

**DEVELOPMENT OF A FUNCTIONAL  
SCREENING IN MELANOCYTES**

by

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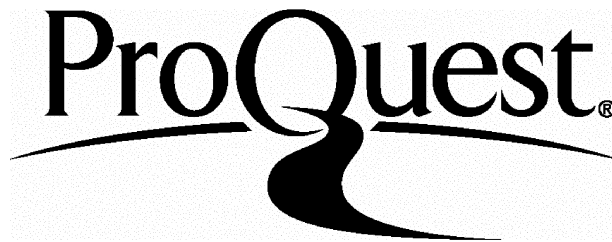
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## Abstract

Melanocytes are very specialised cells that produce melanin, the pigment responsible for skin, hair and retina colour. They originate during embryogenesis from precursor cells called melanoblasts. These are round shaped and unpigmented cells that migrate from the neural crest to the dermis and differentiate in highly pigmented and dendritic melanocytes after they have reached the skin and hair follicles.

Melanoma is a skin cancer that arises from the uncontrolled proliferation of melanocytes. Its incidence is rapidly increasing and it fails to respond to common treatment such as chemotherapy and radiotherapy. While initially it is very pigmented and confined to the upper layers of the dermis, it subsequently invades underneath tissues and forms metastasis that are frequently unpigmented. Indeed it has been suggested that melanoma progression involves a sort of dedifferentiation of the melanocyte into a transformed melanoma cell that morphologically is more similar to a melanoblast than to the melanocyte it originated from.

In this thesis we describe the development, optimisation and execution of a functional screening on the mouse melanocyte cell line Melan-a for the identification of new genes involved in melanocyte differentiation and melanoma progression. To do this, a mouse cDNA library was constructed in the pHMII retroviral vector. The marker chosen for the identification of the positive clones was pigmentation and the screening was based on the selection of genes that provoked the depigmentation of melanocytes. The conditions of the screening were optimised using the *Agouti* and activated *Ras* genes in reconstruction experiments. The positive clones isolated from the screening were characterised for their ability to inhibit melanin production when overexpressed in melanocytes, as well as to confer growth advantages and transformed phenotype.

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*To my wife Patrizia*

## Abbreviations

aa	Amino Acid
$\alpha$ -MSH	Alpha-Melanocyte Stimulating Hormone
ATP	Adenosine Triphosphate (similarly CTP)
bFGF	Basic Fibroblast Growth Factor
bp	Base-pairs
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid
HD	Homeobox Domain
HRP	Horseradish Peroxidase
IRES	Internal Ribosome Entry Site
kb	Kilo-base
LTR	Long Terminal Repeats
MLV	Murine Leukemia Virus
mRNA	Messenger Ribonucleic Acid
NLS	Nuclear Localisation Signal
PAGE	Poly-Acrylamide Gel Electrophoresis

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pHMII	HygroMaRX II Plasmid
PTU	Phenylthiourea
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
TPA	Tetradecanoyl Phorbol Acetate
Tris	Tris (hydroxymethyl) methylamine
Trp-1	Tyrosinase Related Protein 1 (similarly Trp-2)



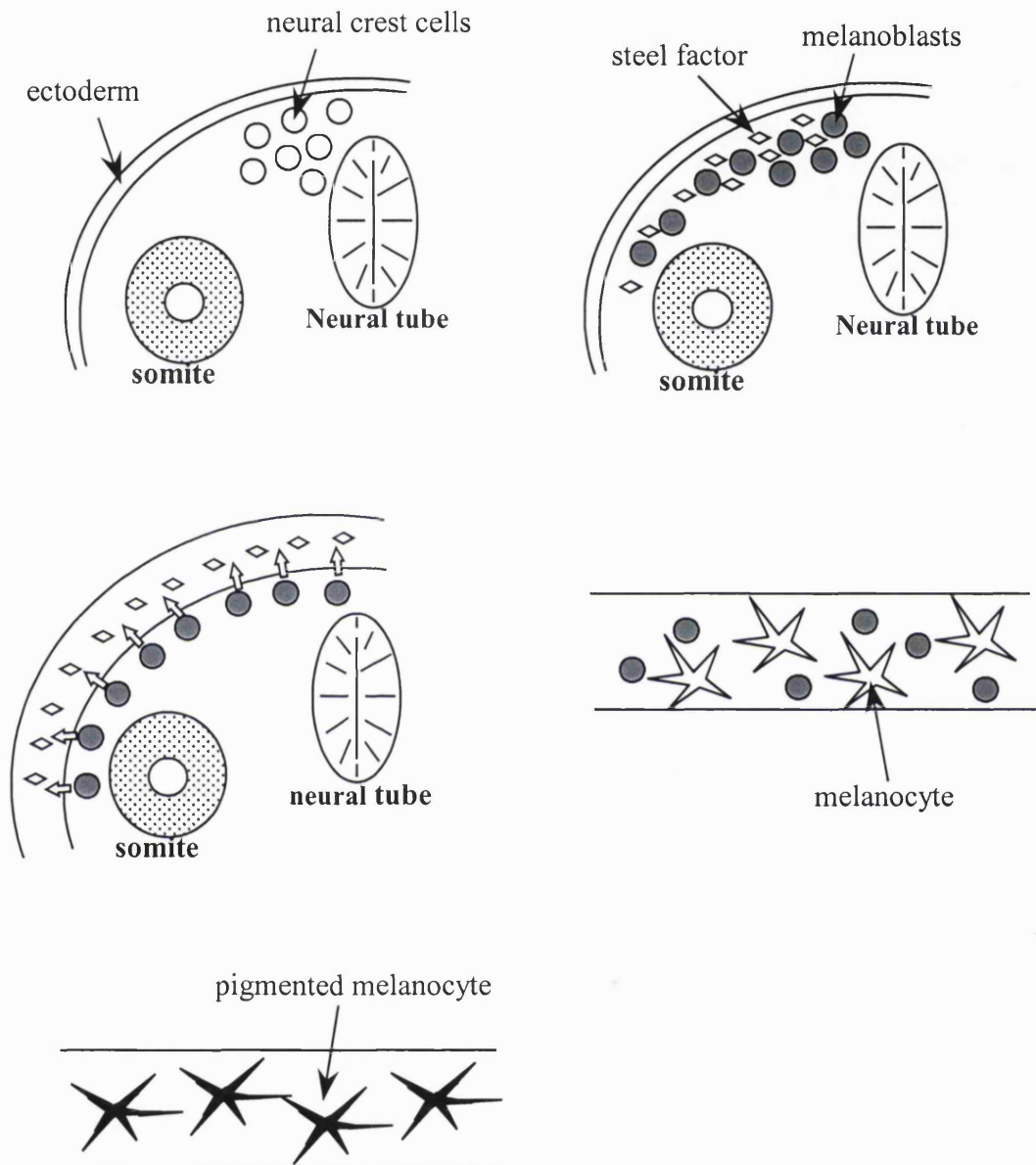
# **1 INTRODUCTION**

## **1.1 Melanocyte Biology**

Melanocytes are very specialised cells that produce melanin, the pigment responsible for skin, hair and retina colour (Spritz and Hearing, 1994). While melanocytes found in the Retina Pigment Epithelium originate from the optic cup, pigment cells of the skin and hair follicle derive from the neural crest (Bennett, 1991). In mouse, during embryogenesis, neural crest cells migrate along different pathways leading to the genesis of a variety of cell lineages including neurons and glia of the peripheral nervous system, gland cells, connective tissue in the craniofacial structures, and melanocytes (Wehrle-Haller *et al*, 1997). Mutations affecting the early stages of neural crest migration and differentiation are likely to affect multiple cell lineages. Thus, mutations in the *Pax3* gene, a transcription factor essential for the correct formation and closure of the neural tube, are lethal if homozygous and result in pigmentation and craniofacial defects when heterozygous (Epstein *et al*, 1991). Similarly, multiple cell lineage defects are observed when mutations occur in the endothelin-3 gene (*End3*) or its receptor, the endothelin receptor-B (*EdnrB*). Indeed mutations in this pathway will result in pigmentation defects associated with megacolon, a pathology caused by the absence of neural crest derived enteric ganglia

(Hosoda *et al*, 1994). Since mutant mice also lack melanocyte precursor cells, it is likely that the Edn3/EdnrB interaction acts at early stages of development, prior to the neural crest cell commitment to either the melanocyte or enteric ganglia lineage (Baynash *et al*, 1994).

Melanocytes originating from the neural crest migrate in a dorsolateral pathway between day 10 and 14 through an area delimited by the neural tube, the somites and the ectoderm known as MSA (Migrating Staging Area) (Manova *et al*, 1991; Yoshida *et al*, 1996). While migrating, they proliferate and commit to the pigment cell lineage by becoming melanoblasts, the melanocytes precursors. Finally, upon reaching their specific location in the dermis and hair follicles, they will fully differentiate in melanin producing melanocytes (Fig 1.1). Although little is known about the regulation of the melanoblasts migratory process to the dermis, it is clear that the Steel factor (SCF) and its receptor c-Kit play a crucial role (Wehrle-Haller *et al*, 1997). The Steel factor is found in two splice variants: a longer form that is cleaved on the outer side of the cell providing a soluble version of the protein, and a shorter form that stays anchored to the cell membrane (Wehrle-Haller *et al*, 1995). Both variants are essential for the survival and correct migration of the melanoblasts through the MSA to the dermis. In particular it is believed that the soluble form is important for initial survival and dispersal of melanoblasts in the early stages of migration, whereas the membrane bound form is critical for survival at later stages. In support of this data is the observation that melanoblasts are initially found on both



**Fig 1.1** Melanoblasts migration in the MSA during embryogenesis and subsequent differentiation in pigment producing melanocytes.  
(Modified from Wehrle-Haller B. and Weston J.A., 1997)

medial and dorsolateral migratory pathways, but only survive at the lateral one where SCF is found (Wehrle-Haller *et al*, 1995).

In addition to the key role played in defining the melanocytic lineage, the SCF/C-Kit pathway is also essential for haematopoiesis and gametogenesis. Indeed mutations in either the *SCF* or the *C-kit* genes often result in pigmentation defects associated to anaemia and sterility (Copeland *et al*, 1990; Besmer *et al*, 1993).

The differentiation of melanoblasts into pigment producing melanocytes involves the expression of melanocytes specific genes, particularly those responsible for melanin production (*Tyrosinase*, *Trp-1* and *Trp-2*), the expression of which greatly depends on the melanocytes-specific transcription factor microphthalmia. (Ganss *et al*, 1994; Yasumoto *et al*, 1994).

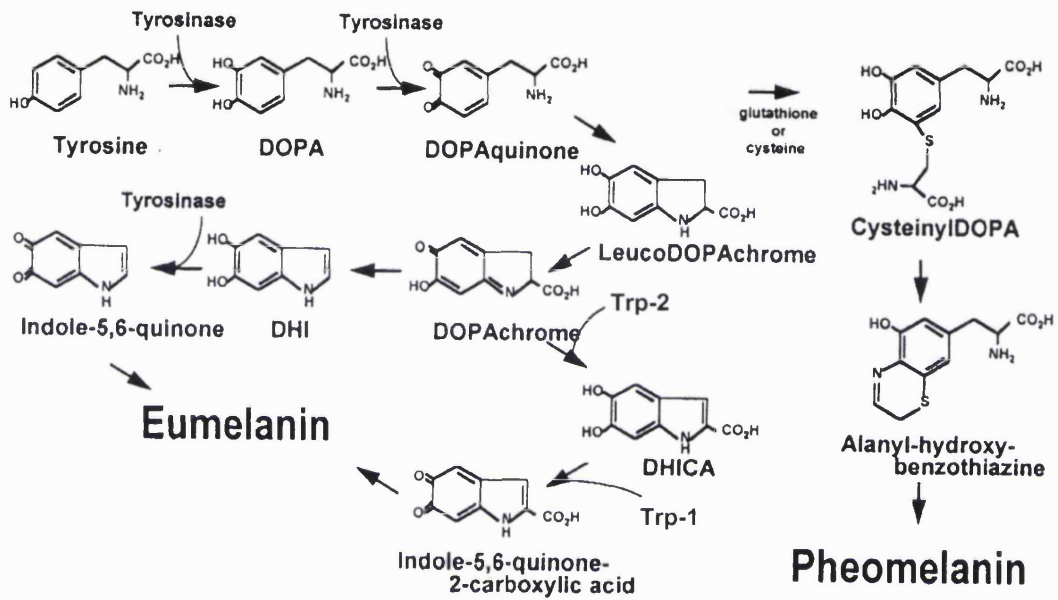
The activation of microphthalmia in melanoblasts causes profound functional and phenotypic changes in the cells, so that the melanocytes that originate have little resemblance to their precursors. The most obvious difference between the two cell types is the high degree of dendricity and pigmentation of melanocytes as opposed to the unpigmented and generally round-shaped melanoblasts (Hirobe *et al*, 1994; Sviderskaya *et al*, 1995). Interestingly, overexpression of microphthalmia in the NIH/3T3 fibroblast cell line induces the expression of melanocyte specific genes like *Tyrosinase* and *Trp-1* (Tachibana *et al*, 1996). It also provokes the formation of dendrites but is not followed by melanin production, probably because NIH/3T3 fibroblasts derive from albino Swiss mice carrying mutated tyrosinase gene.

Microphthalmia is a basic helix-loop-helix transcription factor that drives the expression of the melanogenic enzymes Tyrosinase, Trp-1 and Trp-2 through the

specific binding of a highly conserved element termed the M box (Yasumoto *et al*, 1994). Microphthalmia expression, like the SCF/C-Kit pathway, is not limited to the pigment cells lineage but is also found in different isoforms in other tissues. For example in the heart and in osteoclasts it is transcribed by an alternative promoter, resulting in a molecule which has a different amino-termini and is longer than the one found in melanocytes (Yasumoto *et al*, 1998). This explains why mutations in the microphthalmia gene can have pleiotropic effects like pigmentation defects, small eye size, reduced number of mast cells and osteopetrosis (Hodgkinson *et al*, 1993; Hughes *et al*, 1993).

Pigment production is a very specific function of melanocytes. The synthesis of melanin occurs in specialised cellular organelles called melanosomes, that are believed to be modified lysosomes (Jackson, 1994). Melanosomes display on their membrane the enzymes responsible for melanin production, with their catalytic domain directed towards the lumen of the vesicles. The pigment thus produced is accumulated inside the organelle until its interior is solely occupied by melanin. Melanosomes with accumulated pigment can undergo different fates: those produced by ocular melanocytes will remain in the cells whereas those produced by skin melanocytes will migrate along the dendrites from a perinuclear position to the periphery of the cell (Spritz and Hearing, 1994). Here, through the numerous dendrites of melanocytes, they are secreted and passed on to the surrounding keratinocytes which in turn migrate with the accumulated pigment toward the upper layers of the skin.

The primary substrate for melanin production is the L-Tyrosine amino acid. It is believed that this amino acid enters the melanosome through an as yet unidentified receptor (Gahl *et al*, 1995; Puri *et al*, 2000). Once in the organelle, the amino acid becomes the substrate of the melanogenic enzymes Tyrosinase, Trp-1 and Trp-2. The first two biochemical steps of the reaction are controlled by Tyrosinase, without which no pigment production is possible. Mutations in the *Tyrosinase* gene are known to be associated with type-1 oculocutaneous albinism, a disease characterised by severe reduction or total lack of pigmentation in the skin, hair and eyes (Spritz and Hearing, 1994). The tyrosinase related Trp-1 and Trp-2 enzymes catalyse subsequent steps of the reaction until the final product is formed (Spritz and Hearing, 1994) (Fig 1.2). Melanin can be found in two different forms: the black/brown eumelanin and the yellow/red pheomelanin. Wild-type mice coat colour in the dorsal region is characterised by the so called “agouti” pattern: the base and tip of the hair are black (eumelanin), whereas the middle portion is yellow (pheomelanin). This happens because during the hair growth, the melanocytes present in the hair follicle temporarily switch the production of pigment from the default eumelanin to pheomelanin (Bultman *et al*, 1992). The production of both qualities of pigment begins with the two steps catalysed by tyrosinase, after which the decision of making one or the other type of melanin is taken. Although environmental factors, like availability of sulfhydryls such as cysteine and glutathione, can promote the switch of melanin production, it is clear that there are also enzymatic pathways involved in the making of the decision. In particular the Melanocortin-1 receptor (Mc1r) and its ligands Agouti and  $\alpha$ -Melanocyte Stimulating Hormone ( $\alpha$ -MSH), are important

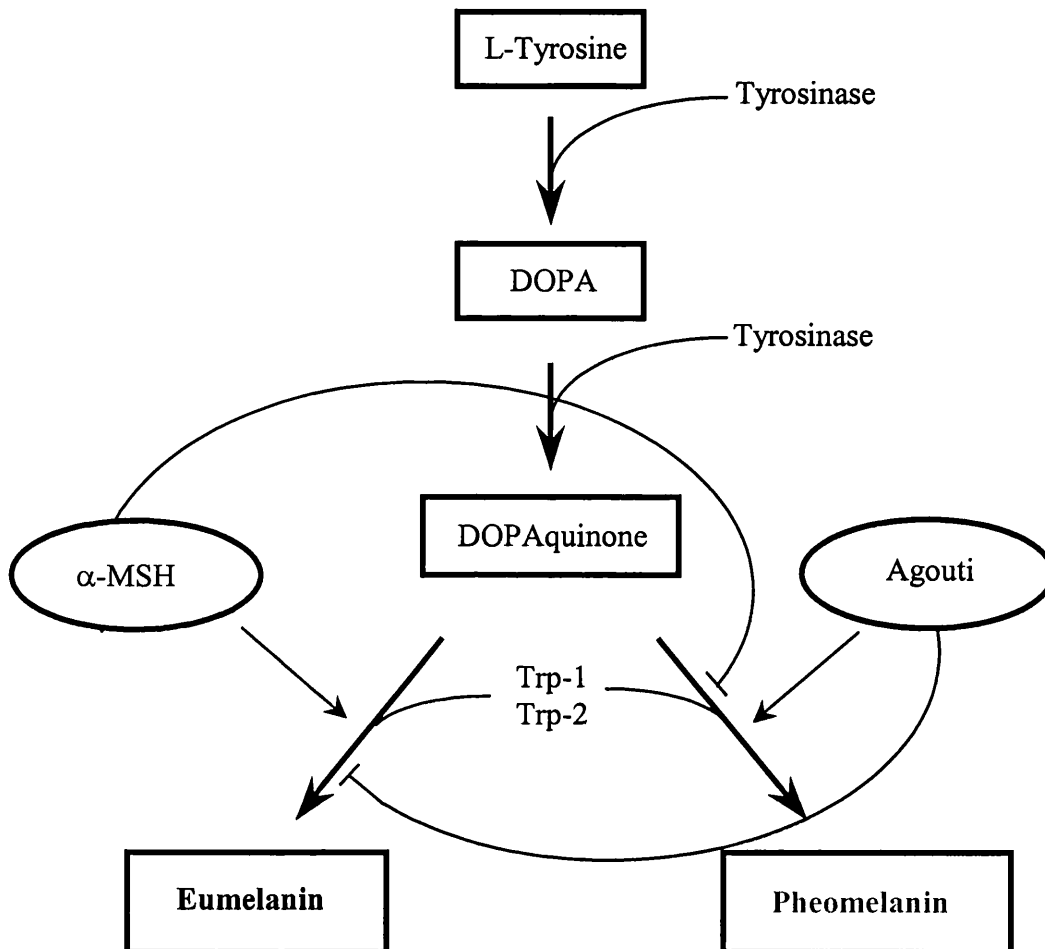


**Fig 1.2** Schematic of melanin biosynthetic pathway  
(Modified from Spritz and Hearing, 1994)

modulators of the pheomelanin versus eumelanin switch (Jordan *et al*, 1998; Ollmann *et al*, 1998).

The Melanocortin-1 receptor is a member of the seven transmembrane receptor family that is specifically expressed by melanocytes and exposed on their plasma membrane (Robbins *et al*, 1993). It normally interacts with  $\alpha$ -MSH, a 13 amino acid peptide derived from post-transcriptional modifications of the *Proopiomelanocortin* gene product. This provokes intracellular signalling mediated by the G-proteins the receptor is coupled to, causing an increase in cytoplasmic cAMP levels which in turn have been shown to cause upregulation and activation of the microphthalmia transcription factor (Bertolotto *et al*, 1998). The result is an increased transcription of the melanogenic enzymes and production of eumelanin. The *Agouti* gene product is a secreted molecule that is expressed by cells in the epidermis but not by melanocytes themselves (Bultman *et al*, 1992; Millar *et al*, 1995). *Agouti* is an antagonist of the Mc1r and its effect on melanocytes is to compete with  $\alpha$ -MSH for binding to the receptor thereby inhibiting its activity and to promote pheomelanin production (Ollmann *et al*, 1998) (Fig 1.3). Indeed mutations in the Mc1r gene that cause inactivation of the receptor, are known to result in mice that are completely yellow, due to a total absence of eumelanin and a constitutive production of pheomelanin (Robbins *et al*, 1993). The same phenotype is observed in mice with dominant mutations in the *Agouti* gene that cause the constitutive expression of the molecule: *Agouti* expression is tightly regulated and is normally limited during the mid growth of the hair (Klebig *et al*, 1995).





**Fig 1.3** Schematic of Eumelanin and Pheomelanin biosynthetic pathways

In addition to the pigmentation defects, the dominant agouti mutations are also associated to abnormal phenotypes such as obesity and diabetes (Bultman *et al*, 1992). This happens because mutations affecting the time-regulated expression of the agouti gene often result in the ectopic expression of the molecule, which is normally limited to the hair follicle. The expression of agouti in the hypothalamus causes the molecule to interact with the Melanocortin-4 receptor known to be involved in the regulation of the animal feeding behaviour and metabolism. Indeed it has been shown that AGRP (Agouti Related Protein), the natural ligand of the Mc4r, is a secreted molecule that is distantly related to agouti (Ollmann *et al*, 1997; Shutter *et al*, 1997). Thus the ectopic and constitutive expression of agouti in the brain has profound consequences on the normal regulation of the animal food intake and weight control.

## **1.2 Melanoma**

Malignant Melanoma is a skin cancer that arises from the uncontrolled proliferation of melanocytes. Although it is the less common among the skin cancers, its incidence is rapidly increasing and it is at the moment one of the fastest growing cancers in the world, especially among the Caucasian population. Indeed it is estimated that in the USA the cumulative lifetime risk of developing melanoma is 1:75 compared with 1:1500 in the 1930s (Rigel *et al*, 1996), and in Australia it has been evaluated to be as high as 1:25 (Marks, 2000). On the other hand mortality rates, mainly thanks to education programmes aimed at raising awareness, have been increasing at a lower pace, the figures being in the range of 1-3 per 100,000 people in the northern hemisphere and 5-10 per 100,000 people in the southern one (Marks, 2000). A likely explanation for such an increase in melanoma in the last decades is social behaviour: melanoma, like any skin cancer, can be triggered by the mutagenic effects of UV rays and in the last 40 years the number of people exposing themselves to sunlight for cosmetic reasons has dramatically increased.

Melanoma can be generally divided in four major clinicopathological categories: superficial spreading, nodular, lentigo maligna and acral lentiginous. These classes of melanoma differ in origin, appearance and aggressiveness but all of them, if unchecked and untreated, can metastasise and cause death.

In addition there are a small percent of melanomas that do not fit in any of the above categories. This includes amelanotic melanoma, which accounts for only 5% of the total incidence and is characterised by a total lack of pigmentation (Perniciaro, 1997). Malignant Melanoma generally presents as a dark patch on the skin as a consequence of melanocyte proliferation. The growth and progression of the disease can be divided in two separate stages, the early horizontal or radial growth phase and the following vertical growth phase (Welch *et al*, 1997). The radial growth phase consists of a localised proliferation of melanocytes that is confined to the upper layers of the skin. At this stage the lesion is not life threatening and can be cured simply by surgically removing the affected area. However, as the disease progresses, melanocytes begin to invade and proliferate in the tissues directly below the epidermis and the disease enters the vertical growth phase. At this point the tumour becomes much more aggressive and very rapidly undergoes metastasis, targeting in particular skin, lymph nodes, lung, liver, brain and the small intestine (Chin *et al*, 1998). Once malignant melanoma has metastasised, the chances of successfully curing the disease decrease dramatically. Indeed melanoma is characterised by metastasis that are extremely resistant to all common treatment procedures such as chemotherapy and radiotherapy (Chin *et al*, 1998). The reason of such behaviour is still poorly understood although some recent studies seem to correlate it with mutations in the tumour cells that inactivate the apoptotic pathways (Schmitt *et al*, 1999; Soengas *et al*, 2001).

The great majority of primary melanomas are readily identifiable as dark patches on the skin that will change in size and colour in time. There are cases when, as the disease progresses, the melanocytes in the lesion change morphology and become less dendritic and pigmented. This is when amelanotic melanoma arises, a phenotype that is usually associated with a more aggressive progression of the disease, although there is some contradictory evidence to suggest that pigmented forms may be equally serious (Adler *et al*, 1997; Orlow *et al*, 1998). To date it is still not clear whether the higher fatality rate associated to amelanotic melanoma is really caused by a more aggressive phenotype of the disease or is a consequence of misdiagnosis of the tumour. Indeed the lack of pigmentation in the lesion, which is the primary marker for melanoma, often causes the tumour to be identified only at later stages when it is difficult to treat (Adler *et al*, 1997).

If amelanotic melanoma is quite rare, unpigmented metastases generated from primary melanomas, even if highly pigmented, are relatively common (Gibson *et al*, 1988; Albino *et al*, 1992). Interestingly, the morphology of the cells forming such metastases is more similar to a melanoblast than it is to the melanocyte from which they originated. Similarities are not only at the macroscopic level, such as lack of pigmentation and poor dendricity, but also at the molecular level. Indeed amelanotic metastatic and primary melanoma cells seem to have downregulated expression or deregulated activity of those melanocyte specific genes responsible for pigment production such as *Tyrosinase*, *Trp-1* and, to a lower degree, *Trp-2* (Orlow *et al*, 1998).

Taken together these data seem to suggest that the passage from melanocyte to melanoma cell, in particular the amelanotic one, involves a dedifferentiation process that ultimately is responsible for the phenotypical changes leading to the undifferentiated morphology of the transformed cells.

### **1.3 Genetics of melanoma**

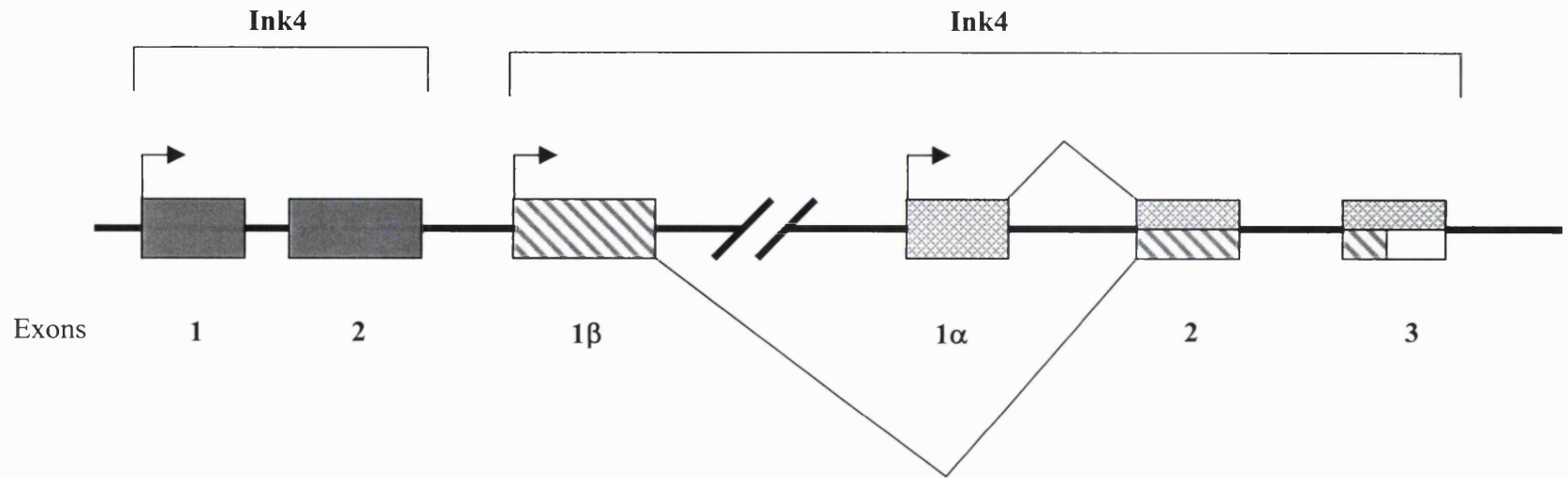
Although the increase in melanoma incidence observed in the last decades can be largely explained by changes in social behaviour and environmental factors, it is clear that genetic predisposition also plays an important role in the onset of the disease. For example, it has been long known that skin colour is an important inheritable factor and the degree of pigmentation of the skin correlates well with the risk of developing melanoma. Indeed it has been shown that, given the same amount of UV rays exposure, fair skinned individuals are more likely to develop the disease than people with dark skin (Barnhill, 1993).

In addition, further evidences supporting a possible genetic predisposition is that about 10% of the patients affected by malignant melanoma present a family history for the disease (Chin *et al*, 1998). Through linkage and molecular studies on both familial and sporadic melanomas in humans, it has been possible to clearly demonstrate that a crucial step towards the onset of the disease is the loss of a locus residing at 9p21 on the short arm of human chromosome 9 (Cannon-Albright *et al*, 1992). Indeed, in the majority of the families presenting a history of melanoma,

members developing the malignancy have a heterozygous mutation on this locus, resulting in loss of heterozygosity for the gene(s) residing in that region. Also, in patients with sporadic melanoma, this locus is very often found with homozygous mutations, reinforcing the belief that a very important tumour suppressor gene is situated on that portion of chromosome 9 (Weaver-Feldhaus *et al*, 1994; Holland *et al*, 1994). Further studies of the locus showed the presence of two genes, *INK4a* and *INK4b*. In addition, both in human and mouse (on chromosome 4), the *INK4a* gene presents a complex genomic organisation resulting in the production of two distinct and unrelated proteins, p16 (Serrano *et al*, 1993) and p19<sup>ARF</sup> (Alternative Reading Frame) (Quelle *et al*, 1995) (**Fig 1.4**). The gene has four exons: E1 $\alpha$ , E1 $\beta$ , E2 and E3. The p16 product is encoded by exons E1 $\alpha$ , E2 and E3. The p19<sup>ARF</sup> starts in exon E1 $\beta$  and continues into the shared exons E2 and E3 with a different reading frame to p16. The *INK4b* gene, on the other hand, is composed of two exons and only gives rise to one product, the p15 protein (Hannon *et al*, 1994). In conclusion, the 9p21 locus in humans and its corresponding locus on mouse chromosome 4, harbours two genes and is responsible for the expression of three different products.

Interestingly, all these proteins have tumour suppressor properties and are involved in the regulation of the cell cycle by inhibiting the G1/S transition.

The first products to be characterised were p15 and p16. These proteins belong to the family of cyclin dependent kinases inhibitors (CDKi), and in particular have been shown to act as negative regulators of CDK4 and CDK6 (Serrano *et al*, 1993; Hannon *et al*, 1994). These cyclin dependent kinases are negative regulators of cell cycle and are involved in the phosphorylation of the retinoblastoma pRB protein (Kato *et al*,



**Fig 1.4** Schematic of the 9p21 locus organization. The Ink4b gene has two exons and encodes for p15. The Ink4a gene is composed of four exons and encodes for both p16 (E1 $\alpha$ , E2, E3) and p19 (E1 $\beta$ , E2 E3) through different promoters, two distinct first exons and an alternative reading frames in the shared exons 2 and 3.  
(From Chin *et al*, 1998)



1993; Meyerson *et al*, 1994). Hypophosphorylated pRB blocks the cell cycle by repressing the expression of genes necessary for the progression of the cell from the resting G1 phase to the DNA synthesis S phase (Weinberg, 1995). Loss of p15 and/or p16 can lead to the inappropriate phosphorylation of pRb, and subsequent entry into S phase.

The p19<sup>ARF</sup> protein is an inhibitor of the *Mdm2* oncogene whose product is involved in p53 degradation, the protein responsible for cell cycle arrest in response to cellular stress resulting in DNA damage (Levine, 1997). Thus p19<sup>ARF</sup> is responsible for stabilising the p53 product. Loss of p19<sup>ARF</sup> can lead to a decrease in p53 levels in the cell impairing its cell cycle arrest activity (Pomerantz *et al*, 1998; Zhang *et al*, 1998). Due to the particular arrangement of the 9p21 locus, that is to express three tumour suppressor products, it is not clear whether predisposition to melanoma requires the loss of all proteins, or a combination of them. The data accumulated to date seem to rule out p15 as the familial melanoma gene product since, whenever loss of p15 is observed, it is always the result of large deletions that also include *INK4a* and mutations associated with melanoma specifically targeting the *INK4b* gene have only been found in one isolated case (Glendening *et al*, 1995). This would suggest that the real familial melanoma gene is *INK4a*, but does not clarify the role played by each of its two products in the onset of the disease. There are data suggesting that only p16 is involved in melanoma predisposition since melanoma prone families have been found with mutations in the E1 $\alpha$  p16 specific exon, leaving p19<sup>ARF</sup> expression and function unaltered (FitzGerald *et al*, 1996; Fagnoli *et al*, 1998).

However the vast majority of *INK4a* mutations found in melanoma affect both p16 and p19<sup>ARF</sup>, impairing both the pRb and the p53 pathways.

This is probably the reason why malignant melanoma, as opposed to the vast majority of aggressive human tumours, presents a relatively low percentage of p53 mutations (Lubbe *et al*, 1994; Papp *et al*, 1996). Malignant melanoma is extremely resistant to both radiotherapy and chemotherapy. This has always been perceived as a contradiction since most melanomas maintain a wild type p53 and should therefore respond to p53 mediated apoptosis following chemotherapy or radiotherapy. Recently it has been shown that *INK4a/ARF* null lymphomas, like the p53 null ones, are extremely aggressive and resistant to chemotherapy (Schmitt *et al*, 1999). This is likely to happen because the *INK4a/ARF* loss of function compromises p53 activity and its capacity to trigger DNA damage mediated apoptosis. It is therefore likely that the same mechanism is found in *INK4a/ARF* null melanomas with wild type p53, accounting for their resistance to chemotherapeutic drugs.

In addition, it has been recently shown that a high proportion of malignant melanomas have no detectable levels of *Apaf-1* gene expression (Soengas *et al*, 2001) because of deletions in the 12q23 locus causing loss of heterozygosity (LOH) and transcriptional repression of the remaining wild type allele through methylation. *Apaf-1* is an important cell-death effector known to be involved, together with cytochrome-c and caspase-9, in the initiation of the p53-mediated apoptosis. Therefore loss of function of *Apaf-1* causes defects in the chain of events leading to cell death following damages induced by chemotherapy. Loss of *Apaf-1* is also observed in many melanoma cell lines and its expression levels correlate well with the cell line

resistance to chemotherapeutic drugs. When Apaf-1 levels are restored in these cells using a retrovirus expressing *Apaf-1* cDNA, the apoptosis pathway is rescued and the sensitivity to the drugs is regained.

Although loss of the *INK4a* gene appears to be an important step in melanoma predisposition and onset, there are other mutations, especially those activating oncogenes, which are thought to be involved in its progression. These include mutations activating the Ras oncogene and those inducing autocrine loops, for example mutations that activate RTKs (receptor tyrosine kinases) (Chin *et al*, 1998). Ras is a monomeric GTPase enzyme that is involved in the signal transduction of many pathways activated by extracellular stimuli such as growth factors, cytokines and hormones (Malumbres *et al*, 1998). Ras mutations causing its constitutive activation have been associated with many tumours, making it one of the most common oncogenes in human cancers. Despite such a crucial role in the onset and progression of many malignancies, its involvement in melanoma has been disputed for some time. Indeed Ras activation was reported to be a very common feature in cultured melanoma cell lines, but relatively rare in non-cultured melanomas (Albino *et al*, 1989). This finding seemed to suggest that the Ras activation observed in cultured melanomas was a consequence of their adaptation to culture conditions rather than being pathologically relevant. On the other hand, there followed other reports describing Ras activation in many primary melanomas and especially in metastatic melanomas (Ball *et al*, 1994; Platz *et al*, 1994; Platz *et al*, 1995). It is now accepted that Ras activating mutations are generally found at later stages of the disease,

suggesting that Ras mutations are involved in the progression of the cancer. However, a mouse model for melanoma has been created expressing activated Ras in melanocytes of an *INK4a* gene knockout mouse (Chin *et al*, 1997). These animals, unlike *INK4a* null mice, rapidly develop spontaneous melanoma with a very high penetrance suggesting that Ras activation can be an initiation step towards the disease rather than being involved only in its progression. Moreover these mice, although they recapitulate some of the main traits of melanomas such as wild type p53, *INK4a* loss of function and Ras activation, are lacking one of the main features of this cancer that is the ability to form metastasis. This seems to suggest that, despite Ras activation having been described as a metastatic event in many malignancies, it may not be sufficient for producing such an aggressive phenotype in melanoma.

Another common event in melanoma progression is the generation of autocrine loops. This is usually achieved through the ectopic expression of growth factors and/or their corresponding receptors that are not normally expressed by melanocytes (Halaban, 1996). Growth factor receptors belong to the Receptor Tyrosine Kinase (RTK) family. These are transmembrane receptors involved in many pathways including growth factors, cytokines and hormones, presenting tyrosine-kinase activity in the cytoplasmic domain. Upon binding of the appropriate ligand on the extracellular domain, the receptor dimerizes causing the autophosphorylation of the cytoplasmic domain. This in turn creates a cascade of events involving intermediate kinases that ultimately results in the signal being transduced to the nucleus where genes will be expressed and proteins activated for the cell to proliferate. In melanoma these

pathways are often activated, either by mutations constitutively activating the receptor, by overexpression of the receptor or by producing the ligand for the receptor. Typical examples are the over-expression of the epidermal growth factor receptor (EGFR) found in late stage melanomas (Koprowski *et al*, 1985; de Wit *et al*, 1992), and the production of bFGF (basic fibroblast growth factor) by virtually all melanoma cells, leading to the induction of an autocrine loop (Halaban *et al*, 1988).

Interestingly, transformation of melanocyte cell lines by oncogenes overexpression not only confers the expected growth advantages such as higher proliferation rate and TPA independence, but also provokes the loss of typical melanocyte markers of differentiation like pigmentation and dendricity. These phenotypical changes have been noticed using different oncogenes such as activated Ras, bFGF, myc, E1A and neu (Dotto *et al*, 1989; Cajal *et al*, 1991; Yavuzer *et al*, 1995; Donatien *et al*, 1996). Although the mechanism leading to this dedifferentiated phenotype is not well understood, it is clear that it ultimately depends on the oncogene dependent down-regulation of the microphthalmia transcription factor (Halaban *et al*, 1996). It is also worth noticing how this *in-vitro* characteristic of cultured melanocytes parallels what is observed during melanoma progression where cells found in metastases are often lacking both pigmentation and dendricity.

## **1.4 Technological Approach**

### **1.4.1 Genetics in mammalian cells**

The use of genetic manipulation aiming at the specific delivery and expression of ectopic genes in a genetically defined organism is a powerful tool for the discovery and characterisation of biological pathways. In particular, over the years, this approach has been extensively applied to relatively simple unicellular organisms such as bacteria and, most importantly, yeast. The use of lower eukaryotes is still the preferred way to study basic biological pathways and it is indeed a very useful approach. There are two main reasons for this. Firstly, almost invariably genes that are found to be important in yeast have a homologue with a similar function in mammalian cells and therefore their characterisation in the lower eukaryote is relevant to the biology of more complex organisms. Secondly, genetics in yeast is technically relatively simple and the experimental procedures in use have been optimised over the years and are very reliable (Botstein, 1988).

Having said that, it is clear that a similar approach directly on mammalian cells could prove even more informative, especially when applied to the characterisation of more cell specific pathways that are not shared by all cell types. For example, the discovery of the cell cycle genes and their characterisation in yeast was extremely useful and the information gathered in this system could very effectively be applied to mammalian cells (Nasmyth, 1996). This is because the cell cycle mechanism is such a fundamental biological activity, that it is well conserved and similar in all eukaryotes,

from yeast to humans. Unfortunately this does not apply for those specialised functions that characterise cells of more complex multicellular organisms. For example the biological pathway leading to melanin production is such a specific activity of pigment cells that it can only be studied and characterised in melanocytes.

When facing the challenge of developing a tool that allows genetic manipulation of mammalian cells in a similar way to what is commonly done in bacteria and yeast, the following requirements need to be fulfilled:

- a. Efficient delivery of the genomic information
- b. Stable delivery of the genomic information
- c. Recovery of the genomic information

Over the years many vectors have been devised for the successful delivery of genomic information in mammalian cells. Normally DNA in mammalian cells is introduced using chemical methods that allow to bypass the physical barrier represented by the cellular membrane. The most commonly used protocols consist in precipitating the DNA in calcium phosphate salts which are in turn internalised by the cells (Graham, 1973), or in enveloping the DNA in liposomal vesicles that can subsequently fuse with the plasma membrane delivering the DNA into the cells (Mannino, 1988).

Although these methodologies can be quite efficient, depending on the cell line used, they have numerous limitations. For example the number of DNA molecules introduced in a single cell is random and it is very common to have multiple

molecules being introduced in one cell. This is not a problem if the DNA molecules introduced in a population are all the same, but it is not acceptable in the case, for example, of cDNA library screening where one has millions of different clones that need to be examined according to the biological properties they confer on the cell. For such an application, one would ideally be able to introduce only one DNA molecule per cell, so that the phenotype observed can be related to a specific transcript.

Another major limitation of common transfection methods is their inherent instability. Unlike vectors used for bacteria or yeast transformation, those employed for mammalian transfections are not recognised by the cell as endogenous genomic information. This means that the plasmids are not duplicated when the cell divides and, over time and cell duplications, the vector DNA is lost resulting in a decrease in the population of transfected cells still carrying the information originally delivered. A way to get around this problem is to introduce into the vector DNA a drug selection marker so that, in the presence of the appropriate drug, cells that lose the vector can be selected against and cannot overtake the transfected population. If the selection is carried out long enough, the result will be to select for a clonal population that have integrated the vector DNA in their genome thereby becoming stably transfected. Unfortunately this is a relatively rare event and it can take up to three months before a stably transfected cell line is produced from a transient transfection.

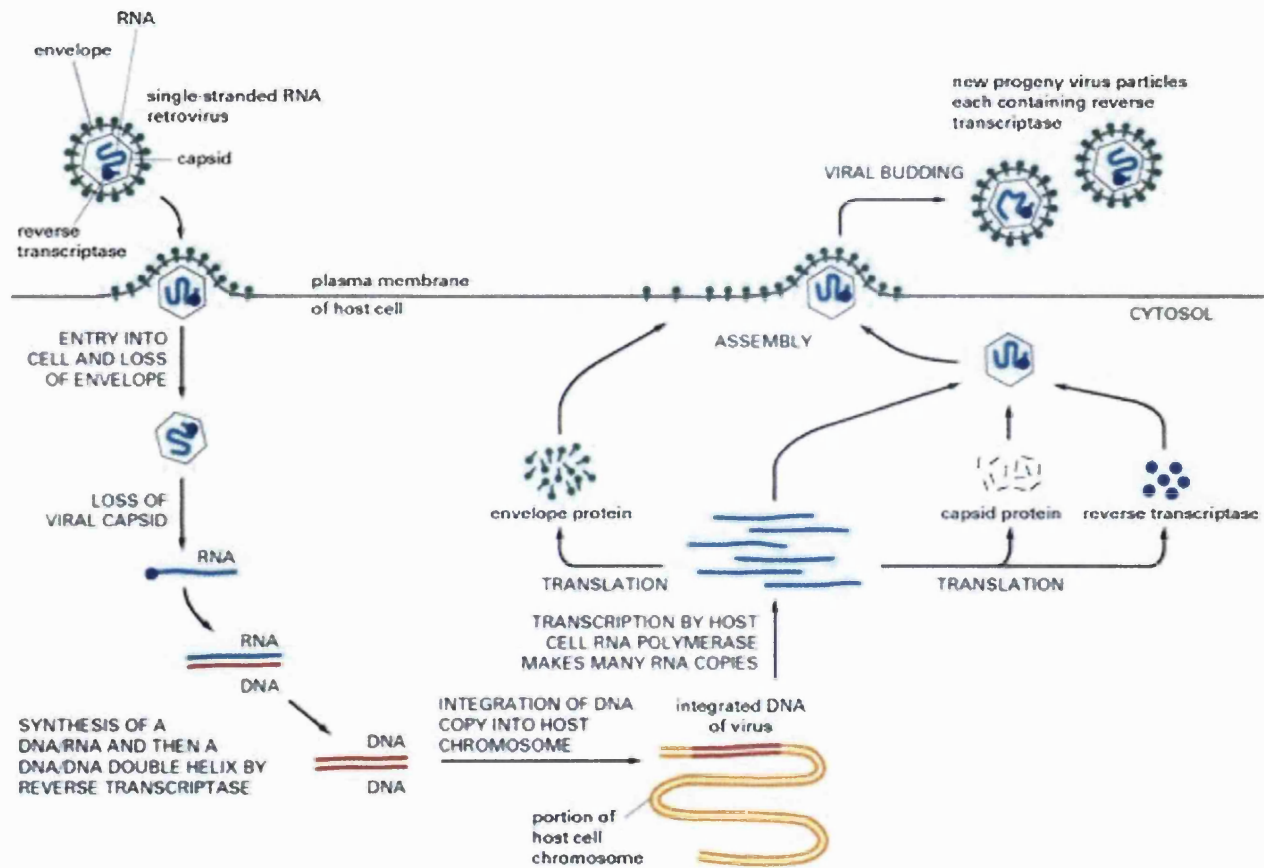
Finally, in order to do genetics in mammalian cells it is fundamental to be able to recover the genetic information originally delivered. This is particularly important in



the case of functional screenings of complex cDNA libraries where nothing is known of the cDNA introduced in the cells and the selection is based on phenotypic changes.

### 1.4.2 Retroviral vectors

Retroviral vectors, especially those based on Murine Leukemia Virus (MLV), have been successfully used in the last few years for genetically modifying mammalian cells and they present a number of advantages over more conventional transfection methods (Hu and Pathak, 2000). First, being based on retroviruses, the delivery of the genetic material goes through an infection step and most cell lines, provided that they are actively dividing, can be efficiently infected by MLV. Second, the number of viruses used on the target cells can be titrated so that statistically there is not more than one infectious event per cell, eliminating the issue of more than one cDNA molecule being introduced in the same cell. Finally, and perhaps most importantly, every single infection event automatically becomes a stable transfection. This is an intrinsic characteristic of the system and is a consequence of the retroviruses life cycle. Retroviruses are so called because, unlike most living organisms, their genome is made of two identical molecules of RNA. Upon infection of the target cell, the viral RNA genome is reverse-transcribed by a retroviral specific protein (the reverse transcriptase) into a double strand molecule of DNA (**Fig 1.5**). This in turn actively integrates into the target cell DNA becoming part of the host genomic information and as such is transmitted to all the cell progeny. This means that a cell infected by a

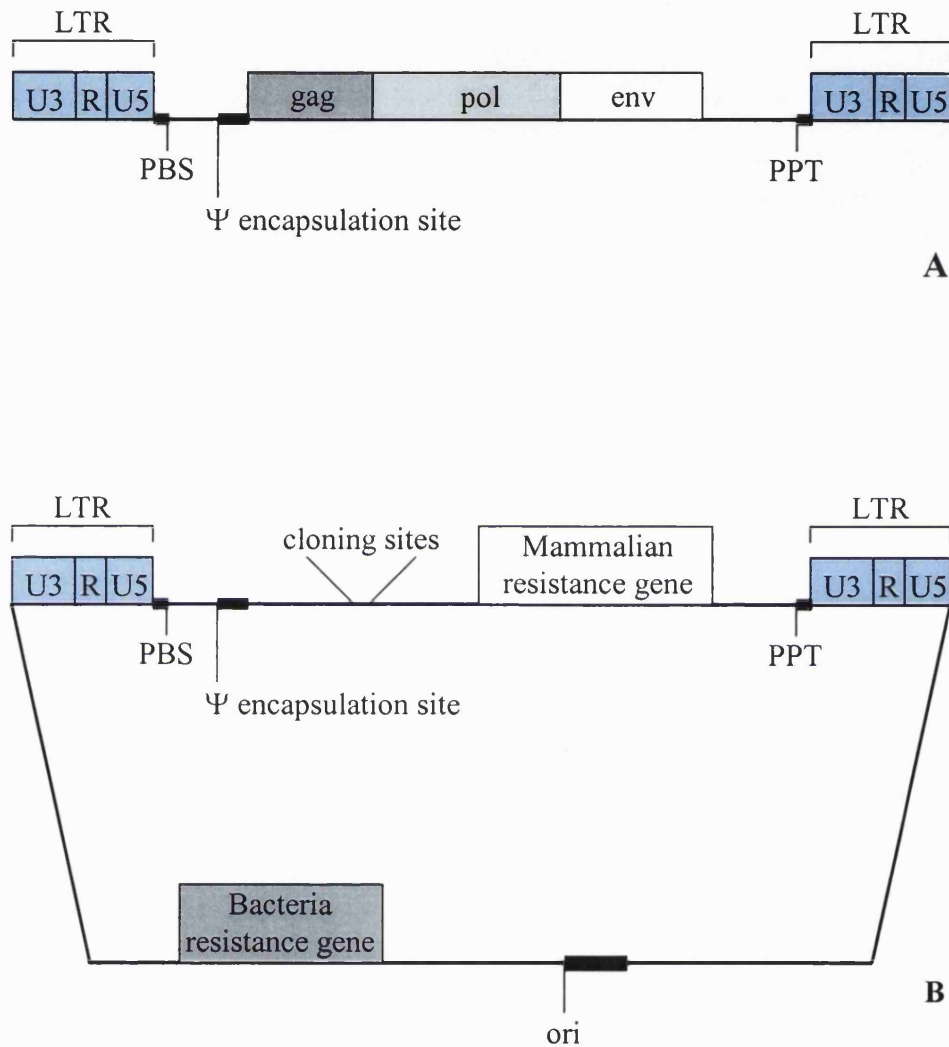


**Fig 1.5** Schematic of retrovirus life cycle

(From "Essential Cell Biology", Alberts B., Bray B., Johnson A., Lewis J., Raff M., Roberts K., and Walter P. 1998)

retrovirus receives a permanent (or stable) genetic modification and gives rise to a clonal population of cells bearing the exact same acquired information. The next step in the retrovirus life cycle is to produce more copies of itself to spread the infection to neighbouring cells. This is achieved through the transcription of the retroviral genomic DNA (called provirus) into RNA molecules that have the double function of being translated into viral proteins necessary for the production of infectious particles, as well as providing the retroviral genome to be encapsulated in the particles themselves (Luciw and Leung, 1992).

The MLV retrovirus genome organisation is very simple and mainly consists of three genes: *gag*, *pol* and *env* (Fig 1.6A). *gag* is responsible for the transcription of the capsid proteins, *pol* transcribes for both reverse-transcriptase and integrase, and *env* encodes for the envelope proteins determining the spectrum of target cells that can be infected. Other important genomic elements are the  $\Psi$  encapsulation site for packaging the RNA viral genome in the capsid, the Primer Binding Site (PBS) and Poly Purine Tract (PPT) necessary for the reverse transcription steps, and two LTRs (Long Terminal Repeats) delimiting the viral genome. These are divided in three regions, U3 (unique 3'), R (repeat) and U5 (unique 5'), with different functions. U3 is essentially a very strong promoter and is responsible for the transcription of the viral genes, R is important for the reverse transcription step and also contains a strong polyadenylation signal and U5 contains sequences facilitating the initiation of reverse transcription (Hu and Pathak, 2000).



**Fig 1.6** A) Schematic of the MLV retrovirus genome  
B) Schematic of a replication-deficient retroviral vector  
(Modified from Hu and Pathak, 2000)

When designing retroviral-based vectors, it is important to ensure that the information necessary for this last part of the retrovirus life cycle to happen, that is the capacity to make copies of itself and to spread the infection, is totally missing (Hu and Pathak, 2000). This is a precaution dictated by safety reasons arising when working with a tool that is extremely efficient in genetically modifying mammalian cells and has a self-replicating potential. In addition, such a limitation in the retroviral vector life cycle is of great advantage when the system is applied to the functional screening of cDNA libraries. Indeed if the retroviral vector could make copies of itself and spread the infection, it would be impossible to titrate the virus number and to avoid multiple infection of the same cell. Considering that every virus potentially carries a different cDNA molecule, the spreading of the infection would result in a single cell being infected by several viral particles leading to different cDNAs being introduced in the same cell.

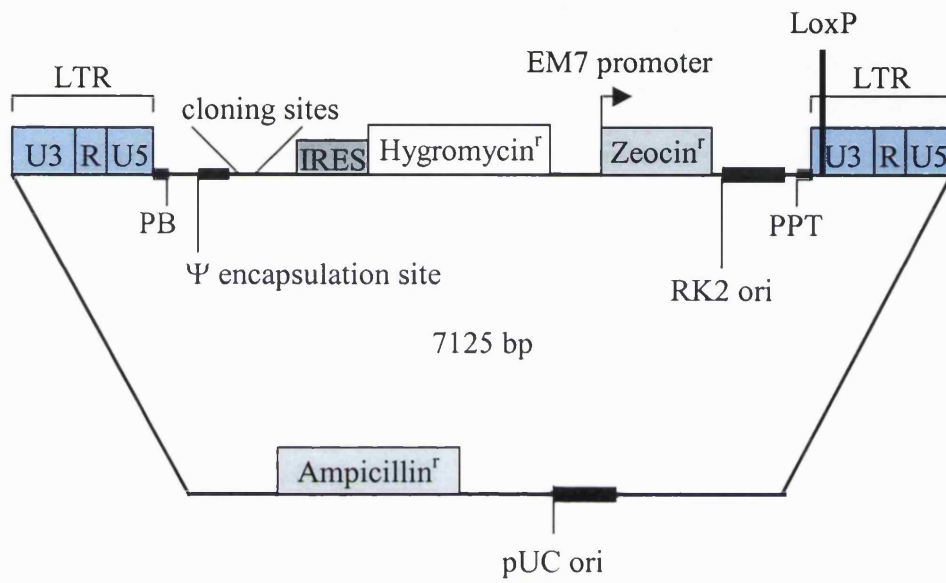
The easiest way to engineer replication-deficient retroviral systems is to divide the viral components that make an infectious particle in two: a vector and a helper construct. The vector is essentially a modified provirus, in the form of a circular plasmid, still bearing all the *cis*-acting elements necessary for gene expression and replication such as LTRs,  $\Psi$ , PBS and PPT sites, but missing the *gag*, *pol* and *env* genes. These are replaced by a multiple cloning sites for the subcloning of genes of interest, a drug selection for mammalian cells and whatever else may be useful for one particular application (**Fig 1.6B**). The helper construct normally consists of a highly transfectable cell line that has been genetically modified to constitutively express the *gag*, *pol* and *env* genes missing in the retroviral vector. Upon transfection of the

retroviral vector into the helper cell line, all the elements for the making of an infectious particle are present in the same cell, and virus production will occur. Obviously, the only RNA genome that can be packaged in such a condition is the one from the retroviral vector so that the virus produced carries the gene of interest and the selectable drug marker but lacks the ability to self replicate, limiting the infectious events to just one (Hu and Pathak, 2000).

### 1.4.3 The MaRX System

The MaRX system is a Moloney Murine Leukemia Virus (MMLV) retroviral-based technology for the genetic manipulation of mammalian cells. It was specifically designed with the intention of allowing cDNA library screenings and, within limits, fulfils the requirements expected when developing a tool for genetics in mammalian cells (Hannon *et al*, 1999). The technology consists of the MaRX (in our case pHygroMaRX II) retroviral vector and the helper LinX cell line. While the packaging cell line is a standard helper 293 human embryonic kidney stably modified to constitutively express the *gag*, *pol* and *env* genes, the pHMII vector presents some unique features that are worth describing.

The vector is ~7.1kb long and consists of a bacterial plasmid, with Ampicillin resistant marker and a pUC origin of replication site for amplification in *E. Coli*, in which a retroviral provirus delimited by LTRs has been inserted (Fig 1.7). The



**Fig 1.7** Schematic of the pHMII retroviral vector

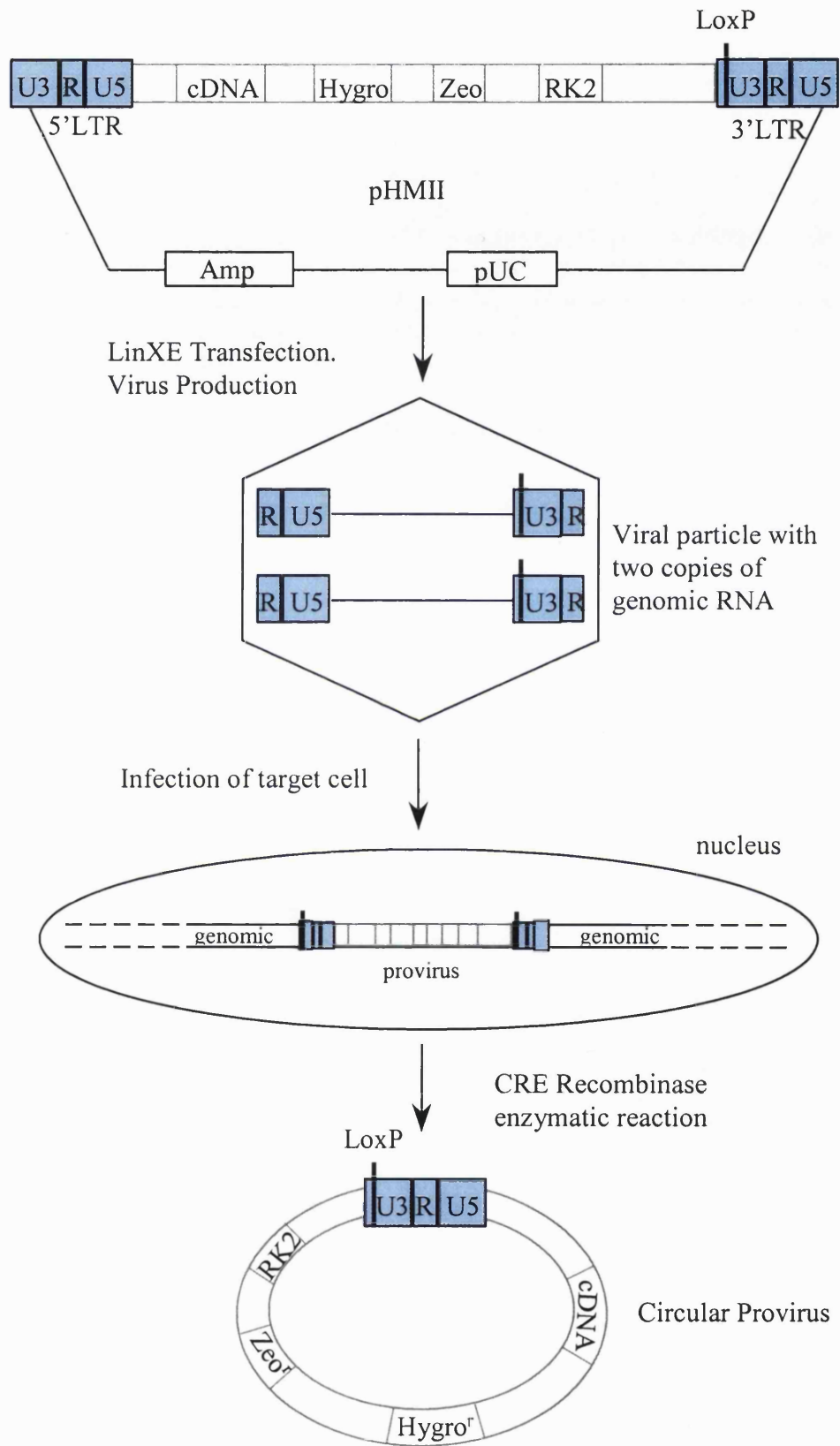
provirus, in addition to the usual *cis* acting elements necessary for replication and packaging, features cloning sites for genes of interest followed by the Internal Ribosome Entry Site (IRES) responsible for the expression of the Hygromycin resistance gene in mammalian cells. It also includes a mini-plasmid composed of the EM7 bacteria promoter for the expression of the Zeocin resistance gene in *E. Coli* and an RK2 origin of replication for amplification in bacteria. When the vector is transfected into the packaging cell line, the 5'LTR is responsible for the transcription of the retroviral vector RNA genome, which comprises everything included between the two Long Terminal Repeats, necessary for virus production. On the other hand, upon infection of the target cell, the same unique RNA molecule serves as a transcript for the expression of both the gene of interest and the Hygromycin resistance gene. While the gene of interest is close to the 5'cap and its translation happens using the normal ribosomal binding mechanisms to the 5' of the RNA, the translation of the Hygromycin resistance cDNA is possible thanks to the IRES sequence that allows the binding of ribosomes in the middle of the RNA molecule (Pelletier and Sonenberg, 1988). The advantage of using the IRES sequence is that two different genes can be expressed by the same transcript, avoiding the necessity of cloning an additional promoter between the two cDNAs.

Finally, the main characteristic of this vector is the presence of a LoxP site in the U3 region of the 3'LTR. LoxP is a 34bp DNA palindromic sequence that is specifically recognised by CRE recombinase (Sternberg and Hamilton, 1981). This is a site-specific recombinase of bacteriophage P1 that actively brings together two *cis* LoxP



sequences and recombines them provoking the excision of whatever is included between the two sites. Although the vector only presents one LoxP site in the U3 region of the 3'LTR, upon infection of the target cell and reverse transcription of the viral genomic RNA, it is copied on the 5'LTR resulting in the DNA provirus being delimited by two identical LTRs both bearing the LoxP site (Fig 1.8). Once the provirus integrates in the host genome, one can recover it by treating the cell genomic DNA with CRE recombinase. This will cause recombination of the LoxP sites and excision of the provirus in the form of a circular plasmid that can be directly transformed into bacteria cells and selected using the Zeocyn marker. The advantage of such a system is that the gene of interest can be recovered without any further manipulation or subcloning steps. Moreover, the circular bacteria plasmid resulting from CRE recombinase reaction is also a functional retroviral vector that can be directly transfected in the packaging cell line for virus production allowing easy retesting and confirmation of potential positive clones.

This methodology has been successfully applied in different cell lines resulting in the isolation of new genes and characterisation of a variety of biological pathways (Sun *et al*, 1998; Hudson *et al*, 1999; Maestro *et al*, 1999; Carnero *et al*, 2000a; Carnero *et al*, 2000b).



**Fig 1.8** Schematic of the pHMII retroviral vector cycle

## **1.5 Aims**

In this thesis we describe the development of functional screenings for the identification of genes involved in melanocyte differentiation and melanoma progression using the MaRX technology.

The strategy is to develop screenings both in melanoblast and melanocyte cell lines using pigmentation as the selection marker for the identification of the positive clones. The choice of pigmentation as the discriminating criteria is dictated by the following observations:

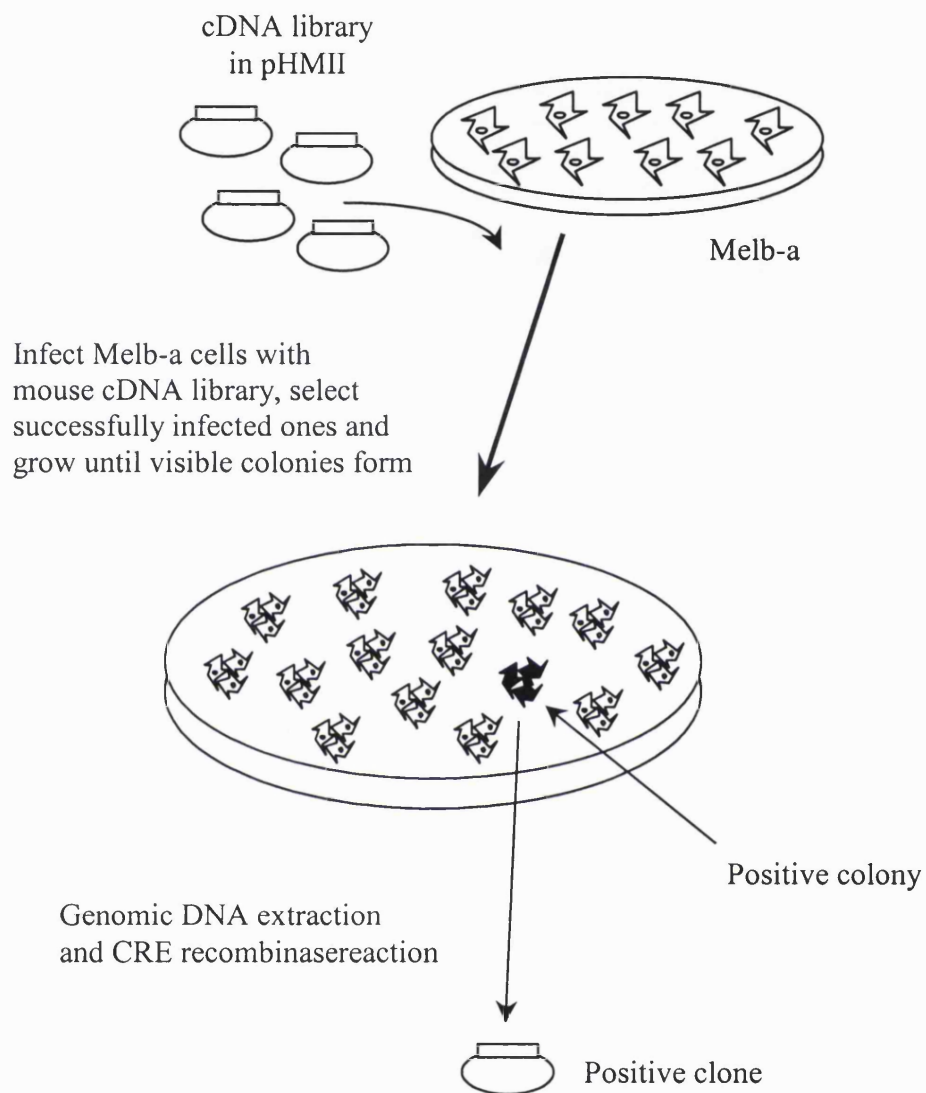
- a. Pigment is an easy to identify marker and it is visible in living cells.
- b. Melanocytes are highly dendritic and pigmented cells that derive from the differentiation of round shaped and unpigmented melanoblasts (Hirobe *et al*, 1994; Sviderskaya *et al*, 1995).
- c. Although melanoma in its early stages is normally very well pigmented, it can lose the capacity of producing melanin, especially when metastasising, leading to an amelanotic phenotype that is normally associated with a more aggressive progression of the disease (Gibson *et al*, 1988; Albino *et al*, 1992).
- d. Melanocyte cell lines have been reported to lose differentiation traits such as dendricity and pigmentation when transfected with oncogenes such as activated Ras, Neu, bFGF, myc and E1A (Dotto *et al*, 1989; Cajal *et al*, 1991; Yavuzer *et al*, 1995; Donatien *et al*, 1996; Halaban *et al*, 1996).

Thus by screening melanocytes for cDNAs that decrease pigmentation and melanoblasts for cDNAs that induce it, we intend to find genes of interest not only for the pigmentation biology itself, but also for melanocyte differentiation and progression towards a transformed phenotype.

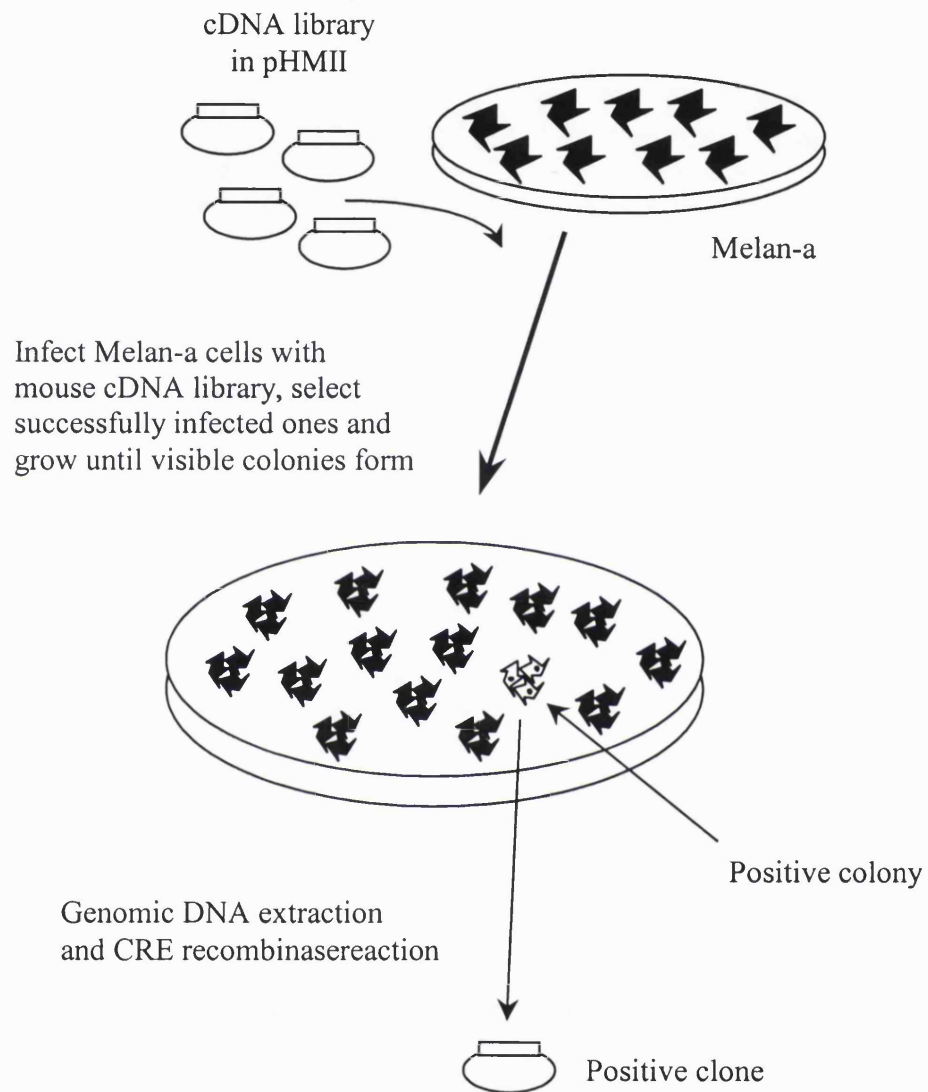
In developing our screenings, we have chosen to use the mouse melanocyte cell line Melan-a and the mouse melanoblast cell line Melb-a (Bennett *et al*, 1987; Svirideskaya *et al*, 1995). The murine system is preferred to the human one because the mouse Melb-a and Melan-a cell lines, together with the specific growing conditions required, are very well established, whereas human melanocytes and in particular human melanoblasts are still very challenging to grow. In addition it is worth noticing that generally human melanocytes are not as pigmented as the mouse Melan-a cell line and a poor pigmentation could decrease the effectiveness of the melanocyte screening raising the chances of selecting false positives.

Another advantage of using Melan-a and Melb-a is that both cell lines have been reported to change pigmentation levels when grown in particular conditions or transduced with specific genes. Thus Melan-a has been shown to lose pigmentation when transduced with E1A or activated H-Ras (Yavuzer *et al*, 1995; Donatien *et al*, 1996) and Melb-a can be induced to differentiate in pigmented melanocytes when grown in conditions promoting an increase in intracellular cAMP levels (Sviderskaya *et al*, 1995).

The general outline of both screenings is to infect cells with an appropriate cDNA library cloned into the pHMII retroviral vector, grow the successfully infected cells long enough to allow colony formation, and isolate the positive clones for genomic DNA extraction and provirus recovery. The definition of a positive clone will depend on the screening carried out. Thus, if screening Melb-a for genes that can induce differentiation, only pigmented colonies will be considered (**Fig 1.9**), whereas if screening for genes that inhibit melanoblasts differentiation, cells cultured in conditions that favour their differentiation will be screened for colonies that remain unpigmented. In the case of the screening on Melan-a, only unpigmented colonies are to be considered potential positive clones (**Fig 1.10**).



**Fig 1.9** Schematic of the functional screening on Melb-a cells

**Fig 1.10**

Schematic of the functional screening on Melan-a cells

## **2 MATERIALS AND METHODS**

### **2.1 Basic molecular biology methods**

#### **2.1.1 Plasmid preparation**

##### **2.1.1.1 Large scale preparation**

Unless otherwise stated, large-scale purifications of plasmid DNA were carried out using the Plasmid Midi Kit (*Qiagen* #12143) following the supplier instructions. Briefly, the pelleted bacterial cells (100ml from an overnight (ON) suspension) were resuspended in 4ml of buffer P1 (100µg/ml RNase A, 50mM Tris-HCl pH8, 10mM EDTA). 4ml of buffer P2 (200mM NaOH, 1% SDS) were added, mixed gently and incubated at room temperature for 5 min. To precipitate proteins, 4ml of cold buffer P3 (3M potassium acetate pH5.5) were added and mixed gently, followed by 10 min incubation in ice. Samples were centrifuged at 10,000g for 30 min at 4°C and supernatants were loaded on a QIAGEN-tip 100 and washed twice with 10ml of buffer QC (1M NaCl, 50mM MOPS, 15% ethanol, pH7). DNA was eluted with 5ml of buffer QF (1.25M NaCl, 50mM Tris-HCl pH8.5, 15% ethanol), precipitated with



0.7 volumes of isopropanol and centrifuged at 10,000g for 30 min at 4°C. The DNA pellet was washed with 2ml of 70% ethanol, air-dried and resuspended in 200µl H<sub>2</sub>O.

#### 2.1.1.2 Small scale preparation

Small-scale purifications of plasmid DNA (minipreps) were carried out using the Quantum Prep minipreps kit (*Biorad #732-6100*) following the supplier instructions. Briefly, 1.5ml of overnight bacteria culture were pelleted in a microfuge tube at 13,000rpm for 45 seconds. Cells were resuspended in 200µl of Cell Resuspension Solution and lysed for 2 minutes with 250µl Cell Lysis Solution. Proteins were precipitated by adding 250µl of Neutralization Solution and centrifuging at 13,000rpm for 5 minutes. Supernatants were loaded on the supplied Spin Filters, added with 200µl of silica resin and microfuged at 13,000rpm for 30 seconds. DNA was washed twice with 500µl Wash Buffer microfuging first 30 seconds and then 2 minutes to completely remove ethanol. Finally the DNA was eluted from the silica resin by adding 100µl H<sub>2</sub>O and microfuging at 13,000rpm for 1 minute.

#### **2.1.2 Restriction enzyme reactions**

Unless otherwise stated DNA digestions were carried out using enzymes supplied by Promega following the supplier's instruction. Generally 10 units of enzyme were used per reaction incubating at 37°C for 2 hours. Digestion fragments were analysed by loading the samples on a 1% agarose gel run at 120 Volts in TAE buffer (40mM Tris-acetate, 1mM EDTA)

### **2.1.3 *E. Coli* competent cells**

#### 2.1.3.1 Chemical competent cells

A single bacteria colony from an LB-agar plate was incubated in 10ml of LB at 37°C overnight. Next morning, 10ml of ON culture were incubated in 100ml fresh LB and grown at 37°C until an OD<sub>600</sub> of 0.5-0.6 was reached.

The culture was left 10 minutes in ice and centrifuged for 10 minutes at 1000g, 4°C. Cells were resuspended in 40ml of ice-cold buffer TfbI (30mM potassium acetate, 50mM MnCl<sub>2</sub>, 100mM KCl, 10mM CaCl<sub>2</sub>, 15% glycerol, pH 5.8) and left in a water/ice bath for 10 minutes. Cells were again centrifuged at 1000g, 10 minutes at 4°C, resuspended in 4ml of ice-cold buffer TfbII (10mM MOPS, 75mM CaCl<sub>2</sub>, 10mM KCl, 15% glycerol, pH 7), aliquoted in 1.5ml microfuge tubes (200µl per aliquot) and stored at -80°C.

#### 2.1.3.2 Electroporation competent cells

A single bacteria colony from an LB-agar plate was incubated in 10ml of LB at 37°C overnight. Next morning, 8ml of ON culture were incubated in 800ml of fresh LB and grown at 37°C until an OD<sub>600</sub> of 0.7 was reached.

The culture was left 10 minutes in ice, centrifuged for 5 minutes at 4,500rpm, 4°C and resuspended in 800ml of distilled water with 10% glycerol. After 10 minutes in ice/water bath, cells were again centrifuged and resuspended in 400ml of H<sub>2</sub>O-glycerol. This last step was repeated twice and followed by resuspension of the pellet in 50ml H<sub>2</sub>O-glycerol. Finally cells were centrifuged 5 minutes at 4,500rpm and

resuspended in ~1ml of H<sub>2</sub>O-glycerol. Before aliquoting the sample in 50µl aliquots and store them at -80°C, the cells concentration was checked by diluting 12.5µl of the sample in 5ml of H<sub>2</sub>O-glycerol. If the OD<sub>600</sub> of the diluted cells was not 0.2, more H<sub>2</sub>O-glycerol was added until the right OD<sub>600</sub> was reached.

#### **2.1.4 Ligations**

Unless otherwise stated, ligations were carried out using 3 units of T4 Ligase (*Promega #M1801*) in 10µl final volume. Generally 50ng of vector were used and enough insert to have a vector/insert molar ratio of 1/3. Reactions were incubated for 2 hours at room temperature (RT) and transformed into either chemical or electro-competent *E.Coli* cells.

#### **2.1.5 Transformations**

##### 2.1.5.1 Heat-shock transformation

Vector DNA (either from plasmid preparations or ligations) was mixed with 100µl of chemical-competent *E.Coli* cells, incubated in ice for 30 minutes and heat-shocked at 42°C for 45 seconds. Cells were incubated with 500µl of fresh LB at 37°C for 1 hour with constant agitation. 200µl of transformed *E.Coli* were plated on LB-agar plates with the relevant antibiotic selection (generally 100µg/ml Ampicillin (*Sigma #A-9518*) or 30µg/ml Zeocin (*Invitrogen #202100*)).

### 2.1.5.2 Electroporation

Vector DNA from plasmid preparations was used directly, whereas ligations were first purified with two Phenol/Chloroform extractions, followed by one Chloroform extraction, an Ethanol precipitation and three 70%-Ethanol washes. The pellet DNA was resuspended in 5µl distilled H<sub>2</sub>O, mixed with 25µl of electro-competent *E. Coli*, and electroporated at 1.8 KV using 1mm gap cuvettes and the “*E. Coli* Pulser” electroporator (*Biorad*). Cells were incubated with 200µl of fresh LB at 37°C for 1 hour and plated on LB-Agar plates supplied with the relevant antibiotic selection.

### **2.1.6 DNA sequencing**

Sequencing reactions were performed using the Beckman Coulter CEQ 2000 Dye Terminator Sequencing Kit (#608000) following the supplier instructions. Briefly, 350ng of ds DNA template were diluted in 6µl of distilled water, denatured at 96°C for 1 minute and immediately transferred to ice. Samples were supplemented with 4pmoles of the relevant sequencing primer, 12µl of reaction mix and adjusted to 20µl with distilled water. PCR conditions were 96°C 20 seconds, 50°C 20 seconds and 60°C 4 minutes for 30 cycles. Samples were transferred to a 1.5ml microfuge tube containing 5µl of Stop Solution (1.2M Na-Acetate pH5.2, 40mM EDTA, 10µg glycogen) and ethanol precipitated. Pellets were washed twice in 70% Ethanol, vacuum dried for 40 minutes, resuspended in 40µl deionised formamide and stored at -20°C until ready to run.

## **2.1.7 Northern Blot**

### 2.1.7.1 Formaldehyde gel

1g of agarose was dissolved in 80ml of distilled water, supplemented with 10ml 10X RNA Buffer (200mM MOPS, 80mM Na-Acetate pH8, 10mM EDTA), 10ml 37% formaldehyde and poured.

### 2.1.7.2 Samples preparation and electrophoresis

10 $\mu$ g of total RNA per sample were supplemented with 4 volumes of denaturation dye (prepared mixing 1ml formamide with 200 $\mu$ l 10X RNA Buffer, 320 $\mu$ l 37% formaldehyde and 8 $\mu$ l 10mg/ml Ethidium Bromide), heated at 65°C for 15 minutes and immediately transferred to ice. Prior to loading on gel, 2 $\mu$ l of RNA loading buffer were added (50% glycerol, 1mM EDTA, 0.25% Bromophenol Blue, 0.25% Xylene Cyanole) and the gel was run at 75 volts for 6 hours.

### 2.1.7.3 Transfer

The gel was rinsed briefly in distilled water and the RNA was transferred to Hybond-N<sup>+</sup> nylon membrane (Amersham-Pharmacia #RPN203B) in 10X SSC buffer for at least 16 hours. The following day the nylon membrane was rinsed in 10X SSC, air dried and the RNA was UV cross-linked to the membrane using the autocross-link program of the UV Stratalinker 1800 oven (*Stratagene*).

#### 2.1.7.4 Hybridisation and results visualisation

##### *- Probe radiolabelling*

50 ng of ds probe DNA (either PCR product or digestion fragment) were mixed with 3  $\mu$ l of 3.3  $\mu$ g/ $\mu$ l of N6 random oligos (5'-NNNNNN-3') and the volume adjusted to 18  $\mu$ l with distilled water. The sample was heated at 100°C for 5 minutes, immediately transferred to ice and incubated 1 hour at 37°C with 20  $\mu$ l 2.5X N6-Buffer (0.5M Hepes pH7, 12.5mM MgCl<sub>2</sub>, 125mM  $\beta$ -mercaptoethanol, 125mM Tris-Cl pH7.5, 80  $\mu$ g/ml BSA, 50  $\mu$ M dGTP, 50  $\mu$ M dCTP, 50  $\mu$ M dTTP), 5  $\mu$ l [ $\alpha$ -<sup>32</sup>P]dATP (*Amersham-Pharmacia* #PB10384) and 2  $\mu$ l Klenow DNA polymerase I (*Promega* #M220A). The reaction was stopped with 50  $\mu$ l of 10mM EDTA and unincorporated nucleotides were removed spinning the sample at 1200rpm for 5 minutes through 500  $\mu$ l of Sepharose Cl-6B resin (*Amersham-Pharmacia* #17-0160-01) previously washed in 500  $\mu$ l of PBS. 1  $\mu$ l of the purified probe was used to measure the probe radioactivity with a  $\beta$ -counter and an equivalent amount of probe corresponding to  $\sim 40 \times 10^6$  cpm was used in the hybridisation step.

##### *- Hybridisation*

The RNA filter was pre-hybridised for a minimum of 2 hours at 65°C in 10ml of HB (5X Denhardt's solution (*Sigma* #D-2532), 5X SSC, 0.5% SDS, 10% Dextran Sulfate) supplemented with 100  $\mu$ g of denatured Salmon Sperm DNA. After pre-hybridisation, the radioactive probe was heated at 95°C for 10 minutes, immediately transferred to

ice and added to the hybridisation solution. The probe was left hybridising overnight at 65°C and the day after the filter was washed three times at 55°C for 15 minutes in Wash Solution (0.2X SSC, 0.1% SDS) and results visualised using the Fuji FLA-2000 phosphorimager.

### 2.1.8 Polymerase Chain Reaction (PCR)

Unless otherwise stated, PCRs were performed on 100ng of template DNA supplemented with 75nmoles dNTPs, 50pmoles of forward and reverse primers, 10µl of 10X buffer (500mM KCl, 100mM Tris-Cl pH9, 1% Triton X-100, 15mM MgCl<sub>2</sub>), 2.5 units of Taq polymerase (*Promega* #M1661) and distilled water to a final volume of 100µl. Agouti and Ras PCRs were performed on 1µg of library DNA.

Amplifications of inserts cloned in pHMII were performed on 1.5µl of overnight bacteria culture in 30µl final volume. Inserts were amplified using vector-specific primers flanking the cloning sites. An initial step at 94°C for 2 minutes was introduced to allow bacteria lysis and release of the target vector DNA. Following is a list of the primers and conditions used for each reaction:

Sample	Primers	Conditions	Cycles
Emx1 Probe	F: gctgcaacccccctcactc R: ggcccgtgcaaaagcagc	94°C 30sec 55°C 30sec 72°C 30sec	30
Emx2 Probe	F: aagcgctgcttcaccatcg R: ctctcgggacttgtgtcg	94°C 30sec 53°C 30sec 72°C 1min	30
GapdH probe	F: taatacgactcactataggg R: attaaccctcactaaag	94°C 30sec 55°C 30sec 72°C 1min	30

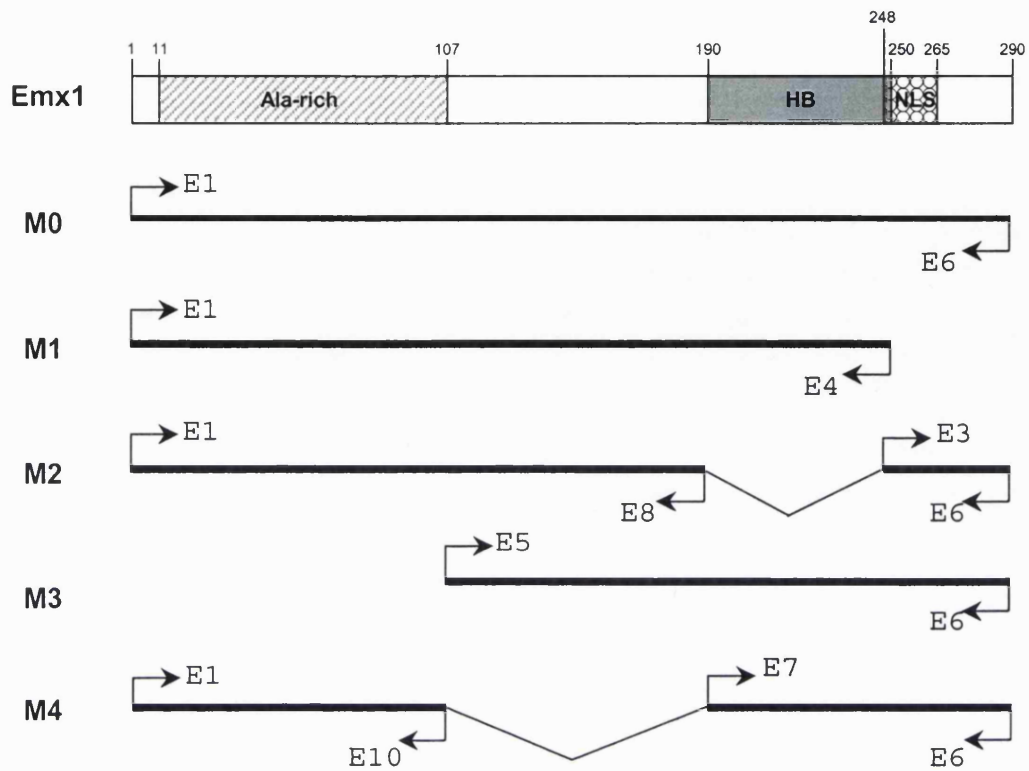
Agouti	<b>F:</b> atggatgtcaccgccta <b>R:</b> tcagcagttggggttgag	94°C 30sec 50°C 30sec 72°C 1min	40
Ras	<b>F:</b> acaagcttgtggtggtgg <b>R:</b> ggacagcacacatttgca	94°C 30sec 50°C 30sec 72°C 1min	40
pHMII cDNA inserts (from overnight culture)	<b>F:</b> tcactccttctctaggcg <b>R:</b> tgggtggaaaataaccgga	94°C 2min  94°C 30sec 50°C 30sec 72°C 2.5min	1  30

### 2.1.9 Generation of mutants

Emx1 mutants were generated by PCR using primers bearing restriction sites to facilitate cloning into the pHMII vector. M0, M1, and M3 PCRs were performed with forward and reverse primers bearing an EcoRI and XhoI site respectively. Mutants M2 and M4 were generated by amplifying the relevant 5' and 3' portions with primers bearing the EcoRI and HindIII, and the HindIII and XhoI cloning sites respectively. Following is a list of the primers used for each mutant and the conditions used for each reaction. Sequences in *italic bold* represent restriction sites: *gaattc* (EcoRI), *ctcgag* (XhoI), *aagctt* (HindIII).

Name	Sequence	Cloning site
<b>E1:</b>	ggg <b>gaatcc</b> atgtgccaggcttgggtgc	EcoRI
<b>E3:</b>	ggg <b>aagctt</b> aaacggcagaagctggaa	HindIII
<b>E4:</b>	ggg <b>ctcgag</b> tcacagcttctgccgtttgta	XhoI
<b>E5:</b>	ggg <b>gaattc</b> atgggcccgtcgctctatgga	EcoRI
<b>E6:</b>	ggg <b>ctcgag</b> ctagtcattggaggtgac	XhoI
<b>E7:</b>	ggg <b>aagctt</b> cgcaagcccaagcggatt	HindIII
<b>E8:</b>	ggg <b>aagctt</b> tgcgaagggcccgtgcaa	HindIII
<b>E10:</b>	ggg <b>aagctt</b> tccatagagcgcgagcggcc	HindIII





Mutant	Primers	Conditions	Cycles
M0	E1 - E6	94°C 30sec 53°C 30sec 72°C 1min	30
M1	E1 - E4	94°C 30sec 53°C 30sec 72°C 1min	30
M2	E1 - E8 E3 - E6	94°C 30sec 53°C 30sec 72°C 1min	30
M3	E5 - E6	94°C 30sec 53°C 30sec 72°C 1min	30
M4	E1 - E10 E7 - E6	94°C 30sec 58°C 30sec 72°C 1min	30

### 2.1.10 Western Blot

Total cell lysates were prepared from a confluent 10cm dish. Cells were trypsinised, washed in 1X PBS and lysed in 1ml of Lysis Buffer (1%SDS, 10mM Tris-Cl pH7.5). Genomic DNA was shredded by sonicating the sample through a 26-gauge needle and the insoluble material was removed by spinning at 13,000rpm for 10 minutes. The supernatant was transferred to a fresh 1.5ml microfuge tube and the protein content was estimated using a colorimetric assay (*Biorad* #500-0006). 20µg of each sample were electrophoresed for 1 hour at 150Volts on a 10% SDS-PAGE gel in Running Buffer (25mM Tris, 250mM glycine pH8.3, 0.1%SDS) and transferred for 1 hour at 400mAmps to a nitrocellulose membrane (*Schleicher & Schuell* #10402096) in Transfer Buffer (25mM Tris, 250mM glycine, 0.1%SDS, 20% Methanol). Filters were preblocked overnight at 4°C in PBS-T (PBS, 0.1% Tween-20) supplemented with 5% nonfat milk. Samples were incubated at RT for 2 hours with primary antibody diluted in PBS-T-Milk and washed 5 times at RT for 10 minutes with 10ml of PBS-T. This was followed by incubation with HRP-conjugated secondary antibody in PBS-T-Milk for 1 hour at RT. Finally the filters were washed 5 times in PBS-T and once in PBS. Results were visualised by chemiluminescence by incubating the filters for 1 minute in ECL reagent (*Amersham-Pharmacia* #RPN2106). Following is a list of antibodies used in this thesis.

<b>Primary</b>	<b>Target protein</b>	<b>Dilution</b>	<b>Supplier</b>
Microphthalmia Ab-1(C5)	Microphthalmia	1:1000	NeoMarkers #MS-771-P1
$\alpha$ PEP7	Tyrosinase	1:500	<i>Dr. Vincent Hearing</i>
$\alpha$ PEP1	Trp-1	1:500	<i>Dr. Vincent Hearing</i>
TRP2(G15)	Trp-2	1:1000	Santa Cruz #sc-10452
AC-74	$\beta$ -actin	1:1000	Sigma #A-5316
<b>HRP-Secondary</b>			
Anti-mouse	Mouse IgG	1:2000	Santa Cruz #sc-2005
Anti-rabbit	Rabbit IgG	1:2000	Santa Cruz #sc-2004
Anti-goat	Goat IgG	1:2000	Santa Cruz #sc-2020

### 2.1.11 Melanin Assay

Cells from a confluent 10cm dish were lysed in 1ml of Lysis Buffer (1%SDS, 10mM Tris-Cl pH7, 100mM EDTA). 10 $\mu$ l were diluted in water to 200 $\mu$ l and used to measure the protein contents with a standard Bio-Rad protein assay. The remaining lysates were treated with H<sub>2</sub>O<sub>2</sub> at a final concentration of 1% for 30minutes at 80°C. Samples were diluted 1 in 5 with water and melanin contents were measured with a spectrophotometer at 410nm. Results were standardised to the protein contents.

## **2.2 Library construction**

### **2.2.1 Vector preparation**

#### **2.2.1.1 CsCl<sub>2</sub> purification**

*E. Coli* cells transformed with the pHMII vector were grown in 1 litre of LB medium overnight at 37°C with constant agitation. The next morning cells were centrifuged for 15 minutes at 3,000rpm, resuspended in 10ml of GTE solution (50mM glucose, 25mM Tris-HCl pH8, 10mM EDTA) and incubated for 10 minutes at RT with 5mg of lysozyme (*Sigma #L-6876*). Lysis of the cells was completed by incubation for 10 minutes on ice with 10ml of P2 buffer (200mM NaOH, 1% SDS). The lysis reaction was neutralised with 10ml of buffer P3 (3M potassium acetate pH5.5) and proteins were precipitated incubating 20 minutes in ice. The sample was centrifuged for 15 minutes at 5,000rpm and the supernatant was collected, mixed with 0.6 volumes of isopropanol and incubated for 15 minutes at RT. Next the sample was centrifuged at 5,000rpm for 15 minutes and the nucleic acid pellet was washed twice with 70% ethanol and resuspended in 3ml TE. The RNA was removed by precipitation following the addition of 3ml of 5M LiCl followed by 10 minutes incubation on ice and 10 minutes centrifugation at 9,000rpm. The supernatant was collected and the DNA precipitated by incubating for 10 minutes on ice with an equal volume of isopropanol and centrifuging the sample for 10 minutes at 10,000 rpm. The DNA pellet was washed twice with 70% ethanol and resuspended in 4ml TE. The sample was supplemented with 5g CsCl<sub>2</sub> and 300µl of 10mg/ml ethidium bromide, transferred to an Ultracentrifuge tube and centrifuged at 55,000rpm overnight. This

step separated the bacterial genomic DNA from the plasmid DNA producing two distinct visible bands. The lower band, containing the plasmid DNA, was harvested using a syringe and transferred to a fresh ultracentrifuge tube. This was filled with a solution of 1.59g/ml CsCl<sub>2</sub> in TE and the sample centrifuged at 55,000rpm for a minimum of 6 hours. The lower visible band was again harvested using a syringe and transferred to a 15ml tube. To eliminate the ethidium bromide from the solution, the sample was washed at least 4 times with 12ml H<sub>2</sub>O-saturated butanol (1:1 H<sub>2</sub>O/butanol solution), and twice with 100% butanol. Plasmid DNA was precipitated by adding 2.5 volumes of cold 100% ethanol and centrifuging for 15 minutes at 10,000rpm. Finally the pellet DNA was washed twice with 70% ethanol and resuspended in 500µl TE.

#### 2.2.1.2 Digestion and electroelution

20µg of pHMII vector purified by CsCl<sub>2</sub> were digested with 40units of EcoRI and XhoI restriction enzymes for 90 minutes at 37°C. The digested vector was loaded on a 1% agarose gel in TAE supplemented with 150ng/ml of ethidium bromide and electrophorated at 100 volts for 30 minutes. The band corresponding to the linearised vector was cut and the DNA was electroeluted in 1ml of TAE using dialysis tubes (*Gibco* #15961). The sample was washed at least 3 times in H<sub>2</sub>O-saturated butanol and twice in 100% butanol to remove residual ethidium bromide, extracted twice in phenol-chloroform and once in 100% chloroform and finally ethanol precipitated and resuspended in 50µl distilled water.

## 2.2.2 cDNA preparation

### 2.2.2.1 RNA extraction

Total RNA was extracted from mouse embryos at days 7 and 15 and adult mouse brain and testis. Tissues were provided by Cold Spring Harbor Laboratory Animal House and the RNA was extracted using the TRIzol reagent (*Gibco #15596*) following the supplier instructions. Briefly, tissues were homogenised in 1ml of TRIzol per 50mg of sample until all debris were dissolved in the solution. Samples were incubated for 5 minutes at RT, mixed vigorously for 15 seconds with 0.2ml of chloroform per ml of TRIzol reagent, and incubated 3 minutes at RT. Separation of RNA from proteins and DNA was performed by centrifuging the samples at 12,000g for 15 minutes at 4°C. The supernatants were transferred to a fresh tube and the RNA was precipitated by mixing the samples with 0.5ml of isopropanol per ml of TRIzol reagent used for the initial homogenisation. Samples were incubated 10 minutes at RT and centrifuged at 12,000g for 10 minutes at 4°C. The RNA pellets were washed twice in 75% ethanol and resuspended in 500µl of DEPC-treated water.

### 2.2.2.2 PolyA<sup>+</sup> enrichment

#### *- Column preparation*

For each sample a Poly-Prep column (*Biorad #731-1550*) was loaded with 0.1g of oligo-dT cellulose (*New England Biolabs #1401*) resuspended in 5ml of 0.1M NaOH. The cellulose was washed with 15ml of 1X Elute Buffer (10mM Tris-Cl pH7.5, 0.5%

SDS, 1mM EDTA) in order to equilibrate the pH to 7-7.5 and then washed with 10ml of 1X Start Buffer (10mM Tris-Cl pH7.5, 0.5% SDS, 1mM EDTA, 500mM LiCl).

*- Samples preparation and enrichment*

2mg of each sample of total RNA were diluted in 2ml of DEPC-treated water and supplemented with 250 $\mu$ l of 10X Elute Buffer (100mM Tris-Cl pH7.5, 5% SDS, 10mM EDTA). Samples were heated at 65°C for 5 minutes, immediately transferred to ice and supplemented with 250 $\mu$ l of 5M LiCl. The RNA was loaded on the Oligo-dT cellulose columns and the eluate was collected and recycled through the columns twice. The PolyA<sup>+</sup> samples were washed first with 15ml of 1X Start Buffer, then with 15ml of 1X Medium Salt Buffer (10mM Tris-Cl pH7.5, 0.5% SDS, 1mM EDTA, 1.5M LiCl) and finally eluted from the column with 1ml 1X Elute Buffer followed by 1ml DEPC-treated water. Samples were again heated at 65°C, transferred to ice and supplemented with 110 $\mu$ l of 10X Elute Buffer and 220 $\mu$ l of 5M LiCl. Columns were reprimed by resuspending and washing the resin in 20ml of 1X Elute Buffer and the samples were loaded again. The eluate was collected and recycled through the resin twice, and the samples were washed in 15ml 1X Start Buffer and 15ml 1X Medium Salt Buffer. The PolyA<sup>+</sup> were eluted with 2ml 1X Elute Buffer followed by 1ml DEPC-treated water, extracted with 1 volume of phenol/chloroform and ethanol precipitated. Pellets were resuspended in 300 $\mu$ l DEPC-treated water and again extracted once with phenol/chloroform, once with chloroform and ethanol

precipitated. Pellets were washed twice in 70% ethanol and resuspended in 40µl DEPC-treated water.

### 2.2.2.3 cDNA synthesis

cDNA synthesis and modifications for subcloning into the pHMII vector were performed using the ZAP Express cDNA Synthesis Kit (*Stratagene #200403*) following the supplier instructions.

#### *- First-strand synthesis*

10µg of enriched PolyA<sup>+</sup> were heated at 65°C for 5 minutes and transferred to ice in order to remove secondary structures in the RNA that could interfere with subsequent steps. The sample was supplemented with 5µl 10x first-strand buffer, 3µl methyl nucleotide mixture, 1µl RNase inhibitor, 2µl linker primer (50-mer with XhoI site (in bold) 5'-ga(x10)actagt**CTCGAG**-PolyT(x18)-3') and DEPC-treated water to 38.5µl. After 10 minutes incubation at RT to allow primer annealing, 1.5µl MMLV-Reverse Transcriptase were added and the reaction was incubated at 37°C for 1 hour. Prior to incubation at 37°C, in order to estimate the efficiency of the first-stand synthesis, a control reaction was prepared by incubating 5µl of the sample with 0.5µl [ $\alpha$ -<sup>32</sup>P]dATP at 37°C for 1 hour. The efficiency of the synthesis reaction was calculated as follows: three round filters (DE-81 Whatman paper) were spotted with 1µl of radioactive control reaction, two immediately before the samples were incubated at 37°C (time 0h) and the third one when the reaction was over (time 1h). The time 1h



filter and one of the time 0h filters were washed three times at RT in 0.25M NaPO<sub>4</sub> for 15 minutes in order to remove unincorporated nucleotides and the level of radioactivity measured on each filter using a  $\beta$ -counter. The efficiency of the reaction was estimated by dividing the incorporated radioactivity (1h washed filter minus 0h washed filter (background)) by the total radioactivity available at the beginning of the reaction (0h unwashed filter).

*- Second-strand synthesis*

The remaining 45 $\mu$ l of the first-strand reaction were supplemented with 20 $\mu$ l 10x second-strand buffer, 6 $\mu$ l dNTP mixture, 2 $\mu$ l [ $\alpha$ -<sup>32</sup>P]dATP, 2 $\mu$ l RNase H, 11 $\mu$ l DNA polymerase I, and 114 $\mu$ l distilled water. The reaction was incubated for 2.5 hours at 16°C and its efficiency estimated in a similar way to that described for the first-strand synthesis.

*- Blunting the cDNA termini*

In order to efficiently ligate the EcoRI polylinkers, the cDNAs were blunted adding 23 $\mu$ l dNTP mix and 2 $\mu$ l *Pfu* DNA polymerase to the second-strand reaction. The sample was incubated at 72°C for 30 minutes and the cDNAs extracted once in phenol/chloroform, once in chloroform and finally ethanol precipitated overnight.

*- EcoRI adapters ligation and phosphorylation*

The next day the pellets were washed twice in 70% ethanol and resuspended at 4°C for 30 minutes in 8µl of EcoRI adapters solution. The adapters were composed of 9- and 13-mer complementary oligonucleotides resulting in an EcoRI cohesive ends with phosphorylated blunted ends to allow ligation to the cDNAs. Ligation of the adapters was carried out incubating the sample at 8°C for 24 hours with 1µl 10x ligase buffer, 1µl 10mM rATP and 1µl T4 DNA ligase. The day after the reaction was terminated by heat inactivation of the ligase at 70°C for 30 minutes and the cohesive ends of the adapters were phosphorylated to allow ligation of the cDNA into the digested vector. This was done by incubating the sample at 37°C for 30 minutes with 1µl 10x kinase buffer, 2µl 10mM rATP, 6µl distilled water and 1µl T4 polynucleotide kinase. The kinase was heat inactivated at 70°C for 30 minutes and the sample allowed to cool at RT on the bench.

*- XhoI digestion of the cDNAs*

The 3' cDNA termini were digested with XhoI by incubating at 37°C for 90 minutes with 28µl XhoI buffer-supplement and 3µl XhoI (40u/µl). Since the first-strand synthesis was performed with methylated dCTP, only the XhoI sites of the linker primers were susceptible to the enzyme digestion, leaving the cDNAs intact. Following restriction enzyme digestion, the sample was ethanol precipitated overnight and the day after the pellet was washed twice in 70% ethanol and resuspended in 16.4µl TE.

#### 2.2.2.4 Size fractionation

In order to select cDNAs according to their size and to remove unincorporated residual nucleotides and EcoRI adapters, the sample was size fractionated by passing it through a Sephacryl S-500 resin (*Amersham-Pharmacia* #17-0613) column.

##### *- Column preparation*

An Econo-column (*Biorad* #737-0521) was connected to a fraction collector, rinsed with column buffer (TE+400mM NaCl) and loaded with S-500 resin diluted 1:5 in column buffer until the bed of the resin reached the top of the column. The resin was washed with 5ml column buffer and to ensure its complete equilibration, 2 $\mu$ l of DNA loading buffer (with Xylene Cyanole and Bromophenol Blue) were mixed with 18 $\mu$ l of column buffer, loaded on top of the resin and run until both dyes disappeared.

##### *- Sample fractionation*

Before loading the sample, the cDNA was heated at 65°C for 5 minutes, transferred to ice and supplemented with 1.6 $\mu$ l 5M NaCl and 2 $\mu$ l DNA loading buffer. Once the sample was loaded, 30 fractions of 200 $\mu$ l each were collected in 1.5ml microfuge tubes and their level of radioactivity measured with a  $\beta$ -counter. In the 30 fractions collected, two peaks of radioactivity were recognisable, the first corresponding to the cDNAs and the second representing unincorporated nucleotides.

2 $\mu$ l of the fractions corresponding to the first peak were run on a 1% TAE agarose gel for 30 minutes at 100 volts. The gel was fixed for 30 minutes in fixing buffer (10%

glycerol, 10% acetic acid), vacuum dried and exposed for 2 hours using the Fuji FLA-2000 phosphorimager. Fractions corresponding to cDNAs bigger than 200bp were pooled together and ethanol precipitated overnight. The day after, the cDNA pellet was washed twice in 70% ethanol and resuspended in distilled water.

### **2.2.3 Ligation of the cDNAs into pHMII and transformation**

600ng of digested vector (as described in 2.2.1.2) were mixed with 600ng of cDNA, 2µl 10x ligase buffer, 1µl T4 ligase (*Gibco* #15224) and distilled water to 20µl. Samples were incubated 24 hours at 16°C, supplemented with 1µl of fresh T4 ligase and incubated at 16°C for an additional 24 hours. The volume of the reaction was brought to 100µl with distilled water, and the sample extracted twice with phenol/chloroform, once with chloroform, and ethanol precipitated using 1µl of glycogen as carrier (*Gibco* #10814-010). The DNA pellet was washed twice in 70% ethanol and resuspended in 20µl of distilled water. The sample was split in 5 aliquots of 4µl that were electroporated in 25µl of DH10β Electromax competent cells (*Gibco* #18290-015) at 1.8 KV using 1mm gap cuvettes and the “*E. Coli* Pulser” electroporator (*Biorad*). All samples were incubated at 37°C for 1 hour with constant agitation in 10ml of SOC media and 1µl plated on an LB-agar plate supplemented with 100µg/ml Ampicillin and 30µg/ml Zeocin to allow calculation of the library complexity. The rest was incubated overnight at 37°C with constant agitation in 4 litres of LB with 100µg/ml Ampicillin and 25µg/ml Zeocin. The morning after a CsCl<sub>2</sub> preparation of the library was performed as described in 2.2.1.1.

## **2.3 Tissue Culture**

### **2.3.1 Cell lines growth and maintenance**

#### **2.3.1.1 Melb-a**

The melanoblast cell line Melb-a was grown in RPMI-1640 medium (*Gibco* #31870) with 10% FCS (*Sigma* #F7524), 2mM L-Glutamine (*Gibco* #25030) and 1X antibiotics (*Gibco* #15240). 20nM TPA (*Calbiochem* #524400), 40pM bFGF (*R&D* #133-FB-025) and 20ng/ml of murine SCF (*Insight Biotechnology* #IB-1084) were added freshly to the medium before use. Cells were incubated in a 10%CO<sub>2</sub> atmosphere at 37°C until 80% confluent, then split 1 in 4 with Trypsin-EDTA (*Gibco* #35400). Differentiation of the cells in pigment producing melanocytes was achieved by changing the medium to DMEM (*Gibco* #41965) with 10% FCS supplemented with 200nM TPA and 1nM [4-Nle, 7-D-Phe]- $\alpha$ -MSH (*Sigma* #M8764) for 10 days. Stocks were prepared in RPMI-7.5% DMSO (*Sigma* #D8779) and transferred to liquid-nitrogen after 24 hours at -80°C.

#### **2.3.1.2 Melan-a**

The melanocyte cell line Melan-a was grown in RPMI-1640 medium with 10% FCS, 2mM L-Glutamine and antibiotics. The medium was also supplemented with 200nM TPA and cells were incubated in a 10% CO<sub>2</sub> atmosphere at 37°C until 80% confluent. Splitting 1 in 4 of the cells was performed with Trypsin-EDTA. When stocks needed to be prepared, cells were first depigmented by adding a tyrosinase inhibitor for at

least 1 week (200 $\mu$ M PTU *Sigma* #P7629). Stocks were prepared in RPMI-7.5% DMSO.

#### 2.3.1.3 Melanoma cell lines

All melanoma cell lines were grown in RPMI-1640 with 10% FCS, 2mM L-Glutamine and antibiotics in a 10% CO<sub>2</sub> atmosphere at 37°C. Cells were split 1 in 4 with Trypsin-EDTA when 80% confluent. Stocks were prepared in RPMI-7.5% DMSO.

#### 2.3.1.4 LinXE

The packaging cell line LinXE was grown in DMEM with 10%FCS and 50 $\mu$ g/ml Hygromycin B (*Calbiochem* #400051). Cells were incubated in a 5% CO<sub>2</sub> atmosphere at 37°C. When confluent, cells were split 1 in 4 with Trypsin-EDTA. Stocks were prepared in DMEM-7.5% DMSO.

### **2.3.2 Cell lines Transduction**

#### 2.3.2.1 Calcium-Phosphate Transfection

Confluent cells were split 1 in 4 and the day after the medium was changed 4h prior to transfection. 25 $\mu$ g of vector DNA were diluted to 450 $\mu$ l with distilled water and supplemented with 50 $\mu$ l of a 2.5M CaCl<sub>2</sub> solution. The sample was thoroughly mixed and 500 $\mu$ l of 2X HBS pH 7.0 were added (50mM HEPES, 1.5mM NaH<sub>2</sub>PO<sub>4</sub>, 280mM

NaCl). HBS was added drop by drop while bubbling the DNA solution by blowing air with a pasteur pipette. Samples were incubated at RT for 30 minutes and added to the target cells. The transfection was carried out at 37°C in a 5% CO<sub>2</sub> atmosphere for 16 hours at the end of which the media was changed. Before selecting successfully transfected cells or assaying enzymatic activity, cells were grown for 48 hours. If the transfection was carried out on LinXE with the intention of producing virus, cells were incubated for 48 hours at 32°C.

#### 2.3.2.2 Retroviral Infection

Target cells (either Melan-a or Melb-a) were seeded at 5x10<sup>4</sup>/ml 24 hours before infection. Virus production was obtained by incubating LinXE transfected with retroviral vector in RPMI-1640 at 32°C for 48 hours. The medium was collected, filtered through a 45µm syringe filter in order to remove cell debris, diluted 1 in 2 with fresh RPMI and supplemented with 8µg/ml Polybrene (*Sigma #H9268*) and 200nM TPA (for Melan-a) or 20nM TPA, 40pM bFGF and 20ng/ml of murine SCF (for Melb-a). The virus-containing medium was applied to the target cells and the infection was carried out at 37°C in a 10% CO<sub>2</sub> atmosphere for 8 hours. At the end of the infection step, the medium was replaced and cells were grown for at least 24 hours before selecting for successfully infected ones (100µg/ml Hygromycin B).

### **2.3.3 $\beta$ -galactosidase staining of cultured cells**

Cells cultured on 10cm dishes were washed twice in 1X PBS, and fixed in glutaraldehyde solution (0.5% glutaraldehyde in 1X PBS) for 15 minutes at RT. Samples were washed three times in PBS/MgCl<sub>2</sub> (1X PBS, 1mM MgCl<sub>2</sub>) and incubated at 37°C for 15 hours with 5ml of staining solution (1X PBS supplemented with 1mM MgCl<sub>2</sub>, 5mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 5mg X-Gal). Stained plates were stored at 4°C.

### **2.3.4 Crystal violet staining of cultured cells**

Cells were fixed in 0.5% glutaraldehyde as described above. This was followed by two washes in 1X PBS and 30 minutes incubation at RT in CV solution (0.25% Crystal Violet in 1X PBS). Excess crystal violet staining was removed with repetitive washes in distilled water. Stained plates were stored at 4°C.

### **2.3.5 Colonies subcloning**

Subcloning of positive colonies was carried out using the filter-lifting technique. Small circles about 3mm in diameter of 3MM paper were sterilised by autoclaving in a 50ml Falcon tube at 121°C for 20 minutes and soaked in 1X trypsin. Dishes carrying the colonies to subclone were washed once in 1X PBS and the soaked filters applied to the target colonies. After 30 seconds, the colony was gently scraped with the filter and transferred to a 24 well plate with 1ml of medium per well. Samples were incubated at 37°C in a 10% CO<sub>2</sub> atmosphere and filters were removed after 24 hours.



### **2.3.6 Soft agar assay**

Soft agar assays were carried out in 6 well dishes. Cells were grown in a top layer of 0.3% agar (called top-agar) lying on a base layer of 0.5% agar (base-agar). The base-agar was prepared as follow: 1ml of 1% Low Melting agarose pre-equilibrated at 45°C was mixed with 1ml of room temperature 2X medium (made from 10X RPMI (*Gibco* #22511-018)) supplemented with 20% FCS, 4mM L-glutamine and 2X antibiotics, and poured in 6 well plates (2ml per well). The 0.5% agar medium was left to solidify at RT for at least 30 minutes. Cells in top-agar were prepared mixing 1ml of 1% agarose at 45°C with 1ml of 2X medium and 1ml of 1X medium containing 10<sup>4</sup> cells. The sample was immediately poured on the base-agar and left at RT to set for at least 30 minutes. Finally samples were supplemented with 1 ml of 1X medium and transferred at 37°C in 10% CO<sub>2</sub> atmosphere. Colony formation was assayed for 21 days during which the medium was changed with 1ml of fresh RPMI every three days.

## **2.4 Screening associated methods**

### **2.4.1 Genomic DNA extraction**

Genomic DNA was purified from cells using the Blood and Cell Culture DNA Midi Kit (*Qiagen* #13343), following the supplier instructions. Briefly, a confluent 10cm dish of cultured cells was trypsinised and pelleted in a 15ml tube. The cell pellet was

washed once in 10ml 1X PBS and resuspended in 2ml of ice cold 1X PBS. Cells were lysed and the nuclei stabilised by incubating the sample for 10 minutes in ice with 2ml of ice-cold buffer C1 (1.28M sucrose, 40mM Tris-Cl pH7.5, 20mM MgCl<sub>2</sub>, 4% Triton X-100) and 6ml of ice-cold distilled water. The nuclei were pelleted by centrifuging at 1300g for 15 minutes at 4°C and lysed by vortexing for 30 seconds in 5ml of buffer G2 (800mM guanidine HCl, 30mM Tris-Cl pH8, 30mM EDTA pH8, 5% Tween-20, 0.5% Triton X-100). Samples were incubated 1 hour at 55°C with 2mg of QIAGEN Protease and loaded on a pre-equilibrated Genomic Tip-100. The genomic DNA was washed twice with 7.5ml of buffer QC and eluted with 5ml of buffer QF pre-heated at 55°C. Precipitation of the genomic DNA was performed by gently mixing the samples with 3.5ml of isopropanol. The resulting visible genomic DNA precipitates were transferred to a 1.5ml tube, washed twice in 70% ethanol and resuspended in 500µl of distilled water.

In order to completely remove melanin from the samples, a further purification was performed by extracting the resuspended genomic DNA with phenol/chloroform three times and with chloroform twice. The purified DNA was finally ethanol precipitated, washed twice in 70% ethanol and resuspended in 50-100µl of distilled water.

#### **2.4.2 CRE recombinase purification**

CRE recombinase was expressed in BL21 *E.Coli* cells transformed with the pET19d vector carrying the recombinase cDNA in frame with a His-Tag. One litre of LB supplemented with 100µg/ml of Ampicillin was incubated at 37°C with 10ml of an

overnight culture of BL21. Cells were grown with constant agitation until the OD<sub>600</sub> reached 0.6. Protein expression was induced by adding IPTG to a final concentration of 1mM and expression of the protein was carried out at room temperature for 16 hours. Cells were centrifuged at 4,500 rpm for 10 minutes at 4°C and the pellet was resuspended in 10ml of Sonication Buffer (50mM NaPO<sub>4</sub> pH8, 300mM NaCl) supplemented with protease inhibitors (*Roche* #1-873-580, 1 tablet per 50ml lysate). Lysis of the cells was performed by sonicating the sample at 4°C five times consecutively for 1 minute with 2 minutes rest. The insoluble material was removed by centrifuging the sample at 10,000rpm for 30 minutes followed by filtration of the supernatant through a 0.45µm syringe filter. Purification of the protein was performed using 2ml (1ml bed volume) of Ni-NTA Agarose metal chelating resin (*Qiagen* #30210) pre-washed three times in sonication buffer. The sample was incubated with the resin for 1 hour at 4°C on a mixer followed by three 10ml washes with Wash Buffer (50mM NaPO<sub>4</sub> pH8, 300mM NaCl, 10% glycerol) separated by centrifugation steps at 800rpm for 5 minutes. Samples were purified further with two washes in Wash Buffer supplemented with 50mM Imidazole and two washes in Wash Buffer supplemented with 100mM Imidazole. Finally the resin was loaded on a Poly-Prep disposable column (*Biorad* #731-1550) and the CRE recombinase eluted in 500µl aliquots with Wash Buffer supplemented with 200mM Imidazole. Fractions were diluted with an equal volume of 100% glycerol and stored at -80°C.

### **2.4.3 CRE recombinase reaction**

10-20 $\mu$ g of genomic DNA were incubated for 6 hours at 37°C with 10 $\mu$ l 10X buffer (500mM Tris-Cl pH7, 330mM NaCl, 100mM MgCl<sub>2</sub>, 1mg/ml BSA, 10mM DTT), 2 $\mu$ l of purified CRE recombinase and distilled water to a final volume of 100 $\mu$ l. Samples were extracted three times with phenol/chloroform, once with chloroform and ethanol precipitated. Pellets were washed twice in 70% ethanol and resuspended in 5 $\mu$ l of distilled water. For each sample, 3 $\mu$ l were electroporated into DH10 $\beta$ -trfA and plated on LB agar with 30 $\mu$ g/ml Zeocin.

### **2.4.4 Cell sorting**

Cells were detached from the dishes by incubating for 2 minutes at 37°C in Trypsin-EDTA. The reaction was stopped with 10ml RPMI-10%FCS and samples were washed once in 1X PBS. Cell pellets were resuspended in 3ml of RPMI-2%FCS and sorted using a Beckman-Coulter ALTRA Cell Sorter. Sorting was performed with a 488nm laser at 1,500cells/sec applying a pressure of 12psi. Positive cells were gated and collected in RPMI-10%FCS, supplemented with 200nM TPA and plated in 10cm dishes.

## **3 RESULTS: LIBRARY CONSTRUCTION AND SCREENING**

The aim of the project was to isolate new genes involved in melanocyte differentiation and melanoma progression through the functional screening of a mouse cDNA library cloned into the pHMII retroviral vector. The project can be divided into the following stages:

- a. Construction of the cDNA library
- b. Reconstruction and optimisation of the screening conditions in Melb-a and Melan-a
- c. Functional screening of the library
- d. Characterisation of the positive clones

The following is a description of the strategies applied, the problem encountered and the solutions proposed to achieve the intended goal.

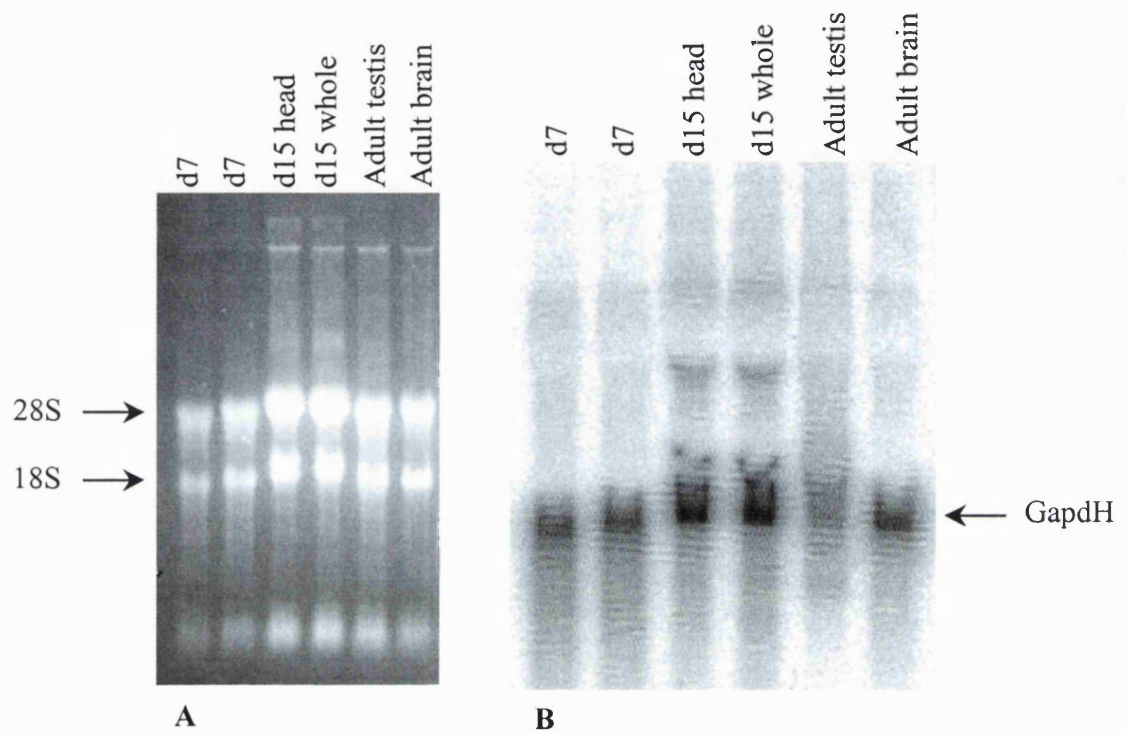
## **3.1 Library Construction**

### **3.1.1 RNA extraction**

In order to be able to screen as many different genes as possible, total RNA was extracted from mouse embryos at different development stages (day 7 and 15) and from mouse adult tissues (head and testis). The choice of using embryos at day 7 and 15 was dictated by the evidence that at these time during embryogenesis, genes should be expressed that control melanoblasts migration from the neural crest to the dermis and their subsequent differentiation into melanocytes (Manova *et al*, 1991).

Adult brain and testis tissues were chosen because they express a vast number of different transcripts, hence enhancing the diversity of the library.

The RNA extraction yielded ~3mg of total RNA per sample. The quality of the RNA was verified by running between 4 and 10µg of total RNA on 1% agarose gel followed by Northern Blot using a <sup>32</sup>P labelled mouse *GapdH* probe (**Fig 3.1**).



**Fig 3.1** A) 1% agarose gel loaded with 4 to 10 $\mu$ g of total RNA. Arrows indicate the position of the 18S and 28S ribosomal RNA bands. B) Northern Blot using a GapdH radioactive probe. The arrow indicate the position of the GapdH mRNA.

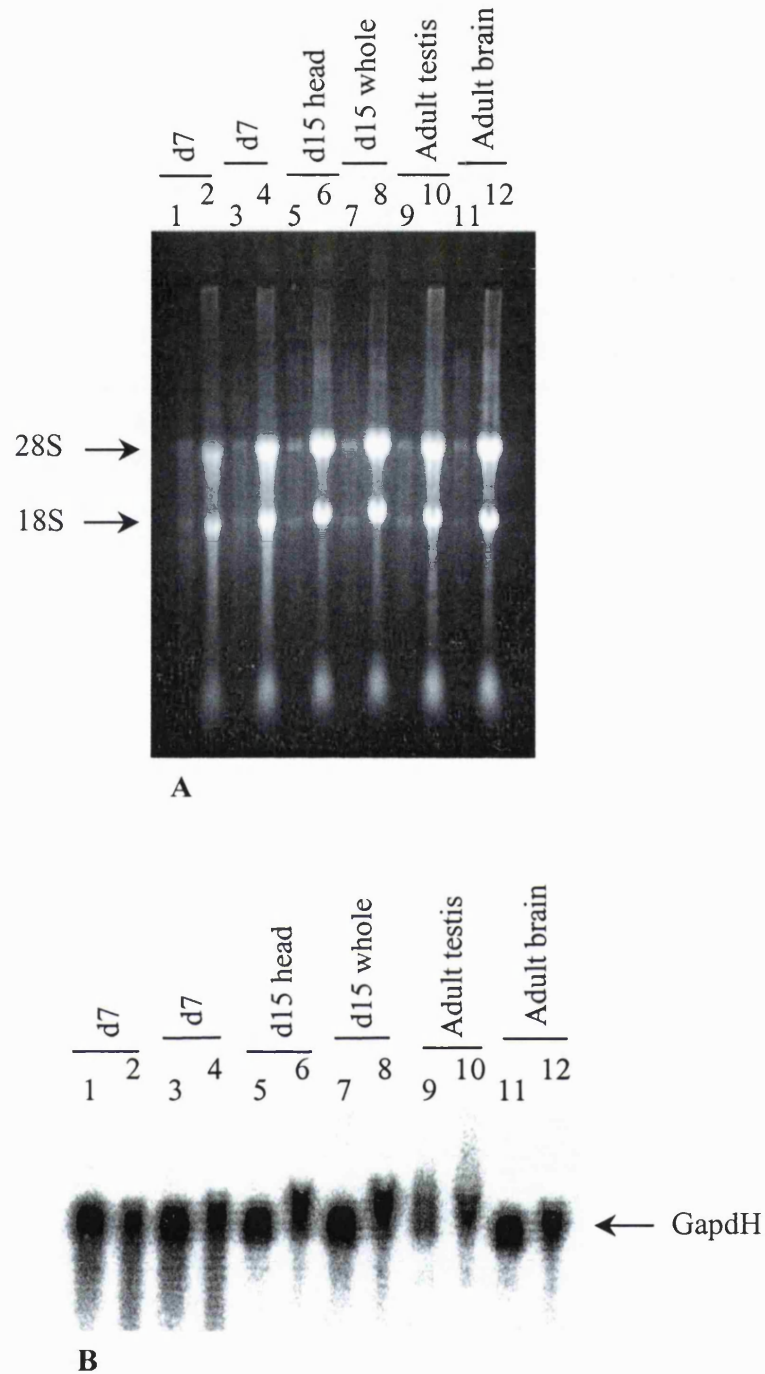
### 3.1.2 PolyA<sup>+</sup> enrichment

In order to synthesise the cDNA for the library construction, the messenger RNA (PolyA<sup>+</sup>) was enriched from the total RNA using an Oligo-dT resin. The enrichment ratio of each sample was assessed by running and blotting 1 µg of enriched PolyA<sup>+</sup> alongside 10 µg of the corresponding total RNA, followed by hybridisation with <sup>32</sup>P labelled *GapdH* probe. The enrichment ratio was estimated to be around 20 fold, as assessed by comparing the signals intensity using a Fuji Phosphorimager (**Fig 3.2**). The mRNA normally represents between 2-4% of the total RNA extracted. This means that a 20 fold enrichment was raising the PolyA<sup>+</sup> ratio to 40-80% of the total RNA, a value considered to be suitable for downstream applications (Watson *et al*, 1985).

### 3.1.3 cDNA synthesis and ligations

cDNA was synthesised starting from 10 µg of PolyA<sup>+</sup> RNA (2 µg of each sample). The cDNA synthesis consists of two main steps: first strand synthesis and second strand synthesis. In each step radioactive dATP was added to the reaction to allow the estimation of the efficiency of the reaction. This was done by spotting on three discs of Whatman paper 1 µl of the reaction. Two were spotted at time zero (no incorporation), and one at time 1 hour (when the reaction was over). The time 1h and



**Fig 3.2**

**A)** Agarose gel loaded with 1 $\mu$ g PolyA<sup>+</sup> (lanes 1, 3, 5, 7, 9 and 11) and 10 $\mu$ g total RNA (lanes 2, 4, 6, 8, 10, and 12). Arrows indicate the position of the 18S and 28S ribosomal RNA bands.

**B)** Northern blot using a GapdH radioactive probe, the arrow indicate the position of the GapdH mRNA.

one time 0h filters were washed in order to remove any unincorporated nucleotide, and the radioactivity values from all filters were used to estimate the efficiency of first strand synthesis. A similar procedure was done when synthesising the second strand. As shown in **Table 1**, there was a ~20% yield for the first strand synthesis and 100% for the second strand one. These values are considered indicative of a successful cDNA synthesis reaction (Watson *et al*, 1985).

The cDNA was first modified at the ends to generate EcoRI and XhoI sites for ease of cloning, and then size-fractionated by running it on a Sephacryl S-500 column. The size fractionation allows separation of the modified cDNAs from the linkers used to create the EcoRI and XhoI sites and the free unincorporated nucleotides. In addition it allows small cDNAs of less than 200bp to be discarded, as they are likely to represent truncated molecules. Thirty 200 $\mu$ l fractions were collected and their radioactive content was measured: two peaks of radioactivity were recognisable. The first, between fractions 8 and 12, corresponded to the synthesised cDNA. The second represented free unincorporated nucleotides. 2 $\mu$ l of fractions 8 to 15 were run on a 1% agarose gel. The gel was then dried and exposed using the Fuji Phosphorimager (**Fig 3.3**). Fractions 8, 9 and 10 were considered to be within the suitable range in size for constructing a full length library, and were therefore precipitated and resuspended in 10 $\mu$ l of water.

600ng of cDNA thus prepared were ligated into 600ng of EcoRI and XhoI digested pHMII vector. After transformation of DH10B electrocompetent cell, the size of the library was estimated to be  $\sim 6 \times 10^6$  clones.

Knowing the percent of incorporated radioactivity during the cDNA strand reaction, the cDNA yield can be calculated as follow:

$$\text{cDNA yield} = \frac{\text{Incorporated (1h washed)} - \text{Background (0h washed)}}{\text{Total Radioactivity (0h unwashed)}} \times \text{moles dATP in reaction} \times 4 \text{ (nucleotides)} \times \frac{\text{Nucleotides}}{\text{MW}}$$

In our case, for the first strand

$$\text{cDNA yield (g)} = \frac{47,202.4 - 1795 \text{ cpm}}{821,550.9 \text{ cpm}} \times 3 \times 10^{-8} \text{ moles dATP} \times 4 \times 330 \text{ (MW)} = 2.18 \times 10^{-6} \text{ g}$$

Since we started with 10 $\mu\text{g}$  of PolyA<sup>+</sup> and we synthesised 2.18 $\mu\text{g}$  of first strand cDNA, the efficiency of first strand synthesis has been 21%.

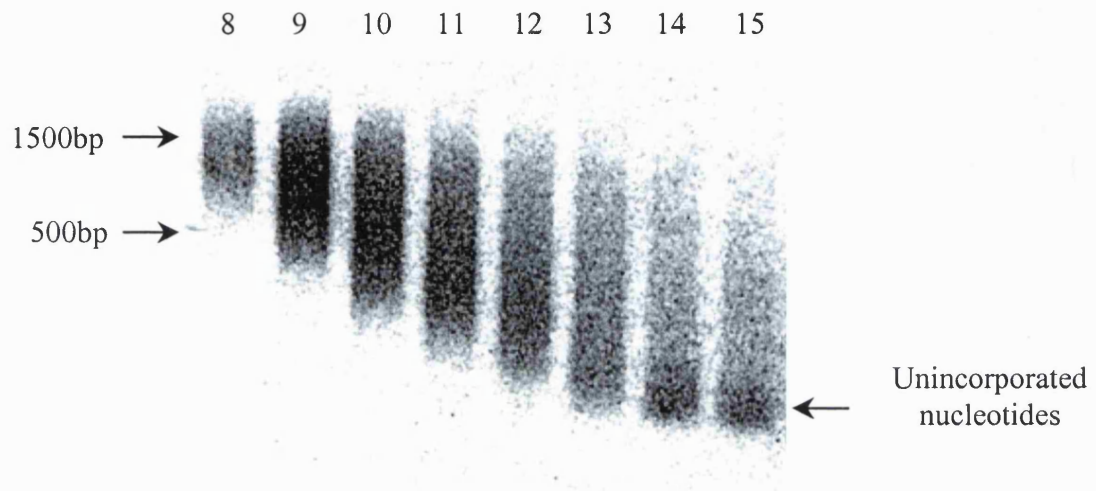
An identical procedure is used to assess the efficiency of second strand synthesis.

In our case:

$$\text{cDNA yield (g)} = \frac{2,005.21 - 460 \text{ cpm}}{52,189.59 \text{ cpm}} \times 6 \times 10^{-8} \text{ moles dATP} \times 4 \times 330 \text{ (MW)} = 2.3 \times 10^{-6} \text{ g}$$

Since we started with 2.18 $\mu\text{g}$  of template (first-strand) and we synthesise 2.3 $\mu\text{g}$  of product (second-strand) it means that virtually all the single strand cDNA from the first reaction was successfully converted into double strand cDNA making the efficiency of the second strand synthesis reaction close to 100%.

**Table 1**



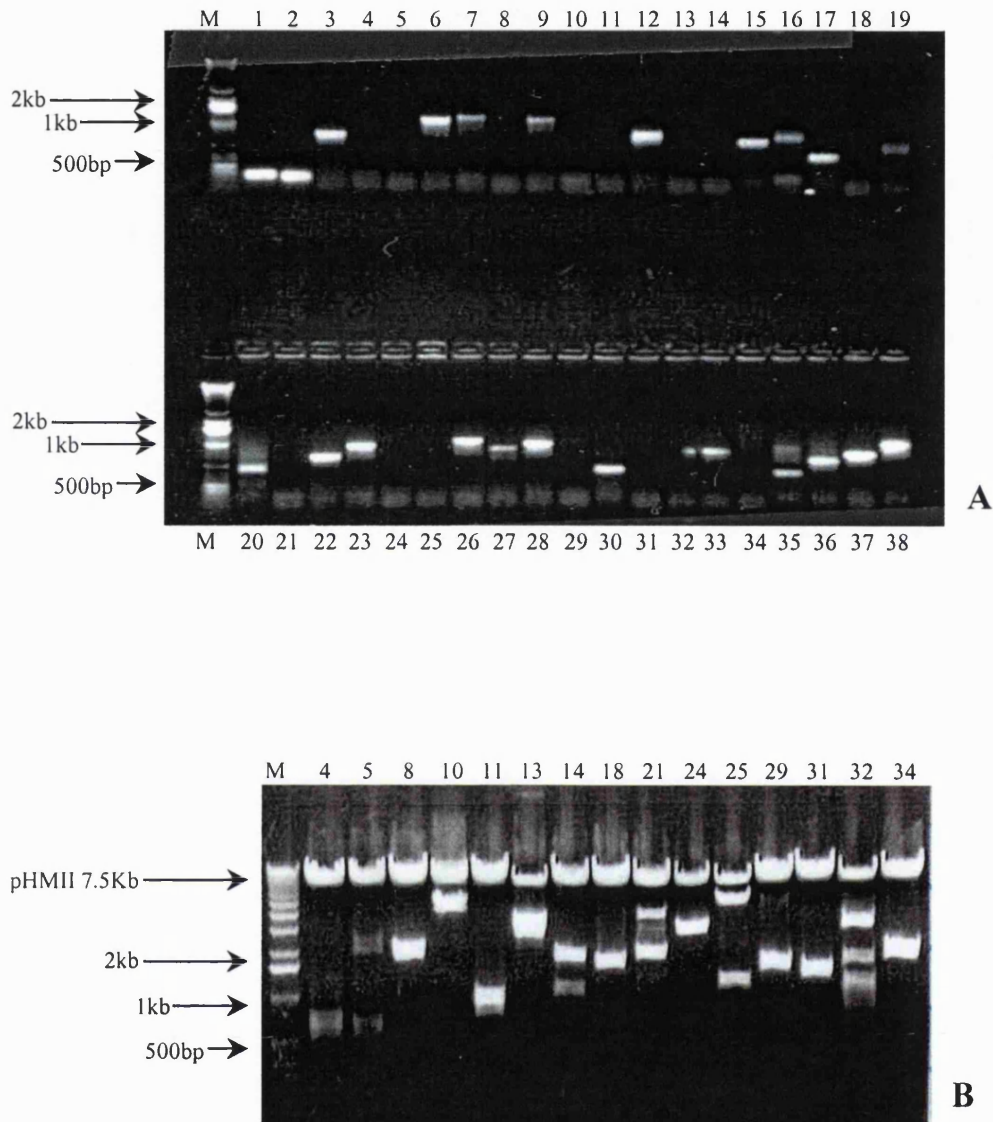
**Fig 3.3** 2 $\mu$ l of fractions 8 to 15 were run on a 1% agarose gel and analysed with a Fuji Phorpshoimager. Only fractions 8, 9 and 10 were used for the library construction.

### 3.1.4 Library quality

The quality of the library was checked by PCR amplification of the inserts of 38 independent clones using vector specific primers immediately flanking the cloning sites. Out of the 38 clones, 15 did not amplify, mainly because the inserts were too large. These were tested by digesting the DNA with EcoRI and XhoI, together with an additional 9 independent clones. Out of the 47 clones analysed 2 had no insert, whereas the remaining 45 had inserts with sizes spanning from 300bp to more than 5kb (Fig 3.4 and Fig 3.5A).

An additional test was performed by amplifying from 1µg of library the cDNA of the two genes used for the reconstruction of the screening: *Agouti* and activated *Ras* (*H-Ras*). In both cases, the amplification of the full length cDNA was successful (Fig 3.5B).

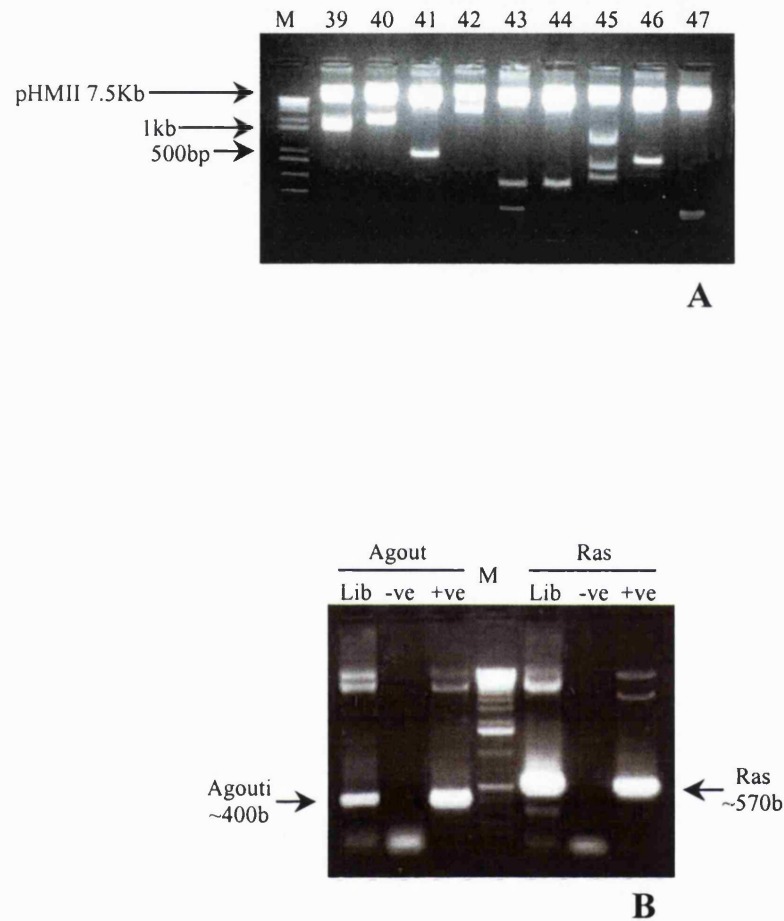
Taken together these results suggest that the library was successfully constructed with 1 out of 23 clones on average being empty vector (~95% recombinant).

**Fig 3.4**

**A)** 1.5 $\mu$ l of ON liquid culture were boiled and the extracted DNA was amplified using primers flanking the cloning sites of the vector.

Two clones had no insert (1, 2), whereas fifteen did not amplify and were tested by restriction enzyme digestion (4, 5, 8, 10, 11, 13, 14, 18, 21, 24, 25, 29, 31, 32, 34).

**B)** Clones that did not amplify in A) were digested with EcoRI and XhoI. All contain an insert.



**Fig 3.5** A) A further 9 independent clones were digested with EcoRI and XhoI. All contain an insert.  
B) PCR amplifications of Agouti and Ras full length sequences were performed on 1 $\mu$ g of library. Templates for the +ve controls were 300ng of pHMII-Agouti and pBabePuro-Ras respectively.

## **3.2 Tissue culture**

### **3.2.1 Colony formation assay**

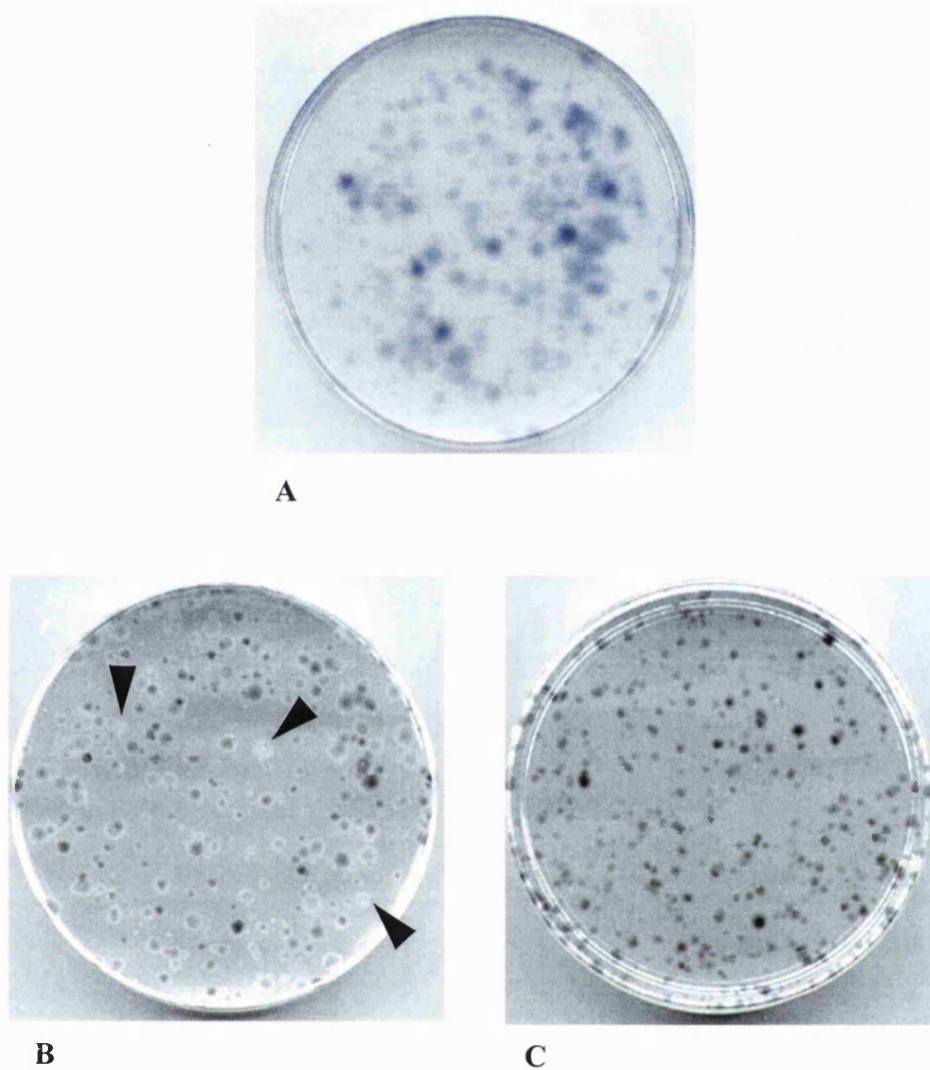
Since the screening is based on selecting clones that confer a different pigmentation to the cells, it was necessary to assess whether Melb-a (melanoblast cell line) and Melan-a (melanocyte cell line) were able to produce independent and distinguishable colonies from a single cell.

The test was performed by plating  $1 \times 10^3$  cells in a 10 cm dish. Both Melb-a and Melan-a in these conditions were able to form ~200 independent colonies in two weeks. The colony formation efficiency for both cell lines was therefore ~20% (**Fig 3.6**).

Morphologically, colonies originating from Melb-a were unpigmented and, although independent and distinguishable, they were not as well defined as those originating from Melan-a. This was probably due to a tendency of Melb-a to migrate and move in the plate.

Melan-a colonies on the contrary were generally very defined and pigmented. The original Melan-a cell line available to us contained a number of naturally unpigmented cells approximately 3% of the total population (**Fig 3.6B**). This would have been a problem during the screening, causing a dramatic





**Fig 3.6** Colony formation assays with  $1 \times 10^3$  cells.  
**A)** Melb-a colonies stained with Crystal Violet dye.  
**B)** Melan-a colonies prior to subcloning step. Arrowheads indicate unpigmented colonies.  
**C)** Melan-a colonies after subcloning step. No unpigmented colonies are visible.

increase in the number of false positives. The problem was solved by subcloning 10 well pigmented Melan-a colonies. Each colony originated a clonal population that was then tested several times in colony formation assays to assess the percent of natural unpigmented cells in the subcloned population. One clone in particular showed no unpigmented colonies out of more than  $1 \times 10^4$  analysed and was chosen for future work (Fig 3.6C).

### 3.2.2 Infectability assay

The screening we intended to carry out uses a cDNA library cloned into pHMII, an MMLV derived retroviral vector. It was therefore necessary to assess the infectability of the Melb-a and Melan-a cell lines we proposed to use.

To carry out the infectability test, the LinXE packaging cell line was transfected with  $25 \mu\text{g}$  of pHMII-LacZ vector. This leads to the production of viral particles carrying the LacZ reporter gene, which can be used to infect both Melb-a and Melan-a.

After 48h from infection, cells were fixed in 0.5% glutaraldehyde and treated with X-Gal. This causes a reaction with the LacZ gene product, conferring a characteristic blue colour only to the cells successfully infected. This assay was also used to assess the LinXE transfection rate which turned out to be ~50%.

From this test, Melb-a was shown to be very poorly infectable: less than 0.1% of the cells stained blue after X-Gal treatment. To improve this ratio, a pBabe-Puro

retroviral vector carrying the cDNA encoding for the ecotropic receptor and the Puromycin resistance gene, was introduced into the cells. The overexpression of the receptor involved in the internalisation of the virus into the cell is known to be helpful in increasing the efficiency of infection in some cell lines. Following Puromycin selection, the Melb-a cells overexpressing the ecotropic receptor were tested for increased infectability using the pHMII-LacZ retroviral vector. In this case, the estimated infection rate did not significantly improve and was still too low for our purposes, and therefore the screening on Melb-a was abandoned.

By contrast Melan-a are efficiently infected at a rate of 15-20%, making them suitable for our screening.

### **3.3 Screening optimisation 1**

The screening we intended to carry out was based on infecting Melan-a cells with a retroviral cDNA library, plating the successfully infected cells at a sufficiently low density to allow single colony formation and selection of the unpigmented (positive) colonies for further characterisation. To make the screening as efficient as possible, all these separate stages were previously optimised.

### 3.3.1 Ras and Agouti reconstruction

To optimise the screening conditions, reconstruction tests were carried out using genes known to provoke the depigmentation of Melan-a. We chose to use two such molecules: *Ras* and *Agouti*.

Activated *Ras* is a known oncogene that has been shown to provoke depigmentation of melanocytes when overexpressed in cell lines like Melan-a. *Ras* is able to affect melanogenesis by inhibiting the Microphthalmia transcription factor, a key regulator of melanocyte differentiation involved in the upregulation of the melanogenic enzymes Tyrosinase, Trp-1 and Trp-2. By using *Ras* oncogene in our reconstruction, we assessed the capacity of our system to detect oncogenes and other molecules that can inhibit the production of pigment when expressed in Melan-a.

*Agouti* is a secreted protein that is known to bind to the Melanocortin-1 Receptor (Mc1r) on the cell surface. This causes intracellular signalling leading to the switch in melanin production from the default eumelanin (black) to pheomelanin (yellow).

Eventually, if the *Agouti* signal is not turned-off, all the black pigment in the cell will be substituted by the yellow one, causing the cell to look unpigmented. By using *Agouti* in the reconstruction, we also assessed the ability of our system to detect both secreted molecules affecting pigmentation, and molecules affecting the quality of the pigment produced rather than its production level.

When Melan-a mouse melanocytes were infected with either the pHMII/*Agouti* or the pHMII/*Ras* constructs, cells became unpigmented in about 10 days, proving that the

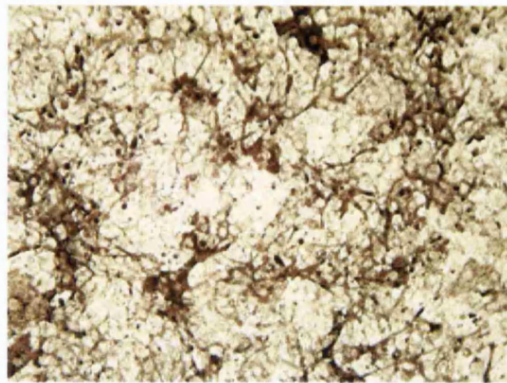
two genes were appropriate to use for reconstruction purposes (**Fig 3.7**). Moreover, both *Ras* and *Agouti* infected melanocytes were able to form unpigmented colonies after 14 days when plated at low density on a 10cm dish (**Fig 3.8**). To rule out the possibility that the effect on pigmentation was given by the retroviral vector itself, a negative control was made by infecting Melan-a with an empty pHMII retroviral vector: only pigmented colonies were formed by these cells.

In conclusion these data suggest that it is possible to select for Melan-a cells carrying a gene affecting their melanogenesis. Moreover these cells will form distinguishable unpigmented colonies that can therefore be subcloned and expanded for further characterisation.

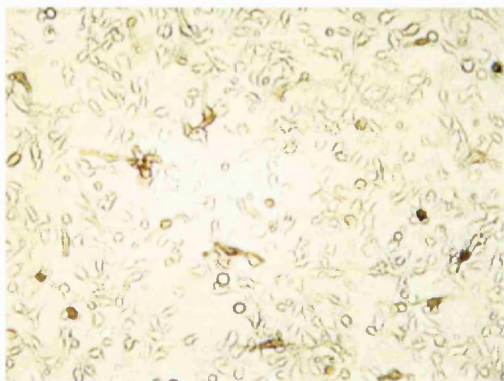
### **3.3.2 CRE recombinase purification and genomic DNA extraction optimisation**

One of the key advantages of using the pHMII system is that, upon selection and expansion of the positive clones, the cDNAs responsible for the observed phenotypes can be recovered for further characterisation.

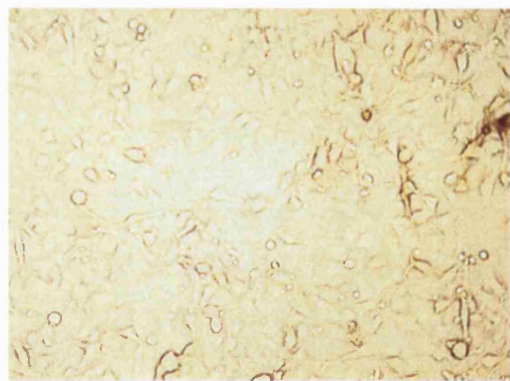
To do so it is necessary to extract the cells' genomic DNA, in which the cDNA of interest is integrated, and treat it with CRE recombinase. This causes the cDNA to be excised from the genome in the form of a plasmid that can in turn be transformed and expanded in bacteria.



Melan-a

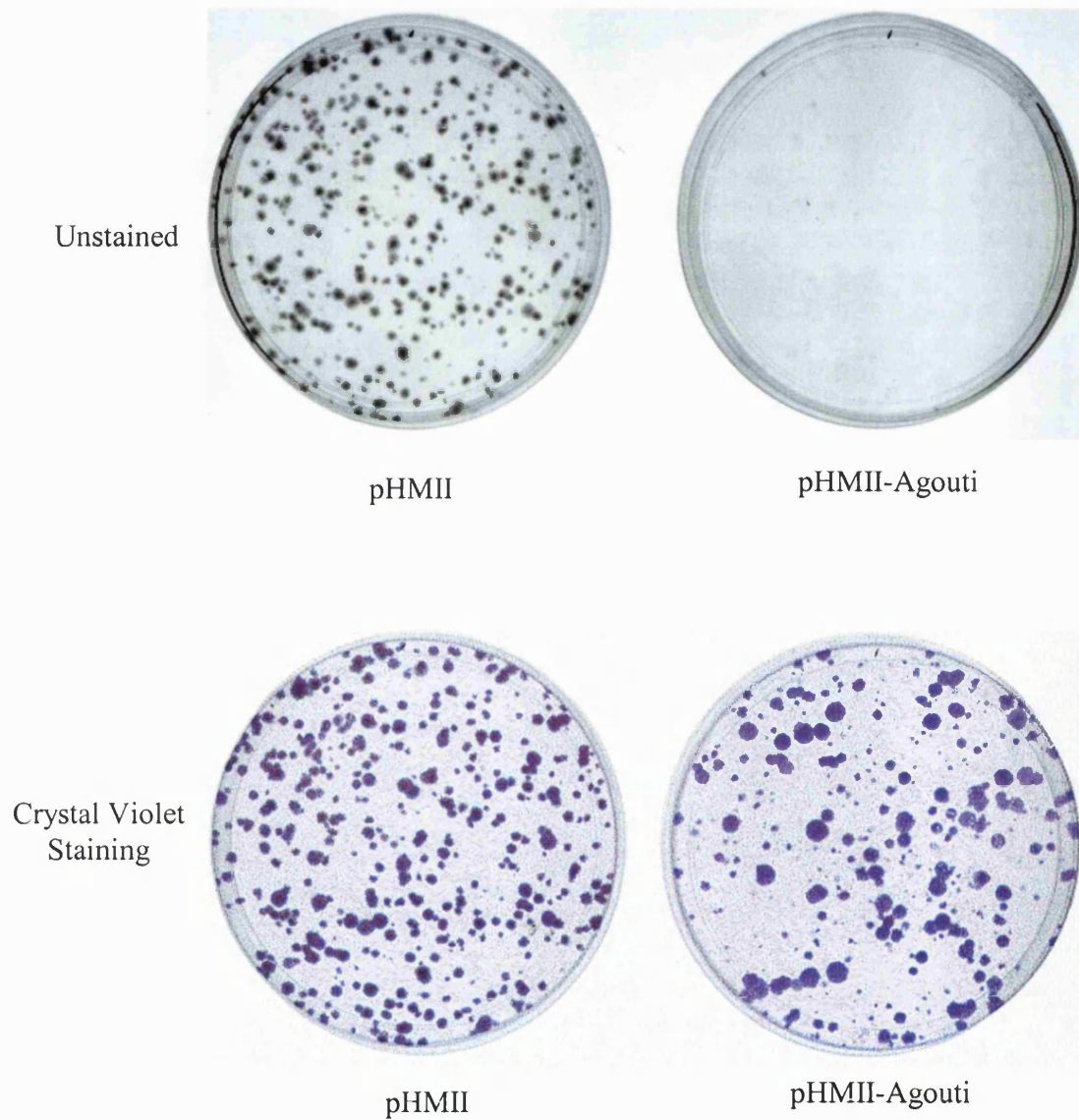


Melan-a/Agouti



Melan-a/Ras

**Fig 3.7** Phenotypic changes induced by Agouti or Ras when overexpressed in the mouse melanocyte cell line Melan-a.

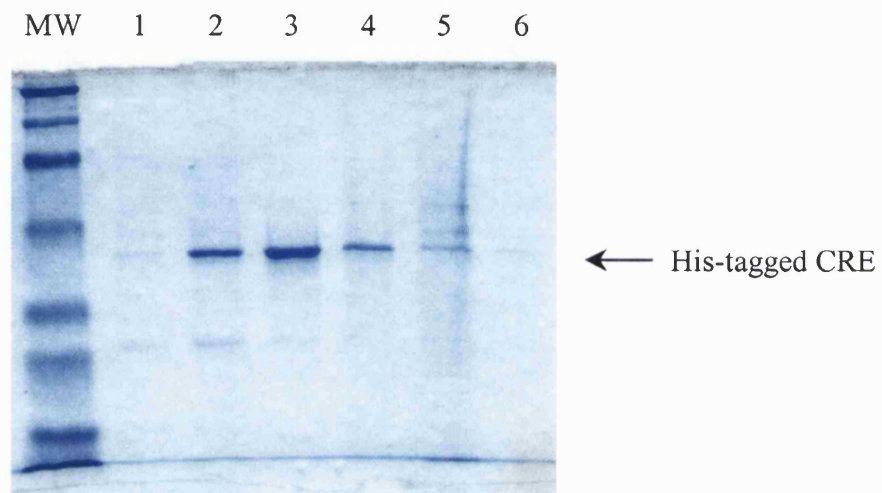


**Fig 3.8** 1500 cells of Melan-a infected with vector only (pHMII) or vector expressing the Agouti cDNA (pHMII-Agouti) were plated on a 10cm dish for 14 days.  
**A)** Unstained plates: only the pigmented colonies are visible  
**B)** Same plates stained with Crystal Violet dye: both pigmented and unpigmented colonies are visible

The first thing we decided to optimise was the CRE recombinase expression and purification in bacteria. Our laboratory had access to the CRE cDNA cloned into the pET19 expression vector. The standard protocol in use included an expression step at 37°C for 4h. This was followed by purification through a metal-chelating resin, the recombinant protein being expressed with a His-Tag in the N-terminus. This method, although providing enough CRE activity for a few reactions, was limiting in the amount of protein produced, indeed below the resolution limits of a standard Coomassie-Blue staining. By changing the expression conditions to RT for 16h and by optimising the sonication step on the bacteria, we were able to purify high amounts of protein (Fig 3.9). Moreover the enzymatic activity of the protein was assessed using the control DNA provided by Novagen when purchasing their commercially available CRE recombinase. The control DNA is a linearized  $\lambda$  phage vector whose core is made of a pET based sequence (with Ampicillin resistance gene) flanked by two LoxP CRE recombination sites.

When the DNA is treated with CRE recombinase, the recombination between the two LoxP sites causes the pET plasmid to be excised and circularised. If the DNA so treated is transformed into bacteria, the number of Ampicillin resistant colonies observed will be proportional to the CRE reaction efficiency. In our test, using 50ng of  $\lambda$  vector DNA, we had 52 colonies in the negative control ( $\lambda$  vector with no CRE), 207 colonies using 1 $\mu$ l of Novagen CRE and more than 2500 colonies using 1 $\mu$ l of CRE purified in our laboratory.





**Fig 3.9** His tagged CRE recombinase was purified on metal-chelating resin. Six 500 $\mu$ l elutions were collected and added to 500 $\mu$ l of 100% glycerol. 10 $\mu$ l of each elution were run on 12% polyacrylamide gel. The position of recombinant CRE recombinase (~40Kda) is indicated. Elutions 2, 3, and 4 were stored at  $-80^{\circ}\text{C}$ .

The reconstruction on the genomic DNA extracted from mammalian cells was carried out on genomic DNA from  $5 \times 10^6$  Melan-a cells infected with pHMII-Agouti. The DNA was purified using a standard protocol consisting of lysing the cells in lysis buffer containing 1% SDS followed by phenol/chloroform extractions. The DNA thus prepared was treated with CRE recombinase and transformed into bacteria.

Unfortunately no colonies were observed suggesting that the CRE recombinase reaction was not successful for this sample. On the contrary a control reaction on genomic DNA extracted from fibroblasts, worked within the efficiency limits of the technique (~1 bacteria colony per 5,000 mammalian cells). This was probably caused by the presence of melanin in the genomic DNA preparation from melanocytes. When cells are lysed, the residual amount of pigment present in the melanosomes is released and can unspecifically associate to nucleic acids and proteins. This is known to inhibit enzymatic reactions such as PCR and reverse-transcription (Giambernardi *et al*, 1998; Eckhart *et al*, 2000). It is therefore plausible that melanin, even at the low concentrations present in unpigmented looking melanocytes, will inhibit any enzyme that has to interact with DNA such as CRE recombinase.

To overcome this problem a new protocol for extracting genomic DNA from melanocytes was developed.

Cells were treated for a week prior to extraction with 200 $\mu$ M PTU (Phenylthiourea, an inhibitor of Tyrosinase) to further reduce their pigment content.

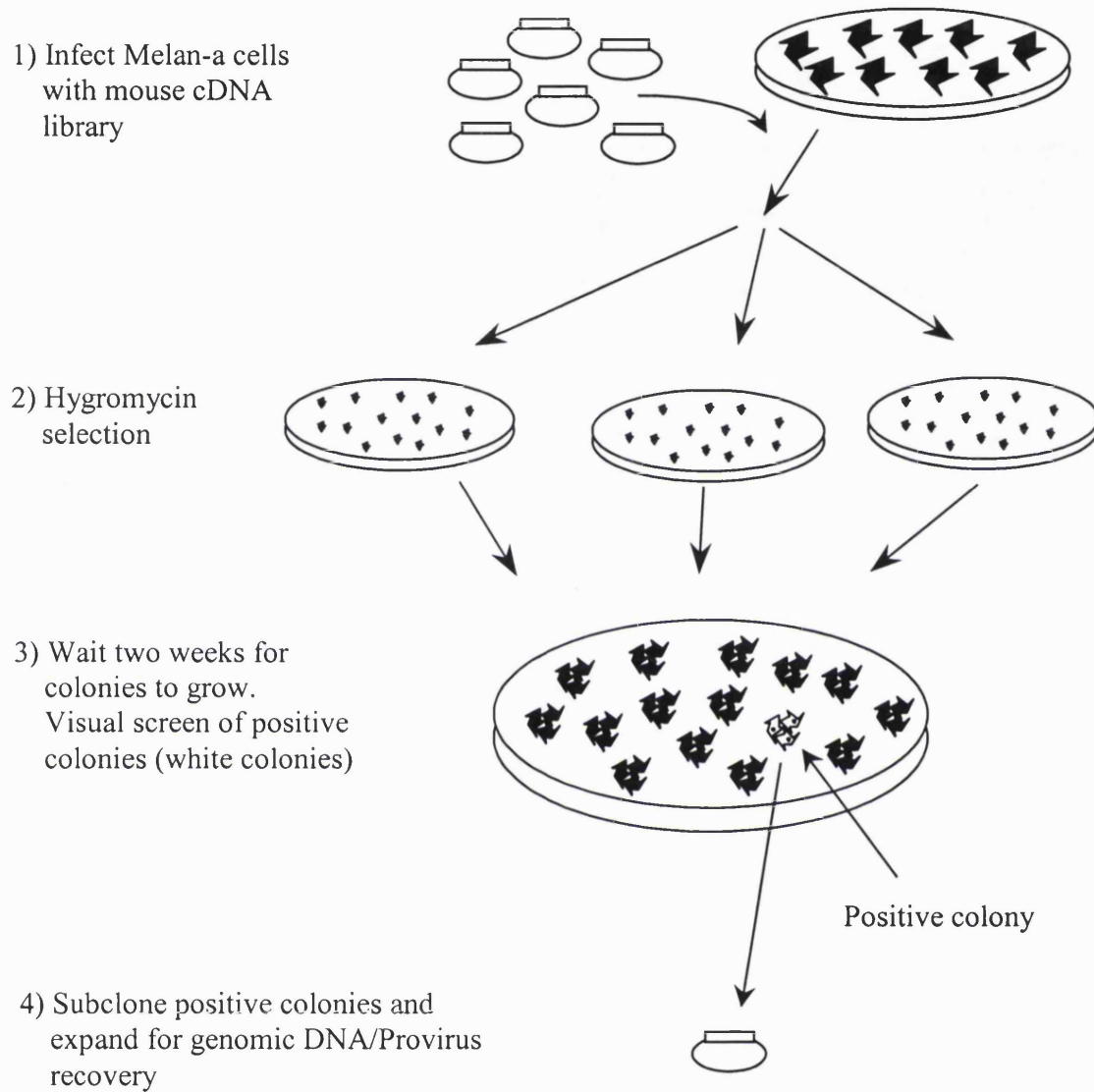
Moreover, the genomic extraction was carried out using QIAGEN genomic DNA columns following the manufacturer's instructions. These include a step to isolate cell nuclei from cell debris, hence reducing the possibility of melanin interacting with

DNA. Finally, genomic DNA was further purified by Phenol/Chloroform extractions and ethanol precipitation.

This protocol proved successful, and the CRE recombinase reaction efficiency on genomic DNA from melanocytes thus prepared was similar to the control one.

### **3.4 Screening 1**

In our first screening attempt, we decided to use 15 cm dishes to screen as many clones as possible. An outline of the screening is schematised in **Fig 3.10**. Following infection with the cDNA library, Melan-a cells were selected using Hygromycin for 10 days and plated at low density ( $3 \times 10^3$  cells per 15 cm dish) in order to have colony formation (~500 colonies per dish). Due to the restricted space in the incubator, only 100 dishes could be seeded at the same time, making the total number of clones tested per screen ~50,000. Three such screenings were consecutively carried out, bringing the total number of clones analysed with this method to 150,000. After two weeks, the unpigmented (positive) colonies were selected by visual screening enhanced with a lightbox. Among the 150,000 clones thus analysed, we subcloned 30 that formed unpigmented, although smaller than the average (~50 cells), colonies. These clones were expanded for 3 weeks until enough cells were available for genomic DNA extraction. The recovered DNA was treated with CRE recombinase and the excised



**Fig 3.10** Schematic of the functional screening in Melan-a cells.

proviruses were re-transfected into LinX cells for virus production. These in turn were used to infect Melan-a and tested for their ability to alter melanocyte pigmentation. Unfortunately none of these 30 clones resulted to be truly positive when retested in melanocytes. It is likely that the original unpigmentation of the colonies was related to their small size. Indeed it is known that the degree of confluency in the plate can affect pigmentation: the more confluent the cells, the more pigment is produced, (Mahalingam *et al*, 1997; Hornyak *et al*, 2000). It is likely that a small colony represents a less confluent environment for its cells <sup>a</sup> than a bigger one, and hence its cells are not as pigmented. It is also worth mentioning that all the clones that did not retest positive became very pigmented during the time that it took to expand them enough to allow genomic DNA extraction and provirus recovery, proving that their initial unpigmentation was more likely to be caused by environmental factors rather than genetic ones.

Taken together these results suggested that only unpigmented colonies with a reasonable size (~130 cells) should be subcloned and considered as potential positives.

Also it became evident that we needed to screen a much higher number of clones in order to isolate real positives. As mentioned before, the screening methodology described above allows the simultaneous screen of 50,000 clones and takes approximately one month from start to finish (that is from initial infection of the melanocytes to the moment the positive colonies are subcloned). Considering that our library has  $\sim 6 \times 10^6$  independent clones and that ideally one should at least screen a

number of clones to match that of the library complexity, it was necessary to develop alternative methodologies to improve the yield and efficiency of the screening.

### **3.5 Screening optimisation 2**

In order to screen a higher number of clones, we had to find a way to enrich the population of library infected melanocytes for cells that could be potentially positive clones, hence enrich for the unpigmented cells. Indeed we felt that this was the only way to enhance the yield of the screening: the biggest limitation in our system at the time was due to restricted incubator space and the laborious final step consisting of the visual screening and selection of the unpigmented colonies. Neither limitations could be changed. Hence the need of enriching the population for potential positives somewhere in the screening process before the limiting steps were reached.

#### **3.5.1 White cells enrichment**

##### **3.5.1.1 Freeze/Thawing method**

When pigmented melanocytes are grown in vitro and DMSO stocks need to be prepared, it is necessary to treat the cells for at least four days with PTU, a reversible inhibitor of tyrosinase, in order to decrease their pigmentation levels. Indeed, it has been shown that the freeze/thawing steps are extremely toxic for pigmented cells and, unless previously depigmented, up to 99% of the melanocytes, depending on their melanin levels, will die in the process, and only the less pigmented one will survive.

We thought that it may be possible to exploit this characteristic to enrich for unpigmented cells in the library infected population. It was decided that the Freeze/Thawing step should be introduced after the 10 days Hygromycin selection of the successfully infected melanocytes had occurred. This way we were minimising the unwanted background composed of the uninfected cell, and at the same time allowing enough time for depigmentation to occur in those cells infected with positive clones.

In order to test the efficiency of enrichment using such a system, unpigmented (Agouti infected) and pigmented melanocytes were mixed in a 1:1,000 ratio (500 unpigmented cells plus  $5 \times 10^5$  pigmented ones) and subjected to a round of Freeze/Thawing. Also, in order to facilitate the recognition of the Agouti infected melanocytes, these cells were infected with a pBabe-Puro retroviral vector carrying the LacZ reporter gene and the Puromycin resistance gene.

Cells were slowly frozen in 7.5% DMSO at  $-80^{\circ}\text{C}$  overnight and left in liquid nitrogen for 24 hours. Finally cells were defrosted and plated into a 10cm dish, left for 24 hours to recover and seeded at low density to allow colony formation for 14 days. Once colonies were formed, plates were fixed and stained with X-Gal. Colonies formed by Agouti/LacZ infected melanocytes became blue under these conditions and were easily identified. Their number was compared to the number of unstained, pigmented colonies, and the rate of enrichment calculated.

This test was carried out several times and, although enrichment always occurred (Table below), it was difficult to obtain consistent results. This probably happens because the Freeze/Thawing selection against pigmented melanocytes only works for

cells with high eumelanin content. As mentioned before, the pigmentation levels of a melanocyte population can be affected by many external factors such as cell confluency and medium pH. This means that it is very difficult to keep all the different variables affecting melanin production under control, and to ensure that the same level of pigmentation is achieved in different populations during different experiments.

Test	Blue Colonies	Black Colonies	Enrichment
1	49	804	x60
2	32	847	x35
3	65	810	x80

Moreover, when the unpigmented versus pigmented cell ratio was decreased to 1:10,000, a figure that is likely to be closer to <sup>that</sup> what found in a real screening, the rate of enrichment fell to only 10 fold, making this method unsuitable for our purposes.



### 3.5.1.2 Cell Sorter

Pigmented and unpigmented melanocytes can be distinguished by a FACS machine because of different light scattering properties (Boissy *et al*, 1989).

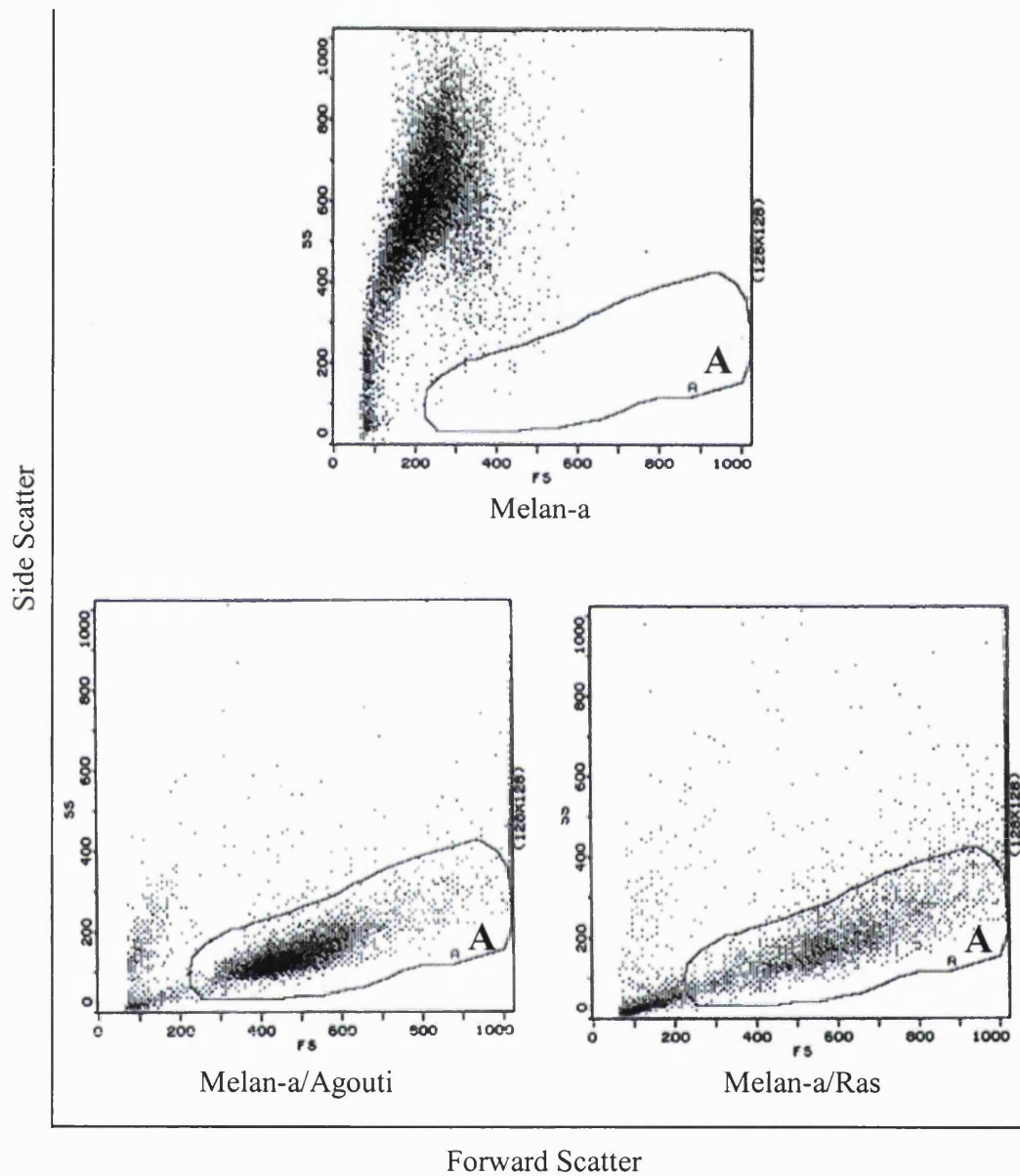
We decided to test the possibility of using a cell sorter to enrich for the unpigmented cells in a melanocyte population.

First we assessed how easily the pigmented and unpigmented population could be separated by a FACS machine. When a cell population is analysed by FACS, results are normally visualised with a two dimensional graph. In our case, two values were measured and plotted: Forward Scatter and Side Scatter. The Forward Scatter value represents the capacity of the examined cell to absorb light: generally it is associated with cell size but it can depend on other characteristics too. The Side Scatter represents the amount of light that is reflected by the cell and it normally depends on the cell granularity.

A typical result obtained when we analysed a population of pigmented melanocytes (Melan-a) is shown in **Fig 3.11**. These cells were characterised by high light absorbency (translated in low Forward Scatter values in the plot) and high light reflection (high Side Scatter values). This was probably due to the presence in the pigmented cell of highly eumelanised melanosomes that have a strong effect on both the absorption and reflection of the laser beam.

On the contrary, all unpigmented melanocytes tested (Agouti or Ras infected melanocytes), were characterised by a lower light absorbency (higher Forward Scatter values) and lower light reflecting properties (lower Side Scatter values, **Fig 3.11**).

Moreover, when the pigmented and unpigmented plots were superimposed, it became



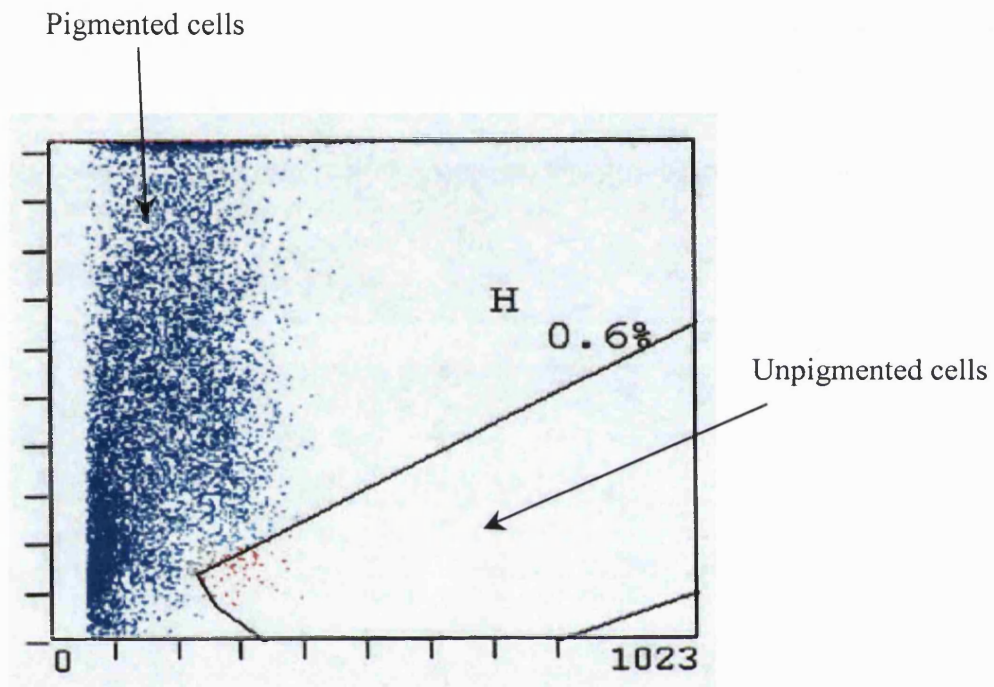
**Fig 3.11** FACS analysis of pigmented (Melan-a) and unpigmented cells (Melan-a/Agouti and Melan-a/Ras). It is possible to select for areas of the graph that will mainly contain unpigmented cells. In this case, when area A is drawn, ~80% of the Agouti or Ras infected melanocytes are included, whereas only 0.5% of the pigmented Melan-a are selected.

obvious that the two populations were falling in different areas with very little overlapping and therefore could be easily sorted with a high degree of purity.

Once established that the cell sorter could efficiently discriminate between pigmented and unpigmented melanocytes, it was necessary to assess both the enrichment ratio when a mixed population was sorted and the viability of the cells after the selection had occurred. Cell sorting using a FACS machine can be a very harsh process on the cells and, depending on the cell line used, a poor ratio of recovery can be observed. In our case a poor survival rate of the selected cells could jeopardise the efficiency of the screening, making this enrichment methodology impractical.

This test was done mixing unpigmented (Agouti-LacZ infected) and pigmented melanocytes in a 1:10,000 ratio (100 unpigmented cells plus  $1 \times 10^6$  pigmented cells). The mixed population was passed through the cell sorter at a speed of 1200 cells per second and the unpigmented ones were gated (Fig 3.12). We collected ~6100 cells of which 5000 were seeded at low density in two 10cm dishes (2x 2500 cells per 10 cm dish). After 14 days colonies were fixed in 0.5% glutaraldehyde and stained with X-Gal. This allowed easy identification of Agouti/LacZ colonies and their discrimination from white colonies given by Melan-a false positive background.

Out of a total of 5000 cells being seeded, 878 colonies were obtained (17.6% colony formation efficiency), of which 27 stained blue and 1 was a white colony that did not react with X-Gal, probably because it generated from a naturally unpigmented Melan-a cells (false positive).



**Fig 3.12** Cell sorting diagram of a 1:10,000 ratio between Agouti/LacZ infected Melan-a and wild type Melan-a. Cells falling in area H were gated and collected (0.6% of the total population).

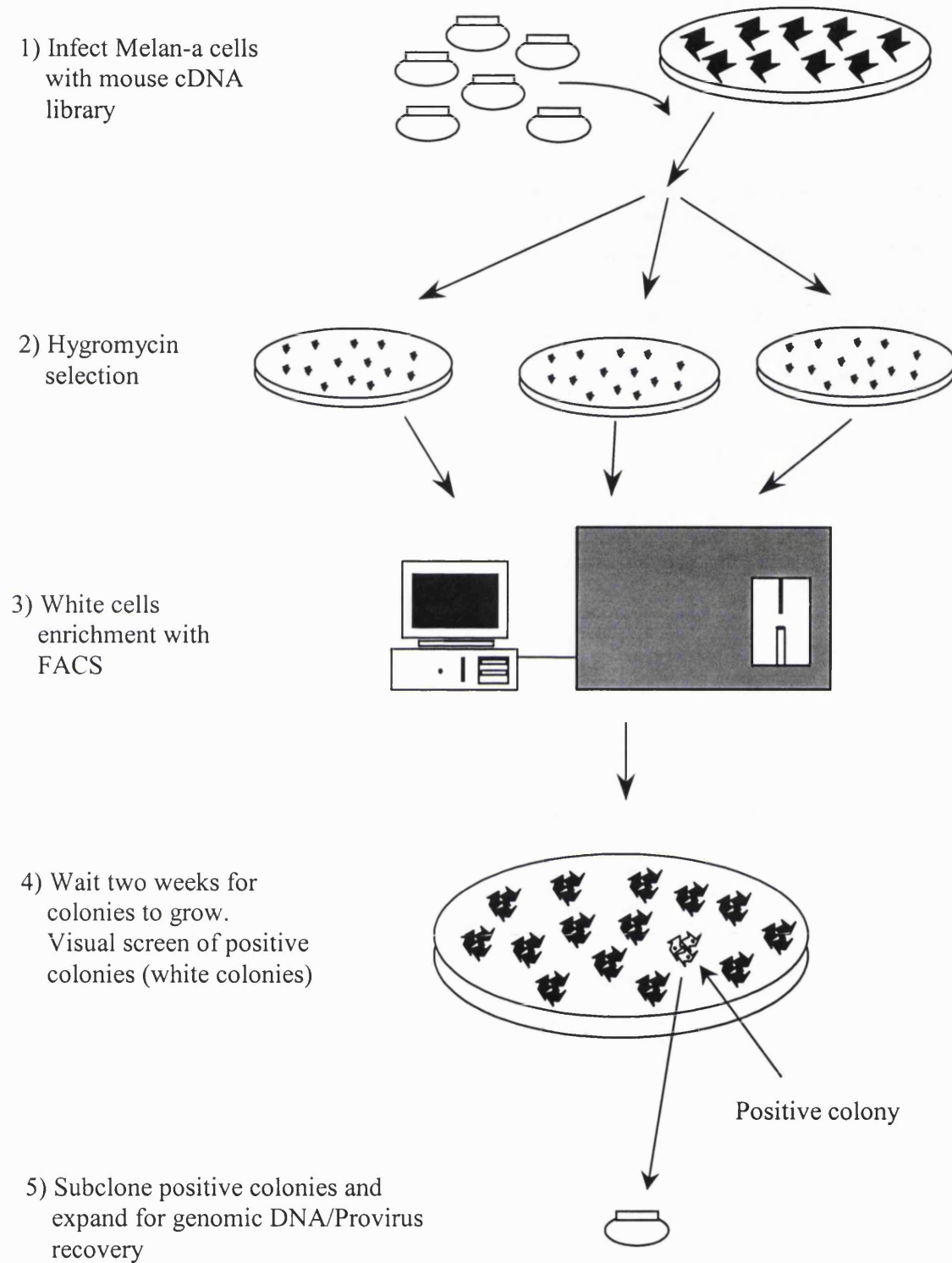
In conclusion, using the cell sorter, we were able to have a ~300 fold enrichment for unpigmented cells in about 15 minutes. The vast majority of the wild-type Melan-a cells that were gated formed pigmented colonies in two weeks and could therefore be recognised as non-positive clones. Moreover, none of the pigmented colonies reacted with X-Gal, showing that all the Agouti infected cells selected formed unpigmented colonies.

Finally, from the total number of colonies recovered, it was obvious that we were keeping the ratio of colony formation efficiency close to the previously observed 20%, meaning that the viability of the cells was not significantly affected by this methodology.

### **3.6 Screening 2**

Having optimised the conditions for white cells enrichment, we decided to apply the Cell Sorter methodology to perform the screening (**Fig 3.13**). We calculated that a single enrichment by FACS would allow us to increase the number of clones tested per screen to  $1.5 \times 10^6$ .

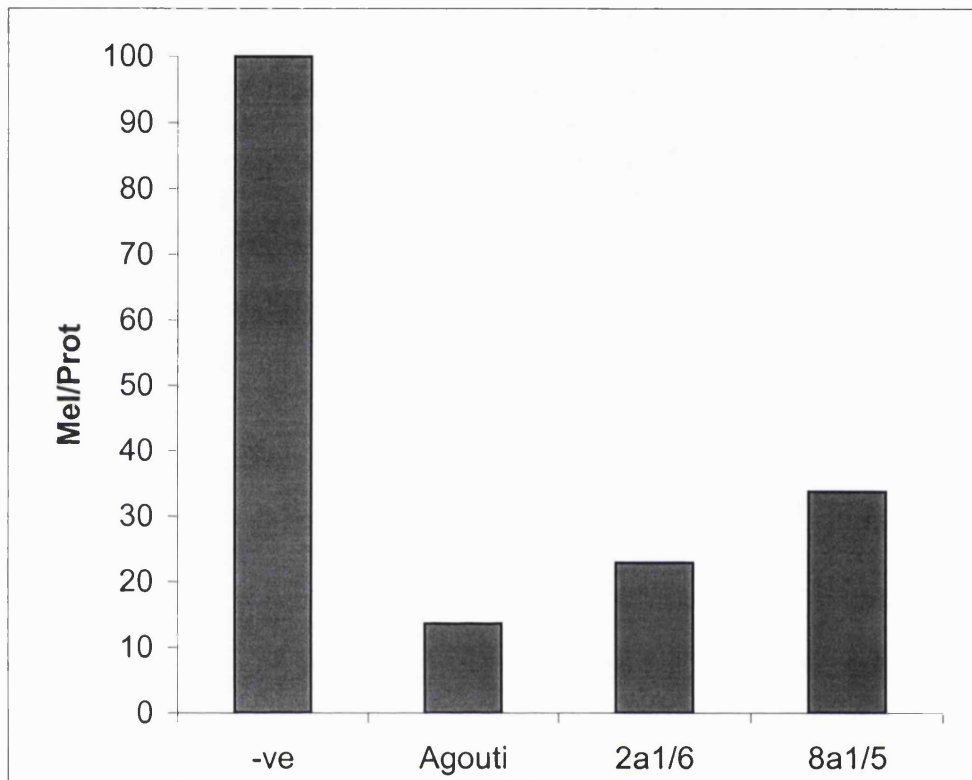
Briefly, twenty 10cm dishes were seeded with  $5 \times 10^5$  Melan-a cells and infected with the mouse cDNA library. Estimating an average infection efficiency of ~15%, this resulted in 75,000 cells infected per plate, meaning that  $1.5 \times 10^6$  clones were tested in total. Successfully infected melanocytes were selected for 10 days with Hygromycin



**Fig 3.13** Schematic of the functional screening on Melan-a using the Cell Sorter

and the surviving cells were passed through the Cell Sorter gating for the unpigmented ones. During the Hygromycin selection, the resistant cells divided ~5 times, so that by the time we were able to pass the cells through the Cell Sorter, the total number of cells per plate was  $\sim 2 \times 10^6$ . This forced us to sort each plate separately, the whole process taking ~30 minutes per sample. The number of cells gated per sample was ~15,000 (~0.8% gating), and these selected cells were plated at low density in six 10cm dishes for colony formation (2,500 cells per 10cm dish), making the total number of dishes 120. After 14 days, the dishes were visually screened using a lightbox and 29 white and good-sized colonies (130 cells per colony) were subcloned. Although most of the clones remained unpigmented during the period of time necessary to expand them enough to allow genomic DNA extraction and provirus recovery, only two (2a1/5 and 8a1/6) retested positively when re-infected into melanocytes and assayed for melanin content (**Fig 3.14**).

Taken together these results suggest that the introduction of the enrichment step in our screening allowed the identification of positive clones by greatly enhancing the number of clones tested. Moreover, the finding of genuine positive clones was a validation of the whole methodology.



**Fig 3.14** Relative melanin content in melanocytes infected with empty pHMII (-ve), Agouti and the two positive clones.

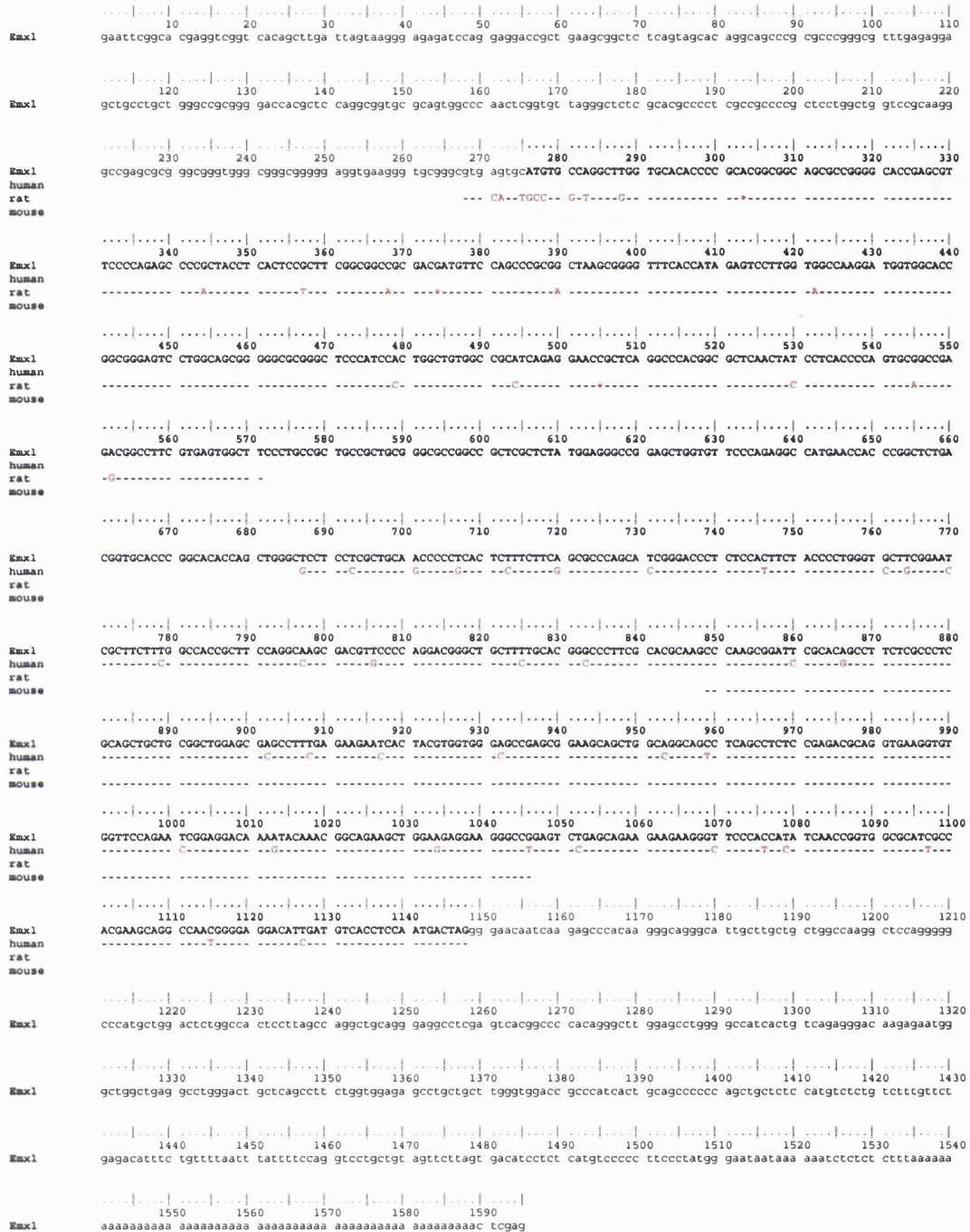


### 3.6.1 Positive clones sequencing and identification

The cDNA of both positive clones was fully sequenced using the Dye terminator methodology. Sequence analysis revealed that the two positive clones were identical except for the length of the PolyA<sup>+</sup> tail, indicating that they were different clones of the same transcript (**Fig 3.15**). This means that using our screening we were able to independently isolate the same gene twice, validating both the technology used and the ability of the gene to cause the phenotype changes selected in our screening when overexpressed in melanocytes.

The gene sequence of the two clones was compared to the available database of the NCBI Blast program. The result was a very good match with the published sequences of the *Emx1* gene. The only mammalian *Emx1* sequences available in the database are 198bp of the mouse *Emx1* homeobox region (100% identity-Simeone *et al*, 1992), 301bp of the 5' rat *Emx1* sequence (94% identity-Robel *et al*, 1995) and the last 462bp of the human *Emx1* coding sequence (92% identity-Simeone *et al*, 1992) (**Fig 3.16**).





**Fig 3.16** Emx1 sequences alignment. Emx1 coding sequence is in capital and bold. Also indicated are the published human, rat and mouse sequences. Dashes represent identical nucleotides, whereas red characters and diamonds indicate mutations and gaps respectively.

## **4 RESULTS: THE *Emx* GENES**

*Emx1* is a mouse homeobox gene homologue of the *Drosophila* gene *empty spiracles* (*ems*).

It was first cloned through the screening of a cDNA library prepared from 11-day mouse embryos using the human *EMX1* cDNA as probe. This in turn had been previously cloned using the *Drosophila ems* cDNA on an 8 weeks human embryos cDNA library. The result of this screening was the identification of two human homologues to the *empty spiracles* fruit fly gene, *EMX1* and *EMX2*, and subsequently of the corresponding mouse genes, *Emx1* and *Emx2* (Simeone *et al*, 1992).

*Empty spiracles* is so called because it is required for the development of the tracheal system in abdominal segment 8 in *Drosophila*, but mutations causing *ems* loss of function also result in the deletion of specific anterior head structures (Dalton *et al*, 1989).

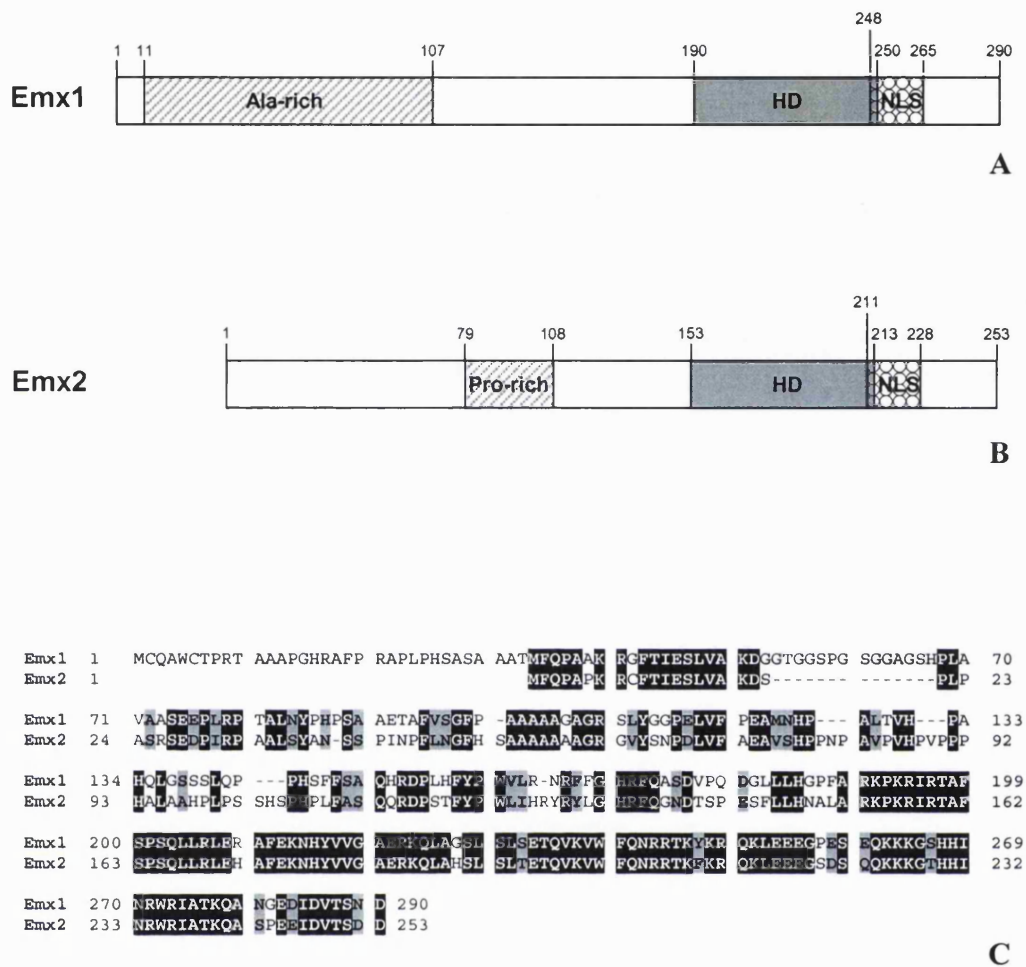
In mouse, the *Emx* genes are expressed during embryogenesis mainly between day 8.5 and 16. Both genes are strongly expressed in the developing rostral brain including the dorsal telencephalon and the olfactory bulbs (Simeone *et al*, 1992) as well as in the developing urogenital system (Pellegrini *et al*, 1997). *Emx1* and *Emx2* have been independently homozygously mutated in mice and the resulting knockout phenotypes characterised (Yoshida *et al*, 1997). Although they clearly have a role in forebrain

development, neither of the two knockouts seem to be able to reproduce the strong head development defects observed in *ems* mutated fruit flies. This could be a consequence of the partially overlapping expression patterns of the two genes in mouse embryos, resulting in a partial reciprocal rescue whenever one of the two is inactivated. In particular *Emx1* mutants seem to have only very mild defects in brain and head development, in accordance with the observation that, while *Emx2* expression does not always overlap with *Emx1*, this almost exclusively happens in *Emx2* positive regions (Yoshida *et al*, 1997). In addition to head development defects, in *Emx2* knockouts the urogenital tract is completely missing and newborns die within a few hours because of kidney failure (Miyamoto *et al*, 1997). To date an *Emx1/Emx2* double mutant has not been produced, but it is reasonable to speculate that such a knockout will have strong head and brain development defects. Indeed it has been reported that *Emx1* and *Emx2* expression depends on the Gli3 transcription factor (Theil *et al*, 1999) and its inactivation results in the downregulation of both homeobox genes leading to the disruption of normal dorsal telencephalon development (Tole *et al*, 2000).

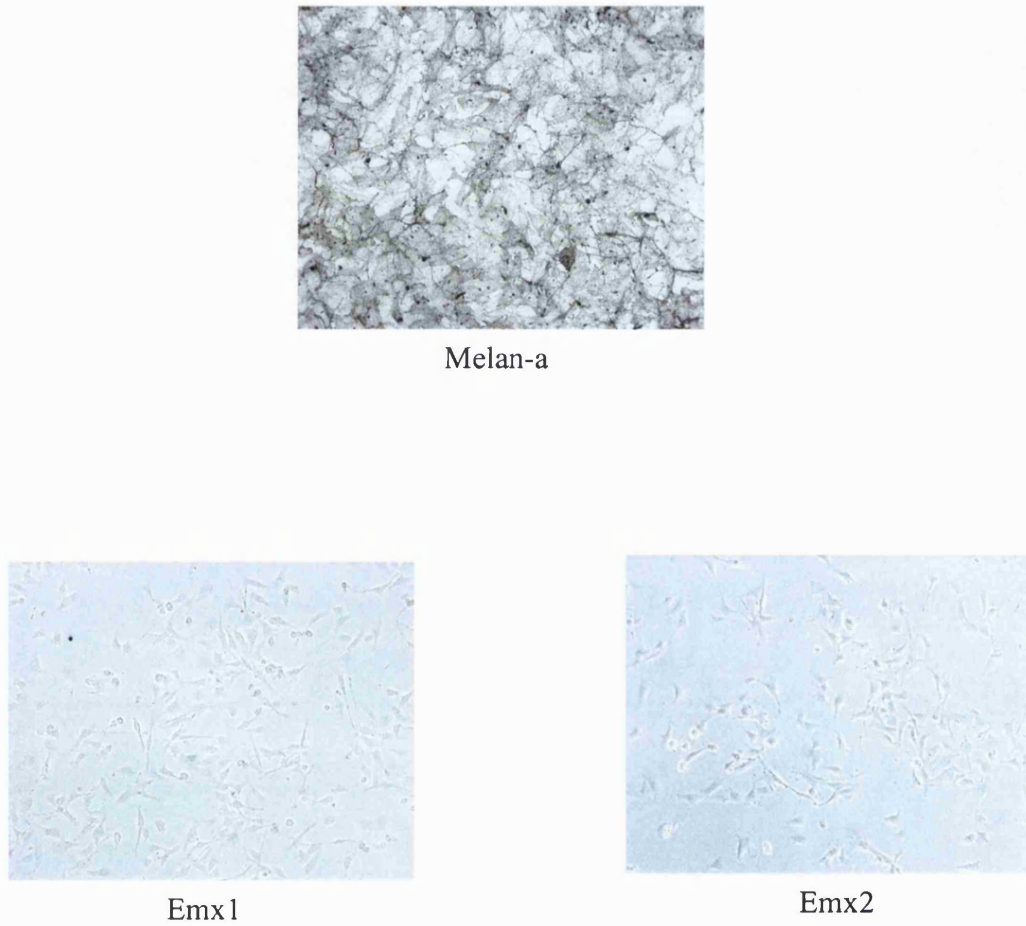
We decided to further characterise the phenotype induced by *Emx1* overexpression in melanocytes by carrying out preliminary tests such as effects on duplication time (growth curves), ability to confer TPA independence and growth in soft agar. These tests were mainly chosen because they represent standard experiments to characterise oncogenes, and it has been shown that when Melan-a cells are transfected with

oncogenes, the depigmentation observed is often associated with TPA independence and the acquired ability to form colonies in soft agar.

As mentioned before, *Emx1* and *Emx2* are highly related and are believed to partially rescue each other in case of inactivating mutations. Indeed, the sequences of our *Emx1* clones show that its coding sequence is 873bp long resulting in a predicted protein of 290aa. The protein is characterised by an alanine-rich region (aa 11-107), a homeobox domain (aa 190-250) and a nuclear localisation signal (aa 248-265) (**Fig 4.1a**). The sequence of the murine *Emx2* cDNA clone provided by Dr. Antonio Simeone, shows a cds of 762bp, resulting in a protein of 253aa presenting a proline-rich region (aa 79-108), a homeobox domain (aa 153-213) and a nuclear localisation signal (aa 211-228) (**Fig 4.1b**). The two proteins are 62% identical and in particular, the C-terminus, including the homeobox domains and the nuclear localisation signals, show 88% identity (**Fig 4.1c**). For these reasons we also decided to investigate *Emx2* in relation to its capacity to alter pigment cells biology when overexpressed in melanocytes. To this purpose, the 2kb fragment corresponding to the *Emx2* cDNA was subcloned in pHMII and transduced into Melan-a. The *Emx2*-infected cells were selected with hygromycin and their pigmentation levels were evaluated. As expected, *Emx2* overexpression resulted in phenotypic changes similar to those observed when overexpressing *Emx1* (**Fig 4.2**). This result was also confirmed performing three independent melanin assays using wild-type and Agouti-infected Melan-a as negative and positive controls respectively. As shown in **Fig 4.3**, a nominal value of 100 was assigned to the pigmentation levels of the negative control (wild-type Melan-a)

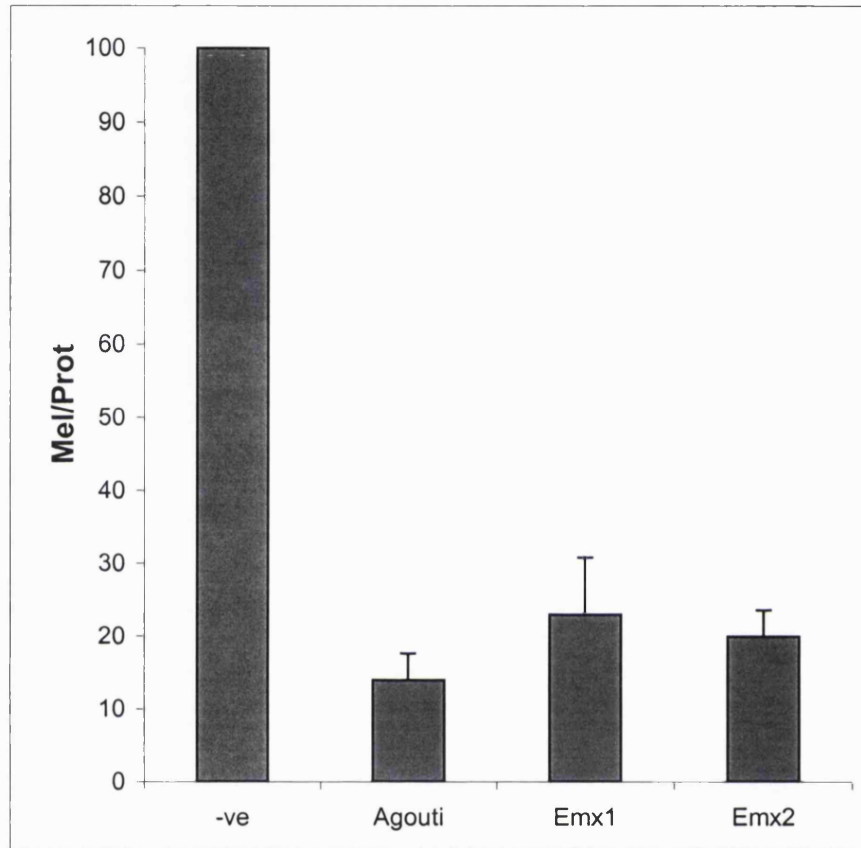


**Fig 4.1** A) Schematic of the predicted Emx1 protein. Indicated are the alanin rich region (aa 11-107), the homeobox domain (HD) (aa 190-250) and the nuclear localisation signal (NLS) (aa 248-265).  
 B) Predicted Emx2 protein. Recognisable are the proline rich region (aa 79-108), the homeobox domain (aa 153-213) and the nuclear localisation signal (aa 211-228).  
 C) Emx1 and Emx2 protein sequence alignment. Identical aa are boxed in black, similar aa are boxed in grey. The overall identity is 62%, whereas the C-Terminus identity, including homeobox domain and NLS, is 88%.



**Fig 4.2** Depigmentation elicited by Emx1 or Emx2 overexpression in Melan-a cells. The phenotypic changes were obvious within 10 days from infection.





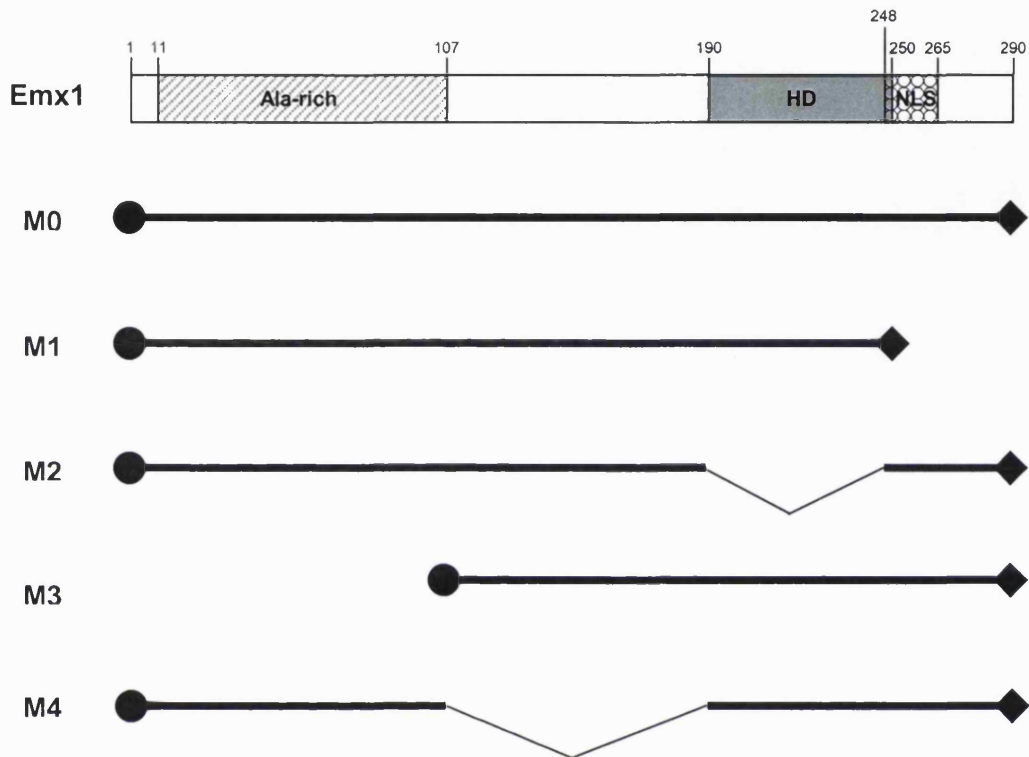
**Fig 4.3** Relative melanin content in Melan-a infected with empty pHMII (-ve control), Agouti (+ve control) and either Emx genes. The depigmentation observed with the two homeobox genes is comparable to the one observed with Agouti.

whereas the remaining samples were expressed as a percentage of this value, enabling us to compare the data obtained from different experiments.

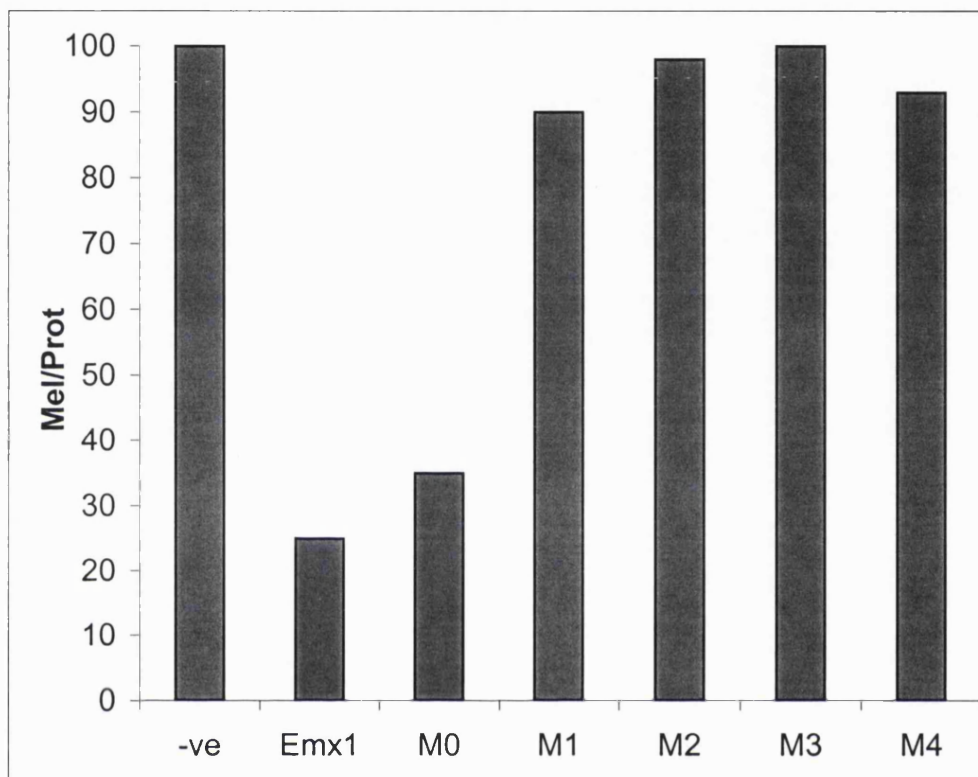
#### **4.1 Full length *Emx1* is required for Melan-a depigmentation**

In order to establish whether *Emx1*-triggered depigmentation in melanocytes was caused by its activity in the nucleus, we generated a mutant lacking the nuclear localisation signal (M1). Also we decided to investigate if any of the recognisable regions of the protein were indispensable for the observed phenotype. We therefore created mutants lacking the homeobox domain (M2), the alanine-rich region (M3), and the 83aa long region comprised between the two (M4) (Fig 4.4). Mutants were generated by PCR, subcloned into pHMII, and sequenced to ensure that no errors were introduced during the polymerase chain reaction step. Finally, the mutated *Emx1* clones were tested for their ability to inhibit pigment production when overexpressed in melanocytes. As positive controls, we used the original *Emx1* clone and the full length PCR amplification of the *Emx1* cds (M0).

Pigment content was assessed performing a melanin assay. As shown in Fig 4.5, both positive controls were able to reduce melanin levels in the cells, whereas none of the mutants was able to provoke melanin synthesis inhibition, suggesting that the full-length protein is required for Melan-a depigmentation.



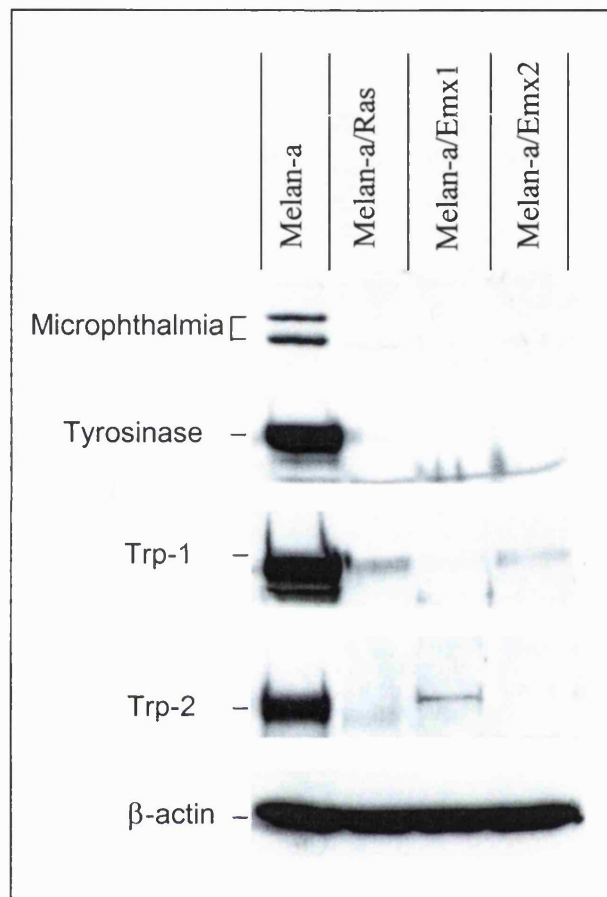
**Fig 4.4** Schematic of Emx1 mutants. M0 represents the full length cds and was used as positive control. M1 lacks the Nuclear Localisation Signal, M2 the homeobox domain, M3 the alanine-rich region and M4 the sequence comprised between the alanine-rich region and the homeobox domain.



**Fig 4.5** Relative melanin content in Melan-a infected with empty pHMII (-ve control), Emx1 (+ve control) and the Emx1 mutants. Also, as an additional positive control, the full length Emx1 cds obtained by PCR was tested (M0). As opposed to what observed with both positive controls, none of the Emx1 mutants were able to elicit depigmentation in Melan-a.

## **4.2 Emx1 and Emx2 downregulate microphthalmia and the melanogenic enzymes**

Pigment production in melanocytes depends on the activity of three main melanogenic enzymes: Tyrosinase, Trp-1 and Trp-2. The expression levels of these enzymes vastly depend on the activity of the transcription factor microphthalmia. Changes in the expression levels of any of these genes, will result in changes in the pigment production and quality. For example it is known that the depigmentation observed in melanocytes overexpressing oncogenes is caused by the downregulation of microphthalmia leading to the downregulation of the melanogenic enzymes and consequently of pigment production (Halaban *et al*, 1996). In order to establish the effect of Emx1 or Emx2 overexpression on the melanogenic enzymes levels, we performed Western blots analysis of cell extracts from wild-type, Ras infected and Emx1 or Emx2 infected Melan-a. As shown in **Fig 4.6**, cells overexpressing either clones had reduced microphthalmia, Tyrosinase, Trp-1 and Trp-2 levels suggesting that, similarly to what observed with oncogenes overexpression, Emx1 and Emx2 cause microphthalmia downregulation and consequently melanocytes depigmentation.



**Fig 4.6** Western blots on 20 $\mu$ g of total cell lysate from wild-type (+ve control), Ras infected (-ve control) and Emx1 or Emx2-overexpressing Melan-a. Blots were probed against microphthalmia (runs as a double band of 52-56 Kda), Tyrosinase, Trp-1 and Trp-2. As a further control, the levels of the housekeeping protein  $\beta$ -actin were assessed.

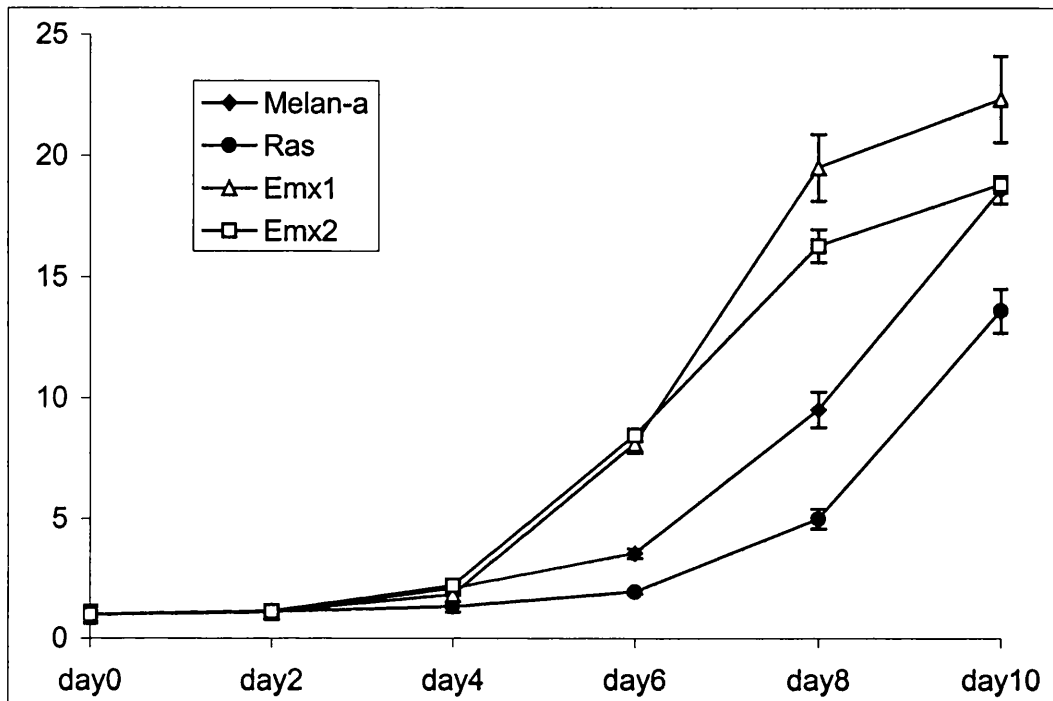
### **4.3 Emx1 and Emx2 overexpression confer growth advantages in melanocytes**

Since the pattern of melanogenic enzymes downregulation is similar to the one observed when overexpressing oncogenes, we decided to investigate whether overexpression of the *Emx* genes could result in growth advantages.

#### **4.3.1 Growth Curve**

First, we decided to establish whether either *Emx* gene overexpression was altering the cell cycle characteristics of the Melan-a cells.

$2 \times 10^3$  melanocytes infected with either *Emx1* or *Emx2* were plated in 24 well plates in triplicate and grown in presence of 200nM TPA. Melan-a infected with empty vector and *Ras* were used as controls. Six different time points were taken (day 0, 2, 4, 6, 8, 10), and at each time point cells were fixed in glutaraldehyde, stained with crystal violet and washed several times in PBS. Cell growth was calculated by reading at 595nm the unwashed crystal violet staining dissolved in 10% Acetic Acid. As shown in **Fig 4.7**, melanocytes expressing either *Emx* genes were growing at a faster rate compared to those infected with empty vector or activated *Ras* oncogene. Indeed, in these conditions, melanocytes infected with *Ras* were the slowest to grow, probably because prolonged high doses of TPA can downregulate PKC activity, an important effector of *Ras* mediated mitogenic properties (Lacal *et al*, 1987).



**Fig 4.7** Standardised growth curves of Melan-a infected with empty pHMII, Ras, Emx1 and Emx2. Cells were grown in the presence of 200nM TPA. Both Emx genes are able to accelerate the cell cycle when overexpressed in Melan-a.



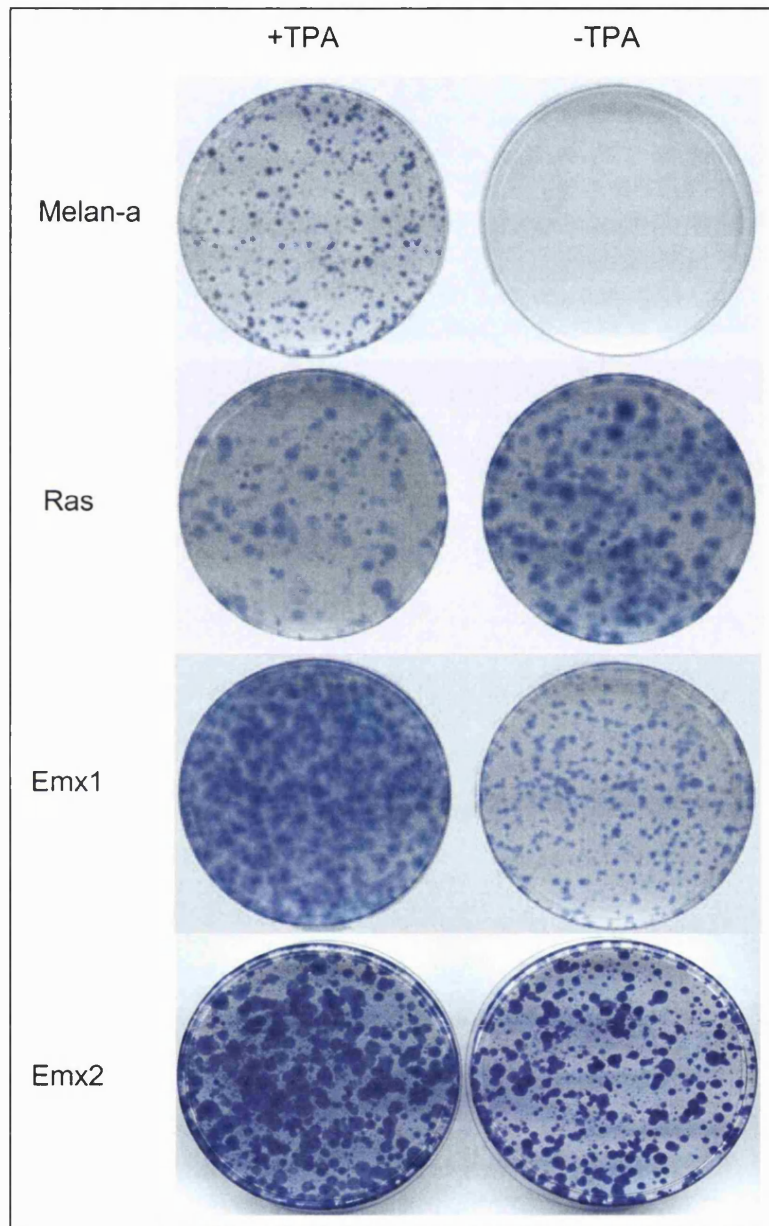
### 4.3.2 TPA independence

Next, we decided to establish whether *Emx1* or *Emx2* overexpression in melanocytes could confer the cells the ability to grow in absence of TPA in the media. This experiment was carried out by plating in triplicate at low density (2000 cells in a 10cm dish) melanocytes infected with empty pHMII (negative control), activated *Ras* (positive control) or the *Emx* clones in media containing 200nM TPA or in absence of the mitogen.

Cells were grown for 14 days and the resulting colonies were stained with crystal violet (Fig 4.8). Following is the mean number of colonies obtained with each sample in both presence and absence of TPA.

Sample	+TPA	-TPA
Melan-a	316	0
Ras-Melan-a	209	220
<i>Emx1</i> -Melan-a	293	266
<i>Emx2</i> -Melan-a	254	243

It was obvious from this experiment that *Emx1* or *Emx2* overexpression in melanocytes was able to render the cells independent of TPA, although they grew at a faster rate when the mitogen was added to the media (Fig 4.8).



**Fig 4.8** Colony formation assay. Wild-type, Ras, Emx1 and Emx2 infected Melan-a were seeded at low density and grown for 14 days with 200nM TPA or in absence of the mitogen.

It is worth noticing that *Emx1* and *Emx2* infected melanocytes were yielding roughly the same number of colonies in both conditions, suggesting that the TPA independence observed was not the result of sporadic mutations but an acquired characteristic shared by the whole population. In addition, worth mentioning is the difference in size of the colonies grown in presence or absence of TPA. Both *Emx1* and *Emx2* infected melanocytes, as opposed to the *Ras* infected ones, grow at a faster rate when the mitogen is added to the media, confirming the data obtained from the growth curves.

#### **4.3.3 Growth in Soft-Agar**

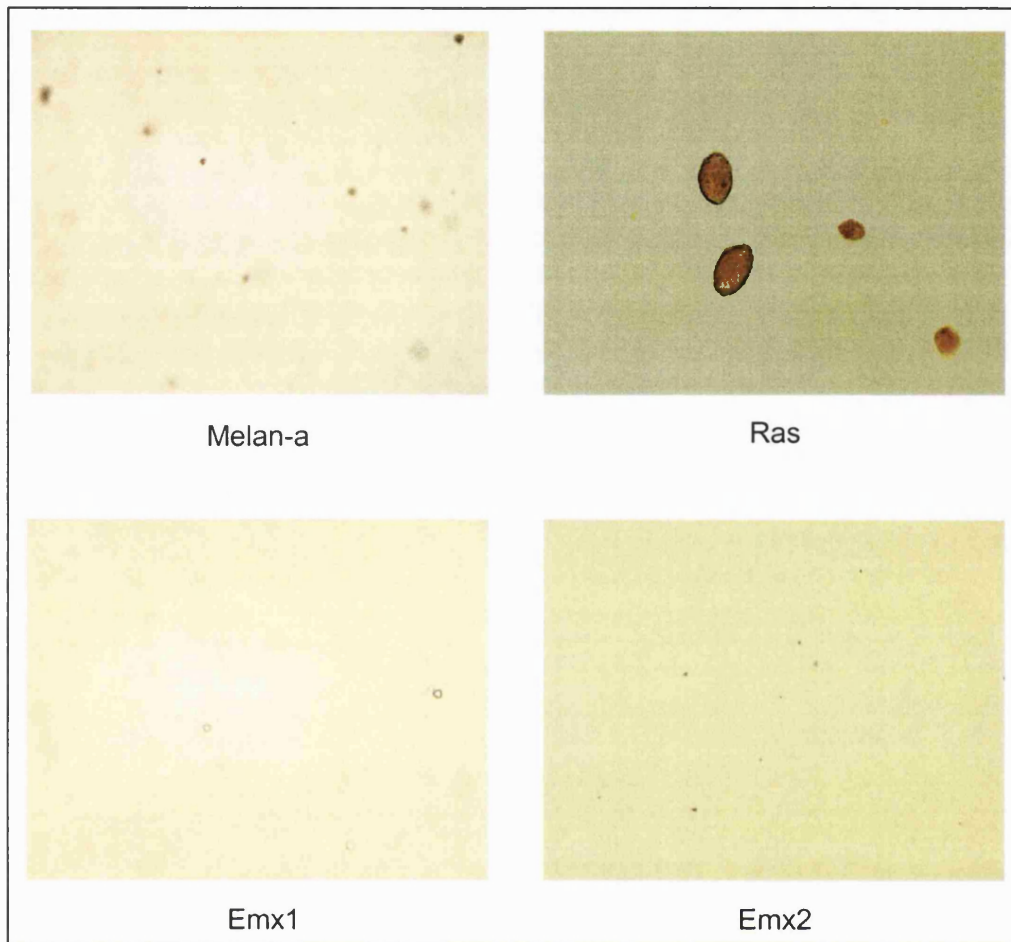
Finally we tested the ability of the *Emx1* and *Emx2* infected melanocytes to form colonies in soft agar. This is a standard test to establish if a cell population has acquired transforming capabilities. Mammalian adherent immortalized cell lines, despite being able to divide indefinitely, can only grow in monolayers and respond to contact inhibition. On the contrary transformed cells (like *Ras* infected Melan-a) are insensitive to the contact inhibition signals and will keep dividing even after they have reached confluence, the only limit being the amount of nutrients available in the media.

The growth in soft agar test mimics the contact inhibition situation: cells are seeded in and surrounded by a matrix composed of 0.3% agar. In these conditions, only transformed cells are able to divide and form visible three-dimensional colonies (as opposed to the monolayer colonies obtained in a plate) in about 21 days.

The test was performed in triplicate seeding  $10^4$  cells in 6 well plates. Melanocytes infected with empty pHMII and *Ras* were used as negative and positive controls respectively.

After 3 weeks, colonies were visible only in the *Ras* infected melanocytes samples (average of 107 colonies/ $10^4$  cells). Neither the negative control nor the *Emx1* or *Emx2* infected cells were able to form colonies in soft agar (Fig 4.9).

In conclusion it appears that overexpression of the *Emx* genes in melanocytes, despite conferring growth advantages such as TPA independence and lower duplication time in presence of TPA, is not causing the cells to lose response to contact inhibition and therefore to acquire a fully transformed phenotype. However, this was not enough to rule out the possibility that *Emx1* or *Emx2* overexpression in melanocytes may play a role in melanoma progression. Indeed the same characteristics are observed with melanocytes overexpressing *bFGF*, a well characterised oncogene involved in virtually all invasive melanomas (Dotto *et al*, 1989; Cajal *et al*, 1991). We therefore decided to check the expression of both *Emx* genes in 9 human melanoma cell lines available to us and in the melanoblast Melb-a cell line.



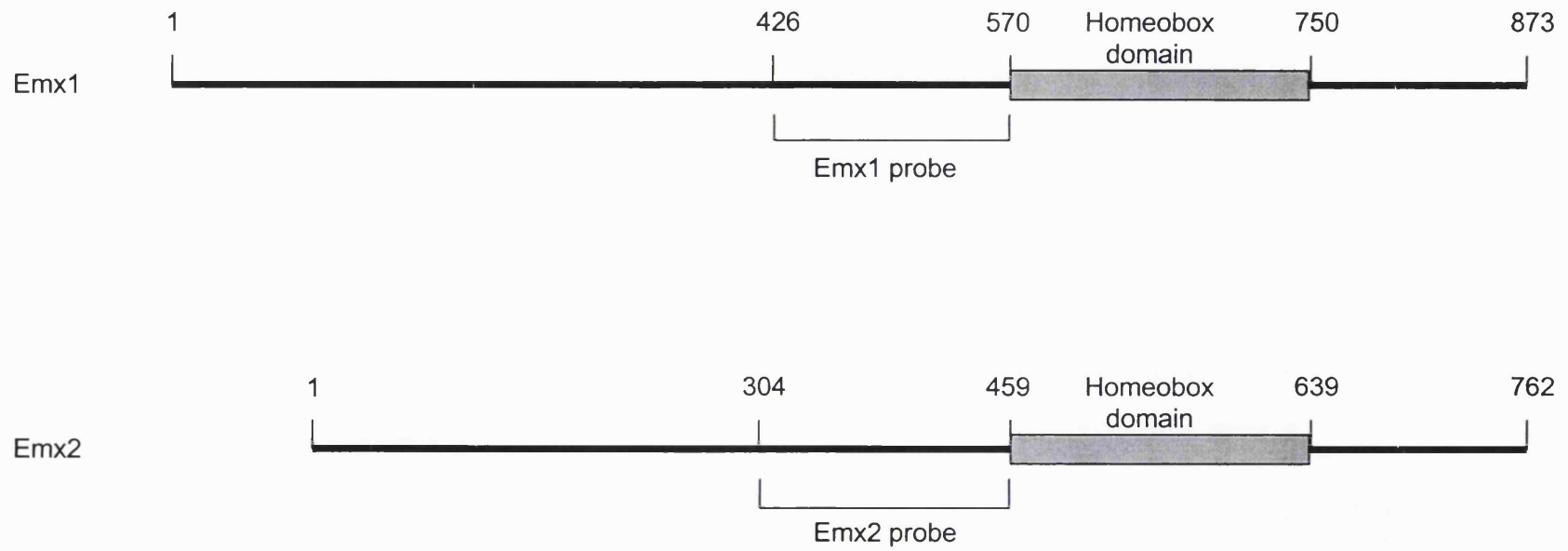
Sample	Colonies/ $10^4$ cells
Ras	107
Melan-a	0
Emx1	0
Emx2	0

**Fig 4.9** Growth in soft agar.  $10^4$  cells were seeded in triplicate in soft agar (0.3%) and left to grow for 21 days. Only Ras-infected melanocytes could form three-dimensional colonies.

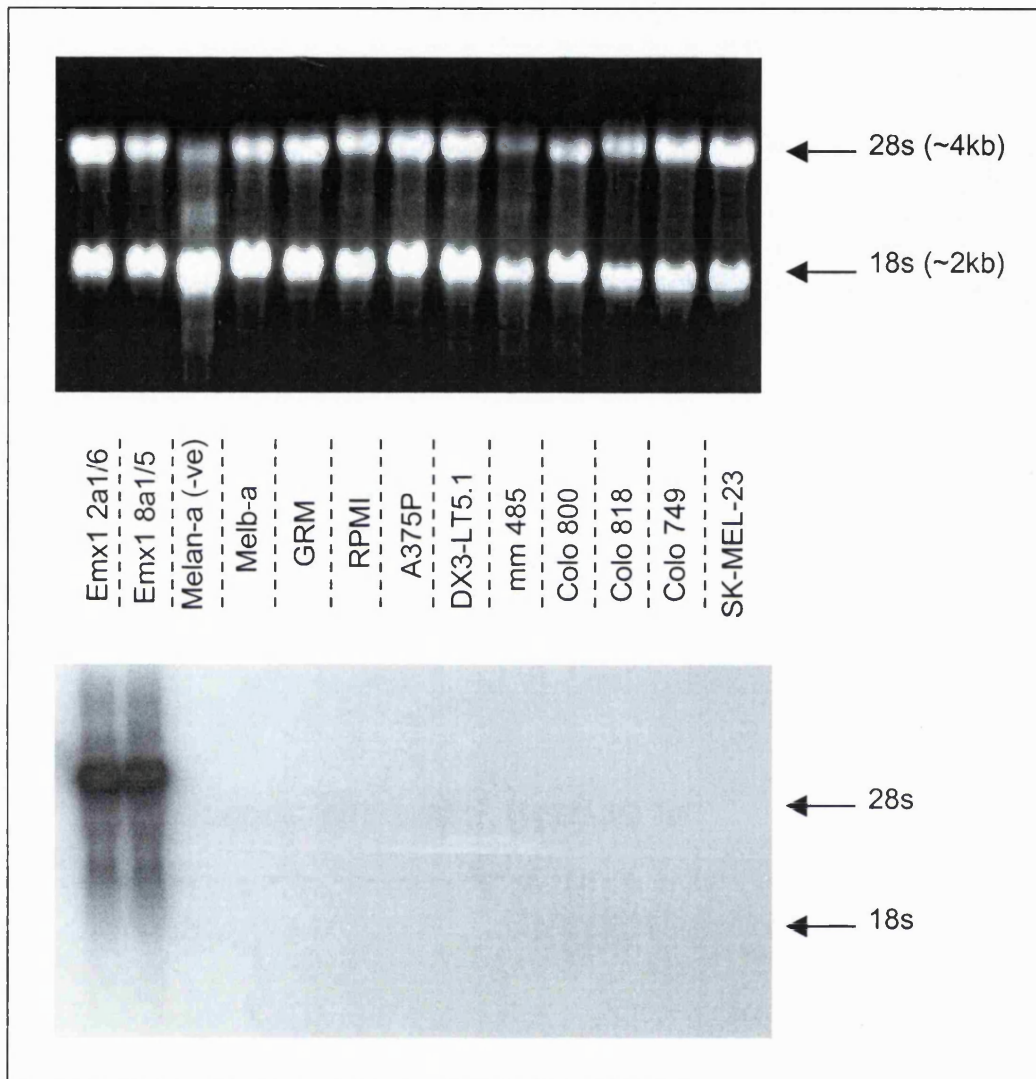
#### **4.4 *Emx1* and *Emx2* expression in melanoma and melanoblast cell lines**

In order to assess the involvement of the *Emx* genes in melanoma progression and melanocytes differentiation, we performed Northern Blots on 10µg of total RNA extracted from 9 human melanoma cell lines and the melanoblasts Melb-a cell line. The two *Emx1* clones (2a1/6 and 8a1/5) and the *Emx2*-infected melanocytes were used as positive controls, whereas wild-type Melan-a was used as negative control. Samples were run on a 1% agarose gel and the Northern Blot was performed using a <sup>32</sup>P-radiolabelled fragment of the *Emx1* or *Emx2* mouse cDNA as probe. In order to avoid problems due to lack of specificity, both probes were designed so that they would not include the Homeobox domain and their sequences were blasted on the NCBI database (Fig 4.10). Moreover, we made sure that the murine probes designed had a high degree of identity with the corresponding human sequence. We calculated that the *Emx1* probe had 98% identity to human and the *Emx2* one had 97%, making them suitable for detecting the human *Emx* genes mRNA.

As shown in Fig 4.11 and Fig 4.12, neither *Emx1* nor *Emx2* are expressed in the melanoma or the melanoblast Melb-a cell lines tested. On the other hand the positive controls present a very clear signal at a relative size of ~7kb. Although both *Emx1* and *Emx2* mRNAs are only ~2kb long, this is the expected size for the transcript driven by the pHMII retroviral vector since the transcription termination site is only found in the 3'LTR of the vector, and therefore the mRNA molecule produced encompasses everything included between the two LTRs.

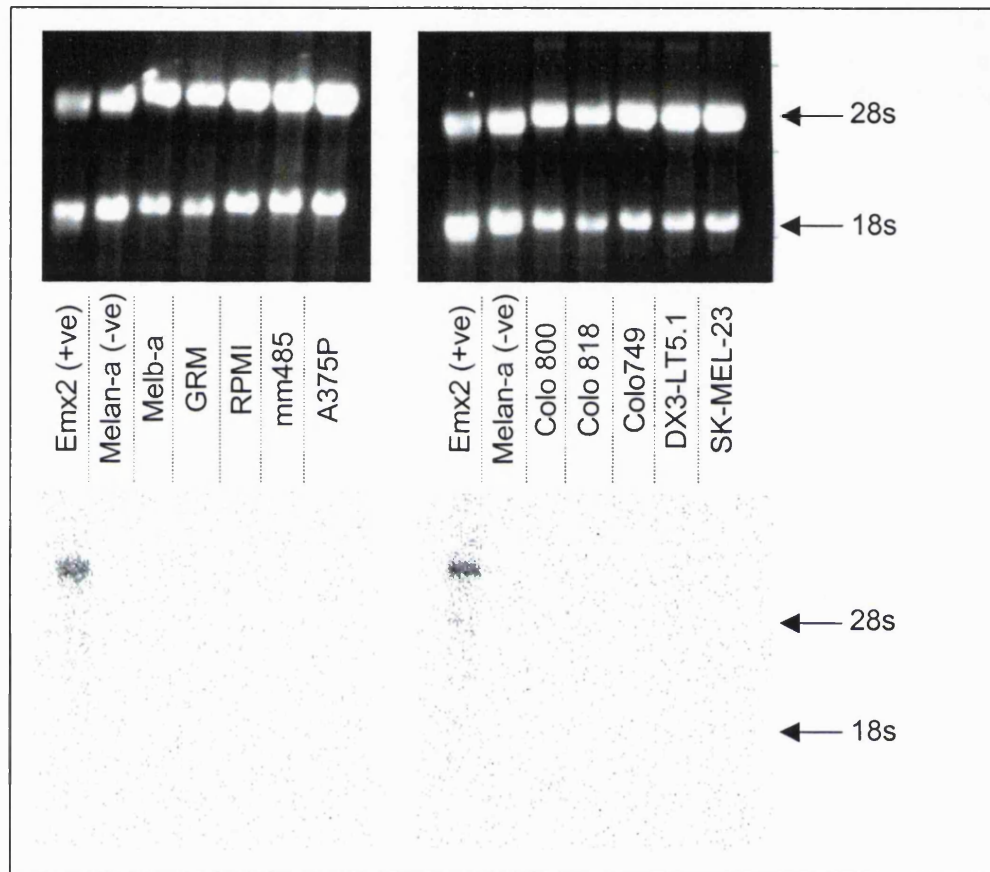


**Fig 4.10** Schematic of the Emx1 and Emx2 probes. To avoid problems with specificity, both probes were designed just before the homeobox domain.



**Fig 4.11** Northern Blot using the Emx1 probe. Clones 2a1/6 and 8a1/5 were used as positive controls.





**Fig 4.12** Northern Blot using the Emx2 probe. Emx2-infected melanocytes were used as positive controls.

## **5 DISCUSSION**

### **5.1 Technological Approach**

In this thesis we describe an original approach towards the identification of new genes involved in melanocyte biology. The use of a functional screening, allowing the selection of cDNAs according to their ability to confer a particular phenotype, is a powerful tool for the understanding of biological pathways. Although this genetic approach has been successfully used with unicellular organisms such as bacteria and yeast, its application on mammalian cells has been limited by technical problems arising when genetically modifying higher eucaryotes. These are mainly the difficulty of achieving a stable genetic modification of mammalian cells and, whenever this happens, the challenge of recovering the genetic information provided. Indeed the vectors used for standard mammalian cells transfections are not recognised by the cells as endogenous DNA, resulting in the loss of the genetic information provided within a few cell divisions. If the vector carries a drug selection and the cells are grown in the presence of the appropriate drug over a period of a few weeks, a population of stably transfected cells will be selected. Unfortunately this not only is a slow process but it also happens through the non-specific integration of the vector in

the mammalian cell genome, making the recovery of the original genetic information provided very difficult and inefficient, often requiring subcloning steps.

The MaRX system developed in our laboratories was specifically designed to allow efficient and stable delivery of genetic material into mammalian cells and its recovery (Hannon *et al*, 1999). As the system is based on the MMLV retroviral technology, the genetic modification of the target cells goes through an infection step. Moreover, the life cycle of the virus is such that every single infection results in a stable genetic modification of the target cell through the integration of the viral genome (provirus) into the cell genome. The MaRX system is also characterised by the presence of a miniplasmid included in the provirus sequence, and a LoxP site for CRE recombinase specific recombination in the 3'LTR. The LoxP site is copied on the 5'LTR during the reverse transcription step following the delivery in the cell of the viral genome RNA. Hence, upon integration in the host cell genome, the provirus is delimited by two LoxP sites that can specifically recombine in the presence of CRE recombinase. This causes the provirus to be excised from the cell genome in the form of a circular vector that can be directly transformed and expanded into bacteria cells without the need for further subcloning steps. Moreover the circular provirus recovered is also a functional retroviral vector that can be directly transfected in packaging cell lines for virus production and retesting of the potential positive clones.

Although the MaRX system has been successfully applied to different cell lines for the characterisation of a variety of biological pathways (Sun *et al*, 1998; Hudson *et al*, 1999; Maestro *et al*, 1999; Carnero *et al*, 2000a; Carnero *et al*, 2000b), proving both

its validity and versatility, it presents some limitations that need to be considered when designing a screening. First of all, the MaRX system being based on an MMLV retrovirus, it can only be used on cells that are actively dividing making it, for example, unsuitable for primary neurons. Moreover the sole fact that a cell line is actively dividing does not guarantee that it will be successfully infected by MMLV, or at a sufficiently high rate. Hence the need of establishing the efficiency of infection of the cell line used for a particular screening. Another limitation of the system when used for cDNA genetic screenings, is the need of using a selective marker that will not kill the cell. This is because once cells have been selected for a particular phenotype, they need to be clonally expanded to ensure that a high enough copy number of genomes is available for the successful CRE/LoxP mediated recovery of the provirus. Indeed the efficiency of provirus recovery is estimated to be around one bacteria colony per 5000 mammalian cells.

## **5.2 Cloning efficiency of cell lines**

For these reasons, when we decided to develop a screening of melanocytes and melanoblasts, we tested their clonability and infectability.

The clonability was assessed by seeding the cells at low density on a 10cm dish. The efficiency of cloning greatly depends on the initial concentration of cells, so that the higher the number of cells seeded, the higher the survival rate. Also the higher the number of cells seeded, the higher the number of clones screened per plate. Having said that, it was also important for our screening to seed cells at a low enough density to ensure the formation of visible and distinct colonies. We therefore decided to assess

the clonability of both cell lines by seeding 1000 cells in a 10cm dish. In these conditions both cell line formed ~200 distinct colonies in 14 days, resulting in a colony formation efficiency of ~20%. These data were considered satisfactory and for screening purposes we decided that the number of cells to seed on a 10cm dish should be between 1000 and 3000. The colony formation experiments were also important for evaluating the homogeneity of the cell lines we intended to use. Indeed it soon became obvious that the Melan-a population presented a high degree of phenotypic heterogeneity, especially when pigmentation was considered. In a colony formation assay, the original population of Melan-a available to us presented a high number of unpigmented cells giving rise to unpigmented colonies. The occurrence of unpigmented colonies was estimated to be 3% of the total and would have resulted in an unacceptably high number of false positives being isolated from the screening. To solve the problem we decided to subclone the original population selecting a few highly pigmented colonies. The selected Melan-a clones were expanded and extensively tested in colony formation assays. This strategy proved successful and one clone was isolated that did not present a single unpigmented colony out of more than  $1 \times 10^4$  examined.

### **5.3 Infectability of cell lines**

Infectability of a cell line by MMLV retroviral based vector is an unpredictable characteristic of the cell and can only be determined empirically. Therefore, the infectability of Melan-a and Melb-a was tested using a pHMII retroviral vector carrying the cDNA of the LacZ gene. Cells that are successfully infected with this

construct express  $\beta$ -galactosidase and develop a distinctive blue colour in the presence of X-gal. Using this assay it was clear that, while Melan-a could be efficiently infected at rates of up to 20%, Melb-a were hardly infectable at all. The first step leading to infection is the internalisation of the viral particle into the target cell (Luciw and Leung, 1992). This happens through the interaction of the viral envelope protein with the so-called ecotropic receptor found on the target cells. The ecotropic receptor gene is ubiquitously expressed in murine cells. It encodes for a transmembrane protein that acts as a basic aminoacid transporter (Wang *et al*, 1991) and its overexpression is known to increase infection rates. We therefore decided to overexpress the ecotropic receptor in Melb-a using the pBabePuro retroviral vector. Unfortunately, this did not significantly improve the infection rate of the melanoblast cell line and the screening on Melb-a had to be abandoned.

#### **5.4 Library Construction**

The screening we decided to perform was based on changes in pigmentation in melanocytes. There were four reasons to choose pigmentation as the discriminating marker. First, pigmentation is easily identified in living cells without the need for further manipulation of the cells. Second, melanocytes are pigmented cells that derive from the differentiation of unpigmented melanoblasts (Hirobe *et al*, 1994; Sviderskaya *et al*, 1995). Third, despite melanoma being very pigmented in its early stages, it often loses the capacity to produce melanin, leading to an amelanotic phenotype that is normally associated with a more aggressive progression of the disease (Gibson *et al*, 1988; Albino *et al*, 1992). Fourth, it has been shown that

melanocyte cell lines become unpigmented when transfected with a variety of oncogenes (Dotto *et al*, 1989, Cajal *et al*, 1991; Yavuzer *et al*, 1995; Donatien *et al*, 1996; Halaban *et al*, 1996). Taken together these data suggested that, since pigment production is a fundamental characteristic of melanocytes, genes affecting their biology are likely to provoke visible changes in melanogenesis. Therefore, by designing a screening based on pigmentation, we intended to develop a tool that would allow the identification of genes affecting melanocytes biology in general, including those involved in their differentiation and transformation.

In order to exploit the potential of the screening as much as possible, we decided to use a library with a high degree of diversity. For this reason the cDNAs cloned into pHMII were synthesised from different sources of mRNA. In particular, the RNA was extracted from mouse embryos at day 7 and 15, and from adult mouse brain and testis. These tissues express many different transcripts and therefore are good sources of RNA for increasing the diversity of the library.

The construction of the library was validated by checking the insert of 47 randomly picked clones. Only two clones were empty (~95% recombinant), whereas the remaining had inserts spanning from 300bp to more than 5kb, indicating that the library was successfully constructed. //

### **5.5 Screening reconstruction and optimisation**

In order to optimise the screening conditions, we reconstructed the experimental procedures using genes known to alter melanogenesis in melanocytes. The genes used in our reconstructions were *Ras* and *Agouti*. There were three main reasons to use

these genes as positive controls to test the system. First, *Ras* being an oncogene, we were testing our system for its ability to detect genes involved in melanocyte transformation. Second, using *Agouti*, we were testing our system both for its ability to detect secreted molecules affecting pigmentation, and for molecules affecting the pigment quality and its production levels. Third, by using genes affecting melanocyte biology in very different ways, we were testing both the validity of using pigmentation as the discriminating marker and the versatility of our system. In both cases, depigmentation of melanocytes was visible within ten days after infection. This was a very important observation because it gave us an indication of the minimum amount of time we had to wait before we could start detecting pigmentation changes. Fortunately, ten days was about the time needed for selecting the successfully infected cells with Hygromycin. Moreover, once selection was over, cells were seeded at low density for colony formation, so that the visual screening for unpigmented colonies was beginning at least 24 days after infection of the cells, giving enough time for the phenotype to develop.

Melan-a infected with pHMII-Agouti were also used to optimise the provirus recovery. The MaRX system is characterised by the capacity of recovering the cDNA transduced into the target cells through a specific CRE recombinase reaction. The efficiency of the recovery depends on two main factors: the activity of the CRE recombinase enzyme and the quality of the substrate, i.e. the target genomic DNA. When performing a screening, it is important to maximise the chances of recovering potential positive genes and therefore we decided to optimise both the expression and



purification of CRE recombinase from bacterial cells, and the genomic DNA preparation. Expression of the enzyme was greatly enhanced by changing the conditions from 4h at 37°C to 16h at RT. Indeed, it is known that a lower expression temperature leads to slower protein synthesis and this in turn can help with the folding and solubility of recombinant proteins. We also optimised the sonication steps in order to maximise the recovery of the recombinant protein. Sonication is an efficient way to lyse cells in the non-denaturing conditions necessary for the recovery of an active protein. On the other hand, extensive sonication can be disruptive to the protein and it is therefore important to find the right balance between efficient lysis of the cells and protein preservation. We found that for our purposes it was important to keep the cells cold at all times, and by sonicating the sample five times for 1 minute with two minutes rest, we obtained a high yield of active recombinase. This procedure proved successful, as shown assessing the activity of the purified recombinant protein on a control vector.

The CRE recombinase purified as described above was used to recover the provirus from Melan-a cells infected with pHMII-Agouti. In parallel, as a positive control, we performed the provirus recovery from fibroblasts infected with the same construct. It soon became obvious that the different origin of the genomic DNA was having a strong effect on the efficiency of the reaction. Indeed, using the same conditions for genomic DNA extraction and subsequent CRE recombinase reaction, we could recover the provirus from the control fibroblasts but were unable to do so from Melan-a. The main difference between the two cell lines was the presence of melanin

in the melanocyte sample. Indeed, even when melanocytes are depigmented because of the overexpression of particular genes like *Agouti* or *Ras*, there is always some remaining melanin left in the melanosomes. This residual pigment, normally confined into specific organelles, upon lysis of the cells is released and can associate with nucleic acids and proteins. This “sticky” property of melanin has been reported by different groups and has been shown to inhibit many enzymatic reactions like PCR and reverse-transcription (Giambernardi *et al*, 1998; Eckhart *et al*, 2000). It was therefore plausible to think that a similar inhibitory effect was the cause of the poor efficiency of provirus recovery observed for Melan-a samples. In order to remove the residual melanin, we optimised the extraction and designed a different method to isolate genomic DNA from melanocytes. First, cells were treated with 200µM PTU for one week before genomic extraction. PTU is a reversible inhibitor of Tyrosinase, the first enzyme involved in melanin synthesis, and is therefore able to reduce pigment levels in melanocytes. This was followed by extraction of the genomic DNA using the QIAGEN genomic DNA Kit, which includes a step to separate the cells nuclei from the remaining organelles reducing the possibility of melanin associating to the DNA. Finally, the genomic DNA was further purified with three phenol/chloroform extractions. At the end of the process, the DNA isolated visibly contained less pigment than the one obtained with previous methodologies. Indeed, when the DNA thus purified was treated with CRE recombinase, the efficiency of provirus recovery was greatly enhanced and similar to the one observed with control fibroblasts, validating the efficacy of this method.

## **5.6 White cells enrichment**

Once all the screening conditions were optimised, we decided to run a screening on 15cm dishes in order to include as many clones as possible. This first attempt was not successful in identifying new genes, but it proved very useful in highlighting the limitations of the screening that needed to be addressed. In particular, we soon realised that the criteria used to define a positive clone were not strict enough, leading to a high ratio of false positives. Indeed, during this screening, 30 unpigmented colonies were subcloned and none proved to be real when retested. When the selected colonies were re-examined, we realised that their unpigmented phenotype was probably a result of their smaller than average size (~50 cells as opposed to ~130 cells for a medium-sized, 14 days old colony). Indeed it has been shown that confluency is an important factor in melanogenesis, and the more confluent melanocytes are, the more pigment is produced (Mahalingam *et al*, 1997; Hornyak *et al*, 2000). It is possible that a small colony represents a more relaxed environment resulting in a lighter pigmentation. In support of this theory, we also noticed that the clones isolated became very pigmented by the time their number was enough to allow a genomic extraction, suggesting that their unpigmented phenotype was a consequence of environmental factors rather than genetic ones. This is in accord with the observation that pigmentation, although being strictly regulated by enzymatic pathways, is also extremely dependent on external factors such as medium pH and confluency. To overcome this problem, we decided to set more stringent parameters so that only unpigmented colonies with a minimum of ~130 cells were to be considered as potential positives.

Another important observation arising from this experiment was that we needed to improve the throughput of the number of clones examined. The set up of the screening was such that only 50,000 colonies could be examined simultaneously, taking approximately one month from start to finish. At this rate, we could have only covered a very small fraction of our library with a complexity of  $6 \times 10^6$  clones. It was therefore imperative to find a way to increase the efficiency of the screening. We could not increase the yield by screening more colonies simultaneously because we were limited both by the space in the incubator, and by the last step of the screening consisting in visually selecting the colonies according to their pigmentation levels. We felt that the only solution was to introduce an enrichment step for potentially positive white cells before seeding at low density for colony formation. To this purpose we tested two different methodologies.

The first was based on the observation that heavily pigmented melanocytes are extremely sensitive to the freezing/thawing steps involved in the long term storage of cells in liquid nitrogen. Indeed when DMSO stocks need to be prepared, it is necessary to treat melanocytes for at least four days with PTU in order to decrease their pigmentation levels. If this process is omitted, up to 99% of the frozen melanocytes will die, and only the less pigmented ones will survive.

We thought that we could exploit this characteristic to enrich the library-infected population of melanocytes for unpigmented cells. To test the feasibility and reproducibility of this approach, we mixed wild type melanocytes with melanocytes infected with *Agouti* and the *LacZ* gene. *Agouti* was used to induce the unpigmented phenotype, whereas *LacZ* was used to allow an easier identification of the positive

(unpigmented) cells. Different ratios of unpigmented versus pigmented melanocytes were used and, following a single freeze/thawing step without PTU treatment, the enrichment ratio was assessed. Unfortunately, this approach did not prove very efficient probably because it only works on cells with high levels of eumelanin. This also explains the low degree of reproducibility observed between independent experiments. As mentioned earlier, pigment production depends on many environmental factors, and therefore it is very difficult to ensure that the same level of pigmentation is achieved in different experiments, accounting for the variability observed.

The second approach was based on the observation that pigmented and unpigmented cells have different light-scattering properties, and therefore can be separated by a cell sorter (Boissy *et al*, 1989). The feasibility of this methodology had to be tested at different levels. First we assessed how easily unpigmented and pigmented cells could be discriminated by the machine. This was done by sorting separately a population of wild-type, Agouti-infected and Ras-infected Melan-a. When the plots of each run were superimposed, it became obvious that the pigmented and unpigmented populations could be discriminated with very little overlapping. Also of great importance to us was the observation that both Agouti-infected and Ras-infected cells were falling in the same area of the plot, meaning that the machine was able to recognise an unpigmented cell regardless of the mechanism leading to depigmentation.

The viability of the cells following the sorting step was also assessed. This was an important observation because an enrichment methodology causing a high rate of cell

death would not have been very practical. Luckily, Melan-a cells did not suffer during the procedure and the sorted cells were as efficient in forming colonies as the unsorted ones. Finally, the enrichment ratio was tested by sorting a mixed population composed of unpigmented (Agouti/LacZ-infected) and pigmented Melan-a at a 1:10,000 ratio (100 unpigmented and  $1 \times 10^6$  pigmented cells). This experiment was very important in optimising the sorting conditions such as speed and region on the plot to be selected. Indeed, we noticed that the accuracy of the machine was affected by the number of cells sorted per second, the best compromise being around 1200 cells/second. Similarly the selection of the gated area had to reflect the best compromise between number of cells recovered and purity. In our case, we were able to draw a region on the graph that was including more than 80% of the unpigmented cells and only 0.5% of pigmented ones. Moreover, the vast majority of the pigmented cells gated were able to form pigmented colonies when seeded at low density and therefore could be recognised as non-positive clones. With these conditions, using a sample of  $1 \times 10^6$  cells, it took about 15 minutes to raise the ratio of unpigmented versus pigmented Melan-a from 1:10,000 to 1:32, resulting in a 300 fold enrichment.

With these data available, and considering that we still had limited space in the incubator, we estimated that by introducing a single enrichment step we could screen simultaneously the equivalent of  $1.5 \times 10^6$  clones.

A second screening was hence carried out, and 29 unpigmented and good-sized colonies (~130 cells per colony) were isolated. The clones were expanded for genomic extraction and contrary to what previously observed, none developed

pigmentation. Despite this, only two retested positive when re-infected into Melan-a and assayed for melanin content. This seems to suggest that the remaining 27 clones were real false positives (as opposed to those found in the first screening), whose depigmentation was the result of genetic factors rather than environmental ones. Since the colony formation efficiency in our system was about 20%, and around 80% of the unpigmented population was gated during the enrichment step, the number of false positives at the moment of sorting was estimated to be ~170. Before sorting, cells were selected with Hygromycin for 10 days, allowing for ~5 cell divisions. Hence, those 170 cells derived from the division of only 6 initial false positive, resulting in a false positive ratio in the original Melan-a population of 1:250,000.

### **5.7 The Emx genes**

The sequence analysis of the positive proviruses revealed that they were independent clones of the same transcript. Indeed the two clones were identical except for the length of the PolyA<sup>+</sup> tail, indicating that they originated during the library construction from different reverse transcriptase reactions of the same mRNA. The finding that the same gene was isolated twice independently was further validating the ability of the clones selected to elicit the unpigmented phenotype when overexpressed in melanocytes. Moreover, this result was very important to us because it was the definitive proof that the methodology we developed, including the screening conditions and the use of a cell sorter, was sound.

The sequence of the cDNA isolated matched the published partial sequences of the *Emx1* homeobox gene. In particular, we found 100% identity to the published 198bp of the mouse *Emx1* homeobox region (Simeone *et al*, 1992), 94% identity to the published 301bp of the 5' rat *Emx1* sequence (Robel *et al*, 1995) and 92% identity to the published last 462bp of the human *Emx1* cds (Simeone *et al*, 1992). It is worth noticing that our sequence was not in accord with the published rat one, the start codon being 99bp upstream to the published rat one, resulting in the predicted size of our protein being 33aa longer. In addition the published rat sequence results in a stop codon after 62aa (position 292 of the published nucleotide sequence and 186 of their putative cds).

*Emx1*, together with *Emx2*, was originally isolated by Simeone *et al* in 1992 in an attempt to find murine homologues to the *Drosophila empty spiracles* gene. Like the fruit-fly counterpart, *Emx1* and *Emx2* are involved in brain development and are prevalently expressed in the cerebral cortex during embryogenesis between days E8.5-E16. Interestingly, neither *Emx1* nor *Emx2* have been associated with melanocytes biology. Since the two proteins are highly related (62% identity) and are believed to partially rescue each other in case of inactivating mutations, we decided to investigate both *Emx1* and *Emx2* in relation to their biological effects when overexpressed in Melan-a. To this purpose, the murine *Emx2* cDNA obtained from Dr Antonio Simeone was subcloned in pHMII and transduced in Melan-a.

First we decide to test and compare the ability of the *Emx* genes to influence melanogenesis. Pigmentation was clearly affected by both *Emx* genes in a very



similar way. In particular our experiments showed that in both cases depigmentation of Melan-a occurred through the downregulation of the *microphthalmia* transcription factor. This was confirmed by the concomitant downregulation of the Tyrosinase, Trp-1 and Trp-2 melanogenic enzymes, whose expression levels depends on *microphthalmia* activity. Since this is the same pattern of depigmentation observed when oncogenes are overexpressed in melanocytes, we decided to assess the ability of the Emx genes to confer growth advantages to Melan-a. In particular we tested the effects on duplication times, TPA dependence and ability to grow in soft agar. Both duplication times and TPA dependence were affected by the Emx genes overexpression. In particular, in the presence of TPA, cells were able to grow at least 50% faster than wild-type Melan-a. Interestingly, in the same experiment, melanocytes infected with activated Ras were slower to grow. This was probably due to the downregulation of PKC, the primary effector of Ras mediated mitogenesis, caused by prolonged exposure to TPA (Lacal *et al*, 1987).

When TPA was withdrawn from the medium, Melan-a cells infected with either Emx gene were still able to grow, although at a slower rate. This was again in contrast with what was observed with both wild-type Melan-a (unable to grow in the absence of TPA) and Ras infected Melan-a that grow faster in the absence of TPA. These results seemed to suggest that, contrary to what seen with activated *Ras*, the TPA independence observed with the Emx genes was partial.

Finally we tested the Emx genes ability to transform Melan-a cells performing a growth in soft-agar experiment. Contrary to what observed with activated *Ras*, neither

*Emx1* nor *Emx2* infected Melan-a could form colonies in soft agar and responded to contact inhibition.

Interestingly, this pattern of induced growth advantages without a fully transformed phenotype was similar to what reported when overexpressing *bFGF* in melanocytes (Dotto *et al*, 1989; Cajal *et al*, 1991).

Since *bFGF* is a very well characterised oncogene involved in melanoma progression, it was possible that the *Emx* genes were playing a similar role. Therefore we decided to assess the expression of both *Emx1* and *Emx2* in human melanoma cell lines.

Moreover, we tested the possibility that the *Emx* genes are involved in melanocyte differentiation by assessing their expression levels in melanoblasts. This was done by performing Northern Blots on total RNA extracted from 9 melanoma cell lines and Melb-a melanoblasts using specific *Emx1* and *Emx2* probes. Since no expression was detected in any of the samples tested, we concluded that the *Emx* genes might not be involved neither in melanocyte differentiation nor melanoma progression.

Although a function of the *Emx* genes in melanocyte biology is not immediately apparent, it is clear that when ectopically expressed in pigmented cells they can perturb levels of melanogenic enzymes, pigmentation characteristics and growth. To further investigate this effect, we decided to create *Emx1* mutants in order to establish which domains of the homeobox protein were essential to provoke the phenotype observed. In particular we created mutants lacking the Nuclear Localisation Signal (M1), the homeobox domain (M2), the alanine rich region (M3) and the portion of protein comprised between the homeobox domain and the alanine rich region (M4).

Since the mutants were created by PCR, the constructs were sequenced to ensure that no errors were introduced during the amplification step, and the full-length cds of Emx1 (M0) was used as positive control. The mutants were tested for their ability to induce depigmentation when transduced into Melan-a cells. From this experiment it was obvious that the full-length protein is required to inhibit pigmentation. In particular, the protein needs to retain the ability to localise in the nucleus and to interact with the DNA. These data suggest that Emx1 plays an active role in causing the depigmentation of Melan-a, either by positively upregulating the transcription of genes involved in microphthalmia downregulation, or by actively inhibiting its promoter or positive regulators. Unfortunately, very little is known about the transcription activity of Emx1 and its physiological targets, making it difficult to speculate on a possible pathway that could connect Emx1 activity with pigment biology.

## **5.8 Future Work**

The identification of the Emx genes physiological targets could facilitate the understanding of the events leading to the phenotype observed when overexpressed in Melan-a. One possibility is that the Emx genes are capable of upregulating bFGF expression in melanocytes. Indeed, it has been shown that bFGF expression in melanoma often depends on the activation of the HOXB7 homeobox gene, and can be inhibited with antisense oligomers specific for the HOXB7 cDNA (Care *et al*, 1996). It is possible that the homeobox Emx genes can trigger a similar event. This could explain the similarities in the phenotype we observed when overexpressing the Emx genes in Melan-a, with that previously reported for melanocytes transduced with bFGF (e.g, depigmentation associated with TPA independence and lack of transformation).

A very important point to note is that so far only a quarter of the whole library complexity has been screened. Now that the screening conditions have been optimised, it would be very exciting to continue the screening of the mouse cDNA library. This would be likely to result in the isolation of more genes that can trigger melanocyte depigmentation, and that may have a crucial role in melanocyte biology.

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