ with the Chi square test.

3.4 Discussion: role of the mTOR pathway in diagnosis and treatment of Osteosarcoma

In this chapter, 162 OS cases were used to evaluate the activation of the mTOR pathway in OS; 87% of them were immunoreactive for p-mTOR, p-S6K or p-RPS6, hereby showing activation of the pathway. The technical limitations and the perspectives for implementing these results in diagnostic tests or therapeutic strategies are discussed here.

3.4.1 Biomarkers selected and technical limitations

3.4.1.1 Technical limitations in phospho-protein detection

As detailed in paragraph 3.2.2.1, mTOR, TSC2, S6K and RPS6 total protein was detected in most cases, even in the absence of phosphorylation. This indicates that the loss of immunoreactivity for the phospho-protein is not caused by a loss of the total protein.

The detection of large phospho-proteins like mTOR (289kDa) by IHC is technically challenging (Tkaczyk et al. 2002), and the phospho-epitope may be lost during tissue processing. This may explain the low number of mTOR positive cases compared to the downstream targets. This can also account for some of the negative results found for TSC2, S6K and RPS6. Moreover, phosphorylation is a transient mechanism (Riemenschneider et al. 2006) and IHC does not reflect the dynamism of these processes. This may explain the discrepancy found between the different molecules, as 55% of cases had activation of both S6K and RPS6, compared to 84% with one or the other of them. Moreover, as phosphorylation of TSC2 is known to accelerate its degradation by a ubiquitin-mediated mechanism (Plas et al. 2003), the proportion of p-TSC2 positive cases may also be underestimated.

The phosphorylation sites chosen for analysis of mTOR, TSC2, S6K and RPS6 were the most commonly reported ones (section 1.2.1.1). However, the study is not exhaustive and phosphorylation of other sites may occur (Cheng et al. 2004, Nellist et al. 2005). This may account for the discrepancies found between total and phospho-protein and between the different biomarkers, especially for p-TSC2 ; this will be developed further in paragraph 3.4.4.4.

To conclude, due to the technical limitations listed above, it is preferable to assess the status of several markers in parallel to determine the activation of the pathway, as was performed in this study. However this may not be practical in a clinical setting.

3.4.1.2 Detection of phospho and total mTOR

The most striking difference between total and phospho-protein expression is seen in mTOR where total mTOR was expressed in 90% of the cases, whereas only 32% were immunoreactive for p-mTOR (Ser^{2448}). As 67% of cases were positive for p-TSC2 and 69% for p-S6K, the molecules upstream and downstream of mTOR respectively, it would have been expected that close to two third of the cases would show mTOR activation. Such discrepancies have been reported by others in renal cell carcinoma and chordoma (Presneau et al. 2009, Youssif et al. 2010).

It is of note that the role of the phosphorylation site Ser^{2448} on mTOR is not well understood (section 1.2.2.1). This phosphorylation is thought to be a feedback signal from S6K and not a result of its activation by Rheb (Chiang et al. 2005). mTOR mutants with an alanine or a glycine replacing Ser^{2448} are still functional. It implies that cases lacking immunoreactivity for p-mTOR may still have the pathway activated.

Moreover, p-mTOR cannot be used as a biomarker to classify patient potentially responsive to Rapamycin. It has been suggested that both mTORC1, which is Rapamycin sensitive, and mTORC2, which is Rapamycin insensitive, can present phosphorylation at Ser^{2448} (Rosner et al. 2010). However, this was not confirmed by another study which found that mTORC2 is phosphorylated at Ser^{2481} rather than at Ser^{2448}(Copp et al. 2009). The detection of total mTOR does not predict patient's response to Rapamycin treatment either, as it cannot distinguish the mTORC1 or 2 complexes.

To conclude, IHC on mTOR is not fully informative for classifying the patients with pathway activation or responsive to Rapamycin or its analogs. The development of mTOR inhibitors active on both complexes, the mTOR blocking agents or TORKinibs, will overcome this problem (Feldman et al. 2009).

3.4.1.3 Pathway activation: which biomarker to select?

Due to technical limitations and to the poorly known role of mTOR phosphorylation, p-mTOR has rarely been included in studies assessing the activation of the mTOR pathway (El-Salem et al. 2007, Gao et al. 2003, Krishnan et al. 2006, Scheper et al. 2008, Wan et al. 2005) ; the use of pS6K (Thr^{289}) is often preferred (Boulay et al. 2004, MacKenzie et al. 2007, Xu et al. 2004). As RSP6 can be activated by pathways other than mTOR, such as the RAS/MEK/ERK pathway via p90S6K (i.e. RSK1) (Roux et al. 2004), it is not usually used as a biomarker for mTOR pathway activation. However, a recent study has shown that p-RPS6 ($Ser^{235/236}$) was a predictive marker of tumour response to mTOR inhibitor treatment in a small cohort of patients that included 3 OS (Iwenofu et al. 2008). This study needs to be confirmed on a larger cohort of cases, but it nonetheless indicates that RPS6 is a potential biomarker for our cohort. Therefore and as the technical limitations also favour the use of multiple markers in IHC screening, the pathway activation was based on the presence of p-mTOR, p-S6K or p-RPS6.

3.4.2 Use of biomarkers in the mTOR pathway as prognostic markers

3.4.2.1 Pathway activation and disease progression

The activation of the pathway did not correlate with response to chemotherapy; this suggests that both PR and GR could benefit from a treatment with mTOR inhibitors. The mTOR pathway is unlikely to play a role in the mechanism of resistance to chemotherapy in OS.

Previous studies from the group of Pr Khanna have shown that activation of the mTOR pathway was associated with metastasis in OS when ezrin was expressed (Wan et al. 2005). A recent study has also demonstrated that total mTOR and total S6K expression was associated with disease progression and prognosis (Zhou et al. 2009). Zhou et al only looked at total protein expression and not protein activation, and studied a relatively small cohort (65 patients), so these results are not fully conclusive. Our data confirmed the association of pathway activation and disease progression in a larger cohort of patients and focused on protein activation rather than expression. In the cases without disease progression reported at the time of the study, 89 of 107 (83%) showed activation of the pathway. As some of those cases were treated in the last

5 years, it possibly includes patients that will present with progressive disease in the near future. These results need to be followed-up in the coming years.

Virtually all cases with disease progression displayed an activated pathway (48 of 51, 94%). Nevertheless, there is a large overlap between patient with and without disease progression and pathway activation (Figure 3.5). The confidence interval found for the relative risk is very wide, and is barely over 1, confirming that the relationship is weakly significant. This result needs to be confirmed on a larger cohort of cases, as only 51 patients had progressive disease in this study. The activation of the pathway may be associated with an increased risk for progressive disease, but it cannot be used as a prognostic marker in the clinic as a high sensitivity (e.g low proportion of false negative) and a high specificity (e.g a low proportion of false positive) are required for this application.

3.4.2.2 p-TSC2 as a predictor of chemotherapy response or disease relapse

The main prognostic marker identified in this study is p-TSC2 (Thr^{1462}), which was found more frequently in PR than in GR (59% versus 40% respectively, p=0.018). The activation of p-TSC2 was also associated with disease progression. The TSC complex integrates the regulation of the mTOR pathway via the Akt or the LKB1 branches. The differences observed for p-TSC2 between these groups were not caused by differences in total TSC1 or TSC2 expression, as no association for these markers was found in PR compared to GR. These results underline that inactivation of p-TSC2 by Akt is associated with a worse prognosis. The IHC results reported in this chapter give a better understanding of the activation of the pathway in PR versus GR, but they have not fully uncovered the mechanism explaining these differences (section 3.3.1).

There is again a large overlap between the PR and GR groups positive for p-TSC2, as for the groups with or without progressive disease (Figure 3.5). The 95% confidence intervals of the relative risk of either being a PR or having progressive disease if p-TSC2 is detected are relatively narrow, as a larger cohort of cases was used for this study. Nevertheless, testing for p-TSC2 as a tool for chemotherapy response or disease progression gives a specificity and a sensitivity below 80%, which is insufficient. Therefore the presence of p-TSC2 is associated with a higher risk of poor response to chemotherapy and of disease progression, giving an insight

into the pathogenesis of OS. But this biomarker cannot be used as a routine tool to classify patients.

3.4.3 Potential use of mTOR inhibitors for Osteosarcoma treatment

As outlined in the Introduction, mTOR inhibitors have shown their potential in OS treatment *in vitro* (Gazitt et al. 2009), *in vivo* (Houghton et al. 2008, Paoloni et al. 2010) and in phase II clinical trials (MacKenzie et al. 2007, Manara et al. 2010, Staddon et al. 2006). However, these studies never included a large cohort of human patients with OS. Our work shows that the pathway is indeed activated in a majority of patients. The use of p-mTOR, p-S6K or p-RPS6 as biomarkers for pathway activation suggests that at least 87% of our cohort would be responsive to mTOR inhibitors. Total mTOR was only absent in 14 of 144 (9.7%) cases and half of those cases showed activation of p-S6K, suggesting that absence of the protein was due to technical limitations. Hence 7 of 144 (4.9%) cases in our cohort are unlikely to be responsive to mTOR inhibitors.

Because Rapamycin and its analogs have shown only partial response in preclinical studies, as they induce cell cycle arrest but not necessarily apoptosis, the use of combination agents has been recommended. Akt was activated in virtually all of our cases, so a combination of Rapamycin and Akt inhibitors could be an option for OS treatment for most patients. Understanding the mechanism leading to the constitutive activation of the pathway will help to optimise further the combination treatment to give to patient with OS, as will be developed in the next paragraph.

3.4.4 Rationale for constitutive pathway activation and other mTOR combination therapies

Assessing the presence of constitutive activation of the mTOR pathway is essential if mTOR inhibitors are to be used in OS treatment. Knowing the genetic background in the pathway will help to improve design of mTOR targeted therapy. Although the study of total mTOR, TSC2, S6K and RPS6 did not show loss of expression that could either account for or rule out constitutive pathway activation, the examination of other biomarkers can be informative and may suggest that other genetic abnormalities are in place. A limitation of this work is that it only focused on protein expression by IHC. This does not rule out the presence of inactivating mutations in the proteins detected. However, as large numbers of OS are currently being sequenced as part of the Human Cancer Genome Project in the Sanger Institute, this aspect was not investigated further.

3.4.4.1 Role of PTEN

Constitutive activation of the mTOR pathway can be achieved by loss of the tumour suppressor gene *PTEN*. Copy number loss of *PTEN* has been reported previously in human OS (Freeman et al. 2008) and in canine OS (Levine et al. 2002) as detailed in the Introduction (section 1.2.3.2). Our data confirm this finding, as 33% of the cases lacked expression of this protein by IHC; 43 of 46 (93%) cases negative for PTEN expression were immunoreactive for p-Akt, as expected. Pathway activation was also present in 32 of 46 (70%) of those cases. As 14 cases showed both loss of PTEN and absence of pathway activation, additional regulatory mechanisms are to be considered downstream of PTEN. Overall, PTEN loss probably accounts for the constitutive pathway activation in 32 of 137 (23%) of the cases studied. These cases would also benefit from combined mTOR and Akt inhibitor therapy.

Interestingly, PTEN has been reported to be phosphorylated by LKB1 (Mehenni et al. 2005a). Although the nature of the interaction between PTEN and LKB1 is unknown, loss of LKB1 could affect the role of PTEN in inhibiting the mTOR pathway. It would therefore be interesting to assess further the role of LKB1 in OS.

3.4.4.2 Role of TKR activation

Activation of TKR can also lead to constitutive activation of the mTOR pathway. This has been reported in OS, and in particular EGFR amplification has been found in some cases (Freeman et al. 2008). It would therefore be interesting to test for amplification or activating mutations in TKR, as they may explain the activation of the mTOR pathway in a proportion of patients. The role of autocrine growth stimulation has also been shown by Mori et al., in a study using conditioned media from mouse OS cell lines (Mori et al. 2008). This suggests that even in the absence of activating mutations, combination therapy of TKR inhibitors and Rapamycin could be an interesting treatment strategy.

3.4.4.3 Ezrin upregulation

Ezrin expression and its correlation to metastasis in OS have been found in many studies (Folio et al. 2009, Kim et al. 2007, Leonard et al. 2003, Ogino et al. 2007, Salas et al. 2007), and were therefore not included in this study. As ezrin acts through the mTOR pathway, its upregulation in metastatic OS could explain why these cases have a constitutive activation of the pathway. However, as the link between ezrin and mTOR activation is not fully understood, this remains speculative. The relationship found between pathway activation and disease progression infers that targeting both ezrin with sorafenib and the mTOR pathway with Rapamycin could be a successful therapeutic strategy. This needs to be investigated further.

3.4.4.4 The role of the TSC complex in mTOR pathway activation

Presence of the complex

As discussed in paragraph 3.4.2.2, the TSC complex seems to have a central role in the regulation of the pathway. Loss of the complex would lead to constitutive activation of the pathway. TSC2 was present in most cases, but TSC1 may play a role in OS pathogenesis as one case with a mutation in this gene was reported in COSMIC (section 1.2.3.2). Loss of TSC1 protein expression has also been observed in 45% of our cases as presented in paragraph 3.2.2.2. There was a discrepancy in the results as 37 of 62 (59%) of the TSC1 negative cases were immunoreactive for p-TSC2. Detection of TSC1 total protein by IHC is technically

challenging, and similar results were found in another study from our group on chordoma (Presneau et al. 2009): TSC1 was detected in 22 of 48 (46%) chordoma cases, but analysis by FISH failed to unveil any deletion of the *TSC1* locus. TSC1 loss may account for a constitutive activation of the pathway but only in the subset of cases negative for p-TSC2, i.e. in 13 of 137 (9.5%). The screening of more OS cases as part of the Cancer Genome Project will also help to evaluate whether TSC1 loss by mutation, which could lead to absence of expression if the epitope for the antibody is lost, is really a frequent abnormality.

Regulation of the TSC complex by other pathways: a role of LKB1?

This study has focused on the Akt-branch of the mTOR signalling pathway (Inoki et al. 2002), and 88 of 120 (73%) cases showed that the mTOR pathway was activated via this branch. However, TSC2 can integrate the signalling from both LKB1 and Akt. As described in the Introduction (section 1.2.2.1), TSC2 can also be activated by AMPK in energy-deprived conditions (Inoki et al. 2003), hereby inhibiting the activation of the mTOR pathway. Hence TSC2 integrates an activation signal of AMPK and the de-activation signal from Akt (Inoki et al. 2009). Absence of LKB1 would prevent the activation of TSC2 by AMPK. Although the equilibrium between the activating and inactivating phosphorylation status of TSC2 is not fully deciphered, loss of LKB1 could induce constitutive mTOR activation, even in the absence of detection of p-TSC2 (Thr^{1462}). Moreover, other signalling pathways can also interact with the TSC complex and phosphorylate alternative sites, such as the Wnt pathway via GSK-3β (Inoki et al. 2006) or the Raf/MEK/Erk pathway (Inoki et al. 2009, Menon et al. 2008) via Erk. As the phosphorylation status of TSC2 has not been studied exhaustively in this work, the involvement of other pathways may explain why activation of mTOR was found in 87% of the cases, with only 73% of them positive for p-TSC2. It would therefore be interesting to study in particular the status of LKB1 in those OS cases, and see how they correlate with the absence of TSC2 inactivation by Akt. This may also explain why no obvious genetic mechanism of activation was found in the pathway as total protein loss rarely occurred.

3.4.5 Conclusion

This is the first screening of a large cohort of patients for the activation of the mTOR pathway. It shows that 87% of the cases expressed p-mTOR, p-S6K or p-RPS6. This may be an underestimation of the pathway activation as almost all cases expressed p-4EBP1. Inactivation of TSC2 by Thr^{1462} phosphorylation is associated with a worse prognosis, giving an insight into OS pathogenesis. Further proof of the role of this pathway in OS was uncovered as loss of PTEN and TSC1 may lead to constitutive pathway activation. The TSC complex seems to have a central role in pathway activation and its regulation may differ between PR and GR. The study of the expression of LKB1 protein can help to understand the regulation of this pathway and will be the focus of the next chapter.

Chapter 4: REDUCED EXPRESSION OF LKB1 IN A SUBSET OF OSTEOSARCOMAS

4.1 Introduction and aims

As presented in the Introduction (section 1.2.3.3), the report that *Lkb1*-deficient mice develop bone-forming tumours (Robinson et al. 2008) begs the question whether loss of this tumour suppressor gene plays a role in the pathogenesis of human OS. LKB1 is associated with pathways that have been implicated in the development of OS (section 1.2.3.3); our finding in Chapter 3 that the mTOR pathway is activated in a large subset of OS also suggests the involvement of *LKB1* in OS pathogenesis (section 3.2.1.6 and 3.4.5). Moreover, the TSC complex seems to have a central role in mTOR pathway regulation in OS, and deciphering its interaction with LKB1 would help to elucidate its role in the constitutive activation of the pathway (section 3.4.4.4).

As discussed in the Introduction (section 1.2.3.3), the tumours detected in the *LKB1*-deficient mice by Robinson et al were difficult to classify, and may represent an osteoblastoma or an osteoblastoma-like OS. Understanding the molecular pathogenesis of both OS and OB and finding new biomarkers that could distinguish the two could ease the diagnosis of such cases.

To investigate this, we aimed to monitor the protein expression of LKB1 in human OS and OB patients. The objectives were:

4. Assess the expression status of LKB1 in human OS cell lines and tumours and OB tumours
5. Investigate whether LKB1 could be used as a prognostic marker
6. Correlate the expression of LKB1 with the mTOR pathway activation in OS

4.2 Expression of LKB1 protein in Osteosarcoma

To assess the expression of LKB1 in OS, the protein was first monitored by WB on a small cohort of cell lines and tumours, as described in the Materials and Methods chapter (section 2.6.5).

4.2.1 WB results in Osteosarcoma cell lines

Fifteen OS cell lines were screened for LKB1 expression using the Ley clone antibody (Table 2.7). The immortalized human osteoblast cell line HOB and fibroblast cell line HF1 were used to model normal lineages. Three of the OS cell lines, U2OS, SaOS2 and MNNG, were tested for LKB1 expression both in *in vitro* cell culture and in *in vivo* xenografts generated by subcutaneous injection of the cell lines in immunocompromised mice (section 2.3). The results of the WB are presented in Figure 4.1.

4.2.1.1 Analysis of LKB1 protein expression

No, or extremely low, reactivity was found for 5 of 15 (33%) cell lines: SaOS2, OSA, MHM, OST and HAL. The MNNG and U2OS cell lines expressed higher levels of LKB1 *in vitro* than *in vivo*, but the reverse was observed for SaOS2.

4.2.1.2 Densitometry quantification

To quantify these results, the method described in section 2.6.5.4 was used and the results are illustrated in Figure 4.2. This scoring confirmed the findings observed above (section 4.2.1.1). The OST cell line obtained a score of 0, the SaOS2, OSA, MHM and HAL cell lines obtained a score of 1, indicating a reduced LKB1 expression compared to the positive controls. OHS and MG63 also scored as 1 with this method; however, the integrated density for MG63 was very close to the cut-off and was therefore considered as positive for LKB1 expression. The other cell lines, including the controls HOB and HF1, had a score of 2 as expected – e.g. expressed a comparable level of protein to the positive control Jurkat.

Interestingly, all three cell lines possessed a lower score *in vivo* compared to *in vitro*. This result is difficult to interpret as the mechanism of regulation of LKB1 in OS is unknown.

To conclude, using the densitometry analysis, 6 of 15 (40%) cell lines showed a reduced expression of LKB1 compared to the positive control.

4.2.2 WB results in Osteosarcoma cases

To assess whether LKB1 was lost in patients with OS, 26 OS cases were studied by WB. One OB was also included in this screen (case S2290). LKB1 was not detected in six cases, S180, S2267, S2257, S2291, S2312 and S2313. High expression of LKB1 compared to the loading control GAPDH was observed in at least two cases, S2314 and S2260.

Using the same densitometry analysis as for the cell lines, described in section 2.6.5.4, 5 of 26 (19%) OS cases scored 0, including two of the cases without visible LKB1 expression and listed above, S180 and S2257. The remaining four of that list, S2267, S2291, S2312 and S2313, obtained a score of 1, meaning they expressed a reduced LKB1 protein expression compared to the positive control; in total 18 of 26 (69%) of the OS cases obtained this score of 1. Of note is that one case, S2447, has a densitometry quite close to the cut off between 1 and 2, and can be considered borderline. The remaining 3 of 26 (12%) cases, including, as expected, S2314 and S2260, obtained a score of 2, characteristic of a high LKB1 protein expression. The OB case score corresponds to a very low or no expression of LKB1 (i.e. 0).

Overall, with this scoring method, 23 of 26 (88%) OS cases were classified as having a reduced relative expression of LKB1 compared to the positive control Jurkat. This result suggests that LKB1 expression is reduced in a large subset of human OS.

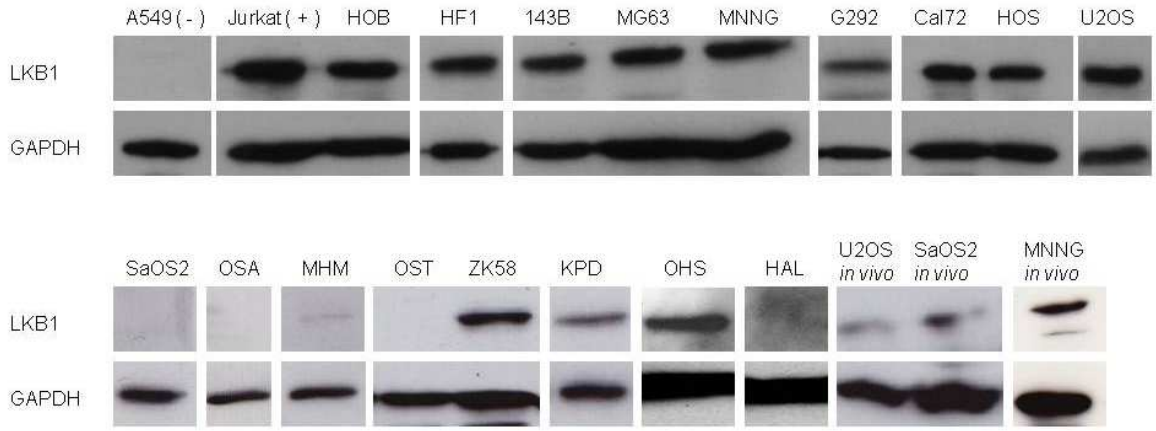


Figure 4.1: Western blot analysis of the expression of LKB1 in OS cell lines.
LKB1 protein is detected at 48kDa and GAPDH at 37kDa. The positive control cell lines Jurkat, HOB and HF1 displayed strong expression and the negative control cell line A549 no expression, as expected. The blotting of the cell lines OHS and HAL was performed by Dr Malihe Eskandarpour.

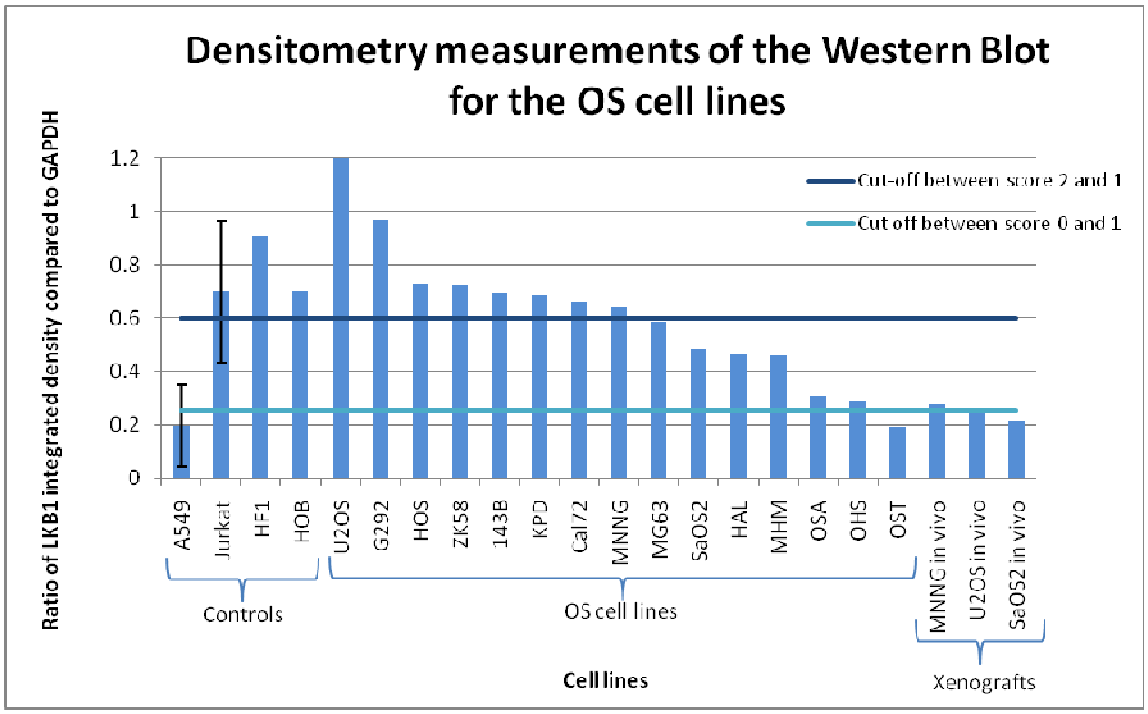


Figure 4.2: Densitometry measurements quantifying the relative LKB1 protein expression compared to GAPDH in OS cell lines.

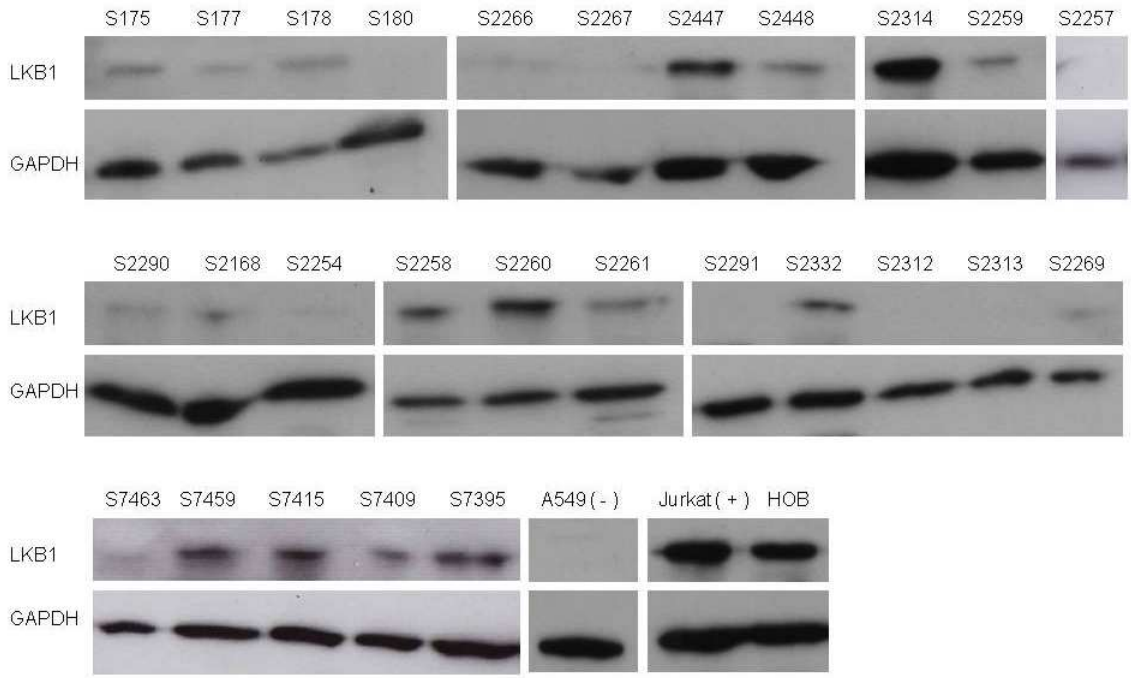


Figure 4.3: Western blot analysis of the expression of LKB1 in OS cases.
LKB1 protein is detected at 48kDa and GAPDH at 37kDa. A549 was used as a negative control, Jurkat and HOB as positive controls.

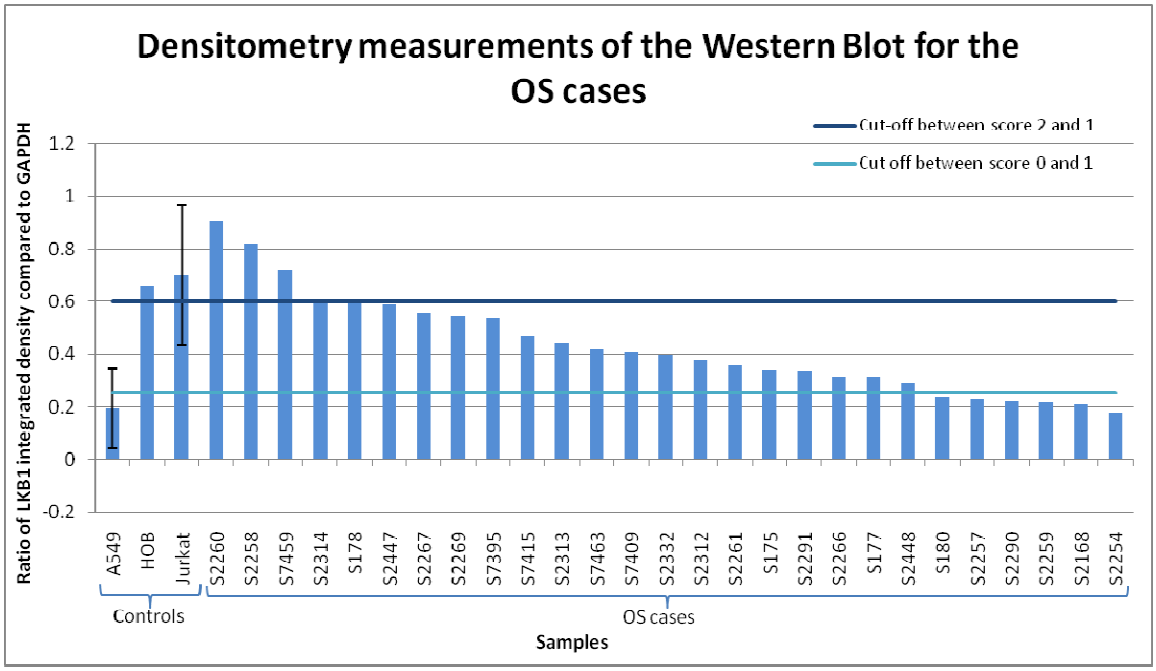


Figure 4.4: Densitometry measurements quantifying the relative LKB1 protein expression compared to GAPDH in OS cases.

4.2.3 IHC results

To confirm the results obtained by WB, LKB1 expression was assessed by IHC as described in the Materials and Methods chapter (section 2.4.2) on the second set of TMA prepared, including 268 OS cases, and on a TMA with 51 OB cases.

4.2.3.1 LKB1 expression in normal control tissues

The Figure 4.5.A shows the results of the IHC for LKB1 in the positive controls, normal lung and normal skeletal muscle – which have been previously tested with this antibody by others (Conde et al. 2007) – and in non-neoplastic reactive bone from five human cases. LKB1 was expressed in non-neoplastic reactive osteoblasts *in vivo*, similar to the results obtained by WB *in vitro* for the HOB non-transformed osteoblastic cell line (Figure 4.1). The validity of the IHC method was thereby confirmed.

4.2.3.2 LKB1 expression in Osteosarcoma

Although present in the nucleus after being translated, LKB1 protein is thought to be mainly active in the cytoplasm, as described in the Introduction (section 1.2.1.1 and Figure 1.10). In accordance with this, LKB1 immunoreactivity was found to be mainly cytoplasmic in OS cases, although focal nuclear expression was detected. No case presented a strong positivity restricted to the nucleus.

The IHC scoring confirmed the findings of the WB, as presented in Table 4.1 (section 4.2.4). Representative microphotographs of a positive and a negative OS cases are shown in Figure 4.5.B. Overall, 153 of 259 (59%) human OS cases presented very low or no immunoreactivity for LKB1, using the scoring method described in the Materials and Methods chapter (section 2.4.2.2).

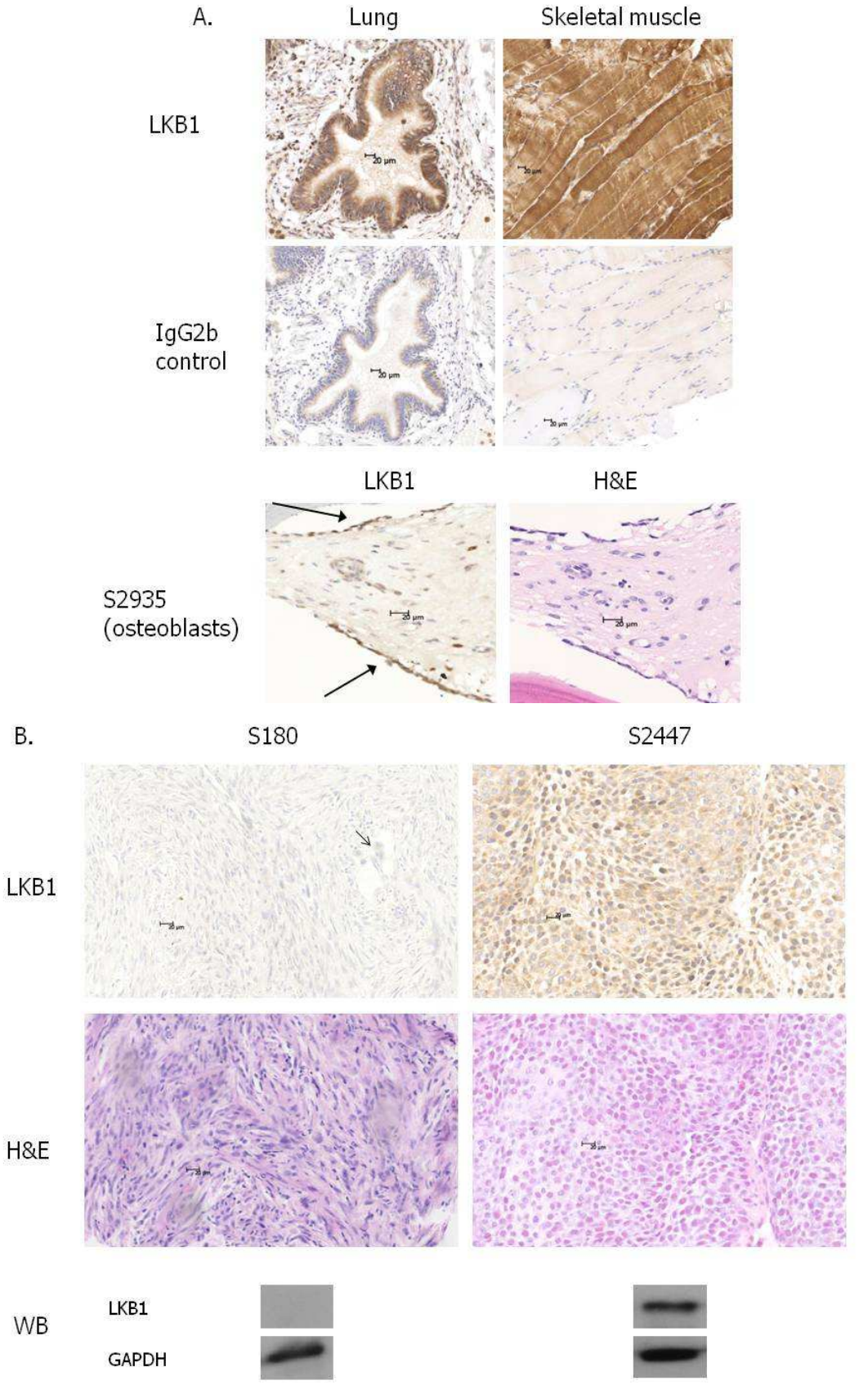
4.2.3.3 LKB1 expression in Osteoblastoma

The LKB1 expression pattern was found to be mostly cytoplasmic in OB, with focal nuclear expression, as in OS (Figure 4.5.C). LKB1 expression was more frequent in OB compared to OS, with 12 of 50 (24%) cases showing no or very low immunoreactivity for LKB1 (Table 4.1). The difference between the two tumours was statistically significant ($p=4.2x10^{-6}$ with the one-tailed Fisher exact test), but as LKB1 loss was detected in both OS and OB cases, it cannot be used as a diagnostic marker to distinguish the tumours.

One patient included in the study presented with an aggressive OB, which recurred less than a year later with features closer to an osteoblastoma-like OS. The malignancy of this case was difficult to classify. Interestingly, LKB1 expression was low in the primary benign tumour and almost negative in the aggressive recurrence. Only two other cases described as aggressive OB were available in the cohort; both were primary tumours and expressed LKB1. Hence LKB1 loss in OB may be associated with an increased aggressiveness of the tumour; this needs to be investigated further in a larger cohort of cases.

Antibody	Detection method	Positive OS cases	Positive OB cases
p-TSC2	IHC	135/238 (57%)	38/43 (88%)*
LKB1	IHC	106/259 (41%)	38/50 (76%)**
LKB1	WB	8/26 (31%)	0/1 (0%)

Table 4.1: Summary of IHC and WB results for OS and OB cases.
*for $p<5x10^{-5}$ and ** for $p<5x10^{-6}$ with the one-tailed Fisher exact test, when compared to the expression in OS.



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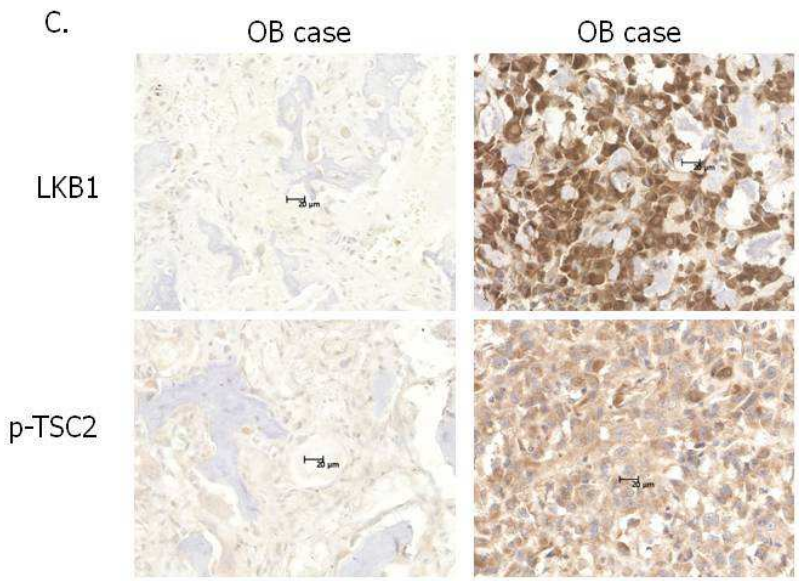


Figure 4.5: Transmitted-light photomicrographs illustrating the IHC results in the controls and OS and OB cases.
A. Immunoreactivity for LKB1 and a mouse IgG2b negative control in normal lung (left) and normal skeletal muscle (right), and immunoreactivity for LKB1 and the corresponding H&E staining of non-neoplastic osteoblasts rimming bone (arrows). No or very low non-specific signal was obtained when using a mouse IgG2b negative control.
B. Immunoreactivity for LKB1 and associated H&E staining and WB results for one positive (right) and one negative (left) OS case. Osteoclasts expressed high levels of LKB1; they provided internal positive controls (arrow), but hampered the assessment of osteoclast-rich cases.
C. Immunoreactivity for LKB1 and p-TSC2 in one negative (left) and one positive (right) OB case.
The bar indicates 20µm (magnification 10x for A. and 20x for B. and C.).

4.2.4 Relationship of IHC and WB

To assess the concordance of the two methods employed to detect LKB1 protein expression, the results of the WB and the IHC were compared on the 27 cases screened by WB. The IHC scoring was assessed on the TMA cores and, when possible, on a section from biopsy or resected tissue from the same case. A score of 0 indicated absence of protein expression, 1 low immunoreactivity and 2 high immunoreactivity, as described in Materials and Methods (section 2.4.2.2).

There was a good association found between the IHC and the WB scoring, with 15 of 27 (56%) showing perfect association (i.e. exactly the same score by IHC and WB), 12 of 27 (44%) showing a good association (i.e. scores differing by 1) and only one case showing opposite results (p=0.044 with the Chi square test). All the 6 of 27 (22%) cases with no protein

expression detected by WB were not immunoreactive for LKB1 by IHC. Moreover, the association between the measured integrated density ratio of LKB1 compared to GAPDH and the score obtained by IHC was statistically significant (p=0.047 using the paired Student's t-test). The results are shown in Figure 4.6.

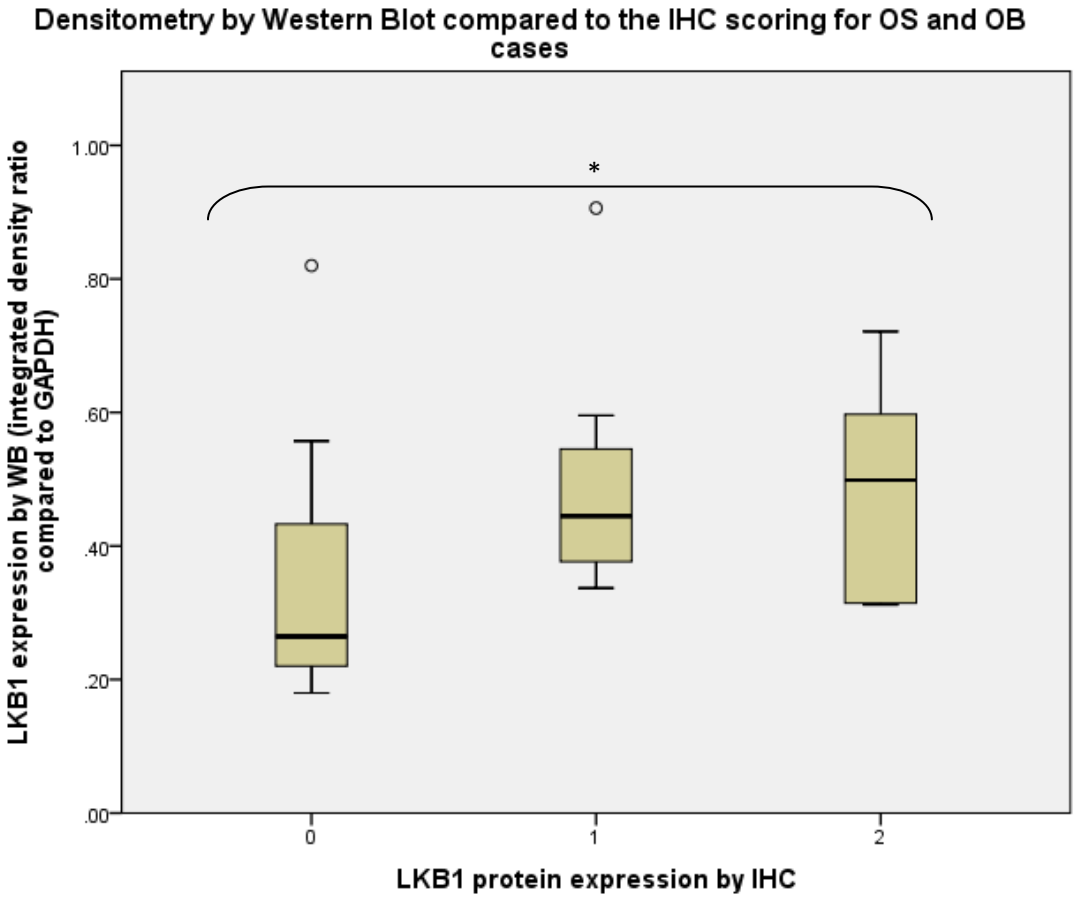


Figure 4.6: Box plot representing the association between the IHC scoring and the densitometry by WB for LKB1 protein in OS and OB cases.
The round points represent the outliers, i.e. the cases with values between 1.5 and 3 times the interquartile range from the upper of lower edge of the box. * for p<0.05 with the paired-Student's t-test.

4.3 Role of LKB1 as a biomarker

As discussed in the Introduction (section 1.1.4.2) and the previous chapter, there is a lack of biomarkers to predict response to chemotherapy or overall prognosis in OS. LKB1 has been associated with metastasis in lung cancer (Carretero et al. 2010). We therefore assessed the relationship between LKB1 loss detected by IHC in OS and OB and clinical features to investigate its use as a prognostic marker.

4.3.1 Relationship with clinical data in Osteosarcoma

The clinical characteristics of the cohort of 268 patients used for the IHC screening are presented in Figure 2.2. There was no relationship found between the expression of LKB1 and the patient age or sex, the histological subtype or grade or the disease progression. However, there was a association between LKB1 expression and the patient chemotherapy status ($p=0.005$ with the Chi square test). More specifically, when comparing PR and GR to chemotherapy, GR tended to lack LKB1 expression more often than PR: 52 of 68 of (77%) GR did not express LKB1 compared to 60 of 104 (58%) PR ($p=0.012$ with the Chi square test), as illustrated in Figure 4.7. In other words, the relative risk of being a GR when LKB1 expression was not detected was 1.74 times higher than being a PR (95% confidence interval [1.12; 2.81]). If the absence of LKB1 expression were to be used as a predictive marker of response to chemotherapy, the sensitivity of this test would be 76.5% (95% confidence interval [67.7%; 84.0%]) and the specificity 42.3% (95% confidence interval [36.6%; 47.2%]).

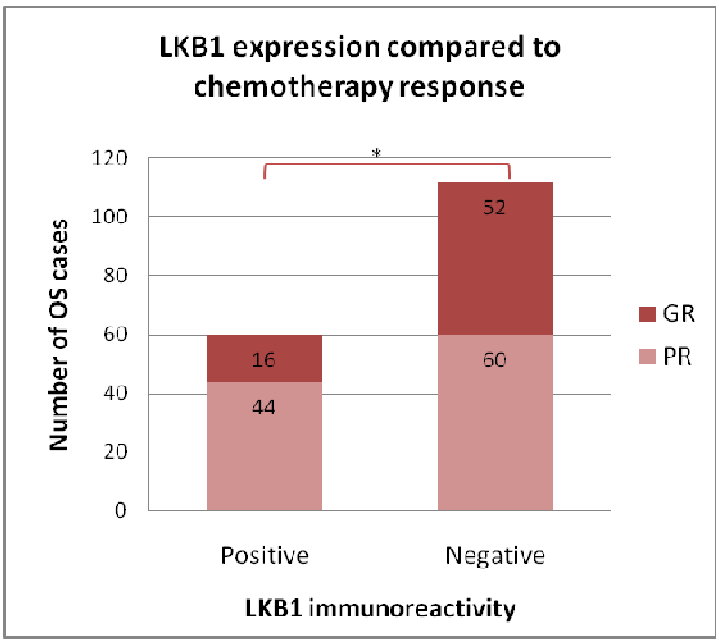


Figure 4.7: Bar chart illustrating the presence of LKB1 expression in PR compared to GR
*for $p<0.05$ with the Chi square test.

4.3.2 Relationship with clinical data in Osteoblastoma

The clinical features of the OB patients included in the TMA are presented in the Materials and Methods chapter, Figure 2.3. No association was found between LKB1 expression in OB and the patient age or sex, or the tumour site or stage with the Chi square or one-tailed Fisher exact tests. The cohort of OB studied is relatively small, so the results need to be confirmed in a larger cohort. Hence, it was not possible to calculate the significance of the association observed above between LKB1 expression and disease aggressiveness (section 4.2.3.3).

4.4 LKB1 and the mTOR pathway

4.4.1 Association between LKB1 and p-TSC2 in Osteosarcoma

As discussed in the previous chapter (section 3.2.1), the mTOR pathway was activated in about 87% of the cases screened, and in 73% of the informative cases, this activation was attributed to Akt, in view of p-TSC2 (Thr^{1462}) being detected. However, as discussed in paragraph 3.4.4.4, at least 32 cases may have presented pathway activation due to inter-dependency to other pathways, or possibly due to the action of LKB1. To confirm the role of LKB1 in the regulation of the activity of the TSC complex, the expression of LKB1 and p-TSC2 (Thr^{1462}) were compared in the cohort of 268 patients with OS (Table 4.1).

A strong association was found between LKB1 and p-TSC2 immunoreactivity ($p=2.51x10^{-5}$ with the Wilcoxon Signed-Rank test), with 86 of 105 (83%) cases lacking LKB1 in absence of p-TSC2 immunoreactivity. In other words, the relative risk of lacking p-TSC2 was 3.56 times higher in the absence of LKB1 (95% confidence interval [2.35; 5.55]). In addition, LKB1 was not detected in 52 of 132 (39%) of the cases that were immunoreactive for p-TSC2. These results are illustrated in the Figure 4.8.

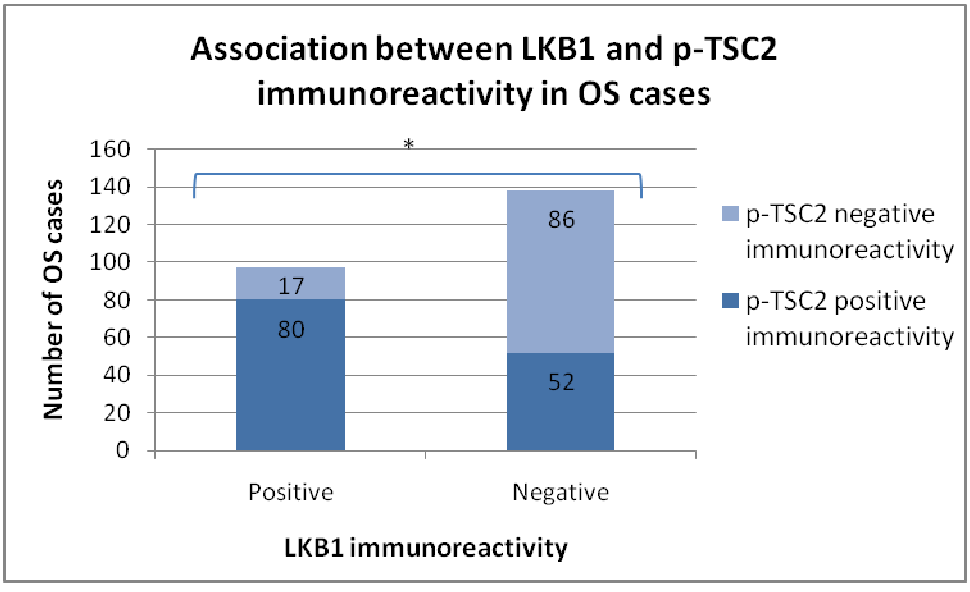


Figure 4.8: Bar chart representing the immunoreactivity of p-TSC2 compared to LKB1 in OS cases.
* for $p<5x10^{-5}$ with the Wilcoxon signed-rank test.

4.4.2 Association between LKB1 and mTOR pathway activation in Osteosarcoma

LKB1 expression was also correlated with mTOR pathway activation, with an even higher significance than the results reported for p-TSC2 in paragraph 3.2.1.5 ($p=1.37x10^{-15}$ with the Wilcoxon Signed-Rank test). LKB1 was not detected in 75 of 133 (56%) of the OS cases showing mTOR pathway activation, whereas only 16 of 91 (18%) LKB1-negative cases did not show pathway activation. Hence, the relative risk of having the pathway activated was 12.78 times higher if LKB1 was absent (95% confidence interval [5.75; 31.67]).

In the first cohort of 162 patients screened for mTOR pathway activation, p-TSC2 loss along with presence of total TSC2 protein could be attributed to LKB1 loss in 27 of 38 (71%) cases. Moreover, 18 of 26 (69%) cases showing absence of p-TSC2 but presence of both the total protein and the activation of the mTOR pathway, lacked LKB1 expression.

Overall, this suggests that loss of LKB1 is strongly associated with activation of the mTOR pathway, with or without the concurrent inactivation of the TSC complex by Akt.

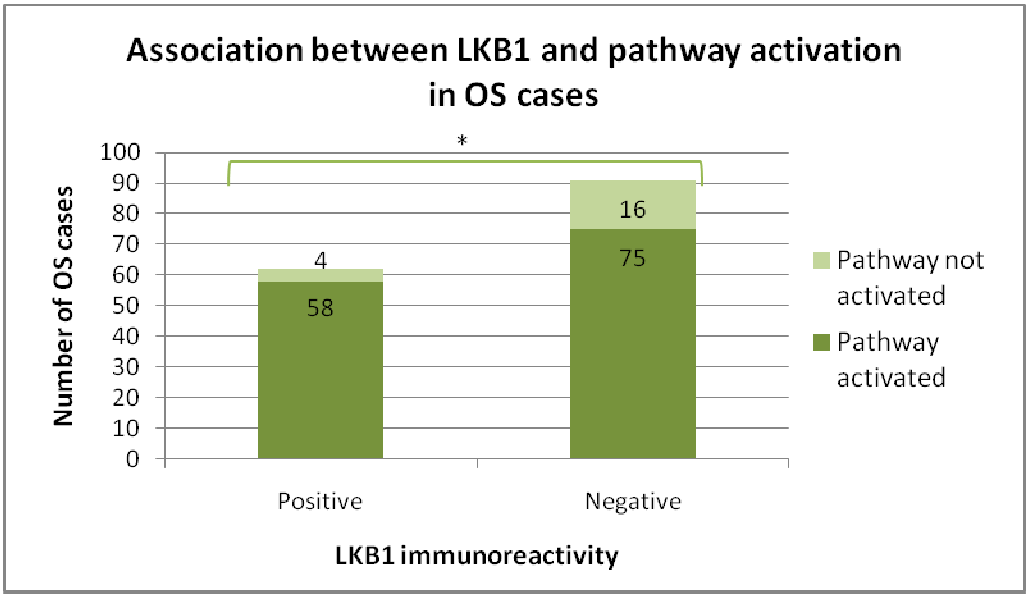


Figure 4.9: Bar chart representing the immunoreactivity of LKB1 compared to the pathway activation in OS cases.
** for $p<5x10^{-15}$ with the Wilcoxon signed-rank test.

4.4.3 Association between LKB1 and p-TSC2 in Osteoblastoma

To compare LKB1 and p-TSC2 expression in OB, p-TSC2 immunoreactivity was assessed by IHC on the OB TMA. The results are illustrated in Table 4.1 and Figure 4.5.C. p-TSC2 expression was detected in a vast majority of OB cases, with 38 of 43 (88%) immunoreactive cases. This proportion was significantly higher than found in OS ($p=3.2x10^{-5}$ with the one-tailed Fisher exact test) but as both OS and OB could express this biomarker, it could not be used for differential diagnosis, as found for LKB1 (section 4.2.3.3).

No association was found between p-TSC2 expression and patient age or sex, or tumour stage. An association was found with tumour site (p=0.018 with the Chi square test). However, some sites were only represented by one patient, so this result may be an artefact and needs to be confirmed in a larger cohort of cases.

We observed a good association between the expression of LKB1 and p-TSC2 (p=0.005 with the one-tailed Fisher exact test), as was described for OS. However, the association for the pair of LKB1 and p-TSC2 scoring was not statistically significant using the Wilcoxon Signed-Rank test, which is not surprising considering the small number of cases available. In cases negative for p-TSC2, absence of LKB1 expression occurred in 4 of 5 (80%) cases. Additionally, 5 of 38 (13%) cases immunoreactive for p-TSC2 lacked LKB1 expression. The relative risk for patients to lack p-TSC2 expression was 15.11 times higher in the absence of LKB1 expression (95% confidence interval [2.61; 97.02]).

4.5 Discussion: potential role for LKB1 in Osteosarcoma and Osteoblastoma pathogenesis

The study of LKB1 protein expression in OS cases showed that a subset of patients showed reduced expression. To a lesser extent, LKB1 expression was also lost in some OB cases. The technical limitations and the perspective of these findings with regards to normal bone and bone tumour biology are discussed here.

4.5.1 Technical limitations and relationship between WB and IHC data

Two methods were chosen to assess the LKB1 protein expression in OS cell line and cases, IHC and WB, to diminish the risk of false positive or false negative results due to the limitation of each of them. However, neither of them tests for the functionality of the protein, but only for the presence of the epitope against which the antibody was raised. It is therefore possible that a number of cases with protein detected by IHC or WB have a non-functional protein, and that the actual number of cases without a normal LKB1 protein expressed is underestimated by this study. Sequencing *LKB1* could confirm whether the protein expressed is functional, as will be developed in the next chapter.

WB is a nearly quantitative method, but with limitations: for protein highly expressed, the band observed becomes saturated and the quantification of the density observed do not remain proportional to the levels of expressed protein. There may also be discrepancies in the measurement obtained from different blots. Therefore, the exposure of the blot was monitored to avoid signal saturation and GAPDH was always used as a loading control. No automated scaning system - which could have facilitated the quantification of the intensity of the bands obtained - could be use for this experiment due to technical limitations. To normalize the results and limit all these effects, the WB densitometry measured for each case and cell line was compared to the value obtained on a large sample of positive and negative controls (n=26).

IHC is not strictly a quantitative method, which hampers its comparison to WB. Moreover, the results of this method can be difficult to interprete. To ensure that all the cases were scored

unequivocally, cases without internal positive or negative controls were excluded. This is particularly important for the study of the absence of a protein. Additionnally, the scoring was validated independently by different pathologists. Cases where the interpretation of the immunoreactivity observed was not reproduced were then considered equivocal cases and were excluded.

To overcome the difficulty of comparing those two methods, a scoring system for both IHC and WB was set up to assess which cases presented a high (score of 2), low (1) or very low/non-existent (0) level of LKB1 protein expression. Although the choice of the cut-off could be considered arbitrary, this enabled statistical quantification of the relationship between the two methods. This relationship proved significant in 26 of 27 (96%) cases (p=0.044, section 4.2.4).

The fact that 12 of 27 (44%) of the cases did not have equal scores could be explained by the limitations of the quantification methods. The WB results are better described using the actual integrated density ratio than the scoring system. To compare this quantity with the IHC scoring, a paired Student's t-test can be used, under the hypothesis that the distribution of the IHC scoring is normal. Under this model, the relationship between the two methods was again significant (p=0.047, section 4.2.4).

The technical limitations of the methods used can also explain the discrepancies observed. Although nearly quantitative, the WB was performed on all the cells present in the cut sections of the sample. To limit false positive results, as normal skeletal muscle expresses LKB1 (Figure 4.5.A) and is usually found in the vicinity of OS tumours, only cases with at least 90% tumour cells were chosen. This was assessed by a pathologist on an H&E every 10 frozen sections cut (section 2.1). However, small numbers of infiltrating normal cells present within the tumour, such as normal osteoclasts – positive for LKB1 – may be present in the tissue used for the protein extraction. This can explain why certain cases had a higher score by WB than by IHC.

As the IHC was assessed on TMAs, only a small fraction of tissue was observed. This method enables the screening of larger number of cases, but as the staining of LKB1 may not be totally homogeneous within the tumour, it may increase the level of false positive or false negative results. To limit this risk, representative areas of the tumour were selected by a pathologist, as detailed in the Materials and Methods chapter (section 2.4.1). Additionally, IHC is not a quantitative method, and the treatment and age of the tissue can influence its

immunoreactivity. This is why internal positive controls were always monitored, to avoid false negative results. However some discrepancies may remain.

4.5.2 Using LKB1 as a biomarker

LKB1 loss has been associated with metastasis in lung cancer when cooperating with Kras (Carretero et al. 2010), probably due to the role of LKB1 in EMT (Roy et al. 2010, Upadhyay et al. 2006)(section 1.2.1.4). Similarly, LKB1 was associated with invasiveness in endometrial carcinoma (Contreras et al. 2008). Therefore, it was interesting to see if LKB1 could be used as a biomarker in OS. There was no association found between LKB1 expression and disease progression or metastasis. This suggests that the function of LKB1 in OS is different to that in lung cancer. LKB1 expression was only found to be associated with patient response to chemotherapy in OS. However, as for p-TSC2 expression in PR versus GR and even though the difference was significant, there was a large overlap between the two groups, as illustrated in Figure 4.7. As in the case of p-TSC2, using LKB1 as a diagnostic test to assess patient response to chemotherapy would give a low sensitivity and a very low specificity. This means that a high number of false positive and false negative results would be obtained, which is not acceptable in this clinical context. To conclude, LKB1 immunoreactivity is associated with a higher risk of poor response to chemotherapy, but cannot be used as a predictive marker of response to treatment.

LKB1 expression also failed to be established as a biomarker in OB. As mentioned in paragraph 4.3.2, LKB1 loss was associated with disease aggressiveness in one case, but further studies are needed to confirm this result. It also fails to distinguish between OB-like OS and aggressive OB as both phenotypes can lack its expression.

4.5.3 Association between LKB1 expression and the mTOR pathway

As discussed in Chapter 3 (section 3.4.4.4), there was insufficient evidence to conclude whether the mTOR pathway was constitutively activated in a subset of cases. Loss of LKB1 expression could account for this activation. As LKB1 mainly interacts with the TSC complex by changing the phosphorylation equilibrium of TSC2 via AMPK, as described in the Introduction (section 1.2.1.1 and 1.2.1.2), the association between LKB1 and p-TSC2 expression was monitored.

4.5.3.1 LKB1 and p-TSC2 protein expression

There was a very strong association found between loss of LKB1 expression and loss of p-TSC2 expression, with highly significant p-values for OS. This supports the hypothesis that LKB1 is involved in the regulation of the TSC complex and in mTOR pathway activation in OS. LKB1 loss could account for the absence of p-TSC2 in 86 of 105 (82%) OS and 4 of 5 (80%) OB cases. Moreover, absence of LKB1 is not incompatible with detection of p-TSC2. As described in the introduction, the TSC complex integrates the signals from both the Akt and the LKB1 branch to regulate the activation of mTOR via its phosphorylation status (Inoki et al. 2002, Inoki et al. 2003, Tee et al. 2002). The signals from both these branches could therefore be concordant. In the absence of LKB1, TSC2 cannot be activated further by AMPK, which contributes to a constitutive activation of mTOR; in that case, p-TSC2 (Thr^{1462}) would not necessarily be detected, even though the pathway would be activated. Additional activation of mTOR via Akt and its phosphorylation of TSC2 would be favoured but would not be necessary. Therefore, absence of LKB1 probably promotes TSC2 phosphorylation by Akt in 53 of 132 (40%) OS and 5 of 38 (13%) OB cases.

4.5.3.2 LKB1 expression and mTOR pathway activation

LKB1, once expressed, is constitutively activated by its binding to its complex, as described in the Introduction (section 1.2.1.1). The action of LKB1 on the mTOR pathway would then be regulated by the AMP to ATP ratio. In energy-deprived conditions, the increase of this ratio would activate AMPK. This would then lead to an inhibition of the mTOR pathway via LKB1 and

AMPK. However, in the absence of LKB1 protein, AMPK could not play its role, even in energy-deprived conditions. This would lead to a constitutive activation of the pathway (Inoki et al. 2003).

LKB1 loss is strongly associated with mTOR pathway activation, with a very low p-value obtained for the statistical correlation test. This was validated with or without taking into account the loss of p-TSC2. Hence our results confirm that LKB1 loss could lead to constitutive activation of the mTOR pathway in OS, in up to 55% of our cohort. As LKB1 expression is ubiquitous (section 1.2.1.1), and as it is considered a tumour suppressor gene (section 1.2.1.5), its absence in OS can be considered pathogenic. However, the genetic background behind this loss needs to be investigated further to confirm its role in mTOR pathway regulation (section 4.5.5 below).

4.5.4 Role of LKB1 in osteoblast biology and Osteosarcoma pathogenesis

Strong expression of LKB1 protein was consistently found in osteoblasts, whether assessed *in vivo* by IHC or *in vitro* in the HOB cell line (Figure 4.1 and Figure 4.5). As described in the Introduction (section 1.1.5.4 and 1.1.5.5), although not proven, there is strong evidence that OS may arise from an osteoblastic lineage, as suggested by the establishment of conditional transgenic mouse models (Berman et al. 2008, Walkley et al. 2008). Robinson et al have shown that LKB1 loss can induce the formation of osteoblastic tumours in a mouse knock-down model (Robinson et al. 2008), which links further OS development to loss of LKB1 in osteoblasts. Our finding that LKB1 protein is not expressed in a large subset of OS strengthens the hypothesis that LKB1 loss within an osteoblast lineage could induce tumourigenesis. The use of conditional mouse models restricted to other cell lineages has already shown that loss of LKB1 could lead to breast, endometrial, lung, pancreatic and liver cancer (Contreras et al. 2008, Ji et al. 2007, McCarthy et al. 2009, Miyoshi et al. 2009, Morton et al. 2010). To our knowledge, there are no reported conditional knock-out or knock-down mice with LKB1 loss restricted to osteoblasts. Establishing such a model would help to investigate further the role of LKB1 in the pathogenesis of OS. As a preliminary study, its functional role could be assessed by using an *in vitro* model to knock-down LKB1 in osteoblasts. This will be discussed further in the Chapter 7.

4.5.5 LKB1, a tumour suppressor gene lost in Osteosarcoma?

As discussed in the Introduction (section 1.2.1.4), LKB1 is considered a tumour suppressor gene, although recent findings suggests it may also play a role when only haploinsufficient. The finding of a reduced expression of LKB1 in a large subset of OS begs the question whether genetic events can lead to the loss of LKB1 protein in OS. Gene deletion, methylation or mutation of *LKB1* have been described in lung cancer (Sanchez-Cespedes et al. 2002) and similar abnormalities have also been reported in other cancers, including gynaecological, liver, colorectal, brain and head and neck (Kenanli et al. 2010, Kim et al. 2004, Sobottka et al. 2000, Trojan et al. 2000, Wingo et al. 2009). Such genetic events could explain the loss of LKB1 protein in OS. It is also possible that the loss of LKB1 is underestimated, as some cases may express a non-functional protein, still detectable by IHC or WB. Therefore, investigating such genetic abnormalities in *LKB1* is essential in order to determine whether its loss is indeed pathogenic, and this will be the focus of the next chapter.

Chapter 5: GENETICS OF *LKB1* IN OSTEOSARCOMA

5.1 Introduction and aims

As reported in the previous chapter, we have identified that LKB1 protein expression is reduced in a large subset of OS: 23 of 26 (88%) cases by WB and 153 of 259 (59%) by IHC (section 4.2.2 and 4.2.3). However, to confirm the role of this tumour suppressor gene in OS pathogenesis, loss of protein expression needs to be associated with a somatic genetic or epigenetic modification. As discussed in the Introduction (section 1.2.1.4), according to the Knudson "two-hits" hypothesis (Knudson, Jr. 1971), loss of function of a tumour suppressor gene is caused by two successive abnormalities or "hits". However, this theory is now under debate, and *LKB1* has been suggested to be pathogenic even when haploinsufficient (section 1.2.1.4). In any case, to our knowledge, the genetic or epigenetic abnormalities in *LKB1* have not been investigated in OS. As presented in paragraph 1.2.3.3, no mutations of *LKB1* have been reported. No changes in mRNA expression were noticed in gene expression microarray screening. Loss of the *LKB1* locus (19p13.3) has been identified in some studies (Tarkkanen et al. 1999, Tarkkanen et al. 1995), but this is not fully conclusive as gain of chromosome 19 is also recorded in others (Zielenska et al. 2004, Zielenska et al. 2001). Overall, the status of *LKB1* in OS is unclear.

Our aim in this chapter was to assess whether genetic abnormalities could account for the loss of LKB1 protein observed in OS and OB cases, and whether *LKB1* was a tumour suppressor or a haploinsufficient gene in OS. To achieve this, the objectives were:

1. Assess the frequency and extent of gene deletion in OS and OB cases and correlate this with protein expression
2. Assess the frequency of gene mutations
3. Assess the expression of *LKB1* at the mRNA level and correlate it with protein expression and genetic abnormalities

5.2 *LKB1* deletion in Osteosarcoma

As the most commonly reported genetic abnormality leading to the loss of *LKB1* is gene deletion (section 1.2.1.4), the presence of *LKB1* locus was first monitored by interphase fluorescent *in situ* hybridisation (FISH) on the first cohort of OS TMAs, including 162 cases, and the OB TMA, including 51 cases, as described in the Materials and Methods chapter (section 2.4.3).

5.2.1 Results of FISH screening

An "in house" BAC probe for the locus of *LKB1*, 19p13.3, was used. No centromeric probe is available for chromosome 19, so a telomeric BAC probe for the 19q arm was used instead.

5.2.1.1 Results for the Osteosarcoma cases

Ninety three of 162 (57%) OS cases were informative by FISH. The results are presented in Table 5.1 and illustrated in Figure 5.1. None of the informative cases presented one or no copies of *LKB1*, meaning that no strict copy number loss occurred. However, this does not exclude a possible loss of one parental allele, followed by a duplication of the remaining one (e.g. copy neutral loss or uniparental disomy). Since only a telomeric probe was available, it is difficult to distinguish this pattern from a copy number gain of the telomeric region. Such an abnormality had occurred in 2 of 93 (2%) cases, which showed two copies of the *LKB1* probe and at least three copies of the telomeric probe in over 20% of the cells. On the contrary, copy number gain of the *LKB1* locus compared to the telomeric region occurred in 14 of 93 (15%) cases. Twenty-eight of 93 (30%) cases were disomic. Interestingly, 61 of 93 (66%) cases showed polysomy (defined as the presence of at least 3 copies of both *LKB1* and telomeric probe in over 20% of the cells) with between 3 to 17 copy number of chromosome 19.

To conclude, we could not detect any gene deletion of *LKB1* by FISH, but genetic abnormalities need to be investigated further to rule out the presence of copy neutral loss or microdeletions.

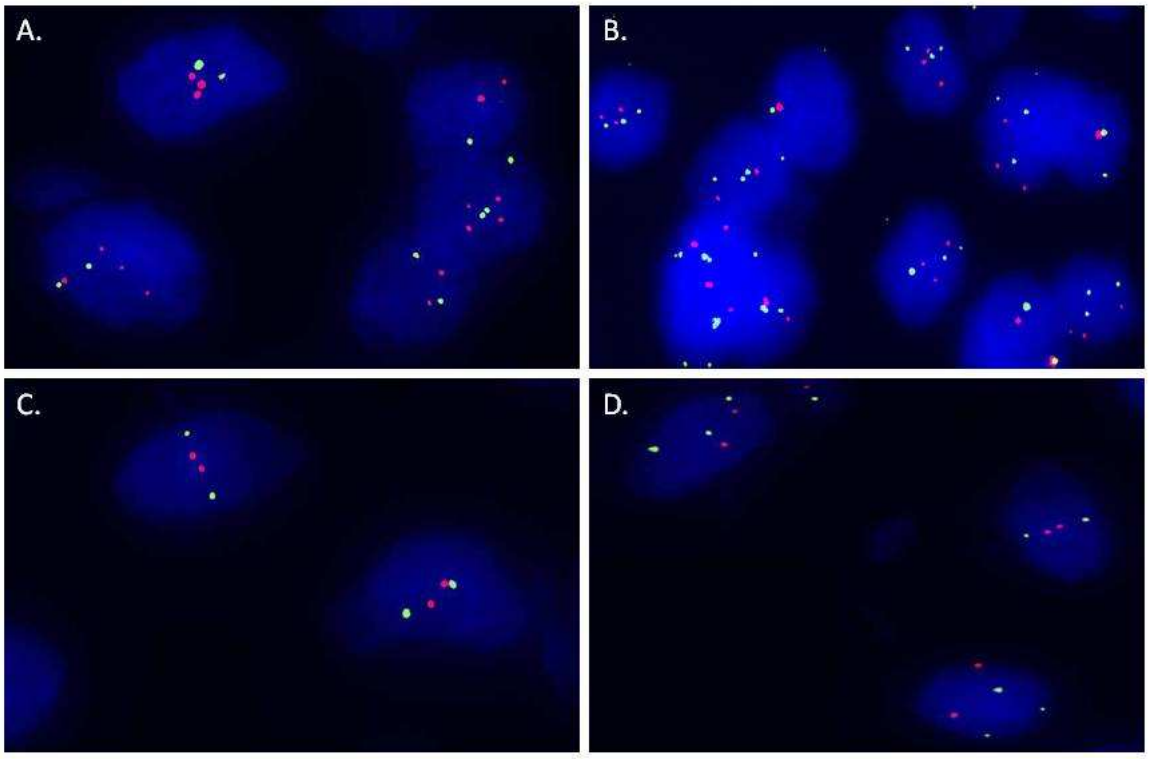


Figure 5.1: Photomicrograph of interphase FISH for LKB1 in OS and OB.
The *LKB1* probe is in red and the telomeric probe in green. The figure includes one OS case with copy number gain of *LKB1* (A.), one OS case with polysomy (B.), one diploid OS case (C.) and one OB case with copy number gain of the telomere 19q (D.) Magnification: x100.

Scoring	OS cases	OB cases
Polysomy	61/93 (66%)	2/24 (8%)
Copy number gain of *LKB1*	14/93 (15%)	0/24 (0%)
Copy number gain of telomere / Copy neutral loss of *LKB1*	2/93 (2%)	8/24 (33%)
Loss of heterozygosity or gene deletion	0/93 (0%)	0/24 (0%)
Disomy	28/93 (30%)	13/24 (54%)

Table 5.1: Results of scoring of the FISH for the OS and OB TMAs.

5.2.1.2 Results for the Osteoblastoma cases

For the OB cohort, 24 of 51 (47%) cases were informative. This ratio is lower than in OS cases. This may be due to the time the paraffin blocks were kept before use; cases dating from more than 5 years were rarely informative. The cohort of OB cases included in the study spans the last 13 years, compared to the last 10 years for OS. Similarly, no case presented copy number loss of *LKB1,* with all 24 informative cases showing two copies of the *LKB1* locus. There were less abnormalities observed in the benign OB compared to the malignant OS, as 13 of 24 (54%) of the cases were disomic. Two of 24 (8%) cases were polysomic, with only three copies of both *LKB1* and the telomere (compared to up to 17 in OS cases). Copy number gain of the telomere (or copy neutral loss of *LKB1*) occurred in 8 of 24 (33%) cases. To conclude, no deletion of *LKB1* was detected by FISH in OB, but this does not exclude the occurrence of copy neutral allelic loss or of microdeletions.

5.2.1.3 Association between protein expression and FISH results

To assess whether the genetic abnormalities (e.g. polysomy, copy number gain of *LKB1* or copy number gain of telomere 19q) were correlated the level of LKB1 protein expression in OS and OB cases, they were compared to the results obtained by IHC, described in the previous chapter (section 4.2.3 and Table 4.1), using the one-tailed Fisher exact test.

No association was found between these abnormalities and the expression of LKB1 protein in OB cases.

For OS however, there was a good association between the presence of polysomy and of LKB1 protein expression by IHC ($p=0.002$). There were 22 of 58 (38%) cases not expressing LKB1 among the polysomic cases (Figure 5.2). Conversely, 23 of 32 (72%) of the cases without polysomy were not immunoreactive for LKB1. In other terms, polysomic cases were 0.53 times as likely (95% confidence interval [0.38; 0.78]) to have lost LKB1 expression than non polysomic cases.

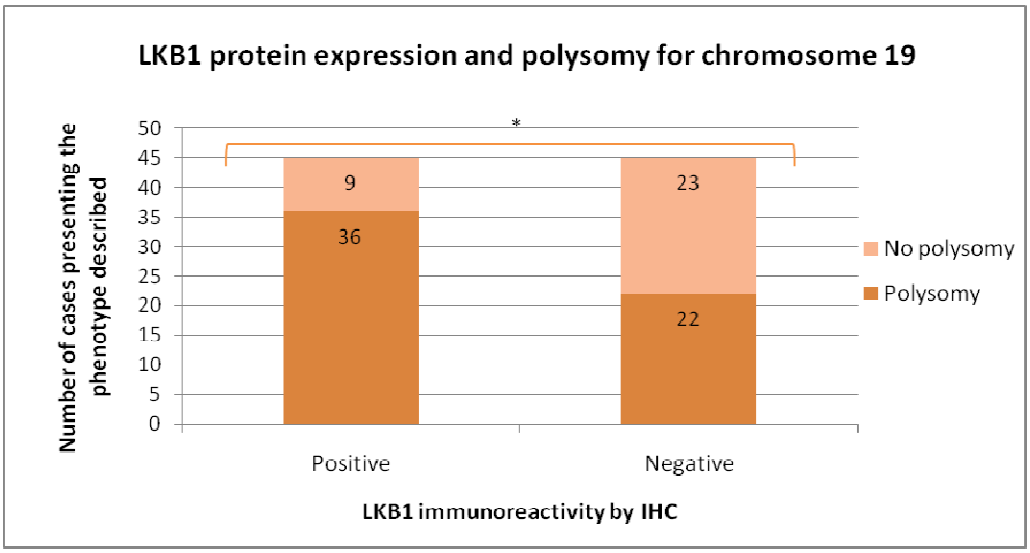


Figure 5.2: Bar chart representing the association between occurrence of chromosome 19 polysomy and the expression of LKB1 protein in patients with OS.
* for p<0.005 with the one-tailed Fisher exact test.

5.2.1.4 Association between FISH results and clinical data

The association between the occurrence of genetic abnormalities (e.g. LKB1 copy number gain, telomeric copy number gain or polysomy) and the clinical characteristics of OS and OB cases, as presented in Figure 2.1 and 2.3, was investigated.

For OB, an association was found between the presence of copy number gain of the telomere and the patient age (p=0.027 with the Mann-Whitney U test). Patients with copy number gain of the telomere tended to be younger at presentation (Figure 5.3). This needs to be confirmed on larger cohorts of cases, as only 8 of 24 cases had this abnormality. No other associations were found with the other genetic abnormalities described and the patient age or sex, or the tumour stage or site using the Chi square or one-tailed Fisher exact test.

For OS, an association was found between the chemotherapy status and the occurrence of polysomy (p=0.001 with the Chi square test). There was a statistically significant association between polysomy and the response to chemotherapy (p=0.002 with the one-tailed Fisher exact test): 2 of 10 (20%) GR were polysomic compared to 27 of 35 (77%) PR (Figure 5.4). The relative risk of polysomy for chromosome 19 was 3.86 times higher in PR compared to GR (95% confidence

interval [1.49; 13.52]). Additionally, there was a significant association between the presence of *LKB1* copy number gain and the tumour site (p=0.044 with the Chi square test). However, this association was barely significant and several sites were only represented by one case. Therefore, this association needs to be confirmed on a larger cohort of cases. No other associations were found between the other genetic abnormalities described and the patient age or sex, the tumour stage, site, response to treatment, grade or subtype or the disease progression.

5.2.1.5 Conclusion

No cases with *LKB1* allelic loss could be identified by FISH in OS and OB cases, with all presenting two copies of the *LKB1* locus. Hence deletion of the locus 19p13.3 cannot account for the loss of LKB1 protein expression in OS. A subset of cases presented two copies of *LKB1* and over 3 copies of the telomere 19q, which can be interpreted as either a copy number gain of the telomeric region or copy neutral loss of *LKB1*, as discussed in section 5.5.1.3. Finally, polysomy for chromosome 19 was frequently recorded in our cohort of cases. To investigate further the possibility of copy neutral loss or microdeletions of the gene, the status of several single nucleotide polymorphisms (SNPs) was monitored.

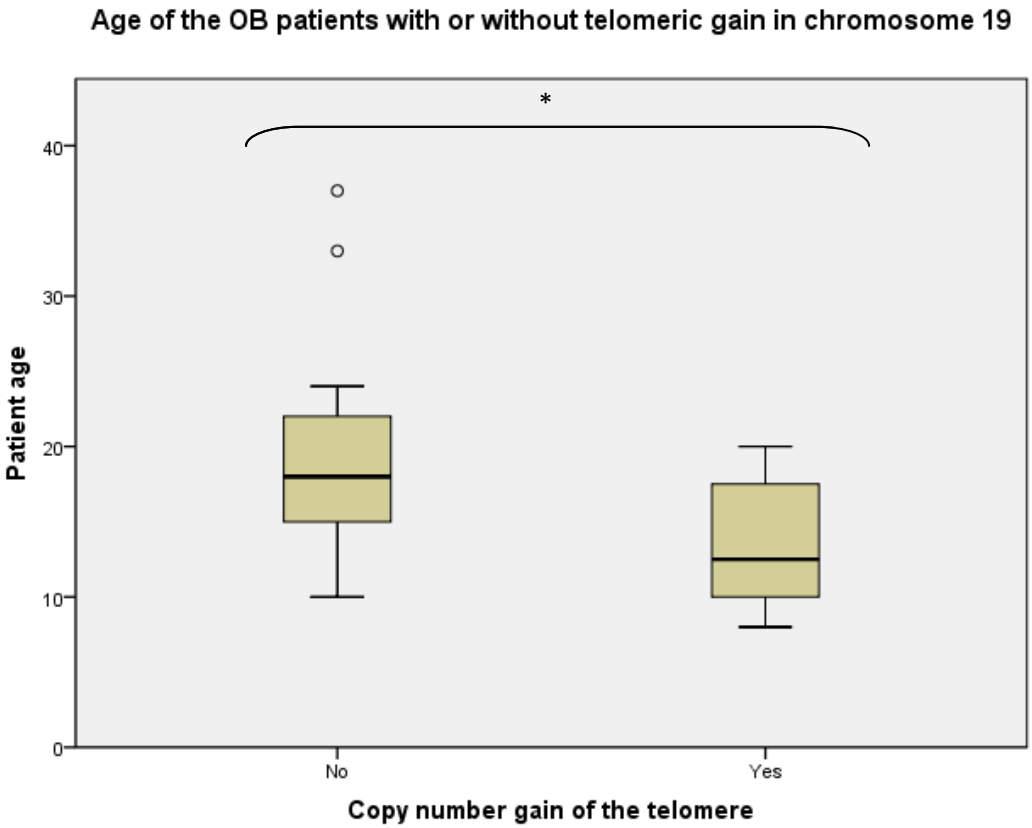


Figure 5.3: Box plot showing the association between the patient age and the occurrence of copy number gain of the telomere of chromosome 19.
* for p<0.05 with the Mann-Whitney U test.

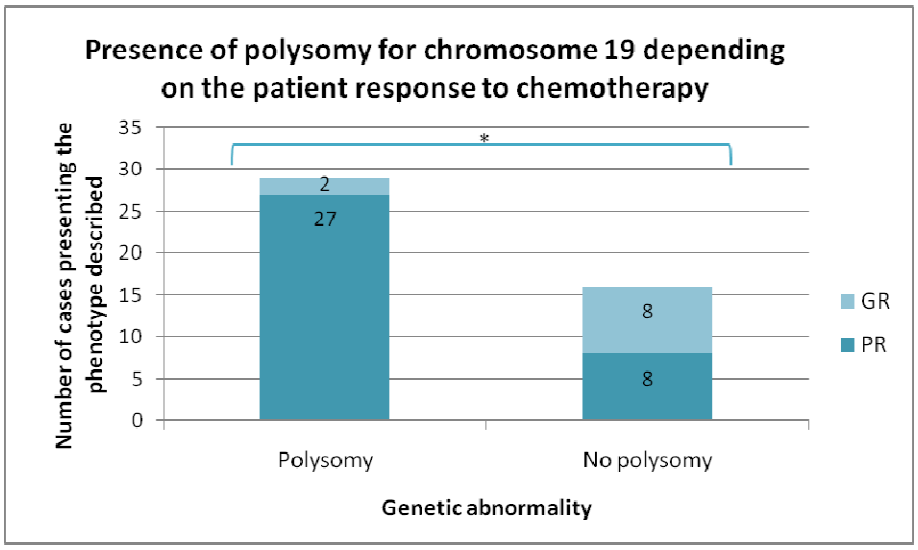


Figure 5.4: Bar chart representing the association between occurrence of chromosome 19 polysomy and response to chemotherapy in patients with OS.
* for p<0.005 with the one-tailed Fisher exact test.

5.2.2 Result of the screening of single nucleotide polymorphism changes

5.2.2.1 Results of SNP screening by restriction digestion and sequencing

To investigate further the occurrence of loss of a parental *LKB1* allele by abnormalities undetectable by FISH, the status of five single nucleotide polymorphisms (SNP), rs56354945 in exon 1, rs2075604 and rs34928889 in intron 2-3, rs2075607 in intron 7-8 and rs59912467 in exon 8 was investigated by a restriction digestion method, as described in the Materials and Methods chapter (section 2.6.3). The samples included 21 OS and 1 OB cases, previously used for the protein screening in Chapter 4. When somatic loss of one parental allele was detected (e.g. normal tissue heterozygous and tumour tissue homozygous), the result was confirmed by sequencing both the tumour and the normal tissue. The status of SNPs in the area sequenced was thus monitored as well. Moreover, when tumours were sequenced for screening of exonic mutations (section 5.3), the status of SNPs covered in the screen was monitored to identify additional cases with non-parental allelic loss. The results are summarized in Figure 5.5.

Among the whole cohort, 9 cases did not present any SNP that were informative. In the remaining 13 cases, 4 of 12 (33%) OS cases presented a parental allelic loss and 1 of 1 OB case at the rs34928889 locus. Interestingly, all five cases presented the same nucleotide change, i.e. loss in the tumour of the G allele compared to the normal tissue being A/G. Overall, 5 of 13 (38%) informative cases presented loss on one parental allele. In two cases, S175 and S2257, the gene deletion was probably more extensive as allelic loss was also detected in intron 7-8 (rs2075608). The study of further SNPS outside *LKB1* could help to determine the exact size of the deletion. On the contrary, in the OB case, S2290, and in case S2258, other SNPs along the gene were heterozygous, suggesting that the deletion may be restricted within the gene.

The remaining 8 of 13 (62%) cases were found to have at least two heterozygous SNPs within the tumour. As not all the SNPs were informative, this does not fully exclude the presence of a microdeletion at those loci.

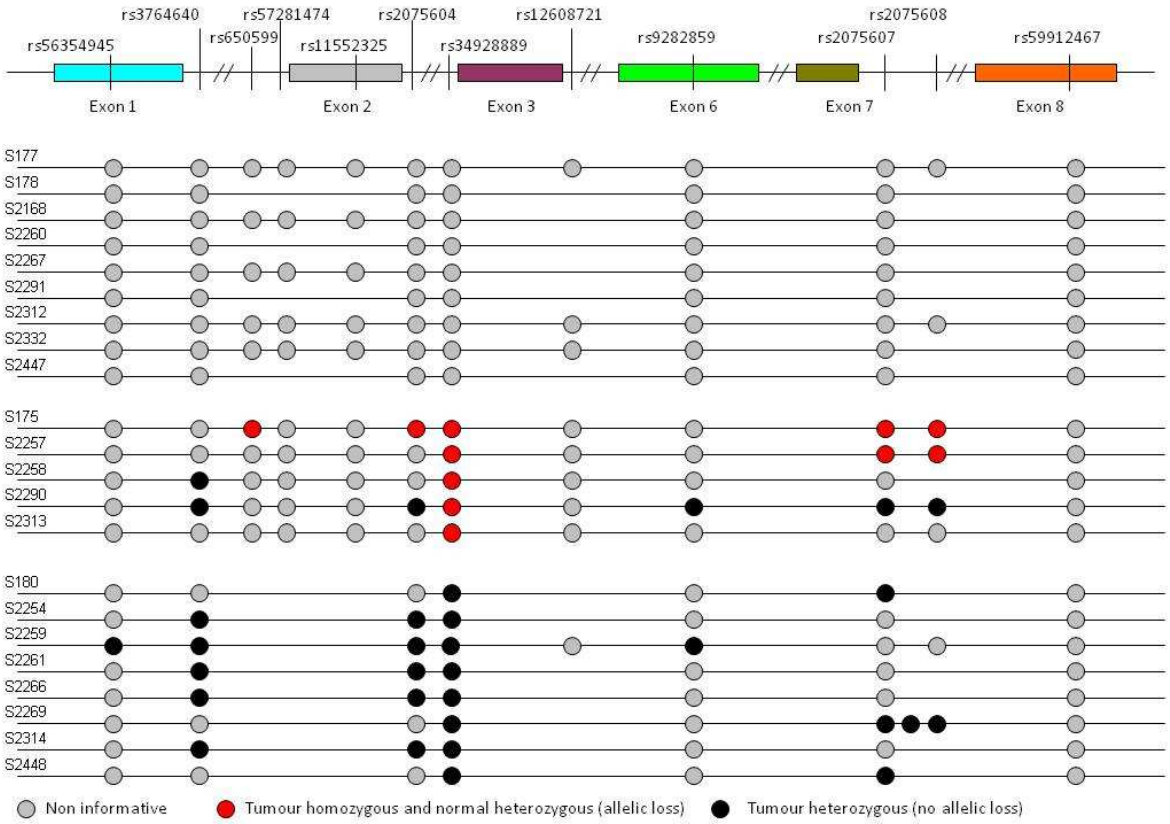


Figure 5.5: Map of the SNP analysed and results of the screening by restriction digestion and sequencing.

5.2.2.2 Results of qPCR

As a large proportion of cases were found to have copy number gain of chromosome 19, loss of one parental allele could be accompanied by a gain of the remaining allele (e.g. copy neutral loss). Indeed, 2 of 5 (40%) cases with parental allelic loss presented polysomy by FISH: S175 presented 30% of its cells with 3 to 5 copies of chromosome 19, and S2258 had 30% with 3 to 10 copies. Hence, to assess whether the microdeletions detected were accompanied by a copy number loss, the number of each allele was quantified by semi-quantitative PCR (section 2.6.6).

As expected, no signal was detected for the allele G on the 5 cases that had lost it somatically as determined by restriction digestion and sequencing. One of 5 (20%) cases, S2257, presented a fold difference for the allele A of less than 1 (0.70±0.27). This means that the allelic loss was accompanied by a copy number loss of *LKB1*. It is of note that this case presented with allelic loss in two other SNPs in intron 7-8. Two other cases presented a fold difference of less than two (S175 with 1.38±0.13 and S2290 with 1.40±0.57); these are not fully conclusive, and it is possible that

only a proportion of cells had a strict copy number loss, with the remaining cells having a copy neutral loss. The remaining two cases had a fold difference around 2 (S2258 with 1.77±0.57 and S2313 with 2.10±0.14), implying copy neutral loss.

5.2.2.3 Association between LOH and protein expression

To assess whether the loss of one parental allele was associated with a reduced protein expression, the integrated densitometry of the band detected by WB was compared among the informative cases with (n=5) or without (n=8) parental allelic loss detected. No statistically significant difference was found between these two groups (p=0.699 with the Mann-Whitney U test)(Figure 5.6). However, the case S2257, with a strict copy number loss of *LKB1*, expressed very low amount of protein by WB and by IHC, with a score of 0 for both methods (Figure 3.3 and Figure 3.4). Among the remaining cases, one (S2258) presented with a high level of protein by WB with a score of 2, but not by IHC (score of 0); two cases presented with a score of 1 (S175 and S2313) and the OB case (S2290) a score of 0.

5.2.2.4 Conclusion

Of the 21 OS and 1 OB cases studied, two presented with a probable copy neutral loss of *LKB1,* two cases were borderline and one had a parental allelic loss with copy number loss, all due to a microdeletion on the allele G in SNP rs3492889.

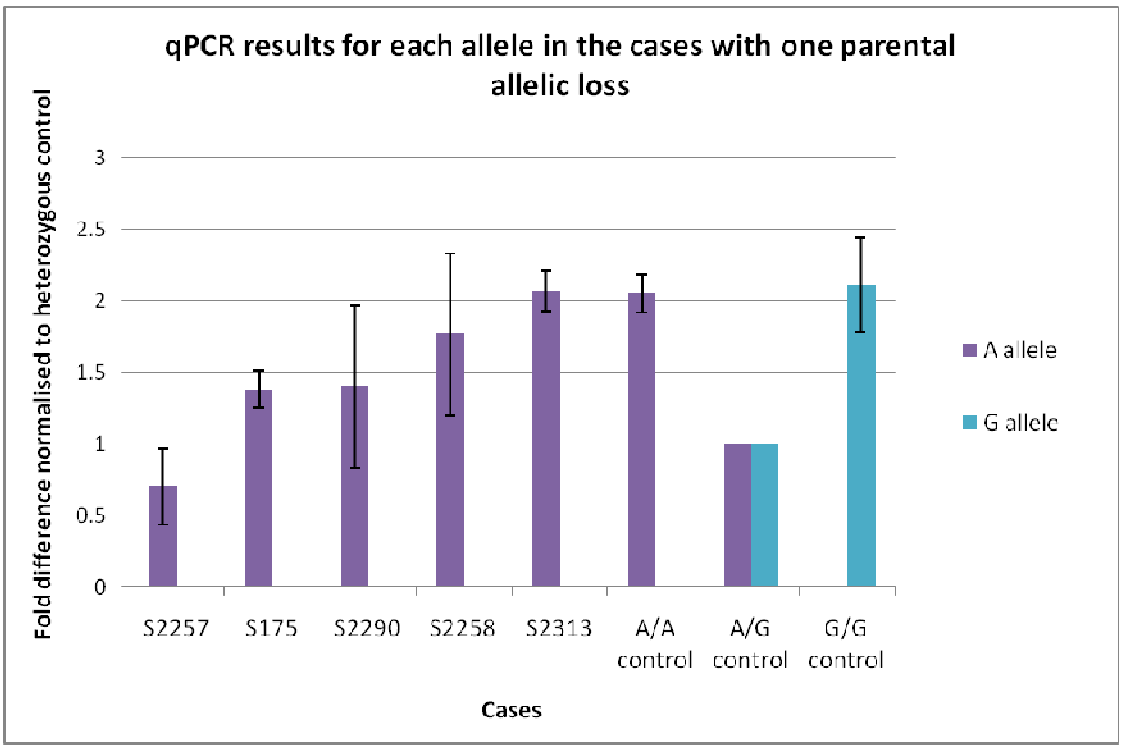


Figure 5.6: qPCR results to quantify whether the loss of one parental allele was a copy neutral loss.

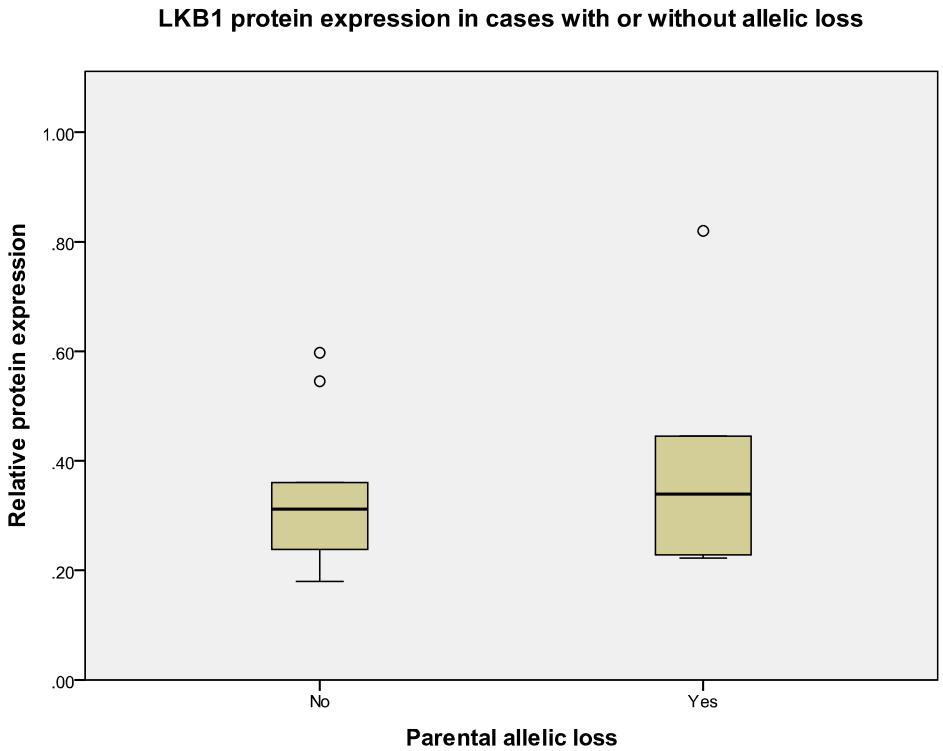


Figure 5.7: Box plot representing the association between LOH and protein expression by WB.
The round points represent the outliers. The relative protein expression is the ratio of the integrated density of the LKB1 band by WB compared to GAPDH.

5.3 Screening of exonic mutations in *LKB1*

As discussed in the previous chapter (section 4.5.5), the detection of LKB1 protein does not exclude loss of its function by mutations that are not affecting the conformation or the presence of the epitope against which the antibody used was raised. To investigate this further, direct sequencing of the 21 OS and 1 OB cases used for the SNP screening was performed as described in the Materials and Methods chapter (section 2.6.1 and 2.6.2) in exons 1, 4, 5, 6 and 8, which are commonly reported to harbour mutations (COSMIC database)(Sanchez-Cespedes et al. 2002). We failed to detect any mutations in these exons in all the cases. We also failed to detect any in exons 2, 3 and 7 in the 5 cases presenting loss of a parental allele. To conclude, *LKB1* point mutation is unlikely to be a common event explaining the loss of protein expression in OS. The genetic loss of one parental allele in a subset of cases was not accompanied by detectable mutation as a second "hit".

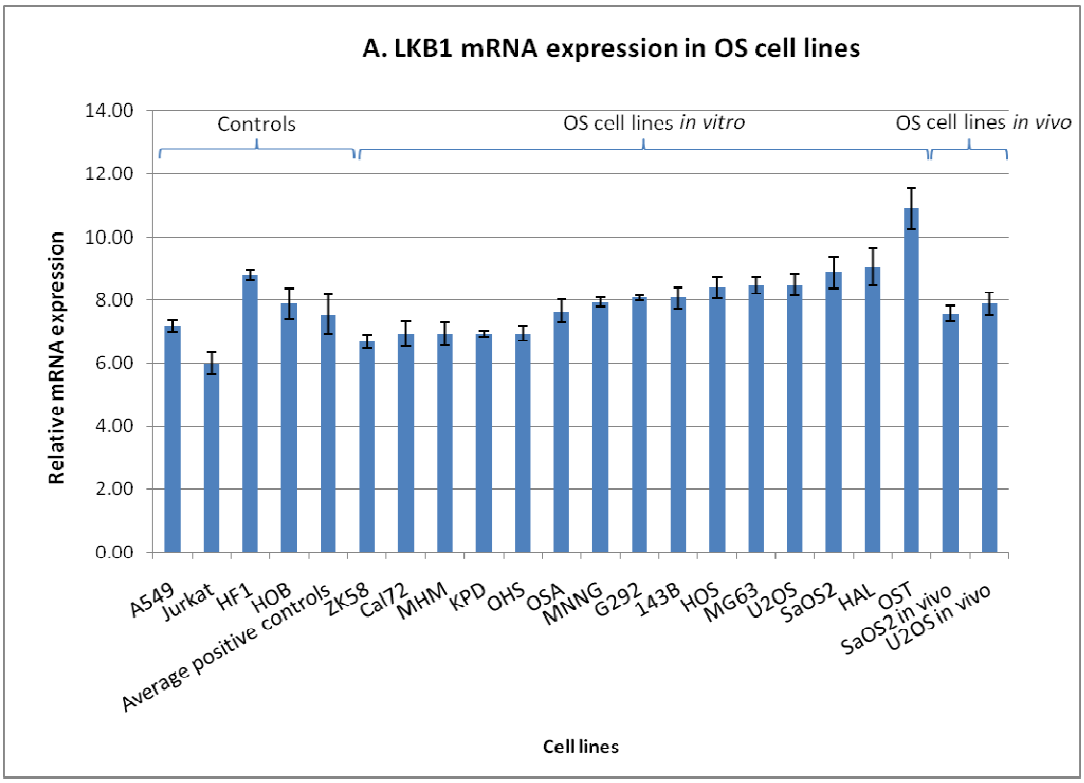
5.4 LKB1 mRNA expression

As detailed in the introduction of this chapter (section 5.1), a "hit" is defined as the loss of expression or function of one parental allele. This can be caused by a mutation or a gene deletion, which were studied in paragraphs 5.3 and 5.2. There was no convincing evidence that both alleles of *LKB1* were inactivated by these mechanisms in the OS cases screened. To confirm whether the observed genetic abnormalities were translated in a differential gene expression, the mRNA level of *LKB1* was assessed by qRT-CPR, as described in the Materials and Methods chapter (section 2.6.4).

5.4.1 Results of the relative mRNA expression in Osteosarcoma cases and cell lines

5.4.1.1 Results of the qRT-PCR screening

The relative mRNA expression of *LKB1*, expressed as the ΔCt of LKB1 compared to β-actin, was calculated for the 15 OS cell lines and the 21 OS and 1 OB cases (Figure 5.8). All the OS and OB cases expressed significant *LKB1* mRNA levels compared to the average of the positive controls (Jurkat, HF1 and HOB cell lines), including case S2257 which showed loss of one parental allele with copy number loss. Only 1 of 15 (7%) OS cell lines, OST, which expressed the lowest level of LKB1 protein (Figure 4.1 and Figure 4.2), revealed low levels of *LKB1* mRNA compared to the average of the positive control cell lines. It is of note that the expression between the cases was quite heterogeneous, with up to two cycle difference between the different samples and cases. The significance of this discrepancy is discussed below (section 5.4.2).



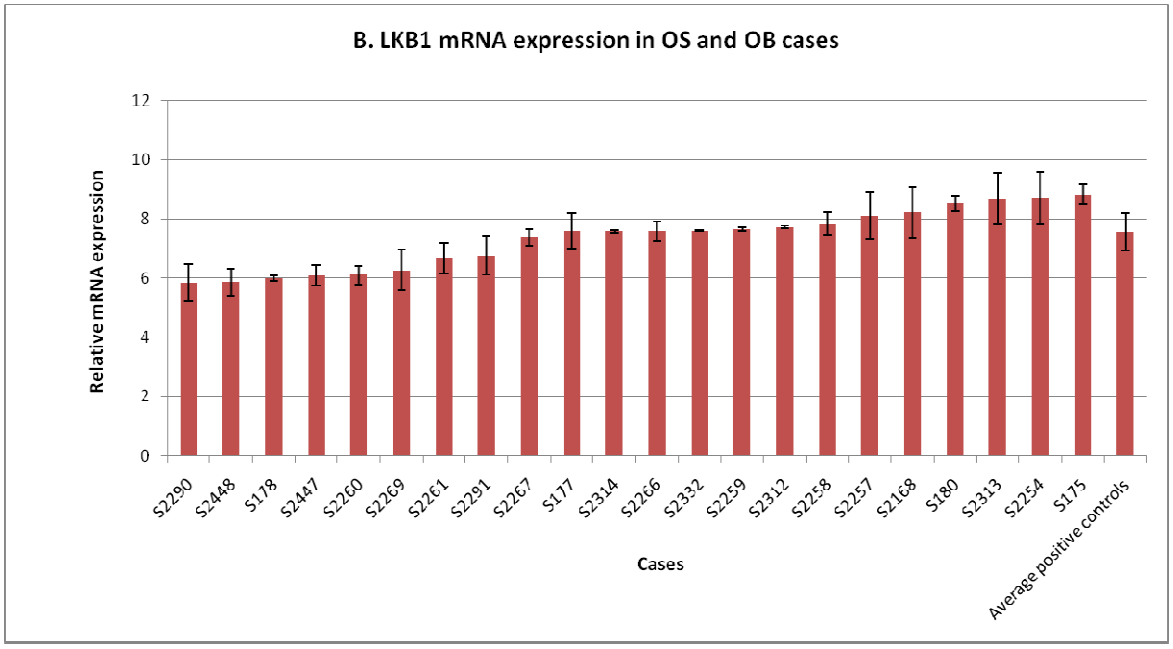


Figure 5.8: LKB1 mRNA expression in OS cell lines (A.) and OS and OB cases (B.)
The relative mRNA expression is expressed as the ΔCt of LKB1 compared to β-actin.

5.4.1.2 Results of a gene expression microarray

To confirm the results obtained by qRT-PCR, the gene expression profile for *LKB1* was monitored on gene expression microarray data (U133A Affymetrix gene expression microarray) previously published by our group (Henderson et al. 2005). The analysis was performed using Cluster and Treview software package(Eisen et al. 1998) , free to download from Eisen's lab at:

- http://rana.lbl.gov/EisenSoftware.htm

The average linkage clustering of 55 cell lines, including 10 OS, and 86 sarcoma cases, including 10 OS, was calculated to compare the signal detected in OS to the standard deviation from the median of all the samples. The result for both *LKB1* probes and their median from the OS samples only is presented in the heatmap in Figure 5.9. In common with the qRT-PCR results, the level of mRNA expression detected in the primary cases and in the cell lines was heterogeneous, and no clear *LKB1* down-regulation was observed with both probes compared to other sarcoma cell lines or cases, except in one cell line (CBOSM1) and one case (513). Eight of 10 (80%) cases expressed levels of LKB1 that were very close to the median on all sarcoma cases. Overall, six of 10 (60%) OS cases and 4 of 10 (40%) OS cell lines had a slight *LKB1* downregulation but the remaining cases showed the inverse pattern, with an upregulation of *LKB1*. Hence, this confirms that *LKB1* expression is not frequently lost at the mRNA level in OS.

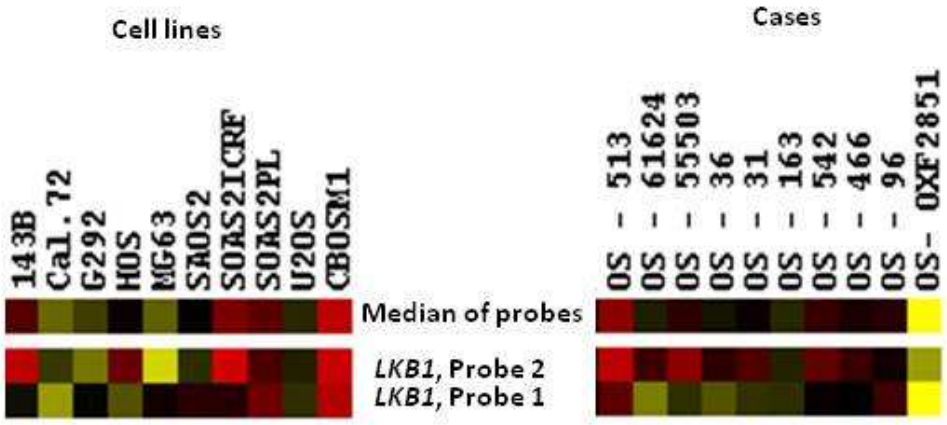


Figure 5.9: Heatmap showing the expression of *LKB1* in OS cell lines and cases.
The red pattern indicates a downregulation of the mRNA expression compared to the standard deviation from the median on all samples, the yellow pattern an upregulation and the black pattern a similar level of expression.

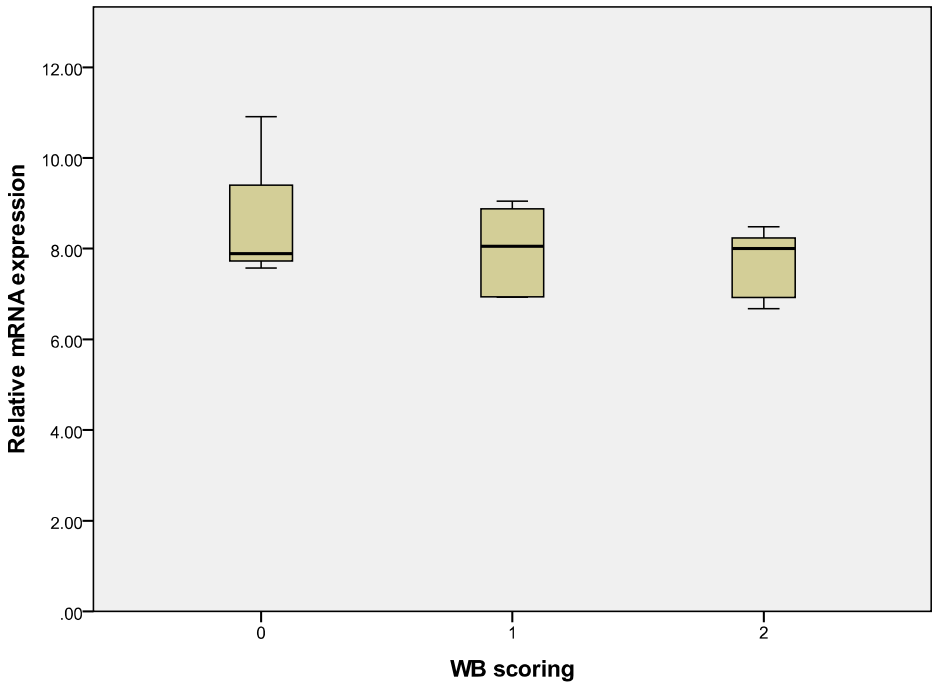
5.4.2 Association between mRNA and protein expression

To explain the discrepancy observed between samples at the mRNA level, the association between the protein and mRNA expression of *LKB1* was studied. *LKB1* relative mRNA expression, i.e. the ΔCt of LKB1 compared to β-actin on qRT-PCR was not normally distributed (p=0.91 with the Shapiro-Wilk normality test). No association was found between protein (expressed as the relative integrated density of LKB1 compared to GAPDH by WB) and mRNA expression using the two-tailed Spearman rho test (p=0.878) for the cell lines. Similarly, no association was found for the 21 OS and 1 OB cases (p=0.145 with the two-tailed Spearman rho test). The same result was obtained when comparing the IHC scoring obtained for the cases with the relative mRNA expression (p=0.239 with the Kruskall-Wallis test). This is illustrated in Figure 5.10. To conclude, the difference observed between the samples at the mRNA level was not translated in a different protein expression. This suggests that LKB1 protein is regulated post-transcriptionnally in OS.

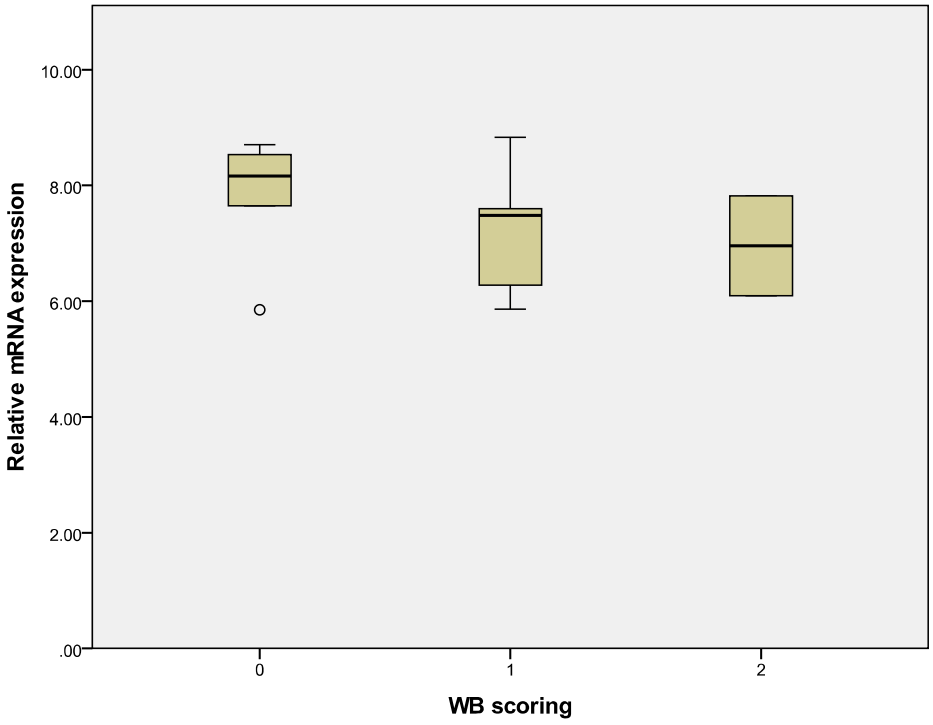
5.4.3 mRNA expression in cases presenting a parental allelic loss

To assess whether the loss of one parental allele resulted in a reduction of mRNA expression, hereby showing a haploinsufficient effect of *LKB1* gene loss, the mRNA expression between cases with or without parental allelic loss was compared. This could only be performed among the 12 informative cases. There was a trend for cases with allelic loss to have a higher ΔCt of LKB1 compared to β-action on qRT-PCR, i.e. to express lower levels of LKB1 mRNA than cases without the loss as presented in Figure 5.11. However, no association was found between these two groups (p=0.298 with the Mann-Whitney U test), suggesting that loss of one parental allele may not be sufficient to induce a significant effect at the mRNA level.

A. LKB1 mRNA expression compared to the protein expresion in OS cell lines



B. LKB1 mRNA expression compared to the protein expression by WB in OS cases



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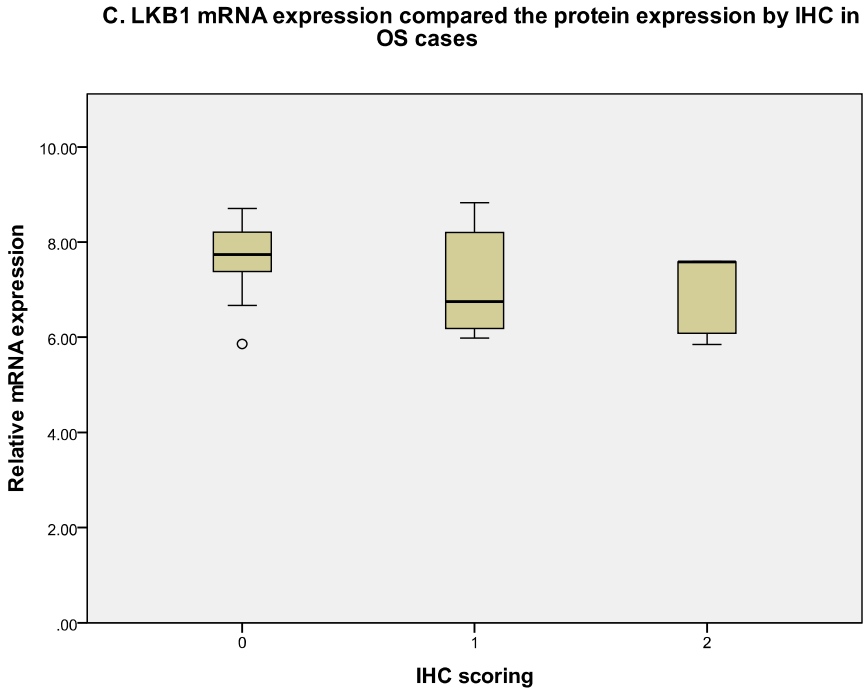


Figure 5.10: Box plots illustrating the association between LKB1 mRNA and protein expression.
The relative mRNA expression is expressed as the ΔCt of LKB1 compared to β-actin, against the WB scoring in OS cell lines (A.), and against the WB (B.) or IHC (C.) scoring in 21 OS and 1 OB cases. The round point represents the outliers.

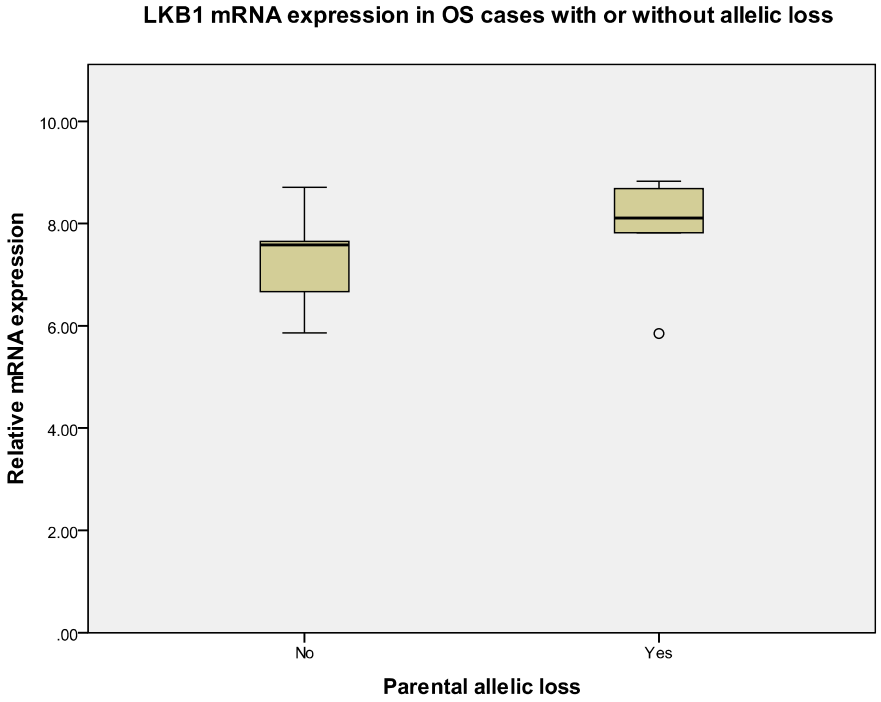


Figure 5.11: Box plot comparing the mRNA expression in OS cases with or without a detected LOH.
The relative mRNA expression is expressed as the ΔCt of LKB1 compared to β-actin on qRT-PCR. The round points represent the outliers.

5.5 Discussion: genomic abnormalities in *LKB1* in Osteosarcoma and post-transcriptional regulation

LKB1 is considered as a tumour suppressor gene due to its role in cancer development in PJS patients, and in lung cancer. In this chapter, we used different methods to assess whether *LKB1* possessed 2, 1 or no "hits" leading to the loss of protein detected by WB and IHC (Chapter 4). The most common genetic abnormality found in sporadic cases of lung cancer is chromosomal loss of *LKB1* locus, 19p13.3 (Rodriguez-Nieto et al. 2009). This is also the most frequently reported "hit" in other cancers (section 1.2.1.4) including colorectal (Trojan et al. 2000), gynaecological (Kato et al. 2004, Wang et al. 1999a), breast (Forster et al. 2000) and brain (Sobottka et al. 2000). We therefore investigated first whether this event occurred in OS.

5.5.1 Genetic abnormalities detected by FISH

Contrary to the findings in other cancers, the study of *LKB1* by FISH did not reveal any case with copy number loss of the locus 19p13.3 but we identified other abnormalities such as polysomy of chromosome 19 in a large proportion of cases.

5.5.1.1 Polysomy of chromosome 19

Genetic changes in OS cases have been extensively screened in the last 10 years, and *LKB1* locus has been reported as both lost (Squire et al. 2003, Stock et al. 2000, Tarkkanen et al. 1999) and gained (Ozaki et al. 2002, Zielenska et al. 2001) by Comparative Genomic Hybridisation or SNP arrays (section 1.2.3.3). Zielenska et al reported gains in chromosome 19 in 8 of 17 (47%) cases and Ozaki et al in 6 of 47 (13%) cases. Our screening by FISH indicated that polysomy of chromosome 19 may be more frequent than previously reported, as 61 of 93 (66%) cases had copy number gains of both *LKB1* and the telomere locus; it was rare in OB, (2 of 24; 8%) and only one additional copy of the chromosome was found. This finding is not surprising as OS often have very complex karyotypes, unlike OB, a benign tumour. Moreover, the tissue used in the TMA was post-chemotherapy for most of the cases, which would favour these abnormalities. The fact that polysomy of chromosome 19 is more frequent in PR compared to GR suggests that either this

pattern is acquired following the chemotherapy treatment of the cells or that the treatment selects for cells with this abnormality. Finally, the polysomic cases were more likely to express LKB1 protein. This is not surprising as polysomic cases were mainly PR, contrary to cases lacking LKB1 protein, which were mainly GR (section 4.3.1). However, this suggests that polysomic cases were unlikely to have lost *LKB1* at the genetic level.

5.5.1.2 Copy number gain of *LKB1*

In addition to the polysomy, copy number gain of LKB1 compared to the telomere was found in 14 of 91 (15%) OS cases. Overall, 75 of 93 (80%) OS cases presented at least three copies of *LKB1* and 2 of 24 (15%) OB cases. This copy number gain may promote the expression of another gene close to the *LKB1* locus. The BAC probe used to detect *LKB1*, RP11-81M8, can be used to detect 60 other genes in the 19p13.3 locus. Among these genes, seven could be relevant to cancer or bone biology: fibroblastic growth factor 22 (*FGF22*), ephrin-A2 (*EFNA2*), follistatin-like 3 (*FSTL3*), ATP-binding cassette sub-family A (ABC1) member 7 (*ABCA7*), ribosomal protein S15 (*RSP15*), MAP kinase interacting serine/threonine kinase 2 (*MKNK2*) and methyl-CpG binding domain protein 3 (*MBD3*). *FGF22* and *EFNA2* are growth factors, and can activate TKR from the fibroblast and ephrin families respectively; however the role of FGF and ephrins in OS development are unknown, even though LOH of *FGFR* has been previously reported (Mendoza et al. 2005). *FSTL3* is involved in bone formation and osteoclast differentiation (Bartholin et al. 2005). *RSP15* is a 40S ribosomal protein family member and is involved in transcription regulation. *MNKN2* could regulate transcription by interacting with eIF4E (Mahalingam et al. 2001), which was detected in 127 of 141 (90%) of our OS cohort (Table 3.1). *MBD3* participates in DNA methylation and has been suggested as a novel therapeutic target in cancer (Sansom et al. 2007). However, to our knowledge, there is no report of the role of any of these genes in OS pathogenesis. Further studies are needed to understand whether the increased copy number of the any of these genes in the locus 19p13.3 promotes tumourigenesis.

5.5.1.3 Copy number gains of chromosome 19 telomere

A very small subset of OS cases, 2 of 93 (2%), and almost a third of OB cases, 8 of 24 (33%), presented an increased copy number of the telomeric probe compared to the LKB1 probe. Therefore, these cases may either present a copy number gain of the chromosome 19 telomere, or a copy neutral loss of *LKB1*. As no centromeric probe is available for chromosome 19, these two patterns cannot be distinguished by FISH. However, it seems more probable that the cases present a copy number gain of the telomere, as it requires only one genetic event. The telomeric BAC probe used for chromosome 19q mapped with zinc finger proteins, which may be involved in transcription regulation. Their role in cancer is unknown. Hence, the effect of the copy number gain of these genes remains to be established.

5.5.1.4 Conclusion

The detection of two copies of *LKB1* locus in all cases and the high occurrence of copy number gain of *LKB1* or polysomy of chromosome 19, which together totalled 75 of 93 (81%) of cases suggests that LKB1 protein loss is unlikely to be caused by strict copy number loss of *LKB1* via the deletion of the whole 19p13.3 locus. However, this does not exclude loss of *LKB1* by other mechanisms undetectable by FISH.

5.5.2 LKB1 loss by microdeletions

5.5.2.1 Technical limitations of the FISH and SNP screening

In the presence of mutations or microdeletion of *LKB1*, followed by mitotic recombination, two copies of *LKB1* would be detected by FISH without the expression of a functional protein. Similarly, the duplication of an allele already presenting a mutation or microdeletion of *LKB1* would be detected as a copy number gain of *LKB1* by FISH, without leading to an increased protein expression. Hence, the results obtained by FISH were not sufficient to conclude whether *LKB1* was lost in OS at the genetic level. Moreover, FISH screening enables assessment of the status of a large cohort of cases as paraffin material is used, but it only assesses the loss of a chromosomal region spanning several genes. Studying the status of *LKB1* at the SNP level enables a more refined picture of the genetic changes in the gene. However, this method, for which DNA was extracted from snap-frozen tumour tissue, cannot as easily be performed on a large scale. As reported by Sanchez-Cespedes et al, small intronic deletions of *LKB1* were frequent in lung cancer (Sanchez-Cespedes et al. 2002) and these cannot be detected by FISH. Therefore, to investigate further the occurrence of LKB1 loss, the status of several SNPs was assessed.

5.5.2.2 Microdeletions in *LKB1* in Osteosarcoma

As mentioned in the paragraph above, the study of the SNP status of *LKB1* proved more informative than the FISH screening. The loss of a parental allele was confirmed in 5 of 12 informative cases (42%), including 4 OS and 1 OB. Interestingly, all cases presented the same loss, of allele G of SNP rs34928889. In at least two cases, these deletions were restricted to a portion of the gene, as both alleles of *LKB1* were detected at other SNPs loci (Figure 5.5). In addition, 1 of 5 (20%) cases presented copy number loss and 4 of 5 (80%) had at least two copies of the remaining allele. These results, in light of the data obtained by FISH, suggest that *LKB1* is indeed lost due to microdeletions within the gene in a small subset of cases.

5.5.3 LKB1 and haploinsufficiency in Osteosarcoma

5.5.3.1 The search for a second "hit"

As a small subset of cases presented a genetic abnormality of *LKB1,* the presence of a second "hit" was investigated, as suggested by the Knudson "two-hit" hypothesis (Knudson, Jr. 1971). However, no further abnormalities were detected. No mutations were found in the 5 cases with loss of a parental allele in exon 1 to 8, where all the mutations were detected in lung cancer (Sanchez-Cespedes et al. 2002), and all the cases expressed a significant amount of mRNA. This also ruled out the possibility of gene silencing by promoter methylation or of large intronic deletion, which would prevent transcription. Hence only one "hit" was detected, the loss of a parental allele by gene deletion with copy number loss in rare cases.

5.5.3.2 Investigating the haploinsufficient effect of *LKB1*

As discussed in the Introduction (section 1.2.1.4) the status of *LKB1* is under debate: contrary to the classical view of the Knudson two-hit hypothesis, recent findings suggest that *LKB1* can play a role as a haploinsufficient gene. This is confirmed by the finding that *Lkb1*$^{+/-}$ mice develop osteogenic tumours (Robinson et al. 2008). Our finding of only one "hit" for *LKB1* suggests that this gene is at best haploinsufficient in OS.

However, no statistically significant association was found between presence of an allelic loss and the amount of LKB1 mRNA and protein expressed. Nevertheless, a trend was observed at the mRNA level (Figure 5.10) and the protein expression in all these cases was reduced compared to the controls.

The cases were compared to the group with no allelic loss detected and at least one SNP showing both parental alleles present. As not all SNPs were informative, it is possible that some cases among this group have an undetected allelic loss. This may explain why no significant association was found. Moreover, only 12 cases were informative, so the results should be confirmed on a larger cohort.

To conclude, *LKB1* may act as a haploinsufficient gene in a small subset of OS cases, rather than a tumour suppressor gene, with 1 of 12 (8%) cases showing loss of a parental allele with copy

number loss of *LKB1*. However, even in this case, this genetic abnormality did not fully explain the absence of LKB1 protein as the mRNA level detected was comparable to the other cases.

5.5.4 LKB1 regulation is post-transcriptional in Osteosarcoma

Although the presence of parental allelic loss in a small subset of cases may contribute to LKB1 reduced protein expression in OS, most cases and cell lines presented comparable levels of mRNA. Only one cell line showed loss of *LKB1* at the mRNA level, OST, suggesting a different mechanism of regulation of *LKB1* expression to primary tumours. Moreover, there was no association found between the level of mRNA and the level of protein expression (Figure 5.10) in primary tumours or cell lines. To conclude, the regulation of LKB1 expression in OS appears to be post-transcriptional. The possible mechanisms of this regulation will be investigated in the next chapter.

Chapter 6: POST-TRANSCRIPTIONAL REGULATION OF *LKB1* IN OSTEOSARCOMA

6.1 Introduction and aims

As concluded from the previous chapter on the genetics of *LKB1* in OS (section 5.5.4), LKB1 protein expression seems to be regulated post-transcriptionally: LKB1 mRNA was detected in all patient tumours and all but one cell line, irrespective to their protein expression, and genetic abnormalities were rare and did not correlate with a decrease in mRNA expression. Therefore, our aim in this chapter was to investigate this post-transcriptional mechanism further. We hypothesised that either LKB1 mRNA translation or LKB1 protein stability was de-regulated in OS. Two mechanisms introduced in section 1.2.1.5 were considered: the control of LKB1 mRNA translation by miRNAs or the control of LKB1 protein stability by changes in its acetylation status caused by the protein SIRT1.

6.1.1 miRNA

As miRNA can inhibit the translation of mRNA (Flynt et al. 2008), they may regulate *LKB1* expression in OS. As presented in the Introduction, there is little known at present on the miRNA profile of OS (section 1.1.5.2) and on the role of miRNAs in connection with LKB1 (section 1.2.1.1). Only one study reports that mRNA translation of MO25, which forms a complex with LKB1 and hereby constitutively activates it, could be regulated by miRNA-451 in glioma cells (Godlewski et al. 2010b, Godlewski et al. 2010a). This finding suggests that miRNAs may at least be implicated in LKB1 regulation indirectly, by targeting its complex.

The objective in the first part of this chapter was:

- Assess whether miRNAs could regulate LKB1 protein expression

6.1.2 Protein stability

As introduced in section 1.2.1.5, SIRT1 is a NAD-dependant deacetylase enzyme that can interact with AMPK to control cell proliferation in energy deprived conditions. Moreover, it can directly modify LKB1 acetylation status (Lan et al. 2008, Zu et al. 2010), thereby disrupting its stability and function. The exact mechanism of this interaction is unknown and conflicting results are published: one study suggests that LKB1 deacetylation by SIRT1 leads to increased kinase activity (Lan et al. 2008) when another demonstrated that it led to LKB1 ubiquitination and degradation (Zu et al. 2010). The effect of SIRT1 on LKB1 may well be context and cell type dependant. There is no report of its role in bone.

The objectives in this second part were:

1. Assess whether de-regulation of SIRT1 was present at the mRNA level
2. Correlate SIRT1 expression at the mRNA level with LKB1 expression at the protein level

The role of LKB1 protein degradation in OS and of SIRT1 de-regulation at the protein level was studied further by Dr Malihe Eskandarpour and will be discussed here briefly.

6.2 miRNA and LKB1 protein expression

To assess the role of miRNAs in the regulation of LKB1 in OS, the miRNA expression profile from the frozen biopsies of 24 patients with OS was determined using an Agilent miRNA microarray, as described in the Materials and Methods chapter (section 2.7). The clinical characteristics of these cases are presented in supplementary data (Table 10.1).

6.2.1 Data mining on miRNA targeting LKB1

We first investigated the bioinformatic target predictions for LKB1 with the online algorithms Targetscan and PITA, as described in the Materials and Methods (section 2.7.4). Fourteen miRNAs were predicted to bind to the 3' UTR of LKB1 with both algorithms (Table 10.2). Among these, two miRNAs were highly expressed in all the 24 OS samples included in the study in the miRNA microarray data: hsa-miR-663 and hsa-miR-93. Additionally, miR-663 was also upregulated in 3 of 3 (100%) negative cases for LKB1 expression by IHC and downregulated in 8 of 19 (42%) positive cases. Similar results were found in the online sarcoma microRNA expression database SMED (Sarver et al. 2010). Hsa-miR-93 expression was high in all tumours without any differential expression between the cases or compared to other sarcomas. Hsa-miR-663 was highly expressed in OS but was either upregulated or downregulated in different OS cases compared to other sarcomas. Following this preliminary screen, 3 cases lacking LKB1 protein expression by IHC and 4 cases showing high expression and not osteoclast-rich (Figure 10.2 in Supplementary data) were selected and the most differentially expressed miRNAs between the two groups were quantified.

6.2.2 Differentially expressed miRNAs in cases with or without LKB1 protein expression

Twenty miRNA probes were identified as differentially regulated between cases expressing or not expressing LKB1 protein by IHC, as shown in the heatmap in Figure 6.1.B. They corresponded to 15 different miRNAs, as some probes hybridised to different regions of the same miRNA, one being a viral miRNA from HIV (hiv1-miR-H1). The latter was probably an artefact and not studied further. As illustrated by the histogram (Figure 6.1.A), the Z-scores were well separated into two groups. The p-values and fold changes obtained for each probe are reported in Table 6.1.

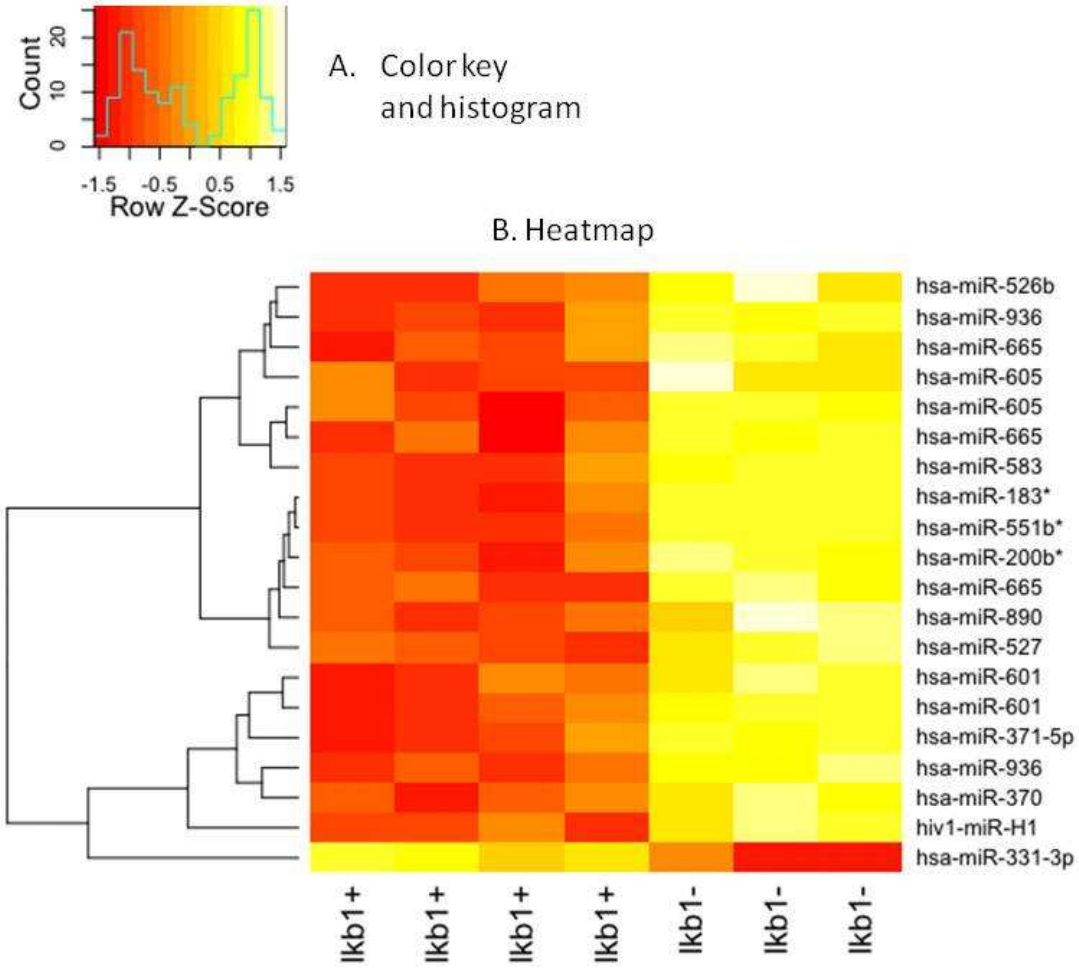


Figure 6.1: Heatmap of the most differentially regulated miRNAs in cases with or without LKB1 protein expression.
The color key and histogram of the Z-score of all cases is presented in A. Yellow indicates an upregulation and red a downregulation. The heatmap of the 20 most differentially regulated miRNAs between cases with (lkb1+) or without (lkb1-) LKB1 protein expression is in B. Each column corresponds to one case.

miRNA ID	p-value	Fold change	LKB1 complex targeted	Prediction scores for LKB1 complex (in PITA ; in Targetscan)	SIRT1 targeted	Prediction scores for SIRT1 (in PITA ; in Targetscan)
hsa-miR-183*	0.0005	1.658524	No		No	
hsa-miR-200b*	0.0011	1.611272	No		No	
hsa-miR-331-3p	0.0011	0.37229	No		No	
hsa-miR-370	0.001	4.41802	MO25	(NA ; -0.23)	No	
hsa-miR-371-5p	0.0009	1.850224	Strad	(-2.49 ; -0.05)	No	
hsa-miR-526b	0.0008	2.810253	MO25	(NA ; -0.21)	Yes	(-0.94 ; -0.12)
hsa-miR-527	0.0009	1.315307	MO25	(-8.87 ; -0.13)	Yes	(-3.38 ; NA)
hsa-miR-551b*	0.0001	1.661746	No		No	
hsa-miR-583	0.0008	2.545766	No		No	
hsa-miR-601	0.0005	3.30947	MO25	(-9.07 ; NA)	Yes	(-3.14 ; -0.46)
hsa-miR-601	0.0016	3.065027	MO25	(-9.07 ; NA)	Yes	(-3.14 ; -0.46)
hsa-miR-605	0.0012	2.39961	No		No	
hsa-miR-605	0.0008	1.695252	No		No	
hsa-miR-665	0.001	1.841779	Strad	(NA ; -0.18)	No	
hsa-miR-665	0.0014	1.612724	Strad	(NA ; -0.18)	No	
hsa-miR-665	0.0006	1.355289	Strad	(NA ; -0.18)	No	
hsa-miR-890	0.0009	1.799754	MO25	(-5.56 ; NA)	No	
hsa-miR-936	0.0002	3.573159	MO25	(-2.43 ; NA)	Yes	(-5.71 ; -0.12)
hsa-miR-936	0.0006	2.851063	MO25	(-2.43 ; NA)	Yes	(-5.71 ; -0.12)

Table 6.1 : List of the 19 most differentially regulated human miRNA probes in cases with or without LKB1 protein expression.
hiv1-miR-H1 was excluded from the study.

6.2.3 Targets of the miRNAs deregulated in cases with or without LKB1 protein expression

6.2.3.1 miRNAs targeting LKB1 mRNA

Hsa-miR-663, identified as a miRNA potentially targeting LKB1 mRNA in OS in the preliminary data-mining study (section 6.2.1) was not significantly deregulated in this cohort of cases. Additionally, none of the most differentially de-regulated miRNAs identified in cases expressing versus not expressing LKB1 protein directly target LKB1, according to bioinformatics predictions with both PITA and Targetscan algorithms. This suggests that LKB1 expression is not directly regulated by these miRNAs in OS.

6.2.3.2 miRNAs targeting LKB1 complex

Interestingly, 8 of the 14 (57%) miRNAs identified in the heatmap in Figure 6.1 were predicted to target either MO25α or Stradα using either PITA or Targetscan algorithms (Table 6.1). Of note is that SIRT1 may also be targeted by 4 of 14 (29%) of these miRNAs, in addition to their targeting of MO25α. Moreover, two miRNAs target HSP90, a chaperone protein which facilitates the formation of LKB1 complex, according to Targetscan but not to PITA algorithm: miR-370 (score of -0.20 for HSP90AA1 and -0.43 for HSP90AB1) and miR-601 (score of -0.13 for HSP90AA1). Hence the miRNA signature identified could influence LKB1 complex stability.

6.2.3.3 Other predicted targets

To investigate whether other genes belonging to the *LKB1* network were targeted by miRNAs of this signature, all their highly conserved targets predicted by Targetscan were listed, totalling 1836 genes with a negative score. Ten of 1836 (0.5%) were predicted as targets of 4 different upregulated miRNAs in the signature, 46 of 1836 (2.5%) by 3 and 224 of 1836 (12%) by 2. Among this list of potential target genes, some were previously reported to interact with LKB1 directly, such as *NUAK1* (targeted by miR-371-5p, score of -0.09 and miR-605, score of -0.36), *PRKAB2* which is the regulatory subunit of AMPK (targeted by miR-605, score of -0.19), both phosphorylated by LKB1 (Figure 1.8), and *PAK1* (targeted by miR-562b, score of -0.16) which was

associated with LKB1 role in cell motility (Deguchi et al. 2010, Zhang et al. 2008). Moreover, two genes associated with OS pathogenesis (section 1.1.5.1) were also included: *RB* (targeted by miR-936, score of -0.16) and *FOSB* (targeted by miR-370, score of -0.03), which is part of the JUN family as is *FOS* (Gamberi et al. 1998, Wu et al. 1990). The online bioinformatics resource DAVID (v6.7, National Institutes of Health, USA) (Dennis, Jr. et al. 2003, Huang et al. 2009) was used to validate further the relevance of the potential target genes obtained.

Targets related to Osteosarcoma and other cancers or bone biology

By searching the genes referenced in the OMIM database via DAVID, we confirmed that several targets were relevant to bone biology, as they were associated with variations in human adult height (n=35, the most frequent OMIM reference), with defects in bone mineralisation or with other bone defects, e.g. osteogenesis imperfecta and osteoporosis. Similarly, several targets were previously associated with an increased risk of various cancers, including colorectal and gastric cancer, lung cancer, hepatocellular carcinoma, breast cancer, thyroid cancer, pancreatic cancer, melanoma, ovarian cancer, brain cancer, prostate cancer, leukemia and lymphoma and rhabdomyosarcoma. We deduced that the target genes obtained are relevant to this study. The network obtained was then investigated further.

Gene Ontology

As a large number of the predicted target genes were associated with cancer and/or bone biology, the ontology of the list of target genes was studied. Regulation of transcription was among the most commonly reported biological process, as it concerned 353 of the 1744 (20.4%) genes identified with DAVID. Again, ontology terms associated with bone biology, such as mesenchymal cell differentiation, regulation of osteoblast differentiation and bone development, were represented. Ontology terms for biological processes associated with LKB1 function, presented in the Introduction (section 1.2.1.2), such as cell migration, apoptosis, metabolism and cell migration were also present. These terms are summarised below (Table 6.2). Once more, this confirms that target genes identified with the miRNA signature are relevant to OS pathogenesis and LKB1 function.

GO ID	GO term	Proportion of genes concerned (%)
GO:0060348	bone development	18 of 1744 (1.0%)
GO:0030278	regulation of ossification	16 of 1744 (0.93%)
GO:0048762	mesenchymal cell differentiation	14 of 1744 (0.81%)
GO:0014031	mesenchymal cell development	14 of 1744 (0.81%)
GO:0060485	mesenchyme development	14 of 1744 (0.81%)
GO:0051216	cartilage development	13 of 1744 (0.75%)
GO:0045667	regulation of osteoblast differentiation	10 of 1744 (0.58%)
GO:0045667	regulation of osteoblast differentiation	10 of 1744 (0.58%)
GO:0032330	regulation of chondrocyte differentiation	6 of 1744 (0.35%)
GO:0006928	cell motion	84 of 1744 (4.8%)
GO:0048870	cell motility	56 of 1744 (3.2%)
GO:0016477	cell migration	55 of 1744 (3.2%)
GO:0030036	actin cytoskeleton organization	40 of 1744 (2.3%)
GO:0010941	regulation of cell death	108 of 1744 (6.3%)
GO:0016265	death	83 of 1744 (4.8%)
GO:0012501	programmed cell death	75 of 1744 (4.3%)
GO:0006915	apoptosis	73 of 1744 (4.2%)
GO:0043069	negative regulation of programmed cell death	57 of 1744 (3.3%)
GO:0060548	negative regulation of cell death	57 of 1744 (3.3%)
GO:0042127	regulation of cell proliferation	120 of 1744 (6.9%)
GO:0008283	cell proliferation	65 of 1744 (3.8%)
GO:0040008	regulation of growth	45 of 1744 (2.6%)
GO:0045597	positive regulation of cell differentiation	41 of 1744 (2.4%)

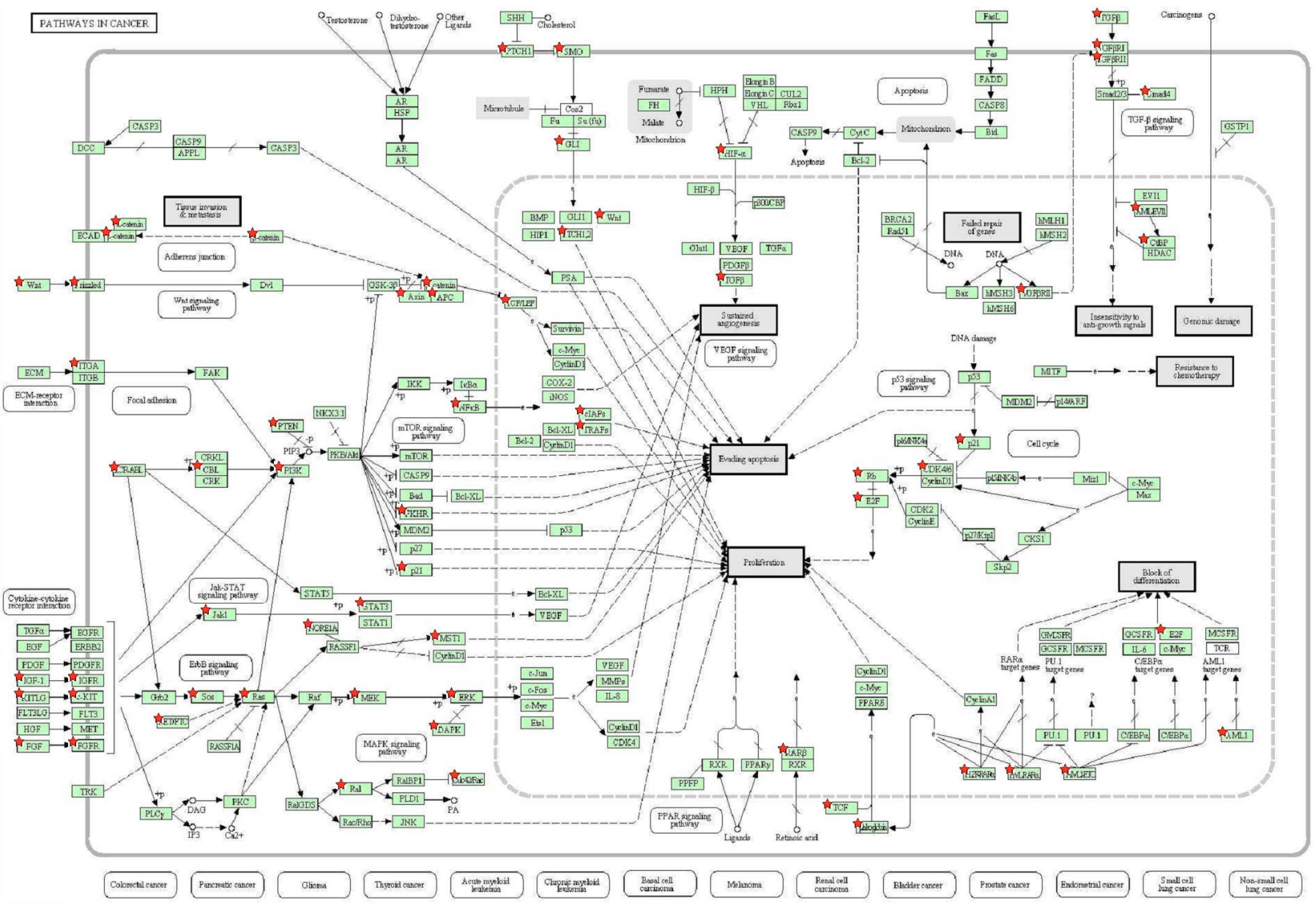
Table 6.2: Gene ontology terms referenced for the genes targeted by the miRNA signature of cases with versus without LKB1 protein expression.
Only the ontologies related to bone biology and LKB1 function are listed.

Pathways implicated by the study

The ontology terms related to the list of target genes suggested that the Wnt, MAPK, RAS and TGF-β signalling cascades are involved in the network obtained. To confirm and integrate these findings, the main pathways de-regulated via the target genes were investigated using KEGG pathway through DAVID: 47 pathways were hereby identified and 14 (30%) of them were related to cancer. The top one was entitled "pathways deregulated in cancer", with 328 hits (Figure 6.2). Additionally, pathways implicated in LKB1 function, such as TGF-β, Wnt and mTOR pathways were among this list, along with MAPK, Hedgehog and cell cycle pathways (Figure 10.3 in Supplementary data).

Legend for figure on the next page:

Figure 6.2: Scheme of the pathways implicated in cancer, highlighting the genes predicted to be targeted by the miRNA signature deregulated between OS cases expressing LKB1 or not.
The scheme was obtained from KEGG pathways. Targeted genes are represented with a red star. Only the highly conserved targets predicted by Targetscan were used for this study.


PATHWAYS IN CANCER
Testosterone
Dihydro-testosterone
Other Ligands
AR
HSP
AR
AR
CASP3
DCC
CASP9
APPL
CASP3
Tissue invasion & metastasis
ECAD
β-catenin
α-catenin
Adherens junction
Wnt
Wnt signaling pathway
Dvl
GSK-3β
Axin
APC
β-catenin
TCF/LEF
ECM
ITGA
ITGB
ECM-receptor interaction
FAK
Focal adhesion
PTEN
NKX3.1
PIP3
PKB/Akt
CRKL
CBL
CRK
CRAHL
PI3K
IKK
IκBα
NFκB
mTOR signaling pathway
mTOR
CASP9
Bad
Bcl-XL
FKHR
MDM2
p27
p21
PSA
Survivin
c-Myc
CyclinD1
COX-2
iNOS
cIAPs
TRAFs
Bcl-2
CyclinD1
Evading apoptosis
Jak-STAT signaling pathway
Jak1
STAT5
STAT3
STAT1
NORE1A
RASSF1
MST1
CyclinD1
Bcl-XL
VEGF
Cytokine-cytokine receptor interaction
TGFα
EGF
EGFR
ERBB2
PDGF
PDGFR
IGF-1
IGFR
KITLG
c-KIT
FLT3LG
FLT3
HGF
MET
FGF
FGFR
TRK
EtbB signaling pathway
Grb2
Sos
Ras
Raf
MEK
ERK
RETPTC
RASSF1A
DAPK
MAPK signaling pathway
PLCγ
DAG
IP3
Ca2+
PKC
RalGDS
Ral
RalBP1
CDC42/Rac
PLD1
PA
Rac/Rho
JNK
c-Jun
c-Fos
c-Myc
Ets1
VEGF
MMPs
IL-8
CyclinD1
CDK4
Proliferation
Sustained angiogenesis
VEGF signaling pathway
BMP
GLI1
Wnt
HIP1
TCH1,2
HIF-β
p300/CBP
Glut1
VEGF
TGFα
PDGFβ
TGFβ
Fumarate
FH
Malate
Mitochondrion
HPH
Elongin B
Elongin C
VHL
CUL2
Rbx1
HIF-α
SHH
Cholesterol
PTCH1
SMO
Microtubule
Cos2
Fu
Su (fu)
GLI
FasL
Fas
FADD
CASP8
Bid
Apoptosis
CASP9
CytC
Mitochondrion
Apoptosis
Bcl-2
TGFβ
Carcinogens
TGFβRI
TGFβRII
Smad2/3
Smad4
TGF-β signaling pathway
GSTP1
EVI1
MLF1/NPM
CtBP
HDAC
BRCA2
FasL1
Failed repair of genes
hMLH1
hMSH2
DNA
DNA
Bax
hMSH3
hMSH6
TGFβRII
Insensitivity to anti-growth signals
Genomic damage
DNA damage
p53 signaling pathway
p53
MDM2
p14ARF
MITF
Resistance to chemotherapy
p16INK4a
p21
Cell cycle
Rb
E2F
CDK4/6
CyclinD1
p15INK4b
Miz1
c-Myc
Max
CDK2
CyclinE
p27/Kip1
CKS1
Skp2
Block of differentiation
GMCSFR
GCSFR
MCSFR
GCSFR
E2F
MCSFR
IL-6
c-Myc
TCR
RARα target genes
PU.1 target genes
C/EBPα target genes
AML1 target genes
CyclinD1
c-Myc
PPARδ
CyclinA1
PU.1
PU.1
C/EBPα
C/EBPα
AML1
PPARγ
RXR
RARβ
RXR
PPFP
PPAR signaling pathway
Ligands
Retinoic acid
TCF
Alkylation
H2RRARα
hMLRARα
hMLHC
Colorectal cancer
Pancreatic cancer
Glioma
Thyroid cancer
Acute myeloid leukemia
Chronic myeloid leukemia
Basal cell carcinoma
Melanoma
Renal cell carcinoma
Bladder cancer
Prostate cancer
Endometrial cancer
Small cell lung cancer
Non-small cell lung cancer

6.2.4 Discussion of the role of miRNAs in LKB1 regulation in Osteosarcoma

As our data suggest that LKB1 is regulated post-transcriptionally in OS (section 5.5.4), we investigated whether miRNAs were deregulated between cases expressing LKB1 (n=4) or not (n=3). Hence 19 differentially regulated miRNA probes from 14 individual miRNAs were identified (Figure 6.1 and Table 6.1).

6.2.4.1 Previous reports on the miRNAs identified in this signature

There are only a few reports on the miRNAs identified in this signature. The most interesting one showed that hsa-miR-183 can inhibit metastasis in lung cancer by its binding to ezrin (Wang et al. 2008). Ezrin have been reported to play a role in OS metastasis via the mTOR pathway, as discussed in the Introduction (section 1.2.3.1), linking this miRNA to the signalling network of LKB1. However, it was its complementary branch that was identified in our signature, hsa-miR-183*. No predictions are available in PITA and Targetscan for this miRNA, so it is unknown whether it can also regulate ezrin expression. Another miRNA in this signature has been previously associated to the mTOR pathway in cancer: it was demonstrated that hsa-miR-331-3p directly targets ERBB2 in prostate cancer, hereby inhibiting PI3K/Akt signalling cascade (Epis et al. 2009). Moreover, this miRNA can directly target E2F1 in gastric cancer (Guo et al. 2010b); this transcription factor is regulated by *RB*, which plays a major role in OS (Figure 1.5).

Several miRNAs from this signature were reported as deregulated in other cancers, such as hsa-miR-370 in malignant cholangiocarcinoma, also proved to target MAP3K8 in the same study (Meng et al. 2008), hsa-miR-527 in breast cancers expressing progesterone receptors (Lowery et al. 2009), hsa-miR-936 in cervical cancer cell lines (Lui et al. 2007) and miRNA-200 family in endometrial endometrioid carcinoma (Lee et al. 2010a).

One study analysed the role of hsa-miR-601 deregulation in a lung carcinoma cell line model, confirming it directly targets NF-kappaB by reporter assay, hereby affecting several signalling pathways (Ohdaira et al. 2009). However, SIRT1, MO25 and HSP90AA1, which were predicted targets for this miRNA (Table 6.1 and section 6.2.3.3), were not included in this work. More

generally, none of the bioinformatics predictions we obtained had been validated or excluded by other groups.

Some reports concerning the miRNAs in this signature have no obvious relation to our study. Hsa-miR-605 has been demonstrated to target SEC24D by reporter assay (Lee et al. 2009), which is involved in vesicle trafficking, while hsa-miR-371-5p was found upregulated in lupus nephritis patients (Te et al. 2010). Hsa-miR-526b has been suggested as a biomarker for pregnancy (Miura et al. 2010).

To conclude, although little data is published on the miRNA signature identified, there is previous evidence that they are related to cancer and can deregulate members of the LKB1 signalling network.

6.2.4.2 miRNAs regulating LKB1 complex

Direct targeting of LKB1

A preliminary data mining study showed that hsa-miR-663 was highly expressed among the 24 OS cases screened. This miRNA has been reported as upregulated in human THP-1 monocytic cells after treatment of reverastrol, and as targeting the JUN family (Tili et al. 2010). Reverastrol (trans-3,4',5-trihydroxystilbene) is an antioxidant and anti-inflammatory agent proposed for cancer prevention; as presented in the Introduction (section 1.2.1.5, paragraph on SIRT1 protein family), it can also be used to prevent LKB1 acetylation (Dasgupta et al. 2007, Lee et al. 2010b, Shin et al. 2009). It was also demonstrated that this miRNA was downregulated in gastric cancer cell lines by qRT-PCR (Pan et al. 2010). But although interesting, this potential target was not found significantly deregulated when comparing the 4 cases expressing LKB1 to the 3 cases not expressing it by IHC. Moreover, none of the miRNAs predicted to target LKB1 by PITA or Targetscan were deregulated in this cohort of cases.

miRNAs have been thought to regulate gene expression by binding complementarily to the 3'UTR of target mRNAs, thereby either inducing mRNA degradation or repressing mRNA translation (Esquela-Kerscher et al. 2006). However, a recent study has shown that over 84% of mammalian miRNAs acted on mRNA by inducing degradation rather than by inhibiting translation (Guo et al. 2010a). This implies that regulation of mRNA by miRNAs by repression of translation only is not

very common in mammals. No association was found in our OS cases between the mRNA and protein expression of LKB1 (section 5.4.2), suggesting that LKB1 mRNA was not degraded in cases lacking LKB1 protein. In the light of this finding and as no differentially expressed miRNA was found in our small cohort, we concluded that LKB1 is unlikely to be regulated by the direct binding of a miRNA to its mRNA.

Indirect targeting: MO25 and Strad

As developed in the Introduction (section 1.2.1.1), MO25 and Strad are essential for LKB1 activity. The binding of LKB1 to its complex will promote its activation by an allosteric mechanism deciphered by Zeqiraj et al (Zeqiraj et al. 2009a), and will enhance the enzyme's activity dramatically (Hawley et al. 2003). Moreover, LKB1 is transported to the nucleus once synthetised; but it can only play its role once it is exported to the nucleus, after its binding in its complex. Finally, mutations detected in complex binding sites on *LKB1* were previously described in cancer or PJS (Boudeau et al. 2004); they impaired the activity of the protein. This suggests that regulation of LKB1 activity may be channeled through the regulation of the complex formation, and that the regulation of either of MO25 or Strad will impact the activity of LKB1.

MO25 has already been reported to be regulated by hsa-miR-451 (Godlewski et al. 2010b, Godlewski et al. 2010a). Interestingly, this miRNA is the most highly upregulated (fold change of 99.4, p-value of 1.15×10^{-20}) when comparing the miRNA signature of OS to other cancers and normal tissue screened by our group (10 neurofibromas, 9 nerve sheath tumours and 4 Schwann cell primary cultures) as illustrated in the Supplementary Data (Figure 10.4 and Table 10.3). This result needs to be confirmed using more appropriate controls, but it implies that hsa-miR-451 may play an important role in OS.

Even though miRNA-451 was not among the most differentially regulated miRNAs in cases expressing LKB1 protein or not, half of them targeted either MO25α or Stradα. This suggests that LKB1 expression in OS may be regulated by the de-regulation of miRNA targeting the rest of the complex. The finding of miRNAs targeting those two proteins in our signature underlines their importance OS. LKB1 expression and activity may then be regulated by miRNA, via their action on the complex.

The stability of LKB1 protein in the absence of its binding to the complex is not fully understood. Godlewski et al report in their supplementary data that LKB1 protein was detected by WB in the absence of MO25α due to its repression by hsa-miR-451 (Godlewski et al. 2010b). However, they also show that MO25β, another isoform, is present; hence LKB1 may be stabilised by formation of the complex with the isoform β (instead of the α) of MO25. It is difficult to predict whether the miRNAs identified in our signature will lead to LKB1 protein degradation by their repression of MO25 and Strad expression; this needs to be investigated further. Establishing the role of MO25 and Strad regulation in LKB1 stability would help to decipher further the contribution to LKB1 regulation in OS.

To conclude, our data suggests that miRNAs may play a role in LKB1 regulation in OS by targeting its complex rather than its mRNA. The expression of MO25 and Strad in OS cases and cell lines needs to be assessed and the degradation of LKB1 protein in their absence needs to be confirmed. Additionally, the target genes used are only predicted bioinformatically and need to be validated by reporter assay. This is currently being assessed by Dr Malihe Eskandarpour as this project was beyond the scope of this study.

Indirect targeting: regulation of the proteins cooperating in complex formation

In addition to the members of the LKB1 complex, the miRNA identified in the signature could also regulate the formation of this complex by inhibiting SIRT1 and HSP90 mRNA translation. The role of SIRT1 is investigated further below (section 6.3). HSP90 stabilises the LKB1 protein and stops its degradation by facilitating the formation of LKB1 complex (Boudeau et al. 2003b) as presented in the Introduction (section 1.2.1.1). The role of the miRNAs identified on these targets needs to be confirmed by reporter assay. However this is beyond the scope of this study.

6.2.4.3 miRNAs targeting the LKB1-related pathway

The relevance of the miRNA signature obtained was then validated further by the finding that the target genes were part of the LKB1 signalling cascade. This was already suggested by the report of the role of hsa-miR-183 on ezrin regulation and of miR-331-3p on ERBB2 and PI3K signaling (section 6.2.4.1). The study of the ontologies of the predicted target genes and of the pathway they belonged to showed that the following pathways were affected: TGF-β – via the gene targets *TGF-β*, TGF-β receptors and SMAD protein family – Wnt – via *Wnt*, *Frizzled* and *β-catenin* – and Src – via *PAK1*. All these signalling pathways are part of LKB1 signalling network (Carretero et al. 2010, Inoki et al. 2006, Katajisto et al. 2008) as detailed in the Introduction (section 1.2.1.2).

Moreover, these miRNAs also target several molecules of the mTOR pathway, the most commonly reported signalling cascade downstream of LKB1, such as: AMPK, PI3K, IRS, RICTOR and RPS6K, but also many growth factors from the IGF, ephrin and FGF family, and TKR, such as IGFR1, FGFR1&2 and ERBB2, which can activate the mTOR pathway upstream of PI3K. The role of miRNAs in the regulation of the mTOR pathway in OS was confirmed by analysing the targets of the miRNAs deregulated in 24 OS cases compared to other sarcomas (Figure 10.4 and Table 10.3 in Supplementary Data). PTEN, TSC1 and CAMKK-β were among these. CAMKK- β can regulate AMPK in response to changes in Ca^{2+} levels as would LKB1 (Carling et al. 2008). We have already reported PTEN and TSC1 protein to be lost in a subset of OS in Chapter 3 (Figure 3.2). Our data suggests a novel regulation mechanism to explain this loss of protein in OS.

Finally, as mentioned in the Introduction (section 1.2.1.2), LKB1 can phosphorylate several downstream kinases, and the coordination between these kinases is not fully understood. Our study implies that miRNAs could regulate the expression of the downstream targets of LKB1, with AMPK and *NUAK1* targeted, offering a new insight into the regulation of LKB1 function.

6.2.5 Conclusion and future work

The data mining performed with OMIM, gene ontology and pathway analysis through DAVID validated the relevance of the miRNA signature. Although the list of targets was not exhaustive as only highly conserved targets from Targetscan were included, many predicted targets were related to bone biology and LKB1 signalling network. This study needs to be validated on a larger cohort of cases, as only a small number of cases were compared, and the predicted targets should be validated by reporter assay. Overall, it demonstrates the feasibility of studying miRNA signatures of OS tumours to decipher the pathogenesis of OS.

To conclude, this study has shown that although miRNAs probably do not directly regulate LKB1 protein expression, they may participate in the regulation of LKB1 and its signalling network by targeting its complex (via MO25 and Strad or proteins involved in complex stability like SIRT1 and HSP90). As the regulation of LKB1 is firstly and predominantly undertaken by its binding to MO25 and Strad, the role of these proteins in the pathogenesis of OS should be further investigated.

6.3 Role of SIRT1 in LKB1 protein stability

6.3.1 SIRT1 expression at the mRNA level

6.3.1.1 Results of the qRT-PCR

To assess whether *SIRT1* plays a role in OS, its expression at the mRNA level was assessed by qRT-PCR. SIRT1 mRNA was detected in all cell lines, although there was a big heterogeneity between the samples (Figure 6.3.A) with 7 cycles difference between the most extreme cases.

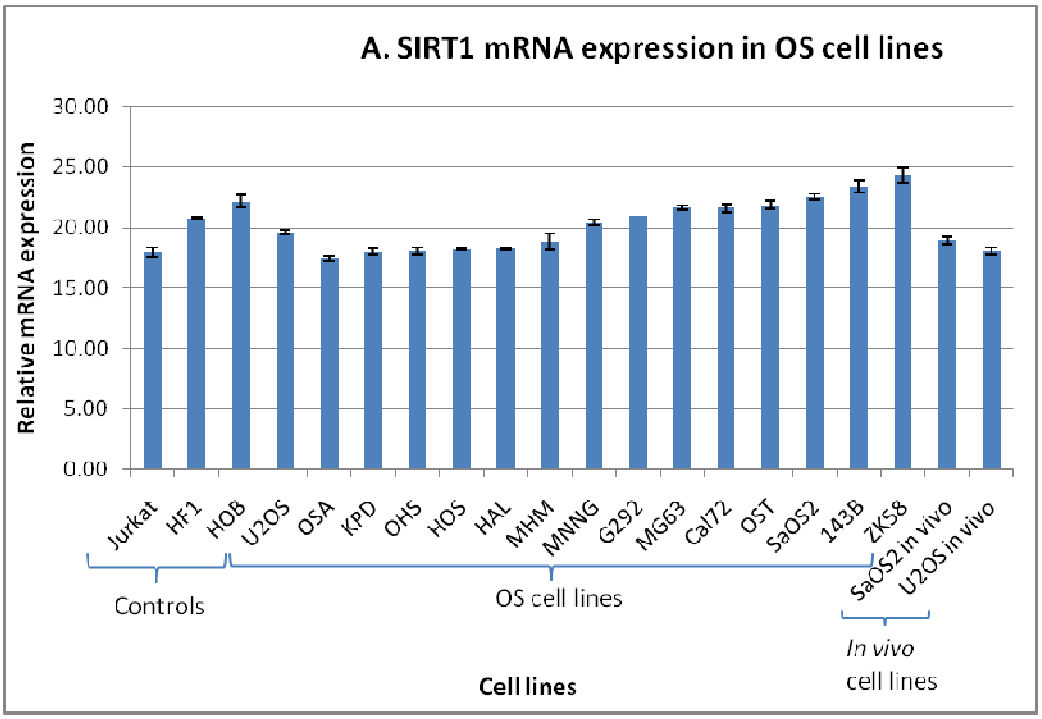
For OS primary tumours, *SIRT1* levels of expression were globally higher than in the cell lines. The case with the lowest mRNA expression, S2269, had a ΔCt very close to the positive control, U2OS (Figure 6.3.B). Hence all cases expressed high levels of *SIRT1* mRNA, showing a different profile than in the cell lines.

6.3.1.2 Association between SIRT1 mRNA and LKB1 protein expression

To assess whether the heterogeneous mRNA expression of *SIRT1* correlated with LKB1 protein expression, the significance of this association was studied using the results of LKB1 protein expression by WB for the cell lines and by WB and IHC for the tissue samples, as discussed in Chapter 4 (section 4.2). The WB data was analysed both using the relative integrated density of the detected bands for LKB1 protein compared to GAPDH and the overall score of the protein expression as defined in paragraph 2.6.5.4. The relative mRNA expression of SIRT1 was expressed as the ΔCt of SIRT1 compared to RPS18.

For cell lines, the relative integrated density of LKB1 and SIRT1 mRNA expression were not normally distributed (p=0.41 and p=0.053 respectively with the Shapiro-Wilk normality test). For the patient samples, SIRT1 relative mRNA expression was also not normally distributed (p=0.87), but the relative integrated densitometry of LKB1 was (p=0.019).

The association between SIRT1 relative mRNA expression and LKB1 protein expression by WB (measured as the relative integrated density) was neither statistically significant for the cell lines nor for the tissue samples (p=0.20 and p=0.86 respectively with the Spearman rho test).



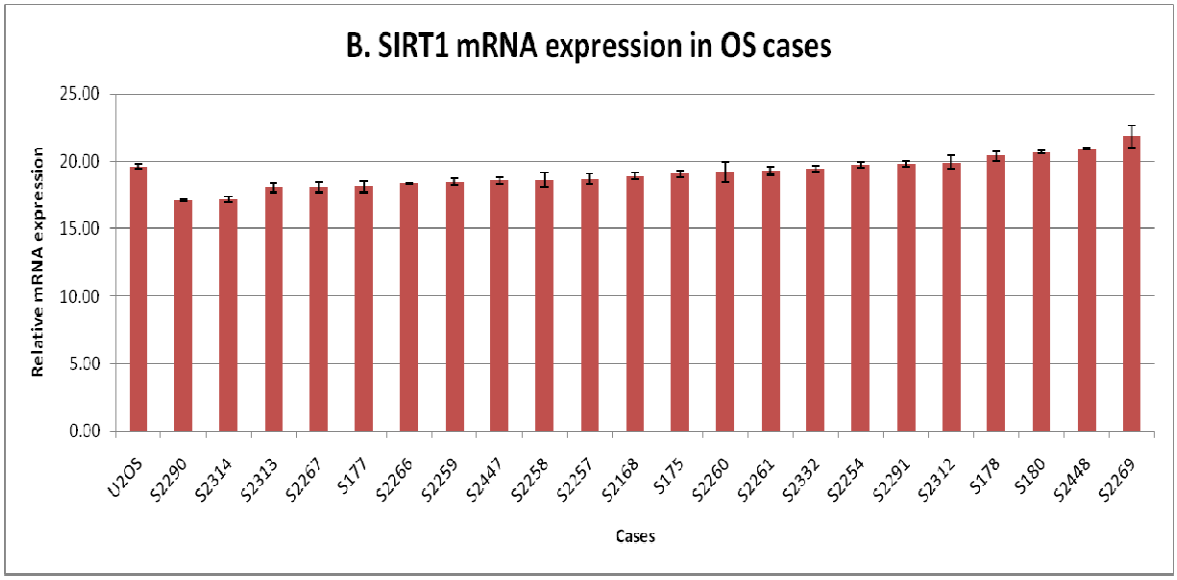


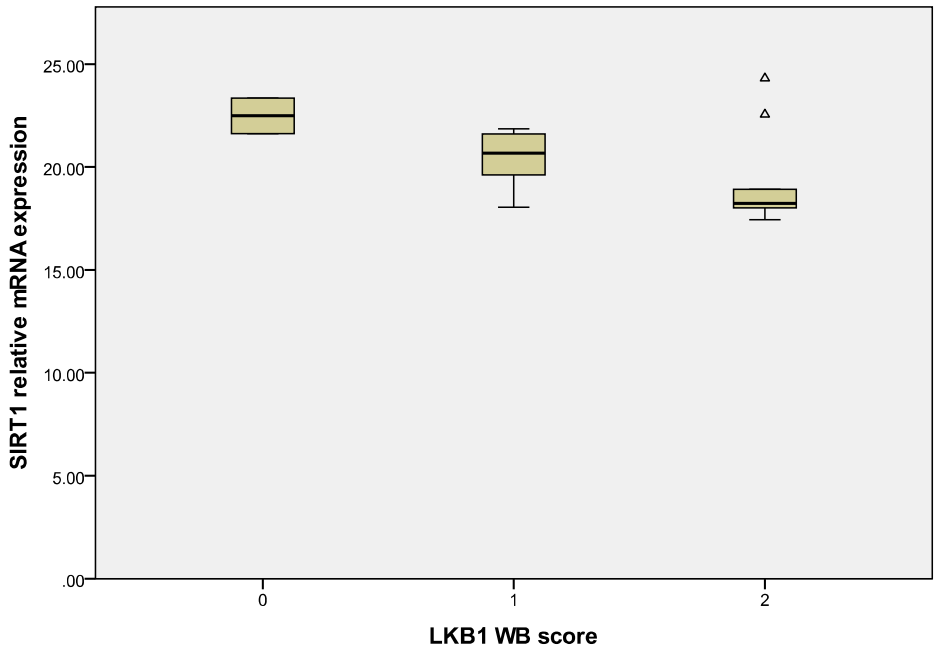
Figure 6.3: Bar chart representing SIRT1 mRNA expression in OS cell lines (A.) and cases (B.)
The relative mRNA expression is expressed as the ΔCt of SIRT1 compared to ribosomal protein 18s. U2OS was used as a positive control for the patient samples and Jurkat, HF1, HOB and U2OS for the cell lines.

Similarly, there was no statistically significant association between SIRT1 relative mRNA expression and the WB score obtained for LKB1 in the cell lines (p=0.12 with the Kruskal-Wallis test), although cell lines expressing lower levels of LKB1 protein tended to express lower levels of SIRT1. The same result was encountered for the patient samples with WB (p=0.94) but not IHC (p=0.017), which displayed a significant association. However, there was a large overlap between the cases expressing low (score of 1) and very low or no (score of 0) LKB1 protein, as illustrated in Figure 6.4.

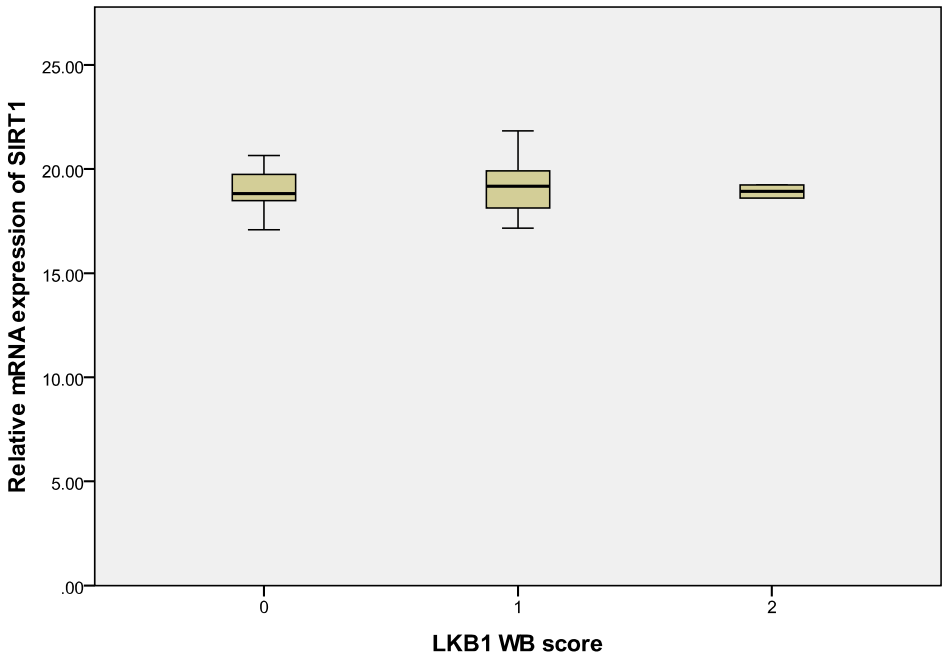
To assess whether the associations remained poor with a larger cohort of cases, the results obtained for the cell lines and the patient samples were pooled. Both SIRT1 relative mRNA expression and LKB1 relative protein expression - measured as the integrated density of the detected bands - were normally distributed (p=0.014 and p=0.010 respectively with the Shapiro-Wilk test). However, the association between the two quantities remained non significant (p=0.77 with the Pearson correlation test). Similarly, no significant association was found between the WB scores of LKB1 and SIRT1 relative mRNA expression of (p=0.82 with the one-way ANOVA test) with the pooled data from the cell lines and the patient samples (Figure 6.4.D).

To conclude, there was no convincing evidence of an association between SIRT1 mRNA expression and LKB1 protein expression.

A. SIRT1 mRNA expression compared to LKB1 protein expression by WB in OS cell lines



B. SIRT1 mRNA expression compared to LKB1 protein expression by WB in OS cases



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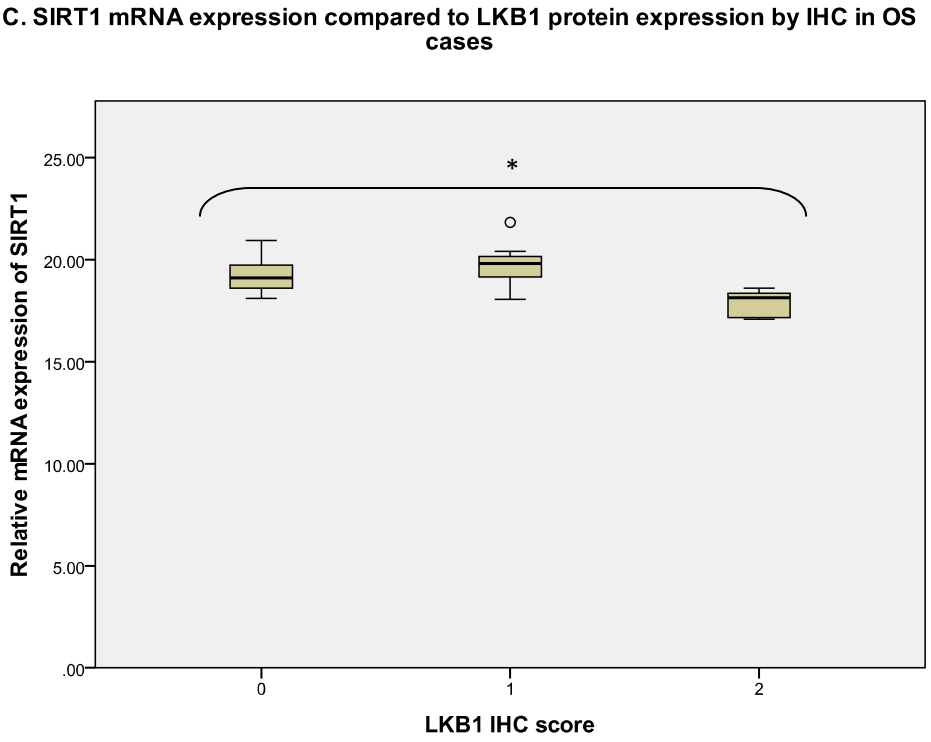


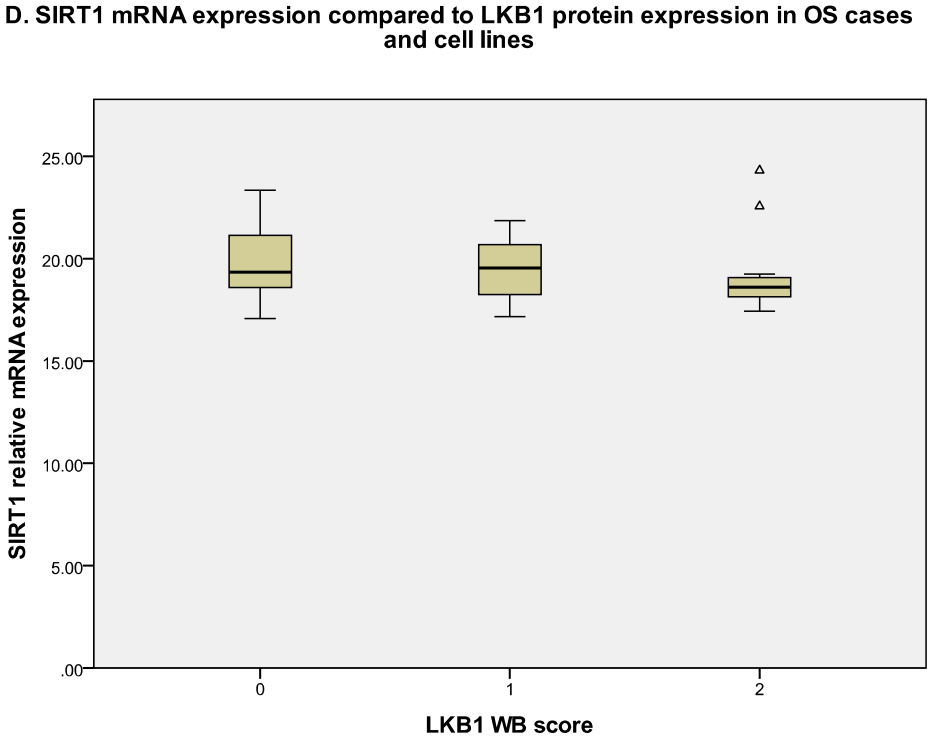


Figure 6.4: Boxplot showing SIRT1 mRNA expression compared to LKB1 protein expression.
Expression of SIRT1 compared to LKB1 expression by WB in OS cell lines (A.), by WB (B.) and IHC (C.) in OS cases, and by WB in OS cell lines and cases combined (D.). The relative mRNA expression is expressed as the ΔCt of SIRT compared to the RPS18 control. The round point represent the outliers and the triangle ones the extreme cases. * for $p<0.05$.

6.3.2 Discussion of the role of SIRT1 and future work

As shown in paragraph 6.2.4.2, the epigenetic screening of a small number of cases expressing different levels of LKB1 protein indicates that *LKB1* post-transcriptional regulation is unlikely to be accounted for by miRNA directly targeting this gene. We therefore hypothesised that LKB1 protein loss was due to a deregulation of the mechanism ensuring protein stability. As *SIRT1* has been previously reported to regulate this process, we investigated expression of this gene in OS cell lines and patient samples.

Our study showed that *SIRT1* is expressed at the mRNA level in all OS cases and cell lines, but at greatly differing levels. As shown in paragraph 6.2.3.2, miRNAs differentially expressed in OS cases with or without LKB1 protein expression could regulate SIRT1 expression. This was confirmed by the study of the miRNA signature of 24 OS cases compared to other sarcomas, with 6 miRNAs from this signature predicted to target this gene by Targetscan. This may account for the different levels of mRNA observed between the cases. However, there is only weak evidence of an association between LKB1 protein expression and SIRT1 mRNA expression (Figure 6.4).

Nevertheless, this preliminary study is not sufficient to exclude a role of SIRT1 in LKB1 regulation in OS. To confirm that LKB1 post-transcriptional regulation is mediated by protein degradation, the restoration of LKB1 expression in presence of proteosome inhibitor in OS cell lines has to be demonstrated. SIRT1 protein expression also needs to be assessed in OS cases and cell lines, and correlated with LKB1 protein expression. To decipher further the interaction between SIRT1 and LKB1, the changes in levels of LKB1 protein expression and protein acetylation in the presence of SIRT1 inhibitors (like sirtinol) or of non coding shRNA against SIRT1 should be detected. This is beyond the scope of this study, and this is currently being investigated by Dr Malihe Eskandarpour.

Chapter 7: ROLE OF *LKB1* IN THE CONTEXT OF NORMAL BONE

7.1 Introduction and aims

As demonstrated in the previous chapters, LKB1 protein expression is reduced in a subset of OS thanks to its post-transcriptional regulation, even though we have not fully deciphered this mechanism in Chapter 6. As presented in the Introduction (section 1.2.1.2), LKB1 can participate in many cell processes. These include cell proliferation by regulating cell metabolism via the mTOR pathway, cell survival by regulating apoptosis or anoikis via the SIK family and p53 pathway and cell migration by regulating cell polarity via the MARK family or PAK1 (Figure 1.9). There has not been any report on the role of LKB1 in the context of normal bone, and it is not known which of these processes are at play in OS. Moreover, as we found that both copies of *LKB1* were not lost at the genetic level (section 5.5.3), decrypting its function in OS would confirm whether it is a driver or passenger event in tumourigenesis. Hence, our aim in this chapter was to investigate the role of *LKB1* in the context of normal bone, to better understand its part in the pathogenesis of OS.

A model was required to achieve this aim. As discussed in the Introduction, there is a line of evidence pointing to the osteoblast lineage as the cancer initiating cells in OS (section 1.1.5.4 and 0). *Lkb1*$^{+/-}$ mice were reported to develop osteogenic tumours which seemed to arise from an osteoblast lineage (Robinson et al. 2008), but in this *in vivo* model the gene was knocked-down in all cell types. Moreover, as mentioned in the Introduction (section 1.2.3.3), a decreased AMPK activity promotes osteoblast differentiation (Kasai et al. 2009). This suggests that loss of *LKB1* in this lineage may induce a deregulation of their differentiation mechanism hereby promoting tumourigenesis. Within the context of normal bone, we therefore focused on the role of *LKB1* in the osteoblast lineage.

The use of conditional knock-down mouse models with loss of *LKB1* restricted to a specific cell lineage has greatly helped our understanding of its role in other cancers, such as breast or endometrial carcinomas (Contreras et al. 2008, McCarthy et al. 2009). As discussed in section 1.1.5.4, no mouse model with *Lkb1* loss restricted to osteoblasts has been generated. Such a model would be very valuable to decipher the role of *LKB1* in OS pathogenesis. But prior to this, we validated our strategy by assessing its function *in vitro* in an immortalised human osteoblast cell line, HOB, by gene knock-down.

The objectives were:

1. Set up an *in vitro* model in the HOB cell line with *LKB1* knocked-down
2. Assess the functional effect of loss with regards to:
    a. Tumourigenic potential
    b. Cell proliferation
    c. Cell migration
    d. Cell survival

7.2 Knock-down validation and clone selection

As presented in Chapter 4, the HOB cell line expresses high levels of LKB1 protein (Figure 4.1). The strategy selected to silence *LKB1* was to induce a permanent knock-down by shRNA using the Open Biosystem pGIPZ human lentiviral shRNAmir library, as described in the Materials and Methods chapter (section 2.8). Four different clones, abbreviated as clones 35, 21, 04 and 03, were tested and compared to the empty vector (EV) and non-silencing (NS) controls (Figure 2.4). For each clone, the virus was titrated to ensure that only one copy was integrated per cell (section 2.8.4). Following infection, the cells were selected with Puromycin at a concentration optimised by kill curve analysis beforehand. The success of this selection was confirmed by flow cytometry: virtually all selected cells were GFP fluorescent.

The efficiency of the knock-down was validated at the mRNA level by qRT-PCR and at the protein level by WB (Figure 7.1). Only clone 03 was found to have a significantly reduced expression of LKB1 mRNA ($p=0.0016$ with the one-tailed Student's t-test) and protein ($p= 0.012$) compared to the EV control. The infection was repeated two more times to confirm these results, and on average, the clone 03 expressed 42% less protein and 50% less mRNA than the EV control. Hence only this clone and the EV control were studied on a functional level.

No changes in morphology were noted in the cell line upon infection, as illustrated in Figure 7.2.

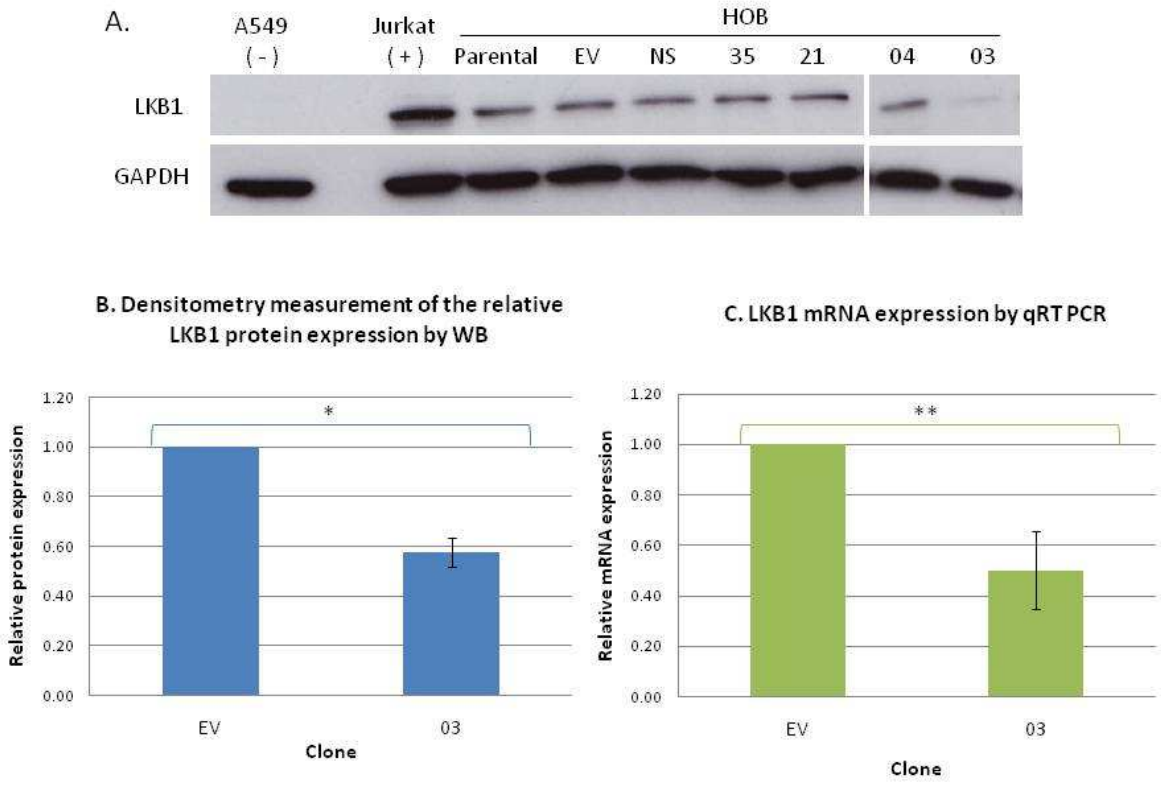


Figure 7.1: Knock-down efficiency at the protein and mRNA level.
A. WB showing LKB1 protein expression obtained after knock-down in the different clones. A549 was used as a negative control and Jurkat as a positive control. B. Densitometry analysis quantifying LKB1 relative expression for clone, 03, compared to GAPDH and normalised to the EV control clone. C. Fold difference of the relative LKB1 mRNA expression for clone 03, compared to β-actin control normalised against the EV control. * for $p<0.05$ and ** for $p<0.005$ with the one-tailed Student's t-test.

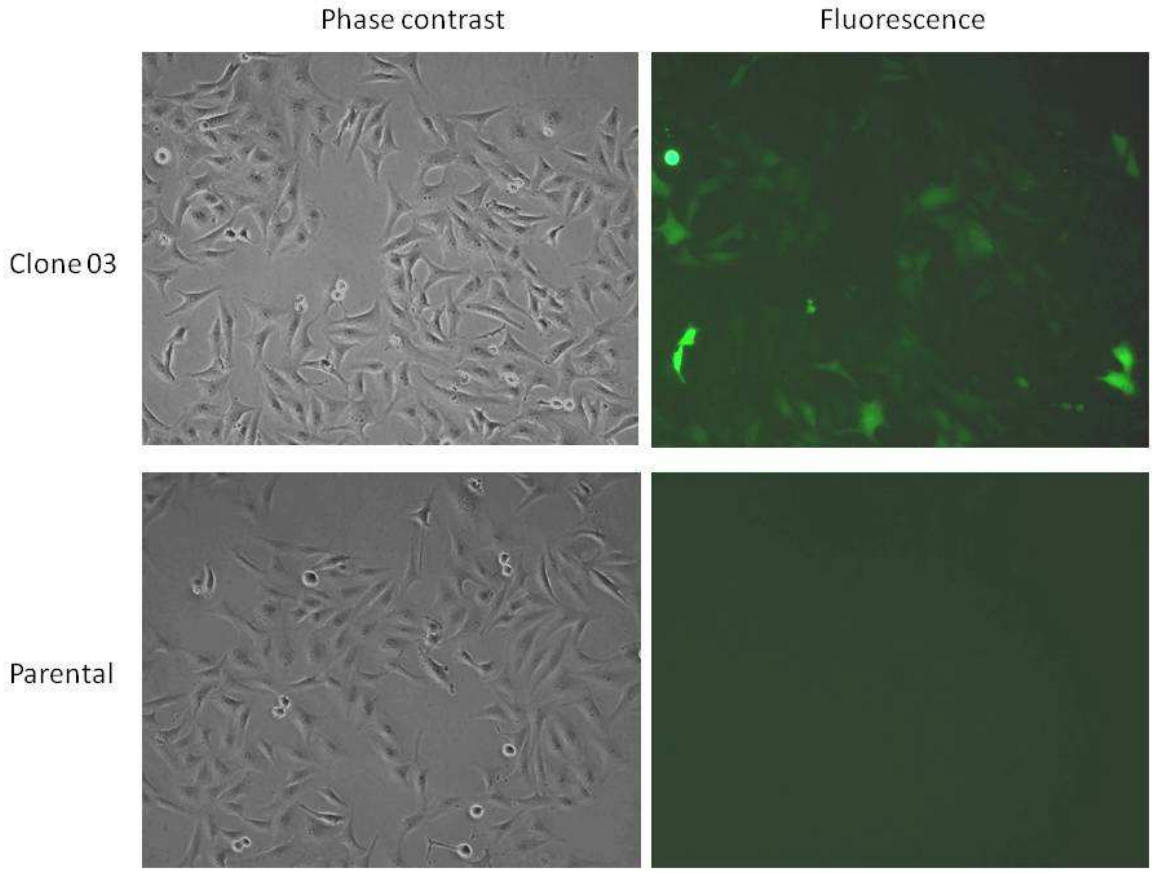


Figure 7.2: Morphology of the HOB cell line with and without LKB1 knock-down.
Both phase contrast and GFP fluorescence are shown. The *LKB1*-knocked down clone 03 and the parental for the HOB cell lines are shown after selection.

7.3 Effect of *LKB1* knock-down on tumourigenicity

To assess whether *LKB*1 loss could induce tumour formation in normal osteoblasts, a soft agarose colony formation assay was performed with the EV and 03 clones, as described in the Materials and Methods chapter (section 2.10.1). No colonies were formed in either of the clones, showing that LKB1 loss was not sufficient to induce anchorage independent growth in this context.

7.4 Effect of *LKB1* knock-down on cell proliferation

To monitor the functional effect of *LKB1* on cell proliferation, three approaches were used, all described in the Materials and Methods chapter: an MTS assay (section 2.10.2.1), a cell cycle assay (section 2.10.2.3) and a growth curve assay (section 2.10.2.2). As LKB1 acts on the mTOR pathway in response to energy deprivation via AMPK, the assays were performed in normal, low glucose and no glucose conditions as described in section 2.10.

7.4.1 Results of the MTS assay

The results of the MTS assay are presented in Figure 7.3. Three different cell densities were used and the experiment was repeated three times. No differences were found with this assay in normal glucose levels and in the absence of glucose. However, in low glucose conditions, clone 03 had an increased proliferation rate compared to the EV control, and the difference was statistically significant at day 1 ($p=0.045$ with the paired one-tailed Student's t-test) and day 3 ($p=0.0065$) with the intermediate cell density. Although the difference was not statistically significant, this trend was also observed at the other time points and densities, particularly at day 2 with the lowest cell density ($p=0.066$) and at day 2 with the intermediate cell density ($p=0.14$).

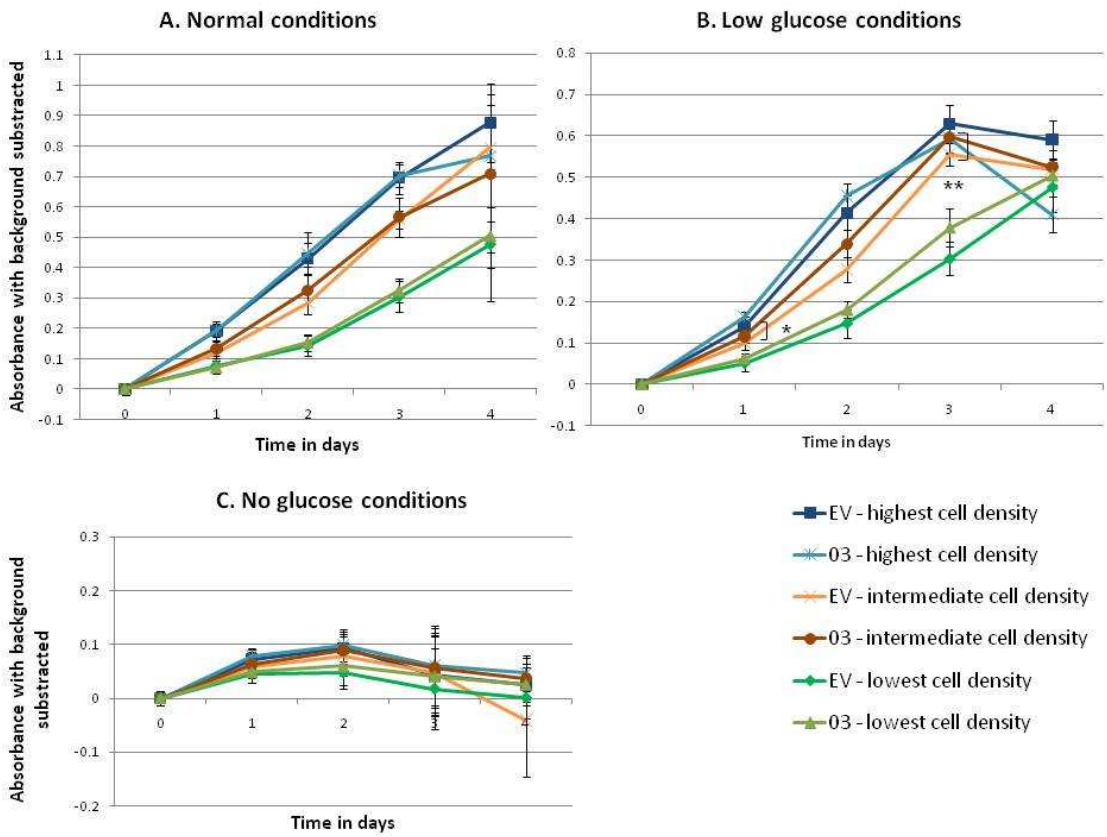


Figure 7.3: Effect of *LKB1* knock-down on cell proliferation recorded by MTS assay.
Three cell densities were plated at the start of the experiments: 3,000 (highest cell density), 2,000 (intermediate) or 1,000 (lowest) cells. The experiment was performed in normal (A.), low glucose (B.) and no glucose (C.) conditions. * for $p<0.05$ and ** for $p<0.01$ with the paired Student's t-test.

7.4.2 Results of the cell cycle analysis

As the differences observed by MTS assay were small (although significant for some time points), other assays were performed to confirm the increased proliferation of clone 03 compared to the EV control. The cell cycle profile of clone 03 and the EV control were obtained in all three conditions. The average proportion of cells in each phase over the three independent experiments performed is presented in Figure 7.4.A and a representative cell cycle profile is given in Figure 7.4.B. Although the differences found were not statistically significant, we consistently obtained an increased proportion of cells in the G2 and S phases, and a lower proportion of cells in the pre-apoptotic population for clone 03 compared to the EV control. Again, the differences were small; on average, there were 5% less pre-apoptotic cells and 4% more proliferative cells in clone 03 compared to the EV control.

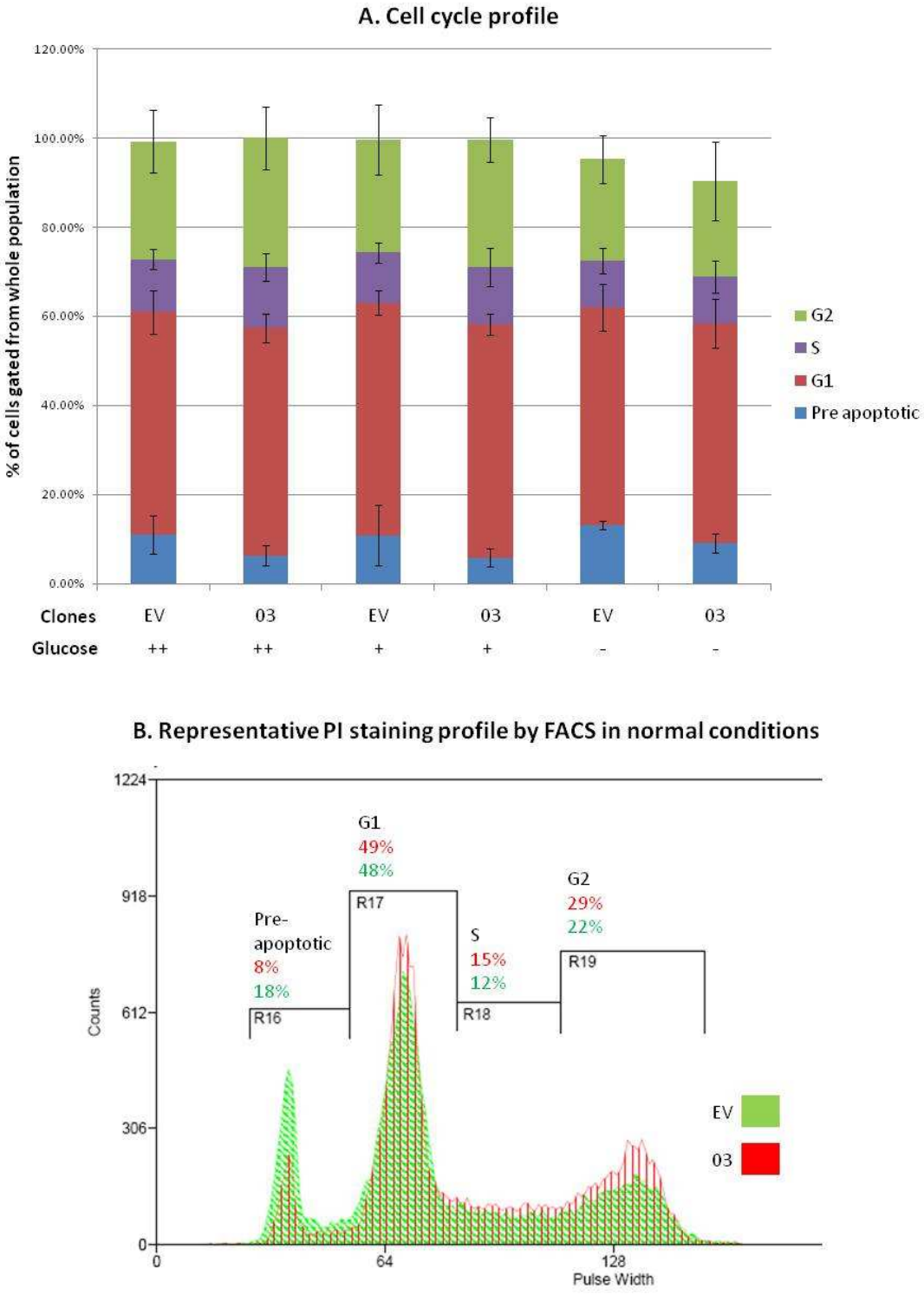


Figure 7.4: Cell cycle analysis for *LKB1* knock-down.
The average proportion of cells over three experiments in pre-apoptotic, G1, S and G2 phases for EV and 03 clones in normal (++), low glucose (+) and no glucose (-) conditions is presented in A. and a representative profile obtained by PI staining and analysed by flow cytometry is given in B.

7.4.3 Results of the growth curve assay

Finally, the cell proliferation in the HOB with *LKB1* knocked-down compared to the EV control was assessed by recording the cell growth curve using the live cell imaging system of the Incucyte (Essex Bioscience). No differences were found in the growth curve in normal conditions and in low glucose over two experiments between clone 03 and the EV control, as illustrated in the representative graphs in Figure 7.5.

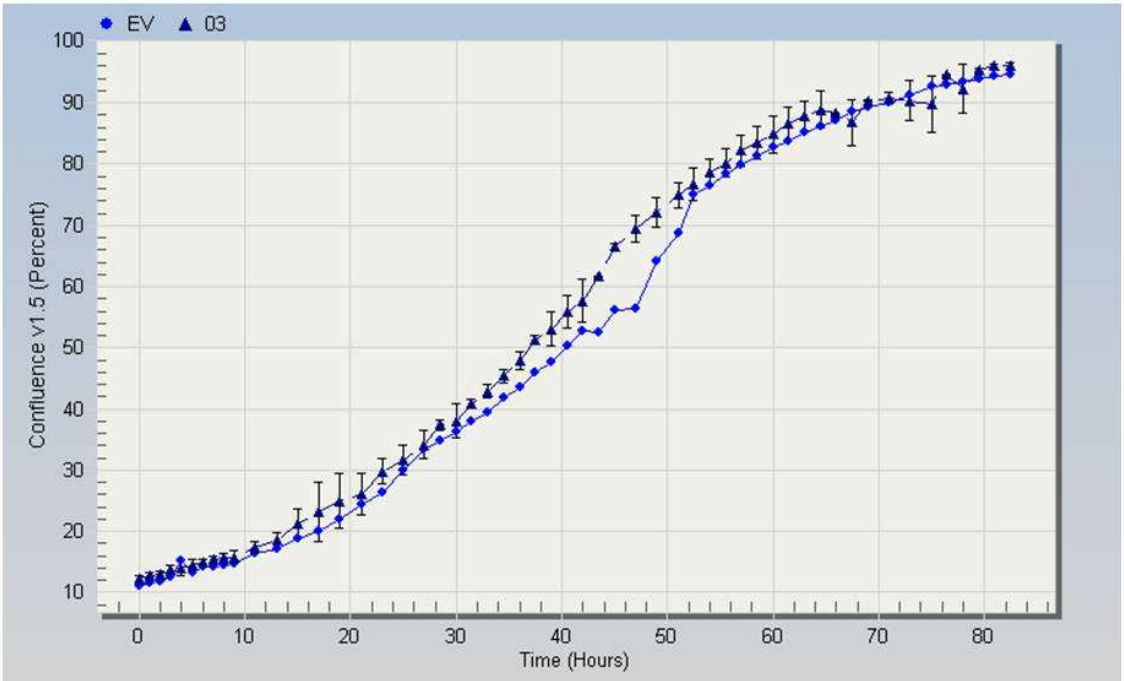
7.4.4 Conclusion on the effect of LKB1 knock-down in cell proliferation

LKB1 knock-down induced an increased cell proliferation in the HOB cell line, as recorded by the increased metabolic activity recorded by MTS assay in low glucose condition. The same trend was recorded by cell cycle analysis with the increased proportion of cells in G2 and S phase in all conditions.

7.5 Effect of *LKB1* knock-down on cell migration

To assess the effect of LKB1 knock-down on cell polarity and hence on cell migration, two tightly linked functions, a scratch assay was performed in all three conditions, as described in the Materials and Methods chapter (section 2.10.3.1). No differences were found in the ability of clone 03 and the EV control to close their wound in three independent experiments, whether the cells were in normal, low glucose or no glucose conditions (Figure 7.6).

A. Growth curve in normal conditions



B. Growth curve in low glucose conditions

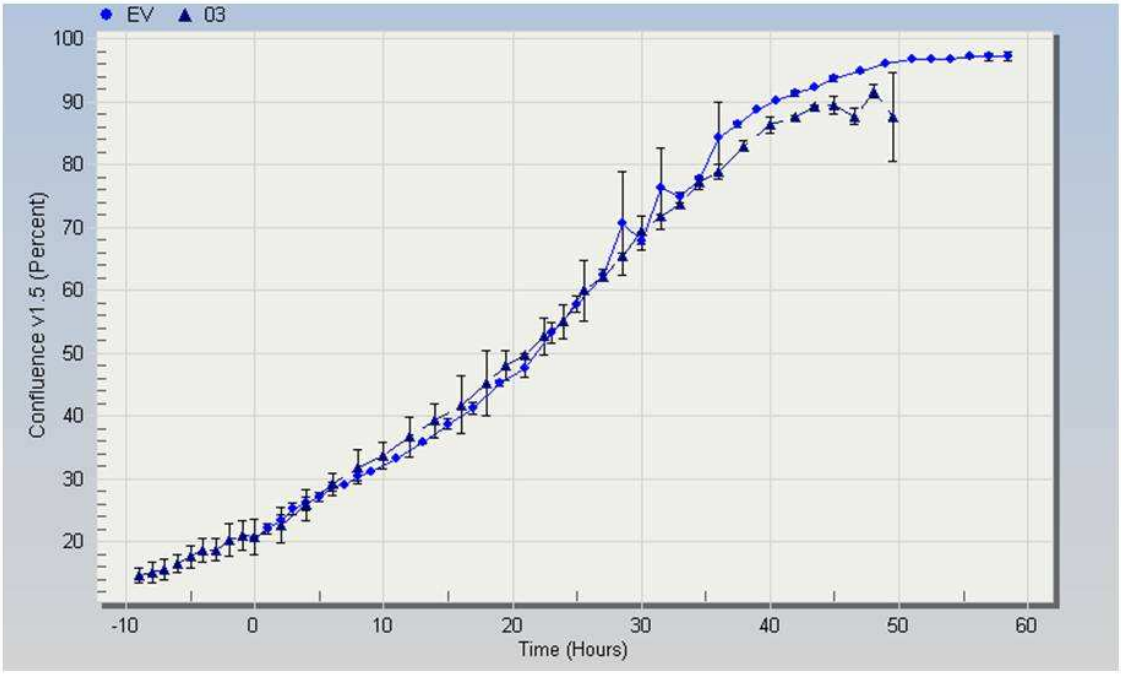
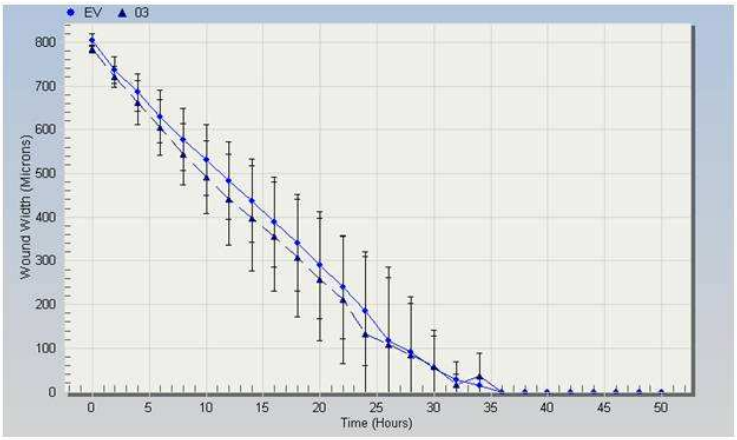
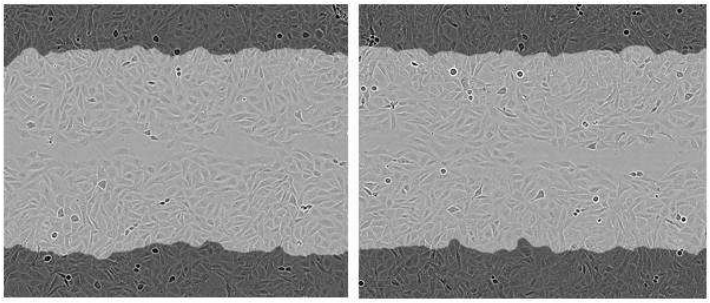


Figure 7.5: Representative growth curves of *LKB1* knock-down in normal (A.) and low glucose (B.) conditions.
The growth curve is represented as the average confluency measured with the Incucyte over time.

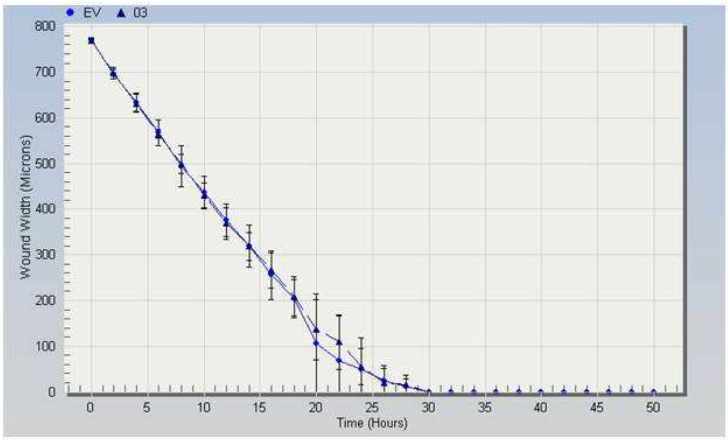
A. Scratch assay in normal conditions



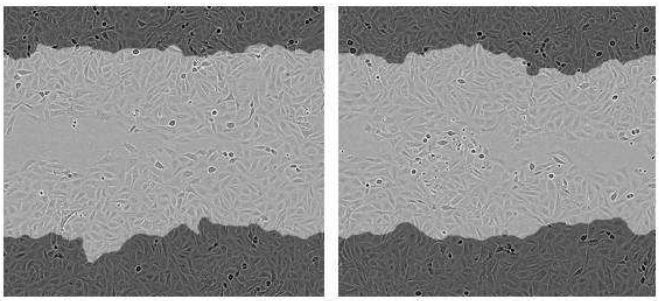
EV 03



B. Scratch assay in low glucose conditions



EV 03



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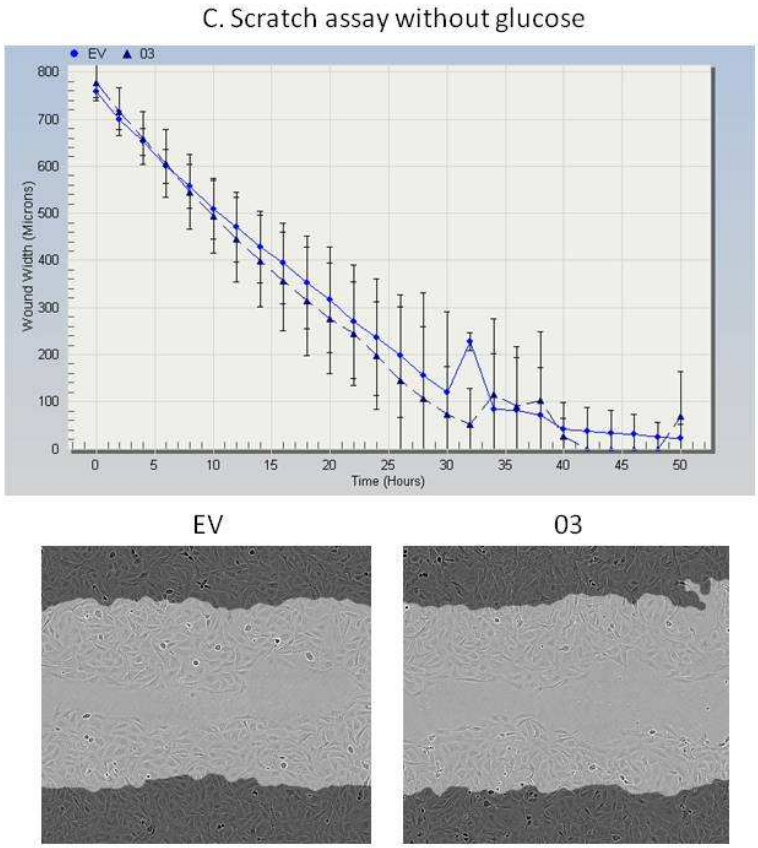


Figure 7.6: Representative graphs of the wound width over time and representative pictures of the wound closure after 24h in normal (A.), low glucose (B.) and no glucose (C.) conditions.

7.6 Effect of *LKB1* knock-down on cell survival

As suggested in Figure 7.4, *LKB1* knock-down promotes cell survival; clone 03 consistently presented a lower proportion of pre-apoptotic cells compared to the EV controls when studying their cell cycle profile in adherent conditions. To confirm these results, the survival potential of clone 03 was assessed in adherent and non-adherent conditions, by determining the proportion of apoptosing cells (early or late) with Annexin V staining by flow cytometry (section 2.10.1). The experiment was performed twice in all three conditions as the differences observed were small and are illustrated in Figure 7.7.

7.6.1 Results of the apoptosis assay in adherent conditions

In adherent conditions, *LKB1* knocked-down cells consistently survived better than the EV control in normal and no glucose conditions; discrepancies were found between the two repeats in low glucose conditions. The magnitude of the differences were the same as the cell cycle analysis, with 3% less cells apoptosing in normal conditions on average, 1.6% in low glucose and 2% without glucose after 2 days of culture. The results obtained with the MTS assay and the observation of the cells with the Incucyte suggested that greater differences were obtained after a longer culture without glucose. To confirm these results, one more time point was performed after 4 days of culture. Indeed, an additional 12% of cells underwent apoptosis in the absence of glucose and in adherent conditions in clone 03 compared to the EV control.

7.6.2 Results of the apoptosis assay in anchorage-independent conditions

In anchorage independent conditions, consistent differences were found again over the two repeats: in normal and low glucose conditions, clone 03 underwent anoikis less frequently than the EV control. In the absence of glucose, however, this pattern was reversed, as clone 03 underwent anoikis more frequently than the EV control. The differences observed were still small, although slightly bigger than in adherent conditions: on average, 8% less apoptotic cells (early and late) were found in clone 03 compared to the EV control in normal conditions and 6% in low glucose, compared to 3% more apoptotic cells without glucose.

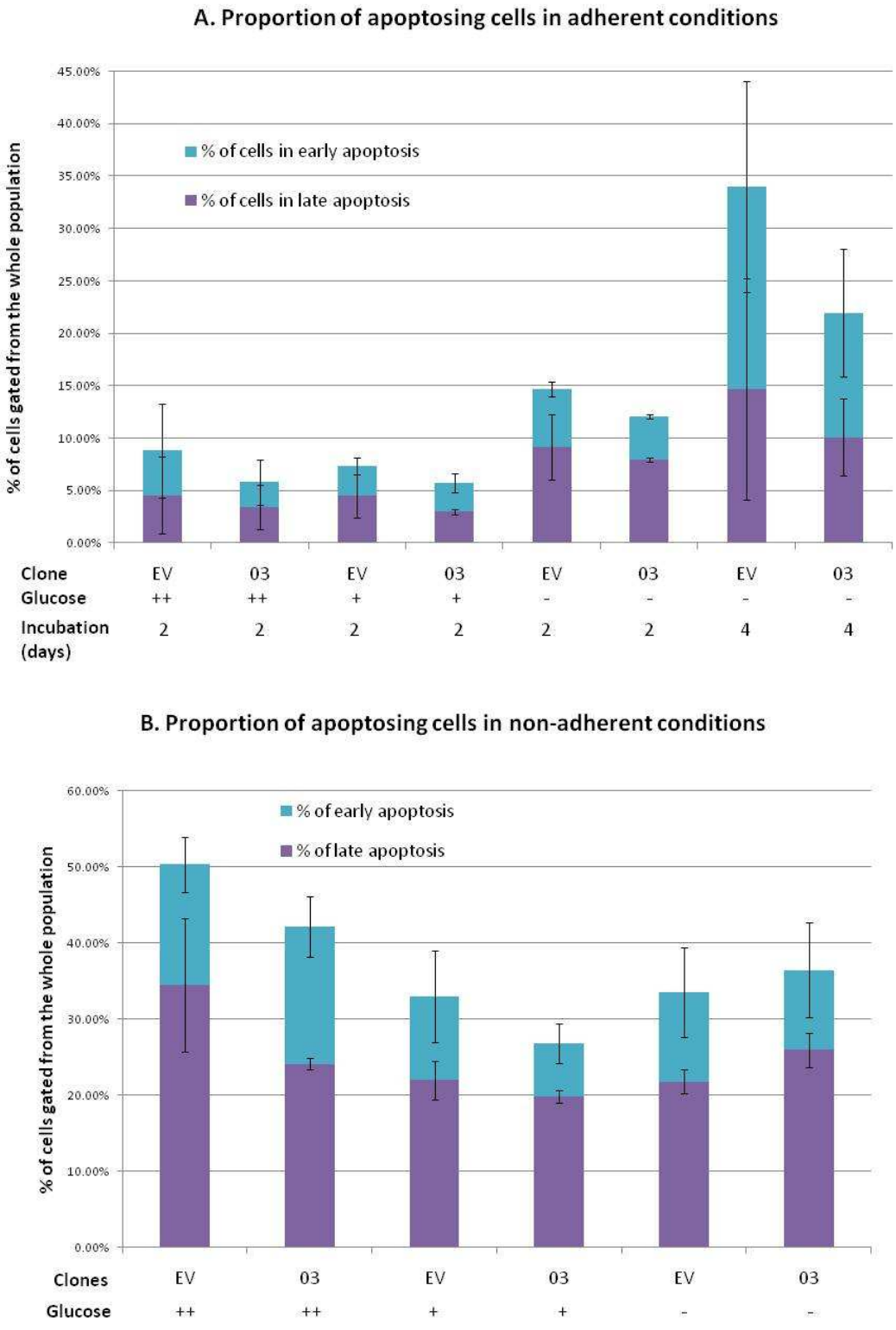


Figure 7.7: Effect of *LKB1* knock-down on apoptosis in adherent (A.) and non-adherent (B.) conditions.
The bar chart represents the proportion of cells in early or late apoptosis as detected by Annexin V and PI staining and recorded by flow cytometry. EV and 03 clones were cultured in normal (++), low glucose (+) or no glucose (-) conditions.

7.7 Discussion

To decipher the role of *LKB1* in the context of normal bone, we have set up an *in vitro* knock-down model to functionally study loss of this gene expression in an osteoblast cell line, HOB.

7.7.1 Methods selected to analyse LKB1 function

As presented in the Introduction, *LKB1* plays a role in a wide range of cellular processes via either its 14 downstream kinases or poorly understood interactions with other pathways such as p53 and p21 (Morton et al. 2010, Zeng et al. 2006), Src (Carretero et al. 2010), TGF-β (Katajisto et al. 2008) or Wnt (Ossipova et al. 2003) pathways (Figure 1.9 and section 1.2.1.2). We therefore focused on the main functions affected by *LKB1*: cell proliferation (via its action on cell metabolism with the mTOR pathway), cell migration (via cell polarity with the MARK family and cell motility with the Src pathway) and cell survival (via apoptosis with SIK and NUAK families or with the p53-p21 pathway). Functional assays were selected accordingly.

LKB1 can affect cell metabolism via AMPK, which then acts on different aspects of cell proliferation by inhibiting protein translation with the mTOR pathway and fatty acid synthesis. The use of the MTS assay, which indirectly infers differences in proliferation by recording the cell metabolism, was very useful in that sense. This may explain why differences were recorded with this assay but not with the growth curve assay.

As discussed in the Introduction (section 1.2.1.2), LKB1 is constitutively activated once bound to its complex, and the regulation of its function seems to be cell type and context dependent. For example, if cells are in energy deprived conditions, LKB1 can induce apoptosis through AMPK (Shaw et al. 2004). In the absence of glucose, NUAK2 can have the same effect (Suzuki et al. 2003). However, when the cells are in anchorage-free conditions, LKB1 is thought to act through SIK1 (Cheng et al. 2009). This implies that the functional assays need to mimic these external stimuli to induce the different effects of *LKB1*. We therefore performed all the assays in normal, low glucose and no glucose conditions.

7.7.2 Conclusion on the effect of LKB1 knock-down in osteoblasts

The functional analysis of the *LKB1* knocked-down HOB cell line compared to its EV control showed a statistically significant effect on cell proliferation. It also tented to affect cell survival. The differences obtained for both these processes were small but consistent. No differences were found for their tumourigenic potential by colony formation assays and for cell migration by scratch assay.

7.7.2.1 Relevance and limitations of the model

As mentioned in the introduction of this chapter (section 7.1), to investigate the effect of *LKB1* in the context of normal bone, the gene was knocked-down *in vitro* in the cell lineage believed to be the cancer initiating cells for OS, osteoblasts.

It is of note that in the *Lkb1*$^{+/-}$ mouse model, the tumours were discovered late in life – suggesting that the process induced by this loss was slow – and had the hallmark of an abnormal osteoblastic proliferation (Robinson et al. 2008). Hence, the finding of an increased cell proliferation *in vitro* in the *LKB1* knock-down model and the magnitude of these proliferation differences fit with the phenotype observed *in vivo*. Significant differences may only arise from the small effect *in vitro* after a prolonged culture time, which cannot be easily modelled in this set up.

The knock-down obtained in the HOB cell line did not totally remove LKB1-protein: low levels were still detected by WB. Hence, this model was very close to the *in vivo* mouse model of Robinson et al, which had only lost one allele of *Lkb1*. Similarly, low levels of LKB1 were detected by WB in a large number of OS cases (Figure 4.3) and our data suggests that this gene may have a haploinsufficient role in OS (section 5.5.3.2). Therefore our model was consistent with the disease presentation and enabled assessment of the haploinsufficient effect of *LKB1* in normal osteoblasts. However, it may also have accounted for the magnitude of the differences observed.

A limitation of the model used is that it focuses on the action of *LKB1* in only one cell lineage. Katajisto et al have shown that the cross-talk between different cell types was essential to the formation of polyps in Peutz-Jeghers syndrome (Katajisto et al. 2008). Loss of LKB1 in the mesenchyme compartment induced a downregulation of TGF-β signalling in these cells. This signal led to an increased proliferation of the epithelial cells in the gut and to the appearance of the

polyps. Our model only considered the effect of *LKB1* on osteoblasts. Osteoclasts in fact cooperate closely with this cell type in the context of normal bone. It has been suggested that targeting osteoclasts could help to control the abnormal osteoblastic activity in OS tumours (section 1.1.6.1). Therefore, it is possible that the effect of *LKB1* in OS tumours is partly mediated via a similar mechanism that disrupts the cross-talk between osteoblasts and osteoclasts. This may explain why studying the effect of LKB1 on osteoblasts only yielded minor differences.

Performing the knock-down of only one gene in an immortalised cell line enables specific study of its effect; however, five genetic alterations are thought to be necessary in humans to induce cancer (Fearon et al. 1990). This may also account for the subtle results obtained, especially in the colony formation assay.

Finally, as reported in section 1.2.1.4, *LKB1* haploinsufficient loss was reported to promote tumourigenecity in mouse models when associated with another genetic event, in lung cancer, pancreatic cancer and liver cancer (Ji et al. 2007, Miyoshi et al. 2009, Morton et al. 2010). It is then possible that an additional genetic event is needed in normal osteoblasts in cooperation with *LKB1* loss to obtain a significant functional effect.

7.7.2.2 Role of environmental conditions for *LKB1* function in osteoblasts

In the panel of assays tested, differences were found depending on the stimulus employed to stress the cells. As mentioned in the previous paragraph (section 7.7.1), *LKB1* role is context dependent, and it may act via different downstream kinases depending on the environmental conditions, through unknown mechanisms.

The effect of LKB1 on cell proliferation by the MTS assay could only be detected in low glucose conditions. This suggests that LKB1 loss only favours cell proliferation in energy deprived conditions.

Interestingly, in anchorage independent conditions, the addition of a second stress changed the response of the cells. LKB1 promoted cell survival in normal glucose and non-adherent conditions, with 8% less apoptotic cells on average. On the contrary, it promoted cell death in energy deprived conditions, with a smaller difference found in low glucose compared to normal glucose conditions (6% difference on average) and even an increased proportion of apoptotic cells (3% on average)

without glucose. This would argue that *LKB1* loss participates in early tumour formation: it can promote cell survival and cell proliferation but may not promote metastasis in OS, contrary to the findings in lung carcinoma (Carretero et al. 2010). Such different roles for *LKB1* in cancer cells depending of environmental stimuli have been reported by Godlewski et al. They showed that in normal glucose conditions, LKB1 could be suppressed in glioma cells via the upregulation of miR-451, leading to an increased cell proliferation. In low glucose conditions, the reverse mechanism was initiated: miR-451 was downregulated, LKB1 expressed and thanks to its downstream kinases, proliferation was repressed via AMPK and migration increased via MARK phosphorylation (Godlewski et al. 2010b). Similarly, in osteoblasts, the permanent loss of *LKB1* promotes cells proliferation even in the absence of glucose but may not be sufficient to help the cells to resist to anoikis. Additional genetic hits may be needed in cooperation with *LKB1* loss to favour resistance to anoikis in the absence of glucose.

7.7.2.3 Conclusion

Our data suggests that LKB1 may drive OS pathogenesis, although it may not be the first hit in tumour formation, as its effect was small in normal osteoblasts. It is likely that other genetic abnormalities cooperating with this loss are needed to obtain a more dramatic effect. To exclude that the small amplitude of the differences found was due to the selection of a normal cell line, another model was set up: the knock-in of the gene in OS cell lines expressing low levels of LKB1 protein. This will be the focus of the next chapter.

Chapter 8: ROLE OF *LKB1* IN THE CONTEXT OF OSTEOSARCOMA

8.1 Introduction and aims

As demonstrated in Chapter 4, there is low or absent expression of LKB1 in a subset of OS cases and cell lines. LKB1 can affect multiple cellular processes (section 1.2.1.2) and the effect of its loss in OS is unknown. Data from the previous chapter using gene knock-down in an immortalised osteoblast cell line suggests that it plays a role in cell proliferation in low glucose conditions; a trend was also observed for a potential role in cell death. However the differences we found in the knock-down experiments were small. Therefore we aimed in this chapter to confirm these results in another model, by directly investigating the role of *LKB1* in the context of OS.

As discussed in the previous chapter (section 7.7.2.1), LKB1 may need to be associated with another genetic abnormality to promote tumourigenecity. The model selected for this study was to knock-in *LKB1 in vitro* in tumourigenic cell lines expressing no or very low levels of the protein. Three cell lines were selected: OST, which expressed no LKB1 protein (score of 0 by WB, Figure 4.2) and no mRNA (Figure 5.8), and OSA and SaOS2, which expressed low levels of LKB1 (score of 1 by WB) and levels of mRNA equivalent to the positive controls.

The objectives were:

3. Set up an *in vitro* model in the SaOS2, OST and OSA cell lines with *LKB1* knocked-in
4. Assess the functional effect of loss of LKB1 with regards to:
    a. Tumourigenic potential
    b. Cell proliferation
    c. Cell migration
    d. Cell survival

8.2 Knock-in validation

To knock-in *LKB1* in the cell lines, two constructs including the human cDNA for either *LKB1* wild type (LKB1 clone) or a kinase dead mutant of *LKB1* (KD clone), K78I, both FLAG-tagged, in a pBABE vector and under the SV40 promoter, were acquired from Addgene (Figure 2.5). The infection and selection were performed as described in the Materials and Methods chapter (section 2.9). The lack of activity of the KD clone has been previously described (Shaw et al. 2004). Both clones were sequenced following selection, to confirm they either had or lacked the KD mutation as expected. An empty-vector construct was prepared to confirm that the infection with the pBABE vector did not alter the endogenous expression of LKB1 protein (section 2.9.2). The infection with the clones KD and LKB1 was performed three times for the SaOS2 and OST cell lines, and once for the OSA cell line.

8.2.1 qRT-PCR and WB results

The efficiency of the knock-in was validated at the mRNA level by qRT-PCR and at the protein level by WB (Figure 8.1).

Very high protein levels were detected in all three cell lines for the LKB1 clone compared to the parental (P) and empty-vector (EV) clones, and compared to the positive control (Figure 8.1.A). As the KD clone has only a point mutation in the active site of the protein that does not alter its three-dimensional structure, high levels of LKB1 were also detected by the antibody used against this construct.

To quantify further LKB1 expression in the different clones, the protein level was monitored regularly during the course of the study (in total twice for OSA and four times for SaOS2 and OST) and once for the other two repeats of the infection. The densitometry of the detected bands for LKB1 compared to GAPDH was calculated. Overall, LKB1 protein expression over all the repeats was 9.2 times higher in SaOS2 KD compared to the respective parental control ($p=0.0026$ with the one-tailed Student's t-test), 7.5 times for SaOS2 LKB1 ($p=0.0078$), 14.8 times for OST KD ($p=0.00063$), 11.4 times for OST LKB1 ($p=0.0027$), 8.8 for OSA KD ($p=0.034$) and 8.7 for OSA LKB1 ($p=0.0010$). These results are illustrated in the bar chart in Figure 8.1.B.

Similarly, very high levels of LKB1 mRNA were detected in the knock-in clones compared to the parental for each cell line in all the infection replicates performed, with a 33 fold increase in SaOS2 KD compared to the parental cell line ($p=0.017$ with the one-tailed Student's t-test), 28 fold in SaOS2 LKB1 ($p=0.020$), 838 fold in OST KD ($p=0.0041$), 1176 in OST LKB1 ($p=0.0028$), 38 fold in OSA KD and 30 in OSA LKB1.

The levels of LKB1 protein and mRNA detected in the EV and P clones were very close to each other for all three cell lines. This confirmed that the pBABE construct used had no endogenous effect on LKB1 expression.

8.2.2 Morphological changes

The OST cell line acquired a more spindled morphology in the LKB1 clone compared to the KD and parental clones (Figure 8.2). This was not detected in the SaOS2 and OSA cell lines; however, these lines already possessed a spindled morphology before the knock-in, which may explain the absence of changes observed.

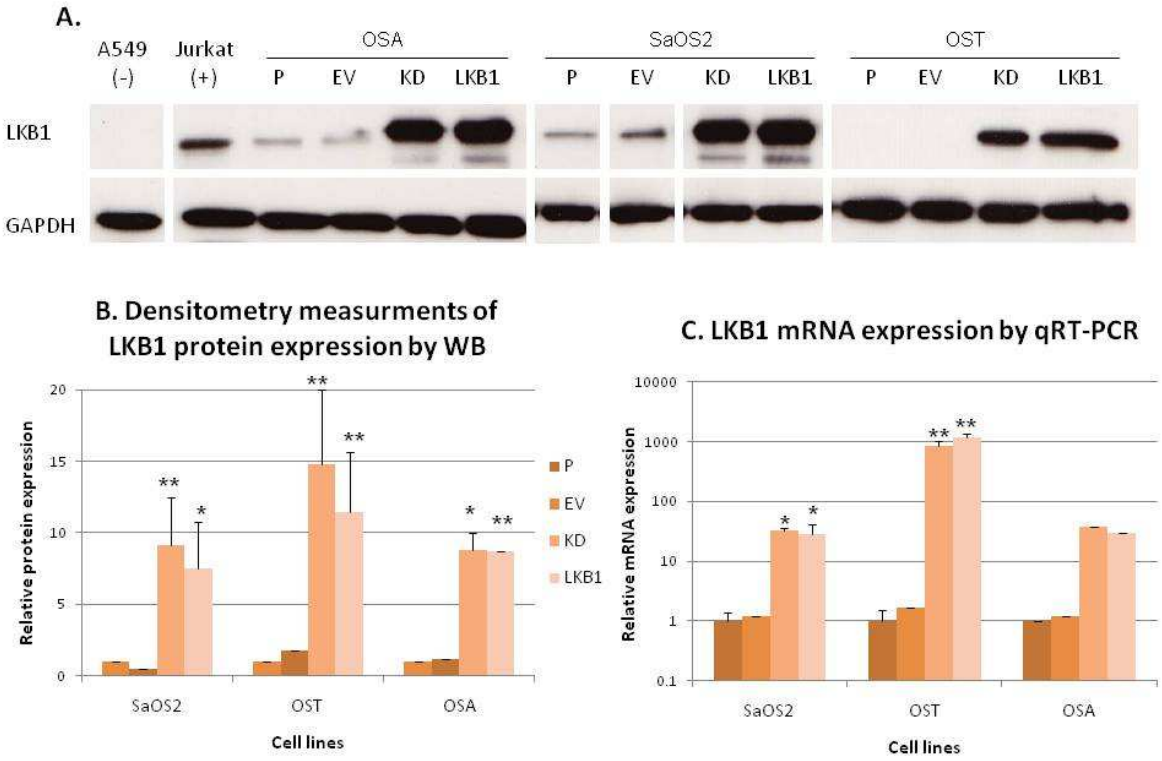


Figure 8.1: Knock-in efficiency at the protein and mRNA levels.
A. WB showing LKB1 expression obtained when *LKB1* was knocked-in in three different OS cell lines, OSA, SaOS2 and OST. A549 was used as a negative control and Jurkat as a positive control. B. Densitometry analysis quantifying LKB1 relative protein expression compared to GAPDH and normalised to the parental (P) expression for each cell line. C. Fold difference of the relative LKB1 mRNA expression compared to β-actin, normalised against parental expression for each cell line. * for $p<0.05$ and ** for $p<0.005$ with the Student's t-test.

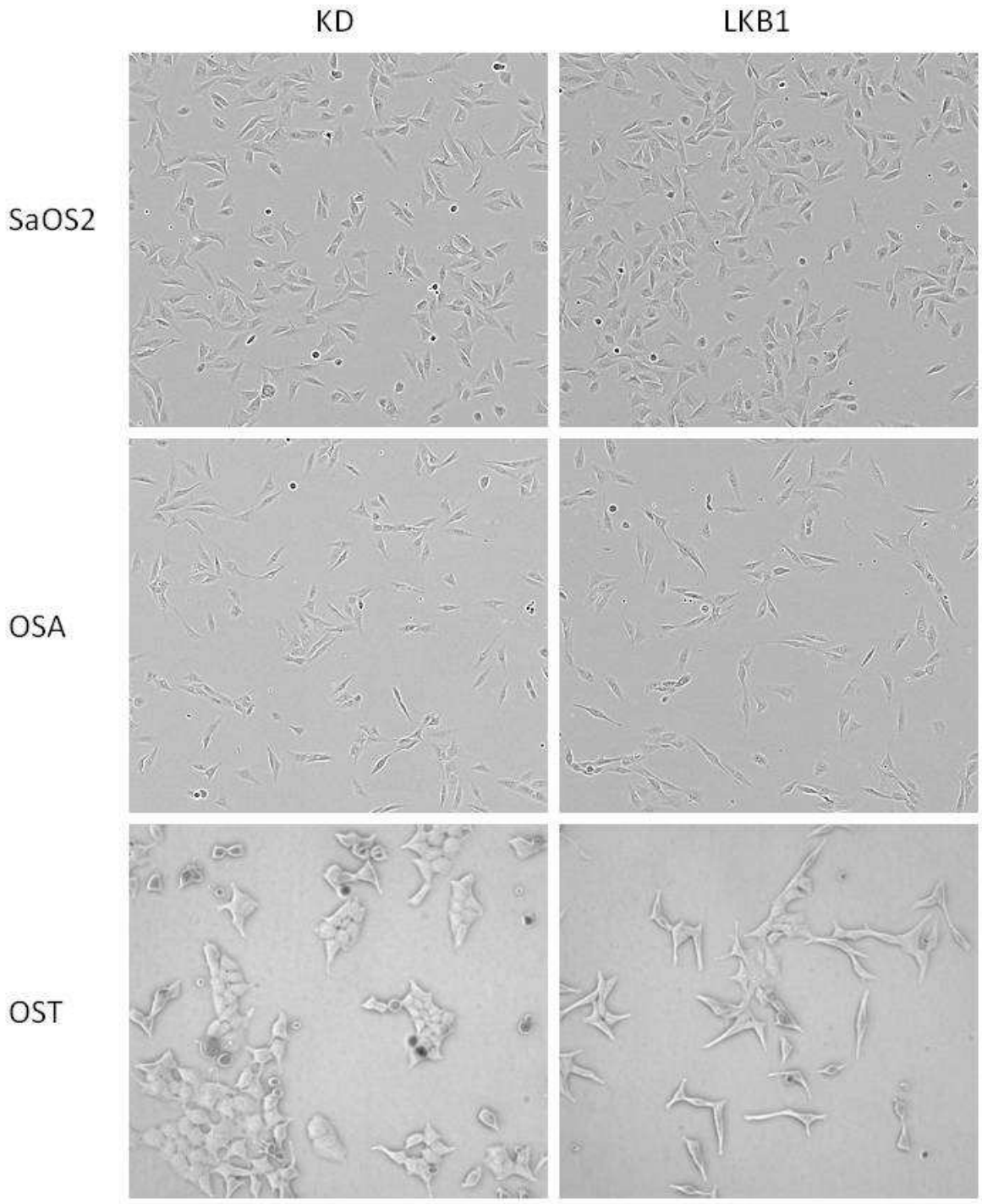


Figure 8.2: Morphology of the *LKB1* knock-in clones for each cell line.
Representative phase contrast pictures (10x) of the kinase dead (KD) or functional (LKB1) clones after selection.

8.3 Effect of *LKB1* knock-in on tumourigenicity

To assess whether *LKB1* was playing a tumourigenic role in OS, the ability of the LKB1 and KD (e.g. the control) clones of the SaOS2 and OST cell lines to form colonies *in vitro* was assessed as described in the Materials and Methods chapter (section 2.10.1). Both clones of both cell lines yielded high number of colonies, which were detectable in less than two weeks. The number of colonies in both clones was close (Figure 8.3); no statistically significant differences were observed between them for all cell lines (p=0.39 for SaOS2 and p=0.089 for OST with the two-tailed paired Student's t-test).

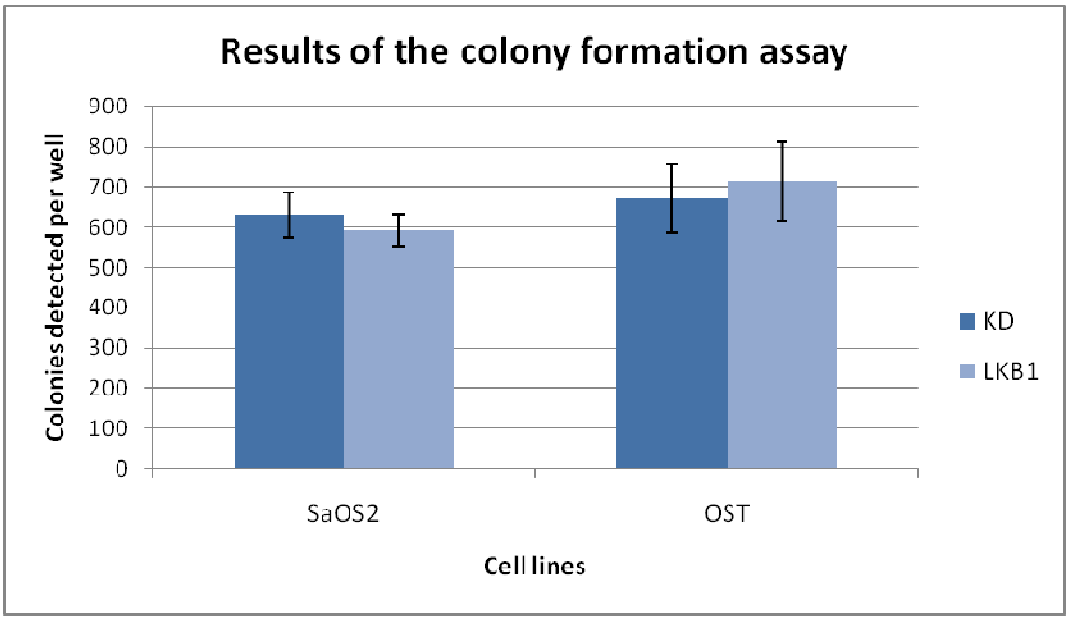


Figure 8.3: Bar chart illustrating the results of the colony formation assay with or without *LKB1* knock-in. Average number of colonies detected per well in three independent replicates. Two wells were seeded for each replicate.

8.4 Effect of *LKB1* knock-in on cell proliferation

As performed in the knock-down study, three approaches were used to assess the functional effect of LKB1 knock-in on cell proliferation in normal, low glucose and no glucose conditions, as described in the Materials and Methods chapter: a growth curve assay (section 2.10.2.2) on all three cell lines, an MTS assay (section 2.10.2.1) and a cell cycle assay (section 2.10.2.3) on the SaOS2 and OST cell lines.

8.4.1 Results of the growth curve assay

8.4.1.1 Growth curve

The growth curve of the KD and LKB1 clones for all three cell lines was monitored in normal and low glucose conditions using a live-cell imaging system, the Incucyte (Essex Bioscience). Consistent differences were observed between the KD and LKB1 clones in at least three replicates for all three cell lines, with a reduced growth rate of the LKB1 clone compared to the KD clone (Figure 8.4). This decreased proliferation was subtle in the SaOS2 cell line, but very dramatic in the OSA cell line.

8.4.1.2 Growth rate quantification

To quantify further this result, the growth rate of the different clones during the exponential growth phase was calculated by linear regression (Figure 8.5.A). During this phase, the growth rate is a constant and can be computed using the density of cells and time points from the Incucyte data. Although the growth rate obtained for the SaOS2 cell lines showed a higher rate in the KD compared to the LKB1 clone in 7 replicates in normal conditions and 4 replicates in low glucose conditions, these differences were not statistically significant ($p=0.56$ with the two-tailed paired Student's t-test in normal conditions and $p=0.37$ in low glucose conditions). However, the differences were significant for the OST and OSA cell lines in normal conditions ($p=0.0017$ and $p=0.0042$ respectively with the one-tailed paired Student's t-test) in 6 and 5 replicates respectively and in low glucose for the OSA cell line ($p=0.046$) in 3 replicates. The same trend was observed in low glucose for the OST cell line but the difference was not significant ($p=0.094$) in 3 replicates.

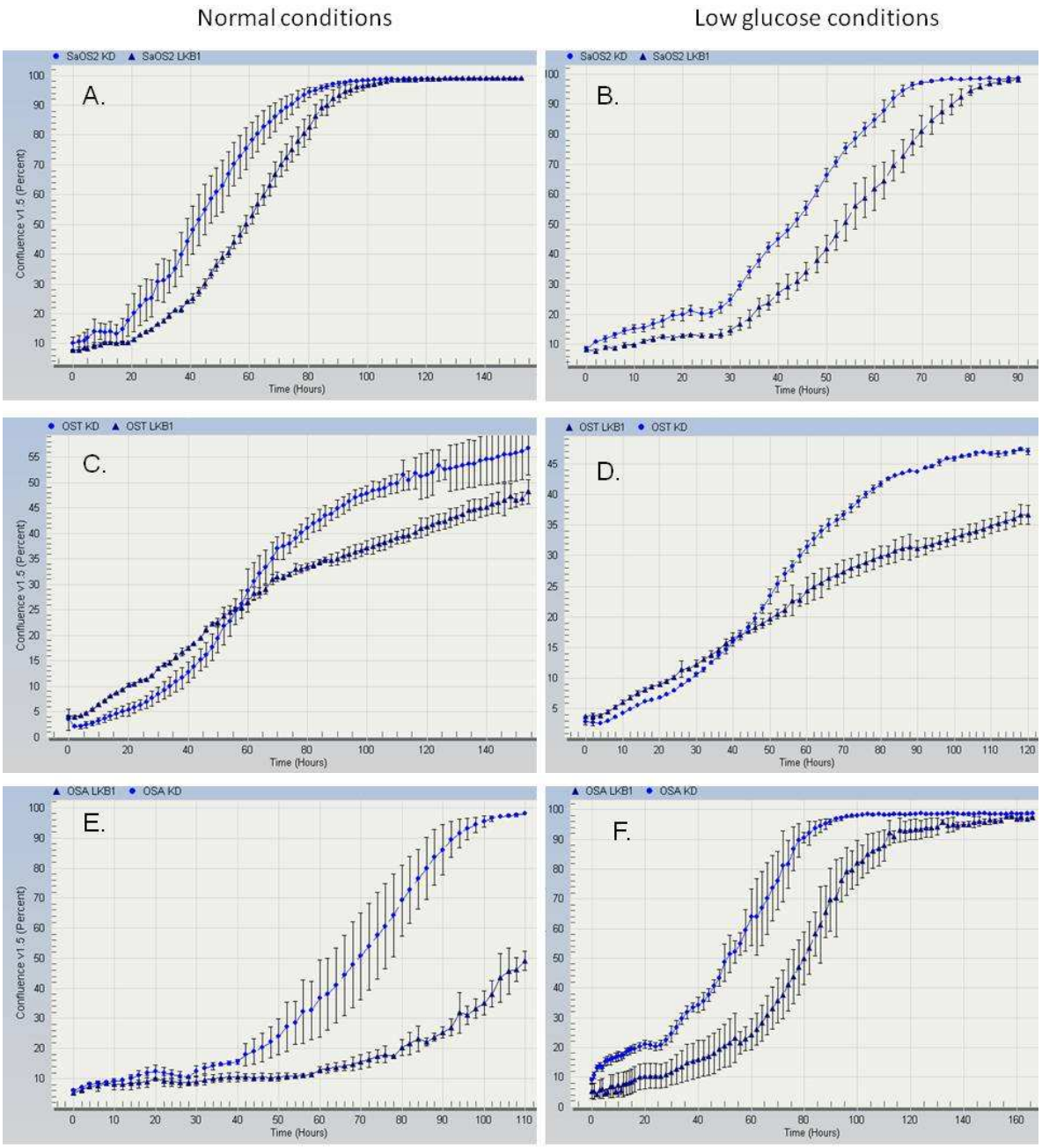


Figure 8.4: Effect of *LKB1* knock-in on proliferation by growth curve recorded with the Incucyte.
The growth curve is represented by the average confluency measured with the Incucyte over time for SaOS2 (A. and B.), OST (C. and D.) and OSA (E. and F.) cell lines in normal or low glucose conditions. One representative curve out of the three replicates performed is presented here. The clone LKB1 is represented by a dark blue triangle, and the clone KD by a light blue circle for every cell line.

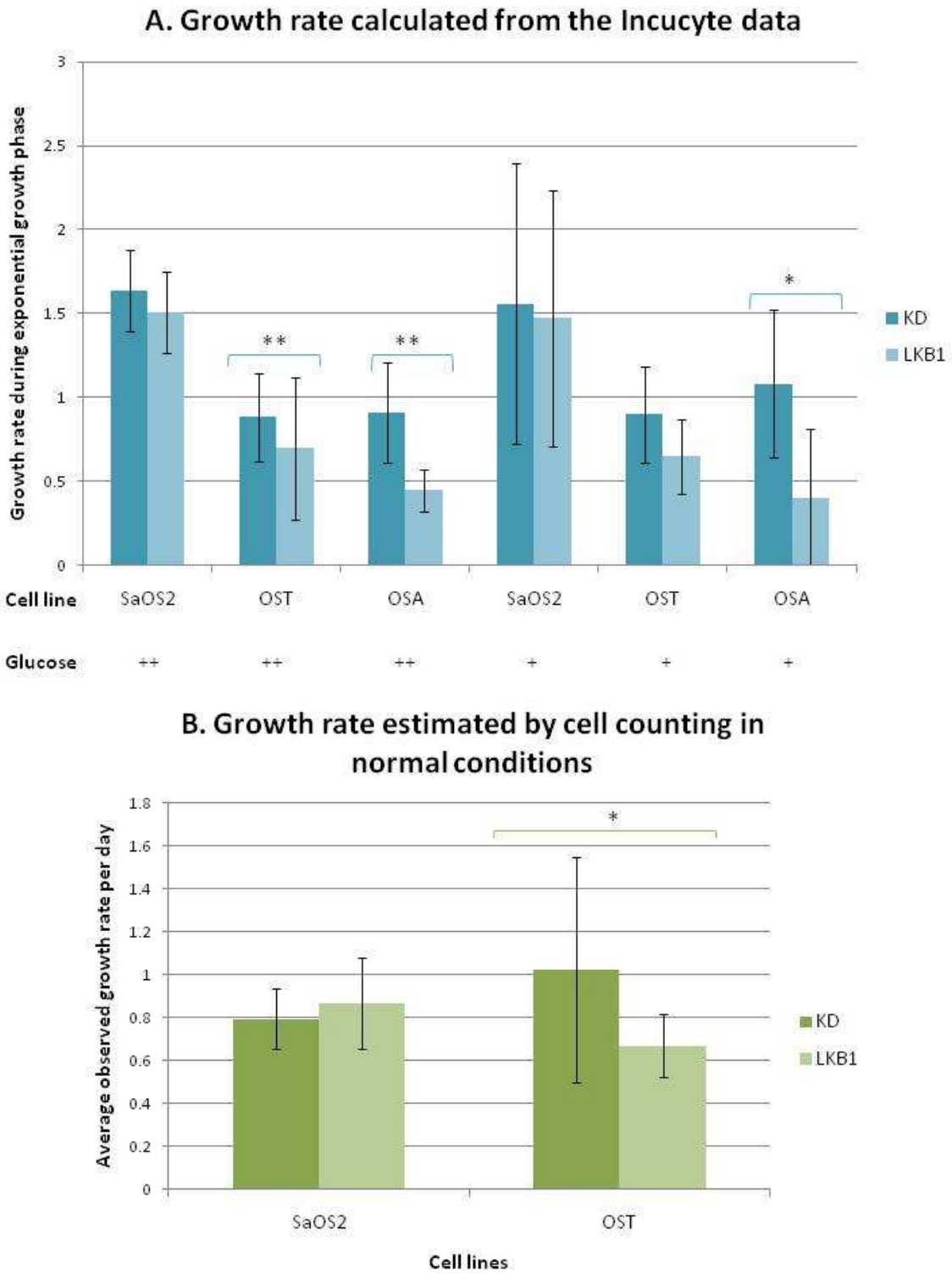


Figure 8.5: Quantification of cell growth rate with or without *LKB1* knock-in.
A. Average growth rate computed by linear regression for SaOS2 (n=7 and n=4 replicates respectively), OST (n=6 and 3 resp.) and OSA (n=5 and 3 resp.) in normal (++) or low glucose (+) conditions. B. Growth rate measured by cell counting during culture in normal conditions for SaOS2 (n=8 experiments) and OST (n=9). * for $p<0.05$ and ** for $p<0.005$ with the Student's t-test.

Similar results were found when the growth rate was calculated with another method, by directly counting the cells. The ratio of the number of cells obtained at near confluence divided by the number of cells seeded and the number of days of growth was computed, to estimate the growth rate per day in normal conditions for the KD and LKB1 clones in the SaOS2 and OST cell lines (Figure 8.5.B). No difference was found for the SaOS2 cell line in 8 replicates (p=0.16 with the two-tailed paired Student's t-test), but it was statistically significant for the OST cell line in 9 replicates (p=0.030 with the one-tailed paired Student's t-test).

8.4.1.3 Validation by BrdU assay

Finally, to validate the results observed on the Incucyte, a BrdU assay was performed in parallel to a growth curve assay as described in the Materials and Methods chapter (section 2.10.2.4). In one replicate, 4.7% more cells were positive for BrdU in the KD clone compared to the LKB1 clone in the OST cell line. The growth curve of the cells monitored with the Incucyte 24 hours before the BrdU experiment also showed that the KD clone had an increased proliferation rate compared to the LKB1 clone (Figure 8.6 below).

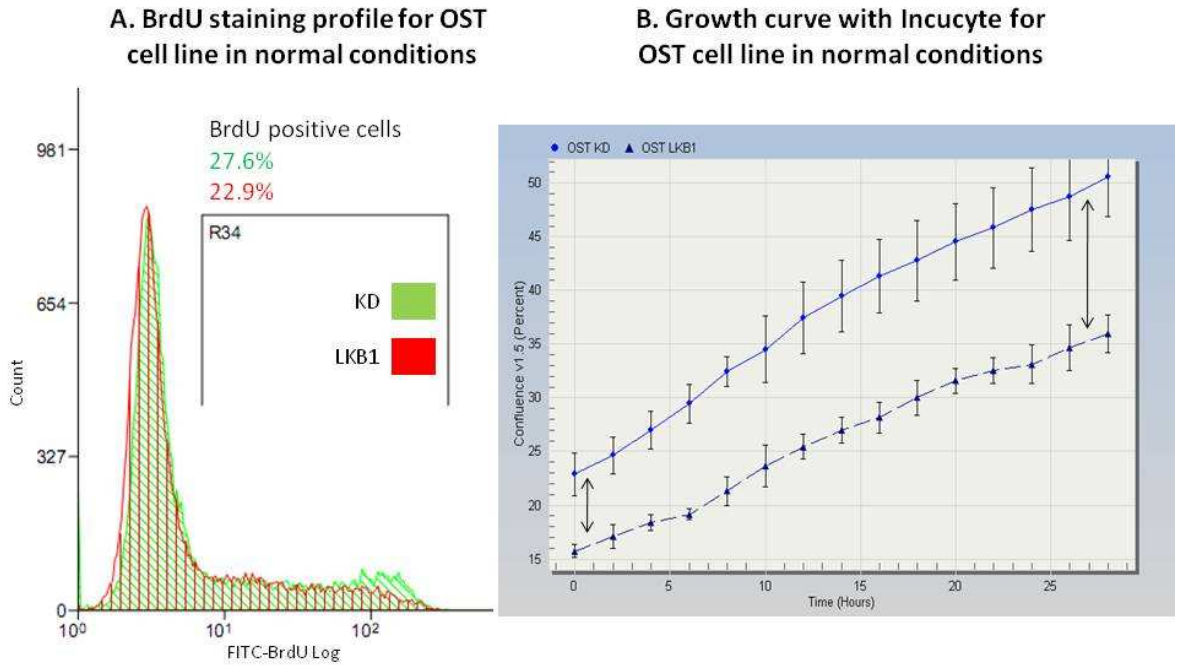


Figure 8.6: Effect of *LKB1* knock-in on proliferation in OST cell line with the BrdU (A.) and the growth curve (B.) assays.

8.4.2 Results of the MTS assay

To elucidate whether the differences in proliferation were also detected by monitoring the cell metabolism, an MTS assay was performed with three cell densities in normal and low glucose conditions, with the SaOS2 and OST cell lines. Virtually no differences were found in the OST cell line. However, the LKB1 clone in the SaOS2 cell line had a decreased metabolism compared to the KD clone in both conditions. These differences could be observed at all cell densities, and were statistically significant at day 2 and 3 in the lowest cell density (p=0.0099 and p=0.023 respectively with the one-tailed paired Student's t-test) and the intermediate cell density (p=0.0034 and p=0.030 resp.) in normal conditions. The same trend was observed in low glucose conditions with significant differences at day 2 and 3 in the lowest cell density (p=0.041 and 0.024 resp.), day 1 in the highest (p=0.039) and close to significance in day 3 in the intermediate (p=0.06).

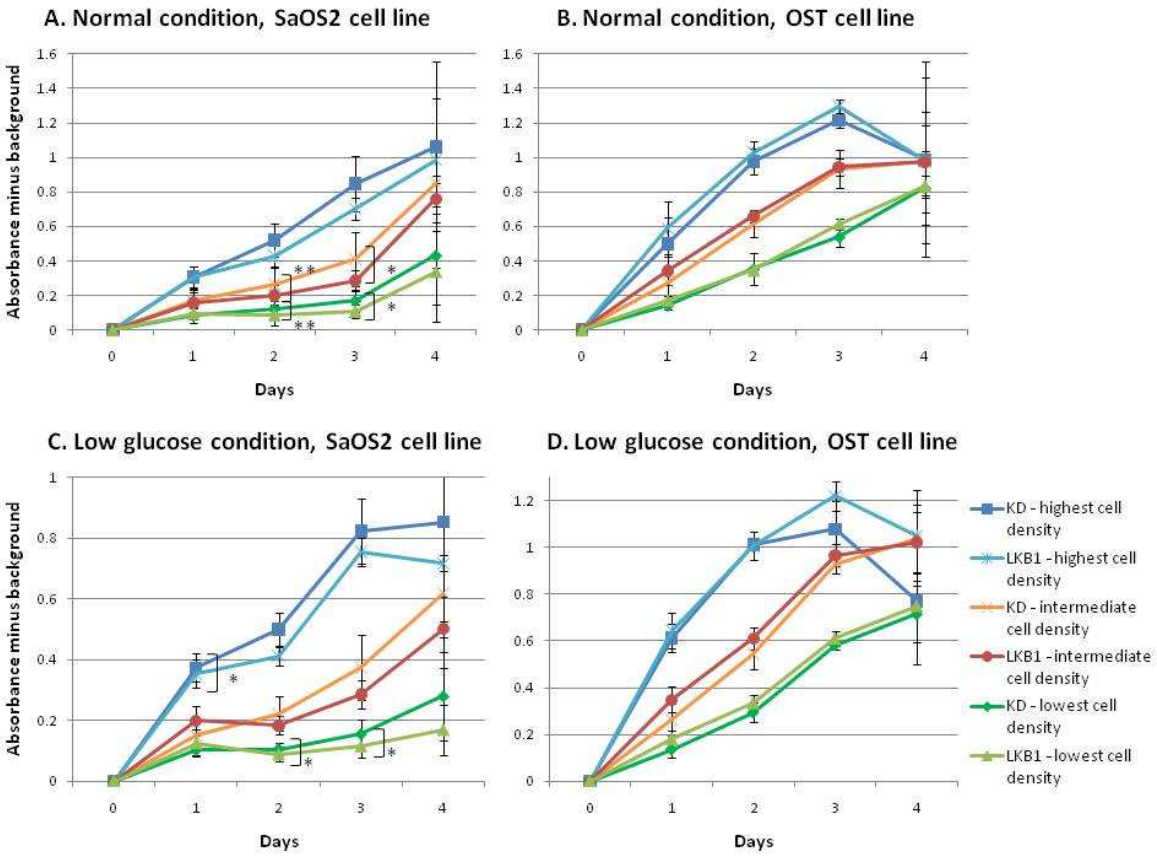


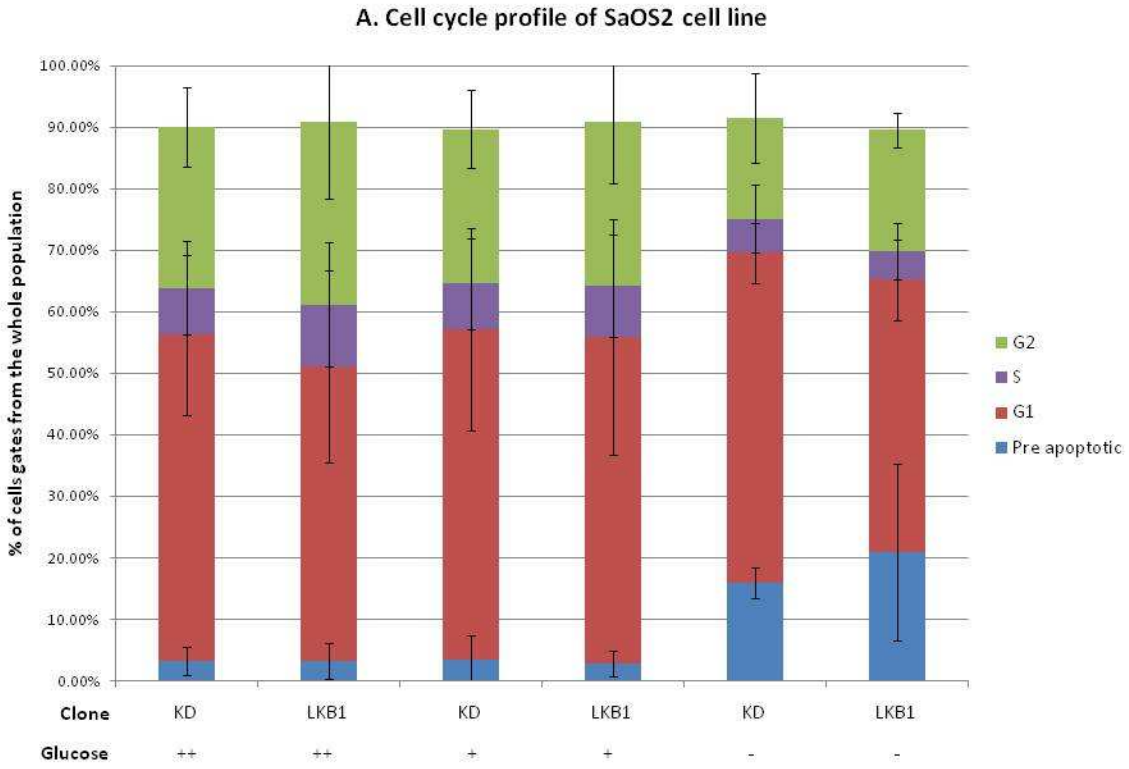
Figure 8.7: Effect of *LKB1* knock-in on cell proliferation by MTS assay.
Three cell densities were plated for each cell line and the experiment was performed in normal or low glucose conditions. * for p<0.05 and ** for p<0.01 with the Student's t-test.

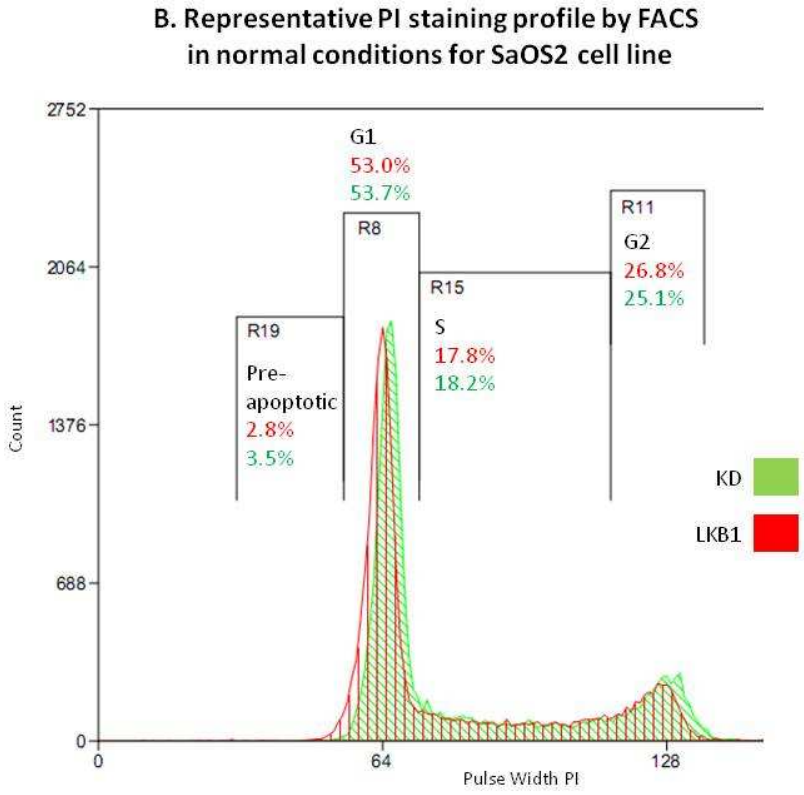
8.4.3 Results of the cell cycle assay

As significant differences were observed by MTS assay after two to three days of culture in SaOS2 but not the OST cell lines, their cell cycle profile was monitored after 2.5 days of culture as previously described. No consistent differences were observed in both cell lines between the KD and LKB1 clone in all three conditions studied (Figure 8.8).

8.4.4 Conclusion for the effect of LKB1 knock-in on cell proliferation

All three cell lines showed reduced cell growth rate as shown with the Incucyte in *LKB1* knocked-in cell lines compared to the KD control. The validity of this method was confirmed by cell counting and BrdU assay on the OST cell line. The functional mechanism underlying these differences differed in the cell lines used, as the LKB1 clone showed a reduced metabolic activity by MTS assay compared to the KD clone in the SaOS2, but not the OST cell line.





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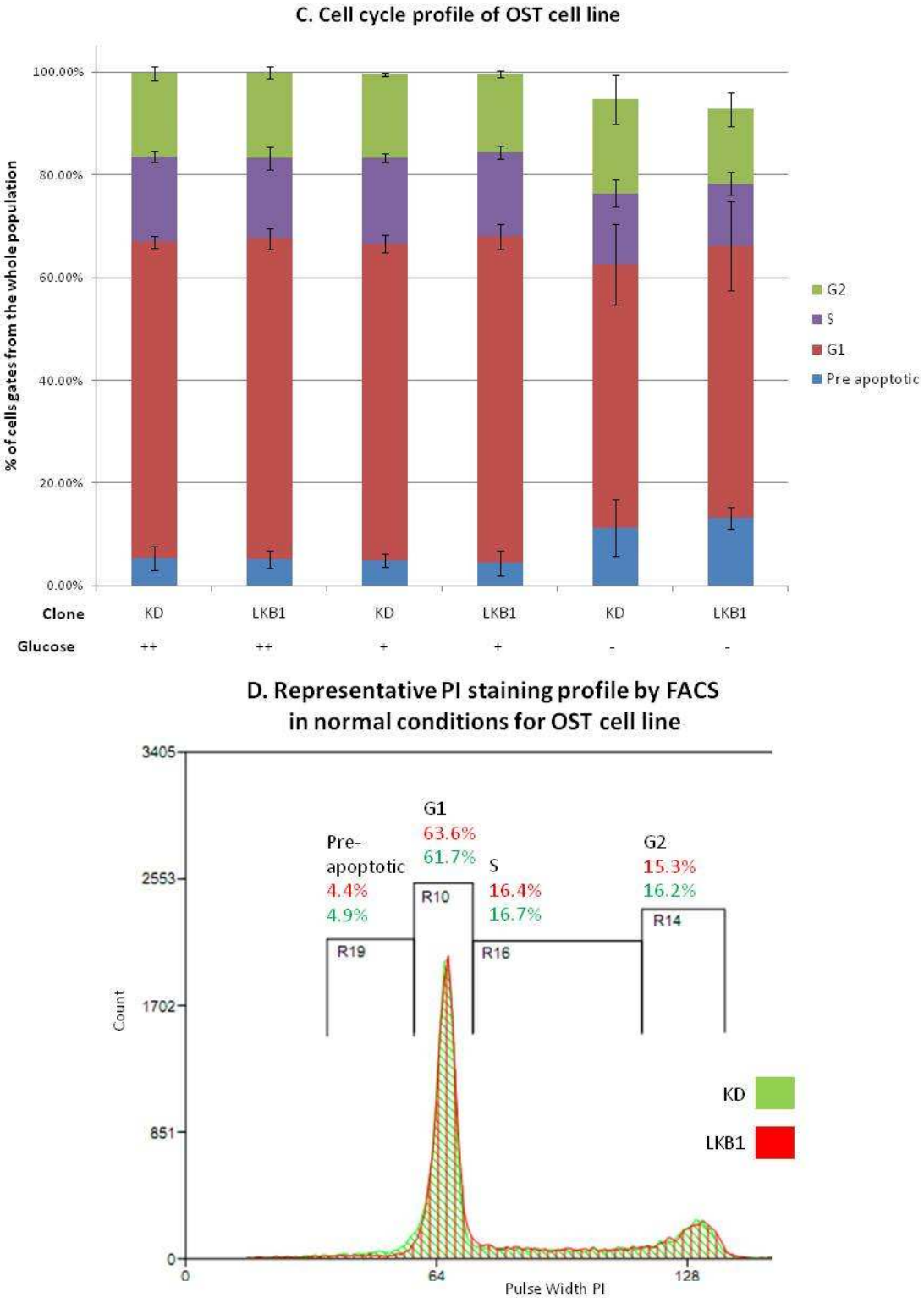


Figure 8.8: Cell cycle analysis of *LKB1* knock-in.
The average proportion of cells over three experiments in pre-apoptotic, G1, S and G2 phases for the KD and LKB1 clones in normal (++), low glucose (+) and no glucose (-) conditions is given for SaOS2 (A.) and OST (C.). A representative profile obtained by PI staining by flow cytometry is also given for each cell line in C. and D.

8.5 Effect of *LKB1* knock-in on cell migration

8.5.1 Results of the scratch assay

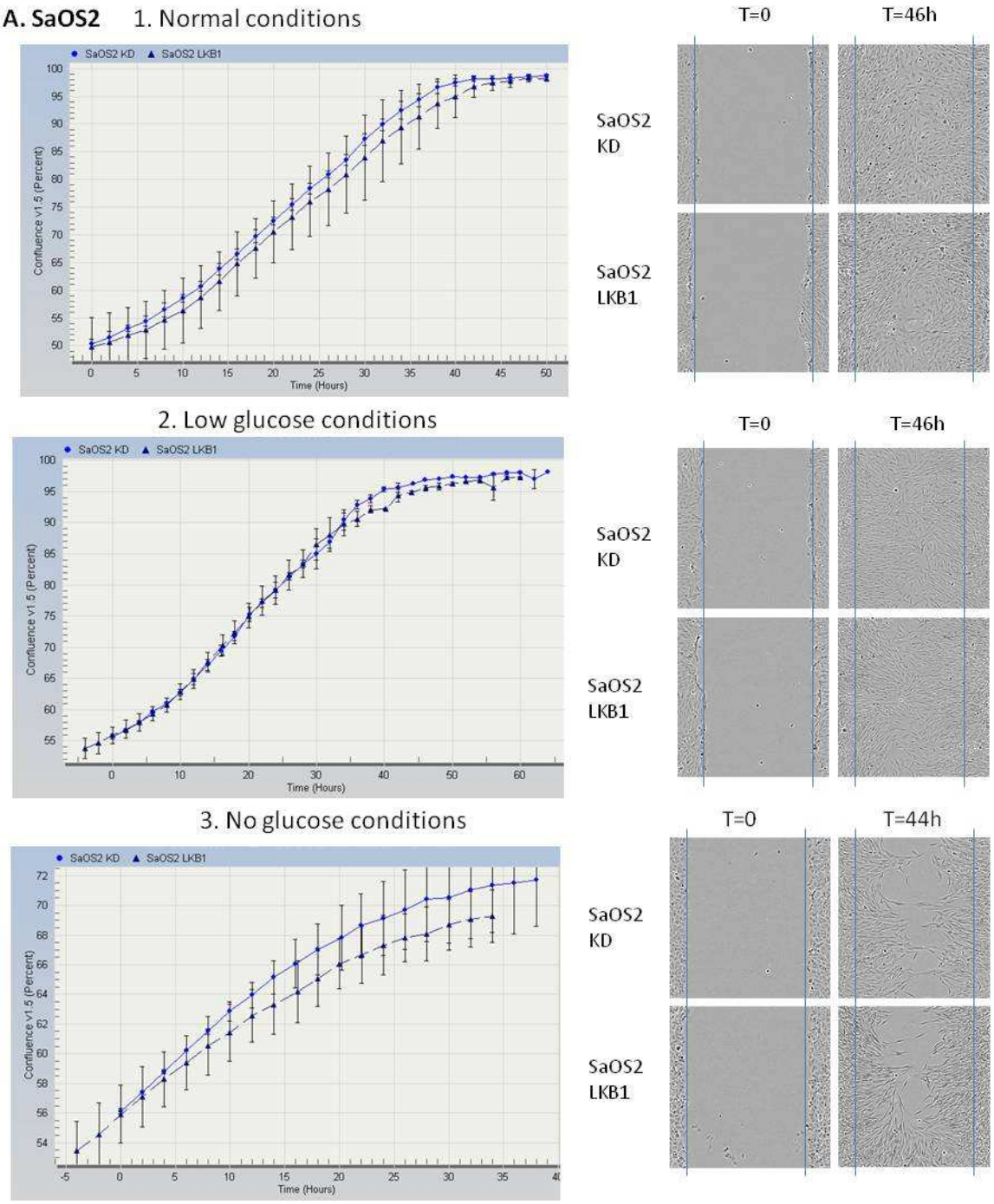
The functional effect of LKB1 on cell migration was first assessed by scratch assay on the SaOS2 and OSA cell lines in all three conditions (Figure 8.9). The OST cell line could not close the wound inflicted with any of the clones prepared. This is due to its limited ability to migrate when grown in adherence. Hence this cell line was not suitable for testing by this method. No reproducible differences were found for the SaOS2 cell line in the four replicates performed. The KD clone consistently closed the wound at a higher rate than the LKB1 clone for the OSA cell line in all conditions.

8.5.2 Results of the transwell assay

As mentioned in section 8.2.2, *LKB1* knock-in induced a morphological change in the OST cell line, leading to a more spindle appearance of the LKB1 clone compared to the KD clone (Figure 8.2). This could be due to a disruption in the cell polarity induced by LKB1 re-expression and may influence its migration ability. Therefore, the transwell assay described in section 2.10.3.2 was used to assess the functional effect of *LKB1* on migration in the OST and SaOS2 cell lines. An increased number of cells were detected in the KD clone compared to the LKB1 clone in the OST cell line in all five replicates; although the results were not statistically significant, the p-value obtained was close to 0.5 ($p=0.056$ with the one-tailed paired Student's t-test). On the contrary, a reduced number of cells were found to migrate through the transwell for the clone KD in the SaOS2 cell line compared to the LKB1 clone, and the difference obtained was significant ($p=0.048$). This is illustrated in Figure 8.10.

8.5.3 Conclusion on the effect of LKB1 knock-in on cell migration

As observed for cell proliferation, the effect of *LKB1* knock-in was cell line dependent. Indeed, re-expression of the gene in the OSA and OST cell lines induced a decreased migration potential by scratch or transwell assays, whereas migration of the LKB1 clone was enhanced compared to the KD clone in the SaOS2 cell line by transwell assay.



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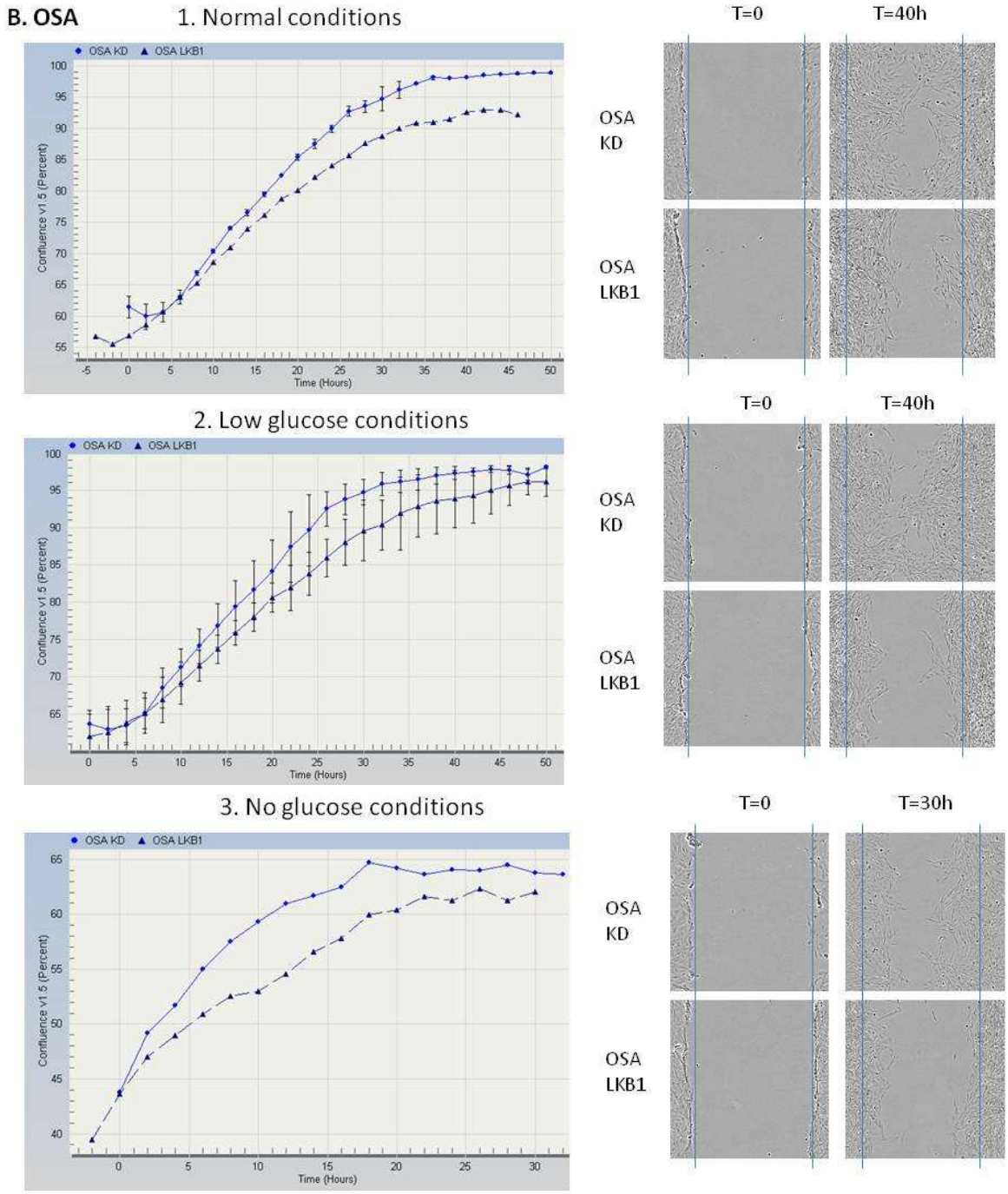


Figure 8.9: Representative graphs and pictures of the scratch assay for SaOS2 (A.) and OSA (B.) cell lines. Graph of the well confluency recorded with the Incucyte and representative pictures at the start (t=0) and at the end (t=30 to 46h) in normal (1.), low glucose (2.) and no glucose (3.) conditions.

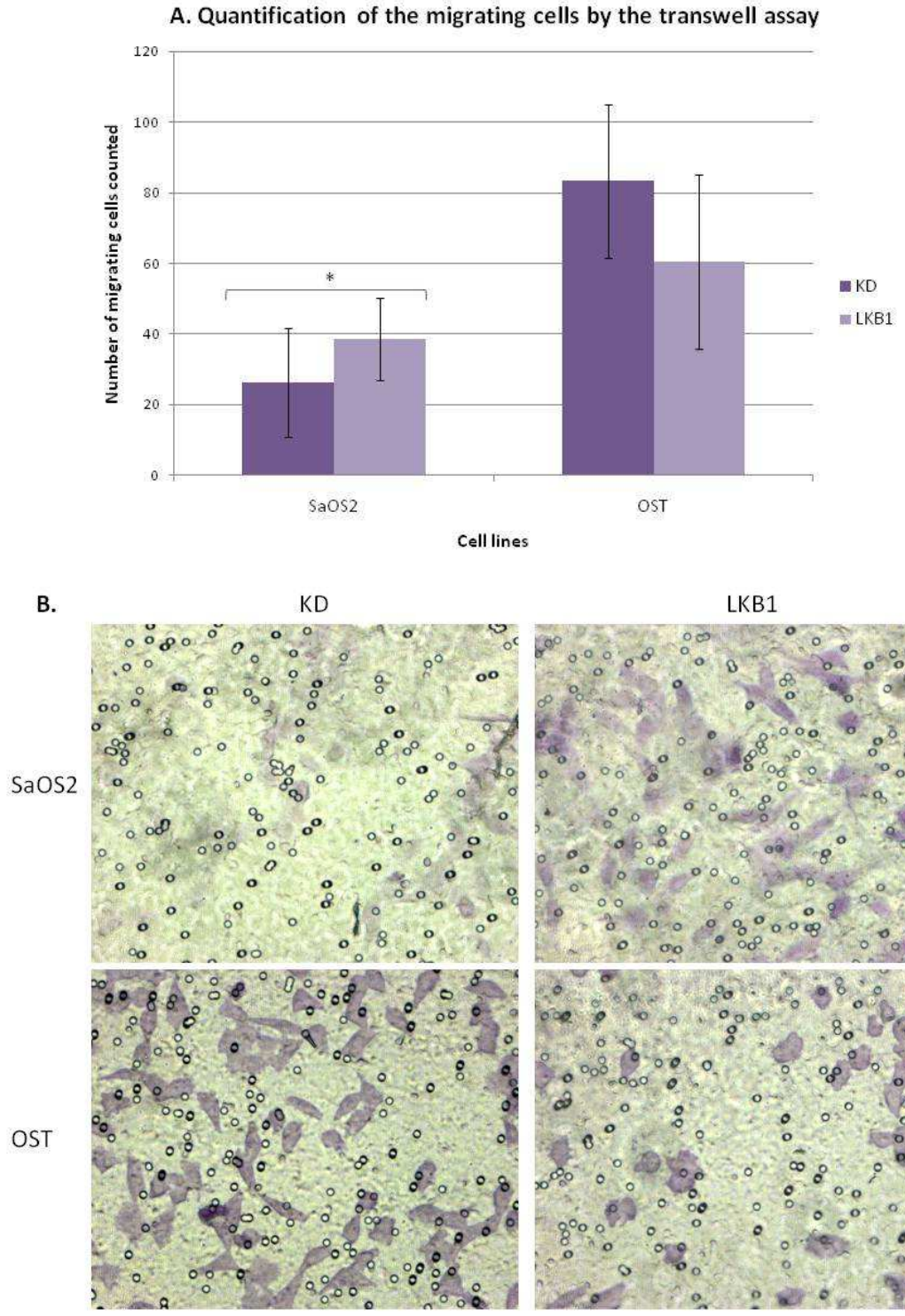


Figure 8.10: Cell migration assessed by the transwell assay.
A. Graph illustrating the average number of migrating cells counted in 4 pictures in each well (n=5) with or without *LKB1* knock-in. B. Representative pictures of the migrating cells stained with crystal violet (magnification 10x). * for $p<0.05$ with the one-paired Student's t-test.

8.6 Effect of *LKB1* knock-in on anoikis

The functional effect of *LKB1* knock-in on cell survival was monitored, as it was affected by *LKB1* knock-down in the HOB cell line (Figure 7.7). As no consistent differences were observed in the proportion of pre-apoptotic cells in the cell cycle analysis of both SaOS2 and OST in all three conditions (Figure 8.8), the survival of the LKB1 clone compared to the KD clone was only assessed in anchorage independent conditions, as described in section 2.10.1.

As found for cell proliferation and migration (section 8.4.4 and 8.5.3), the functional effect of *LKB1* knock-in in the SaOS2 cell line were dissimilar to that in the OST cell line. In normal and low glucose conditions, the LKB1 clone consistently presented an increased proportion of apoptosing cells compared to the KD clone in the SaOS2 cell line (on average 8% and 4% respectively). On the contrary, in the OST cell line, the proportion of apoptosing cells decreased in the LKB1 compared to the KD clone in these two conditions (of 4% and 2% respectively). These results were statistically significant in low glucose for the SaOS2 cell line ($p=0.039$ with the one-tailed paired Student t-test) and in normal conditions for the OST cell line ($p=0.032$). In the absence of glucose, both cell lines tended to survive better in non-adherent conditions when *LKB1* was re-expressed, but these differences were not significant (Figure 8.11).

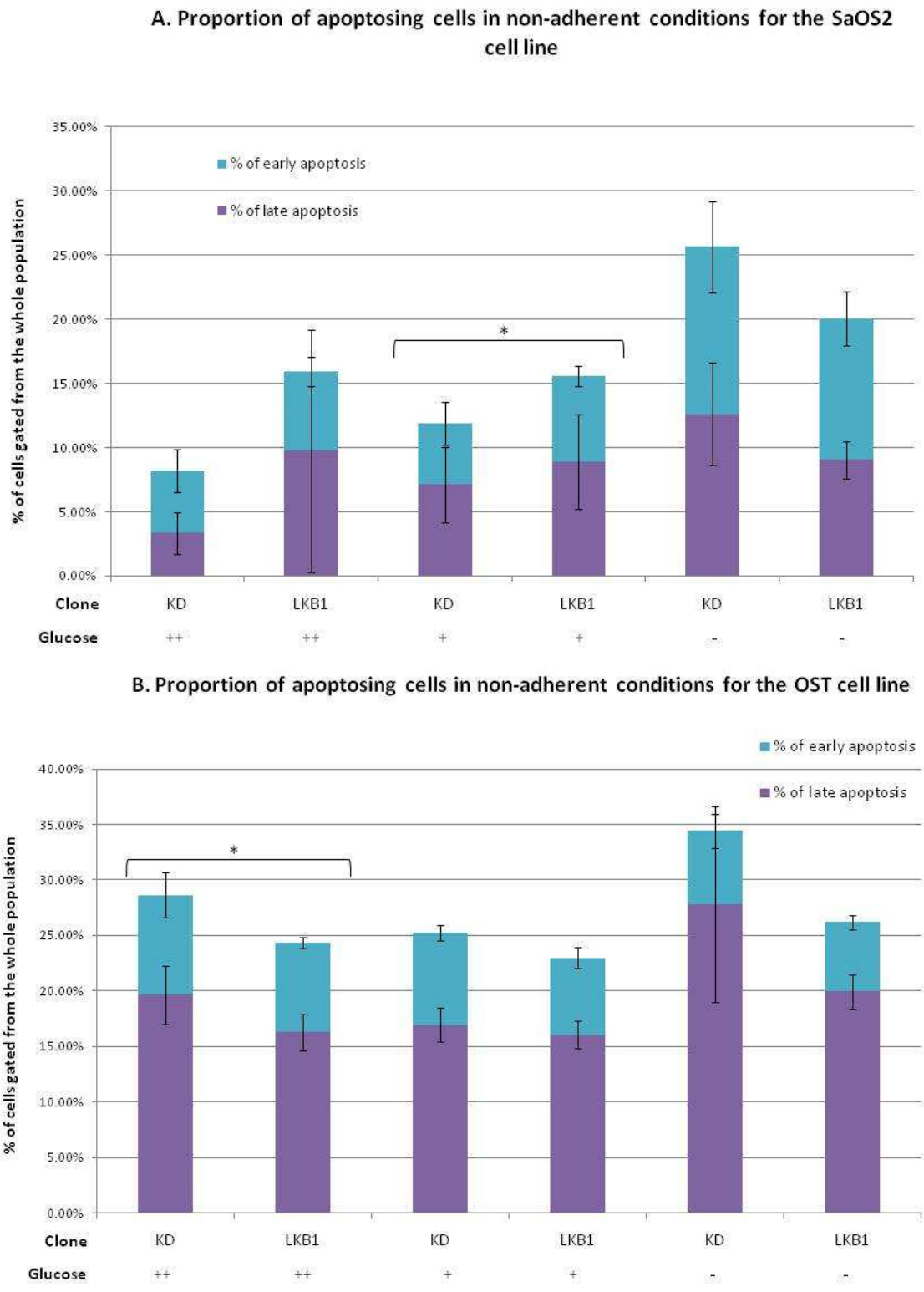


Figure 8.11: Effect of *LKB1* knock-in on anoikis in the SaOS2 (A.) and OST (B.) cell lines.
The bar chart represents the proportion of cells in early or late apoptosis as detected by Annexin V and PI staining recorded by flow cytometry. The cell lines were cultured in non-adherent conditions in normal (++), low glucose (+) or no glucose (-) conditions. * for $p<0.05$ with the Student's t-test.

8.7 Discussion

The knock-down of *LKB1* in immortalised osteoblasts reported in the previous chapter suggests that LKB1 plays a functional role in OS pathogenesis by disrupting cell proliferation and cell survival. To confirm these results, the gene was knocked-in in three *LKB1*-deficient OS cell lines. The effect of this was to induce a decrease in cell proliferation in all three cell lines tested. However, the amplitude and the mechanism of this functional effect differed between the cell lines, and opposite effects were seen in cell migration and cell survival depending on the cell line. Therefore, it raises the question of the value of these cell lines as a model and underlines the importance of the genetic context where loss of *LKB1* occurs.

8.7.1 LKB1 functional role in cell proliferation

8.7.1.1 Use of panel of assays and discrepancies observed

LKB1 has been shown to play a role in cell growth mainly via AMPK (Inoki et al. 2003) as presented in the Introduction (Figure 1.9). We confirmed this function of LKB1 in OS by monitoring cellular growth curves in the KD compared to the LKB1 clones in the three knocked-in cell lines SaOS2, OST and OSA (Figure 8.4). These results were validated by estimating the growth rate in the two clones for all cell lines, with statistically significant differences for OST in normal conditions and for OSA in normal and low glucose conditions (Figure 8.5). Furthermore, the results in normal condition in the OST cell line were supported by the evaluation of the growth rate by cell counting (Figure 8.5) and by a BrdU assay (Figure 8.6).

Nevertheless, the magnitude of these differences varied in each cell line; the effect in SaOS2 was quite minor compared to OSA and OST, and although the same trend was observed in the growth curves, the decrease in the growth rate in SaOS2 was not statistically significant. Furthermore, significant differences were observed by MTS assay for the SaOS2 cell line, but not with the OST cell line (Figure 8.7). Similarly, small differences had been observed in the *LKB1* knocked-down cell line of normal osteoblasts HOB by MTS assay rather than by the growth curve assay in the previous chapter.

No effects were seen by cell cycle analysis on the three cell lines. This may be due to the fact that the assay was not performed during the peak of the differential cell growth as it was performed

after 60 hours of growth, whereas the growth curves showed significant differences after 24 to 36 hours of culture. It may also be because of reduced sensitivity of this assay, compared to the BrdU assay, which did show differences.

It can be concluded that LKB1 re-expression altered cell proliferation in all three cell lines, but that the underlying mechanism leading to this may differ between them.

8.7.1.2 Possible mechanisms leading to the differences observed

LKB1 is known to act mainly on cell proliferation by its action on cell metabolism via the phosphorylation of its downstream kinase AMPK (Shaw et al. 2004). Loss of *LKB1* can then induce a disruption of the coordination between cellular growth and external stimuli – like glucose starvation – a hallmark of cancer. But *LKB1* has also been reported to act via the TGF-β pathway, as Katajisto et al have demonstrated that loss of *LKB1* in the mesenchymal compartment of the guts in mice led to abnormal epithelial proliferation (Katajisto et al. 2008). Therefore it is possible that LKB1 loss in OS is enforced either via the AMPK or the TGF-β pathways, depending of the genetic context. The parallel observed between the SaOS2 and HOB cell lines suggests that in these cells, LKB1 is only acting via its main pathway and affects cell proliferation by controlling cell metabolism, yielding a rather minor effect. On the contrary, the effect in the OST cell line may be channelled through the TGF-β pathway as no differences were detected in the MTS assay. To confirm this, the effect of *LKB1* loss should be studied with a systematic approach and the downstream players in each cell line need to be investigated. These processes should be deciphered further but this is beyond the scope of this study.

Overall, we have confirmed the results found in the previous chapter in the context of normal bone, and LKB1 exerts an effect on cell proliferation in normal conditions and in starvation in OS cell lines.

8.7.2 Cooperation of LKB1 with another gene?

8.7.2.1 Discrepancies between cell lines point to the implication of another gene

It was observed that *LKB1* knock-in had different effects depending on the cell line used as a model. For cellular growth, as discussed in the previous paragraph, it may be accounted for by an involvement of either AMPK or TGF-β signaling pathways, although this needs to be confirmed. Hence the cell proliferation data imply that the role of *LKB1* is dependent on the genetic context in the cell lines. This could also explain why the effect of *LKB1* re-expression is more dramatic in some lines compared to others. Therefore, we hypothesise that it is probable that *LKB1* loss is cooperating with another genetic abnormality that enhances its effect on proliferation in the OSA and OST cell lines; this additional hit may not be present in SaOS2, where the effects observed on cell proliferation were minor and caused by a different mechanism.

Different responses were found for the functional effect of LKB1 re-expression in the SaOS2 and the OST or OSA cell lines for cell migration and cell survival. They were more dramatic than that observed for cell proliferation, with opposite results for cell migration and cell survival in non-adherent conditions in normal and low glucose conditions. This suggests further that the effect of *LKB1* depends on the genetic context and that another genetic abnormality may cooperate with *LKB1* loss.

8.7.2.2 Possible gene candidates cooperating with *LKB1*

As discussed in paragraph 8.7.1.2, cooperation between LKB1 and the TGF-β pathway may account for the differences observed on a proliferation level.

Additionally, LKB1 has been reported to promote apoptosis by several mechanisms, including by its action on p53 via its downstream kinases AMPK, SIK and NUAK, depending on the environmental stimuli (section 1.2.1.2). However, the action of LKB1 on the p53 pathway could also be via its interaction with p21 (Zeng et al. 2006). The status of AMPK, SIK, NUAK and p21 is unknown in the three OS cell lines used in this study. The cooperation of *LKB1* with a member of the p53 pathway may explain the differences observed in the anoikis experiment between the SaOS2 and OST cell line. p53 has been reported as mutated in SaOS2 but the OSA and OST cell

lines are wild type (Asada et al. 1999, Ottaviano et al. 2010), so these different genetic background may also account for the discrepancies obtained.

Furthermore, expression of LKB1 in OS cells has previously been reported to induce apoptosis via TRAIL (TNF-related apoptosis inducing ligand) due to its interaction with DAP3 (death associated protein 3)(Takeda et al. 2007). This could explain why LKB1 re-expression induces apoptosis in the SaOS2 cell line, whereas OST may have abnormalities preventing the activation of TRAIL-mediated apoptosis. However, this needs to be confirmed.

It is of note that SaOS2 has been reported as dependent on Src for anoikis, a mechanism which is involved in metastasis (Diaz-Montero et al. 2006). As cooperation between Src and LKB1 to promote metastasis has been shown in lung cancer (Carretero et al. 2010), such a mechanism could also be at play in the SaOS2 rather than in the OST cell line. The status of Src and its pathway is unknown in OSA and OST and its role needs to be confirmed in our model.

Concerning the role of *LKB1* in cell migration, it has been reported either to phosphorylate the MARK family - and by changes to cell polarity to alter migration (Figure 1.9) - or interact with PAK1 in cooperation with Src, as mentioned in the Introduction (Carretero et al. 2010, Deguchi et al. 2010). As reported above, Src has already been shown to play a role in anoikis in SaOS2, so this may also explain the discrepancies observed for the migration assays. To conclude, cooperation between LKB1 and Src that promotes cell migration and cell survival may be in place in some of the OS cell lines selected for the knock-in experiments.

The cooperation of *LKB1* with another gene, to enhance its effect, has been reported in other cancers (section 1.2.1.4): with Kras to promote metastasis in lung cancer (Carretero et al. 2010, Ji et al. 2007) and cell growth and survival in pancreatic cancer (Morton et al. 2010) and with β-catenin to promote cell growth in hepatocellular carcinoma. No activating mutations in Kras have been reported in OS, but drugs indirectly targeting its pathway have been reported to have an effect in OS. Indeed, Sorafenib decreased tumour growth, angiogenic activity and metastasis *in vitro* and *in vivo*, possibly by targeting Erk (Pignochino et al. 2009) and RANK-Fc was shown to limit cell migration and invasion also by targeting Erk *in vivo* (Akiyama et al. 2010). β-catenin has been reported as not activated in OS cases (Cai et al. 2010), although this is under debate as mentioned in the Introduction (section 1.1.5.3).

Hence, there are multiple candidates that may cooperate with LKB1 to account for the discrepancies observed. Further studies are needed to elucidate which of these are at play in the cell lines used and more generally in OS cases, but this is beyond the scope of this study.

8.7.3 Implications for new therapeutic strategies in Osteosarcoma

We have demonstrated in this chapter that LKB1 promotes cell proliferation in OS by knock-in *in vitro* in OS cell lines. This confirms that *LKB1* plays a role in OS pathogenesis. As it is a tumour suppressor gene, designing a therapy is more complex than for an oncogene and should restore the gene function. By deciphering which downstream effectors are involved, it may be possible to design activators of these kinases. For example, activators of AMPK have already been tested, such as metformin, used to treat diabetes and proposed for treatment of lung cancer (Memmott et al. 2010); it could prove useful to control cell proliferation in OS cases with reduced LKB1 expression. Similarly, the design of activators of SIK or MARK families could help to counter the functional effect of LKB1 loss on anoikis and cell migration. Further studies are warranted first on the LKB1 network to assess which kinase to target in OS.

The discrepancies observed between the cell lines indicates that *LKB1* may cooperate with another gene. Investigating this cooperation could help to design new treatments to target both LKB1 and its partner's pathways.

We have already demonstrated that LKB1 loss in OS cases correlated with mTOR activation, suggesting that cases with loss of this protein should be treated with drugs targeting mTOR. Moreover, Carretero et al have discovered that LKB1 and Src cooperate in lung cancer to promote cell proliferation and cell migration by a coordinated action on the mTOR and PAK1 pathways (Carretero et al. 2010). Src also possibly cooperates with LKB1 in OS, as suggested in paragraph 8.7.2.2. A drug targeting Src, dasatinib, is available but effects have only been seen *in vitro* in the SaOS2 and U2OS cell lines (Shor et al. 2007, Spreafico et al. 2008a), but not *in vivo* (Hingorani et al. 2009). This may be due to a compensation mechanism between the two partners. Therefore, the effect of dasatinib may be enhanced if the pathways downstream of LKB1 were simultaneously targeted. For example, the use of a combination of Rapamycin and dasatinib may prove more successful than each of them alone. Alternatively, activators of AMPK in combination with dasatinib should be efficient as cooperating agents.

Other candidate genes cooperating with LKB1 include Kras via the Erk pathway, TGF-β and its pathway or p21 via the p53 pathway. As for Src, deciphering the role of LKB1 within its network and its cooperation with this pathway may help to design new therapies in OS, or to enhance the effect of existing molecules such as Sorafenib or Rank-Fc, whose effect on OS via Erk has already been reported (section 8.7.2.2 and 1.1.6.1).

To conclude, a better understanding of LKB1 and its network in OS will help to design new therapeutic strategies that could, depending on the genetic context of the cells, help to control cell proliferation, cell migration and cell survival.

Chapter 9: CONCLUSION AND FUTURE WORK

9.1 Summary

The objective of the thesis was to assess the role of *LKB1* in OS pathogenesis, with the long term aim of identifying the factors driving this rare and aggressive disease.

As the mTOR pathway is the most widely reported downstream pathway constitutively activated by *LKB1* loss and as it provides potential therapeutic targets currently under review in OS, the first result chapter focused on its status in this sarcoma (Chapter 3). The pathway was found to be activated in a vast majority of OS cases, with 137 of 158 (87%) cases positive by IHC for p-mTOR, p-S6K or p-RPS6. This confirms the potential usefulness of mTOR pathway inhibitors such as Rapamycin and its analogs for the treatment of OS. Activation of the pathway was associated with disease progression. The TSC1/2 complex seemed to play a central role in the status of the pathway in OS. Activation of TSC2 by Akt was detected in 67% of the cases, and associated with disease progression and chemotherapy response, the latter result being confirmed by IHC in a larger cohort of cases. Nevertheless, p-TSC2 could not be used as a prognostic marker to predict chemotherapy response. TSC2 can integrate the signals from Akt of LKB1 to regulate mTOR activation and a subset of cases showed mTOR activation without TSC2 activation (32 of 120, 27%). TSC1 protein was not detected by IHC in a subset of cases (13 of 137, 9.5%), probably leading to constitutive activation of the pathway. Mutations of this gene have been reported by the Sanger Institute as part of the Cancer Genome Project. Constitutive activation of the pathway could also be accounted for by loss of PTEN in our cohort, as previously reported. Overall, the role of the TSC complex in the mTOR pathway activation pointed to a possible involvement of LKB1.

Therefore, the second result chapter focused on evaluating the expression of this tumour suppressor gene at the protein level in OS cases and cell lines (Chapter 4). It was indeed reduced in a subset of OS cases and cell lines, as confirmed by two different techniques, IHC and WB. Six of 15 (40%) OS cell lines showed low or no protein expression by WB, 23 of 26 (88%) OS cases by WB and 153 of 259 (59%) by IHC, and the association between the two methods was good. A strong association was found between loss of LKB1 protein expression and p-TSC2 detection, and loss of LKB1 was also strongly associated with mTOR pathway activation. This confirmed its

role in the constitutive activation of the pathway. Better understanding of this network in OS can help to improve the design of therapies targeting this pathway. LKB1 could not be used as a biomarker although its loss was more frequently observed in OS with a good response to chemotherapy. No other relationships were found with the clinical data.

The following result chapter (Chapter 5) aimed at confirming the pathogenic role of *LKB1* by assessing its status as a tumour suppressor or as a haploinsufficient gene in OS. Genetic abnormalities were investigated using a panel of techniques. It was concluded that LKB1 regulation was post-transcriptional in OS and that only a small number of cases exhibited haploinsufficient loss of LKB1, with 1 of 12 (8%) having confirmed LOH with copy number loss by qPCR. No differences were detected by FISH, showing that loss of the whole 19p13.3 locus was very rare, but microdeletions of *LKB1* at the SNP level was present in 5 of 12 (42%) cases. No additional hit was found; no mutations were detected and mRNA was present in all cases, excluding the occurrence of silencing by gene methylation.

To investigate the mechanism leading to the post-transcriptional regulation of *LKB1* in OS, as established in Chapter 5, two hypotheses were tested: a regulation via miRNA or via decreased protein stability. A direct regulation of LKB1 mRNA by miRNA was excluded by comparing the most deregulated miRNA in three cases expressing no LKB1 protein compared to four cases expressing it at high levels. However, miRNA affecting the LKB1 complex directly by targeting Stradα and MO25α or its stability by targeting SIRT1 and HSP90 were deregulated; this suggests that they could play a role in LKB1 protein expression in OS. This result was validated by data mining: the predicted targets of the identified miRNA were relevant to bone biology or to the LKB1 network. This validated the feasibility of using patient miRNA signatures to investigate the pathogenesis of OS. Concerning the second hypothesis studied in Chapter 6, SIRT1 has been shown to influence LKB1 stability by acetylation and its expression was studied at the mRNA level by qRT-PCR. No relationship was found between LKB1 and SIRT1 mRNA expression, but this cannot exclude its role in LKB1 post-transcriptional regulation. The exact mechanism of *LKB1* post-transcriptional regulation was not fully deciphered, although direct targeting by miRNA was excluded.

Consequently, the role of LKB1 as a driver of OS pathogenesis was confirmed by showing its functional role first in the context of normal bone (Chapter 7). The model used was a permanent knock-down of *LKB1* by shRNA in a human immortalised osteoblast cell line. No effects were

found on tumourigenecity by colony formation assay in soft agarose and on cell migration. However, a reduced *LKB1* expression in osteoblasts led to an increased proliferation in low glucose conditions by MTS assay. Knocked-down cells also tended to have a higher proportion of proliferating cells (i.e. in G2 and S phase) in all conditions by cell cycle profile. Small effects were also found in cell survival by Annexin V and PI staining, with a tendency for decreased cell death in adherent and non-adherent conditions.

The differences found in Chapter 7 were small, although in accordance with the results reported for *Lkb1* haploinsufficient loss *in vivo* in a mouse model. Therefore, another model was used as a validation in the last result chapter: the functional effect of *LKB1* knock-in was assessed in three OS cell lines. As in the knocked-down model, differences were recorded in all cell lines for cell proliferation, with a slower growth for *LKB1* knocked-in cells monitored by growth curve with the Incucyte. These results were validated by cell counting and BrdU assay for the OST cell line. The mechanism leading to cell proliferation appeared to differ in the SaOS2 and OST cell line, with differences noted by MTS assay for the first but not the latter. Discrepancies were also found between SaOS2 and the other cell lines for cell migration by scratch and transwell assays and for cell survival in non-adherent conditions by Annexin V and PI staining. This suggests that LKB1 loss may be cooperating with another genetic event in some cell lines.

To conclude, this work has demonstrated that LKB1 protein expression is lost in OS by a post-transcriptional mechanism, and this promotes OS pathogenesis mainly by inducing an increased cell proliferation, via the mTOR pathway and possibly other signalling networks.

9.2 Future work

9.2.1 New therapeutic strategies

The data presented in this thesis could lead to several new projects. The identification of the role of mTOR and LKB1 in OS pathogenesis can be implemented into the design of innovative treatments. The effort already initiated for the use of mTOR targeted therapy to treat OS needs to be pursued. Moreover, our work has shown that several combination therapies could enhance the effect of Rapamycin, which only induces cell cycle arrest but not apoptosis *in vitro* and *in vivo* as presented in the Introduction (section 1.2.3.2). Targeting simultaneously mTOR with Rapamycin and its analogs and Akt, TKR with dedicated inhibitors or ezrin and Erk with Sorafenib could give better results; this needs to be tested in clinical trials. As loss of *LKB1* can constitutively activate mTOR, the treatment of patients with this abnormality with both Rapamycin and AMPK activators like metformin can also be considered. Similarly, dasatinib, which targets Src, has been reported to have limited effects *in vivo* (section 1.1.6.1); it could be enhanced in cases lacking LKB1 protein expression by re-establishing its function using Rapamycin or metformin. Pre-clinical studies and clinical trials are also warranted to validate this therapeutic strategy.

The study of the mTOR pathway also suggested that PR and GR to chemotherapy may have different mechanisms of activation of the mTOR pathway, caused more frequently by over-activation of Akt in PR and loss of LKB1 in GR. This suggests that different therapeutic design should be considered in these two groups, and this has to be studied further.

9.2.2 Post-transcriptional regulation of LKB1

We have shown that LKB1 protein expression is unlikely to be regulated by direct binding of miRNA to its mRNA. The role of miRNA predicted to target the members of the LKB1 complex, Stradα and MO25α, needs to be confirmed on a larger cohort. Additionally, the targets were predicted bioinformatically and need to be confirmed by reporter assay.

Preliminary results have not shown any relationship at between *SIRT1* and *LKB1* expression at the mRNA level, but this does not exclude a role of this gene in *LKB1* post-transcriptional regulation. To confirm this is mediated by a decreased protein stability, LKB1 protein expression

should be monitored in cell lines treated with proteosome inhibitors. SIRT1 protein expression should also be assessed in OS cases and cell lines, and LKB1 expression in the presence of the SIRT1 inhibitor sirtinol or of siRNA against SIRT1 mRNA needs to be evaluated. Finally, determining the acetylation status of LKB1 in these models would help to uncover the role of SIRT1.

9.2.3 LKB1 and its network

The functional analysis of *LKB1* knock-down in osteoblasts and knock-in in OS cell lines has demonstrated its role in cell proliferation, but has also raised new questions. The effects noted were rather small and discrepancies were noted between the different cell lines. This suggests that *LKB1* may be cooperating with another gene and its function needs to be understood within its network. LKB1 can de-regulate the TGF-β pathway, as demonstrated for the formation of polyps in the gut (Katajisto et al. 2008), and it would be interesting to investigate further the role of this pathway in connection of LKB1 in OS. LKB1 can also interact with several downstream kinases and understanding the effect of its loss at a molecular rather than only at a functional level would help to design new therapies. This potential partner and the downstream targets of *LKB1* could be identified by gene expression microarray or protein arrays. It would be interesting to understand the role of *LKB1* loss not only within osteoblasts, but also with regards to the cross-talk between osteoblasts and osteoclasts, as promising therapeutic strategies targeting osteoclasts are currently under development in OS.

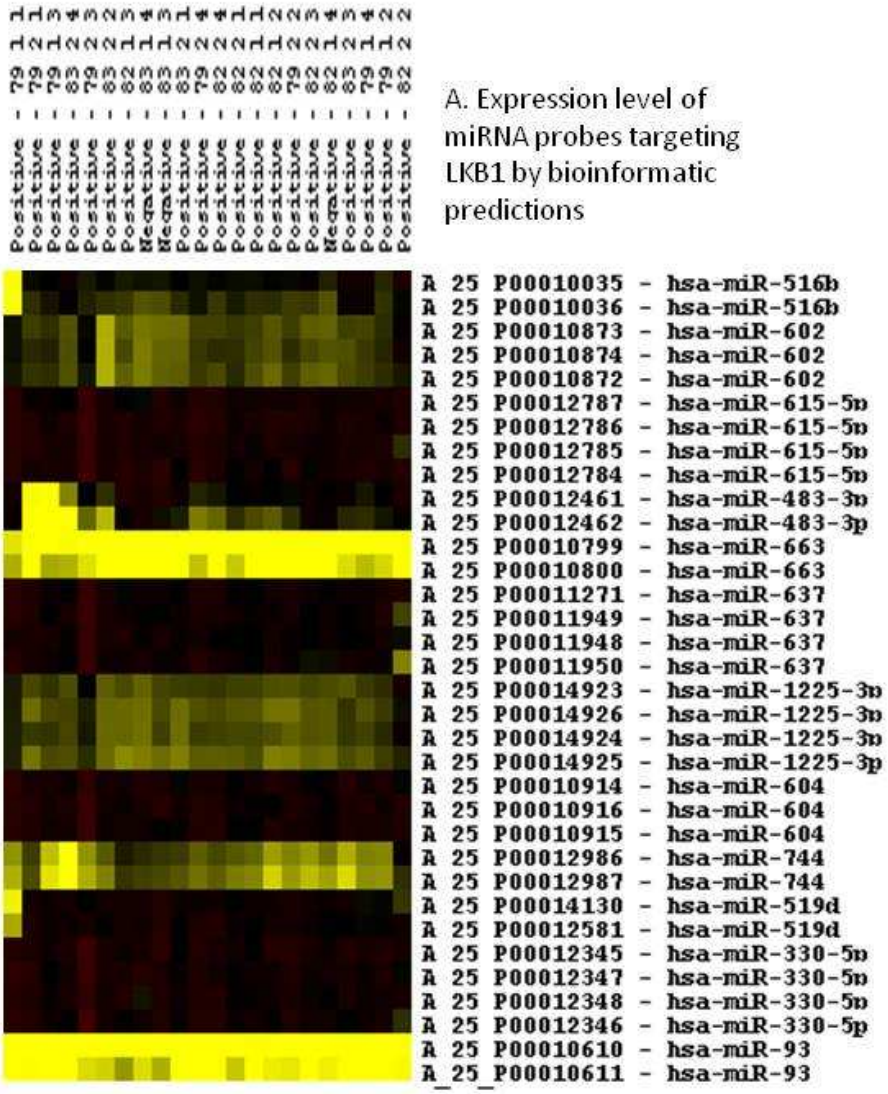
Chapter 10: SUPPLEMENTARY DATA

Case ID	Response to chemo therapy	Patient in EURAMOS	Patient age	Patient sex	Subtype	Tumour site	LKB1 status
79_1.1	Excellent	Yes	17	F	Osteoblastic	Distal femur	
79_1.2	Excellent	Yes	23	M	Osteoblastic	Proximal tibia	
79_1.3	Excellent	Yes	10	F	Telangiectatic	Distal femur	
79_1.4	Excellent	Yes	16	F	Osteoblastic	Distal femur	+
79_2.1	Very poor	Yes	11	M	Mixed (including chondroblastic areas)	Femur	
79_2.2	Very poor	No	16	M	Periosteal, with mixed osteoblastic and chondroblastic areas	Distal femur	
79_2.3	Very poor	Yes	13	M	Mixed (fibroblastic and osteoblastic)	Distal femur	
79_2.4	Very poor	No	56	F	Mixed (osteoblastic and telangiectatic)	Distal tibia	
82_1.1	Good	Yes	12	F	Osteoblastic	Distal femur	
82_1.2	Very poor	No	16	M	Osteoblastic	Vertebra T12	
82_1.3	Good	No	10	M	Osteoblastic	Distal femur	
82_1.4	Excellent	Yes	15	NA	Osteoblastic	Distal femur	–
82_2.1	Poor	No	13	M	Osteoblastic or osteoblastoma-like	Proximal tibia	+
82_2.2	Good	Yes	8	F	Mixed (osteoblastic and chondroblastic)	Distal femur	+
82_2.3	Poor	Yes	15	F	Mixed (osteoblastic and chondroblastic)	Distal femur	
82_2.4	Good	Yes	15	F	Mixed (osteoblastic and chondroblastic)	Proximal tibia	+
83_1.1	Poor	No	14	M	Osteoblastic	Distal femur	
83_1.2	Very poor	No	19	F	Parosteal	Distal femur	
83_1.3	Excellent	No	12	F	Fibroblastic	Distal femur	–
83_1.4	Poor	No	15	F	Conventional	Femur	–
83_2.1	Good	No	11	F	Chondroblastic	Distal femur	
83_2.2	Poor	Yes	16	M	Chondroblastic	Proximal femur	
83_2.3	Excellent	No	14	M	Osteoclast rich	Proximal tibia	
83_2.4	Good	Yes	14	F	Mixed (osteoblastic and telangiectatic)	Distal radius	

Table 10.1: Clinical characteristics of the 24 cases used in the miRNA microarray.
The last column indicates the result of the IHC for the seven cases with high (+, n=4) or no (–. n=3) LKB1 protein expression and used to identified miRNA associated to LKB1 regulation.

miRNA ID	Prediction with PITA	Prediction with Targetscan
hsa-miR-93	-0.25	-0.28
hsa-miR-330-5p	-0.38	-0.25
hsa-miR-483-3p	-7.59	-0.16
hsa-miR-516b	-9.38	-0.16
hsa-miR-519d	-1.00	-0.28
hsa-miR-602	-8.77	-0.04
hsa-miR-604	-1.28	-0.12
hsa-miR-615-5p	-8.58	-0.26
hsa-miR-637	-4.60	-0.02
hsa-miR-663	-5.74	-0.46
hsa-miR-744	-1.27	-0.14
hsa-miR-1225-3p	-3.46	-0.02
hsa-miR-1268	-7.63	-0.06
hsa-miR-1299	-11.93	-0.14

Table 10.2: Bioinformatic predictions of miRNA targeting LKB1 with PITA and Targetscan.



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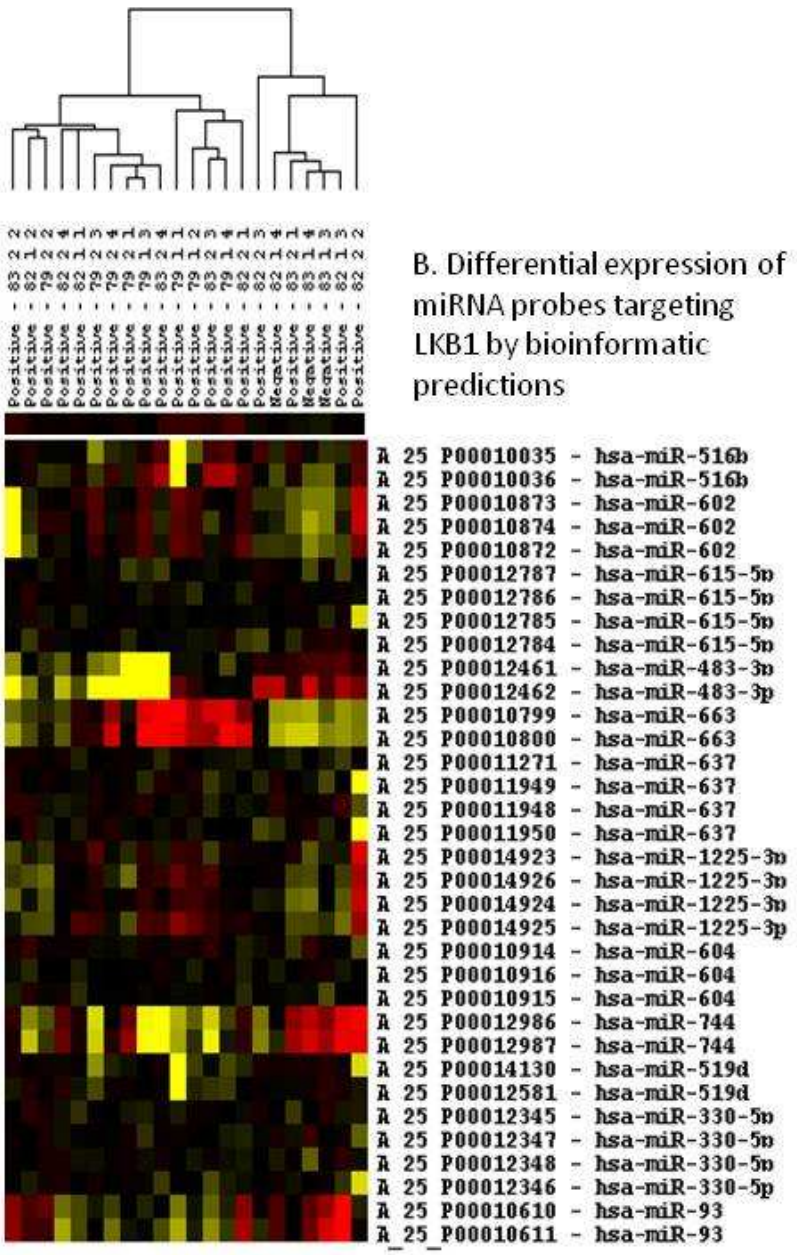


Figure 10.1: Heatmaps showing the overall level of expression (A.) and the differential expression (B.) of miRNA probes predicted to target LKB1 in 22 OS cases.
Yellow indicates an upregulation compared to the median, red a downregulation and black no differences. Each line represents a miRNA probe and each column one OS case. The result for LKB1 protein expression by IHC is indicated as Positive or Negative for each case, next to the case ID.

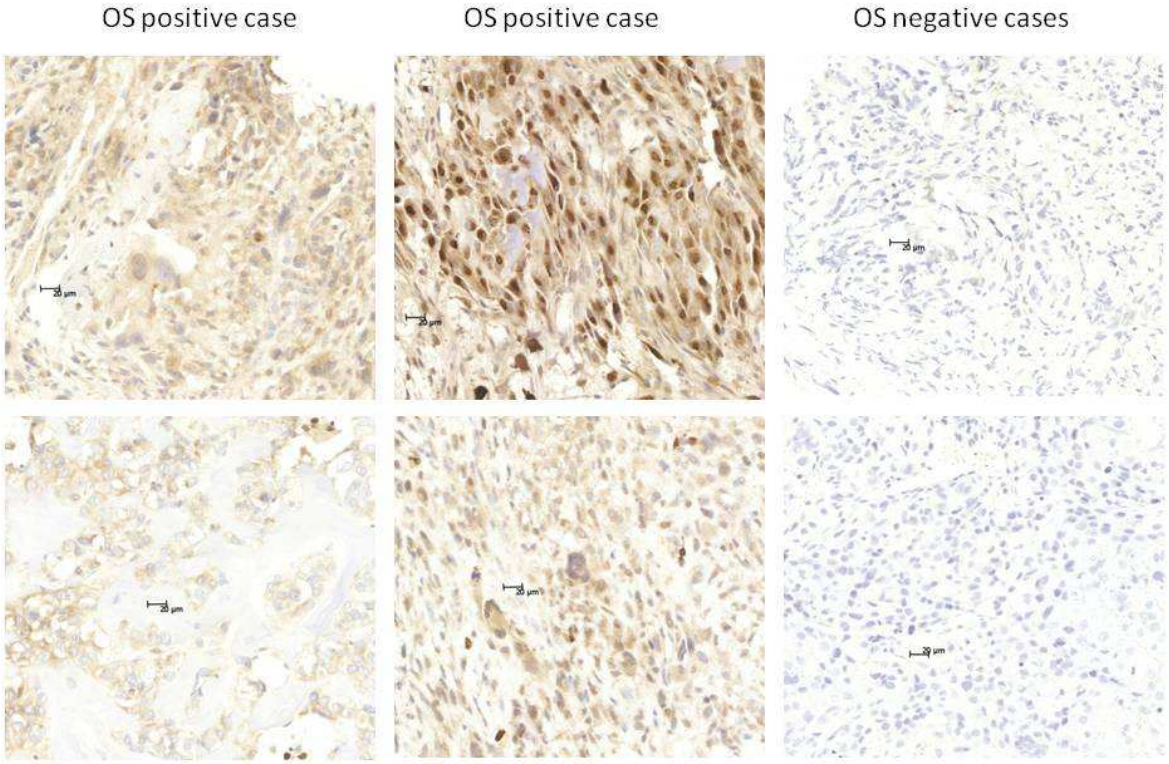
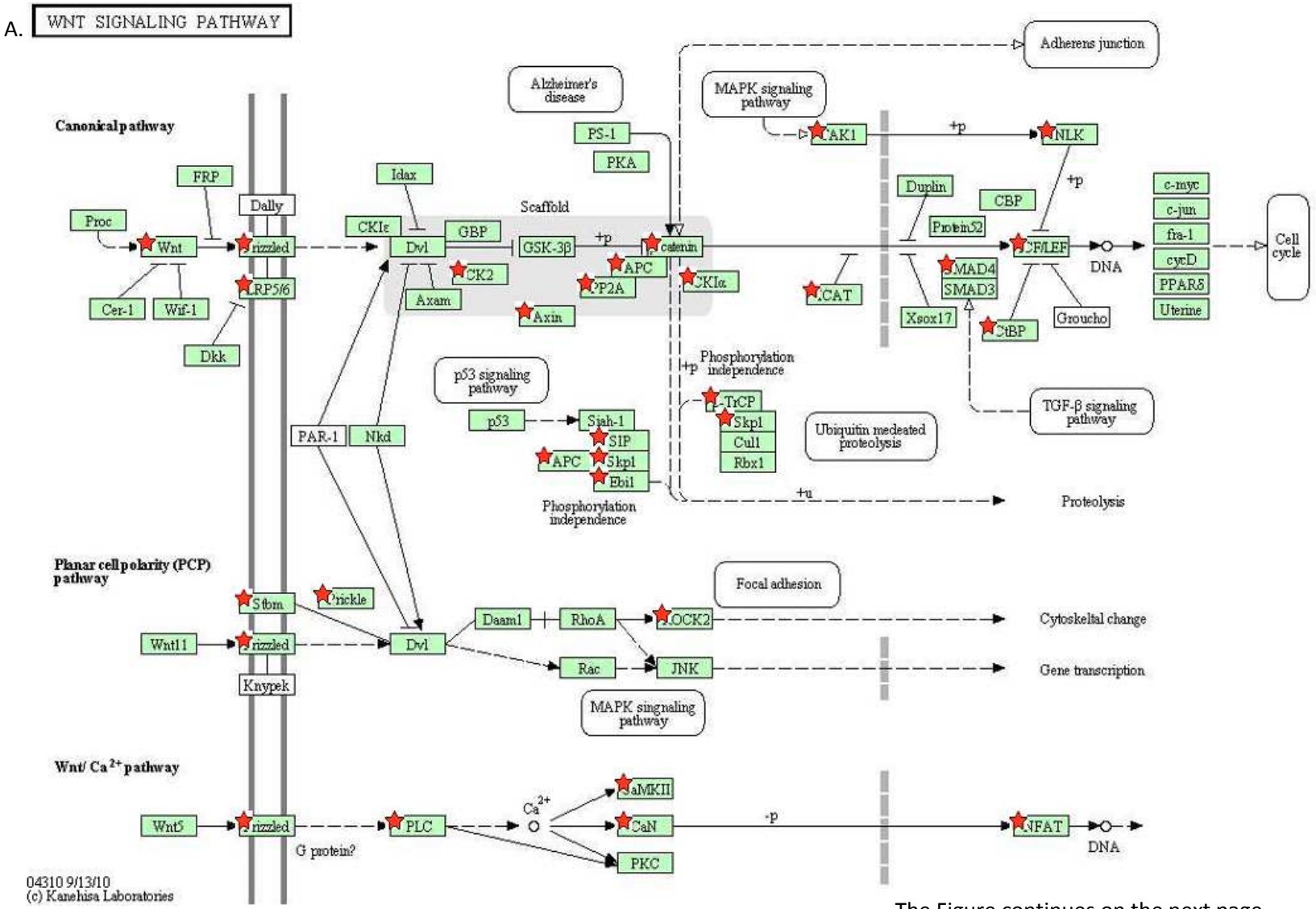


Figure 10.2: LKB1 immunoreactivity of the human OS biopsy cases selected for in the miRNA microarray. Magnification 20x. The bar represents 20µm.

A.



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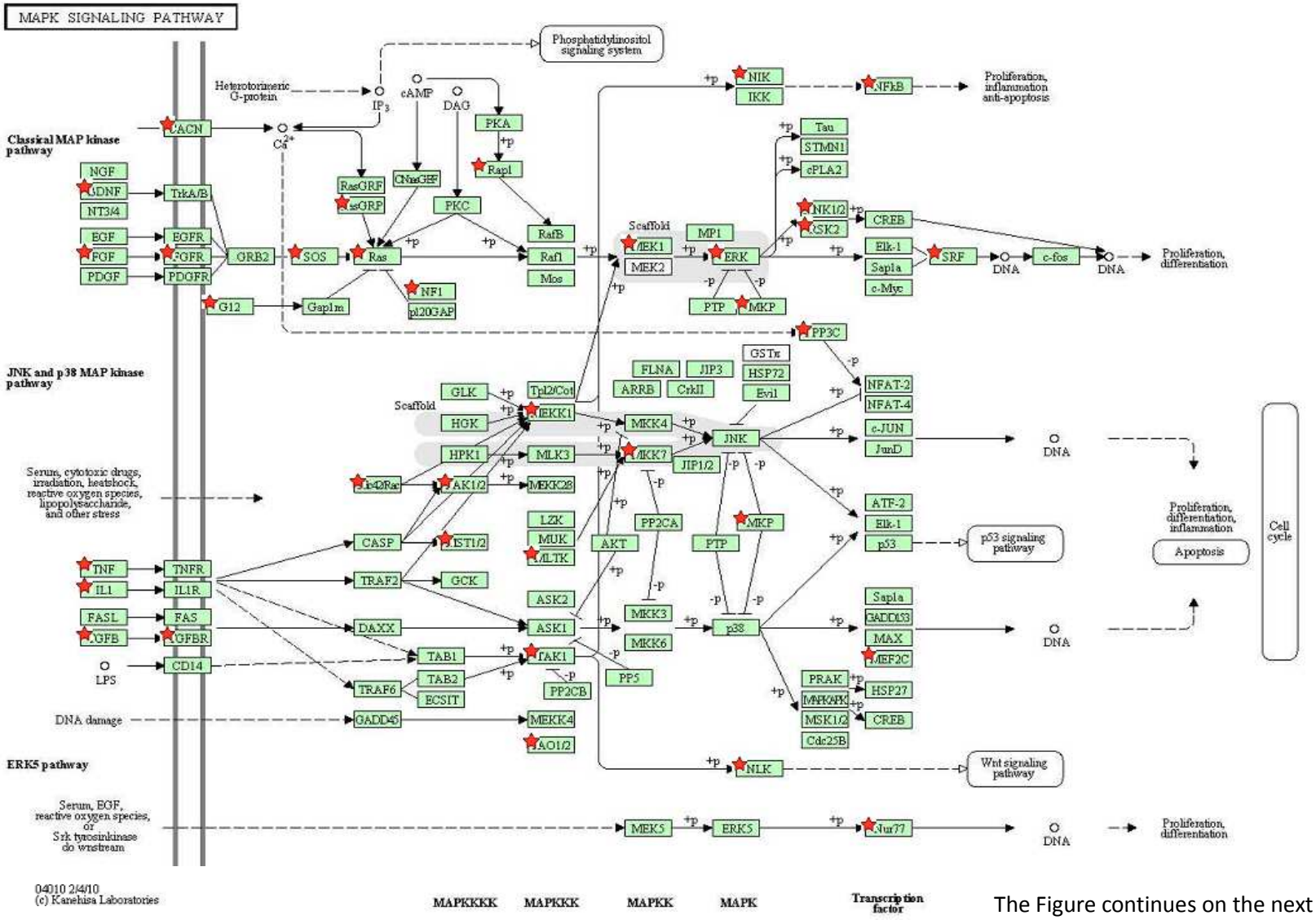
B.



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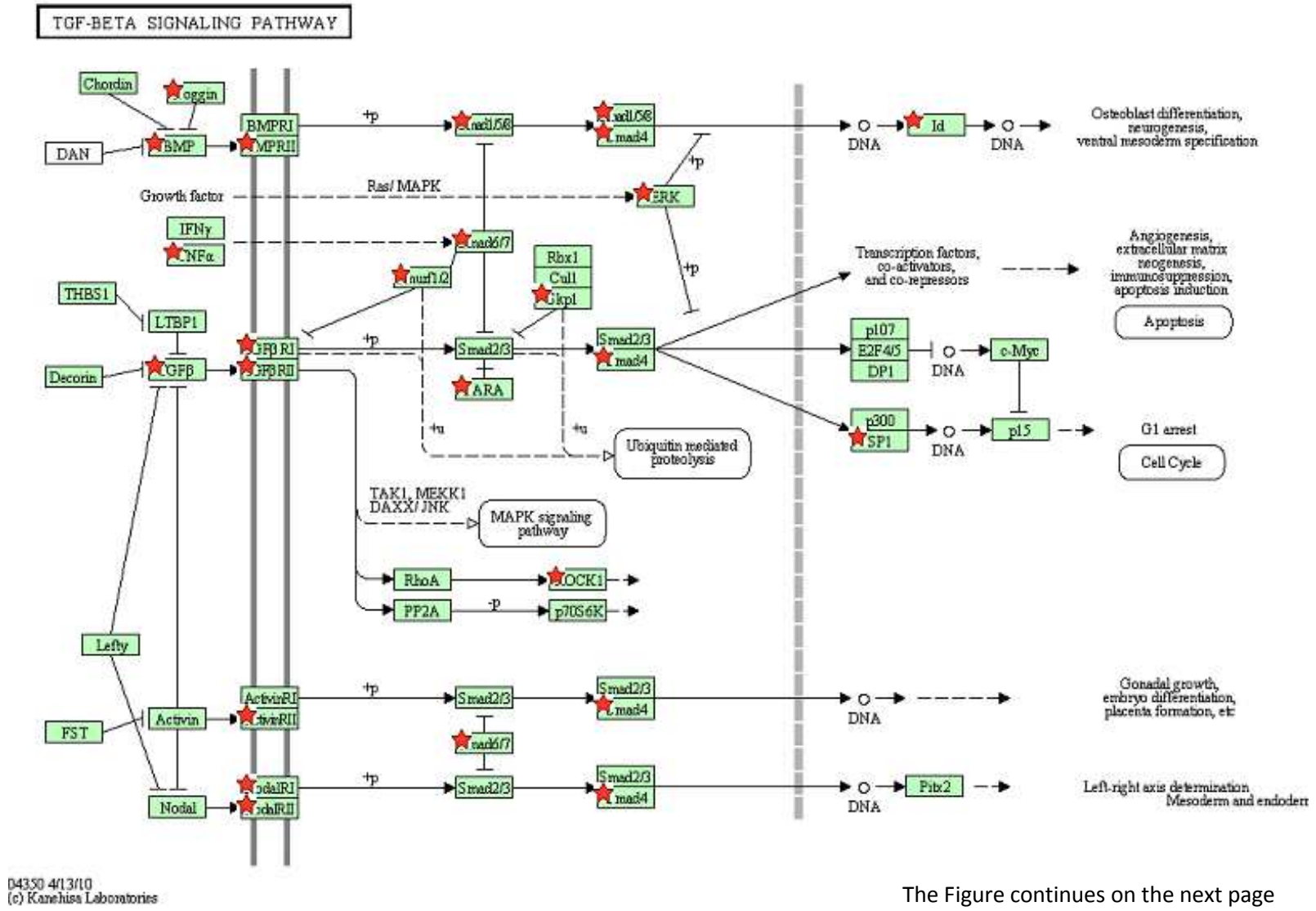
C.



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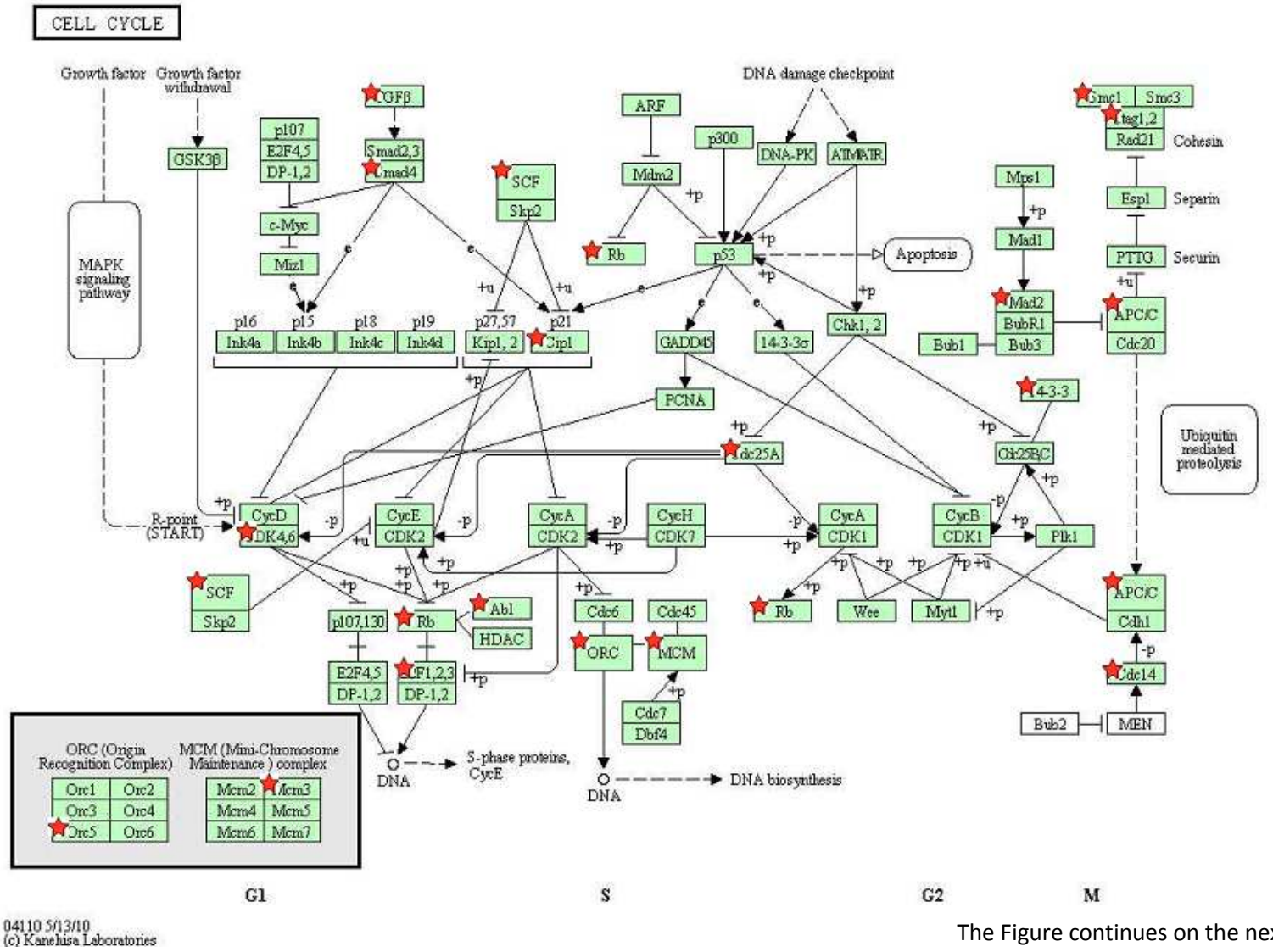

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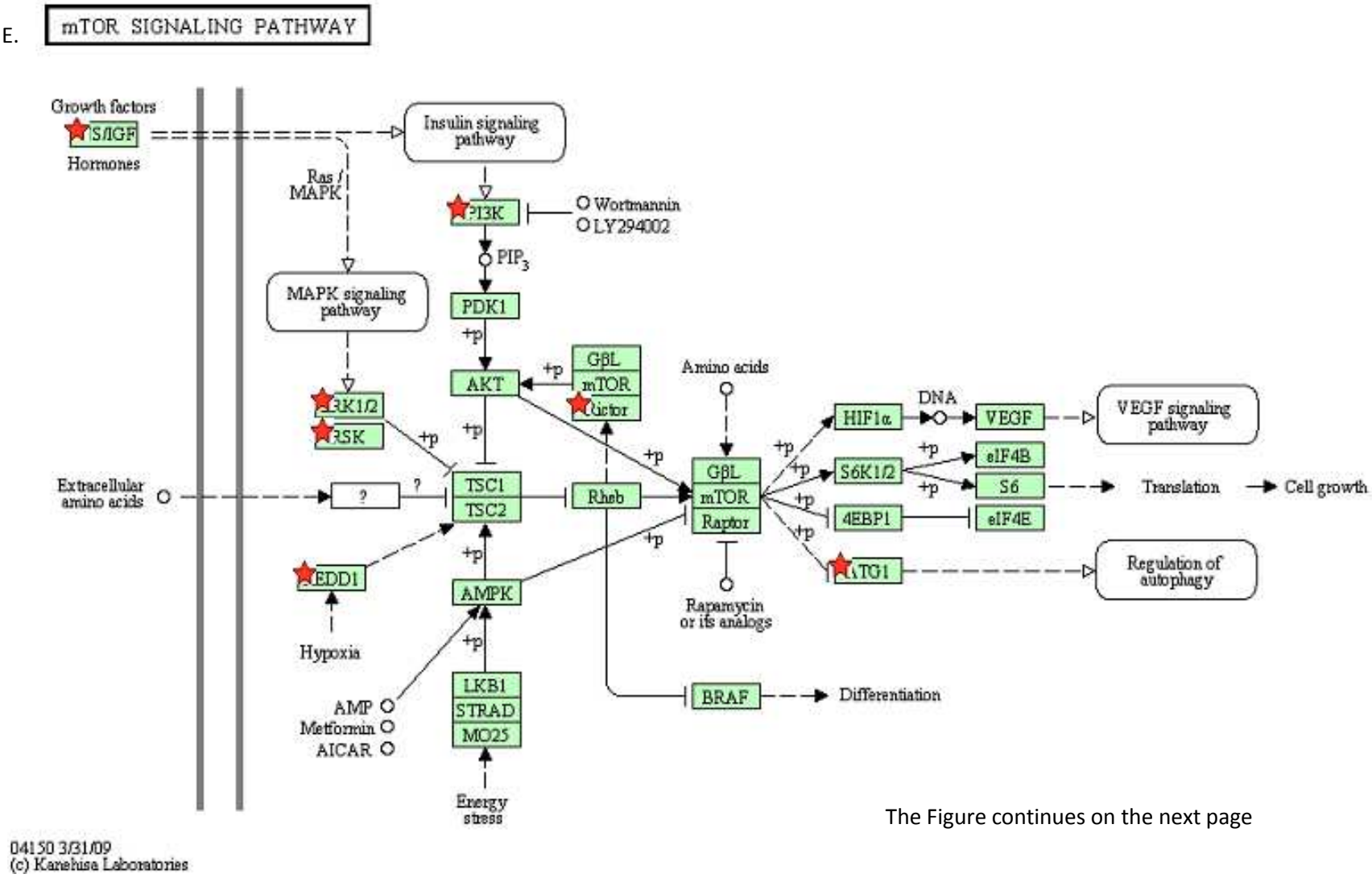
E.



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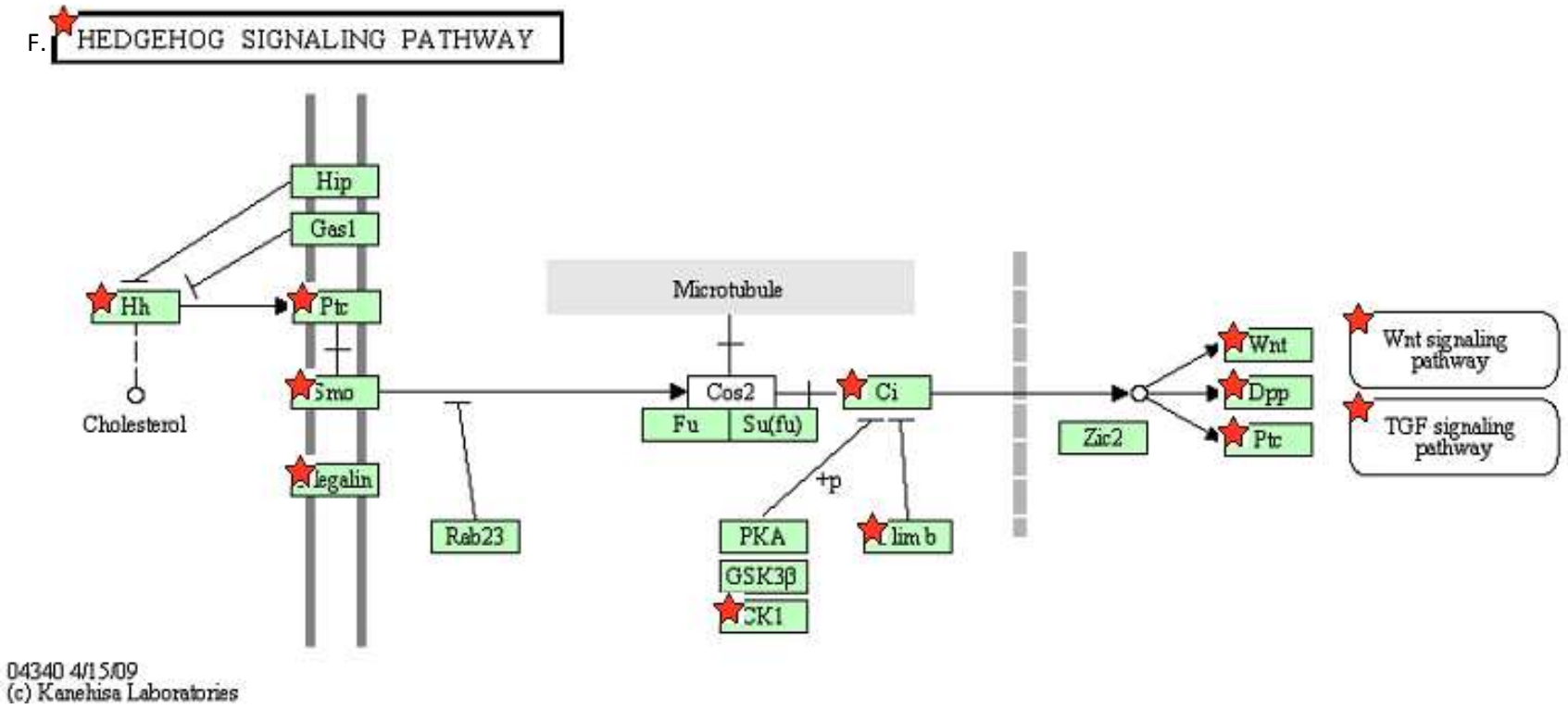

Figure 10.3: Scheme of the Wnt (A.), MAPK (B.), TGF-β (C.), cell cycle (D.), mTOR (E.) and Hedgehog (F.) signaling pathways, highlighting the genes predicted to be targeted by the miRNA signature deregulated between OS cases expressing LKB1 or not.

The scheme was obtained from KEGG pathways. Targeted genes are represented with a red star. Only the highly conserved targets predicted by Targetscan were used for this study.

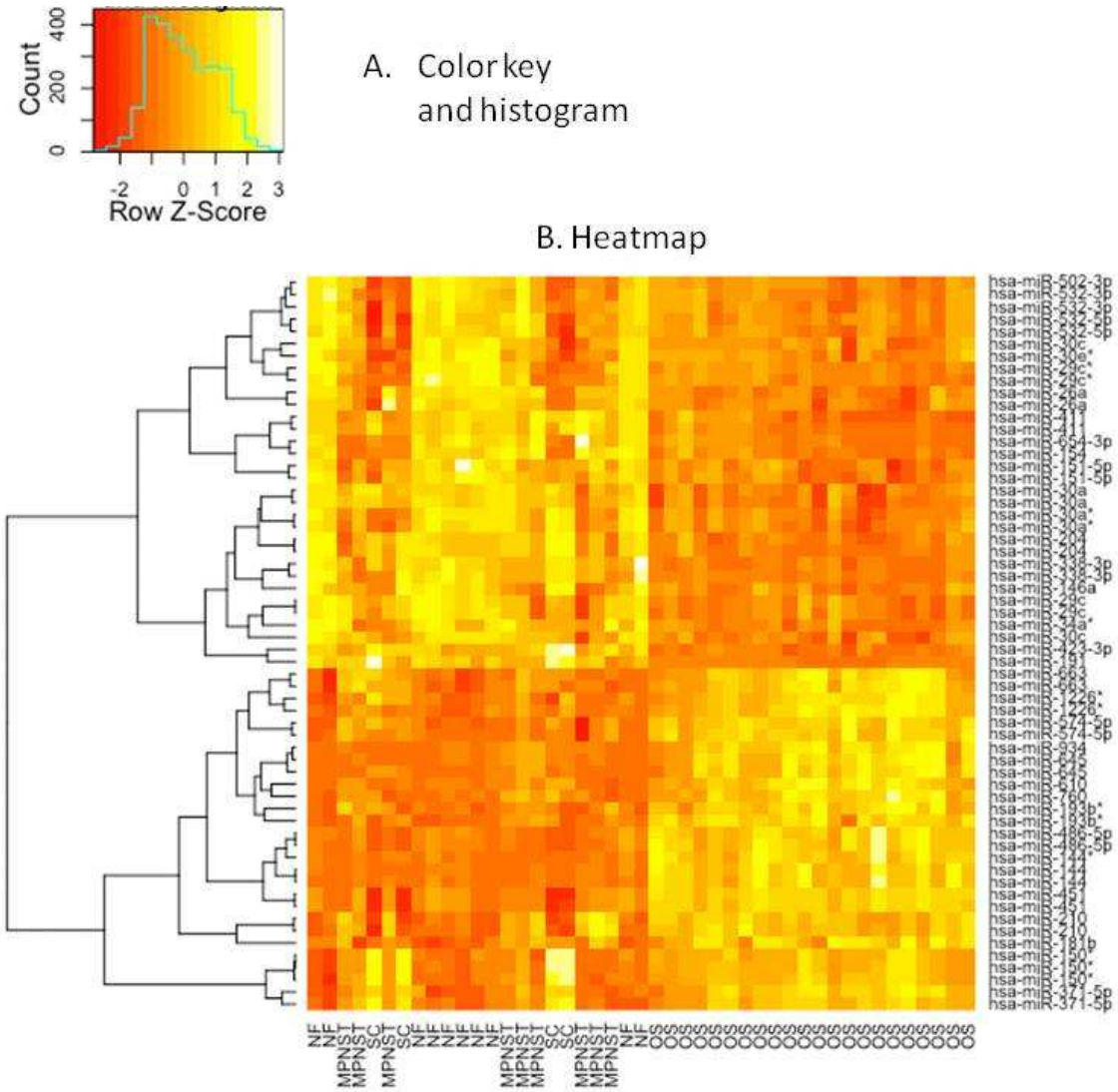

Figure 10.4: Heatmap of the most differentially expressed miRNA in OS compared to other cell types.
The color key and histogram of the Z-score of all cases is presented in A. Yellow indicates an upregulation and red a downregulation. The heatmap of the 60 most differentially regulated miRNAs between OS and other cell types, including Schwann cells (SC), neurofibroma (NF) and malignant nerve sheath tumours (MPSNT) is in B. Each column corresponds to one case.

miRNA ID	p-value	Fold change
hsa-miR-9	3.30E-09	0.166508
hsa-miR-30a	3.56E-11	0.28864
hsa-miR-125b	1.60E-09	0.278629
hsa-miR-126	1.17E-20	6.20034
hsa-miR-144	4.79E-14	9.62298
hsa-miR-144*	2.64E-13	7.24893
hsa-miR-181a	3.92E-09	3.457053
hsa-miR-181a*	2.51E-09	3.135951
hsa-miR-196b	1.45E-08	3.405563
hsa-miR-199a-5p	7.15E-11	7.05517
hsa-miR-199b-3p	1.42E-08	5.38909
hsa-miR-204	4.80E-11	0.083874
hsa-miR-210	2.24E-10	3.345699
hsa-miR-214	1.26E-10	6.69369
hsa-miR-338-3p	1.26E-12	0.108618
hsa-miR-378	4.19E-10	3.118598
hsa-miR-411	1.18E-09	0.327722
hsa-miR-451	1.15E-20	99.4036
hsa-miR-486-5p	5.40E-14	16.1354
hsa-miR-601	3.55E-10	3.511062
hsa-miR-645	1.11E-10	3.730048
hsa-miR-765	1.48E-08	3.708748
hsa-miR-934	1.46E-11	6.25161
hsa-miR-1226*	6.37E-10	3.238614

Table 10.3: List of the 24 most differentially regulated miRNA in OS compared to MPNST.

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