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Activation of Human Endogenous Retrovirus K and cellular modifications in human melanoma cell lines: gene expression analysis

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Grazie….!

Dedication

I would like to dedicate this work to my loving parents Ali and Haya, my dear husband Waleed and to my little angel Lyan.

ABSTRACT

HERV-K has been linked extensively with human melanoma supported by the oncogenic ability of other human and animal retroviruses and by the detection of HERV-K particles and transcripts in human melanoma. In an attempt to approach better understanding of human melanoma pathogenesis, a cellular system has been developed at the University of Rome, Tor Vergata, that consist of original adherent melanoma cell line, named TVM-A12, and two non-adherent cell line obtained either by cloning, named $Clone_{sp}$, or by stressful cultural condition i.e. 1% FBS instead of 10%, named TVM-$A12_{sp}$. It was observed that transition of melanoma cell line TVM-A12 from adherent to non adherent phenotype under stressful cultural condition was accompanied by decrease expression of melanocyte differentiating antigen (Melan A/MART-1) and HLA class I and higher colony forming ability in soft agar assay. Furthermore, this transition was associated with increased expression of HERV-K env mRNA and protein and could be inhibited by down regulation of HERV-K expression using RNA interference. Therefore, the purpose of this study was to provide further characterization for better understanding of the genetic bases of increased malignant behaviour and HERV-K activation. Methods used include microarray technology followed by real-time PCR confirmation. Results of microarray test showed modulation in gene expression that favours increased malignant potential in non-adherent cells compared to parental cells. In addition it showed very similar gene expression profile in $Clone_{sp}$ and TVM-$A12_{sp}$. Real-time PCR confirmation showed transient up regulation of transcription factor BHLHB2 and MYC prior to detachment. TVM-A12 grown in stressful cultural conditions started to show up regulation of PTEN, VEFGA, CSK, PITCH1, FOXG1A, and TP53 which continue to rise in non adherent cells and in $Clone_{sp}$. Once detached, they showed up regulation of WNT3, MYCN, MYCL1, BTK, CCND2, WNT2, TIMP3, IRF3, GTF2I, CTNNB1, E2F1, ARHGAP5, ARHGEF5, GPR39, and ITGB4. On the other hand, adherent TVM-A12 1%, TVM-$A12_{sp}$ and $Clone_{sp}$ showed down regulation of ANXA7, CTNNA1, NME1, RRM1, CDKN1A, XRCC6, HDAC, TRAM1, CD59 and TOB1 in comparison to parental TVM-A12 cells. This study describes, for the first time, a unique cellular system that demonstrate the association between peak rise in HERV-K env expression and acquisition of a more malignant phenotype thus providing a useful tools for better understanding of human melanoma pathogenesis and for studying the effect of pharmacological agents and genetic modulators on HERV-K expression and melanoma progression.

CHAPTER 1. INTRODUCTION

1.1 RETROVIRUSES

1.1.1. General properties of Retroviruses

Retroviridae is a big and rapidly growing virus family, characterized by two main features: the ability to convert genetic information from genomic viral RNA into DNA and to integrate into the host genome the DNA copy of viral RNA. In fact, genomic RNA is converted into a complementary copy (cDNA) in a process called reverse transcription, using the enzyme reverse transcriptase (RT), and the DNA copy of viral RNA is integrated in the host genome, using the enzyme integrase.

The ability of the virus to be integrated within the host genome and to replicate as a cellular gene, using cellular machinery, is a key factor for its persistence and evasion from the immune mechanisms.

1.1.2. Structure of retroviruses

Retroviruses are composed of an outer envelope made of lipid bilayer, derived from the membrane of infected cells, in which virally encoded glycoproteins were embedded. The number of these glycoproteins varies in different retroviruses and among different isolates of the same virus but the significance of this variation is not clearly understood. Viral glycoproteins play a significant role in the initial stage of infection through cellular receptor recognition and therefore in the determination of host range and of target cells and tissues within infected host. Matrix proteins separate viral envelope from viral capsid. Capsid is made of proteins and it encloses viral RNA in associated with nucleoproteins. Viral capsid also contains the viral enzymes RT, protease and integrase (Wagner *et al.* 2004*)* (Figure1.1).

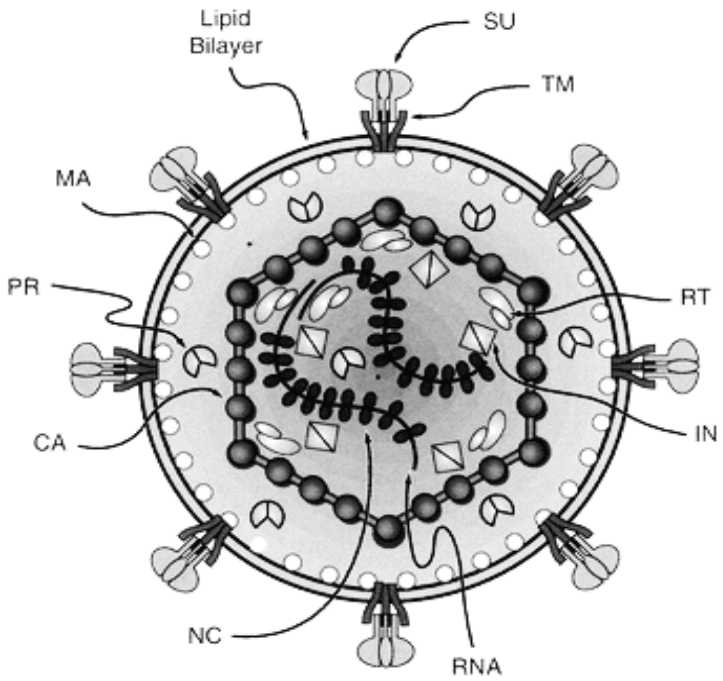

Figure 1.1. **Diagrammatic representation of retrovirus structure.**

CA=capsid, NC=nucleocapsid, IN=integrase, RT=reverse transcriptase, TM= transmembrane, SU= surface component of envelope protein, MA= matrix protein, and PR= protease.

The morphology of the viral core is an important feature used in classifying retroviruses in to four main morphological forms: type A, B, C, and D particles (Goff 2001) (Figure 1.2).

Type A particles represent the immature form of the virus, which is mainly seen in mutant viruses with defective proteolysis of viral protein precursors and in infectious viruses, early in infection, prior to budding and maturation. They have doughnut like appearance due to the electron lucent center, surrounded by one or two concentric rings. Mature viruses on the other hand displays one of three morphological forms: type B particle has an eccentrically placed rounded core, type C particle has a centrally located rounded or slightly angular core and type D particle has a characteristic cylindrical or bar shaped core. Some retroviruses display morphology not belonging to any of these morphological forms like HIV-1 (Human Immunodeficiency Virus type one), which has a cone shaped core.

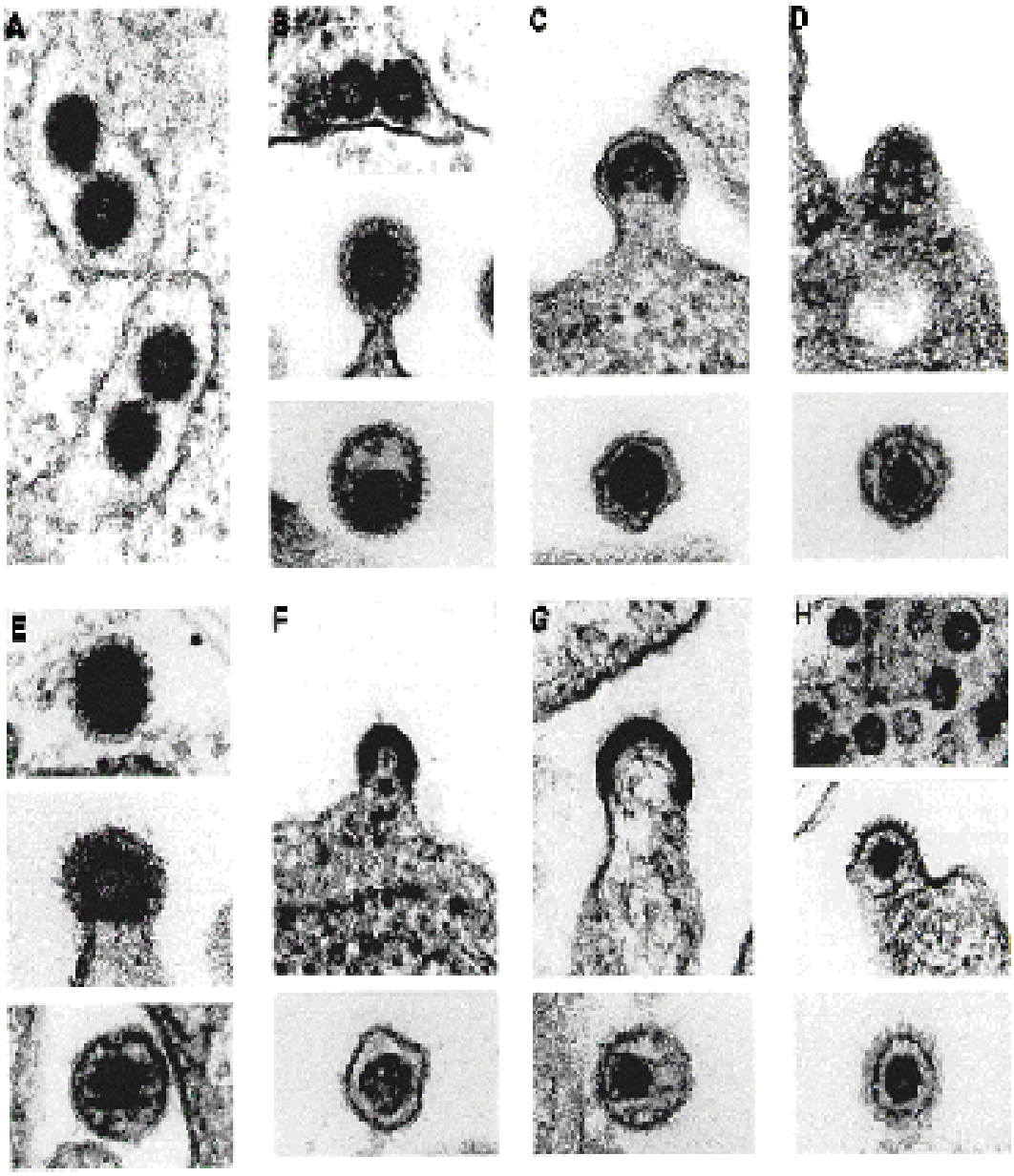

Figure1.2. Morphological forms of retroviruses.

(A) immature intracisternal type A particle, (B) type B particle with eccentric core seen in mouse mammary tumor virus, (C) and (D) type C particle with central core seen in murine leukemia virus and avian leucosis virus, (E) type D particle of mason Pfizer monkey virus, (F) type C particles seen in bovine leukemia virus, (G) rod or cone shaped core in bovine immunodeficiency virus, (H) spumavirus (Goff 2001).

1.1.3. Genomic composition of retroviruses

Retroviruses are RNA viruses composed of diploid genome of two copies of positive sense non-segmented single stranded RNA, carries 7-13 kilo bases and joined at 5` end by a self complementary region called dimmer linkage structure. The significance of having two copies is not clearly understood but it is thought to act as a biological buffer that compensate for the high rate of error of reverse transcriptase (Wagner *et al*. 2004). Retrovirus genome is composed of the following genes and functional elements:

R - U5 - PBS - *gag* - *pol* - *env* – PPT - U3 - R - A_n

Description of each gene and gene segment is shown in Table 1.1.

Table 1.1. Genome composition of retroviruses (Wagner *et al*. 2004).

Genes and functional elements	***Description***
R	20-250 bases sequence repeated at both ends
U5	75-200 bases unique sequence at the 5` end with transcription signal and is involved in viral integration
PBS	Primer binding site, which is the site for binding of cellular tRNA for reverse transcription
Gag	Encodes for capsid, matrix and nucleoprotein
Pol	Encode the enzymes reverse transcriptase and integrase
Env	Encodes the surface and transmembrane component of envelope protein
PPT	Polypurine tract which is a short stretch of A and G nucleotides that is resistant to degradation and is used as primer during cDNA synthesis
U3	Unique sequence at the 3` end involved in gene expression
A_n	Poly A region

1.1.4. Replication of retroviruses

Replication cycle of retroviruses start with receptor recognition on target cell followed by receptor mediated membrane fusion. Within the cytoplasm, partial uncoating of the virus occurs and reverse transcription of the viral genomic RNA start within the partially uncoated virus. Replication of the virus genome involves a step of reverse transcription to synthesize negative sense complementary DNA, followed by the synthesis of positive sense complementary DNA and the formation of long terminal repeat (LTR) made of U3, R, and U5 at both ends as shown in Figure 1.3. Following that, cDNA migrates into the nucleus by different mechanisms depending on retrovirus type: oncornaviruses require nuclear membrane breakdown, which occurs during cell division, while lentiviruses enter even in a non-dividing cell. Within the nucleus, cDNA integrate into the cellular genome, using the enzyme integrase, forming a provirus. Full length viral RNA can be produced from the provirus, by cellular machinery. Newly produced viral RNA migrates to the cytoplasm, where it can be either spliced to generate mRNA encoding various virus proteins or encapsidated to generate a new virus particle. Viral envelope proteins are incorporated in the cell membrane and immature viral particle buds through the plasma membrane taking an outer envelope of cellular lipid bilayer, in which virally encoded proteins are incorporated. After budding, maturation of the virion occurs by cleavage of precursor proteins (Wagner *et al*. 2004; Goff 2001).

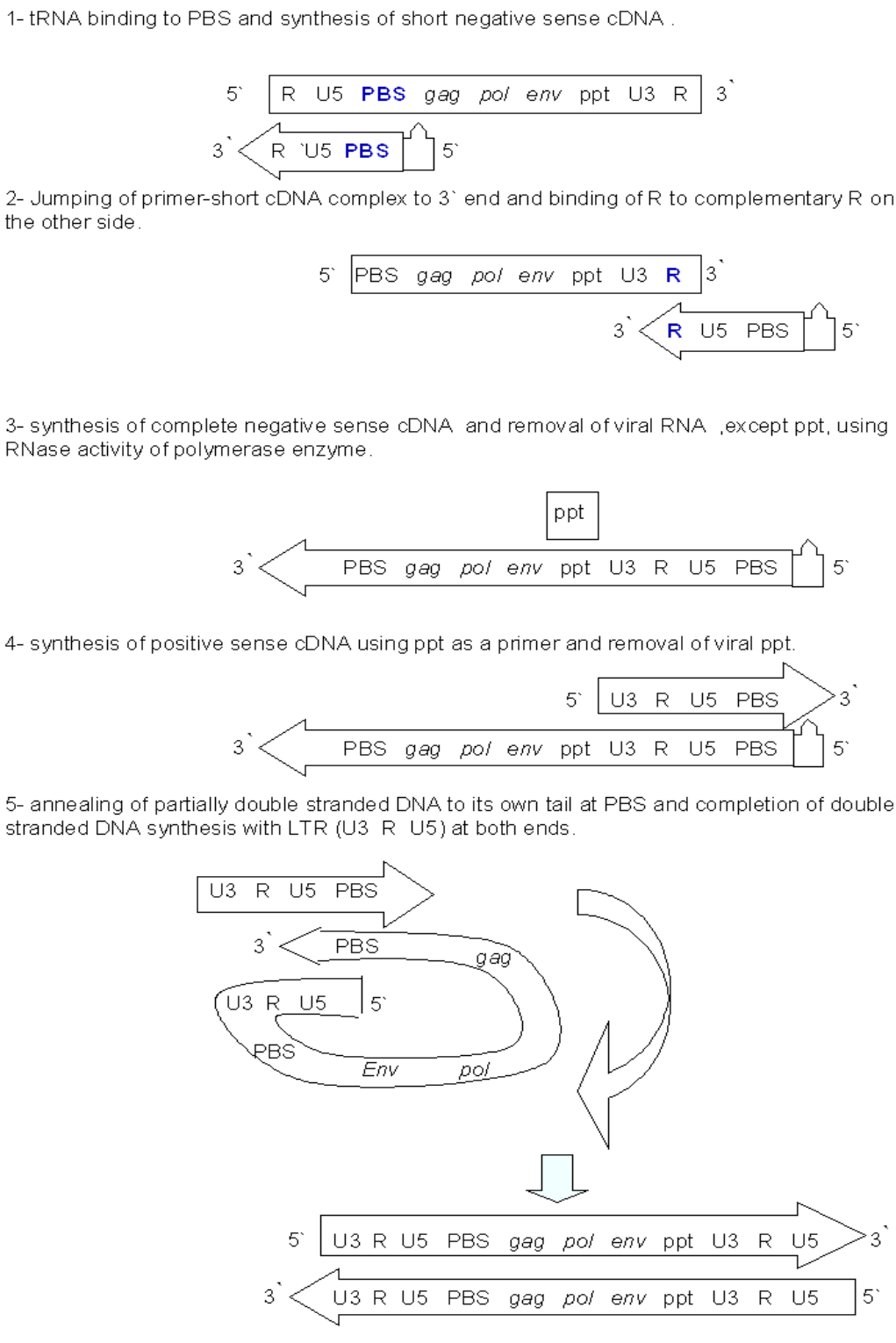

Figure 1.3. Mechanism of cDNA synthesis and formation of LTR (long terminal repeat) PBS= primer binding site, R= repeat sequence, PPT= Polypurine tract (Wagner *et al*. 2004).

1.1.5. Classification of Retroviruses

Different classification systems have been used to describe retroviruses. The simplest one classifies retroviruses according to genome composition: simple retroviruses have genome encoding only the proteins essential for replication (*gag, pol* and *env* gene products) while complex retroviruses encode also an array of regulatory proteins.

Other classification system is based on the pathogenesis, distributing retroviruses in to three categories: *Oncornaviruses*, *Lentiviruses* and *Spumaviruses*. *Oncornaviruses*, as the name implies, have oncogenic potential but some members cause only benign diseases. *Lentiviruses* cause infections with characteristic long incubation periods and potentially fatal diseases while *Spumaviruses* have been shown to cause benign infections (Goff 2001).

The most recent classification system, described by the international committee on taxonomy of viruses (ICTV), classifies retrovirus in to two subfamilies, *Orthoretrovirinae* and *Spumaretrovirinae*. *Orthoretrovirinae* includes six genera: *Alpharetroviruses*, *Betaretroviruses*, and *Gammaretroviruses*, considered as simple retroviruses, and *Deltaretrovirus*, *Epsilonretrovirus* and *Lentivirus*, described as complex retrovirus. Subfamily *Spumaretrovirinae* contains only the genus *Spumavirus*, which includes complex retroviruses. All genera display C type morphology except three genera: *Betaretrovirus* which has B or D type morphology, *Lentivirus* which has cylindrical or conical core, and *Spumavirus* which displays immature morphological form, with characteristic central uncondensed core.

It must be stressed that classification of *Retroviridae* is still incomplete with many retroviruses being unclassified including human endogenous retroviruses (Table 1.2)

Table 1.2. Classification of *Retroviridae* according to ICTV*.

Subfamily	*Genus*	Host	Example
Orthoretrovirinae	*Alpharetrovirus*	Avian	**Avian leukemia virus**
	Betaretrovirus	Mice, primates and sheep	**Mouse mammary tumor virus**
	Gammaretrovirus	Mammals, reptiles and birds	**Murine leukemia virus**
	Deltaretrovirus	Mammals	**Human T lymphotropic virus**
	Epsilonretrovirus	Fish and reptiles	**Walleye dermal sarcoma virus**
	Lentivirus	Mammals	**Human immunodeficiency virus type 1 and 2**
Spumaretrovirinae	*Spumavirus*	Mammals	**Human foamy virus**
Unclassified retroviruses			**Human endogenous retrovirus** **Murine endogenous retrovirus** **Avian endogenous retrovirus**

* This table is not meant to be comprehensive of all retroviruses. A complete comprehensive listing of retroviruses is available on The International Committee on Taxonomy of Viruses website.

1.2. HUMAN ENDOGENOUS RETROVIRUS (HERV)

1.2.1 General properties of HERVs

Retroviruses usually infect somatic cells. When the cell is proliferating, the insertion is present at the same chromosomal location in all progeny cells and vanishes with the last cell of the clone, which might not occur until the host dies. Occasionally however, a retrovirus can infect a cell belonging to the germ line. Any offspring, which develop from an infected germ-line cell, carry the provirus in every single nucleated cell of the body (at the same chromosomal position) and this proviral element is then passed to the descendants according to Mendelian laws. Retroviruses that became part of the host genome in this way are referred to as endogenous retroviruses (ERVs), and the transmission mode, from one host generation to the next, has been termed "vertical" to distinguish it from the "horizontal" spread of exogenous retroviruses. Endogenous retroviruses closely resemble infectious retroviruses integrated into the genome of our ancestors at least 25 million years ago and are detected in all vertebrates studied to date **(**Bannert and Kurth 2006).

The obligatory integration step of the retroviral replication cycle allows the incorporation of the viral genome (provirus) into the host genome and retro transposition or reinfection of the germline can generate further insertions augmenting the number of a particular lineage in the genome (Gifford and Tristem 2003).

A complete ERV "provirus" (i.e., the retroviral genome integrated into the host cell genome) shares the same genomic structure of an exogenous retrovirus: four viral genes (*gag, pro, pol* and *env)* flanked by two long terminal repeats (LTRs). The *gag* gene encodes for the major viral structural protein, while *pro* and *pol* encode for the viral enzymatic machinery, necessary for the viral replication cycle. The *env* gene encodes for the envelope glycoprotein that is inserted in the lipid bilayer of the cell membrane to form the viral envelope and mediates entry of the virus into susceptible cells. The LTRs contain enhancer elements that direct expression of the viral genes. ERVs are destined to extinction if their expression brings deleterious consequences for the host. Thus, their

persistence in the host genome is the result of a fine balance reached throughout evolution which usually renders them replication defective due to the accumulation of mutations, deletions, rearrangements and methylation (Boeke and Stoye 1997).

The recent sequencing of the human genome has revealed that over 40% of human DNA sequences belong to the category of repeated or retrotransposable elements including endogenous retroviruses (Weiss 2006). Retroviral sequences (HERVs) comprise up to 8% of the host genome (International Human Genome Sequencing Consortium 2001), most of which are defective, due to mutations, deletions or termination signals within coding sequences (Lower 1999) and only very few have been shown to express functional proteins (Bannert and Kurt 2004).

It is unclear whether they were retained because they performed or perform a useful biological function and thus persisted during evolution. Even if the exact function and the importance of HERV in human genome is not very well understood, it is thought to play an important role in genome modeling and plasticity through gene transposition and cellular protection from other retrovirus infection, by receptor interference. In addition, HERV-W envelop gene on chromosome 7 encode the protein syncytin, which is thought to play a key role in placenta formation (Ruprechta *et al.* 2008).

HERV sequences have the potential to be integrated in any location of the genome and consequently may alter the structure and function of other genes. Therefore, they can obviously be involved in genetic disorders. Mainly, they can disrupt genes at their integration site and generate truncated proteins or other isoforms by alternative splicing, or they may even regulate in an abnormal way the expression of particular transcripts by means of the new inserted LTRs, which may harbour promoter sequences.

Epigenetic control is notably involved in silencing most of these genetic elements but certain environmental factors such as viruses are known to dysregulate their expression in susceptible cells. More particularly, embryonal cells with limited gene methylation are most susceptible to uncontrolled activation of these mobile genetic elements by, e.g., viral infections (Perron and Lang 2009).

In contrast to humans, several animals contain ERVs highly related to exogenous retroviruses (Boeke and Stoye 1997; Arnaud *et al.* 2008) including mouse mammary

tumour virus (MMTV), feline leukaemia virus (FeLV), and avian leukaemia virus (ALV), which are currently active and infect mice, cats and chickens, respectively. The most striking example is represented by the koala, in which a retrovirus is currently undergoing the process of endogenisation since it still displays many of the features of an exogenous virus such as the ability to produce infectious viral particles and variability in proviral copy number and sequence (Tarlinton *et al*. 2006).

1.2.2. Classification of HERVs

Approximately 30 HERV families have been identified in the human genome (Katzourakis and Tristem 2005) all composed of *gag*, *pro*, *pol* and *env* genes, flanked by two long terminal repeat (LTR) regions (Boeke and Stoye 1997). They are present in a variable copy number and are classified according to the single letter amino acid code for the tRNA specificity of the primer binding site used to initiate reverse transcription (Bannert and Kurth 2004). Up to date, HERV have not been included in the retrovirus taxonomy since most sequences are fragmented or incomplete**.**

Classification of endogenous retroviruses is still troublesome and currently HERV are classifies according to similarity to exogenous retroviruses in to three classes (Gifford and Tristem 2003) (Table 1.3). **Class I** shows great homology to *Gammaretroviruses* and *Epsilonretrovirus* and contains 18 HERV families, with large copy number in the human genome, almost all, are defective. **Class II** (also called HERV-K superfamily): shows homology to *Betaretroviruses*, includes 4 HERV families and is present in a copy number less than the other two classes. **Class III**: shows homology to *Spumaretroviruses* and include 4 HERV families with large copy number.

Table 1.3. HERV families (Gifford and Tristem 2003)

HERV family	Primer	Copy number
Class I		
HERV Z69907	**ND***	**30**
HERV-ADP	**tRNA ^{Thr}**	**60**
HERV-E	**tRNA ^{Glu}**	**85**
HERV-F	**tRNA ^{Phe}**	**15**
HERV-F type b	**tRNA ^{Phe}**	**30**
HERV-FRD	**tRNA ^{His}**	**15**
HERV-H	**tRNA ^{His}**	**660**
HERV-H49C23	**No LTRs**	**70**
HERV-I	**tRNA ^{Ile}**	**85**
RRHERV-I	**tRNA ^{Ile}**	**15**
ERV-9	**tRNA ^{Arg}**	**70**
HERV-F type c	**tRNA ^{Phe}**	**ND**
HERV-P	**tRNA ^{Pro}**	**70**
HERV-R	**tRNA ^{Arg}**	**15**
HERV-R type b	**tRNA ^{Arg}**	**15**
HERV-T	**tRNA ^{Thr}**	**15**
HERV-W	**tRNA ^{Trp}**	**115**
HERV-XA	**tRNA ^{Phe}**	**15**
Class II		
HERV-K.HML1-4	**tRNA ^{Lys}**	**170**
HERV-K.HML5	**tRNA ^{Ile}**	**45**
HERV-K.HML6	**tRNA ^{Lys}**	**70**
HERV-K.HML9	**ND***	**ND**
Class III		
HERV-L	**tRNA ^{Leu}**	**575**
HERV-S	**tRNA ^{Ser}**	**70**
HERV-U2	**ND***	**ND**
HERV-U3	**ND***	**ND**

*ND= not determined

The endogenous retrovirus HERV-K is the most recent (Barbulescu *et al*. 1999; Turner *et al.* 2001) and transcriptionally active endogenous retroviral family (Ruda *et al.* 2004; Seifarth *et al.* 1998; Wang-Johanning *et al.* 2001; Yi *et al*. 2001) and the members of HML-2 subgroup are human specific (Turner *et al*. 2001).

Genome-wide screening has revealed high levels of insertional polymorphism in the HERV-K (HML-2) family (Belshaw *et al.* 2005) demonstrated by the presence of insertionally polymorphic HERVs proviruses only in a proportion of the human population.

Only members of the HERV-K (HML-2) provirus family have open reading frames (ORFs) for all viral genes, and have therefore received attention in relation to diseases (Tonjes *et al*. 1996; Mayer *et al.* 1999; Bannert and Kurth 2004). Some of them have retained the capacity to form retroviral-like particles, as demonstrated in human teratocarcinoma, germ-cell tumours, melanoma, breast cancer and in megakaryocytes from patients with essential thrombocythemia (Boller *et al*. 2008; Buscher *et al*. 2005; Lower *et al*. 1993; Bieda *et al.* 2001; Morgan and Brodsky 2004; Muster *et al*. 2003; Seifarth *et al*. 1998), in the plasma of lymphoma patients (Contreras-Galindo *et al.* 2008) and possibly in human placenta (Kalter *et al*. 1973; Dirksen and Levy 1977, Wilkinson *et al.* 1994). However, up to date, the infectivity of these retroviral-like particles are not yet been demonstrated.

The HERV-K proviruses exist in two forms: type I and type II (Lower *et al*. 1993; Lower *et al.* 1995). Type I proviruses have a 292-bp deletion at the boundary of the *pol* and *env* genes and the viruses encode the Np9 protein (Armbruester *et al.* 2002) whereas type 2 proviruses are complete and the viruses produce Rec (an HIV-1 Rev protein equivalent) and Env proteins, which both have been implicated in tumorogenesis (Denne *et al.* 2007).

1.3. HERV AND DISEASES

1.3.1. Pathological role of HERVs

In humans, exogenous retroviruses are known to cause a number of diseases, such as immunodeficiency, neurological disease and cancer.
While endogenous retroviruses are firmly established pathogens in other species, the human endogenous retroviruses may well be considered as emerging pathogens.

The vast majority of the HERVs sequences are defective, although mRNAs from several families are differentially but ubiquitously expressed in a number of cell types and tissues (Forsman *et al.* 2005; Seifarth *et al.* 2005; Yi *et al*. 2006). HERV expression levels vary even between individuals (Andersson *et al.* 1996) and they may cause disease in some individuals and not in others due to the existence of HERV polymorphisms (Moyes *et al.* 2007). Some HERV mRNAs and a few HERV-encoded proteins are expressed in placental or embryonic tissues and may have essential functions here (Muir *et al*. 2004).

HERVs family have been also implicated in the pathogenesis of human diseases including type 1 diabetes (Marguerat *et al.* 2004), autoimmune diseases such as rheumatoid arthritis, (Herrmann *et al.* 1998; Reynier *et al.* 2009), psoriasis (Molès *et al.* 2005) and systemic lupus erythematosis (Adelman and Marchalonis 2002; Pullmann *et al.* 2008). Recently, an increasing number of reports suggested the association of the HERVs with neuropathological conditions such as multiple sclerosis (MS) and schizophrenia. In particular HERV-H/F (Christensen *et al*. 1998; Christensen *et al.* 2000) and HERV-W (Perron *et al.* 1997; Garson *et al.* 1998) virions have been detected in plasma, sera and cerebrospinal fluid (CSF) of MS patients and increased levels of HERV-H, HERV-K and HERV-W RNA in MS brain (Johnston *et al*. 2001). HERV-W env and gag proteins have also been found in brain tissue from MS patients and HERV antibodies reactive against specific HERV epitope have also been detected in sera and CSF samples of MS patients and cell-mediated immune responses have been reported

(Christensen *et al*. 2005). Furthermore, HERV-W mRNA has been detected in CSF and post-mortem samples obtained from schizophrenic patients (Karlsson *et al*. 2001).
HIV-1 infection has been also associated with increased HERV-K expression. HERV-K RNA has been detected in the plasma of HIV-1 infected individuals but not in hepatitis C virus (HCV) infected patients or in seronegative controls (Contreras-Galindo *et al.* 2006). The level of HERV-K titre was found to correlate significantly with that of HIV-1, and was found to be high in HIV-1 patients with non-suppressive HAART therapy and undetectable or low in HIV-1 patients with effective suppressive HAART therapy (Contreras-Galindo *et al.* 2006).). Moreover, a longitudinal study following HERV-K titer in HIV-1 infected individuals has shown a correlation between HERV-K expression level and treatment failure and viral rebound. More specifically, the increase in HERV-K expression was detected before the onset of treatment failure and viral rebound indicating that HERV-K expression might be a useful predictor of HIV-1 treatment failure (Contreras-Galindo *et al.* 2007). The increase in HERV-K expression in HIV-1 patients might have a role in the disease pathogenesis and might also be related to HIV-associated cancer (Contreras-Galindo *et al.* 2007)

The HERV-K is the most recent (Barbulescu *et al*. 1999) and transcriptionally active endogenous retroviral family (Wang-Johanning *et al.* 2001; Tönjes *et al*. 1996) and therefore received particular attention in relation to diseases (Bannert and Kurth 2004).

Different HERV families have been found to be expressed in various human pathologies (Stauffer *et al*. 2004) (Table1.4.). HERV-E mRNA was detected by reverse transcription PCR in 38.8% of prostatic cancer but not in normal specimens (Wang-Johanning *et al.* 2003). HERV-W mRNA encoding *env* gene product syncytin 1 was found to be over expressed in endometrial cancer specimens and is thought to play an important role in cell-cell fusion in endometrial carcinoma (Strick *et al*. 2007) and in cell-endothelial fusion in breast cancer (Bjerregaard *et al*. 2006). In addition, HERV-K, HERV-E, and ERV-3 *env* mRNAs were detected in ovarian cancer and not in control and anti HERV antibodies were also detected in a high percentage of patients sera (55%, 40%, and 30% respectively) (Wang-Johanning *et al*. 2006). HERV-K transcripts were detected at high levels in the plasma of patients with lymphoma and breast carcinoma, which decreased with treatment and HERV-K like particles were also

observed using electron microscopy (Contreras-Galindo *et al.* 2008). Furthermore, HERV-K *rec*, which originate from *env* transcript, were detected in germ cell tumors and it was found that the induction of *rec* expression in transgenic mice induced developmental disturbances and carcinoma in situ (Galli *et al.* 2005) giving the first evidence that the induction of HERV-K *rec* expression might have an essential role in tumorogenesis in transgenic mice.

The mere expression of retroviral transcripts in these pathologies does not implicate a causal association and their exact roles in these processes need to be further evaluated.

Table 1.4. HERV transcript detected in various human tissues.

Tissue type	HERV protein/mRNA	Technique used	References
Ovarian cancer cell line	HERV-K, HERV-E and ERV-3 *env* protein	Immunohisto-chemistry	Wang-Johanning *et al.* 2006
Seminoma	HERV-K *gag* protein	Immunohisto-chemistry	Sauter *et al.* 1995
Teratocarcinoma cell line (Tera1,2102EP,GH,NCCIT)	HERV-K *gag* protein	Immunoblotting	Bieda *et al.* 2001
Endometrial carcinoma	HERV-W env product syncytin-1 mRNA	RT-PCR	Strick *et al.* 2007
Melanoma (primary, metastatic, and cell line)	HERV-env K, protein gag	Immunohisto-chemistry	Buscher *et al.* 2005
Breast cancer cell line and specimens	HERV-K *env* mRNA HERV-W *env* protein	RT-PCR Immunoblotting	Wang-Johanning *et al.* 2001 Bjerregaard *et al.* 2006
Prostatic cancer	HERV-E *env* mRNA	RT-PCR	Wang-Johanning *et al.* 2003
Lymphoma	HERV-K mRNA	RT-PCR	Contreras-Galindo *et al.* 2008
Placenta (BeWo choriocarcinoma cell line model of human placenta trophoblast)	ERV-3 *env* mRNA	RT-PCR	Lin *et al.* 1999
CSF and post mortem brain samples obtained from schizophrenic patient	HERV-W mRNA	RT-PCR	Karlsson *et al.* 2001
Sera and CSF of multiple sclerosis patients	HERV-H/F, W env mRNA	RT-PCR	Christensen *et al.* 2005

1.3.2. Virological etiology of cancer

The etiology of cancer can be summarized in three words: radiation, chemical agents and viruses.

Approximately 20% of human cancers have been attributed to viral infection, and other cancers may also have a viral component (Weiss 2001).

A number of human viruses have been implemented in the causation of a number of human cancers including hepatitis B and C virus as a causative agent of hepatocellular carcinoma (Koike 2007), EBV (Epstein Barr Virus) as a causative agent of Burkitt's lymphoma, nasopharyngeal carcinoma and some of the lymphomas that complicate AIDS (Young and Murray 2003) and HPV (Human Papilloma Virus) as a cause of cervical cancer (Schiffman *et al*. 2007). Furthermore, retroviruses have been found to be causative agents in a number of animal as well as human cancers.

Exogenous retroviruses are carcinogenic throughout the animal kingdom, including marine invertebrates, birds, marsupials and a wide variety of placental mammals (Romalde *et al* 2007; Martin *et al* 1997).

These retroviruses are usually oncogenic through common indirect pathways, such as integration of the virus adjacent to a cellular oncogene, or incorporation of a host oncogene within the retroviral genome, or through more complex interactions involving viral LTRs and host regulatory pathways, such as tumour suppression genes (Lower *et al*. 1999; Hayward *et al.* 1981). In human HTLV-1 (human T-cell lymphoma/leukemia-1) virus is the only exogenous virus which has been proven with no doubt to be involved in the causation of human T-cell lymphoma/leukaemia (Huang *et al*. 2009) In addition HIV-1 (human immunodeficiency virus-1) has been associated with an increase incidence of opportunistic cancers which could be due to impaired immune control of cancer or due to insertional mutagenesis seen in non-Hodgkin's lymphoma (Carbone 2002).

1.4. HERV-K AND HUMAN MELANOMA

1.4.1. HERV-K particles and human melanoma

The association between HERV and human melanoma gained attention since 1968 with the discovery of the possibility of transmitting hamster melanoma using cell free ultra filtrate along with the detection of virus like particle (Epstein *et al*. 1968). This discovery drew the attention toward the causal association between hamster melanoma and the still unknown virus particle. This was followed briefly in 1972 by the first published study on human melanoma in which virus particles were detected in primary malignant melanoma and in metastasis to regional lymph nodes (Birkmayer *et al*. 1972). The particles were described as spherical or slightly ovoid measuring 90-120 nm in diameter with an electron dense core measuring 50-70 nm surrounded by three layered membrane with occasional projections. The observation of the similarity between these particles and those found in hamster melanoma strongly suggested a role of these particles in human melanoma.

Thereafter, two different study groups (Birkmayer *et al*. 1974; Balda *et al.* 1975) have detected viral particles in primary and metastatic melanoma tissues, which were characterized by high-molecular-weight RNA and RNA-dependent DNA polymerase activity, suggestive of retroviruses. Since then, no infectious virus particles were isolated and the exact nature of the virus was not known. In an attempt to rescue the virus using a helper virus, Zavada *et al*. 1986 used mouse NIH-3T3 cells carrying molony mouse leukemia virus (MSV and MLV complex) and fused them with human melanoma cell carrying the virus to be rescued. The filtrate of the fused cells was infectious to both mouse and human cells indicating the rescue of an unknown human virus. More recently, virus-like particles have been demonstrated in melanoma cell line supernatants, by electron microscopy observation. They were shown to have RT activity, package sequences with 95.5-97% homology to HERV-K 108 (HML-2 HOM) and 93-95% homology to HERV-K 113 and to contain gag and env proteins (Muster *et al.* 2003, Buscher *et al.* 2005). No RT activity was detected in supernatants from cultured human neonatal melanocytes, indicating that the production of particles containing RT is exclusive to melanoma. However, these viral particles were found to be defective and noninfectious.

Further sequencing studies showed that particles derived from different melanoma cell lines and even those produced by the same cell line have sequence variability (Hirschl *et al.* 2007).

1.4.2 HERV-K mRNA and protein expression in human melanoma

High copy numbers of the HERV-K 108 *pol* sequence was demonstrated in primary lymph node metastases and cutaneous metastases, by *in situ* hybridization. Retroviral proteins gag, Rec and env were found to be expressed in melanoma cell line and all primary melanoma, lymph node and cutaneous metastases, by immunofluorescence.

The expression of HERV-K transmembrane envelope (TM), Rec and Np9 proteins has also been studied (Buscher *et al.* 2006) because of the particular interest of Rec and Np9 as potentially oncogenic and TM as putatively immunosuppressive. HERV-K Rec and Np9 mRNA were also found to be expressed in melanoma (39% and 29% respectively) and melanoma cell line (40% and 21% respectively) and Rec protein were detected in 14% of melanoma and no Np9 protein were detected (Buscher *et al.* 2006). More importantly, the interaction of Np9 and Rec with Promyelocytic Leukaemia Zink Finger (PLZF) tumour suppressor has been found to remove the inhibitory effect of PLZF on c-myc proto-oncogene leading to it's over expression and subsequent carcinogenic effect (Denne *et al.* 2007). The expression of TM protein was detected in approximately 50% and HERV-K gag in approximately 70-80% of primary and metastatic melanoma samples. Env, Rec and Np9-specific antisera were also generated, characterized and used to study protein expression in metastatic melanoma and melanoma cell lines.

1.4.3. HERV-K and human melanoma: immunological evidences

Interest in the potential association of retrovirus with human melanoma was reignited when a HERV-K antigen, presented in association with the HLA class I molecule on autologous tumor cells of a patient with melanoma, was shown to be targeted by CD8+ T-cytotoxic lymphocytes (Schiavetti *et al*. 2002). The antigenic peptide, comprising nine amino acids, was found to be encoded by a short ORF in the *env*

region of a defective HERV-K provirus. The gene encoding the antigen, designated HER-K-MEL, was expressed in most samples of cutaneous and ocular melanoma tested, but also in the majority of benign naevi and in some normal skin samples.

Furthermore the causal association between HERV and human melanoma was highly supported by a multicentre case-control study which revealed significant associations between reduced risk of melanoma and history of certain severe infections and/or history of vaccinations with bacille Calmette-Guerin (BCG) and/or vaccinia virus in childhood (Krone *et al.* 2005; Krone *et al*. 2003)). This can be explained by the fact that BCG and vaccinia virus have antigenic determinants homologous in their amino acids sequences with the melanoma antigen HERV-K-MEL, encoded by a human endogenous retrovirus K, which is expressed in about 95% of malignant melanocytes.

In addition, yellow fever vaccine, with antigenic determinant homologous to HERV-K MEL antigen was found to reduce the risk of melanoma 10 years after vaccination (Mastrangelo *et al*. 2008).

Furthermore, animal experimental studies have shown that knocking down the melanoma-associated retrovirus (MelARV), using RNA interference (RNAi), resulted in the rejection of tumor cells in immunocompetent mice. Re-expression of the MelARV *env* gene in the knocked-down cells partially ablated tumor rejection, thus indicating that the *env* gene contributes to tumor immune escape (Mangeney *et al*. 2005).

Regarding human melanoma, there is evidence that the expression of HERV-K proteins may induce humoral immune response, with diagnostic and prognostic value.

Antibodies specific for HERV-K TM and env proteins were detected in some sera from patients with melanoma, however HERV-K Rec and Np9-specific antibodies were not detected in the same patients, by Western blot analyses (Buscher *et al*. 2006; Humer *et al*. 2006)

1.4.4. Biological events in the progression of human melanoma

Melanoma is a complex genetic disease, the management of which requires an in-depth understanding of the biology underlying its initiation and progression.

Malignant melanoma arises from epidermal melanocytes, specialized pigmented cells, predominantly present in the skin. Cutaneous melanocytes originate from highly motile neural-crest progenitors that migrate to the skin during embryonic development and their homeostasis is regulated by epidermal keratinocytes (Slominski *et al*. 2004).

The Clark model (Clark *et al.* 1989), proposed for the progression of melanoma, describes the histological changes that accompany the progression from normal melanocytes to malignant melanoma (Figure 1.4).

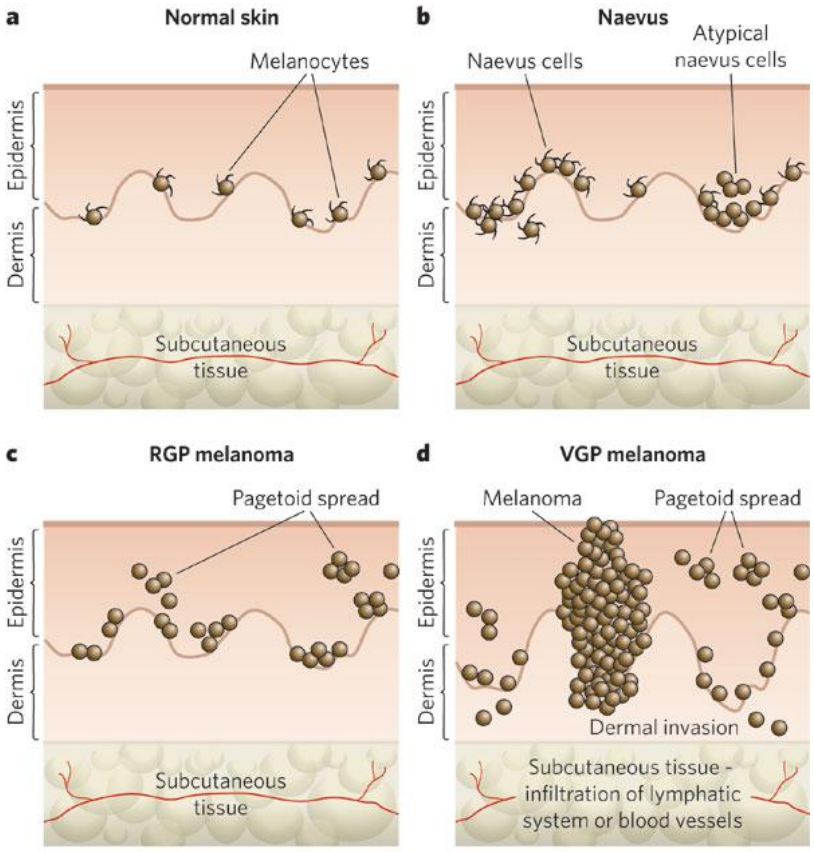

Figure 1.4. The Clark model: progression of melanocyte transformation (Gray-Schopfer *et al.* 2007)

Melanomas are histologically classified into five distinct stages: common acquired and congenital nevi without dysplasia, dysplastic nevi, Radial-Growth-Phase (RGP) melanoma, Vertical Growth Phase (VGP) melanoma and metastatic melanoma.

RGP melanoma grow laterally and remains largely confined to the epidermis, while VGP melanoma invades the upper layers of the epidermis and penetrates into the underlying dermis and subcutaneous tissues forming expansile nodules of malignant cells. It is believed that the crucial step in the evolution to malignant melanoma is the transition from RGP to VGP melanoma.

Metastatic melanoma, on the other hand, develops when tumour cells dissociate from the primary lesion, migrate through the surrounding stroma, and invade bloodstream or lymphatic system to form a tumour at a distant site (Haass *et al.* 2005).

1.4.5. Molecular changes in the progression of human melanoma

The histological changes described in the Clark model have been correlated with particular gene mutations that affect molecular signalling, which in turn contribute to the progression from normal melanocyte to melanoma.

Molecular biological bases of human malignant melanoma development can be classified as either genetic, which include mutation, deletion, amplification or translocation, or epigenetic transcriptional modulation, which does not involve modification of DNA sequence. For the last years, the study of the genetic modulation that overlies melanoma development and progression has been facilitated by the availability of commercial microarray technique.

Between 1996 and 2006, more than 129 DNA microarrays have been reported. These studies were used in an attempt to differentiate early melanoma stages (RGP), from advanced stages (VGP) and to provide novel diagnostic and prognostic markers. However, solid conclusions could not be based on all of these studies, due to the chance of false positive results and the fact that not all results were reproducible. Nevertheless, many reviewers have adopted more rigorous approach to validate gene

expression analysis data, using multiple testing controlled analyses and have highlighted the most common genetic events that underline either the development of melanoma from melanocyte or the progression of melanoma from RGP into VGP which is thought to be the most critical step in predicting prognosis.

Comprehensive strategies, such as comparative genomic hybridization and mutation analysis by gene resequencing, have identified some of the crucial cell-signalling pathways in this disease.

In accordance with other cancers, human melanoma demonstrates a wide range of genetic modification that favors increase cellular proliferation, inhibition of apoptosis, epithelial mesenchymal transmission and cell migration and interference with cell senescence (Bennett 2008). Figure 1.5 Demonstrates the connections between molecular pathways and risk factors for melanoma, the different steps of neoplastic transformation and the patterns of molecular changes.

Stimulation of melanocyte proliferation was found to be associated with activating mutation of oncogenic Ras or BRAF or increase copy number of CCND1. The Ras/Raf/MEK/ERK pathway is a key regulator of melanoma cell proliferation, with ERK being hyperactivated in up to 90% of human melanomas by the production of autocrine growth factors or in rare cases by mutational activation of growth- factor receptors such as c-Kit (Willmore-Payne *et al*. 2005).

BRAF is a proto-oncogene that link Ras to MAPK (Mitogen Activated Protein Kinase) pathway and therefore leads to activation cascade that ultimately leads to cellular proliferation and survival. The MAPK pathway is dysregulated in most melanomas and current understanding of the MAPK pathway in melanoma biology support the concept of distinct groups of molecular and genetic abnormalities in melanomas, related to type of sun exposure and body site.

BRAF and Ras activating mutations are thought to be one of the most common mutations, which are found in around 70% of melanoma (Quatresooz *et al.* 2009).
Interestingly, BRAF mutation is seen mostly in melanoma arising from skin with intermittent sun exposure and not in skin with chronic sun exposure (Hoek 2007) but

occur at a similar frequency in benign nevi and in primary and metastatic melanomas (Pollock *et al.* 2003).

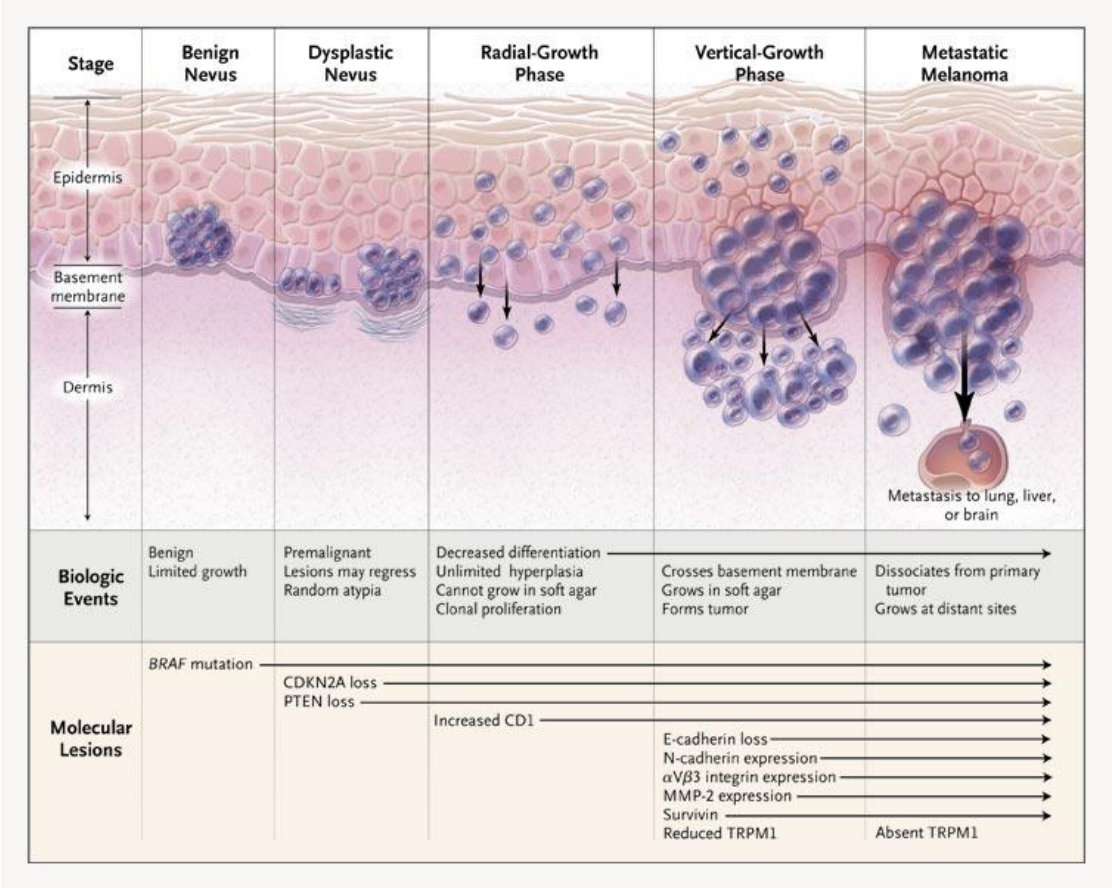

Figure 1.5. Biologic events and molecular changes in the progression of melanoma (Miller and Mihm 2006). At the stage of the benign nevus, *BRAF* mutation and activation of the mitogen-activated protein kinase (MAPK) pathway occur. The cytological atypia in dysplastic nevi reflect lesions within the cyclin-dependent kinase inhibitor 2A (CDKN2A) and phosphatase and tensin homologue (PTEN) pathways. Further progression of melanoma is associated with decreased differentiation and decreased expression of melanoma markers regulated by microphthalmia-associated transcription factor (MITF). The vertical-growth phase and metastatic melanoma are notable for the striking changes in the control of cell adhesion. Changes in the expression of the melanocyte-specific gene melastatin 1 (*TRPM1*) correlate with metastatic propensity, but the function of this gene remains unknown. Other changes include the loss of E-cadherin and increased expression of N-cadherin, αVβ3 integrin, and matrix metalloproteinase 2 (MMP-2).

In addition, activation of β-catenin oncogenic pathway via activating mutation of β-catenin itself, methylation or mutation of APC or activation of AKT3 can transcriptionally activate MYC and CCND1, inhibit apoptosis and activate microphthalmia-associated transcription factor (MITF), which is a key regulator of melanocyte gene expression. Suppression of retinoblastoma protein pathway (RB) via CCND1, CDK4, or RB1 mutation could by itself activate clonal expansion regardless of MAPK pathway (Bennett 2008).

Microphthalmia-associated transcription factor (MITF) is a transcription factor considered the master regulator of melanocyte biology, partly because it regulates the expression of melanogenic proteins such as tyrosinase, silver homologue (GP100) and melanoma-associated antigen recognized by T cells-1 (MART-1, also known as melan-A). MITF is expressed in most human melanomas and its target genes are diagnostic markers for this disease. Furthermore, continued MITF expression is essential for melanoma cell proliferation and survival (Levy *et al.* 2006; Widlund *et al.* 2003). MITF is expressed at significantly lower levels in melanoma cells than in melanocytes, and increased MITF levels reduce melanoma cell proliferation even in the presence of oncogenic BRAF. High MITF levels reduce melanoma cell tumorogenicity (Selzer *et al.* 2002).

Decreased or absent pigmentation accompany the progression from nevus to melanoma. Expression of the melastatin 1 (*TRPM1*) gene, whose function is unknown, is also controlled by MITF (Miller *et al.* 2004). The mechanism of decreased expression of these genes is a puzzle because MITF is present in nearly all melanomas (Granter *et al.* 2002). Although MITF causes differentiation and cell-cycle arrest in normal melanocytes, melanoma cells do not have these characteristics. Recently, a large-scale search for genomic changes in melanoma found an increased copy number (4 to 119 copies per cell) of a region of chromosome 3 that includes the *MITF* locus. This increase was accompanied by the increased expression of MITF protein. The over expression of both MITF and BRAF could transform primary cultures of human melanocytes, implicating *MITF* as an oncogene. Notably, *MITF* amplification occurs most frequently in tumours that have a poor prognosis and is associated with resistance to chemotherapy (Garraway *et al.* 2005).

On the other hand, anti-apoptotic effect in human melanoma can be achieved by loss of apoptosis effecter APF1, PI3K activation via Ras, or NFKB activation via BRAF (Bennett 2008). NFKB can be also activated by cytokines produced by activated white blood cells and melanocyte or by hypoxia inducible factor α (HIFα) induced by hypoxic skin condition.

Cell senescence, which is seen in nevi, is unfavourable to human melanoma development and overcoming it is a key factor in melanoma development. Two main pathways control cell senescence: P16-RB and P53. P16-RB was found to be more relevant in human melanoma. P16, which facilitate cell senescence by acting on telomerase, is encoded by CDKN2A, which in turn is thought to be one of the two genes most commonly affected by inactivating mutation in cancer in general along with P53. Unlike BRAF, which is commonly mutated in melanoma arising from skin with intermittent sun exposure, CDKN2A is commonly mutated in mucosal and acral melanomas along with CDK4 (Quatresooz *et al*. 2009).

In human melanocytes, mutant BRAF protein induces cell senescence by increasing the expression of the cell-cycle inhibitor of kinase 4A (INK4A) (Michaloglou *et al*. 2005). INK4A limits hyperplastic growth caused by a *BRAF* mutation. The arrest of the cell cycle caused by INK4A can, however, be overcome by mutations in INK4A itself, as well as other cell-cycle factors. Thus, BRAF is implicated in several aspects of melanoma induction and progression.

Acquisition of potential for invasion and distant metastasis has been an interesting object of study of many researchers and although a big list of genes has been found differentially expressed in RGP and VGP melanoma, only few have been found to be significant. Non-canonical WNT pathway has a high correlation with invasive and metastatic melanoma. Furthermore, NFKB was found to play a role in activating transcription factor SNAI1 which repress E-cadherin and induce N-cadherin, which in turn activate proteases (matrix metalloproteinase -2 (MMP2) and tissue plasminogen activator (t-PA)) facilitating epithelial mesenchymal transition, cell migration and invasion (Bennett 2008).

Cadherins are multifunctional transmembrane glycoproteins that sustain cell-to-cell contacts, form connections with the actin cytoskeleton, and influence intracellular

signalling. Cadherins are divided into three subtypes: E (epithelial), present in polarized epithelial cells in the epidermis, including melanocytes and keratinocytes; P (placental) and N (neural), found in mesenchymal cells in the dermis. The intracellular domain is associated with a large protein complex that includes β-catenin and forms structural links with bundles of actin filaments (Miller and Mihm 2006).

The major adhesion mediator between keratinocytes and normal melanocytes in the epidermis is E-cadherin, which disappears during melanoma progression (Tang *et al*. 1994). Instead of E-cadherin, melanoma cells express N-cadherin, characteristic of invasive carcinoma, which allows them to change cellular partners. Melanoma cells adhere through N-cadherin to fibroblasts and endothelial cells by gap junctions (small channels for electrolyte transport) (Hsu *et al.* 2000; Danen *et al.* 1996; Hsu *et al*. 1996). Progression from the radial-growth phase to the vertical-growth phase of melanoma is therefore marked by the loss of E-cadherin and the expression of N-cadherin (Danen *et al*. 1996). Besides these changes in cell adhesion, decreased E-cadherin expression (Gottardi *et al*. 1997) and aberrant N-cadherin expression increase the survival of melanoma cells by stimulating β-catenin signalling (Qi *et al.* 2005).

Cell that have lost epithelial differentiation, as manifested by the loss of functional E-cadherin, show increased mobility and invasiveness. Keratinocytes can no longer control melanoma cells that have lost E-cadherin and when melanoma cells are forced to express E-cadherins and are co-cultured with keratinocytes, they dramatically change: melanomas adhere to keratinocytes, no longer express invasion-related molecules and lose their invasive capacities (Hsu *et al.* 2000).

N-cadherin is also a survival factor for melanoma cells as they migrate through the dermis and a major adhesion receptor, when melanoma cells adhere to each other (Li *et al.* 2001; Perils *et al.* 2004).

Similarly integrins are cell surface adhesion molecules playing an important physiologic role in cell adhesion, growth, proliferation, migration, and apoptosis (Tucker 2006).

CHAPTER 2. AIM OF THE STUDY

According to the Clark model of tumorogenesis, (Clark *et al*. 1989) the most critical moment in the development of a melanoma would be the moment at which clonal evolution generates a cell with "vertical-growth phase" (VGP) properties i.e. a melanoma cell whose progeny can enter the deeper dermis rather than growing only in or near the epidermis. That is because VGF melanomas are believed to be competent of metastasis so the tumour would then proceed to spread and kill the patient if untreated.

Human melanoma has been linked to HERV-K expression but the timing of HERV-K expression and its significance in cancer progression is still unclear. The family of HERV-K has been subdivided into type 1 and type II proviruses. Type I proviruses are characterized by a 292 bp deletion at the *pol-env* boundary and an ORF for the nonstructural protein Np9. Type II, the undeleted prototype, has an intact *env* sequence and encodes the accessory protein Rec. Both proteins were found expressed in tumours and transformed cell lines and evidence has accumulated that they play a role in cancer development. Rec and Np9 directly bind to the promyelocytic leukemia zinc finger (PLZF) protein and inhibit the transcriptional inhibitory function of PLZF on MYC promoter thus promoting cellular proliferation (Denne *et al.* 2007). Furthermore, the presence of anti-HERV-K antibodies in early stages of melanoma was shown to be a marker of reduced survival (Hahn *et al.* 2008). In a published work, done at the University of Rome, Tor Vergata, HERV-K was reported to support the *in vitro* transition of melanoma cells from adherent to a more malignant, non adherent phenotype when exposed to stress conditions (Serafino *et al.* 2009).

In that paper a cellular system was described including 3 types of cells: original adherent human melanoma cell line (TVM-A12), and two derived anchorage independent rounded cells, growing in suspension, obtained by limiting dilution ($Clone_{sp}$) and by serum starvation (1% of foetal calf serum in the culture medium) ($TVM\text{-}A12_{sp}$). Anchorage independent suspended cells ($Clone_{sp}$ and $TVM\text{-}A12_{sp}$) have been characterized, showing a more malignant phenotype in comparison to original adherent cells (TVM-A12) in the form of decreased expression of melanoma differentiation antigen (Melan A/MART-1), loss of HLA-I expression, and increased

ability to grow in semi-solid agar. More importantly, these phenotypic and functional modifications were accompanied by the activation of HERV-K expression and massive production of viral-like particles. Down-regulation of HERV-K expression by RNA interference prevented the transition from the adherent to the non-adherent growth phenotype in low serum condition.

Aim of this study is to provide further characterization of the cellular system at the genetic level for better understanding of the genetic modification associated with the phenotypically increased malignant potential and also to look for genes modification that might have a role in the induction of HERV-K expression. Such identification of basic genetic modification provide better understanding of the process of carcinogenesis and shed light on possible drug targets that can be used in the management of human malignant melanoma.

To understand the transcriptional activity of HERV-K in melanoma and their role in cancer progression we investigated the gene expression profile of melanoma cells derived from the same parental cell line (TVM-A12) by different stress conditions, i.e. limiting dilution ($Clone_{sp}$) and serum starvation (1% FBS in the culture medium) ($TVM\text{-}A12_{sp}$). Cells obtained by both conditions were characterized by high levels of expression of HERV-K, corresponding to a phenotype of more malignant cells.

With this in mind the objectives were to:

1. Perform an initial screening of gene expression of a large number of genes commonly modified in human cancer using commercially available microarray tests that include tumor suppressor genes involved in apoptosis, cell expression cycle, cell growth and differentiation, cell mobility and signal transduction.

2. Determine the general theme of genes modification in the cellular system and identify genes with special importance to human melanoma progression and HERV-K expression.

3. Select genes, which show significant modification either up regulation or down regulation and perform real-time PCR confirmation.

4. Determine whether phenotypically more malignant suspended cells (TVM-A12$_{sp}$ and Clone$_{sp}$) have a more malignant genotype in comparison to the original adherent cell line TVM-A12.

CHAPTER 3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Cell cultures

- The human melanoma cell line TVM-A12 was established as monolayers from a melanoma lesion of a patient with metastatic melanoma, obtained at the presentation of the disease (Melino *et al*. 1993).
- RPMI 1640 medium, Trypsin 0.05% EDTA 0.02% in PBS, Dulbecco's Phosphate buffered saline (PBS), Penicillin-streptomycin 100X and L-glutamine solution 100X (EuroClone, Milan, Italy)
- Fetal bovine serum (FBS, EuroClone, Milan, Italy)
- Forma direct heat CO_2 Incubator (Thermo Fisher, USA)

3.1.2. Microarray

- Nucleospin RNA II isolation kit (MACHEREY-NAGEL, Germany)
- EuroGold total RNA kit (EuroClone, Milan, Italy)
- Smartspec plus Spectrophotometer (Bio-Rad, Rome, Italy)
- True labelling-AMP 2.0 cRNA synthesis and labelling kit (GA-030), ArrayGrade™ cRNA Cleanup Kit, SuperArray Oligo GEArray® Microarrays, GEAhyb Hybridization solution (H-01), Chemiluminescent Detection Kit (D-01) and Microarray Membranes, Oligo GEArray® Human Cancer Microarray, OHS-802 (SuperArray Bioscience Corporation, USA)
- Biotin-11 UTP (Perkin Elmer, Germany)

3.1.3. Reverse transcription and Real-time Polymerase chain reaction (PCR)

- I Script cDNA synthesis kit, IQ SYBR Green Supermix, and C1000 Thermal Cycler (CFX96 Real-time System, Bio-Rad, Rome, Italy)
- High Capacity cDNA reverse transcription kit (Applied Biosystem, USA)

- Primer pairs (Table 3.1.) (PRIM, Milan Italy)

3.2. METHODS

3.2.1. Cell culture

- TVM-A12, a human melanoma adherent continuous cell line, obtained at the University of Rome Tor Vergata from metastatic melanoma lesion was maintained and grown in RPMI 1640 with 10% (v/v) heat inactivated FBS (fetal bovine serum) supplemented with penicillin (100 IU/ml), streptomycin (100mg/ml) and L-glutamine (2 mM) and incubated at $37^{o}C$ in a humidified 5% CO_2 atmosphere. Adherent cells were passed every other day using trypsin and 0.02% EDTA solution in PBS.
- $Clone_{sp}$ was isolated by limiting dilution techniques, at 1 cell/well in 96 well plate, from the TVM-A12 human melanoma cell line, maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS (fetal bovine serum), penicillin (100 IU/ml), streptomycin (100mg/ml) and L-glutamine (2 mM). $Clone_{sp}$ was characterized by a completely lost of adherence growth and a stably modified phenotype as big suspended clumps, even when maintained in RPMI 1640 complete medium supplemented with 10% (v/v) FBS.
- $TVM\text{-}A12_{sp}$ suspended cell line was obtained by shifting the parental TVM-A12 melanoma cell line from optimal growth factor conditions (10% FBS) to low-serum condition (i.e.,1% FBS). TVM-A12 were seeded at density of $2X10^5$ cells in T25 cm^2 flask and grown in 10% FBS. After 24 hours the medium was changed to 1% FBS and cells were observed for detachment for one week.

3.2.2. RNA extraction and quantification

- Cell pellets obtained by washing with PBS and subsequent sedimentation using centrifugation 1000g, were used for RNA extraction using Nucleospin RNA II extraction kit according to manufacturer's instruction. RNA was extracted from original adherent TVM-A12 (TVM-A12 10%), adherent TVM-A12 growing in 1% serum (TVM-A12 1%), suspended TVM-A12 (TVM-

$A12_{sp}$), and clone cells growing in suspension in 10%FBS culture medium ($Clone_{sp}$).

- RNA quantification using spectrophotometry at wavelength 260nm was done. The ratio of absorbance at A260:A280 was used to determine the quality of isolated RNA. Isolated RNA was stored at -80^{o}C.

3.2.3. Microarray

- Microarray membranes testing 440 genes involved in human cancer, including genes involved in apoptosis, cell cycle, cell growth and differentiation, cell mobility and signal transduction, were used to test all cells in the cellular system (including original adherent TVM-A12, adherent TVM-A12 in 1% FBS, suspended TVM-$A12_{sp}$, and $Clone_{sp}$, obtained by limiting dilution technique). A total of 3µg of isolated RNA with A260:A280 ratio in the range (1.6-1.9) was used for cRNA synthesis and labelling with biotin using true labelling-AMP 2.0 cRNA synthesis and labelling kit. It must be emphasised that 4 hours incubation was used for cRNA synthesis, amplification and labelling. This was followed by cRNA purification using clean up kit as instructed by manufacturers. Purified cRNA was used for hybridization overnight with subsequent chemiluminescence staining and imaging on chemiluminesence film according to manufacturer's instructions.
- Images obtained were uploaded in to an online image data acquisition and analysis suite (GEArray Expression analysis suite, http://www.superarray.com) for analysis. Spot intensity, for each gene, was PUC18 plasmid (negative control) DNA background subtracted and normalized by the software using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta actin (ACTB) as housekeeping control genes. Gene expression modification in all cells of the cellular system in comparison to original adherent TVM-A12 was determined as fold change (ratio of spot signal detected in test sample over control sample). Genes with fold change ≤0.6 were considered down regulated and those ≥ 1.5 were considered up regulated.
- Microarray analysis was done in triplicate for each cell condition, using samples from three different experiments.

3.2.4. Reverse transcription and Real time PCR

- A total of 500 ng of isolated RNA from each sample was reverse-transcribed, in a total volume of 20µl, using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions.
- Primer pairs for selected genes were designed using primer express software using the following criteria: primer length between 16 and 24 bases, primer's melting temperature between 58°C and 61°C, GC% contents in the range 42-67 % and amplicon length between 65 and 110 bases (Table 3.1).

Table 3.1. Primer pairs designed for real-time PCR.

Gene symbol	Gene bank Accession n°	Forward Primer	Reverse Primer
GUSB	NM-000181	CAGTTCCCTCCAGCTTCAATG	ACCCAGCCGACAAAATGC
ARHGAP5	NM-001173	TGGCTCCTCCACCACCTTC	GCACCATTGGTTCCACCAAC
ARHGEF5	NM-005435	TCCCTGGTTCTCACAGGACAG	GCAACATTCCCACCAGGTCT
BRCA2	NM-000059	AAACATCCACTCTGCCTCGAA	AACACGCAGAGGGAACTTGG
CTNNB1	NM-001904	GCGTACTGTCCTTCGGGCT	ATGGCAGGCTCAGTGATGTCT
E2F1	NM-005225	AGGGCATCCAGCTCATTGC	CCAGCCACTGGATGTGGTTC
GPR39	NM-001508	TGTGACATTGGCCGTATGCT	GCCATGATCCTCCGAATCTG
ITGB4	NM-000213	ACCGCGTGCTAAGCACATC	TTCTGAGCGGGTCAGTGAGTT
PTEN	NM-000314	AATGTTCAGTGGCGGAACTTG	ACCTTTAGCTGGCAGACCACA
TRAM1	NM-014294	GCGATTCGCAAGAAAAGCAC	GAATTCGTGGCTCAGCACTG
VEGFA	NM-003376	ATCACCATGCAGATTATGCGG	TCCTATGTGCTGGCCTTGG
VHL	NM-000551	CGGAGCCTAGTCAAGCCTGA	GCGACCTGACGATGTCCAG
FOXG1A	X74143	CACCCCATGCCCTACAGCT	GTTGTTGCCCAGCGAGTTTT
BHLHB2	NM-003670	GCGACACGGACACAGACAGT	AGTCGCCCTTCTCCGATTCT
BTK	NM-000061	AGACTGCTGAACACATTGCCC	GCCAGATGAGGCCTGTAGAGA
CD59	NM-000611	TGCTGCAAGAAGGACCTGTG	TGTCCCACCATTTTCAAGCTG
CSK	NM-004383	AAACAAGGTGGGCCGTGAG	CGCTTCTGGACGTAGTTGGC
MYCL1	NM-005376	GGAGGATTTCTACCGCTCCAC	CACCAGCTCGAATTTCTTCCA
MYCN	NM-005378	ACCTTCGGTCCAGCTTTCTCA	TTACCAACTCCGGCACGTG
PTCH1	NM-000264	GCGCTGTCTTCCTTCTGAACC	GGACCATCACAATGATCCCG
WNT2	NM-003391	ATGCCATCTCCTCAGCTGGA	GGCTACAGGCCCTGGTGAT
WNT3	NM-030753	CTGGCTACCCAATTTGGTGG	GAGATGTGTACTGCTGGCCCA
CCND2	NM-001759	GGTGCTCCTCAATAGCCTGC	GATCCGTCACGTTGGTCCTG
ANXA7	NM-004034.2	CTGCGCCTGGAGGTTATC	GAGGAACTCCGGGATAGGAT
CTNNA1	NM-001903.2	CGCACCATTGCAGACC	GTTCAGCTGGTGGCAGTAGA
NME1	NM-000269.2	CACTCAGGGCCGGTAGTT	GAGTCTGCAGGGTTGGTCTC
RRM1	NM-001033.3	CAAGGTCGTGTCCGCAAAG	GGTGCCTGTTTCCGTCTGA
TIMP3	NM-000362.4	GGACGCCTTCTGCAACTC	TGGTGTAGACCAGCGTGC
CDKN1A	NM-000389.3	TGTCACCGAGACACCACTG	CGTGGGAAGGTAGAGCTTG
XRCC6	NM-001469.3	GTACGCTGGGCAAGTTCAC	AGTGCTTGGTGAGGGCTT
HDAC1	NM-004964.2	GAACCTTAGAATGCTGCCG	CGCTTGTCAGGGTCGTCT
MYC	NM-002467.3	CTACCCTCTCAACGACAGCA	CGTCGAGGAGAGCAGAGAAT
TOB1	NM-005749.2	CATAAGTGACCCAGCCTCATCA	GGGCTTACAGCAGCAGAGTGA
TP53	NM-000546.4	GGCCATCTACAAGCAGTCAC	CGGATAAGATGCTGAGGAGG
IRF3	NM-001571.4	ACACATACTGGGCAGTGAGC	GCCTCCTTCCTTGTCCTTG
GTF2I	NM-001518.3	GCTCTCGAGTCCATGTGTAAAGAA	ACACGTCTGTTTCATACACTGCAA
FRAP1	NM-004958.3	GGAGCTGCTGAAGGACTCAT	AGCATTGAAGAGATCCCTGG

- Amplification of specific PCR products was detected using the IQ SYBR Green Supermix. Quantitative Real time PCR (Q-PCR) was done in triplicate for each sample, in a total reaction volume of 25µl containing 200 ng of cDNA, 150 nM forward and reverse primer and 1X IQ SYBR Green

Supermix. Samples were heated at 95°C for 3 minutes and subjected to 39 cycles of PCR amplification, each cycle consisting of 95°C for 10 seconds and 60°C for 45 seconds. Within each experiment, no template control (NTC) and housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as reference, were run in parallel to verify any contamination and determine amplification efficiency.

- Real time PCR data were viewed and analysed using Bio-Rad CFX manager software. The software allowed determination of Primer specificity using melting curve analysis. Melting curve, which is also known as dissociation curve, ideally shows a single peak at a specific melting temperature, which corresponds to the temperature at which all PCR products dissociate and release bound SYBR-Green dye. Melting curve, which shows single, clean peak of florescence and repeatedly at the same melting curve indicate single PCR product. While that which shows multiple peaks at different melting temperatures, indicate multiple PCR products, which can be due to primer dimers, contaminating DNA, or non reported splice variant of the gene of interest. Primer pairs, which showed non-specific amplification, were excluded and only those, which showed specific and repeatable PCR products, were used.
- Real-time PCR results were displayed as Ct (cycle threshold), which is the number of cycle at which florescence signal crosses the threshold, which in turn is determined as an amount of florescence significantly greater than the background. Fold change in gene expression was determined using the calculation method instead of standard curve method due to the large number of genes to be tested. Fold change in expression was calculated as $2^{-\Delta\Delta Ct}$.

  ΔCt = Ct of every gene tested - Ct of housekeeping gene (GUSB)

  $\Delta\Delta Ct$= ΔCt of test sample* - ΔCt of control sample **

  Fold change = $2^{-\Delta\Delta ct}$

*Test samples include adherent TVM-A12 1% FBS, suspended TVM-$A12_{sp}$, and Clonesp.

** Control sample is the original TVM-A12, growing in 10% FBS.

CHAPTER 4. RESULTS

4.1. BACKGROUND

In previous studies conducted at the University of Rome, Tor Vergata, it has been shown that stress condition such as serum deprivation in melanoma cell line TVM-A12, which grows as adherent cells in monolayer, causes detachment of cells associated with major morphological and behavioural changes (Serafino *et al*. 2009). Non-adherent cells also known as TVM-A12$_{sp}$ have morphological and behavioural similarities to Clone$_{sp}$, which was derived from melanoma cell line TVM-A12 by limiting dilution. Melanoma derived non-adherent cells, both TVM-A12$_{sp}$ and Clone$_{sp}$, have a higher proliferation rate and decreased expression of HLA class I and melanocyte differentiating antigen (Melan A/MART-1) suggesting a more malignant phenotype. In addition, they tend to grow in clumps and show a significant increase in human endogenous retrovirus (HERV) production in comparison to adherent TVM-A12. More specifically, it was observed that HERV-K *env* gene expression, detected by real-time PCR, was highest in adherent TVM-A12 grown in 1% FBS prior to detachment and once they detached in 1% FBS, the level of HERV-K *env* gene expression was reduced to a lower level which is still higher than that observed in parental TVM-A12 10 % FBS and is similar to that observed in Clone$_{sp}$, obtained by limiting dilution (Figure 4.1).

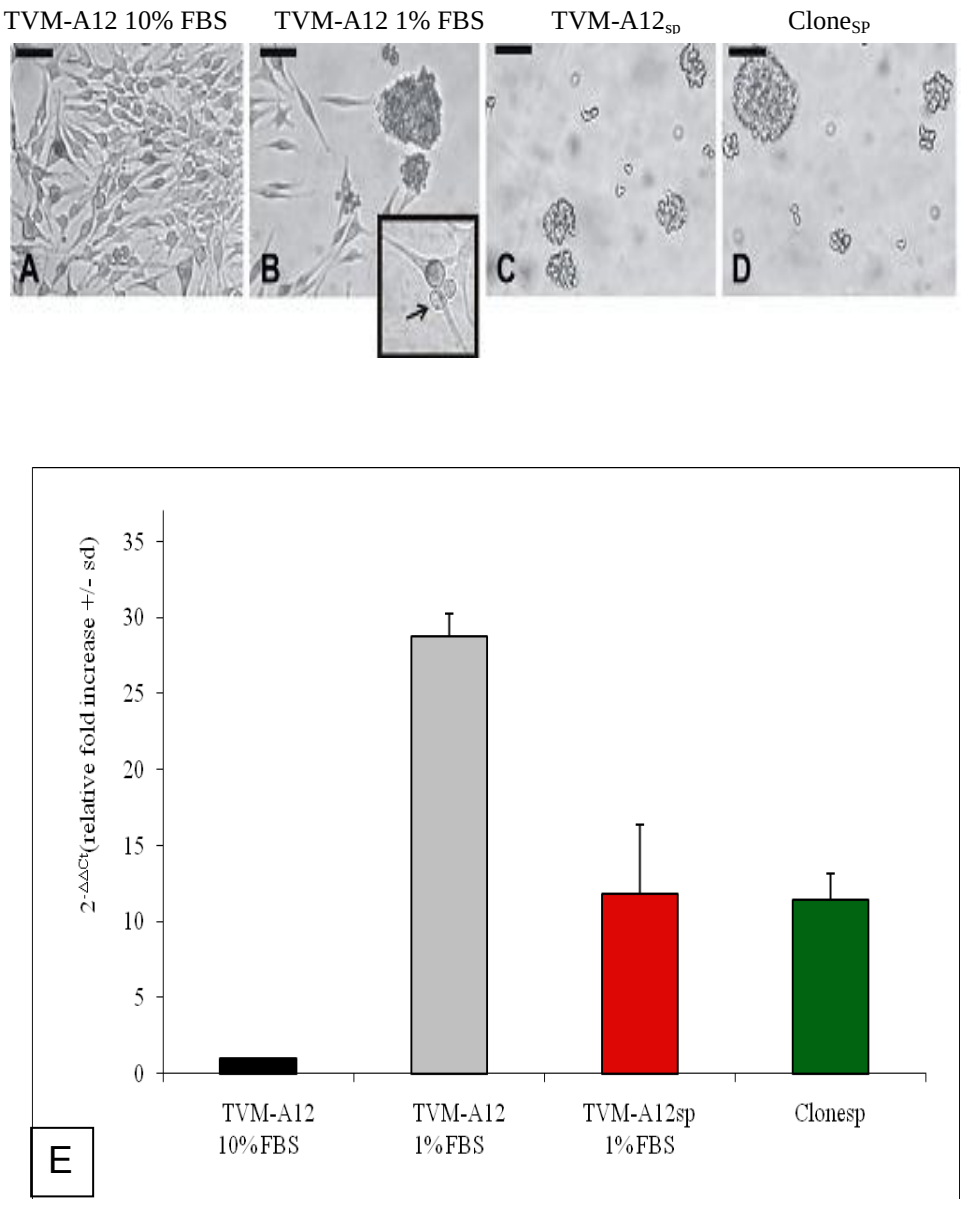

Figure 4.1. The association between peak rise in HERV-K env expression and morphological modification (Serafino *et al.* 2009) . (A–D) Phase contrast microscopy of: TVM-A12 culture grown in 10% FCS (A); TVM-A12 in 1% FCS stress conditions for 4 days (B); clusters of TVM-A12sp cells growing in suspension in low serum conditions separately from the residual adherent monolayer cells (C); Clonesp cells (D).(E) Real-time RT-PCR analysis of env gene expression; levels of env mRNA in TVM-A12 cells cultured in 1% FCS, TVM-A12sp and Clonesp were normalized versus GUSB mRNA levels and compared to the env gene expression in TVM-A12 cells cultured in 10% FCS

The observed peak of HERV-K *env* gene expression marks the point at which morphological modifications start to take place. Interestingly, the process of detachment in serum deprived medium (RPMI with 1% FBS) was found to be HERV-

K-dependent because interference with HERV-K expression using RNA interference causes blockage of this process indicating that HERV-K expression might play a role in the induction of a more malignant phenotype in human melanoma Therefore, understanding the basic molecular genetic changes that underline the morphological modification and the increased HERV-K expression under stressful cultural condition and in $Clone_{sp}$ provide better understanding of the association between increased HERV-K expression and the acquisition of a more malignant phenotype in human melanoma. In addition, it provides a cellular model that demonstrates this association and facilitates *in vitro* study of the effect of various pharmacological and genetic factors on HERV-K expression and melanoma progression. In order to achieve that, gene expression analysis using microarray technology followed by real-time PCR confirmation was used.

4.2. GENE EXPRESSION ANALYSIS USING MICROARRAY

General overview of the pattern of gene modulation in melanoma parental cell line TVM-A12, during and after detachment, in starvation condition (1% FBS) and after limiting dilution, was done using microarray technology, that allows simultaneous detection of the level of expression of 440 genes, on a membrane, all at once, providing a molecular "fingerprint" or "signature" of our cells and also a vision about the pathways involved in the acquisition of a more malignant phenotype. This Oligo GEarray profiles the expression of 440 genes, involved in human cancer, including genes involved in apoptosis, cell cycle, cell growth and differentiation, cell mobility and signal transduction. Relative gene expression levels were calculated as the ratio of the mean values of gene expression in TVM-A12 1%, $TVM\text{-}A12_{sp}$ and $Clone_{sp}$ in comparison to TVM-A12 grown in 10% FBS. Changes in gene expression were expressed as a fold increase/decrease and the cut off, to indicate the fold induction or inhibition of gene expression in comparison with parental TVM-A12 cell line, were $\geq$ 1.5 or $\leq$ 0.6 fold-changes, respectively.

Results obtained by microarray analysis showed that during the transition from adherent (TVM-A12 cells grown in optimal cultural condition, represented by 10% FBS, and named TVM-A12 10%) to non adherent ($TVM\text{-}A12_{sp}$ cells grown in

starvation cultural condition, represented by 1% FBS, and identified as TVM-A12 1%) 116 genes were modulated, and most of them were down regulated (95 of 116 genes) (Table 4.1, comparison between TVM-A12 1% and TVM-A12 10%). Continuing in the transition, i.e. when the cells stably grow in suspension (TVM-$A12_{sp}$ 1% FBS), many other genes were down regulated (Table 4.1, comparison between TVM-$A12_{sp}$ 1% and TVM-A12 10%). Indeed, the total number of modified genes was 166, of which 164 were down regulated. This observation was probably due in part to the stressful cultural condition in which cells were growing. Moreover, the comparison of the genes that were modulated at the beginning of transition, when cells grow in adhesion but with clusters of round-shaped cells detached from the adherent monolayer, and those that were modulated in cells growing in suspension, indicated that most of these genes were the same (data not shown).

Furthermore, $Clone_{sp}$, obtained by limiting dilution, showed modulation of 152 genes in comparison to parental TVM-A12 and the majority of these genes were again down regulated (151 out of 152) (Table 4.1). However, the observed modulation in gene expression in $Clone_{sp}$ cannot be related to stressful cultural conditions because $Clone_{sp}$ is grown in optimal cultural conditions.

Interestingly, the pattern of gene modulation in non-adherent cells TVM-$A12_{sp}$ and $Clone_{sp}$ was very similar and by comparing the two, only 8 genes were up regulated in $Clone_{sp}$ in comparison to TVM-$A12_{sp}$ indicating a high homology in mRNA expression in the two cell lines (Table 4.2).

The above results showed that during transition from adherent to non-adherent phenotypically more malignant phenotype, a generalized pattern of suppression of genes involved in cancer was observed. Furthermore, melanoma-derived non-adherent cells, whether obtained by cloning or cultural starvation condition have a very similar gene expression profile.

In particular, during the transition from adherent to non-adherent phenotype, 95 genes were down regulated including tumor suppressors, negative regulators of metastasis and invasion and pro-apoptotic,and 21 genes were up regulated including positive regulators of metastasis and invasion, anti-apoptotic, transcription factors, and genes

involved in cell growth and differentiation (Table 4.3). In addition, they showed down regulation of 95 genes including tumour suppressors, negative regulators of metastasis and invasion, and pro-apoptotic genes (Table 4.4).

Suspended TVM-A12$_{sp}$ cells, on the other hand, showed up regulation of 2 genes, one anti-apoptotic gene (VHL) and one gene involved in cell cycle regulation (CCND2) (Table 4.5), and down regulation of 164 genes involved in inhibition of metastasis and invasion, tumor suppressor genes, genes involved in antiviral immune response, proapoptotic genes and genes involved in cell growth, differentiation and motility (Table 4.6).

Table 4.1. Number of modified genes using microarray technology.

Comparisons	Number of modified genes	Up regulated	Down regulated
TVMA12 1% FBS / TVM-A12 10% FBS	116	21	95
TVMA12$_{sp}$ 1% FBS / TVM-A12 10% FBS	166	2	164
Clone$_{sp}$ / TVM-A12 10% FBS	152	1	151
Clone$_{sp}$ / TVM-A12$_{sp}$ 1% FBS	8	8	0

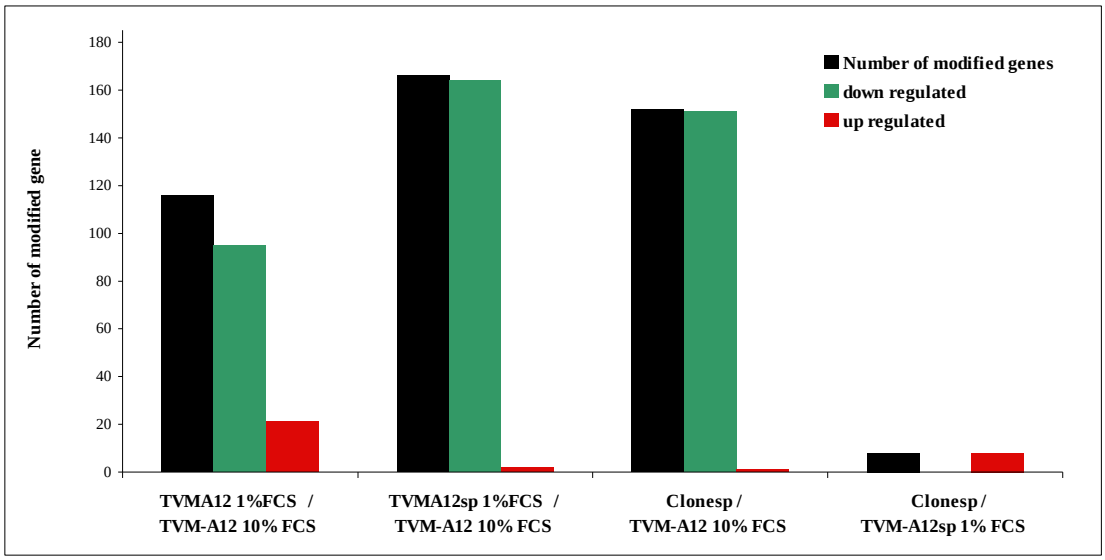

Figure 4.2. Diagrammatic representation of the number of modulated genes using microarray technology. Number of up and down regulated genes in TVM-A12 growing in 1% FBS, TVM-A12$_{sp}$ growing 1% FBS and in Clone$_{sp}$ in comparison with TVM-A12 growing in 10% FBS. The last three histograms are the comparison between Clone$_{sp}$ and TVM-A12$_{sp}$ growing in 1% FBS

Table 4.2. Comparison of gene expression analysis of TVM-A12$_{sp}$ versus Clone$_{sp}$

Gene group function	Gene name	Accession number	Fold change
Cancer related genes			
	AARS Alanyl-tRNA synthetase	NM_001605	**1.615608**
	ASNS Asparagine synthetase	NM_183356	**1.526959**
	TUFM Tu translation elongation factor, mitochondrial	NM_003321	**2.234994**
	VIL2 Villin 2 (ezrin)	NM_003379	**2.269054**
	XRCC1 X-ray repair complementing defective repair in Chinese hamster cells 1	NM_006297	**1.634014**
Anti-apoptotic			
	AKT1 V-akt murine thymoma viral oncogene homolog 1	NM_005163	**1.512497**
Pro-apoptotic			
	TNFRSF10B Tumor necrosis factor receptor superfamily, member 10b	NM_003842	**1.551864**
	TNFRSF1A Tumor necrosis factor receptor superfamily, member 1A	NM_001065	**2.060942**

Table 4.3. Up regulated genes in TVM-A12 grown in RPMI 1% FCS, prior to detachment.

Gene group function	Gene name	Accession number	Fold change
Positive regulator of invasion and metastasis			
	VEGFA Vascular endothelial growth factor A	NM_003376	**2.67**
	WNT2 Wingless-type MMTV integration site family member 2	NM_003391	**1.5**
	ARHGEF5 Rho guanine nucleotide exchange factor (GEF) 5	NM_005435	**2.14**
	TRAM1 Translocation associated membrane protein 1	NM_014294	**3.51**
	ITGB4 Integrin, beta 4	NM_000213	**3.66**
	BRCA2 Breast cancer 2, early onset	NM_000059	**10.37**
Genes with Anti-apoptotic function			
	VHL Von Hippel-Lindau tumour suppressor	NM_000551	**2.17**
	GPR39 G protein-coupled receptor 39	NM_001508	**4.24**
	BTK Bruton agammaglobulinemia tyrosine kinase	NM_000061	**7.06**
	E2F1 E2F transcription factor 1	NM_005225	**1.75**
Transcription factors			
	E2F1 E2F transcription factor 1	NM_005225	**1.75**
	BHLHB2 Basic helix-loop-helix domain containing,class B2	NM_003670	**1.73**
Genes involved in cell cycle			
	E2F1 E2F transcription factor 1	NM_005225	**1.75**
	PTEN Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	NM_000314	**1.61**
	VHL Von Hippel-Lindau tumour suppressor	NM_000551	**2.17**
	BRCA2 Breast cancer 2, early onset	NM_000059	**10.37**
	CSK C-src tyrosine kinase	NM_004383	**5.52**
	E2F1 E2F transcription factor 1	NM_005225	**1.75**
	PTEN Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	NM_000314	**1.61**

Table 4.3 continue

Gene group function	Gene name	Accession number	Fold change
Genes involved in cell growth and differentiation			
	ARHGEF5 Rho guanine nucleotide exchange factor (GEF) 5	NM_005435	**2.14**
	MYCN V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	NM_005378	**2.61**
	WNT3 Wingless-type MMTV integration site family, member 3	NM_030753	**3.84**
	VHL Von Hippel-Lindau tumour suppressor	NM_000551	**2.17**
Genes involved in cell-matrix adhesion			
	ITGB4 Integrin, beta 4	NM_000213	**3.66**
Genes involved in signal transduction			
	CD59 CD59 molecule, complement regulatory protein	NM_000611	**4.69**
	WNT2 Wingless-type MMTV integration site family member 2	NM_003391	**1.5**
	WNT3 Wingless-type MMTV integration site family member 3	NM_030753	**3.84**
	CD59 CD59 molecule, complement regulatory protein	NM_000611	**4.69**
	WNT2 Wingless-type MMTV integration site family member 2	NM_003391	**1.5**
	WNT3 Wingless-type MMTV integration site family member 3	NM_030753	**3.84**
	GPR39 G protein-coupled receptor 39	NM_001508	**4.24**
	BTK Bruton agammaglobulinemia tyrosine kinase	NM_000061	**7.06**
	CSK C-src tyrosine kinase	NM_004383	**5.52**
	ARHGAP5 Rho GTPase activating protein 5	NM_001173	**2.17**
	CTNNB1 Catenin (cadherin-associated protein), beta 1,88k	NM_001904	**1.59**

Table 4.4. Down regulated genes in TVM-A12 grown in RPMI 1% FBS prior to detachment.

Gene group function	Gene name	Accession number	Fold change
Negative regulator of metastasis and invasion			
	CANX Calnexin	NM_001746	**0.40**
	ANXA7 Annexin A7	NM_004034	**0.23**
	RHOA Ras homolog gene family, member A	NM_001664	**0.56**
	CTNNA1 Catenin, cadherin associated protein, alpha 1, 102kDa	NM_001903	**0.41**
	HSPA5 Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	NM_005347	**0.31**
	NME1 Non-metastatic cells 1, protein (NM23A)	NM_000269	**0.55**
Negative regulator of metastasis and invasion			
	RRM1 Ribonucleotide reductase M1 polypeptide	NM_001033	**0.32**
	TIMP1 TIMP metallopeptidase inhibitor 1	NM_003254	**0.45**
	TIMP3 TIMP metallopeptidase inhibitor 3	NM_000362	**0.30**
Tumour suppressor genes			
	BARD1 BRCA1 associated RING domain 1	NM_000465	**0.14**
	NF2 Neurofibromin 2 (bilateral acoustic neuroma)	NM_000268	**0.40**
Genes with pro-apoptotic function			
	TNFRSF1A Tumour necrosis factor receptor superfamily, member 1A	NM_001065	**0.21**
	TNFRSF1B Tumour necrosis factor receptor superfamily, member 1B	NM_001066	**0.22**
	CUL1 Cullin 1	NM_003592	**0.22**
	TIMP3 TIMP metallopeptidase inhibitor 3	NM_000362	**0.30**
	SIVA1 SIVA1, apoptosis-inducing factor	NM_006427	**0.23**
	GSPT1 G1 to S phase transition 1	NM_002094	**0.37**
	RAP5A RAB5A, member RAS oncogene family	NM_004162	**0.54**

Table 4.4 continue

Gene group function	Gene name	Accession number	Fold change
Genes involved in cell cycle			
	TBRG4 Transforming growth factor beta regulator 4	NM_004749	**0.56**
	CUL1 Cullin 1	NM_003592	**0.22**
	CCND3 Cyclin D3	NM_001760	**0.43**
	CCNE1 (Cyclin E1)	NM_001238	**0.23**
	CDC20 Cell division cycle 20 homolog (S. cerevisiae)	NM_001255	**0.21**
	CDK4 Cyclin-dependent kinase 4	NM_000075	**0.47**
	CHAF1A Chromatin assembly factor 1, subunit A (p150)	NM_005483	**0.25**
	MCM2 MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae)	NM_004526	**0.16**
	TAF1 TAF1 RNA polymerase II, TATA box binding protein (TBP) associated factor, 250kDa	NM_004606	**0.40**
	NF2 Neurofibromin 2 (bilateral acoustic neuroma)	NM_000268	**0.40**
	NME1 Non-metastatic cells 1, protein (NM23B)	NM_000269	**0.55**
	AXL AXL receptor tyrosine kinase	NM_001699	**0.33**
	CDC25B Cell division cycle 25 homolog B (S. pombe)	NM_004358	**0.54**
	CLK1 CDC-like kinase 1	NM_004071	**0.30**
	FRAP1 FK506 binding protein 12-rapamycin associated protein 1	NM_004958	**0.39**
	GSPT1 G1 to S phase transition 1	NM_002094	**0.37**
	MYBL2 V-myb myeloblastosis viral oncogene homolog (avian)-like 2	NM_002466	**0.48**
Genes involved in cell growth and differentiation			
	AXL AXL receptor tyrosine kinase	NM_001699	**0.33**
	CBFB Core-binding factor, beta subunit	NM_001755	**0.43**
	CDK4 Cyclin-dependent kinase 4	NM_000075	**0.47**
	IGFBP3 Insulin-like growth factor binding protein 3	NM_000598	**0.52**
	RAF1 V-raf-1 murine leukemia viral oncogene homolog 1	NM_002880	**0.36**

Table 4.4 continue

Gene group function	Gene name	Accession number	Fold change
Genes involved in cell growth and differentiation			
	RHOA Ras homolog gene family, member A	NM_001664	**0.56**
	RHOC Ras homolog gene family, member B	NM_004040	**0.41**
	SMO Smoothened homolog (Drosophila)	NM_005631	**0.18**
	CDC25B Cell division cycle 25 homolog B (S. pombe)	NM_004358	**0.54**
	CLNS1A Chloride channel, nucleotide-sensitive, 1A	NM_001293	**0.40**
	CUL1 Cullin 1	NM_003592	**0.22**
	NF2 Neurofibromin 2 (bilateral acoustic neuroma)	NM_000268	**0.40**
	NME1 Non-metastatic cells 1, protein (NM23B)	NM_000269	**0.55**
	TBRG4 Transforming growth factor beta regulator 4	NM_004749	**0.56**
	TIMP1 TIMP metallopeptidase inhibitor 1	NM_003254	**0.45**
Genes involved in cell motility- matrix metalloproteinases inhibitor			
	TIMP1 TIMP metallopeptidase inhibitor 1	NM_003254	**0.45**
	TIMP3 TIMP metallopeptidase inhibitor 3	NM_000362	**0.30**
Genes involved in signal transduction			
	CBLB Cas-Br-M (murine) ecotropic retroviral transforming sequence b	NM_170662	**0.32**
	FZD2 Frizzled homolog 2 (Drosophila)	NM_001466	**0.33**
	STC1 Stanniocalcin 1	NM_003155	**0.37**
	SMO Smoothened homolog (Drosophila)	NM_005631	**0.18**
	TLE1 Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	NM_005077	**0.58**
	RGS19 Regulator of G-protein signalling 19	NM_005873	**0.26**
	PDPK1 3-phosphoinositide dependent protein kinase-1	NM_002613	**0.51**
	PRKAR1A Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	NM_002734	**0.55**
	RAF1 V-raf-1 murine leukemia viral oncogene homolog 1	NM_002880	**0.36**
	RHOA Ras homolog gene family, member A	NM_001664	**0.56**
	TCF7L2 Transcription factor 7-like 2 (T-cell specific, HMG-box)	NM_030756	**0.29**

Table 4.5. Up regulated genes in suspended TVM-A12$_{sp}$ and Clone$_{sp}$.

Gene group function	Gene name	Accession number	Fold change TVM-A12$_{sp}$	change Fold Clone$_{sp}$
Genes with Anti-apoptotic function				
	VHL Von Hippel-Lindau tumour suppressor	NM_000551	**1.72**	-
Genes involved in cell cycle				
	CCND2 cyclin D2	NM_001759	**1.64**	1.8

Table 4.6 Down regulated genes in suspended TVM-A12$_{sp}$ and Clone$_{sp}$.

Gene group function	Gene name	Accession number	Fold change TVM-A12$_{sp}$	Fold change Clone$_{sp}$
Negative regulator of metastasis and invasion				
	CANX Calnexin	NM_001746	**0.22**	**0.39**
	ANXA7 Annexin A7	NM_004034	**0.08**	**0.15**
	RHOA Ras homolog gene family, member A	NM_001664	**0.56**	**0.56**
	CTNNA1 Catenin (cadherin-associated protein), alpha 1, 102kDa	NM_001903	**0.08**	**0.13**
	HSPA5 Heat shock 70 kDa protein 5, glucose-regulated protein, 78kDa	NM_005347	**0.18**	**0.34**
	NME1 Non-metastatic cells 1, protein (NM23B)	NM_000269	**0.45**	**0.52**
	RRM1 Ribonucleotide reductase M1 polypeptide	NM_001033	**0.31**	**0.31**
	TIMP1 TIMP metallopeptidase inhibitor 1	NM_003254	**0.08**	**0.09**
	TIMP3 TIMP metallopeptidase inhibitor 3	NM_000362	**0.21**	**0.23**
	CCND1 cyclin D1	NM_053056	**0.10**	**0.14**
	CDKN1A Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	**0.06**	**0.06**

Table 4.6. continue

Gene group function	Gene name	Accession number	Fold change TVM-A12$_{sp}$	Fold change Clone$_{sp}$
Negative regulator of metastasis and invasion				
	XRCC6 X-ray repair complementing defective repair in Chinese hamster cells 6 (Ku autoantigen, 70kDa)	NM_001469	**0.05**	**0.05**
	HDAC1 Histone deacetylase 1	NM_004964	**0.13**	**0.18**
	IER3 Immediate early response 3	NM_003897	**0.06**	**0.08**
	MYC V-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467	**0.41**	**0.4**
	PRDX2 Peroxiredoxin 2	NM_005809	**0.13**	**0.24**
Tumour suppressor genes				
	BARD1 BRCA1 associated RING domain 1	NM_000465	**0.19**	**0.28**
	NF2 Neurofibromin2 (bilateral acoustic neuroma)	NM_000268	**0.06**	**0.09**
	TOB1 Transducer of ERBB2, 1	NM_005749	**0.05**	**0.06**
	TP53 Tumour protein p53 (Li-Fraumeni syndrome)	NM_000546	**0.04**	**0.04**
Antiviral immune response				
	IRF3 Interferon regulatory factor 3	NM_001571	**0.13**	**0.2**
	GTF2I General transcription factor II	NM_001518	**0.14**	**0.2**
Genes with pro-apoptotic function				
	TNFRSF1A Tumour necrosis factor receptor superfamily, member 1A	NM_001065	**0.28**	**0.58**
	TNFRSF1B Tumour necrosis factor receptor superfamily, member 1B	NM_001066	**0.16**	**0.4**

Table 4.6. continue

Gene group function	Gene name	Accession number	Fold change TVM-A12$_{sp}$	Fold change Clone$_{sp}$
Genes with pro-apoptotic function				
	CUL1 Cullin 1	NM_003592	**0.05**	**0.06**
	TIMP3 TIMP metallopeptidase inhibitor 3	NM_000362	**0.21**	**0.23**
	SIVA1 SIVA1, apoptosis-inducing factor	NM_006427	**0.05**	**0.05**
	GSPT1 G1 to S phase transition1	NM_002094	**0.09**	**0.15**
	RAP5A RAB5A, member RAS oncogene family	NM_004162	**0.33**	**0.32**
	TNFRSF10B Tumour necrosis factor receptor superfamily, member 10B	NM_003842	**0.46**	**-**
	TP53 Tumour protein p53 (Li-Fraumeni syndrome)	NM_000546	**0.04**	**0.04**
	NME3 Non-metastatic cells 3	NM_002513	**0.37**	**0.53**
	NOTCH2 Notch homolog 2 (Drosophila)	NM_024408	**0.11**	**0.17**
	CDKN1A Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	**0.06**	**0.06**
	IER3 Immediate early response3	NM_003897	**0.06**	**0.08**
Genes involved in cell cycle				
	TBRG4 Transforming growth factor beta regulator 4	NM_004749	**0.17**	**0.2**
	CUL1 Cullin 1	NM_003592	**0.05**	**0.06**
	CCND3 Cyclin D3	NM_001760	**0.12**	**0.14**
	CCNE1 Cyclin E1	NM_001238	**0.04**	**0.05**
	CDC20 Cell division cycle 20 homolog (S. cerevisiae)	NM_001255	**0.28**	**0.41**
	CDK4 Cyclin-dependent kinase 4	NM_000075	**0.03**	**0.05**
	CHAF1A Chromatin assembly factor 1, subunit A (p150)	NM_005483	**0.05**	**0.1**

Table 4.6.contiune

Gene group function	Gene name	Accession number	Fold change TVM-A12$_{sp}$	Fold change Clone$_{sp}$
Genes involved in cell cycle				
	MCM2 MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae)	NM_004526	**0.1**	**0.12**
	TAF1 TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250kDa	NM_004606	**0.09**	**0.1**
	NF2 Neurofibromin 2 (bilateral acoustic neuroma)	NM_000268	**0.06**	**0.09**
	NME1 Non-metastatic cells 1, protein (NM23A)	NM_000269	**0.45**	**0.52**
	AXL AXL receptor tyrosine kinase	NM_001699	**0.06**	**0.11**
	CDC25B Cell division cycle 25 homolog B (S. pombe)	NM_004358	**0.16**	**0.24**
	CLK1 CDC-like kinase 1	NM_004071	**0.06**	**0.15**
	FRAP1 FK506 binding protein 12-rapamycin associated protein 1	NM_004958	**0.04**	**0.1**
	GSPT1 G1 to S phase transition 1	NM_002094	**0.09**	**0.15**
	MYBL2 V-myb myeloblastosis viral oncogene homolog (avian)-like 2	NM_002466	**0.13**	**0.17**
	MYC V-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467	**0.41**	**0.4**
	CDKN1A Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	**0.06**	**0.06**
	NOTCH2 Notch homolog 2 (Drosophila)	NM_024408	**0.11**	**0.17**
	TP53 Tumour protein p53 (Li-Fraumeni syndrome)	NM_000546	**0.04**	**0.04**

Table 4.6. continue

Gene group function	Gene name	Accession number	Fold change TVM-A12$_{sp}$	Fold change Clone$_{sp}$
Genes involved in cell cycle				
	ATM Ataxia telangiectasia mutated (includes complementation groups A, C and D)	NM_000051	**0.10**	**0.13**
	CCND1 Cyclin D1	NM_053056	**0.10**	**0.14**
	PLK2 Polo-like kinase 2 (Drosophila)	NM_006622	**0.46**	**0.54**
	NME2 Non-metastatic cells 2, protein (NM23B)	NM_002512	**0.33**	**0.48**
	PDGFB Platelet-derived growth factor beta polypeptide (simian sarcoma viral, v-sis, oncogene homolog)	NM_002608	**0.21**	**0.3**
Genes involved in cell growth and differentiation				
	NOTCH4 Notch homolog 4 (Drosophila)	NM_004557	**0.11**	**0.16**
	AXL AXL receptor tyrosine kinase	NM_001699	**0.06**	**0.11**
	CBFB Core-binding factor, beta subunit	NM_001755	**0.08**	**0.1**
	CDK4 Cyclin-dependent kinase 4	NM_000075	**0.03**	**0.05**
	IGFBP3 Insulin-like growth factor binding protein 3	NM_000598	**0.16**	**0.28**
	RAF1 V-raf-1 murine leukemia viral oncogene homolog 1	NM_002880	**0.1**	**0.13**
	RHOA Ras homolog gene family, member A	NM_001664	**0.56**	**0.56**
	RHOC Ras homolog gene family, member C	NM_004040	**0.03**	**0.04**
	SMO Smoothened homolog (Drosophila)	NM_005631	**0.21**	**0.27**
	GDF15 Growth differentiation factor 15	NM_004864	**0.14**	**0.08**
	CDC25B Cell division cycle 25 homolog B (S. pombe)	NM_004358	**0.16**	**0.24**

Table 4.6. continue

Gene group function	Gene name	Accession number	Fold change TVM-A12$_{sp}$	Fold change Clone$_{sp}$
Genes involved in cell growth and differentiation				
	CDKN1A Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	**0.06**	**0.06**
	CLNS1A Chloride channel, nucleotide-sensitive, 1A	NM_001293	**0.05**	**0.06**
	CUL1 Cullin 1	NM_003592	**0.05**	**0.06**
	NF2 Neurofibromin 2(bilateral acoustic neuroma)	NM_000268	**0.06**	**0.09**
	NME1 Non-metastatic cells 1, protein (NM23A)	NM_000269	**0.45**	**0.52**
	TBRG4 Transforming growth factor beta regulator 4	NM_004749	**0.17**	**0.2**
	TIMP1 TIMP metallopeptidase inhibitor 1	NM_003254	**0.08**	**0.09**
	NOTCH2 Notch homolog 2 (Drosophila)	NM_024408	**0.11**	**0.17**
	CCND1 Cyclin D1	NM_053056	**0.10**	**0.14**
	IER3 Immediate early response 3	NM_003897	**0.06**	**0.08**
	IGFBP4 Insulin-like growth factor binding protein 4	NM_001552	**0.24**	**0.35**
	MYC V-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467	**0.41**	**0.4**
	NPM1 Nucleophosmin,nucleolar phosphoprotein B23, numatrin	NM_199185	**0.07**	**0.13**
	GAS6 Growth arrest-specific 6	NM_000820	**0.08**	**0.1**
	GDF15 Growth differentiation factor 15	NM_004864	**0.14**	**0.08**
	CAPN1 Calpain 1, (mu/I) large subunit	NM_005186	**0.17**	**0.31**
	CAPNS1 Calpain, small subunit 1	NM_001749	**0.09**	**0.14**

Table 4.6. continue

Gene group function	Gene name	Accession number	Fold change TVM-A12$_{sp}$	Fold change Clone$_{sp}$
Genes involved in cell growth and differentiation				
	CDC2L5 Cell division cycle 2-like 5 (cholinesterase-related cell division controller)	NM_003718	**0.21**	**0.26**
	NME2 Non-metastatic cells 2, protein (NM23B)	NM_002512	**0.33**	**0.48**
	TOB1 Transducer of ERBB2, 1	NM_005749	**0.05**	**0.06**
Genes involved in signal transduction				
	CBLB Cas-Br-M (murine) ecotropic retroviral transforming sequence b	NM_170662	**0.06**	**0.07**
	FZD2 Frizzled homolog 2 (Drosophila)	NM_001466	**0.06**	**0.1**
	MYD88 Myeloid differentiation primary response gene (88)	NM_002468	**0.23**	**0.21**
	STC1 Stanniocalcin 1	NM_003155	**0.05**	**0.07**
	SMO Smoothened homolog (Drosophila)	NM_005631	**0.21**	**0.27**
	DVL1 Dishevelled, dsh homolog 1 (Drosophila)	NM_004421	**0.04**	**0.07**
	TLE1 Transducin-like enhancer of split 1 (E (sp1) homolog, Drosophila)	NM_005077	**0.49**	**0.57**
	GNB2 Guanine nucleotide binding protein (G protein), beta polypeptide 2	NM_005273	**0.26**	**0.34**
	RGS19 Regulator of G-protein signalling 19	NM_005873	**0.08**	**0.09**
	GNA13 Guanine nucleotide binding protein (G protein), alpha 13	NM_006572	**0.21**	**0.23**
	GRB2 Growth factor receptor-bound protein 2	NM_002086	**0.12**	**0.17**
	PDPK1 3-phosphoinositide dependent protein kinase-1	NM_002613	**0.09**	**0.17**
	PRKAR1A Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	NM_002734	**0.2**	**0.44**

Table 4.6. continue

Gene group function	Gene name	Accession number	Fold change TVM-A12$_{sp}$	Fold change Clone$_{sp}$
Genes involved in signal transduction				
	RASGRF1 Ras protein-specific guanine nucleotide-releasing factor 1	NM_153815	**0.46**	**0.54**
	RAF1 V-raf-1 murine leukemia viral oncogene homolog 1	NM_002880	**0.1**	**0.13**
	NOTCH2 Notch homolog 2 (Drosophila)	NM_024408	**0.11**	**0.17**
	NOTCH4 Notch homolog 4 (Drosophila)	NM_004557	**0.11**	**0.16**
	RHOA Ras homolog gene family, member A	NM_001664	**0.56**	**0.56**
	SIAH2 Seven in absentia homolog 2 (Drosophila)	NM_005067	**0.06**	**0.08**
	RHOC Ras homolog gene family, member C	NM_004040	**0.03**	**0.04**
	TCF7L2 Transcription factor 7-like 2, T-cell specific, HMG-box	NM_030756	**0.2**	**0.26**
	PDGFRA Platelet-derived growth factor receptor, alpha polypeptide	NM_006206	**0.13**	**-**
	AKT1 V-akt murine thymoma viral oncogene homolog 1	NM_005163	**0.49**	**-**
	SMO Smoothened homolog (Drosophila))	NM_005631	**0.21**	**0.27**
	ILK Integrin-linked kinase	NM_004517	**0.56**	**-**
	RHOB Ras homolog gene family, member B	NM_004040	**0.26**	**-**
Genes involved in cell motility and adhesion				
	FN1 Fibronectin 1	NM_002026	**0.08**	**0.1**
	GNA13 Guanine nucleotide binding protein (Gprotein), alpha 13	NM_006572	**0.21**	**0.23**
	ILK Integrin-linked Kinase	NM_004517	**0.56**	**-**
	TIMP1 TIMP metallopeptidase inhibitor 1	NM_003254	**0.08**	**0.09**
	TIMP3 TIMP metallopeptidase inhibitor 3	NM_000362	**0.21**	**0.023**

4.3 REAL TIME PCR

Few genes with special correlation to current study involved in human melanoma growth, invasion and metastasis and to viral replication and transcription were selected for real-time PCR confirmation. A total of 36 genes were selected and the levels of their expression were determined using TVM-A12 melanoma cell line as control line, and GUSB as the house-keeping gene (Table 4.7) (See appendix).

Table 4.7. Fold change in gene expression in comparison to adherent TVM-A12 cell line obtained by real-time PCR

Fold change in expression ($2^{-\Delta\Delta CT}$)				
Gene name	**TVM-A12 1%**	**TVM-A12$_{sp}$**	**Clone$_{sp}$**	**Gene function**
BHLHB2	2.03	0.15	0.566	Transcription factor, anti apoptotic
MYC	1.655	0.326	0.183	Negative regulator of invasion
PTEN	1.473	467.9	2645	Tumour suppressor, up regulation lead to activation of HIV-1transcription
VEGFA	1.965	284	1727	Positive regulator of invasion
CSK	3.344	4089	58521	Inhibits tumour proliferation, migration and invasion
PITCH1	1.486	624.9	7692	Tumour suppressor
FOXG1A	5.823	115	274.7	The increase in hepatoblastoma was associated with undifferentiated state.
TP53	4.154	1843.6	36654.4	Tumour suppressor
VHL	0.008	54.57	328.4	Antiapoptotic
WNT3	0.703	100.6	1420	Cell growth and differentiation
MYCN	0.241	7.516	72.34	Amplification associated with poor outcome neuroblastoma regulate VEGF expression, oncogene
MYCL1	0.344	2.676	11.37	Oncogene
BTK	0.748	2.949	9.574	Antiapoptotic
CCND2	0.364	2.258	11.45	Drives cell cycle G1 to S
WNT2	0.133	2.943	10.17	Positive regulator of invasion
TIMP3	0.343	0.744	1.755	Negative regulator of invasion, Proapoptotic
IRF3	0.293	0.809	1.358	Innate antiviral response
GTF2I	0.302	0.824	3.253	Induce HIV-1 latency
FRAP1	0.365	0.97	7.046	Promotes growth and invasion

Table 4.7. continue

Fold change in expression ($2^{-\Delta\Delta CT}$)				
Gene name	**TVM-A12 1%**	**TVM-A12$_{sp}$**	**Clone$_{sp}$**	**Gene function**
CTNNB1	0.945514	1.172835	8.07	Promotes NOTCH1 mediated melanoma growth
E2F1	0.571	0.587	3.666	Antiapoptotic, transcription factor
ARHGAP5	1.25	0.292	1.555	Trasduction signal
ARHGEF5	0.792	0.221	1.917	Oncogene
GPR39	0.575	0.411	5.043	Antiapoptotic
ITGB4	0.526	0.808	5.673	Positive regulator of invasion, cell matrix adhesion; promote early intravascular metastasis
ANAXA7	0.392	0.194	0.354	Negative regulator of invasion in melanoma
CTNNA1	0.765	0.111	0.309	Negative regulator of invasion
NME	0.129	0.039	0.038	Negative regulator of invasion
RRM1	0.353	0.092	0.132	Negative regulator of invasion
CDKN1A	0.337	0.165	0.891	Negative regulator of invasion
XRCC6	0.199	0.222	0.348	Negative regulator of invasion in melanoma
HDAC1	0.45	0.198	0.176	Negative regulator of invasion
TOB1	0.857	0.507	0.415	Tumour suppressor
TRAM1	0.72	0.311	0.769	Positive regulator of invasion, significant predictor of lymphnode metastasis
CD59	0.378	0.09	0.332	Increase in tumours (help escape from complement mediated Ab cytotoxicity), decrease in HIV infection
BRCA2	0.55	0.335	0.908	Positive regulator of invasion, DNA repair, cell cycle regression

Statistical analysis for real-time PCR data was done using SPSS.12. software. Out of the 36 genes tested 16 genes showed significant modulation with p value < 0.05 including: CTNNB1, E2F1, PTEN, VEGFA, VHL, CSK, PITCH1, ARHGEF5, MYCL1, FOXG1A, BHLHB2, BTK, CCND2, TP53, GTF2I, and FRAP1.

CHAPTER 5. DISCUSSION

Human melanoma is a highly invasive tumour that has been linked extensively with HERV-K expression. Although there are theoretical and laboratory evidences to implicate HERV-K in melanomagenesis, it is not yet known whether the expression of HERV-K mRNA and proteins and the formation of viral particles are epiphenomena associated with tumorogenesis or are etiological agents playing a significant role in melanoma induction or progression. Given their ubiquity in the population, it is difficult to assign a pathological role to HERVs investigated so far, but insertionally polymorphic HERVs merit further investigation as they might represent novel genetic risk factor for human melanoma. Importantly, there has been a recent drive towards molecular classification of melanoma as a result of molecular genetic studies demonstrating specific genotype-phenotype correlation (Fecher *et al.* 2007). Recently, a significant correlation of ultraviolet radiation and melanoma site to BRAF/NRAS gene mutation and other genetic events has been demonstrated using array based comparative genomic hybridization.

Investigation of the association of insertionally polymorphic HERVs with melanoma, and correlation of HERV expression in primary melanoma with clinicopathological subtype, site and Breslow thickness may add to this molecular classification.

Oncogenic virus cause cancers by inducing genetic mutations directly or by expressing oncoproteins and there is little direct evidence that HERVs act trough these mechanisms. Further functional analyses of the HERV-K accessory proteins Rec and Np9 are required to ascertain whether they are important in the causation of melanoma.

5.1. MICROARRAY-BASED GENE EXPRESSION ANALYSIS OF ADHERENT TVM-A12

Preliminary screening of the general pattern of genetic modification in adherent TVM-A12 melanoma cell line and TVM-A12-derived non-adherent cells (TVM-A12$_{sp}$ and Clone$_{sp}$) was done using microarray technology. Prior to detachment, adherent TVM-A12 grown in RPMI 10% FBS, showed modulation of 116 gene involved in various

functions including invasion and metastasis potential, apoptosis, tumour suppression, cell growth and differentiation and cell motility. Of these, 21 genes were up regulated, including 4 anti apoptotic genes (VHL, GPR39, BTK, E2F1), 2 transcription factors (E2F1, BHLHB2), 7 positive regulator of metastasis and invasion (VEGFA, WNT2, ARHGEF5, TRAM1, ITGB4, BRCA2, and FOXGIA), and a number of genes involved in cell cycle, growth and differentiation and motility (Table 4.3).. At the same time, adherent TVM-A12 1% FBS showed down regulation of 95 genes including 2 tumour suppressors (BARD1 and NF2), 7 pro apoptotic (TNFRSF1A, TNFRSF1B, CUL1, TIMP3, SIVA1, GSPT1 and RAP5A), 9 negative regulator of invasion and metastasis (CANX, ANXA7, RHOA, CTNNA1, HSPA5, NME1, RRM1, TIMP1, and TIMP3) and a large number of genes involved in cell cycle, growth and differentiation, signal transduction and cell motility (Table 4.4). The above pattern indicate that serum deprivation causes genetic modification in melanoma cell line in favor of decrease tumour suppression, decrease apoptosis and increase invasive and metastatic potential along with stimulation of a number of transcription factors.

5.2. MICROARRAY-BASED GENE EXPRESSION ANALYSIS OF NON ADHERENT TVM-A12$_{sp}$ AND CLONE$_{sp}$

Melanoma derived non-adherent TVM-A12$_{sp}$ showed modulation of 166 genes of which only 2 were up regulated and 164 genes were down regulated. Up regulated genes seen in microarray test were the anti apoptotic gene VHL, and the cell cycle regulator CCND2 (Table 4.5). On the other hand, non adherent cells showed down regulation of 4 tumour suppressor genes (BARD1, NF2, TOB1, and TP53) 16 negative regulator of metastasis and invasion including the 9 genes down regulated in adherent TVM-A12 along with CCND1, CDKN1A, XRCC6, HDAC1, IER3, MYC, and PRDX2. They also showed down regulation of IRF3, which play a role in innate antiviral immune response (Doyle *et al*. 2002), and also act as tumour suppressor (Kim *et al*. 2003), and GTF2I , which was found to induce HIV-1 latency in response to T-cell activation (Chen *et al*. 2005). In addition, non adherent cell showed down regulation of 13 pro apoptotic genes including the 7 pro apoptotic genes down regulated in adherent TVM-A12 along with TNFRSF10B, TP53, NME3, NOTCH2,

CDKN1A, and IER3 (Table 4.6). Again, the pattern of modulation seen in non adherent TVM-A12$_{sp}$ is in favour of decrease tumour suppression, decrease apoptosis, and promote more malignant phenotype which was expected to be seen in anchorage independent, rapidly growing TVM-A12$_{sp}$.

Clone$_{sp}$, derived from TVM-A12 by limiting dilution, showed a pattern of genetic modulation very similar to TVM-A12$_{sp}$, obtained by serum deprivation, with modulation of 152 genes. Out of the 440 genes tested, only 14 genes were modulated in TVM-A12$_{sp}$ but not in clone$_{sp}$ indicating that serum deprivation produces a cell type phenotypically as well as genotypically very similar to clone$_{sp}$ (Table 4.5, Table 4.6).

5.3. CONFIRMATION OF GENE EXPRESSION USING REAL-TIME PCR

Due to the large amount of data obtained from microarray technology and due to the relative decreased sensitivity and specificity of microarray technique, which is based on hybridization, in comparison to real-time PCR, confirmation of the level of expression of selected genes was done using real-time PCR (Table 4.7). It was observed that, before detachment, TVM-A12 grown in RPMI 1% FBS showed transient up regulation of transcription factor BHLHB2, which becomes down regulated in suspended TVM-A12$_{sp}$ and in Clone$_{sp}$. BHLHB2 is a DNA binding protein that was found to bind E box within LTR, causing inhibition of E box and further removal of its inhibitory effect on HIV-1 and HTLV-1 transcription (Terme *et al.* 2009). The fact that BHLHB2 has an effect on retroviruses transcription could lead to the conclusion that it might be involved in the activation of HERV-K transcription required to induce morphological modification in TVM-A12 grown in serum deprived medium. At the same time adherent TVM-A12 grown in RPMI 1% FBS started to show up regulation of PTEN, VEGFA, CSK, PITCH1, FOXG1A and TP53 which continue to rise in suspended TVM-A12 and even higher in Clone$_{sp}$. The up regulation of tumour suppressor genes PTEN, PITCH1 and TP53 were not expected but can be explained by the fact that PTEN up regulation was found to activate human immunodeficiency-1 (HIV-1) transcription (Cook *et al.* 2002) and therefore might have a role in the activation of Human Endogenous Retrovirus-K transcription, and the role of TP53 is not clearly known in human melanoma as it was observed to be

normal or strongly expressed in many melanoma (Bennett 2008). VEGFA (vascular endothelial growth factor A) play an important role in promoting tumour invasion and metastasis and was found to correlate significantly with melanoma progression (Brychtova *et al*. 2008). Finally, FOXG1A, has been associated with maintenance of undifferentiated state in hepatoblastoma tumour and was found to be higher in tumours with embryonal and small cell component in comparison to pure fetal hepatoblastoma (Adesina *et al*. 2007).

Out of the 36 genes tested using real-time PCR, 26 genes were down regulated in adherent TVM-A12 1% FBS including 7 negative regulator of invasion and metastasis (ANXA7, CTNNA1, NME1, RRM1, CDKN1A, XRCC6 and HDAC1) and TOB1 tumour suppressor which started to show down regulation that continued to progress in non adherent TVM-$A12_{sp}$ and also in $Clone_{sp}$. Down regulation of adhesion molecule CTNNA1 and DNA repair gene XRCC6 expression were associated with melanoma progression (Bonitsis *et al*. 2006; Korabiowska *et al*. 2004). On the other hand, down regulation of ANXA7, NME1, RRM1, CDKN1A, and HDAC1 were associated with poor prognosis in a number of malignancies including: ANXA7 in gastric cancer, NME1 in lung and nasopharyngeal carcinoma, RRM1 in lung cancer, CDKN1A in prostatic, rectal and hepatocellular carcinoma, and HDAC1 in breast cancer (Hsu *et al*. 2008; Ma *et al*. 2008; Liu *et al*. 2008; Gautam *et al*. 2003; Roy *et al*. 2008; Hu *et al*. 2008; Kao *et al*. 2007; Zhang *et al*. 2005).

HDAC inhibitors are currently proposed as adjuvant therapy in HIV-1 infection due to the fact that they induce activation of HIV expression in latently infected cells and therefore, aids in the elimination of virally infected reservoirs (Demonte *et al*. 2004). Various HDAC inhibitors are available including Valproic acid, currently used for epilepsy and bipolar disorders, phenybutyrate, which is used in β chain haemoglobinopathies, and more recently, SAHA (vorinostat) which is currently approved by the FDA (Food and Drug Administration) for the treatment of cutaneous T cell lymphoma. The fact that HDAC inhibitor induces HIV transcription along with the fact that HERV-K has been associated with HIV multiplication leads to the conclusion that HDAC inhibitors might also activate HERV-K expression since the effect of HDAC inhibitors is general on cellular transcription and not specific for HIV-1 induction. These facts go along with the observation that increased HERV-K

expression in melanoma derived non-adherent cells is associated with significant down regulation of HDAC.

Adherent TVM-A12 1% FBS also showed down regulation of membrane inhibitor of reactive lysis CD59, which is commonly found to be increased in tumours to help them escape complement mediated antibody cytotoxicity (Fishelson *et al.* 2003) but interestingly this gene was found to be decreased in HIV infection (Schmitz *et al*. 1995). In addition, all the genes up regulated in non adherent cells were transiently down regulated in adherent TVM-A12 prior to detachment except CTNNB1, which promote NOTCH1 mediated melanoma growth and metastasis (Balint *et al*. 2005), which was found to be normal in adherent TVM-A12 and non adherent TVM-$A12_{sp}$ and up regulated in $Clone_{sp}$.

Non adherent TVM-$A12_{sp}$ and $Clone_{sp}$, on the other hand, showed up regulation of anti apoptotic gene VHL and BTK along with cell cycle regulator cyclin D2 (CCND2) which drives the cells from G_1 to S phase of cell cycle. Furthermore, they showed up regulation of MYCN whose amplification was found to be associated with poor outcome in neuroblastoma (Moreau *et al*. 2006). WNT2 and WNT3 were also up regulated and they play roles in promoting tumour invasion and metastasis and in cell growth and maintenance respectively (Cheng *et al*. 2005; Mazieres *et al*. 2005).

$Clone_{sp}$ in particular showed up regulation of transcription factor and anti apoptotic gene E2F1, along with FRAP1, CTNNB1, ARHGEF5, and ITGB4 which were found to promote invasion and metastasis (Lasithiotakis *et al.* 2008; Karbowniczek *et al*. 2008; Takahashi *et al*. 2008; Gujra *et al*. 2008; Van der Velden *et al.* 2003) and also ARHGEF5 oncogene (Debily *et al*. 2004)

$Clone_{sp}$ also showed up regulation of IRF3, which play a role in innate antiviral immune response (Doyle *et al*. 2002) and GTF2I, which was found to induce HIV1 latency in response to T cell activation (Chen *et al*. 2005).

Genetic alterations in melanoma have been extensively studied and evidently, not all melanomas carry somatic mutations in the investigated genes.

In conclusion, human malignant melanoma is a highly malignant tumour with poor prognosis and inadequate response to available therapeutic modalities. More recently,

a trend for molecular classification of human melanoma has emerged, which demonstrated phenotype-genotype association. In continuation to previous work done at the University of Rome, Tor Vergata, in which the transition of melanoma cell line TVM-A12 from adherent to non adherent phenotype under stress condition was associated with loss of Melan A/ MART-1 and HLA class I expression indicating more malignant behavior and increased HERV-K *env* gene and particles production. HERV-K has been linked extensively with human melanoma but its timing and significant is not yet fully understood. It was observed that onset of morphological modification under stress condition coincide with the peak increase in HERV-K *env* gene expression and that interference with HERV-K expression abolish morphological transition under stress condition. current study has demonstrated that the peak increase in HERV-K env expression, which proceed the morphological transition, was associated with certain genetic modulation in gene expression namely, transient activation of transcription factor BHLHB2 along with other modulations previously observed by other researcher to be involved in the acquisition of a more malignant phenotype such as up regulation of VEGFA and FOX1A and down regulation of ANXA7, NME1, RRM1, CDKN1A, and HDAC. Down regulation of HDAC and up regulation of tumour suppressor PTEN is proposed to have a role in the activation of HERV-K expression as explained earlier. Therefore the use of HDAC inhibitor, which is currently proposed as an adjuvant therapy for HIV, based on its ability to activate the expression of HIV-1 in latently infected reservoirs cell need to be carefully tested for its role in the activation of HERV-K., which could have deleterious effects in HIV patients. Furthermore, the acquisition, in the cell line studied, of the ability to grow stably in suspension under stress conditions and in $Clone_{sp}$ was associated with the activation of anti apoptotic genes VHL and BTK along with cell cycle regulator CCND2. In addition, suspended cells showed modulation of other genes found previously to be involved with more malignant behavior such as, the up regulation of MYCN and WNT2. $Clone_{sp}$, obtained by limiting dilution, showed up regulation of transcription factor E2F1, up regulation of a number of positive regulators of invasion and metastasis namely, FRAP1, CTNNB1, ARHGEF5, and ITB4 and GTF2I which was found to induce HIV-1 latency in response to Tcell activation.

Therefore, this study describes, for the first time, a unique cellular system which demonstrates the association between increased HERV-K expression and the

acquisition of more malignant genotype and that can be a useful tool for better understanding of the pathogenesis human melanoma and the effect of pharmacological agents and genetic modulators on HERV-K expression and melanoma progression.

CHAPTER 6. REFERENCES

Adelman M.K., Marchalonis J.J. (2002). Endogenous retroviruses in systemic lupus erythematosis: candidate lupus viruses. Clinical Immunology **102**: 107-116.

Adesina A.M., Nguyen Y., Guanaratne P., Pulliam J., Lopez-Terrada D., Margolin J., Finegold M. (2007). FOXG1 is overexpressed in hepatoblastoma. Human Pathology **38**: 400-409.

Andersson M.L., Medstrand P., Yin H., Blomberg J. (1996). Differential expression of human endogenous retroviral sequences similar to mouse mammary tumor virus in normal peripheral blood mononuclear cells. AIDS Research Human Retroviruses. **12**: 833-840.

Armbruester V., Sauter M., Krautkraemer E., Meese E., Kleiman A., Best B., Roemer K., Mueller-Lantzsch N. (2002). A novel gene from the human endogenous retrovirus K expressed in transformed cells. Clinical Cancer Research. **8**: 1800-1807.

Arnaud F., Varela M., Spencer T.E., Palmarini M. (2008). Coevolution of endogenous betaretroviruses of sheep and their host.Cell Mol Life Sci. **65**: 3422-3432.

Balda B.R., Hehelmann R., Cho J.R., Spiegelman S. (1975). Oncornavirus-like particle in human skin cancer. Proc. Nat. Acad. Sci. **72**: 3697-3700.

Balint K., Xiao M., Pinnix C.C., Soma A., Veres I., Juhasz I., Brown E.J., Capobianco A.J., Herlyn M., Liu Z-J. (2005). Activation of Notch1 signaling is required for β-catenin–mediated human primary melanoma progression. Clinical investigation **115**: 3166–3176.

Bannert N., Kurth R. (2004). Retroelements and the human genome: new perspectives on an old relation. Proc Natl Acad Sci U S A. **101** Suppl 2: 14572-14579.

Bannert N., Kurth R. (2006). The evolutionary dynamics of human endogenous retroviral families. Annu Rev Genomics Hum Genet. **7:** 149-173.

Barbulescu M., Turner G., Seaman MI., Deinard AS., Kidd KK., Lenz J. (1999). Many human endogenous retrovirus K (HERV-K) proviruses are unique to humans. Current Biology **9**: 861-868.

Belshaw R., Dawson AL., Woolven-Allen J., Redding J., Burt A., Tristem M. (2005). Genomewide screening reveals high levels of insertional polymorphism in the human endogenous retrovirus family HERV-K (HML2): implications for present-day activity. Journal of Virolog **79**: 12507-12514.

Bennett D.C. (2008). How to make a melanoma: what do we know of the primary clonal events? Pigment Cell Melanoma Res.**21**: 27-38.

Bieda K., Hoffmann A., Boller K. (2001). Phenotypic heterogeneity of human endogenous retrovirus particles produced by teratocarcinoma cell lines. Journal of General Virology **82**: 591- 596.

Birkmayer G.D., Balda B.R., Miller F. (1974). Onocorna- viral information in human melanoma. European Journal of Cancer **10**: 419-424.

Birkmayer G.D., Balde B.R., Miller F., Braun-Falco O. (1972). Virus like particles in metastasis of human malignant melanoma. Naturwissenschaften **58**: 369-370.

Bjerregaard B., Holck S., Christensen I.J., Larsson L-I. (2006). Syncytin is involved in breast cancer-endothelial cell fusions. Cellular and Molecular Life Sciences **63**: 1906-1911.

Boeke M. and Stoye J.P. (1997). Retrotrasposons, endogenous retroviruses, and the evolution of retroelements. In: Retroviruses. Coffin J.M., Hughes S.H., Varmus H.E. (Eds). Cold Spring Harbor Laboratory Press, New York. pp 343-346.

Boller K., Schönfeld K., Lischer S., Fischer N., Hoffmann A., Kurth R., Tönjes RR. (2008). Human endogenous retrovirus HERV-K113 is capable of producing intact viral particles. J Gen Virol. **89**: 567-572.

Bonitsis N., Batistatou A., Karantima S., Charalabopoulos K. (2006). The role of cadherin/catenin in malignant melanoma. Experimental oncology **28**: 187–193.

Brychtova S., Bezdekova M., Brychta T., Tichy M. (2008). The role of vascular endothelial growth factors and their receptors in malignant melanoma. Neoplasma **55**: 273-279.

Büscher K., Hahn S., Hofmann M., Trefzer U., Ozel M., Sterry W., Löwer J., Löwer R., Kurth R., Denner J. (2006). Expression of the human endogenous retrovirus-K transmembrane envelope, Rec and Np9 proteins in melanomas and melanoma cell lines. Melanoma Res. **16**: 223-234.

Buscher K., Trefzer U., Hofmann M., Sterry W., Kurth R., Denner J. (2005). Expression of human endogenous retrovirus K in melanoma and melanoma cell line. Cancer Research **65**: 4172- 4180.

Carbone A. (2002). AIDS-related non Hodgkin's lymphomas: from pathology and molecular pathogenesis to treatment. Human Pathology **4**: 392-404.

Chen J., Malcolm T., Estable M.C., Roeder R.G., Sadowski I. (2005). TFII-I Regulates Induction of Chromosomally Integrated Human Immunodeficiency Virus Type 1 Long Terminal Repeat in Cooperation with USF. Journal of virology **79**: 4396–4406.

Cheng X-X., Wang Z-C., Chen X-Y., Sun Y., Kong Q-Y., Liu J., Li H. (2005). Correlation of Wnt-2 expression and b-catenin intracellular accumulation in Chinese gastric cancers: relevance with tumour dissemination. Cancer Letters **223:** 339–347.

Christensen T. (2005). Association of human endogenous retroviruses with multiple sclerosis and possible interactions with herpes viruses. Rev Med Virol **15**: 179-211.

Christensen T., Dissing Sørensen P., Riemann H., Hansen HJ., Møller-Larsen A. (1998). Expression of sequence variants of endogenous retrovirus RGH in particle form in multiple sclerosis. Lancet **352**: 1033.

Christensen T., Dissing Sørensen P., Riemann H., Hansen HJ., Munch M., Haahr S., Møller-Larsen A. (2000). Molecular characterization of HERV-H variants associated with multiple sclerosis. Acta Neurol Scand. **101**: 229-238.

Clark W.H. Jr., Elder D.E., Guerry D. 4th, Braitman L.E., Trock B.J., Schultz D., Synnestvedt M., Halpern A.C. (1989). Model predicting survival in stage I melanoma based on tumor progression. J Natl Cancer Inst. **81**: 1893-1904.

Contreras-Galindo R., González M., Almodovar-Camacho S., González-Ramírez S., Lorenzo E., Yamamura Y. (2006). A new Real-Time-RT-PCR for quantitation of human endogenous retroviruses type K (HERV-K) RNA load in plasma samples: increased HERV-K RNA titers in HIV-1 patients with HAART non-suppressive regimens. Journal of Virological Methods **136**: 51-57.

Contreras-Galindo R., Kaplan M.H., Leissner P., Verjat T., Ferlenghi I., Bagnoli F., Giusti F., Dosik M.H., Hayes D.F., Gitlin S.D., Markovitz D.M. (2008). Human Endogenous Retrovirus K (HML-2) elements in the plasma of people with lymphoma and breast cancer. Journal of Virology **82**: 9329-9336.

Contreras-Galindo R., López P., Vélez R., Yamamura Y. (2007). HIV-1 infection increases the expression of human endogenous retroviruses type K (HERV-K) *in vitro*. AIDS Research Human Retroviruses **23**: 116-122.

Cook J.A., August A., Henderson A.J. (2002). Recruitment of Phosphatidylinositol 3-Kinase to CD28 Inhibits HIV Transcription by a Tat-Dependent Mechanism. The journal of immunology **169**: 254-260.

Danen E.H., de Vries T.J., Morandini R., Ghanem G.G., Ruiter D.J., van Muijen G.N. (1996). E-cadherin expression in human melanoma. Melanoma Res **6**:127-131.

Debily M.A., Camarca A., Ciullo M., Mayer C., El Marhomy S., Ba I., Jalil A., Anzisi A., Guardiola J., Piatier-Tonneau D. (2004). Expression and molecular characterization of alternative transcripts of the ARHGEF5/TIM oncogene specific for human breast cancer. Human Molecular genetics **13**: 323-334.

Demonté D., Quivy V., Colette Y., Van Lint C. (2004). Administration of HDAC inhibitors to reactivate HIV-1 expression in latent cellular reservoirs: implications for the development of therapeutic strategies. Biochemical Pharmacology **15**: 1231-1238.

Denne M., Sauter M., Armbruester V., Licht J.D., Roemer K., Mueller-Lantzsch N. (2007). Physical and functional interactions of human endogenous retrovirus proteins Np9 and rec with the promyelocytic leukemia zinc finger protein.Journal of Virology **81**: 5607-5616.

Dirksen E.R , Levy J.A. (1977). Virus-like particles in placentas from normal individuals and patients with systemic lupus erythematosus. J Natl Cancer Inst. **59**: 1187-1192.

Doyle S.E., Vaidya S.A., O'Connell R., Dadgostar H., Dempsey P.W., Wu T.T., Rao G., Sun R., Haberland M.E., Modlin R.L.,and Cheng G. (2002). IRF3 Mediates a TLR3/TLR4-Specific Antiviral Gene Program. Immunity **17**: 251–263.

Epstein W.L., Fukuyama K., Benn M. (1968). Transmission of a pigmented melanoma in golden hamsters by a cell-free ultrafiltrate. Nature **219**: 979-980.

Fecher L.A., Cummings S.D., Keefe M.J., Alani R.M. (2007). Toward a molecular classification of melanoma. Journal of Clinical Oncology **25**: 1606- 1620.

Fishelson Z., Donin N., Zell S., Schultz S., Kirschfink M. (2003). Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. Molecular Immunology **40**: 109-123.

Forsman A., Yun Z., Hu L., Uzhameckis D., Jern P., Blomberg J. (2005). Development of broadly targeted human endogenous gammaretroviral pol-based real time PCRs Quantitation of RNA expression in human tissues. Journal of Virological Methods **129**: 16-30.

Galli U.M., Sauter M., Lecher B., Maurer S., Herbst H., Roemer K., Mueller-Lantzsch N. (2005). Human endogenous retrovirus rec interferes with germ cell development in mice and may cause carcinoma in situ, the predecessor lesion of germ cell tumors. Oncogene **24**: 3223–3228.

Garraway L.A., Widlund H.R., Rubin M.A., Getz G., Berger A.J., Ramaswamy S., Beroukhim R., Milner D.A., Granter S.R., Du J., Lee C., Wagner S.N., Li C., Golub T.R., Rimm D.L., Meyerson M.L., Fisher D.E., Sellers W.R. (2005). Integrative

genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature **436**: 117-122.

Garson J.A., Tuke P.W., Giraud P., Paranhos-Baccala G., Perron H. (1998). Detection of virion-associated MSRV-RNA in serum of patients with multiple sclerosis. Lancet. **351**(9095): 33.

Gautam A., Li Z-R., Bepler G. (2003). RRM1-induced metastasis suppression through PTEN-regulated pathways. Oncogene **22**: 2135–2142.

Gifford R., Tristem M. (2003). The evolution, distribution and diversity of endogenous retroviruses. Virus Genes **26**: 291-315.

Goff S.P. (2001). Retroviridae: the retroviruses and their replication. In: Fundamental virology. Knipe D.M., Howley P.M (Eds). Lippincott Williams and Wilkins. pp 843-894.

Gottardi C.J., Wong E., Gumbiner B.M. (1997). E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling characterisctics of the normal human conjunctiva and cornea. Eye **11**: 607-612.

Granter S.R., Weilbaecher K.N., Quigley C., Fisher D.E. (2002). Role for microphthalmia transcription factor in the diagnosis of metastatic malignant melanoma. Appl Immunohistochem Mol Morphol **10**: 47-51.

Gray-Schopfer V., Wellbrock C., Marais R. (2007). Melanoma biology and new targeted therapy. Nature **445**: 851-7.

Gujra T.S., Veelen W.V., Richardson D.S., Myers S.M., Meens J.A., Acton D.S., Dun˜ach M., Elliott B.E., Ho¨ppener J.W.M., Mulligan L.M. (2008). A novel RET kinase–B-catenin signaling pathway contributes to tumorigenesis in thyroid carcinoma. Cancer Research **68**: 1338–1346.

Haass N.K., Smalley K.S., Li L., Herlyn M. (2005). Adhesion, migration and communication in melanocytes and melanoma. Pigment Cell Res. **18**: 150-159.

Hahn S., Ugurel S., Hanschmann K-M., Strobel H., Tondera C., Schadendorf D., Löwer J., Löwer R. (2008). Serological Response to Human Endogenous Retrovirus K in Melanoma Patients Correlates with Survival Probability. AIDS Research and Human Retroviruses **24**: 717-723.

Hayward W.S., Neel B.G., Astrin S.M. (1981). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature. **290**: 475-480.

Herrmann M., Neidhart M., Gay S., Hagenhofer M., Kalden J.R. (1998). Retrovirus-associated rheumatic syndromes. Curr Opin Rheumatol. **10**: 347-354.

Hirschl S., Schanab O., Seppele H., Waltenberger A., Humer J., Wolff K., Pehamberger H., Muster T. (2007). Sequence variability of retroviral particles derived from human melanoma cells, Melanoma associated retrovirus. Virus Research **123**: 211-215.

Hoek K.S. (2007). DNA microarray analyses of melanoma gene expression: a decade in the mines.Pigment Cell Res **20**: 466-484.

Hsu M.Y, Meier F.E, Nesbit M., Hsu J.Y., Van Belle P., Elder D.E., Herlyn M. (2000). E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and downregulates expression of invasion-related adhesionreceptors Am.J Pathol **156:** 1515-1525.

Hsu M.Y, Wheelock M.J, Johnson K.R., Herlyn M. (1996). Shift in cadherin profiles between human normal melanocytes and melanoma J. Invest. Dermatol Symp. Proc. **1**: 188-194.

Hsu P-I., Huang M-S., Chen H-C., Hsu P-N., Lai T-C., Wang J-L., Lo G-H., Lai K-H, Tseng C-J., Hsiao M. (2008). The significance of ANXA7 expression and its correlation with poor cellular differentiation and enhanced metastatic potential of gastric cancer. Journal of Surgical Oncology **97**: 609–614.

Hu T-H., Tai M-H., Chuah S-K., Chen H-H., Lin J-W., Huang H-Y., Chou Y-P., Yi L-N., Kuo C-M., Chanchien C-S. (2008). Elevated p21 expression is associated with

poor prognosis of rectal stromal tumors after resection. Journal of Surgical Oncology **98**: 117–123.

Huang J., Ren T., Guan H., Jiang Y., Cheng H. (2009). HTLV-1 Tax is a critical lipid raft modulator that hijacks IkappaB kinases to the microdomains for persistent activation of NF-kappaB. J Biol Chem. **284**: 6208-6217.

Humer J., Waltenberger A., Grassauer A., Kurz M., Valencak J., Rapberger R., Hahn S., Löwer R., Wolff K., Bergmann M., Muster T., Mayer B., Pehamberger H. (2006). Identification of a melanoma marker derived from melanoma-associated endogenous retroviruses.Cancer Res. **66**: 1658-1663.

International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genoma. (2001) Nature **409**: 860-921.

Johnston J.B., Silva C., Holden J., Warren K.G., Clark A.W., Power C. (2001). Monocyte activation and differentiation augment human endogenous retrovirus expression: implications for inflammatory brain diseases. Ann Neurol. **50**: 434-442.

Kalter S.S., Helmke R.J., Heberling R.L., Panigel M., Fowler A.K., Strickland J.E., Hellman A. (1973). Brief communication: C-type particles in normal human placentas. J Natl Cancer Inst. **50**: 1081-1084.

Kao J-T., Chuah S-K., Huang C-C., Chen C-L., Wang C-C., Hung C-H., Chen C-H, Wang J-H., Lu S-N., Lee C-M., Changchien C-S., Hu T-H. (2007). P21/WAF1is an independent survival prognostic factor for patients with hepatocellular carcinoma after resection. Liver international **27**: 772-781.

Karbowniczek M., Spittle C.S., Morrison T., Wu H., Henske E.P. (2008). mTOR is activated in the majority of malignant melanomas. Journal of investigative dermatology **128:** 980–987.

Karlsson H., Bachmann S., Schroder J., McArthur J., Torrey E.F., Yolken R.H. (2001). Retroviral RNA identified in the cerebrospinal fluid and brain of individuals with schizophrenia. PNAS **98**: 4634-4639.

Katzourakis A., Tristem M. (2005). Phylogeny of human endogenous and exogenous retroviruses. In: Retroviruses and primate genome evolution. Sverdlov (Ed). Landes Bioscience pp. 186-203.

Kim T.Y., Lee K-H., Chang S., Chung C., Lee H-W., Yim J., Kim T.K. (2003). Oncogenic Potential of a Dominant Negative Mutant of Interferon Regulatory Factor 3. The journal of biological chemistry **278**: 15272- 15278.

Koike K. (2007). Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways. J Gastroenterol Hepatol. Suppl **1**: 108-111.

Korabiowska M.,Bauer H., Quentin T., Stachura J.,Cordon-Cardo C., Brinck U. (2004). Application of new in situ hybridization probes for Ku 70 and Ku 80 in tissue microarray of paraffin-embedded malignant melanoma: correlation with immunohistochemical analysis. Human pathology **35**: 210-216.

Krone B., Kolmel K.F., Henz B.M., Grange J.M. (2005). Protection against melanoma by vaccination with Bacille- Calmette-Guerin (BCG) and/or vaccinia: an epidemiology- based hypothesis on the nature of melanoma risk factor and its immunological response. European Journal of Cancer **41**: 104-117.

Krone B., Kölmel K.F., Grange J.M., Mastrangelo G., Henz B.M., Botev I.N., Niin M., Seebacher C., Lambert D., Shafir R., Kokoschka E.M., Kleeberg U.R., Gefeller O., Pfahlberg A. (2003). Impact of vaccinations and infectious diseases on the risk of melanoma--evaluation of an EORTC case-control study. Eur J Cancer. **39**: 2372-2378.

Lasithiotakis K.G., Sinnberg T.W., Schittek B., Flaherty K.T., Kulms D., Maczey E., GarbeC., Meier F.E. (2008). Combined inhibition of MAPK and mTOR signaling inhibits growth, induces cell death, and abrogates invasive growth of melanoma cells. Journal of investigative dermatology **128:** 2013–2023.

Levy C., Khaled M., Fisher D.E. (2006). MITF: master regulator of melanocyte development and melanoma oncogene. Trends Mol Med **12**: 406-414.

Li G., Satyamoorthy K., Herlyn M. (2001). N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cell Cancer Res. **61**: 3819-3825.

Lin L., Xu B., Rote N.S. (1999). Expression of endogenous retrovirus ERV-3 induces differentiation in BeWo, a choriocarcinoma model of human placental trophoblast. Placenta **20**: 109–118.

Liu S.,Sun Y., Tian D., He Y., Zeng L., He Y, Ling C., Sun S. (2008). Downregulated NM23-H1 expression is associated with intracranial invasion of nasopharyngeal carcinoma. British Journal of Cancer **98**: 363 – 369.

Lower R. (1999). The pathogenic potential of endogenous retroviruses: facts and fantasies. Trend in Microbiology **7**: 350-356.

Löwer R., Boller K., Hasenmaier B., Korbmacher C., Müller-Lantzsch N., Löwer J., Kurth R. (1993). Identification of human endogenous retroviruses with complex mRNA expression and particle formation. Proc Natl Acad Sci U S A. **90**: 4480-4484.

Löwer R., Löwer J., Tondera-Koch C., Kurth R. (1993). A general method for the identification of transcribed retrovirus sequences (R-U5 PCR) reveals the expression of the human endogenous retrovirus loci HERV-H and HERV-K in teratocarcinoma cells. Virology **192**: 501-511.

Löwer R., Tönjes R.R., Korbmacher C., Kurth R., Löwer J. (1995). Identification of a Rev-related protein by analysis of spliced transcripts of the human endogenous retroviruses HTDV/HERV-K J Virol. **69**: 141-149.

Ma W., Chen J., Xue X., Wanga Z., Liu H., Wanga T., Bai Y., Tang S-C., Zhou Q. (2008). Alteration in gene expression profile and biological behavior in human lung cancer cell line NL9980 by nm23-H1 gene silencing. Biochemical and Biophysical Research Communications **371**: 425–430.

Mangeney M., Pothlichet J., Renard M., Ducos B, Heidmann T. (2005). Endogenous retrovirus expression is required for murine melanoma tumor growth in vivo. Cancer Research **65**: 2588-2591.

Marguerat S., Wang WY., Todd JA., Conrad B. (2004). Association of human endogenous retrovirus K-18 polymorphisms with type 1 diabetes. Diabetes. **53**: 852-854.

Martin J., Herniou E., Cook J., Waugh O'Neill R., Tristem M. (1997). Human endogenous retrovirus type I-related viruses have an apparently widespread distribution within vertebrates. J Virol. **71**: 437-443.

Mastrangelo G., Kroneb B., Faddaa E., Bujaa A., Grangec J.M., Rausaa G., Vriesd E., Koelmele K. (2008). Does yellow fever 17D vaccine protect against melanoma?. Vaccine **8645**: 1-4.

Mayer J., Sauter M., Rácz A., Scherer D. Mueller-Lantzsch N., Meese E. (1999). An almost-intact human endogenous retrovirus K on human chromosome 7. Nature **21**: 257-258.

Mazieres J., You L., He B., Xu Z., Twogood S., Lee A.Y., Reguart N., Batra S., Mikami I., Jablons D.M. (2005). Wnt2 as a new therapeutic target in malignant pleural mesothelioma. International journal of cancer **117**: 326–332.

Melino G., Sinibaldi-Vallebona P., D'Altri S., Annichiarico- Petruzzelli M., Rasi G., Catani M.V., Tartaglia R.L., Vernole P., Spagnoli L.G., Finazzi-Agrò A., Garaci E. (1993). Characterization of three melanoma cell lines (TVM-A12, TVM-A197, TVM-BO) sensitivity to lysis and effect of retinoic acid Clin. Chem. Enzymol. Commun. **6**: 105–119.

Michaloglou C., Vredeveld L.C, Soengas M.S, Denoyelle C., Kuilman T., Van der Horst C.M., Majoor D.M., Shay J.W., Mooi W.J., Peeper D.S. (2005). $BRAF^{E600}$-associated senescence-like cell cycle arrest of human naevi. Nature **436**: 720-724.

Miller A.J., Mihm M.C. (2006). Melanoma. New England Journal of Medicine. **355**: 51-65.

Miller A.J., Du J., Rowan S., Hershey C.L., Widlund H.R., Fisher D.E. (2004). Transcriptional regulation of the melanoma prognostic marker melastatin (TRPM1) by MITF in melanocytes and melanoma. Cancer Res **64**: 509-516.

Miller A.J., Mihm M.C. (2006). Melanoma. N Engl J Med. **355**: 51-65.

Molès J.P., Tesniere A., Guilhou J.J. (2005). A new endogenous retroviral sequence is expressed in skin of patients with psoriasis. Br J Dermatol. **53**: 83-89.

Moreau L.A., McGrady P., London W.B., Shimada H., Cohn S.L., Maris J.M., Diller L., Look A.T., George R.E. (2006). Does MYCN amplification manifested as homogeneously staining regions at diagnosis predict a worse outcome in children with neuroblastoma?a children's oncology group study. Clinical Cancer Research **12**: 5693- 5697.

Morgan D., Brodsky I. (2004). Human endogenous retrovirus (HERV-K) particles in megakaryocytes cultured from essential thrombocythemia peripheral blood stem cells. Exp Hematol. **6**: 520-525.

Moyes D., Griffiths D.J., Venables P.J. (2007). Insertional polymorphisms: a new lease of life for endogenous retroviruses in human disease. Trends Genet. **23**: 326-333.

Muir A., Lever A., Moffett A. (2004). Expression and functions of human endogenous retroviruses in the placenta: an update. Placenta **25**: 16-25.

Muster T., Waltenberger A., Grassauer A., Hirschl S., Caucig P., Romirer I., Födinger D., Seppele H., Schanab O., Magin-Lachmann C., Löwer R., Jansen B., Pehamberger H., Wolff K. (2003). An endogenous retrovirus derived from human melanoma cells. Cancer Research **63**: 8735-8741.

Perils C.,Herlyn M. (2004). Recent advances in melanoma biology. The Oncologist **9**: 182-187.

Perron H., and Lang A. (2009). The Human Endogenous Retrovirus Link between Genes and Environment in Multiple Sclerosis and in Multifactorial Diseases Associating Neuroinflammation. Clinical Review Allergy Immunology **39**: 51-61.

Perron H., Garson J.A., Bedin F., Beseme F., Paranhos-Baccala G., Komurian-Pradel F., Mallet F., Tuke P.W., Voisset C., Blond J.L., Lalande B., Seigneurin J.M.,

Mandrand B. (1997). Molecular identification of a novel retrovirus repeatedly isolated from patients with multiple sclerosis. The Collaborative Research Group on Multiple Sclerosis. Proc Natl Acad Sci U S A. **94**: 7583-7588.

Pollock P.M., Harper U.L., Hansen K.S., Yudt L.M., Stark M., Robbins C.M., Moses T.Y., Hostetter G., Wagner U., Kakareka J., Salem G., Pohida T., Heenan P., Duray P., Kallioniemi O., Hayward N.K. Trent J.M., Meltzer P.S. (2003). High frequency of BRAF mutations in nevi. Nat Genet **33**: 19-20.

Pullmann R. Jr., Bonilla E., Phillips P.E., Middleton F.A., Perl A. (2008). Haplotypes of the HRES-1 endogenous retrovirus are associated with development and disease manifestations of systemic lupus erythematosus. Arthritis Rheum **58**: 532-540.

Qi J., Chen N., Wang J., Siu C.H. (2005). Transendothelial migration of melanoma cells involves N-cadherin- mediated adhesion and activation of the beta-catenin signaling pathway. Mol. Biol. Cell. **16**: 4386-4397.

Quatresooz P., Pierard G E., Pierard-Franchimont C. Mosan Study Group of Pigmented Tumors. (2009). Molecular pathways supporting the proliferation staging of malignant melanoma. Int J Mol Med. **24**: 295-301.

Reynier F., Verjat T., Turrel F., Imbert P.E., Marotte H., Mougin B., Miossec P. (2009). Increase in human endogenous retrovirus HERV-K (HML-2) viral load in active rheumatoid arthritis. Scand J Immunol. **70**: 295-299.

Romalde J.L., Luz Vilariño M., Beaz R., Rodríguez J.M., Díaz S., Villalba A., Carballal M.J. (2007). Evidence of retroviral etiology for disseminated neoplasia in cockles (Cerastoderma edule). J Invertebr Pathol. **94**: 95-101.

Roy S.,Singh R., Agarwal C., Siriwardana S., Sclafani R., Agarwal R. (2008). Downregulation of both p21/Cip1 and p27/Kip1 produces a more aggressive prostate cancer phenotype. Cell cycle **12**: 1828-1835.

Ruda V.M., Akopov S.B., Trubetskoy D.O., Manuylov N.L., Vetchinova A.S., Zavalova L.L., Nikolaev L.G., Sverdlov E.D. (2004). Tissue specificity of enhancer and promoter activities of a HERV-K(HML-2) LTR. Virus Res.**104**: 11-16.

Ruprechta K., Mayerb J., Sautera M., Roemerc K., Mueller-Lantzscha M. (2008). Endogenous retroviruses and cancer. Cellular and Molecular Life Science **65**: 3366-3382.

Sauter M.,Schommer S., Kremmer E.,Remberger K., Do¨ Lken G., Lemm I., Buck M., Best B., Neumann-Haefelin D., Mueller-Lantzsch N. (1995). Human endogenous retrovirus K10: expression of Gag protein and detection of antibodies in patients with seminomas. Journal of Virology **69:** 414-421.

Schiavetti F., Thonnard J., Colau D., Boon T., Coulie P.G. (2002). A Human endogenous retroviral sequence encoding an antigen recognized on melanoma by cytolytic T lymphocytes. Cancer Resreach **62:** 5510-5516.

Schiffman M., Castle P E., Jeronimo J., Rodriguez A C., Wacholder S. (2007). Human papillomavirus and cervical cancer. Lancet. **370**: 890-907.

Schmitz J., Zimmer J.P., Kluxen B., Anes S., Bogel M., Gigii I., Schmitz H. (1995). Antibody-dependent complement-mediated cytotoxicity in sera from patients with HIV-1 infection is controlled by CD55 and CD59. Journal of clinical investigation **96**: 1520-1526.

Seifarth W., Baust C., Murr A., Skladny H., Krieg-Schneider F., Blusch J., Werner T., Hehlmann R., Leib-Mösch C. (1998). Proviral structure, chromosomal location, and expression of HERV-K-T47D, a novel human endogenous retrovirus derived from T47D particles. J Virol.**72**: 8384-8391.

Seifarth W., Frank O., Zeilfelder U., Spiess B., Greenwood A.D., Hehlmann R., Leib-Mösch C. (2005). Comprehensive analysis of human endogenous retrovirus transcriptional activity in human tissues with a retrovirus-specific microarray. J Virol. **79**: 341-352.

Selzer E., Wacheck V., Lucas T., Heere-Ress E., Wu M., Weilbaecher K.N., Schlegel W., Valent P., Wrba F., Pehamberger H., Fisher D., Jansen B. (2002). The melanocyte-specific isoform of the microphthalmia transcription factor affects the phenotype of human melanoma. Cancer Res **62**: 2098-2103.

Serafino A., Balestrieri E., Pierimachi P., Matteuci C., Moroni G., Oricchio E., Rasi G., Mastino A., Spadafora C., Garaci E., Sinibaldi Vallebona P. (2009). The activation oh human endogenous retrovirus K (HERV-K) is implicated in melanoma cell malignant transformation. Exp. Cell Res. **315**: 849-862.

Slominski A., Tobin D.J., Shibahara S., Wortsman J. (2004). Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev.**84**: 1155-1228.

Stauffer Y., Theiler G., Sperisen P., Lebedev Y., Jongeneel V. (2004). Digital expression profile of human endogenous retroviral families in normal and cancerous tissue. Cancer Immunity **4**: 2-19.

Strick R., Ackermann S., Langbein M., Swiatek J., Schubert S.W., Hashemolhosseini S., Koscheck T., Fasching P.A., Schild R.L., Beckmann M.W., Strissel P.L.(2007). Proliferation and cell–cell fusion of endometrial carcinoma are induced by the human endogenous retroviral Syncytin-1 and regulated by TGF-β. Journal of molecular medicine **85**: 23-38.

Takahashi Y., Nishikawa M., Suehara T., Takiguchi N.,Takakura Y. (2008). Gene silencing of b-catenin in melanoma cells retards their growth but promotes the formation of pulmonary metastasis in mice. International journal of cancer **123**: 2315–2320.

Tang A., Eller M.S., Hara M., Yaar M., Hirohashi S., Gilchrest B.A. (1994). E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes *in vitro* J Cell Sci **107**: 983-992.

Tarlinton R.E., Meers J., Young P.R. Retroviral invasion of the koala genome. (2006). Nature. **442**: 79-81.

Terme J.M., Calvignac S., Dodon M.D.,Gazzolo L., Jordan A. (2009). E box motifs as mediators of proviral latency of human retroviruses. Retrovirology **6**: 81-85.

Tönjes R.R., Löwer R., Boller K., Denner J., Hasenmaier B., Kirsch H., König H., Korbmacher C., Limbach C., Lugert R., Phelps R.C., Scherer J., Thelen K., Löwer J.,

Kurth R. (1996). HERV-K: the biologically most active human endogenous retrovirus family. J Acquir Immune Defic Syndr Hum Retrovirol.**13**: 261-267.

Tucker G.C. (2006). Integrins: molecular targets in cancer therapy. Curr Oncol Rep. **8**: 96-103.

Turner G., Barbulescu M., Su M., Jensen-Seaman M.I., Kidd K.K., Lenz J. (2001). Insertional polymorphisms of full-length endogenous retroviruses in humans. Curr Biol. **11**: 1531-1535.

Van Der Velden P.A., Zuidervaart W., Hurks M.H.M.H., Pavey S., Ksander B.R., Krijgsman E., Frants R.R., Tensen C.P., Willemze R., Jager M.J., Gruis N.A. (2003). Expression profiling reveals that methylation of TIMP3 is involved in uveal melanoma development. International Journal of Cancer **106**: 472-479.

Wagner E.K., Hewlett M.J. (2004). Basic virology. Blackwell publishing. pp 357-376.

Wang-Johanning F., Liu J., Rycaj K., Huang M., Tsai K., Rosen D.G., Chen D.T., Lu D.W., Barnhart K.F., Johanning G.L. (2006). Expression of multiple human endogenous retrovirus surface envelope proteins in ovarian cancer. International Journal of Cancer **120**: 81–90.

Wang-Johanning F., Frost A.R., Jian B., Azuero R., Lu D.W., Chen D.T., Johanning G.L. (2003). Detecting the expression of human endogenous retrovirus E envelope transcript in human prostate adenocarcinoma. Cancer **98**: 187-197.

Wang-Johanning F., Frost A.R., Johanning G.L., Khazaeli M.B., LoBuglio A.F., Shaw D.R., Strong T.V. (2001). Expression of human endogenous retrovirus K envelope transcript in human breast cancer. Clinical Cancer Research **7**: 1553-1560.

Weiss R.A. (2001). Retroviruses and cancer. Curr Sci **81**: 528-534.

Weiss R.A. (2006). The discovery of endogenous retroviruses. Retrovirology**. 3:** 67-68.

Widlund H.R., Fisher D.E. (2003). Microphthalamia-associated transcription factor: a critical regulator of pigment cell development and survival. Oncogene **22**: 3035-3041.

Wilkinson D.A., Mager D.L., and Leong J.C. (1994). Endogenous human retroviruses. In: The retroviridae. Levy J.A. (Ed) Plenum Press, New York. pp.465-535

Willmore-Payne C., Holden J.A., Tripp S., Layfield L.J. (2005). Human malignant melanoma: detection of BRAF- and c-kit-activating mutations by high-resolution amplicon melting analysis. Hum Pathol. **36**: 486-493.

Yi J.M., Kim H.M., Kim H.S. (2001). Molecular cloning and phylogenetic analysis of the human endogenous retrovirus HERV-K long terminal repeat elements in various cancer cells.Mol Cells. **12**: 137-141.

Yi J.M., Kim H.M., Kim H.S. (2006). Human endogenous retrovirus HERV-H family in human tissues and cancer cells: expression, identification, and phylogeny. Cancer Letters **231**: 228-239.

Young L.S., Murray P.G. (2003). Epstein-Barr virus and oncogenesis: from latent genes to tumours. Oncogene **22**: 5108-5121.

Zavada J., Zavadova Z., Russ G. (1986). Rescue of presumptive viral information from human cell by a helper oncovirus. Journal of General Virology **67**: 1561-1569.

Zhang Z., Yamashita H., Toyama T., Sugiura H., Ando Y., Mita K., Hamaguchi M., Hara Y., Kobayashi S., Iwase H. (2005). Quantitation of HDAC1 mRNA expression in invasive carcinoma of the breast. Breast Cancer Research and Treatment **94**: 11–16.

Appendix 1. Cycle threshold obtained by real-time PCR of the house keeping gene GUSB in 4 cell types tested.

Gene	Cell type	Ct 1	Ct 2	Ct 3	Ct4	Ct 5	Ct 6	Mean
GUSB	**TVM-A12 10% FBS**	25.19	25.18	26.39	25.65	25.77	27.81	25.99
GUSB	**TVM-A12 1% FBS**	26.41	26.61	26.81	26.87	26.7	26.59	26.66
GUSB	**TVM-A12$_{sp}$**	34.37	34.2	35.73	34.06	33.9	34.13	34.39
GUSB	**CLONE$_{sp}$**	38.12	36.58	37.44	38.53	36.2	36.06	37.15

Appendix 2. Cycle threshold obtained by real-time PCR of 36 target genes in 4 TVM-A12 control cell lines (TVM-A12 10% FBS).

Target gene	TVM-A12 10% FBS (1)	TVM-A12 10% FBS (2)	TVM-A12 10% FBS (3)	TVM-A12 10% FBS (4)	Mean
CTNNB1	22.07	22.07	21.03	20.81	21.495
E2F1	26.75	26.43	25.77	25.43	26.095
PTEN	26.25	26.15	26.51	26.25	26.29
TRAM1	25.31	25.32	26.14	25.82	25.6475
VEGFA	28.14	27.97	27.58	27.3	27.7475
VHL	26.69	26.41	26.32	26.02	26.36
CD59	25.86	25.65	26.41	26.26	26.045
WNT3	33.22	34.89	34.56	32.12	33.6975
CSK	28.81	28.62	27.48	27.31	28.055
PITCH1	29.9	29.85	29.78	29.73	29.815
ARHGAP5	28.29	28.06	30.13	30.48	29.24
ARHGEF5	29.26	29.08	28.86	28.79	28.9975
GPR39	28.99	28.61	31.99	31.68	30.3175
ITGB4	32.01	31.53	29.07	29.05	30.415
BRCA2	28.01	27.85	28.82	29.17	28.4625
MYCN	34.71	33.55	33.42	32.66	33.585
MYCL1	32.61	32.2	31.99	32.6	32.35
FOXG1A	36.45	40	34.93	36.4	36.94
BHLHB2	27.12	27.1	25.19	25.35	26.19
BTK	31.43	31.07	30.4	30.15	30.76
CCND2	32.69	32.71	32.28	31.75	32.36
WNT2	32.24	32.28	31.99	32.25	32.19
ANXA7	26.78	26.73	27.25	27.28	27.01
CTNNA1	24.92	24.54	25.18	25.42	25.01
NME1	23.07	23.04	24.58	24.51	23.80
RRM1	25.61	25.92	25.37	25.44	25.59
TIMP3	26.29	27.13	27.62	27.63	27.17
CDKN1A	24.08	24.02	28.01	26.78	25.72
XRCC6	21.61	21.53	25	24.86	23.25
HDAC1	26.46	26.3	25.69	25.57	26.01
MYC	25.84	25.61	25.19	25.04	25.42
TOB1	27.49	27.33	27.1	27.04	27.24
TP53	28.1	28.3	27.17	27.54	27.78
IRF3	28.39	28.14	29.72	29.55	28.95
GTF2I	27.41	27.1	27.18	27.14	27.21
FRAP1	29.23	29.54	28.39	29.08	29.06

Appendix 3. Cycle threshold obtained by real-time PCR of 36 target genes in 4 TVM-A12 grown in 1% FBS (TVM-A12 1% FBS).

Target gene	TVM-A12 1% FBS (1)	TVM-A12 1% FBS (2)	TVM-A12 1% FBS (3)	TVM-A12 1% FBS (4)	Mean
CTNNB1	22.89	22.86	21.57	21.65	22.24
E2F1	28.39	28.27	26.95	26.67	27.57
PTEN	26.59	26.76	26.15	26.09	26.39
TRAM1	26.51	26.56	27.06	27.02	26.78
VEGFA	27.83	27.85	27.11	26.97	27.44
VHL	27.93	28	40	40	33.98
CD59	27.92	27.9	28.24	28.4	28.11
WNT3	34.87	35.45	34.49	34.68	34.87
CSK	26.73	26.57	27.26	27.36	26.98
PITCH1	30.04	30.07	29.82	29.71	29.91
ARHGAP5	28.06	27.93	31.01	31.34	29.58
ARHGEF5	30.22	30.2	29.73	29.85	30
GPR39	31.11	31.08	32.54	32.4	31.78
ITGB4	32.81	32.37	31.46	31.39	32.01
BRCA2	29.44	29.4	30.53	30.6	29.99
MYCN	38.7	38.65	33.61	34.26	36.30
MYCL1	37.38	35.03	33.25	32.57	34.56
FOXG1A	36.23	37.01	33.15	33.89	35.07
BHLHB2	25.74	25.78	25.94	25.88	25.83
BTK	31.73	32.01	31.63	32.02	31.85
CCND2	37.17	36.59	31.88	32.29	34.48
WNT2	31.94	32.31	38.8	40	35.76
ANXA7	29.04	29.17	29.37	29.87	29.36
CTNNA1	26.31	26.32	26.45	26.54	26.40
NME1	26.70	26.70	27.68	27.65	27.18
RRM1	27.75	27.61	28.37	28.62	28.09
TIMP3	29.5	29.04	30.15	30.17	29.72
CDKN1A	27.07	27.01	29.55	29.54	28.29
XRCC6	24.76	24.82	28.25	28.49	26.58
HDAC1	28.17	28.55	27.88	28.03	28.16
MYC	25.93	25.76	25.62	25.47	25.70
TOB1	28.99	29.15	27.83	27.89	28.47
TP53	27.03	26.96	26.47	26.44	26.73
IRF3	31.14	31.86	31.97	31.93	31.73
GTF2I	29.93	29.99	29.76	30.07	29.94
FRAP1	31.99	31.84	31.08	31.15	31.52

Appendix 4. Cycle threshold obtained by real-time PCR of 36 target genes in TVM-A12 1% FBS suspended cells (TVM-A12$_{sp}$)

Target gene	TVM-A12$_{sp}$ (1)	TVM-A12$_{sp}$ (2)	TVM-A12$_{sp}$ (3)	TVM-A12$_{sp}$ (4)	mean
CTNNB1	30.22	30.05	29.18	29.21	29.66
E2F1	35.09	35.76	34.89	35.31	35.26
PTEN	26.36	26.58	25.2	25.14	25.82
TRAM1	35.81	35.61	35.12	36.39	35.73
VEGFA	28.4	28.45	27.63	27.51	27.99
VHL	29.14	29.37	28.71	28.74	28.99
CD59	36.1	35.55	40	40	37.91
WNT3	35.62	34.85	35.18	36.13	35.44
CSK	24.1	24.2	24.67	24.86	24.45
PITCH1	28.93	28.95	29	28.83	28.92
ARHGAP5	39.33	40	39.29	39.04	39.41
ARHGEF5	40	39.03	40	39.27	39.57
GPR39	40	40	40	40	40
ITGB4	37.05	39.44	40	40	39.12
BRCA2	40	35.64	38.12	40	38.44
MYCN	40	39.92	38.5	37.88	39.07
MYCL1	38.27	40	40	39.05	39.33
FOXG1A	37.09	36.91	40	40	38.5
BHLHB2	35.38	36.32	37.59	40	37.32
BTK	37.32	37.24	37.72	38.13	37.60
CCND2	40	40	39.29	39.04	39.58
WNT2	40	36.13	40	40	39.03
ANXA7	40.00	40.00	35.49	36.92	38.10
CTNNA1	40.00	39.22	34.3	34.15	36.92
NME1	40.00	40.00	34.67	34.12	37.20
RRM1	40	40	35.08	35.94	37.76
TIMP3	35.17	36.06	37.16	36.9	36.32
CDKN1A	39.28	35.69	36.59	36.65	37.05
XRCC6	34.17	33.11	34.67	34.66	34.15
HDAC1	40	40	34.23	34.05	37.07
MYC	40	40	31.58	31.49	35.77
TOB1	40	40	34.27	33.52	36.95
TP53	25.05	25.46	25.89	26.23	25.66
IRF3	40	40	35.88	36.06	37.99
GTF2I	37.48	36.86	35.45	35.07	36.22
FRAP1	38.85	38.75	36.51	37.22	37.83

Appendix 5. Cycle threshold obtained by real-time PCR of 36 target genes in $clone_{sp}$

Target gene	$Cl_{sp}(1)$	$Cl_{sp}(2)$	$Cl_{sp}(3)$	$Cl_{sp}(4)$	Mean
CTNNB1	29.86	30.68	28.8	29.21	29.63
E2F1	35.58	36.11	34.61	35.21	35.37
PTEN	25.65	25.91	26.24	26.51	26.07
TRAM1	36.41	36.81	37.51	38	37.18
VEGFA	28.16	28.5	27.85	28.09	28.15
VHL	28.97	29.84	28.87	28.95	29.15
CD59	39.96	37.77	37.44	40	38.79
WNT3	35.51	32.04	35.75	34.23	34.38
CSK	23.27	23.5	23.21	23.52	23.37
PITCH1	28.13	28.2	27.82	28.1	28.06
ARHGAP5	40	40	39.04	40	39.76
ARHGEF5	38.86	38	40	40	39.21
GPR39	40	36.56	40	40	39.14
ITGB4	36.27	40	40	40	39.06
BRCA2	39.03	40	40	40	39.75
MYCN	40	39.71	36.9	37.65	38.56
MYCL1	40	40	40	40	40
FOXG1A	40	40	40	40	40
BHLHB2	40	40	36.36	36.31	38.16
BTK	39.02	40	37.27	38.35	38.66
CCND2	40	39.99	40	40	39.99
WNT2	40	40	40	40	40
ANXA7	40.00	40.00	40	40	40.00
CTNNA1	35.89	40.00	39.67	37.25	38.20
NME1	40.00	40.00	40	40	40.00
RRM1	40	40	40	40	40.00
TIMP3	35.72	36.66	39.01	40	37.85
CDKN1A	33.75	35.77	40	40	37.38
XRCC6	32.81	32.25	40	40	36.27
HDAC1	40	40	40	40	40.00
MYC	40	37.45	40	40	39.36
TOB1	40	40	40	40	40.00
TP53	23.42	23.83	24.54	24.64	24.11
IRF3	40	40	40	40	40.00
GTF2I	36.33	36.36	37.06	38.24	37.00
FRAP1	36.84	37.66	37.74	38.7	37.74