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Identification and characterization of proteins involved in tumor formation and progression

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Table of contents

1. List of abbreviations

APC	adenomatous polyposis coli
aRMS	alveolar rhabdomyosarcoma
ATF1	activating transcription factor 1
ATM	ataxia telangiectasia mutated
bHLH	basic helix-loop-helix
βigH3	TGF-β induced gene product 3
cdk	cyclin dependent kinases
CHN	chondrosarcoma
CHOP	CCAAT/enhancer-binding protein homologous protein
CKI	cdk inhibitor proteins
CNS	central nervous system
CRIP	cysteine-rich intestinal protein
CRP	cysteine-rich protein
Del 1	developmentally regulated endothelial cell locus 1
E1AF	E1A enhancer-binding factor
ERG	ETS-related gene
eRMS	embryonal rhabdomyosarcoma
EST	expressed sequence tags
ETS	avian erythroblastoma virus transforming sequence
ETV1	ETS translocation variant 1
EWS	Ewing's sarcoma
FEV	fifth Ewing variant
FHL	four and a half LIM domain protein
FKHR	forkhead related gene
FLI-1	friend leukemia virus integration site 1
HD	homeodomain

INK4	inhibitors of cdk4
kDa	kilo dalton
KO	knockout mouse
LHX	LIM homeobox protein
LIMK	LIM kinase protein
MDM2	murine double minute 2
MITF	microphtalmia-associated transcription factor
ODN	oligonucleotide
OP	octapeptide
p53	tumor suppressor protein of 53 kDa
PAX	paired box
PCNA	proliferating cell nuclear antigen
PD	paired domain
PDGFRα	platelet-derived growth factor receptor α
PNET	primitive neuroectodermal tumor
Rb	retinoblastoma
RBP56	RNA-binding protein 56
RMS	rhabdomyosarcoma
RT-PCR	reverse transcriptase polymerase chain reaction
SSX	synovial sarcoma X breakpoint
SYT	synovial sarcoma translocation
TGF-β	tumor growth factor β
TPR-1	tyrosinase-related protein 1
WT1	Wilms' tumor suppressor gene 1

2. A) Summary

Cancer is a disease that increasingly plays a predominant role in our society. Despite a large gain of information in cancer biology our knowledge is far from being complete, and treatment options are still very limited. Rhabdomyosarcoma (RMS), a pediatric tumor of skeletal muscle origin, usually has a bad prognosis and tends to metastasize very early. At the molecular level it is known that alterations in two fundamental groups of genes, oncogenes and tumor suppressor genes, are responsible for the onset of cancer in general. To identify novel candidate tumor suppressor genes a subtractive hybridization procedure was previously performed between human myoblasts and the RMS cell line RD. This strategy resulted in the identification of 48 independent cDNAs that are downregulated in the tumor cells, 40% of which were coding for unknown genes. In this work we first extended the investigation to other RMS cell lines, where we found that half of the investigated cDNAs were consistently reduced in all RMS compared to normal cultured myoblasts. Thereafter, one interesting clone coding for an unknown gene, was selected and cloned. It was found to belong to the LIM domain protein family and was called DRAL.

As further selection criteria of unknown genes, we then took advantage of a RD subline expressing a temperature sensitive version of the p53 tumor suppressor protein in which wt p53 functions could be achieved by drop of the temperature, since p53 is mutated in this cell line. Interestingly, it was again DRAL that could specifically be induced by wt p53 in this system. Induction of endogenous DRAL through p53 expression could be confirmed by ionizing radiation treatment of normal myoblasts. Finally, by cloning the promoter of DRAL and partial characterization of the genomic structure, we could identify four potential p53 binding sites, which further strengthens the notion that DRAL is responsive to p53. Surprisingly, attempts to stably express DRAL in several cell lines failed. Hence, transient transfection experiments were performed and revealed an increase in the proportion of apoptotic cells among the DRAL expressing cells, suggesting that overexpression of DRAL can induce apoptosis. To gain insight into mechanisms possibly involved in the role of DRAL, transfection experiments using FLAG-tagged DRAL revealed that DRAL localizes to the cytoplasm, nucleus, as well as focal adhesions. The different subcellular localizations is a typical feature of LIM domain proteins. Hence, it is likely that DRAL interacts with different partner proteins according to its subcellular localization.

As in vitro models for RMS, we established two new RMS cell lines, RUCH-2 and RUCH-3, from primary tumor material. The RUCH-2 cell line, which spontaneously progressed in vitro to a metastatic cell line, was then used to identify potential marker genes for metastatic progression. Changes between early and late cell passages were analyzed by cDNA microarray, which contained 588 known tumor related genes, resulting in 92 genes with altered expression levels. These genes can now be further investigated in primary tumor samples to identify significant markers for tumor progression.

Finally, deregulated expression of the transcription factor PAX3 was previously found in several neoplasms, including RMS and Ewing's sarcoma. We identified an additional PAX3 expressing tumor type, melanoma. Indeed, we detected expression of PAX3 in the majority of the melanomas analyzed both by RT-PCR and in situ hybridization. In addition, specific downregulation of PAX3 expression through antisense oligonucleotide-based treatment in cultured melanomas resulted in apoptosis of PAX3 expressing cell lines, whereas PAX3 negative melanoma lines showed no cell death. Thus, expression of PAX3 in melanomas might be required for tumor survival and expansion, and might represent an ideal target for tumor specific treatment.

In summary, this work describes cloning of DRAL, a potential tumor suppressor gene which is induced by p53, establishment and characterization of the RMS cell line RUCH-2 which showed spontaneous metastatic progression, and discovery of the aberrant expression of PAX3 and its inhibitory function of apoptosis in melanoma.

2. B) Zusammenfassung

Krebs ist eine Krankheit, die in unserer heutigen Gesellschaft eine immer wichtigere Rolle einnimmt. Trotz der Datenflut an biologischen Erkenntnissen über Krebs, ist unser Wissen noch lange nicht vollständig, und Behandlungsmethoden sind bis heute noch sehr limitiert. Das Rhabdomyosarkom (RMS) ist ein pediatrischer Tumor muskulären Ursprungs, der schnell metastasiert und eine schlechte Prognose hat. Auf molekularer Ebene ist bekannt, dass Veränderungen in zwei Gruppen von Genen, den Onkogenen und den Tumorsuppressorgenen, die Grundlage zur Krebsentstehung bilden. Um neue potentielle Tumorsuppressorgene zu identifizieren wurde eine subtraktive Hybridisation zwischen humanen Myoblasten und der RMS Zellinie RD durchgeführt. Diese Strategie führte zur Identifizierung von 48 unabhängigen, "herunter regulierten" cDNAs, wobei 40% dieser cDNAs für unbekannte Gene kodierten. In dieser Arbeit wurde zuerst die Untersuchung auf weitere RMS Zellinien erweitert, wobei sich bestätigte, dass die Hälfte der analysierten Klone in allen untersuchten RMS vermindert exprimiert werden. Danach wurde ein interessanter, für ein unbekanntes Gen kodierender Klon ausgewählt und kloniert. Dieses Gen kodiert für ein Protein, das zu der LIM-Domänen-Protein-Familie gehört und wurde DRAL benannt.

Ein weiteres Selektionskriterium für unbekannte Gene ist deren Induzierbarkeit durch wt p53. Wir benutzten für diese Untersuchungen eine RD-Subzellinie, die ein induzierbares, temperatur-sensitives p53 Gen exprimiert (da die RD Zellinie kein funktionelles p53 Protein hat). Bei Temperaturerniedrigung exprimiert diese Zellinie ein funktionell normales wt p53 Genprodukt. Interessanterweise konnten wir DRAL als ein neues p53 induzierbares Gen identifizieren. Die Induktion endogener DRAL Expression konnte zusätzlich durch Bestrahlung von humanen Myoblasten bestätigt werden. Schlussendlich konnten wir durch die Klonierung des DRAL-Promoters zeigen, dass vier potentielle p53 Bindungsstellen in der untersuchten Sequenz vorhanden sind. Sämtliche Versuche stabile Zellklone zu erhalten, die DRAL exprimieren, scheiterten in allen benutzten Zellinien. Um diesen Aspekt genauer zu untersuchen, wurden transiente Transfektionsexperimente durchgeführt. Dabei konnten wir beobachten, dass vornehmlich DRAL exprimierende Zellen apoptierten, woraus man schliessen kann, dass ektopische DRAL Expression Apoptose induziert. Um zusätzliche Erkenntnisse zur Funktion von DRAL zu gewinnen, wurde dessen intrazelluläre Lokalisation untersucht.

Mittels FLAG-markiertem DRAL konnte gezeigt werden, dass DRAL sowohl im Zytoplasma und Zellkern, als auch in den fokalen Adhesionspunkten lokalisiert ist. Das Auftreten unterschiedlicher intrazellulärer Lokalisationen ist ein typisches Bild von LIM-Domänen-Proteinen. Es ist wahrscheinlich, dass die unterschiedlichen Lokalisationen von DRAL innerhalb der Zelle durch die Interaktion mit anderen Proteinen zustande kommt.

Um Modelle zur Untersuchung von RMS zu haben, wurden zwei neue Zellinien aus primären RMS Geweben etabliert, RUCH-2 und RUCH-3. Mit Hilfe der RUCH-2 Zellinie, welche sich in vitro spontan zu einer metastasierenden Zellinie entwickelte, konnten potentielle Markergene für Metastasierung gefunden werden. Veränderte Genexpression zwischen frühen und späten RUCH-2 Passagen wurde anhand eines cDNA Mikroarrays, der 588 bekannte Tumorgene beinhaltet, untersucht. Dieser Assay resultierete in 92 unterschiedlich exprimierten Genen. Um daraus mögliche signifikante Marker für Tumorprogression zu identifizieren, müssen diese Gene in einem weiteren Schritt in primären Tumorgeweben untersucht werden.

Deregulierte Expression des Transkriptionsfaktors PAX3 ist in verschiedenen Tumoren, wie RMS und Ewing's Sarkoma bekannt. Es gelang uns einen neuen Tumortyp zu finden, der PAX3 exprimiert, nämlich Melanome. Durch RT-PCR Analysen als auch durch in situ Hybridisationen konnten wir zeigen, dass die Mehrzahl der Melanome PAX3 exprimieren. Zusätzlich konnnten wir zeigen, dass gezielte Verminderung der PAX3 Expression durch anti-sense Oligonukleotide nur die PAX3 positiven Melanome zur Apoptose zwingt aber nicht die PAX3 negativen Melanome. Die Expression von PAX3 scheint somit eine wichtige Rolle für das Überleben und die Expansion von Melanome zu spielen, deshalb könnte PAX3 ein ideales Ziel für tumorspezifische Behandlung bieten.

Zusammenfassend wurde in dieser Arbeit DRAL als ein neues potentielles Tumorsuppressorgen kloniert, das durch p53 induzierbar ist, weiter wurde eine RMS Zellinie etabliert, die sich in vitro spontan zu einer metastasierenden Zellinie entwickelte, und zuletzt konnten wir zeigen, dass Melanoma PAX3 exprimieren, dessen Expression die Zellen vor Apoptose schützt.

3. Introduction

In a multicellular organism, normal cells reproduce only when instructed to do so by other cells in their vicinity. Such collaboration ensures that each tissue maintains a given size. During our lifetime about 10^{16} cell divisions take place in the body. Because nothing is perfect, sometimes during replication errors occur with an estimated rate of about 10^{-6} mutations per gene per cell division. Thus, in the lifetime of any individual human being, every single gene is likely to have undergone mutation on about 10^{10} occasions. Luckily, human cells are remarkably adept at the repair of genetic damage. Moreover, the body is proficient at eliminating individual cells in which this repair has failed. This backup system, present in each cell, provokes the cell to commit suicide (undergo apoptosis) if some of its essential components are damaged or if its control systems are deregulated. Another defense against runaway proliferation, quite distinct from the apoptotic program, is the limited amount of cell divisions for each cell. All this is orchestrated by a complex signal-transducing circuitry reaching from the extracellular space through the cytoplasm to the cell cycle clock in the nucleus, the central regulator of cell proliferation. Cell cycle is the executive decision maker of the cell, and it apparently runs amok in virtually all types of cancer.

3.1 Cancer

Cancer is a disturbance of the most fundamental rules of behavior of cells in a multicellular organism. It is not a single disease but it is many diseases. Cancer begins as a single cell that progresses to a full malignancy. Today it is well accepted that cancer is a malady of genes (Bishop and Weinberg 1996; Cooper 1995). With increased longevity of humans, cancer plays a more and more predominant role in modern diseases. Despite large gains of knowledge in cancer biology, treatment options are still very limited. Primary neoplasms can now be treated with considerable success, however this is not true for the actual key player in disease lethality, the metastases. During the last decades the use of nonspecific cytotoxic drugs and radiation therapy were pushed to great extremes in the hope of curing cancer. In that time, investigators have made astonishing progress at the molecular level. This molecular understanding of cancer is now hoped to offer new strategies with which the challenge of cancer and metastases might be confronted in a specific way (Hong and Sporn 1997). No one can predict exactly when therapies targeted to the molecular alterations in cancer cells will find wide use, but the effort is now under way. A quite different approach to lower cancer incidence is to limit the external causes, which cause more than half of all cancer deaths. It is simpler to prevent cancer than to treat it. Exogenous causes of cancer are mainly due to behavior, such as cigarette smoking or dietary habits, infectious agents (tumor viruses), or physical and chemical carcinogens. For example, at least one major form of human cancer, carcinoma of the lung, has been attributed persuasively to the complex mixture of carcinogens found in cigarette smoke (Trichopoulos, et al. 1996). Further, our augmented sunbathing lifestyle during the last ten years has increased the incidence of skin melanomas by 4-8% each year (Koh, et al. 1993).

Chromosomal abnormalities in tumors were recognized a long time ago, but their relevance has only recently become clearer. There are three main structural cytogenetic changes: deletions, translocations, and inversions. Deletions often result in loss of a tumor suppressor gene and will not be discussed further. The breakpoints of many different and specific translocations and inversions (herein grouped as chromosomal

translocations) have been cloned and some general principles have emerged from the study of the associated genes. Distinct translocations in leukemias and solid tumors lead to the activation of proto-oncogene products or, more commonly, to creation of tumor specific fusion proteins. These proteins are often transcription factors and thus disruption of transcriptional control plays a major role in tumorigenesis. Fusion proteins formed after chromosomal translocations are common and often tumor specific in a range of tumor types and are therefore potential targets for therapy design. Further, avoidance of apoptosis plays an important role in cancer (Reed 1999).

The vast majority of mutations in cancer are somatic and are found only in an individual cancer cell. However, about 1% of all cancers arise in subjects with an unmistakable hereditary cancer syndrome (Fearon 1997). These patients carry a particular germline mutation in every single cell of their body. The familial cancer syndrome described by Li and Fraumeni, due to mutations in the p53 gene, which results in various tumors in children and adults, is one example (Li and Fraumeni 1969).

3.1.1 The multistep nature of cancer

Tumorigenesis is now widely accepted to involve a multistep process in which several genetic lesions have to accumulate in a single cell in order to produce a fully malignant state, including the ability to metastasize. This "multistep" concept of cancer was first described by Vogelstein & Kinzler, by studying the progression of colorectal cancer (Vogelstein and Kinzler 1993). During the long incubation period, the prospective cancer cells undergo a succession of genetic changes that promote clonal selection of cells with increasingly aggressive behavior. The activation of proto-oncogenes or inactivation of tumor suppressor genes has been recognized to represent these genetic lesions. These two gene classes, which together constitute only a small proportion of all genes, play major roles in triggering cancer.

It is still not clear if the genes must be damaged in a particular order, or if the mere summation of events is sufficient to cause malignancy.

3.1.2 Oncogenes

More than 100 proto-oncogenes have been identified and the number will likely continue to grow (Green, et al. 1996; Hunter 1991). Many proto-oncogenes are members of gene families that have been generated by duplication and subsequent diversification during the course of evolution (e.g. myc, ras) (Hunter 1997). A lot of proto-oncogenes code for proteins that relay growth stimulating signals from outside the cell to the nucleus. The growth of a cell becomes deregulated when a mutation in one of its proto-oncogenes occurs (thereafter called oncogenes), keeping an otherwise silent pathway continuously active. At least three ways are known by which oncogenes can allow cells to grow without stimulation by external growth factors. First by autocrine stimulation (e.g. PDGF), second by constitutively activated or overexpressed receptors releasing stimulatory signals into cells even without growth factor binding (v-erb-B), and third by abnormal transducer in the cascade releasing growth-stimulating signals (without activated receptors) (e.g. ras).

3.1.3 Tumor suppressor genes

Tumor suppressor genes, in contrast to proto-/oncogenes, generally antagonize cell proliferation. They are involved in tumorigenesis by loss-of-function mutations. The resulting loss of functional tumor suppressor proteins deprives the cell of crucial brakes that prevent inappropriate growth e.g. tumorigenesis. About a dozen tumor suppressor genes have been identified (Cowell 1992; Hooper 1998), with the retinoblastoma (Rb) gene being the first tumor suppressor gene proposed to has this function in 1973 (Comings 1973). The gene was finally cloned and its tumor suppressor activity was shown in 1986 (Friend, et al. 1986). Individuals with the inherited syndrome familial retinoblastoma have a germline mutation in Rb, and consequently develop cancer of the retina (Knudson 1971).

More recently, Kinzler & Vogelstein have subdivided the tumor suppressor genes into different groups, namely the "gatekeepers", the "caretakers", and the "landscapers" (Kinzler and Vogelstein 1998). Inactivation of gatekeepers leads to a permanent

imbalance of cell division over cell death contributing directly to the neoplastic growth of the tumors. They normally function to prevent runaway growth and are responsible for maintaining a constant cell number in renewing cell populations. Members of this class are for example p53, Rb, and APC. Restoration of the missing gatekeeper gene suppresses the neoplastic growth. In contrast, inactivation of a caretaker gene results in a greatly increased mutation rate and is equivalent to a constant exposure to mutagens (e.g. ATM). The restoration of caretaker function will not affect tumor growth. Finally, inactivation of landscaper function will lead to abnormal microenvironment.

p53

The p53 gene product was first recognized as a 53 kDa nuclear phosphoprotein that interacts with SV40 large T antigen in SV40-transformed cells (Lane and Crawford 1979). Its tumor suppressor function was later detected, being the second tumor suppressor gene found. Now, 20 years after the discovery of p53, an enormous quantity of knowledge about this protein and its different functions has been obtained. The p53 protein is usually found as a tetramer in cells. Its half-life is short, about 20-30 minutes, and the expression of this protein is maintained at low, often undetectable levels in normal (untreated) cells. Levels and/or activity of p53 increase in response to DNA damaging agents (UV, IR), hypoxia, oncogenic stimuli, redox stress, cell adhesion, and altered ribonucleotide pools. Further, it appears that complexes formed between normal and mutated polypeptides are inactive. The presence of an excess of mutated p53 polypeptides within a cell therefore blocks normal p53 function, and it is this interference with normal p53 activity that results in cell transformation (general p53 reviews (Amundson, et al. 1998; El-Deiry 1998; Giaccia and Kastan 1998; Harris 1996; Ko and Prives 1996; Levine 1997)).

p53 plays a key role in cell cycle regulation, apoptosis, and other cellular functions by activating or repressing a large number of different genes. Therefore, a major role in suppressing tumorigenesis is attributed to p53, because functional p53 protein is absent in every second cancer case. The p53 pathway is even more implicated in cancer because

aberrant or absent expression of regulatory proteins (e.g. mdm-2 or ARF) or downstream molecules are known to occur in tumors. The vast majority of p53 mutations found in tumors map within the domain for sequence-specific DNA binding. The p53 protein is a transcription factor that binds in a sequence-specific manner to particular sites in the genome and activates or represses transcription of target genes. Important functions mediated by p53 are G1 arrest, DNA repair, G2/M arrest, apoptosis, and anti-angiogenesis. Until now about 50 p53 inducible genes are known. The search for p53 target genes, however, is not yet complete and new ones will certainly be discovered, since experimental evidence predicts 200 - 300 p53 consensus sites in the human genome (Tokino, et al. 1994). Some of the known p53 inducible genes are listed in table 1 and grouped into different categories according to their functions: G1 arrest, DNA repair, G2/M arrest, apoptosis, and anti-angiogenesis. Therefore, for example, cells deficient in p53 fail to arrest in the G1 phase of the cell cycle following exposure to DNA damaging agents. Some genes are involved in several categories and are therefore listed more than once. Not mentioned are all the genes which are repressed by p53, like bcl-2 a cell death inhibitor protein (Miyashita, et al. 1994). The final outcome of p53 activation depends on many factors, and is mediated largely through the action of the target genes transactivated by p53. Still, it is not known why some cells enter into apoptosis following p53 expression while others undergo prolonged growth arrest.

Surprisingly, the regulation of p53 function remains poorly understood. Since p53 is a key protein, it is not surprising that multiple mechanisms regulate its activity and there are almost certainly cell type specific differences in the mechanisms used, e.g. ionizing radiation, hypoxia, redox, adhesion, and oncogenes.

For several years it was suspected that p53 had to be a member of a gene family, because p53 knockout mice had only a slight increase in tumor incidence as compared to normal animals. Just recently two p53 homologues were found, namely p63 (p51) and p73 (Kaghad, et al. 1997; Osada, et al. 1998; Trink, et al. 1998; Yang, et al. 1998). It is not clear whether they have redundant roles with p53, however so far no mutations of these genes in human tumors have been identified.

Table 1: Target genes upregulated by p53

involved in	gene	Ref.
G1 arrest	cyclin G1	[1]
	p21	[2]
	cyclin D1	[3]
	Wip1	[4]
DNA repair	p48	[5]
	GADD45	[6]
	p21	[2]
	PCNA	[7]
G2/M arrest	GADD45	[6]
	14-3-3σ	[8]
Apoptosis	bax	[9]
	Fas	[10]
	DR5	[11]
	TRID	[12]
	PAG608	[13]
	IGF-BP3	[14]
Anti-angiogenesis	thrombospondin-1	[15]
	GD-Aif	[16]

[1] Okamoto et al. (1994) *Embo J* 13:4816-4822. Zauberman et al. (1995) *Oncogene* 10:2361-2366. [2] El-Deiry et al. (1993) *Cell* 75:817-825. [3] Spitkovsky et al. (1995) *Oncogene* 10:2421-2425. [4] Fiscella et al. (1997) *Proc Natl Acad Sci U S A* 94:6048-6053. [5] Hwang et al. (1999) *Proc Natl Acad Sci U S A* 96:424-428. [6] Kastan et al. (1992) *Cell* 71:587-597. [7] Morris et al. (1996) *Proc Natl Acad Sci U S A* 93:895-899. [8] Hermeking et al. (1997) *Mol Cell* 1:3-11. [9] Miyashita et al. (1994) *Oncogene* 9:1799-1805. [10] Owen-Schaub et al. (1995) *Mol Cell Biol* 15:3032-3040. [11] Wu et al. (1997) *Nat Genet* 17:141-143. [12] Sheikh et al. (1999) *Oncogene* 18:4153-4159. [13] Israeli et al. (1997) *Embo J* 16:4384-4392. [14] Buckbinder et al. (1995) *Nature* 377:646-649. [15] Dameron et al. (1994) *Science* 265:1582-1584. [16] Van Meir et al. (1994) *Nat Genet* 8:171-176.

3.1.4 Cell cycle: cyclins and cell cycle regulation

Since it is essential for the organism to identify and eliminate cells growing inappropriately, apoptosis and proliferation are tightly coupled, and cell cycle regulators can influence both cell division and cell death. The duration of cell cycle varies

from one cell type to another, lasting eight minutes for fly embryos to over a year for some mammalian liver cells (Alberts, et al. 1994). In proliferating mammalian cells, the cell cycle is typically divided into four phases: G1 (gap 1), S (DNA replication, S = synthesis), G2 (gap 2), and M (mitosis) (Fig. 1). In the G1 phase, a cell may continue to grow and enter the S phase, or escape the cell cycle by becoming inactive (G0), differentiate, or begin apoptosis. Two cell cycle checkpoints exist, the first at the G1/S boundary and the second at the G2/M transition point. Cell cycle progression can be blocked at any of these checkpoints in response to the status of either the intracellular or extracellular environment.

Two types of proteins are of central importance in the regulation of the cell cycle: the cyclins, so called because they vary in abundance through the cell cycle, and the cyclin-dependent kinases (cdk), which are regulated by the binding of cyclins. Cdks (9 have been identified so far in mammalian cells) allow progression through the different phases of the cell cycle by phosphorylating substrates. Their kinase activity is dependent on the presence of the activating subunits, the cyclins (16 mammalian cyclins have been identified). All cyclins contain a common domain known as the cyclin box, which is used to bind and activate cdks (Yang and Kornbluth 1999). Not all cyclins and cdks function in regulating the cell cycle, however. Other functions identified for cyclins and cdks include regulation of transcription, DNA repair, differentiation, and apoptosis. In addition to cyclin binding, other levels of regulation exist for controlling cdk activity during the cell cycle. The phosphorylation of cdk subunits can regulate the kinase activity either positively or negatively. Further, the ubiquitin-mediated proteolysis plays a crucial role in cell cycle control by targeting cyclins and other regulators for destruction at key points during the cell cycle. This provides a strong irreversible directionality to the cell cycle, by eliminating certain factors. Additionally, other proteins have been found whose association leads to the inhibition of cdk activity, the cdk inhibitor proteins (CKI). Two separate families of CKI proteins have been found, the p21 family and the INK4 (inhibitors of cdk4) family. Members of the p21 family (p21, p27, p57) can act on most cyclin/cdk complexes. The other family of CKI, the INK4 family (p16, p15, p18, p19), specifically interacts with cdk4 and cdk6. This

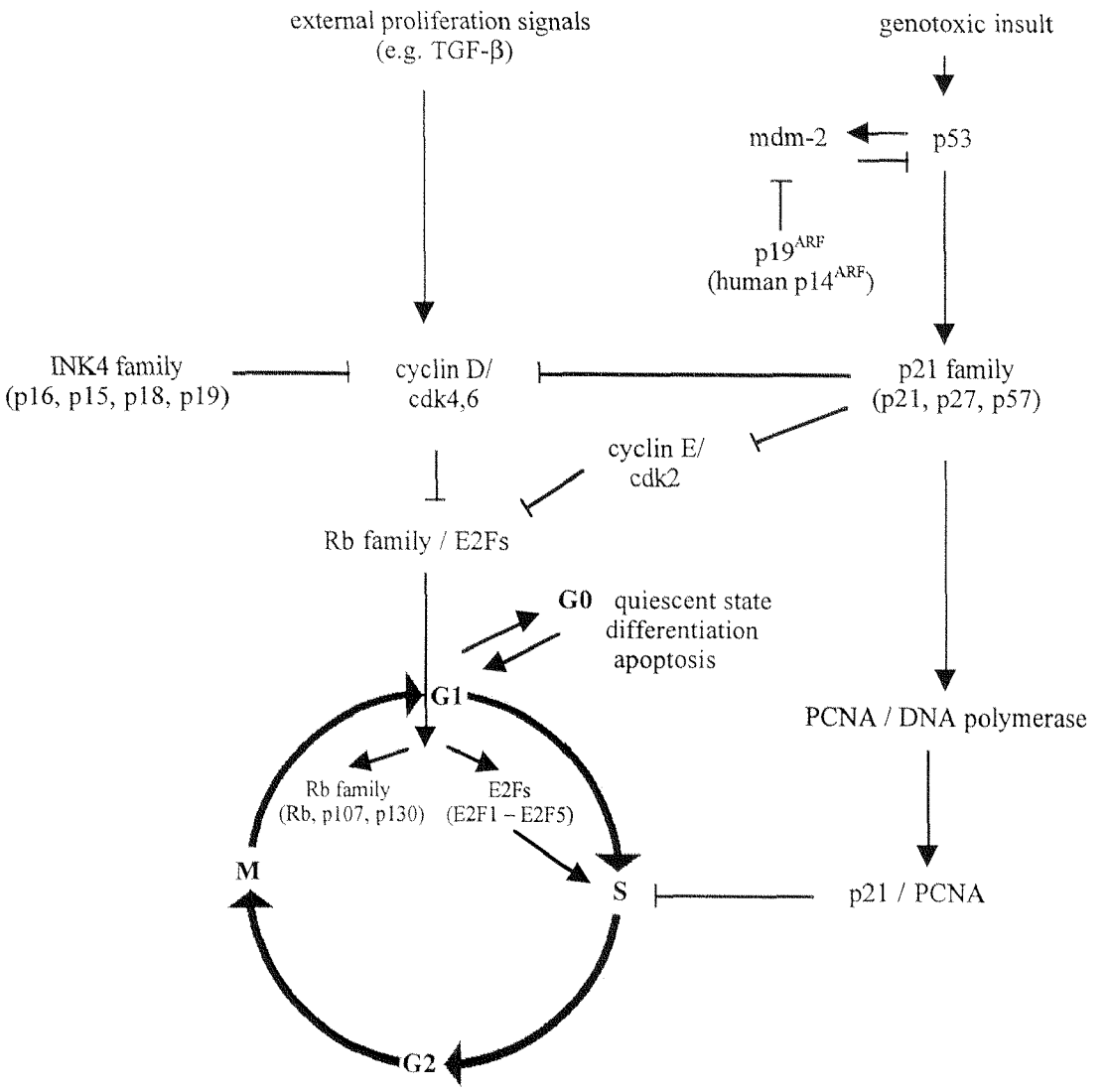


Figure 1. Major proteins involved in cell cycle progression and regulation. Many of them are found to be mutated or deregulated in cancer, leading to an uncontrolled cell cycle progression.

binding prevents the association with cyclin D (Elledge and Harper 1994; Hunter and Pines 1994). The INK4 family members have been found to be inactivated by mutation, deletion, or methylation in human tumors (Sharpless and DePinho 1999). Further, cyclin D1 gene amplification was found in several different carcinomas (Sato, et al. 1999; Shinozaki, et al. 1996), and is implicated in a translocation associated with B-cell lymphoma (Nakamura, et al. 1997).

The expression/activation of cyclin D/cdk4,6 complex, triggered by the presence of mitotic growth factors, controls the reentry of resting G0 cells into the G1 phase of cell cycle. The transcription of genes necessary for S phase, for example, is regulated by cyclin D/cdk4,6 dependent phosphorylation of the Rb protein family (Rb, p107, p130) (see Fig. 1). This phosphorylation results in the dissociation of E2F from Rb, leading to the expression of E2F-regulated genes (e.g. cyclin E, cyclin A, cdk1, thymidine kinase, c-myc). The E2F family consists of five related transcription factors with some differences in biochemical properties. E2F1, E2F2, and E2F3 bind preferentially Rb, whereas E2F4 and E2F5 predominantly associate with p107 and p130. It is likely that the different E2Fs have different functions or are used to different extents in specific tissues. Through the activation of E2F, cyclin E is the next cyclin to be induced during the progression of cells through the G1 phase. Cyclin E associates with cdk2, and this kinase complex is required for the G1/S transition of cells. Cyclin E/cdk2 complex participates in maintaining Rb in the hyperphosphorylated state and thus takes part in a positive feedback loop for the accumulation of active E2F. Therefore, the absence of cyclin E and/or the inhibition of the cyclin E/cdks complex by p21 family members or p53 will cause the cell cycle to be arrested at the G1/S checkpoint. Further, viral-transforming proteins (e.g. SV40 large T antigen) have been shown to inactivate both Rb and p53, thus leading to inappropriate cell proliferation and tumor formation (Cowell 1990). Additionally, it is known that overexpression of cyclin E correlates with advanced grades and stages of tumors, explained by the fact that constitutive cyclin E overexpression results in chromosomal instability (Spruck, et al. 1999). This indicates that downregulation of cyclin E/cdk2 kinase activity following the G1/S phase transition may be necessary for the maintenance of karyotypic stability.

Although very few inactivations of p21 are found in human cancers, p21 is implicated in tumorigenesis through its regulation by p53. p21 can additionally inhibit cell cycle progression by inhibiting DNA synthesis through proliferating cell nuclear antigen (PCNA) binding. The binding of p21 inhibits the ability of PCNA to function in DNA replication but not in DNA repair. As shown in figure 1, in tumors containing amplified mdm-2 genes, and wild type p53 genes, normal p53 proteins are expressed. However, normal p53 function is effectively inhibited by its association with overexpressed mdm-2 protein. Further, mdm-2 expression itself can be blocked by $p19^{ARF}$ (called $p14^{ARF}$ in humans) (Pomerantz, et al. 1998; Sherr 1998; Zhang, et al. 1998), and finally mutations in $p19^{ARF}$ block p53 function. The fate of the G1 arrested cells depends on the presence or absence of cyclin A, and only in the absence of cyclin A can the cells return to G0 and redifferentiate. Otherwise, cells die via an apoptotic pathway. The DNA replication in S phase and the transition to the G2 phase is regulated by the activation of cyclin A/cdk2 complex and PCNA. The main regulator of the G2/M transition is the cyclin B/cdk1 complex. Any perturbation of these regulators will result in arrest at G2/M. Cells arrested at G2/M checkpoint lack the ability to redifferentiate and die via apoptosis (Johnson and Walker 1999).

3.1.5 Pediatric tumors

Most neoplasms can be divided into three major groups: sarcomas, carcinomas, and leukemias/lymphomas. Sarcomas and leukemias/lymphomas are malignant tumors of mesenchymal origin. The word "sarcoma" was derived from the Greek word meaning "fleshy tumor", and " carcinoma" from the Greek word meaning "crab-like tumor". Although many soft tissue sarcomas are well described by the Greek term, some are firm and fibrous, and others are markedly vascular. Carcinomas arise from epithelial cells of either endodermal or ectodermal origin. Finally, the last group, the leukemias and lymphomas, develop from blood cells, and from cells of the lymphoid system respectively.

That cancer in children differs markedly in many ways from cancer in adults is well-known. Of major importance in any consideration of childhood cancer are age incidence and histologic type of tumors. Figure 2 shows the proportional difference in incidence between childhood and adult cancers, whereby an average was taken for the pediatric cancers, whose percentage changes according to age.

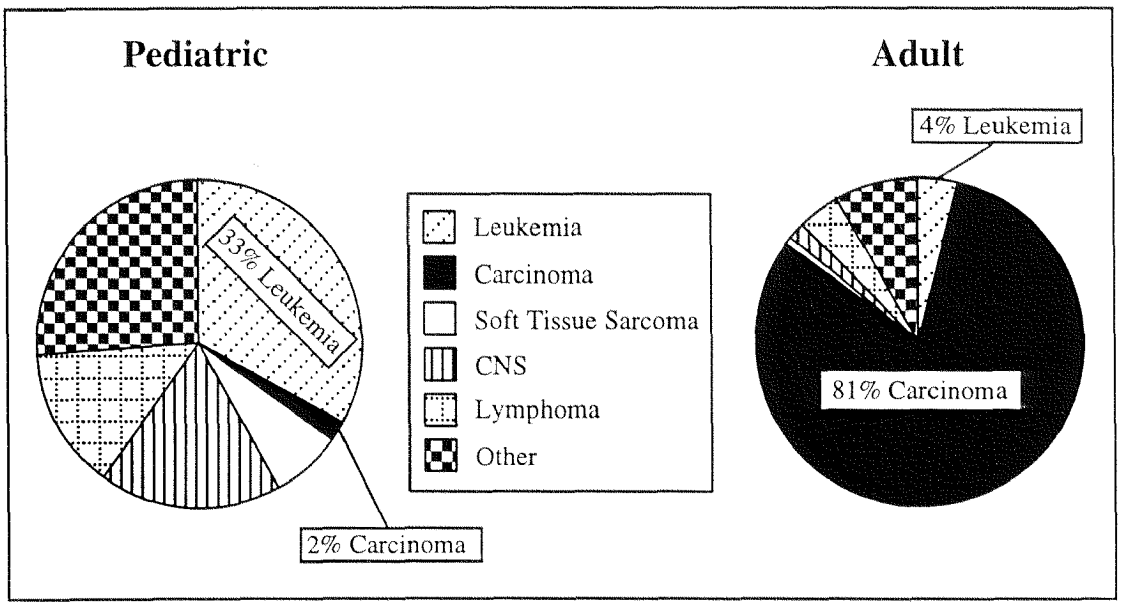


Figure 2. Pediatric versus Adult Cancer. The relative proportion of tumor types in children and adults is depicted. Indicated is the most frequent tumor of each group.

Perhaps the most striking difference between childhood and adult tumors is the over 80% occurrence of carcinomas in adults, compared to the less than 6% carcinoma in children. Furthermore in children, leukemias account for a third of all cancers compared to only 4% of leukemias found in adults. Finally, soft tissue sarcomas are ten times more frequent in children (7% of all tumors) than in adults. The incidence of CNS tumors and lymphomas is higher in children with 18% versus 14% compared to 1.7%

versus 4.4% in adults. Other tumors still account for 26% in children and 8.2% in adults, demonstrating a greater diversity in the type of tumors in children as compared to adults (Triche 1997).

Thus, there is a large disproportion in the occurrence of the major types of cancer between children and adults. The major tumor forms in children likely represent an acquired intrauterine genetic abnormality or, less commonly, an inherited genetic predisposition. For example, a clear case of intrauterine tumorigenesis is retinoblastoma with approximately 90% of sporadic and 10% of inherited tumors. Leukemias represent a large group of different tumors with a multitude of translocations (for review see (Rabbitts 1994; Sanchez-Garcia 1997)). Gross-scale alterations in genes are more relevant for tumorigenesis in leukemia and lymphoma than in any other case. For example, translocation in T-cell acute leukemias involve three major categories of DNA-binding transcription factor proteins (basic helix-loop-helix, LIM, and homeobox). In all cases, however, a T-cell receptor gene is implicated in the activation of these various transcription factors. Although leukemias represent the largest proportion of childhood cancers, they will only be briefly mentioned here, and a few specific translocations and their actions are described later on.

Soft tissue tumors

Over 50% of the human body weight is soft somatic tissue and can be subject to a wide variety of tumors. Pediatric soft tissue sarcomas are a group of tumors that originate from primitive mesenchymal tissue and account for 7% of all childhood tumors (Pappo and Pratt 1997). Rhabdomyosarcoma, tumors of striated muscles, accounts for more than one half of all cases of soft tissue sarcomas in children. The remaining non-rhabdomyosarcomatous soft tissue sarcomas account for approximately 3% of all childhood tumors. Although they can develop in any part of the body, non-rhabdomyosarcomatous soft tissue sarcomas arise most commonly in the trunk and extremities and are much more common in adults than in children.

Classification of soft tissue tumors

There are over 50 different varieties of tumors and tumor-like growth of soft somatic tissues. Unfortunately, despite considerable progress over the years, there is still no universally accepted classification system for soft tissue sarcomas. Various systems are constantly being refined and improved. There are at least 34 different malignant variants, and almost yearly there are new additions to this family of tumors (Dei Tos and Dal Cin 1997; Enzinger and Weiss 1995; Sreekantaiah, et al. 1994). The histologic classification is clearly a major advance toward uniform evaluation of these tumors. However, there are some major practical problems: some sarcomas have multiple cell types present in different areas of the tumor, and many tumors are so undifferentiated that it is close to impossible to subclassify them into their histogenic type. Therefore, molecular markers will be very important in the future (e.g. RT-PCR, array screening).

Each of the histological categories is divided into a benign and malignant group, but this subdivision does not imply that malignant soft tissue tumors tend to originate from their benign counterparts. In fact, malignant transformation of benign soft tissue tumors is an extremely rare event, with the exception of the occasional transformation of neurofibroma to malignant schwannoma. The various tumor types are named according to the histological type of the predominant cellular type, e.g. the resemblance of the tumor to normal tissue or its embryonal counterpart. Table 2 represents a classification of the most common soft tissue sarcomas. The known tumor specific gene alterations are also listed and might be of great help to classify the tumors correctly. The search for additional tumor specific markers is far from complete and there is a tremendous need for those markers.

Table 2: Classification of Soft Tissue Sarcomas

Type of tissue	Malignant tumors		Characteristic cytogenetic events	Fusion gene
striated muscle	rhabdomyosarcoma:	embryonal		
		alveolar	t(1;13)(p36;q14) t(2;13)(q35;q14)	PAX7/FKHR PAX3/FKHR
smooth muscle	leiomyosarcoma		t(12;14), del(1p)	
fibrous tissue	dermatofibrosarcoma protuberans		t(17;22)(q22;q13)	COL1A1/PDGFB
	infantile fibrosarcoma		+8, +11, +17, +20	
fibrohistiocytic tumors	malignant fibrous histiocytoma		1q11-12, der(19p)	
adipose tissue	liposarcoma:	myxoid & round cell	t(12;16)(q13;p11) t(12;22)(q13;q12)	FUS/CHOP EWS/CHOP
		pleomorphic	hyperdiploid, complex	
		well differentiated	ring chromosome 12	
blood and lymph vessels	lymphangiosarcoma			
	angiosarcoma			
	Kaposi's idiopathic sarcoma			
	granulation cell sarcoma			
	hemangiopericytoma		t(12;19)(q13;q13.3) t(13;22)(q22;q13.3)	
synovial mesothelium	synovial sarcoma (malignant synovioma)		t(X;18)(p11.2;q11.2)	SYT/SSX1 or SYT/SSX2
	epithelioid sarcoma			
peripheral nervous system	malignant schwannoma			
	malignant neuroepithelioma		t(11;22)(q23-24;q11-12)	
	malignant neurilemoma			
heterotopic bone and cartilage	extraskeletal osteosarcoma			
	extraskeletal chondrosarcoma:	myxoid	t(9;22)(q22;q12) t(9;17)(q22;q11)	EWS/CHN RBP56/CHN
		well-diff.		
	extraosseous Ewing's sarcoma/PNET		t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(17;22)(q12;q12) t(2;22)(q33;q12)	EWS/FLI-1 EWS/ERG EWS/ETV1 EWS/E1AF EWS/FEV
undifferentiated mesenchyme	malignant mesenchymoma			
	myxoma			
unknown histogenesis	desmoplastic small round cell tumor		t(11;22)(p13;q12)	EWS/WT1
	alveolar soft parts sarcoma		17q25 abnormalities	
	malignant granular cell myoblastoma			
	epithelioid sarcoma			
	malignant extrarenal rhabdoid sarcoma			
	clear cell sarcoma (melanoma of soft parts)		t(12;22)(q13;q12)	EWS /ATF1

Rhabdomyosarcoma

Rhabdomyosarcoma (RMS), a solid tumor of skeletal muscle origin, is the most common soft tissue sarcoma occurring in children, accounting for 4-8% of all childhood cancers (Pappo, et al. 1999). RMS is most frequently found in children aged between 1-4 years, with over 60% of cases manifesting before age 10. Most RMS occur sporadically and are of unknown cause. However, RMS can occur as part of a pattern of familial cancer. This might include the Li-Fraumeni syndrome (Li and Fraumeni 1969) and the Beckwith-Wiedemann syndrome (Mannens, et al. 1996), which shows a higher incidence of RMS than other neoplasms.

RMS may develop at any site in the body. Figure 3 A (taken from (Arndt and Crist 1999)) shows the primary tumor's anatomic location in percentage of the 2747 RMS cases analyzed (Pappo, et al. 1995). The highest rate is found in the genitourinary tract with 24% of the cases, followed by 19% in the limbs, 16% in the paramenigeal area, 10% in the head and neck region, and finally 22% tumors in other areas. The typical histologic appearances of the two most frequent RMS types are shown in figure 3 B and C. Precise determination of the primary site and the extent of disease at presentation is crucial, because these features are strongly associated with prognosis and the optimal approach to treatment varies according to these features. The therapy of this tumor made substantial progress in the last 30 years, resulting in an increase in the five-year survival rates from about 25% in 1960 to 75% in 1995. This is largely due to the Intergroup Rhabdomyosarcoma Study Group, which was formed in 1972 and conducted four sequential studies between 1972 and 1998 that included more than 3000 patients.

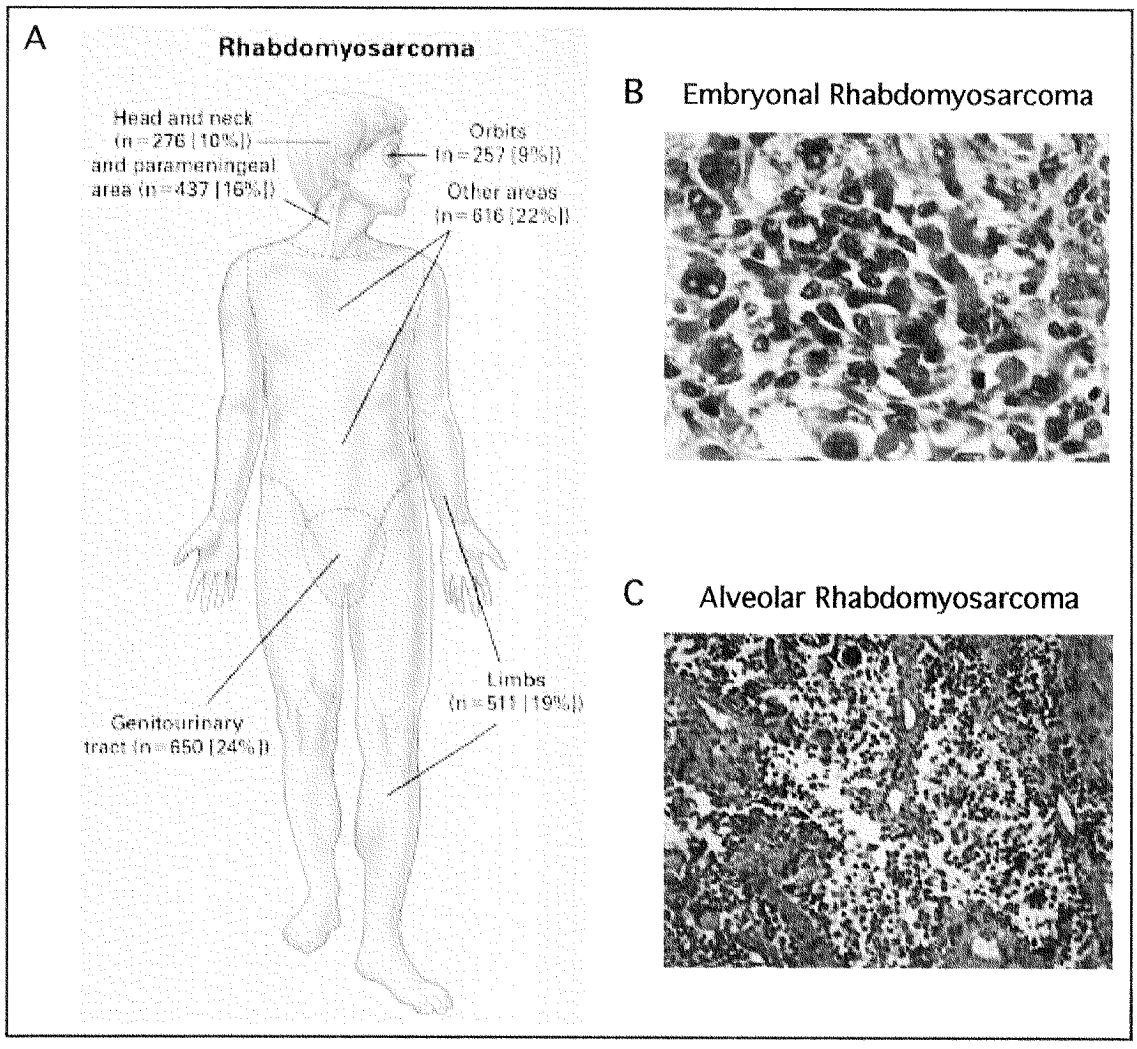


Figure 3. Localization and Histology of RMS. A) The primary sites of RMS are shown in numbers and percentage of cases. B) The histologic appearance of embryonal RMS. This spindle cell tumor is composed of small, elongated cells with plump pink cytoplasm in a loose stromal background. C) A classical view of an alveolar RMS, with neoplastic cells lining and lying freely within the alveolar spaces. There are multinucleated malignant giant cells.

Most RMS share a pattern of expression of muscle-specific genes like normal fetal skeletal muscle, including the MyoD family of muscle-specific regulatory factors (Dias, et al. 1990). Expression of MyoD (called Myf3 in humans) is generally accepted to represent a commitment of the cells to the myogenic lineage and thus serves as a useful marker for identifying skeletal muscle precursor cells. The resemblance of

rhabdomyosarcoma to normal fetal skeletal muscles prior to innervation is the basis for the classification. The tumors vary widely in histological appearance, depending on the degree of cellular differentiation, extent of cellularity, and growth pattern (Enzinger and Weiss 1995). They are generally classified into four histological categories: embryonal, botryoid, alveolar, and pleomorphic RMS (Asmar, et al. 1994; Newton, et al. 1995).

Embryonal RMS (eRMS)

The most common type is the eRMS (shown in Fig. 3 B), which accounts for 50-60% of all cases, and has a favorable prognosis. eRMS is most frequently seen in the head and neck, and genitourinary regions. Tumor cells are most frequently aneuploid (1.1 to 1.8x normal DNA content) (Pappo, et al. 1993). eRMS has no tumor specific translocations, but generally has a loss of heterozygosity (LOH) at chromosome 11p15.5, suggesting an inactivation of an as yet unidentified tumor suppressor gene (Scrable, et al. 1987). Other mutations or amplifications of tumor suppressor genes (e.g. p53, N-ras, K-ras, and N-myc) are frequently observed and may be involved in the pathogenesis of RMS or may represent secondary events related to progression of the tumors (Felix, et al. 1992; Kouraklis, et al. 1999; Stratton, et al. 1989). It is known that most eRMS have deregulated expression of PAX3 and/or PAX7 (Schäfer, et al. 1994), which is also true for the botryoid subtype.

Botryoid RMS

Accounting for about 6% of all RMS, botryoid RMS represents a morphological variant of eRMS with usually the best prognosis of all RMS and is almost exclusively found in children under 4 years of age in the genitourinary area. Only this subtype has a characteristic gross appearance with its grapelike clusters of tumor arising from a mucosal-lined area.

Alveolar RMS (aRMS)

This extremely malignant subgroup of RMS represents about 16-20% of all RMS cases afflicting mostly adolescents and young adults. aRMS is composed largely of poorly differentiated round or polygonal tumor cells that frequently show central loss of cellular cohesion and formation of irregular „alveolar" spaces, simulating cells of the respiratory alveolus (example shown in Fig. 3 C). They are most frequently found in the extremities or the trunk. These tumors are commonly near tetraploid (2x normal DNA content). Chromosomal studies on aRMS identified two alternative translocations. These translocations juxtapose the transcription factors PAX3 or PAX7 with the forkhead related gene (FKHR) to generate PAX3/FKHR and PAX7/FKHR chimeric genes, which encode fusion proteins (Barr 1997). Almost all aRMS have one of these specific translocations. The more prevalent t(2;13)(q35;q14) translocation, encoding PAX3/FKHR, was detected in 60-70% of the aRMS (Barr, et al. 1993; Barr, et al. 1998; Galili, et al. 1993; Shapiro, et al. 1993). The variant translocation t(1;13)(p36;q14), encoding PAX7/FKHR, is found in 15-30% of aRMS (Biegel, et al. 1991; Davis, et al. 1994). Both translocations appear to be highly specific markers for aRMS and have not been associated with any other tumor type.

Pleomorphic RMS

The pleomorphic RMS represents the rarest type of RMS, about 1-3% of the cases, and is most often found in the extremities or the trunk. Usually, it is not found in children, but occurs in patients over 30 years of age. Pleomorphic RMS can be multicentric and arise at many sites within the same muscle group.

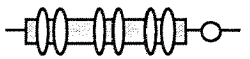
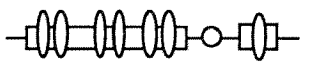
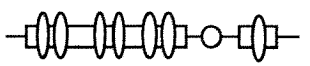
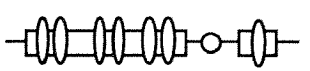
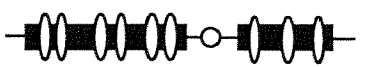
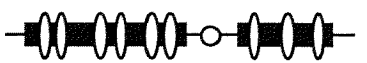
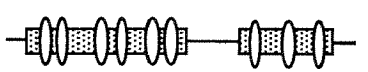
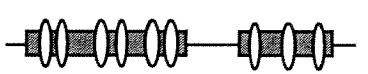
A recently published excellent review by Merlino & Helman summarizes all the possible pathways implicated in RMS formation (Merlino and Helman 1999).

3.2 PAX

The nine members of the human PAX gene family encode a group of transcription factors that play fundamental roles in embryonic development. They are expressed in a highly specific spatial and temporal pattern (general reviews for PAX proteins (Chalepakis, et al. 1993; Dahl, et al. 1997; Mansouri, et al. 1996)). PAX proteins are grouped in four subfamilies. All PAX proteins share a common DNA binding domain of 128 amino acids, called a paired domain (PD), originally identified in the drosophila segmentation genes (paired, gooseberry-distal, and gooseberry-proximal) (Bopp, et al. 1986; Noll 1993). Selected members also contain an additional DNA binding domain, the so-called homeodomain (HD), which is in some members truncated. Additionally, members of the classes II and III have a conserved octapeptide (OP) (see Table 3).

Loss-of-function mutations in these genes have been associated with several human genetic diseases, including PAX3 in Waardenburg syndrome I & III, PAX2 in renal-coloboma syndrome, and PAX6 in aniridia. Several mouse natural mutants are known for PAX1, PAX2, PAX3, and PAX6. Their phenotype could partially be confirmed by the corresponding knock out mice (KO). KO animals have been generated for all Pax members, except for Pax3, for which only several different natural mouse mutants, called splotch (Sp), are available (Epstein, et al. 1991). In general, Pax -/- mice are not viable due to the lack of those organs or cell populations where the corresponding Pax protein is normally expressed (see table 2) (Mansouri, et al. 1998; Mansouri, et al. 1996; Peters, et al. 1998; Sosa-Pineda, et al. 1997; St-Onge, et al. 1997; Torres, et al. 1996; Urbanek, et al. 1994; Wilm, et al. 1998).

Table 3: PAX genes: Structure, localization, expression, association with mouse mutants, human syndromes and cancer

Class	Gene	Gene structure PD OP HD	Chromosomal localization[a]	Sites of expression[b]	Mouse mutants	Human syndromes	Tumors
I	PAX1		20p11.2	sclerotome, thymus	undulated (un) KO-skeletal abnormalities		
	PAX9		14q12-q13	sclerotome, pharyngeal pouches	KO-lacking pharynigeal pouch derivatives and teeth		
II	PAX2		10q24.3-q25.1	kidney, urogenital CNS, eye, inner ear	Krd KO-lacking kidney	renal-coloboma syndrome	Wilms' tumor, renal cell carcinoma
	PAX5		9p13	pro B-cell, CNS	KO-lacking B-cells, brain defects		lymphoma, glio-blastoma, transitional cell carcinoma
	PAX8		2q12-q14	kidney, CNS, follicular cells of the thyroid gland	KO-lacking C-cells of the thyroid		Wilms' tumor, thyroid cancer
III	PAX3		2q35	dermomyotome, neural crest, CNS	Splotch (Sp)	Waardenburg syndrome I & III	RMS, Ewing's sarcoma, melanoma
	PAX7		1p36	dermomyotome, CNS	KO-neural crest defects		RMS Ewing's sarcoma
IV	PAX4		7q22-qter	Pancreas	KO-lacking pancreatic β-cells		
	PAX6		11p13	eye, pancreas, CNS	small eye (sey) KO-lacking pancreatic α-cells	aniridia	

[a] human chromosomal localization, [b] only most prominent sites of expression are indicated, ⬭ helix structure, PD paired domain, OP octapeptide, HD homeodomain KO knockout mice

3.2.1 PAX genes and cancer

It has been shown that some PAX genes are able to transform mouse fibroblasts in culture, leading to foci formation and tumor growth in nude mice (Maulbecker and Gruss 1993). Further, aberrant PAX gene expression has been associated with several human tumors, where they are expressed or involved in translocations. Until now, only the members of class II and class III PAX genes have been associated with neoplasms (see Table 3), no occurrence of any anomaly of class I and class IV PAX genes has been reported in neoplasms (for review see (Schäfer 1998)).

Class II PAX genes involved in cancer

Normally PAX2 and PAX8 are expressed at different stages during renal morphogenesis, but are absent in differentiated tissues. In contrast they are expressed in Wilms' tumors (Dressler 1996; Dressler and Douglass 1992; Poleev, et al. 1992). Both PAX genes are known to have different splice variants. Analysis of Wilms' tumors showed that several alternatively spliced transcripts of both PAX genes are present, but no correlation of any of the splice variants with tumorigenesis could be found (own investigations). Additionally, PAX2 expression was also found in renal cell carcinomas (Gnarra and Dressler 1995), and PAX8 is expressed in thyroid cancers (Fabbro, et al. 1994). The third class II PAX gene, PAX5, has been implicated in several tumors. PAX5 expression has been recently correlated with poorly differentiated tumors of the bladder (transitional cell carcinoma) (Adshead, et al. 1999). Similarly, increased expression of PAX5 has been associated with higher grade of malignancy in brain tumors (Stuart, et al. 1995), but so far no further publications have either confirmed PAX5 expression or the association with malignancies. Additionally, PAX5 was shown to be involved in two specific chromosomal translocations, first the t(9;14)(p13;q32) found in about 2% of all non-Hodgkin lymphomas. Thereby, PAX5 is fused to the

immunoglobulin heavy chain (IgH) locus, bringing the potent Eμ enhancer close to the PAX5 gene (Busslinger, et al. 1996; Iida, et al. 1996). Second, PAX5 is involved in a complex t(2;9;14)(p12;p13;q32) translocation present in a closely related non-Hodgkin's lymphoma referred to as splenic marginal zone lymphoma. This translocation links a switch-Sμ promoter of the IgH locus to the exon 1B of the PAX5 gene (Morrison, et al. 1998).

A possible mechanisms by which class II PAX genes might contribute to tumor development is by inhibition of p53 dependent transactivation. All three PAX proteins were shown to bind directly to an untranslated site within the first exon of p53 (Stuart, et al. 1995). Furthermore, the Wilms' tumor suppressor gene WT1 has been shown to be a direct target of PAX2 as well as PAX8 (Dehbi and Pelletier 1996; McConnell, et al. 1997). Additional PAX8 targets might be the cell adhesion molecule N-CAM, and proto-oncogene bcl-2 (Hewitt, et al. 1997; Holst, et al. 1997).

Recently, it has been shown that the partial homeodomain of PAX5 interacts with the TATA-binding protein (TBP), the DNA binding subunit of the general transcription complex TFIID, and the retinoblastoma (Rb) protein. Thereby, PAX5 is linked through TBP to the basal transcription machinery, and its activity can be controlled by the cell cycle-regulated association with Rb (Eberhard and Busslinger 1999). An equal interaction was shown for PAX6 with TBP and Rb (Cvekl, et al. 1999).

Class III PAX genes involved in cancer

Aberrant expression of this class of PAX genes was thought to be specific for RMS, either by its overexpression or by its specific translocation with the transcription factor FKHR in aRMS (Barr 1999). Both fusion proteins, PAX3/FKHR and PAX7/FKHR, have been previously described in aRMS. More recently, deregulated PAX3 and/or PAX7 expression was found in Ewing's sarcoma in the absence of the specific translocations (Schulte, et al. 1997). In this report PAX3 expression was further found

in a peripheral neuroectodermal tumor and a glioblastoma cell line. Finally, we could demonstrate an aberrant expression of PAX3 in melanomas (see section publications/manuscripts).

Recently, interaction of hDaxx with PAX3, PAX7, and PAX3/FKHR, but not with PAX4, was shown. Only the transcriptional activity of PAX3, not PAX3/FKHR, is repressed through hDaxx interaction (Hollenbach, et al. 1999). Further, it was shown that the OP and HD are necessary for the interaction. As shown in table 3 only class II and class III PAX genes have these elements. Therefore, it might be possible that the inhibition of transcriptional activity of the class II and class III PAX genes is missing in tumors, leading to an inappropriate transcription of target genes.

A known direct target of PAX3 is the tyrosine kinase receptor c-met, which might also be activated by PAX7 (Epstein, et al. 1996). Further, the association of Rb with the previously mentioned PAX5 and PAX6 proteins was also demonstrated for PAX3 (Wiggan, et al. 1997).

To gain more insight into the role of these class III PAX genes in tumors, we developed an antisense oligonucleotide-based strategy to specifically downregulate either PAX3 and PAX3/FKHR, or PAX7 expression in tumor cells. Antisense treatment resulted in a substantial increase of apoptosis in RMS cell lines (Bernasconi, et al. 1996) as well as in melanoma cell lines (see publications/manuscripts). PAX3 and PAX7 seem therefore to play a protective role in cells, probably by stimulating specific survival pathways.

How the PAX genes are (re)expressed in cancer is not solved and might bring some insight into the molecular mechanisms leading to neoplasia.

3.3 LIM domain proteins

All proteins having a LIM domain belong to the family of LIM domain proteins. This protein family is present in mammals, amphibians, flies, worms, yeasts and plants. The LIM domain is a cysteine-rich sequence motif composed of two specialized zinc fingers that is found in a variety of proteins with diverse functions and subcellular distributions. This family of proteins includes transcription factors, proto-oncogene products, and components of adhesion plaques and the actin-based cytoskeleton. The LIM domain proteins show a high homology among species. The primary role of LIM domains appears to be protein/protein interactions (Dawid, et al. 1998; Sanchez-Garcia and Rabbitts 1994). LIM domains interact with other LIM domains either by forming homodimers or heterodimers (Feuerstein, et al. 1994; Schmeichel and Beckerle 1994). Furthermore, interactions between LIM domains and other protein dimerization domains are known to occur (Valge-Archer, et al. 1994). Over 62 human proteins have been identified so far that display one to five copies of the LIM motif. A search of the non-redundant sequence database (NRDB) at the end of July 1999 resulted in a list of 224 proteins containing a LIM domain.

3.3.1 Definition and structure of the LIM motif

The LIM motif was first identified by the alignment of the protein sequences of three developmentally important transcription factors, Lin-11 (C. elegans) (Freyd, et al. 1990), Isl-1 (rat) (Karlsson, et al. 1990), and Mec-3 (C. elegans) (Way and Chalfie 1988). The acronym was derived from these three proteins. The consensus pattern is defined as C-XX-C-X_{17-19}-H-XX-C-XX-C-XX-C-X_{16-20}-C-XX-(C/H/D) (Dawid, et al. 1995). The LIM domain consists of two loops, each co-ordinating a zinc ion with four residues. Both zinc fingers are invariably separated by two residues (Figure 4).

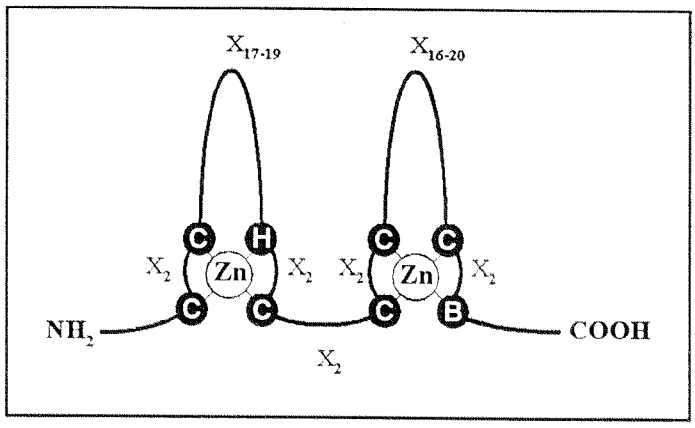


Figure 4. Structure of the LIM domain. The amino acid indicated with B can be either a C, H, or D.

Although the global structure of the LIM domain is unique, the tertiary folding of its C-terminal zinc-binding module bears a striking similarity to that of the DNA-binding zinc fingers of the class I nuclear receptor and GATA-1 transcription factors (Pérez-Alvarado, et al. 1994; Sanchez-Garcia and Rabbitts 1994). However, until now there is no published experimental evidence demonstrating a direct interaction between a LIM domain and either DNA or RNA.

3.3.2 Classification of LIM domain proteins

Several classification systems have been proposed, mainly based on the type of domains present beside the LIM sequence. Based on the sequence similarities among LIM domains and on the overall structure of the protein, they can be divided into the following classes as shown in figure 5:

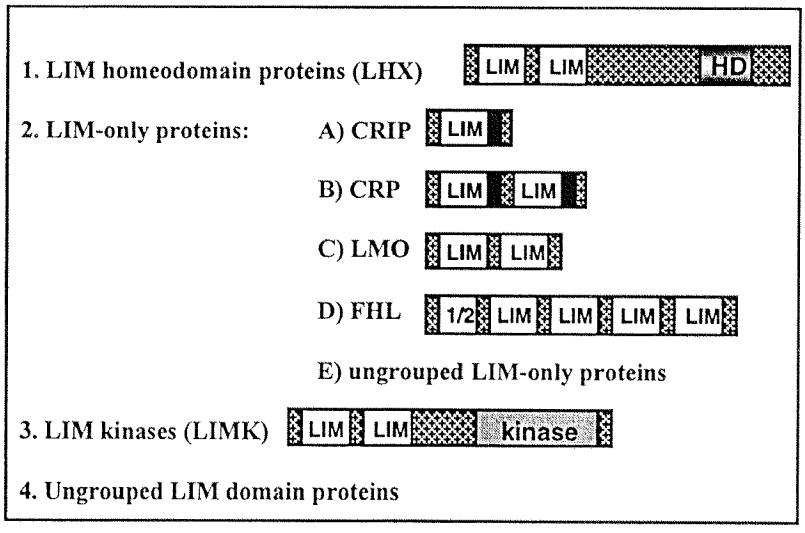


Figure 5. Classification of LIM domain proteins. LIM domains in LHX proteins, and in LMO and LIMK, are related by sequence, whereas sequence similarity between LHX and LMO proteins are greater. Group 2A and B proteins have related LIM domains and contain only a short additional conserved region (black box).

LHX

The first class, the LIM homeodomain proteins (LHX), consists always of two LIM domains and a homeodomain (HD). Until now nine LHX proteins have been identified, all of which have a nuclear localization. They are involved in the control of cell lineage determination and pattern formation during development. In the current view, the LIM domains of the LHX proteins function as a negative regulatory domain by inhibiting the DNA binding mediated by the homeodomain. After binding of cofactors, transcription is activated, and synergism with other transcription factors is promoted. Indeed, cofactors have been isolated, namely NLI/Ldb1/CLIM2 and Ldb2/CLIM1, which until now have been found to interact solely with nuclear LIM proteins, namely LHX proteins and LMO proteins to potentiate transactivation of downstream genes (Agulnick, et al. 1996; Dawid 1998; Jurata, et al. 1996). These cofactors probably confer time and space specificity to the regulatory action of LHX proteins.

LIM-only proteins

The second class, the LIM-only proteins, consists nearly exclusively of LIM domains. They can be further subdivided into five different groups as shown in figure 5. To avoid confusion in the terminology, it needs to be mentioned that LMO is not an abbreviation for LIM-only protein, but it is a name given to certain proteins composed of two LIM domains (2C), which were previously known as rhombotins or TTGs. LMO will be described more precisely later. The first subgroup (2A) of LIM-only proteins consists of CRIP (cyteine-rich intestinal) proteins. CRIP proteins have only a single LIM domain followed by a short additional conserved glycine-rich region (Garcia-Barcelo, et al. 1998). This short additional region is also found in the second subgroup of LIM-only proteins, the CRPs (cyteine-rich proteins) (2B). Three members are known: CRP1, CRP2/ESP1, and CRP3/MLP (Louis, et al. 1997). CRP2 was identified by a subtractive hybridization screen between normal and transformed cells. Subsequent studies revealed that CRP2 mRNA is undetectable in a variety of oncogenically and chemically transformed cells (Weiskirchen, et al. 1995) suggesting a role in tumorigenesis. CRP proteins are found in the nucleus as well as in the cytoplasm. They are known to distribute along the actin cytoskeleton and at integrin-rich sites of adhesion in cultured cells (Arber, et al. 1994; Sadler, et al. 1992). CRPs have also been implicated in promoting muscle differentiation. The crucial involvement of CRPs in myogenic differentiation has been confirmed by CRP3-/- mice. The postnatal lethality of CRP3-/- mice is caused by heart failure that is associated with disorganization of cardiac muscle myofibrils (Arber, et al. 1997). The fourth subgroup of LIM-only proteins (2D) consists of proteins with four and a half LIM domains (FHL). The proteins belonging to this family have just recently been discovered (see section 3.3.2.6). Finally, all other LIM-only proteins belong to the "ungrouped" subclass (2E).

LIMK

The third class of LIM proteins function as kinases and are grouped into the LIM kinases (LIMK) class. The proteins consist always of two LIM domains and a kinase

domain. Linkage between LIMK and human disease was demonstrated by fluorescence in situ hybridization (FISH) and PCR analysis strongly suggesting that LIMK1 hemizygosity in humans is a good candidate for the unexplained neurologic features of Williams syndrome (Frangiskakis, et al. 1996; Tassabehji, et al. 1996).

Ungrouped LIM domain proteins

Finally, the fourth class of LIM domain proteins, the "ungrouped LIM domain proteins" is a quite heterogeneous class with one to five LIM domains and additional protein sequences with an unknown motif. To this group belong the well investigated proteins zyxin, paxillin, and PINCH (Beckerle 1997).

LMO proteins

Today, a total of four LMO proteins are known, composed mainly of two LIM domains. The first LMO proteins, LMO1 and LMO2, have been discovered as putative oncogenes because of their association with chromosomal translocations in T-cell leukemias. The current molecular model proposes that transcription of target genes normally not involved in T-cells contribute to tumorigenesis. The transcription of these genes is induced by the LMO proteins, which are aberrantly expressed by specific chromosomal translocations (Rabbitts 1998).

LMO1

Several names exist for this protein, for example rhombotin 1 (RBTN1), rhombosine 1 (RHOM1), and T-cell translocation gene 1 (TTG1). LMO1 was identified by analyzing the translocation t(11;14)(p15;q11) found in a subset of childhood T-cell acute lymphoblastic leukemia (T-ALL) (Boehm, et al. 1990). The translocation involves the TcR-δ gene on 14q11 and LMO1 on 11p15, resulting in ectopic expression of LMO1, normally expressed in rhombomeres of the developing hindbrain.

LMO2

LOM2 is also known as rhombotin 2 (RBTN2), rhombotin-like 1 (RBTNL1), rhombosine 2 (RHOM2), and T-cell translocation gene 2 (TTG2). This protein was found by analyzing two translocation breakpoints of childhood T-ALL. Breaks at the small region of 11p13 occur in both types of translocation, t(11;14)(p13;q11) or t(7;11)(q35;p13) and involve either TcR-δ on 14q11 or TcR-β on 7q35 activating LMO2 expression. Although rearrangements involving the LMO2 gene occur in T-ALL and are specific for this subsets of tumors, LMO2 is not expressed in normal T-cells. The function of LMO2 has been well investigated and will be therefore more extensively mentioned. Usually, LMO2 is expressed in fetal liver and erythroid cell lines. LMO2 is one of several master regulators of erythroid precursor cell differentiation. The Ldb1/LMO2 complex was shown to maintain erythroid precursors in an immature state. Lmo2 knock out mice revealed that this protein is required for yolk sac erythropoiesis. Lmo2 -/- mice die around embryonic day 9-10 (Warren, et al. 1994). Experiments using Lmo2 -/- cells showed that Lmo2 is required at a point in hematopoiesis before bifurcation of myeloid and lymphoid precursors in adult mouse hematopoiesis. The similar phenotypes of the Lmo2, Tal1, and Gata-1 null mice suggest that these three genes are closely related, perhaps having synergistic roles in erythroid differentiation, whereby the Gata -/- mice is less affected, suggesting later implication of Gata-1 in erythopoiesis. Indeed, several studies showed direct interaction of Lmo2 protein with the basic helix-loop-helix protein Tal1/Scl, the GATA DNA-binding protein Gata-1, and NLI/Ldb1 protein. On the other hand Tal1 is known to interact with the E2A gene products E12 or E47. Furthermore, in vitro binding site selection (CASTing assays; cyclic amplification and selection of targets) led to the identification of a complex involving Lmo2 and also including Tal1, E47, Gata-1, and Ldb1 (Wadman, et al. 1997). This erythroid complex binds to a bipartite DNA motif consisting of E-box and GATA consensus sequences in which the Tal1-E47 component binds to the E-box and Gata-1 binds to the GATA-site. These data strongly support the idea that Lmo2 acts as a bridging molecule bringing together the different DNA binding factors in this erythroid

complex. An analogous DNA-binding complex has been identified in T-cells of Lmo-expressing transgenic mice. In this case, however, a novel complex is formed which recognizes a dual E-box motif, apparently via two bHLH dimers linked by Lmo2 and Ldb1 proteins. These target genes may well be different from those genes normally targeted by Lmo2 proteins in normal cells (Grütz, et al. 1998). This aberrant LMO2 action is thought to be also present in the T-ALL, where LMO2 is ectopically expressed. Therefore the bridging effect of Lmo2 and/or a sequestering effect seem to be important in the development of T-ALL. These molecular models might partially explain, although not sufficiently, previously obtained results. Several mice models were described overexpressing either Lmo1 or Lmo2. The long latency (over 6 months), leading to clonal T-ALL in the transgenic animals, indicates that additional mutations are necessary, either in oncogenes or tumor suppressor genes, before overt disease appears. Further, ectopic expression of Tal1 gene does not lead to leukemia development in mice, nor shows any perturbation of T-cell development. This is intriguing, because Tal1 was initially found by analyzing the T-ALL translocation t(1;14)(p34;q11). The Lmo2-Tal1 double transgenic mice model, which exhibits an accelerated tumor development, indicates a synergistic effect in tumor formation for these two proteins and is therefore well described by molecular models (Larson, et al. 1996). Interestingly, it was shown that the Drosophila Lmo gene homologue (Dlmo), when mutated, causes wing defects that can be modified by the dosage of drosophila gene Chip (equivalent to Ldb1), suggesting that Chip can work by titrating binding partners (Shoresh, et al. 1998).

So far, no data exist about any possible involvement of Ldb1 in tumors.

In summary, LMO2 expression is required in early hematopoiesis, but aberrant LMO2 expression at a latter time point leads to tumor formation by bridging and /or sequestering several DNA binding factors.

LMO3

LMO3 is also named rhombotin 3 (RBTN3), rhombosine 3 (RHOM3), and rhombotin-like 2 (RBTNL2). There is almost no information available on this gene. LMO3 is located on human chromosome 12p12-13 and is mainly expressed in the brain. Until now it has not been implicated in any translocation (Boehm, et al. 1991).

LMO4

LMO4 has been cloned by screening mouse embryonic lambda expression libraries (Kenny, et al. 1998). It is highly expressed in the T lymphocyte lineage, cranial neural crest cells, somite, dorsal limb bud mesenchyme, motor neurons, and Schwannoma cell progenitors. The function and its possible involvement in cancer are not known yet. LMO1, LMO2, and LMO4 were found to have distinct expression patterns in adult tissues. Therefore, they might have similar roles in their distinct tissues.

Four and a half LIM domain proteins (FHL)

Proteins consisting of four and a half LIM domains belong to this subgroup of LIM-only proteins. Chronologically it is the latest defined subgroup of LIM proteins with sizes between 31-35 kDa. Unfortunately, the proteins have several names. The latest attempt of renaming and numbering them as FHL proteins is confusing, because in the literature this abbreviation was already used for at least two other purposes, namely familial hemophagocytic lymphohistocytosis (FHL) or factor H-like protein 1 (FHL1). Therefore it would be better to call them FHLIM proteins, but they will be called FHL in this text according to the available literature. The precise role of these proteins is not known and further investigation is necessary. Furthermore, little is reported about their intracellular distribution.

DRAL

This 32 kDa LIM domain protein was first discovered by our group using a subtractive hybridization procedure between cultured myoblasts and a rhabdomyosarcoma cell line (see under publication/manuscript). DRAL expression is downregulated in the tumorigenic cell lines. It was the first cDNA present in the database, coding for four and a half LIM domains. The occurrence of a half LIM domain was new. Like the other FHL proteins, DRAL has now additional names, like FHL2 or SLIM3 (Chan, et al. 1998). Expression of DRAL is observed in several tissues but the main expression is found in heart. The human chromosomal localization is on 2q12-14. DRAL has an extremely high sequence homology between different species.

SLIM1

This protein, also called SLIM (skeletal LIM protein) or FHL1, is located on human chromosome Xq26-27.2, and has highest expression in skeletal muscle. SLIM1 mRNA was also found in heart, hindbrain, neural tube, and somites. The cardiac expression of SLIM1 was restricted to the proximal ascending aortic arch and atria, seen from embryonic day 8.5-11 on to adulthood (Brown, et al. 1999). SLIM1 is highly conserved between species. The genomic structure of the human SLIM1 was solved (Greene, et al. 1999). Wayne et al. showed that ectopic expression of HOX11 in NIH 3T3 mouse fibroblasts leads to upregulated SLIM1 levels (Greene, et al. 1998). Using a yeast two-hybrid system, the putative murine homologue of SLIM1 (KyoT), was found to interact with RBP-J, a transcription factor (Taniguchi, et al. 1998). KyoT was shown to encode at least two alternatively spliced transcripts, KyoT1 and KyoT2. KyoT1 encodes four and a half LIM domains and is the actual murine counterpart of SLIM1, KyoT2 lacks the two C-terminal LIM domains and has a frameshift in the last exon. This 27 new C-terminal amino acids mediate binding to RBP-J/RBP-J_K/Su(H) DNA binding protein, a mammalian homologue of suppressor of hairless (Su(H)). RBP-J interacts directly with the Notch receptor involved in cell lineage commitment, displacing RBP-J from DNA and thereby inhibiting transcription. Therefore, only KyoT2, not KyoT1, was shown to

interact physically with the RBP-J. Thereby, KyoT2 is a novel negative regulatory molecule, repressing the RBP-J mediated transcriptional activation by EBNA2 and Notch1 by competing with them for binding to RBP-J. Using stably transfected F9 embryonal carcinoma cells KyoT1 expression was detected in the cytoplasm and in the nucleus for KyoT2. An analogous, alternative spliced transcript has now been shown to occur in human, called SLIMMER, missing the last two C-terminal LIM domains (Brown, et al. 1999). Transfection experiments showed for SLIMMER a predominant nuclear localization. In contrast, SLIM1 localizes to the cytoplasm and associates with focal adhesion and actin filaments. Hence, it is the first time that a FHL protein is involved in transcriptional regulation.

SLIM2

This was the second FHL protein found with highest tissue expression in skeletal muscle. This protein is also called FHL3. SLIM2 is localized on chromosome 1p34.2-p32.3. SLIM1 and SLIM2 are differentially expressed in skeletal muscle (Morgan and Madgwick 1999). This was demonstrated by fusion of C2C12 mouse myoblasts in culture. There SLIM2 expression is temporarily downregulated during differentiation; in contrast, SLIM1 levels are low at beginning but are transiently upregulated during differentiation.

ACT

ACT (activator of CREM in testis) was identified by a yeast two hybrid screen a CREM (cAMP responsive element modulator) binding partner. Until now only the murine cDNA is cloned. ACT, as its name suggests, is expressed predominantly in testis and activates CREM. Immunohistochemistry localized ACT expression more precisely to round and elongated spermatids. Coexpression of CREM and ACT is found in a tissue- and developmentally regulated manner (Fimia, et al. 1999).

FHL4

This protein was discovered by sequence homology search for FHL proteins, and represents an alignment of several EST clones, extended by PCR. Until now, only the murine cDNA has been characterized. The highest expression of FHL4 is found in testis, like ACT. In situ hybridization localized its expression to the semiferous tubules. The observed variable intensity of FHL4 across these tubules suggests that it might be associated with the development and maturation of spermatozoa (Morgan and Madgwick 1999).

The knowledge of the FHL protein family is far from complete, indicating a need for further studies.

4. Subjects of investigation

The goal of this research project was to better understand the changes occurring upon neoplastic transformation in one given cell type and to identify potentially new and specific marker genes. We chose RMS, the most common pediatric soft tissue sarcoma, as a model system. To characterize the changes occurring upon neoplastic transformation on the molecular level, a subtractive hybridization procedure was performed previously between normal human myoblasts and the RMS cell line RD. 48 genes were initially found to be downregulated in RMS, whereby 40% of them were coding for unknown genes.

The first goal of this project was to saturate the screening of the subtractive cDNA library and to determine whether the differential expression pattern might be extrapolated to other RMS cells or even other neoplasms.

Further, one interesting clone of unknown sequence was selected based on a number of different criteria, among them induction by wt p53, and further characterized to gain more information on its possible role in normal and tumor cells.

To obtain more insight into the molecular basis of RMS, it is necessary to have well characterized in vitro models (cell lines). Hence, another goal of this project was to establish novel cell lines, follow their progression in culture, while monitoring gene expression on the molecular level.

In a final approach, the significance of a potentially novel oncogene, PAX3, was addressed. Expression of PAX3 is known to occur in RMS but might also be found in other tumors. Since during development PAX3 is also expressed in melanocytes, PAX3 expression might also be detected in melanomas. This hypothesis was investigated using primary melanoma cultures in collaboration with the Department of Dermatology of the University Hospital in Zurich.

5. Experimental part (publications and manuscripts)

5.1 Isolation of genes differentially expressed in human primary myoblasts and embryonal rhabdomyosarcoma

(Int J Cancer 1996; 66:571-577)

Int. J. Cancer: 66, 571–577 (1996)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

ISOLATION OF GENES DIFFERENTIALLY EXPRESSED IN HUMAN PRIMARY MYOBLASTS AND EMBRYONAL RHABDOMYOSARCOMA

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Using a subtractive hybridization method, we have cloned 48 cDNAs which are expressed in human primary myoblasts but down-regulated in the embryonal-rhabdomyosarcoma (RMS) cell line RD. Twenty-nine sequences could be identified as coding for previously known gene products, while 19 encode unknown proteins. Twelve clones coding for known proteins that were highly down-regulated in the RD cells were chosen for further analysis on Northern blots containing additional normal and RMS cells. The expression pattern of TGF-β-induced gene product-3 (βigH3), inhibitory G-protein alpha sub-unit ($G\alpha_{i2}$), osteoblast-specific factor-2 (OSF-2), 22-kDa smooth-muscle protein (SM22), clone A3351 (homologous to mouse talin), testican, thrombospondin-1 and thrombospondin-2 suggests involvement of these proteins in the genesis of the neoplastic phenotype. Among the clones with unknown sequence, several are identical or homologous to expressed sequence tags or known cDNAs, such as integrins or laminin. These results suggest that several isolated clones might have an important role in the determination or maintenance of the normal phenotype, and thus their loss is possibly involved in the progression of malignancy.

The establishment of specialized cell types during development involves commitment of multipotential stem cells to specific lineages and the subsequent activation of distinct gene-expression programs leading to terminal differentiated cells. In muscle cells, the process of differentiation is tightly regulated by a class of transcription factors belonging to the helix-loop-helix family of proteins. These factors, MyoD (Davis *et al.,* 1987), myogenin (Edmonson and Olson, 1989), Myf5 (Braun *et al.,* 1989) and MRF4 (Rhodes and Konieczny, 1989), can activate skeletal-muscle gene transcription when expressed ectopically in a variety of non-muscle cells. Moreover, double knock-out experiments have shown that inactivation of either myogenin (Hasty *et al.,* 1993) or both MyoD and Myf5 (Rudnicki *et al.,* 1993) results in mice lacking any muscle-specific gene products.

Rhabdomyosarcoma (RMS) are small-cell tumors of skeletal muscle phenotype that show poor differentiation toward skeletal muscle, despite expressing myogenic transcription factors in various degrees (Tonin *et al.,* 1991). They can be grouped into 2 major histological categories. Alveolar RMS (aRMS) is characterized molecularly by a translocation involving chromosomes 2 and 13, t(2;13)(q35;q14), resulting in the generation of a fusion protein between the transcription factor PAX3 at 2q35 and a fork head domain gene (FKHR) at 13q14 (Galili *et al.,* 1993). The occurrence of this translocation in the majority of aRMS suggests direct involvement of the fusion protein in tumorigenicity. In contrast, less is known about the molecular mechanisms underlying the development of the more prevalent embryonal RMS (eRMS); one characteristic feature is a consistent loss of heterozygosity on chromosome 11p15 (Scrable *et al.,* 1989). Furthermore, in the embryonal RMS cell line RD it has been demonstrated that both the *p53* tumor-suppressor gene and the *ras* oncogene are mutated (Stratton *et al.,* 1990; Felix *et al.,* 1992). In addition, there is some evidence that these cells are deficient in a factor required for MyoD activity and myogenesis (Tapscott *et al.,* 1993), but the picture is far from being complete. Therefore, it would be of great interest to identify additional molecules that are absent or mutated in these tumor cells.

Subtractive hybridization has proven to be an effective method of isolating cDNAs corresponding to transcripts that are differentially expressed in 2 types of cells. We applied a subtractive hybridization procedure between human primary myoblasts and embryonal RMS cells in order to obtain a catalogue of molecules down-regulated in these tumor cells. We isolated 91 cDNA clones encoding for 29 different transcripts of known sequences and 19 of unknown sequences. The expression of some transcripts in various tumor and normal cells, together with normal and RMS tissue, were investigated.

MATERIAL AND METHODS

Cell lines and primary cell culture

The human RMS cell line RD and primary human lung fibroblasts MRC-5 were obtained from the ATCC (Rockville, MD). The human embryonal RMS Rh1, and the alveolar Rh18 and Rh30 cell lines were a generous gift from Dr P. Houghton (St. Jude Research Hospital, Memphis, TN). Primary human myoblasts B6M and A33 were isolated as described (Schäfer *et al.,* 1994) from autopsy material of a 6-month-old boy (gastrocnemius) and of a 33-week-old boy (quadriceps) respectively. All the RMS cell lines and the MRC-5 lung fibroblasts were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% FCS. Human primary myoblasts were grown in Ham's F-10 supplemented with 20% FCS (growth medium, GM) and differentiated by switching to DME, 2% horse serum, 2.5×10^{-6} M dexamethasone, 10^{-6} M insulin (fusion medium, FM). All media contained 100 U/ml penicillin and 100 μg/ml streptomycin.

cDNA cloning

Total RNA was prepared according to Chirgwin *et al.* (1979). mRNA was isolated from total RNA by chromatography on oligo-dT cellulose (Boehringer, Mannheim, Germany), and treated with 0.4 U/μg RQ1 DNAse (Promega, Madison, WI) in order to avoid genomic DNA contamination when constructing the libraries.

A subtractive library was constructed adapting a published procedure (Boll *et al.,* 1993). Double-stranded cDNA, synthesized from A33 mRNA using random hexamer oligonucleotides, was treated with T_4 DNA polymerase to produce blunt ends and ligated to T-adapters (TLLS: 5′-TTTCTTCATTGTCGACTCAGTCC-3′ and TLLA: 5′-GGACTGAGTCGACAATGAAG-3′). The RD mRNA was similarly worked up and ligated to 5′-end biotinylated D-adapters (DLLS: 5′-TTTCCACTCCGTGCACTTGACTT-3′ and DLLA: 5′-AAGTCAAGTGCACGGAGTGG-3′). cDNAs were amplified by PCR (25 cycles of 30 sec, 95°C, 1 min 50°C, 2 min 72°C, using as primer TLLS or DLLS respectively) and 1.5 μg A33 cDNA was hybridized with a 10-fold excess of RD cDNA (in 0.75 M NaCl,

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25 mM HEPES, pH 7.6, 50 mM EDTA, 0.1% SDS for 18 hr at 68°C). The biotinylated material was removed by incubation with streptavidin followed by phenol extraction (Sive and St John, 1988), which removes all the RD cDNAs plus any hybridized A33 sequences. Six cycles of subtraction were performed with PCR, as described above, every 2 cycles to replenish the cDNA. The remaining myoblast-specific cDNA was cloned into the plasmid pCR II (TA Cloning Kit, Invitrogen, San Diego, CA). The subtracted library was screened by Northern-blot analysis, and differentially expressed clones were sequenced in a single run using the M13 universal primer.

DNA analysis

DNA sequences were determined using the Autoread sequencing kit in conjunction with an A.L.F. DNA sequencer (Pharmacia, Uppsala, Sweden). DNA sequences were analyzed with the genetic computer group (GCG, University of Wisconsin) program package on a VAX computer. Database searching was carried out with the program FASTA.

Northern-blot analysis

Total RNA was run on 1% agarose gels in the presence of 2.2 M formaldehyde and transferred to Nytran nylon membranes by capillary transfer (Schleicher and Schuell, Dassel, Germany). Insert DNA excised from plasmids by SalI was labeled by random priming (prime-a-gene, Promega) and hybridized with filter-bound RNA in 50% formamide buffer. Final washing was performed in 0.75 × SSC, 0.1% SDS at 65°C for 30 min. Primary-tumor total RNA (FR) was a gift from Dr. M. Hany.

RESULTS

Isolation and primary characterization of differentially expressed clones

Subtractive hybridization is a powerful tool for isolating molecules expressed in one cell type but absent from another. Therefore, we utilized this technique to identify cDNAs present in human primary myoblasts (A33) but absent in the embryonal RMS cell line RD, in order to better understand the mechanisms involved in the generation of this neoplastic phenotype.

cDNA derived from primary human myoblasts was depleted of sequences present in the RMS cells through 6 cycles of subtractive hybridization (see "Material and Methods"). The high number of cycles performed was expected to almost completely remove cDNAs having common origin. The remaining myoblast-specific cDNA was cloned into a plasmid. The entire inserts excised from the single clones were used as probes to perform Northern-blot analysis to identify differentially expressed clones. As an example, Figure 1 displays the analysis of plasminogen-activator inhibitor (PAI-1), TGF-β-induced gene product-3 (βigH3), tissue inhibitor of metalloproteinase-3 (TIMP-3) and fibronectin-1 on Northern blots containing total RNA isolated from A33 and RD cells. Thrombospondin-1 and IGFBP-5 are not detectable in RD cells, whereas TIMP-3 and fibronectin-1 are expressed at a low but still visible level in the tumor cells. Screening of 206 clones on similar Northern blots revealed that 91 of them (44%) were differentially expressed in the subtractive library (Table I). The 91 differentially expressed clones were sequenced in a single run and compared with the GenEmbl nucleic acid databank. A sequence was regarded as known when the identity found was greater than 95%, allowing for a limited number of sequencing errors in a single run. Seventy-two clones have a sequence that is already present in the databank, and were therefore indicated as "known". Due to repetitions, the 72 clones of known sequence represented 29 different molecules. The remaining 19 clones had a novel sequence, were homologous to expressed sequence tags (ESTs) or shared limited identity to known proteins. These clones were indicated as "unknown" (see below).

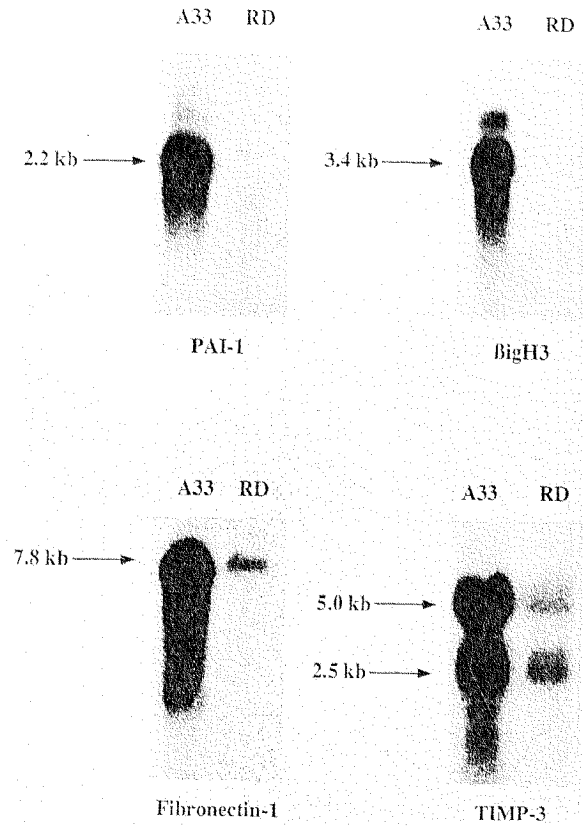

FIGURE 1 – Northern-blot analysis of 4 isolated clones. Total RNA (5 μg) from the indicated cells was loaded in each lane. A33 are primary human myoblasts, RD are human RMS cells. Four clones from the subtracted library were digested with SalI; the inserts were labeled by random priming and used for hybridization. PAI-1, plasminogen activator inhibitor-1; βigH3, TGF-β-induced gene product-3; TIMP-3, tissue inhibitor for metalloproteinase-3. The length of the transcripts is indicated.

TABLE I – RESULTS OF SUBTRACTIVE HYBRIDIZATION

Total screened clones[1]	206
Differentially expressed clones	91
Clones of known sequence:[2]	72
independent clones[3]	29
Clones of unknown sequence:	19
independent clones	19

[1]The clones were screened by Northern blot analysis containing total RNAs derived from RD RMS cells and A33 human primary myoblasts.–[2]A sequence was indicated as known when the identity to cDNAs in the Gene/EMBL databank was greater than 95%.–[3]Due to repetitions the 72 clones of known sequence represent 29 different molecules.

The known clones were divided into classes based on the known or proposed subcellular localization of the proteins they encode for (Table II). The majority of the proteins identified are located extracellularly, including many molecules involved in the building of the extracellular matrix. Also found were molecules that are localized at the cell membrane, in the cytoplasm and in the nucleus. This indicates that our screen included molecules expressed in all compartments of the cell. The fact that most of the clones found code for components of the extracellular matrix probably correlates

TABLE II – DIFFERENTIALLY EXPRESSED CLONES WITH KNOWN SEQUENCE

Cellular localization	Encoded protein	% identity[1] to human cDNAs	Number of clones found	Proposed function	Selected references[2]
Extracellular	Aggrecan	> 95%	4	Extracellular matrix	[1]
	Collagen I, alpha-1	> 95%	5	Extracellular matrix	[2]
	Collagen I, alpha-2	> 96%	5	Extracellular matrix	[3]
	Collagen V, alpha-1	97.9%	1	Extracellular matrix	[4]
	Collagen VI, alpha-2	96.6%	1	Extracellular matrix	[5]
	Collagen VI, alpha-3	97.3%	1	Extracellular matrix	[6]
	Fibrillin-1	> 98%	8	Extracellular matrix	[7]
	Fibronectin-1	> 95%	9	Extracellular matrix	[8]
	Insulin-like growth factor binding protein 5 (IGFBP-5)	97.9%	1	Modulator of IGF and IGF-receptor interactions	[9]
	Lysyl oxidase	> 97%	2	Cross-linking of collagen molecules	[10]
	Plasminogen activator inhibitor-1 (PAI-1)	> 97%	4	Protease inhibitor	[11]
	Testican	96%	1	Cell adhesion	[12]
	Tissue inhibitor for metalloproteinase-3 (TIMP-3)	99.4%	1	Protease inhibitor	[13]
	TGF-β induced gene product 3 (BigH3)	> 96%	2	Mediator of TGF-beta effects?	[14]
	Thrombospondin-1	> 94%	3	Inhibition of angiogenesis	[15]
	Thrombospondin-2	98.7%	1	Inhibition of angiogenesis	[16]
Cell membrane	Integrin alpha-5	> 95%	2	Receptor for fibronectin	[17]
	LDL receptor related protein	98.8%	1	Receptor	[18]
	Osteoblast specific factor-2 (OSF-2)	> 97%	2	Adhesion molecule?	[19]
	Platelet derived growth factor receptor	> 96%	2	Receptor	[20]
	Tyrosine kinase receptor	> 99%	2	Receptor	[21]
Cytoplasm	Cytoskeletal gamma-actin	96.3%	1	Cytoskeleton	[22]
	Filamin	> 97%	4	Actin binding	[23]
	G_i protein alpha-2 subunit ($G\alpha_{i2}$)	96.7%	1	Adenylate cyclase inhibiting GTP binding protein	[24]
	MKK3	95.2%	1	Signal transduction	[25]
	Nonmuscle myosin heavy chain	> 96%	2	Cytoskeleton	[26]
	RhoC	98.8%	1	GTPase	[27]
	22 kDa smooth muscle protein (SM22/WS3)	> 97%	3	Ca^{2+} binding protein	[28]
Nucleus	CDEI binding protein	98.3%	1	Function in the cell cycle?	[29]

[1]Percent of sequence identity between the differentially expressed clones and Gen/EMBL cDNAs.–[2]1. Baldwin, C.T. *et al., J. Biol. Chem.*, **264,** 15747–15750 (1989); 2. Bernard, M.P. *et al., Biochemistry,* **22,** 5213–5223 (1983); 3. Kuivaniemi, H. *et al., Biochem. J.,* **252,** 633–640 (1988); 4. Takahara, K. *et al., J. biol. Chem.*, **266,** 13124–13129 (1991); 5. Chu, M.L. *et al., EMBO J.,* **8,** 1939–1946 (1989); 6. Chu, M.L. *et al., EMBO J.,* **9,** 385–393 (1990). 7. Maslen, C.L. *et al., Nature (Lond.)* **352,** 334–337 (1991); 8. Kornblihtt, A.R. *et al., Proc. Nat. Acad. Sci. (Wash.)* **80,** 3218–3222 (1983). 9. Kiefer, M.C. *et al., J. biol. Chem.*, **226,** 9043–9049 (1991). 10. Kenyon, K. *et al., J. biol. Chem.,* **268,** 18435–18437 (1993); 11. Wun, T.C. *et al., FEBS Lett.,* **210,** 11–16 (1987); 12. Alliel, P.M. *et al., Eurol. J. Biochem.,* **214,** 347–350 (1993); 13. Apte, S. *et al., Genomics,* **19,** 86–90 (1994); 14. Skonier, J. *et al., DNA Cell Biol.,* **11,** 511–522 (1992); 15. Lawler, J. and Hynes, R.O., *J. Cell Biol.,* **103,** 1635–1648 (1986); 16. LaBell, T.L. and Byers, P.H., *Genomics,* **17,** 225–229 (1993); 17. Argraves, W.S. *et al., J. Cell Biol.*, **105,** 1183–1190 (1987). 18. Herz, J.J. *et al., EMBO J.,* **7,** 4119–4127 (1988); 19. Takeshita, S. *et al., Biochem. J.,* **294,** 271–278 (1993); 20. Claesson-Welsh, L. *et al., Mol. cell. Biol.,* **8,** 3476–3486 (1988); 21. Janssen, J. *et al., Oncogene,* **6,** 2113–2120 (1991); 22. Erba, H.P. *et al., Mol. cell. Biol.,* **8,** 1775–1789 (1988); 23. Gorlin, J.B. *et al., J. Cell Biol.,* **111,** 1089–1105 (1990); 24. Didsbury, J.R. *et al., FEBS Lett.,* **211,** 160–164 (1987); 25. Derijyrd, B. *et al., Science,* **267,** 682–685 (1995); 26. Toothhaker, L.E. *et al., Blood,* **78,** 1826–1833 (1991); 27. Chardin, P. *et al., Nucleic Acids Res.,* **16,** 2717 (1988); 28. Nishida, W. *et al., Biochem. Int.,* **23,** 663–668 (1991); 29. Von der Kammer, H. *et al., Genomics,* **20,** 308–311 (1994).

with their expression levels. We observed a correlation between the expression level of a molecule and the likelihood to identify it in the subtractive library. For example, fibrillin-1 and fibronectin-1 were detected 8 and 9 times respectively, which correlates with the high expression observed in Northern blots (data not shown).

Clones homologous to expressed sequence tags or with partial identity to known molecules

Among the 19 clones with unknown sequence, 9 do not share any homology with sequences in the databank; 2 clones are highly homologous to mouse talin and Cyr61 (clones A3351 and A33210 respectively) and 1 to rat jagged (A33111). Homologies ranging from 86.1 to 90.9% strongly indicate that we identified part of the human homologs of these murine sequences. Four clones show only partial homology to known cDNAs (clones A3321, A33108, A33187 and A33292). The remaining 3 are identical (clones A3335, A3358, and A33199) to ESTs (Table III). Clone A3321 shows 57% homology in 356 bp to human integrin alpha-2 in the region between residues 280 and 630. This corresponds to the location of the repeated domains I and II and the first part of the I-domain, a metal-binding site (Hogg *et al.,* 1994). Clone A33108 has 62.5% homology in 192 bp to residues 1840–2030 of rat integrin alpha-1, a region including a binding site for divalent cations (Ignatius *et al.,* 1990). The length of transcripts we detected in Northern-blot analysis is similar to the length of the published cDNA sequences, 4 kb for integrin alpha-1 and 5.3 kb for integrin alpha-2 (Ignatius *et al.,* 1990). These results suggest that the 2 clones could represent parts of new isoforms of integrin alpha subunits. Clones A33187 and A33292 have 62.5% homology in 180 bp to the human laminin B1 chain and 66.7% homology in 264 bp to the human voltage-gated sodium channel respectively. Again, these results indicate that we may have isolated new members of the respective protein families. However, proof of this hypothesis awaits full-length cloning of the corresponding cDNAs. One clone with unknown sequence (A3335) was used as probe for screening a cDNA library derived from human primary myoblasts to isolate its full-length cDNA. This cDNA codes for a novel LIM-domain protein (data not shown).

Expression pattern of differentially expressed clones in additional normal and tumorigenic cells

To determine whether differential expression was limited to the cells used for the subtraction experiment, or could be expanded to other RMS cells, extended Northern-blot analysis of selected differentially expressed clones was performed. For this purpose, the expression of 13 clones in various RMS tumor and normal cells, as well as in adult muscle tissue and RMS tissue, were tested. Figure 2 shows the expression pattern of 2 of the clones tested, 22-kDa smooth-muscle protein (SM22) and TGFβ-induced gene product 3 (βigH3). SM22 was found to be expressed in the primary myoblasts (A33 and B6M), human lung fibroblasts (MRC-5), and to a lower extent in the adult muscle tissue. In the RMS cell lines RD and Rh18 residual expression was detected, whereas in Rh1, Rh30 and the RMS tissue no expression was observed. βigH3 transcripts are present in all normal cells tested, residual expression is present in the RD cells, while the other tumor cells as well as the adult tissue fail to express detectable levels of βigH3 mRNA. Table IV summarizes the expression pattern of all clones tested. βigH3, $G\alpha_{i2}$, OSF-2, SM22, A3351 (talin), testican, thrombospondin-1 and thrombospondin-2 were designated as consistent differentially expressed clones. They are expressed exclusively in the normal cell types, except in some cases where residual expression was detected in the RMS cell lines (*e.g.,* SM22 in RD and Rh18). Since the level of residual expression was very low when compared with the expression in normal cells (Fig. 2), these clones were still considered as consistently differentially expressed. The others, CDEI-binding protein, Cyr61, IGFBP-5, A33111 (jagged) and integrin alpha-5 were not consistently differentially expressed. In all cases, the molecules were found to be expressed in one or more tumor cells at a non-negligible level. We conclude from these expression patterns that 8 of the molecules analyzed are consistently differentially expressed and therefore might be involved, although possibly as a secondary effect, in rhabdomyosarcoma tumorigenicity.

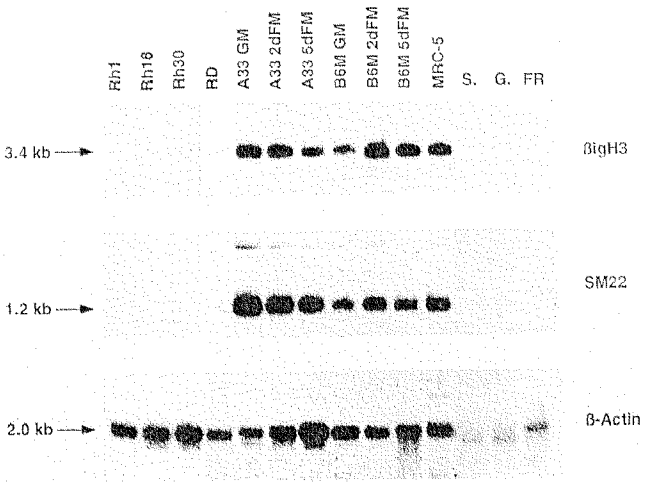

FIGURE 2 – Extended Northern-blot analysis of βigH3 (TGF-β-induced gene product) and SM22 (22-kDa smooth-muscle protein) transcripts. Total RNA (5 μg) from the indicated cells or tissue was loaded on each lane. Inserts from the corresponding clones were isolated, labeled by random priming and used as probes for hybridization. Rh1, Rh18, Rh30 and RD are RMS cell lines; B6M and A33 are human primary myoblasts; MRC-5 are human primary lung fibroblasts. S, soleus; G, gastrocnemius; FR, RMS tumor tissue. GM cells cultivated in growth medium; 2dFM, 5dFM, cells grown in fusion medium for 2 or 5 days respectively. As control for RNA quantities the blots were re-hybridized with a β-actin probe.

TABLE III – DIFFERENTIALLY EXPRESSED CLONES WITH UNKNOWN SEQUENCE

Clone	Closest homology	Identity/bp[1] (%)	Accession number[2]
A3321	human integrin alpha-2	57%/356	Z50167
A3335	EST[3]yb60a04.r1	99.6%/284	HS37727
A3351	mouse talin	90.9%/397	Z50170
A3358	EST 47E04	95.1%/223	Z24831
A33108	rat integrin alpha-1	62.5%/192	Z50157
A33111	rat jagged	89.2%/158	Z50166
A33187	human laminin B1 chain	65.6%/180	Z50158
A33199	EST 01678	96.5%/202	M78088
A33210	mouse Cyr61	86.2%/304	Z50168
A33292	human sodium channel, alpha sub-unit	66.7%/264	Z50169

[1]Percent of sequence identity between the differentially expressed clones and Gen/EMBL cDNAs within the length of the region of identity.–[2]Genbank/EMBL sequence database accession numbers for the indicated clones are given.–[3]EST, expressed sequence tag.

DISCUSSION

We describe here the generation, by subtractive hybridization, of a catalog of molecules down-regulated in tumor cells but expressed in their normal counterparts. We chose to use subtractive hybridization instead of differential RNA display (Liang and Pardee, 1992) because of the high "noise level" of false positives clones observed with this method when comparing only 2 cell types (Liang *et al.,* 1993). In addition, subtractive hybridization can capture coding portions of expressed genes

TABLE IV – NORTHERN-BLOT ANALYSIS OF 13 DIFFERENTIALLY EXPRESSED CLONES

Clone tested[1]	Normal cells[2]			RMS[3] cell lines			Normal adult tissue		Soleus	RMS[3] tissue
	A33	B6M	MRC-5	RD	Rh1	Rh18	Rh30	Gastrocnemius		
Consistent[4]										
A3351 (Talin)	++	+	++	−	+/−	n.d.	+/−	+	+/−	−
βigH3	++	++	++	+/−	−	−	−	−	−	−
$G\alpha_{i2}$[6]	+	+	+	+/−	+/−	n.d.	+/−	−	+/−	+/−
OSF-2[7]	++	++	+	−	−	n.d.	−	−	−	−
SM22	++	++	++	+/−	−	+/−	−	+	+	−
Testican	++	++	−	−	−	−	−	−	−	−
Thrombospondin-1	++	++	+	−	−	−	−	−	−	−
Thrombospondin-2[6]	+	+	−	−	−	n.d.	−	−	−	−
Not consistent[5]										
A33111 (jagged)[6]	+	+	−	−	+	n.d.	+/−	+/−	+/−	+/−
CDEI-binding protein	++	++	+	+/−	+	n.d.	−	+	+	+
A33208	++	++	++	−	−	n.d.	+	−	+	−
IGFBP-5	++	++	++	+/−	+	+	−	+	+	+
Integrin alpha-5[6]	+	+	+	+/−	+	n.d.	+/−	+/−	+/−	+/−

++, level of expression similar to that in A33 primary myoblasts; +, level of expression lower than in A33 primary myoblasts; +/−, residual expression; −, no expression detected. n.d., not done.–[1]IGFBP-5, insulin-like growth-factor-binding protein; βigH3, TGF-β-induced gene product-3; OSF-2, osteoblast-specific factor-2; $G\alpha_{i2}$, G_i-protein alpha sub-unit; SM22, 22 kDa smooth-muscle protein.–[2]A33 and B6M are human primary myoblasts, MRC-5 are human lung fibroblasts.–[3]RMS, rhabdomyosarcoma.–[4]Consistent differentially expressed clones are expressed only in normal cell types, except for residual expression in some RMS cells.–[5]Non-consistent differentially expressed clones are expressed in one or more RMS cells.–[6]To A33 primary myoblasts a + instead of a ++ was assigned because of low expression levels.–[7]A decrease in expression level during differentiation of muscle cells *in vitro* was observed.

making their identification easier. This contrasts with differential RNA display, which favors the isolation of 3′-untranslated regions. The adaptation of a published subtractive hybridization procedure (Boll *et al.*, 1993) allowed us to construct a highly enriched subtracted library, in which nearly 50% of the clones are differentially expressed. The efficiency of this method was surprisingly high, compared with the results reported by other groups. The number of clones differentially expressed ranged between 5 and 15% in other studies (Austruy *et al.*, 1993; Boll *et al.*, 1993; Schraml *et al.*, 1994), indicating that the method of subtractive hybridization is unfavorable for the isolation of large numbers of cDNAs. In contrast, we were able to identify numerous differentially expressed molecules and generate a large catalogue of genes down-regulated in tumor cells. Moreover, the use of PCR in the hybridization protocol led to relative equalization of the abundance of individual cDNAs. This allowed the isolation of genes expressed also at low levels, as demonstrated by Northern-blot analysis, *e.g.*, of clone A33111 (jagged) (data not shown).

Unlike other authors who performed subtractive hybridization of tumors or tumor cells and the corresponding adult tissues (Austruy *et al.*, 1993; Schraml *et al.*, 1994), we chose RMS cells and primary human myoblasts as subtraction partners, in an attempt to minimize the differences between the 2 cells used for the experiment. The phenotype of the RMS cell line RD suggests that the cells are derived from myogenic progenitors committed to the myogenic fate but arrested at an early stage in the differentiation pathway. They closely resemble muscle at the embryonic stage and have therefore only limited expression of genes associated with terminal differentiation (like the proteins building the contractile apparatus, *e.g.*, skeletal-muscle-specific actin and myosin) (Tonin *et al.*, 1991). Consequently, the isolation of genes specifically expressed upon terminal differentiation, as in adult muscle tissue, but not necessarily related to tumorigenesis, was avoided by using primary myoblasts derived from satellite cells and also mimicing embryonal muscle cells.

Some of the clones we identified encode proteins already described in relation to tumorigenesis, for example βigH3 (Skonier *et al.*, 1994), integrin α5 (Giancotti and Ruoslahti, 1990), $G\alpha_{i2}$ (Rudolph *et al.*, 1995), thrombospondin-1 (Sheibani *et al.*, 1995) and plasminogen activator inhibitor-1 (Laug *et al.*, 1993). Hence, the results of our subtractive hybridization experiment are significant in this respect and suggest that other hitherto unrelated molecules identified might also be connected to tumorigenesis.

To test whether the clones initially found are down-regulated in cells other than those used for subtraction, we performed Northern-blot analysis with RNA isolated from various normal and tumorigenic cells. Eight clones, namely A3351 (talin), βigH3, $G\alpha_{i2}$, OSF-2, SM22, testican, thrombospondin-1 and thrombospondin-2, showed differential expression, *i.e.*, were not detectable in all tumor cells tested and present in several normal cells. Interestingly, of these βigH3, $G\alpha_{i2}$ and thrombospondin have been functionally investigated and linked to different aspects of malignant progression (see above). By this criterion, the remaining clones can be regarded as very good candidates for further functional studies. Two of these clones, SM22 and A3351 (talin), were found to be expressed in mature muscle tissue, suggesting a role in the maintenance of the post-mitotic, differentiated state. The others were not detectable in adult tissue, suggesting down-regulation of expression upon muscle maturation. Since a similar expression pattern was also observed for the myogenic transcription factors MyoD and Myf5, we hypothesize that these proteins are expressed during muscle development and might have a role in the regulation of normal cell growth.

Regarding their possible function, 3 of the candidate tumor-suppressor genes might be involved in signaling pathways, which are often defective in tumor cells. The TGF-β-induced gene product βigH3 encodes a secreted protein which inhibits cell attachment and suppresses the growth of Chinese hamster ovary cells in nude mice (Skonier *et al.*, 1992). Its anti-adhesion activity suggests that it may be a component of the extracellular matrix, which exerts its anti-proliferative effects by binding to cell-surface components (integrins) (Skonier *et al.*, 1994). SM22 (WS-3) is a cDNA encoding a cytosolic protein with a putative Ca^{2+}-binding site (Thweatt *et al.*, 1992). Interestingly, its expression is strikingly up-regulated in non-dividing senescent fibroblasts. Since intracellular concentration of free Ca^{2+} plays a pivotal role as second messenger in a variety of cellular processes, including gene expression, DNA synthesis and

mitosis, this protein may play a role in the generation of the neoplastic phenotype.

Cell adhesion is a critical process in normal cell development and homeostasis. Tumor cells have altered cell-adhesion behavior (reviewed in Hynes and Lander, 1992), contributing to their ability to metastasize and to grow without contact inhibition. Two of our candidate tumor-suppressor genes encode proteins that might have a role in cell-adhesion phenomena. Osteoblast-specific factor-2 (Takeshita *et al.,* 1993) shares some homology to fasciclin I, a homophilic adhesion protein involved in neuron-growth-cone guidance during development of *Drosophila* and grasshopper embryos (Zinn *et al.,* 1988; Elkins *et al.,* 1990). Testican, a proteoglycan isolated from testis, is related to several protein families involved in cell-cell and cell-matrix interactions (Alliel *et al.,* 1993). The absence of these 2 proteins in rhabdomyosarcoma cells may contribute to tumor progression.

Our results demonstrate that a large number of genes are down-regulated in RD tumor cells. Hence, there might exist one common mechanism, or several, orchestrating this repression of specific genes, as exemplified by the basic helix-loop-helix myogenic transcription factors coordinating activation of muscle-specific genes. Two molecules that influence transcription directly or indirectly are found to be altered in RD rhabdomyosarcoma cells, namely, transcription factor *p53* and the signal-transduction protein ras. Indeed, wild-type *p53* can inhibit angiogenesis through direct transcriptional stimulation of thrombospondin-1 (Dameron *et al.,* 1994). Therefore, the fact that RD cells express a mutated *p53* could directly relate to down-regulation of thrombospondin-1. In this regard, it will be interesting to see whether other down-regulated molecules might be targets of the *p53* transcription factor. The status of *p53* is known for RD and Rh30 cells, which both express only mutated protein, and for Rh18 where *p53* is normal (Felix *et al.,* 1992). However, it is likely that more than one mechanism is responsible for down-regulating "normal" genes in tumor cells.

In conclusion, we have identified several molecules not previously associated with the development of neoplastic cells. The majority of these proteins have crucial functions in cellular processes such as cell adhesion, angiogenesis, signal transduction and formation of extracellular matrix.

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5.2 Subtractive cloning and characterization of DRAL, a novel LIM-domain protein down-regulated in rhabdomyosarcoma

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Subtractive Cloning and Characterization of DRAL, a Novel LIM-Domain Protein Down-Regulated in Rhabdomyosarcoma

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ABSTRACT

A subtractive cloning procedure was used to characterize the molecular changes involved in transformation of normal myoblasts to rhabdomyosarcoma (RMS) cells. Here we describe the cloning of DRAL, a novel LIM-domain protein expressed in primary myoblasts but down-regulated in the RMS cell line RD. DRAL is a LIM-only protein with five LIM domains whereby one LIM domain consists only of the second half of the consensus motif. Interestingly, down-regulation of DRAL was not confined to the RD RMS cells, but was a phenomenon extended to other RMS cell lines of both embryonal and alveolar subtype, and to some breast cancer cell lines. Analysis of the expression pattern in normal human tissues revealed that DRAL is expressed at high levels in the heart, suggesting an important function in the specification of the terminally differentiated phenotype of heart muscle cells. Immunofluorescence studies using an antibody directed against recombinant DRAL localized the protein predominantly in the nucleus of cultured cells. On the basis of these results, we conclude that down-regulation of DRAL correlates with the tumor phenotype of RMS cells.

INTRODUCTION

TUMORIGENESIS IS A MULTISTEP PROCESS characterized both by the somatic activation of cellular oncogenes and the somatic or germline mutation of tumor suppressor genes. It is now well established that the process leading to malignant transformation of a normal cell requires the accumulation of multiple heritable alterations (Weinberg, 1989; Fearon and Vogelstein, 1990). A number of tumor suppressor genes have been isolated so far by molecular cloning and in several cases tumor suppressor activity has been demonstrated in functional assays (for review, see Stanbridge, 1992; Knudson, 1993).

Rhabdomyosarcomas (RMS) are pediatric solid tumors of skeletal muscle phenotype that can be grouped into two major histological categories—embryonal and alveolar RMS. The two subtypes have distinct and exclusive molecular features. Alveolar RMS is characterized by a chromosomal translocation t(2;13)(q35;q14) leading to a structural rearrangement of the *PAX3* gene (Barr *et al.*, 1993). In contrast, there is no specific translocation for embryonal RMS known; instead a consistent loss of heterozygozity for chromosome 11p15 has been observed (Scrable *et al.*, 1987, 1989), involving so far unknown genes. The well-characterized embryonal RMS cell line RD has been shown to express a mutated p53 tumor suppressor gene (Felix *et al.*, 1992) and an activated N-*ras* oncogene (Stratton *et al.*, 1990). Moreover, RD cells are known to have poor differentiation capability, despite expressing several markers of myogenesis, including the myogenic transcription factors myoD and myogenin (Davis *et al.*, 1987; Wright *et al.*, 1989).

The LIM domain contains a sequence motif of about 60 amino acids found in zinc-finger-like proteins. The conserved cysteine-rich motif is present in an heterogeneous group of proteins from both animals and plants, suggesting important functional features. The LIM motif sequence forms two adjacent

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zinc fingers with the consensus sequence C-X_2-C-X_{17-19}-H-X_2-C-X_2-C-X_2-C-X_{16-20}-C-X_2-C/H/D (Wang *et al.,* 1992), which is able to coordinate two metal ions and to engage in homodimer and heterodimer formation with LIM domains and other protein motifs. According to Dawid *et al.* (1995), LIM domain proteins can be grouped into four classes depending on the additional domains they contain, namely LIM-homeodomain proteins, LIM-only proteins, carboxy-terminal LIM domain proteins, containing three to four LIM domains at their carboxyl terminus associated with other distinct domains, *e.g.,* Paxillin and Zyxin (Sadler *et al.,* 1992; Turner and Miller, 1994), and proteins with LIM domains and other recognized motifs of varied structure, *e.g.,* LIMK (Mizuno *et al.,* 1994).

A major class is formed by LIM-only proteins, consisting of proteins containing one to five LIM domains and little else. Among them we can find the members of the rhombotin family (RBTN-1, RBTN-2, and RBTN-3) (Boehm *et al.,* 1991; Foroni *et al.,* 1992) and MLP (Arber *et al.,* 1994) with two LIM-domains, zyxin with three (Sadler *et al.,* 1992), hic-5 with four (Shibanuma *et al.,* 1994), and PINCH with five domains (Rearden, 1994). Two members of the rhombotin family, RBTN-1 and RBTN-2, were discovered as putative oncogenes because of their association with chromosomal breakpoints in T-cell acute lymphoblastic leukemia (Boehm *et al.,* 1991; Fisch *et al.,* 1992; McGuire *et al.,* 1992). This observation suggests that LIM-only proteins might be involved in the regulation of cell proliferation. Moreover, a null mutation for RBTN-2, which is also expressed in the erythroid lineage, is embryonic lethal due to a failure of erythroid development (Warren *et al.,* 1994), implying a critical role of this protein in erythropoiesis. A comparable role has been assigned to MLP and the *Drosophila* analog DMLP1 in muscle development. In fact, although overexpression of MLP stimulated differentiation in myogenic cell lines, antisense experiments prevented this process, suggesting a critical role for MLP in myogenic differentiation (Arber *et al.,* 1994).

We applied a subtractive hybridization procedure between the human embryonal RMS cell line RD and human primary myoblasts to isolate genes down-regulated in the tumor cells. One rationale for this experiment was that RD cells seem to lack a factor necessary for normal differentiation (Tapscott *et al.,* 1993). Here we describe the isolation and characterization of a novel cDNA encoding a LIM domain protein whose expression is suppressed in several RMS cells.

MATERIALS AND METHODS

Cell lines and primary cell culture

Human RMS cell lines RD, primary human lung fibroblasts MRC-5, and NIH-3T3 mouse embryonal fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). The human embryonal and alveolar RMS cell lines Rh1, Rh18, and Rh30 were a generous gift of Dr. P. Houghton (St. Jude Children's Hospital, Memphis, TN). Primary human myoblasts B6M and A33 were isolated as described (Schafer *et al.,* 1994) from autopsy material of a 6-month- (gastrocnemius) and a 33-week-old boy (quadriceps), respectively. All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). The human primary myoblasts were grown in Ham's F-10 supplemented with 20% FCS (growth media, GM) and differentiated by switching to DMEM, 2% horse serum, 2.5×10^{-6} *M* dexamethasone, 10^{-6} *M* insulin (fusion media, FM). All media contained 100 U/ml penicillin and 100 μg/ml streptomycin.

Construction of cDNA library

Total RNA was prepared from A33 primary human myoblasts according to Chirgwin *et al.* (1979). mRNA was isolated from total RNA by chromatography on olido(dT) cellulose (Boehringer). The mRNA was treated with DNase to avoid genomic DNA contamination when constructing the library.

To construct a human primary myoblast library, double-stranded cDNA was synthesized from A33 mRNA using both oligo(dT) and random hexamer primers in a ratio of 15:1 (Timesaver cDNA Synthesis Kit, Pharmacia). The cDNA was ligated into *Eco* RI predigested Lambda ZAP Express arms (Stratagene) and packaged using the Gigapack II packaging extract as described by the manufacturer (Stratagene). Titration and amplification of the library were carried out as described; one round of amplification was performed to obtain a titer of 4×10^8 pfu/ml.

Isolation and sequencing of DRAL cDNA

A33-35, a clone from the subtracted library (Genini *et al.,* 1996) that did not show any homology to sequences in the databank (GenEmbl), was chosen for further analysis. DRAL full-length cDNA was isolated by screening the A33-derived library with A33-35. Positive plaques were excised *in vivo* with the Ex-assist helper phage as described (Stratagene). Three independent clones were obtained; 35.1 and 35.3 were sequenced using universal and gene specific primers (A3335-1 and A3335-2).

The cloning of 5′ sequences of DRAL was performed using a rapid amplification of cDNA ends (RACE) protocol according to Wey *et al.* (1994). The polyadenylated 5′ DNA pool was amplified using the RACE primers RACETOT, RACE-O, and RACE-I and the gene-specific primers DRAL-O and DRAL-I.

Oligonucleotides

Oligonucleotides and their sequences are as follows: A3335-1, 5′-GGGTGAGAAAGAAAACATA-3′; A3335-2, 5′-AGCCGAATTCCAGCACACT-3′; 35EX5′, 5′-ACGGGATCCCCACTGAGCGCTTTGACTGC-3′; 35EX3′, 5′-ACGCCCGGGGAATTCAGATGTCTTTCCCACA-3′; DRAL-O, GCTTCCCACACTCCTCGCAG; DRAL-I, ACACCACGCAGTAGGGCGTC.

Southern and Northern blot analysis

Southern and Northern blot analysis were performed according to standard procedures (Sambrook *et al.,* 1982).

Bacterial expression of DRAL and antibody production

An 840-bp fragment of DRAL was amplified by the polymerase chain reaction (PCR) with the oligonucleotides 35EX5′ and 35EX3′ and cloned into the *Bam* HI, *Sma* I sites of the prokaryotic expression vector pGEX-3X (glutathione-*S*-trans-

ferase gene fusion system, Pharmacia). The recombinant GST-DRAL fusion protein was induced by addition of 0.5 m*M* isopropyl-β-thiogalactoside. The cells were lysed after 6 hr of induction and the GST-DRAL protein was purified with glutathione Sepharose beads. Recombinant DRAL was obtained after cleavage of GST-DRAL bound to glutathione Sepharose beads with endoprotease factor Xa over 48 hr. The supernatant was used for Western blot analysis.

Five hundred micrograms of GST-DRAL protein in 500 μl of emulsion containing 50% TiterMax (Vaxcel, Inc.) in PBS was used to immunize a rabbit, which was thereafter boosted twice with 250 μg of GST-DRAL protein in intervals of 3 weeks. Two weeks after the last immunization, the rabbits were exsanguinated.

Preparation of tissue and cell extracts and Western blot analysis

The tissue samples were treated with 5 vol/wt extraction buffer (4 *M* urea, 1 *M* KCl, 2 m*M* EDTA, 50 m*M* Tris-HCl pH 7.5) and mixed in 1:1 ratio to sample buffer 2× (20% Glycerin, 0.2% $NaDodSO_4$, 5% β-mercaptoethanol, 50 m*M* Tris-HCl pH 6.8). A total of 10^6 cells were treated with 100 μl of extraction buffer and mixed with sample buffer as described above. Ten microliters were run on a $NaDodSO_4$-PAGE: 5%T(3%C) stacking gel pH 6.8, 12%T(3%C) running gel pH 8.6 by 30 mA/cm^2 at room temperature in an electrophoresis buffer containing 100 m*M* Tris, 100 m*M* Taurine, 0.1% $NaDodSO_4$ pH 8.6. Electroblotting to a polyvinylidene difluoride membrane (Tropifluor, Tropix) was performed in transfer buffer (20 m*M* Tris, 20 m*M* Taurine, 20% methanol, 1 m*M* EDTA) by 50V, 1.5 mA/cm^2 at room temperature. The membrane was then incubated with rabbit-anti-DRAL antibodies, diluted 1:20,000 in blocking buffer, containing 0.2% I-Block (Tropix) and 0.1% Tween 20 in phosphate-buffered saline (PBS) pH 7.3, washed in the same buffer, and incubated with a goat-anti-rabbit Fc conjugated with avidin together with a streptavidin-alkaline phosphatase complex (Avidix, Tropix), both diluted 1:20,000 in blocking buffer. The membrane was washed in assay buffer (0.1 *M* diethanolamine, 1 *M* $MgCl_2$ pH 10) and the light reaction was triggered by CSPD (Tropix). A biotinylated molecular weight marker was used as a standard.

Transient transfection of cells and immunofluorescence

C2C12, NIH-3T3, RD, and RH30 cells were transiently transfected with DRAL cDNA under the control of a cytomegalovirus (CMV) promoter element (pSCT-DRAL). Two days after transfection, the cells were fixed in 3% formaldehyde, followed by treatment with methanol or with 0.5% Triton-X100 in PBS. The samples were incubated with affinity-purified anti-DRAL antiserum at a dilution of 1:75 in DME + 3% HS for 45 min at 37°C and then incubated with a fluorescein-conjugated goat–anti-rabbit antibody (Cappel) at a dilution of 1:200 in DME + 3%HS for 45 min at 37°C. The cells were viewed through a Zeiss fluorescence microscope.

In vitro *translation*

The TNT Coupled Reticulocyte Lysate System (Promega) with T7 RNA polymerase was used to transcribe and translate the DRAL cDNA. The synthesized proteins were analyzed by $NaDodSO_4$ gel electrophoresis ($NaDodSO_4$-PAGE) followed by autoradiography. Prestained $NaDodSO_4$-PAGE standards (Bio-Rad) were used to estimate the size of the proteins.

Chromosomal localization

In situ hybridization on chromosome preparation was carried out as described (Mattei *et al.*, 1985). The insert from clone A33-35, corresponding nucleotides 64–451 of the full-length cDNA, was used as probe.

RESULTS

Isolation of DRAL cDNA

The discovery of genes expressed in normal cells but down-regulated during tumorigenesis is of great interest for a better understanding of the complex mechanisms leading to neoplasia. At present, several molecules involved in this process have been identified but the picture is far from being complete. Therefore, we applied a subtractive hybridization procedure where cDNA prepared from human primary myoblasts (A33) was depleted of sequences also found in RMS cells (RD) (Genini *et al.*, 1996). Clone A33-35, one of the 19 clones with unknown sequence, was chosen for further analysis in virtue of the particularly strong differential expression shown in Northern blots containing only A33 and RD total RNAs (data not shown).

To isolate the full-length cDNA corresponding to A33-35, we screened a library derived from human primary myoblasts with a probe corresponding to the entire insert from clone A33-35. Three independent clones were obtained, and the longest was sequenced on both strands. Because this cDNA was, by comparison with A33-35, not complete at its 5′ end, a RACE experiment was performed to obtain the complete sequence. By this strategy, two overlapping clones were generated. The complete nucleotide sequence of the novel cDNA, referred to as DRAL (for Down-regulated in Rhabdomyosarcoma LIM protein) is displayed in Fig. 1. The clone isolated from the primary myoblasts library spans the region between bp 103 and 1,433, and contains the complete coding sequence of DRAL. The remaining 102 bp on the 5′ end derive from the RACE experiment. The original A33-35 sequence comprises nucleotides 64–451 of the DRAL cDNA. The length of the composite cDNA, 1,433 nucleotides plus a poly(A) tail, is in agreement with the RNA size estimated from Northern blot analysis (approximately 1,500 nucleotides; see below, Fig. 4). Amino acid prediction of the primary translation product, coded by nucleotides 139–975, gives raise to a protein of 279 amino acids with a calculated molecular weight of 32 kD. This coincides with the molecular weight of the *in vitro* translation product (Fig. 2A). A search in the GenEmbl nucleic acid and the Swiss-prot protein data banks indicated that DRAL encodes a new member of the LIM domain family of proteins (for review, see Sanchez-Garcia and Rabbits, 1994), containing four complete LIM domains and an amino-terminal half-domain corresponding to the second part of the consensus. For comparison, sequence alignments of the LIM domain portions of DRAL with

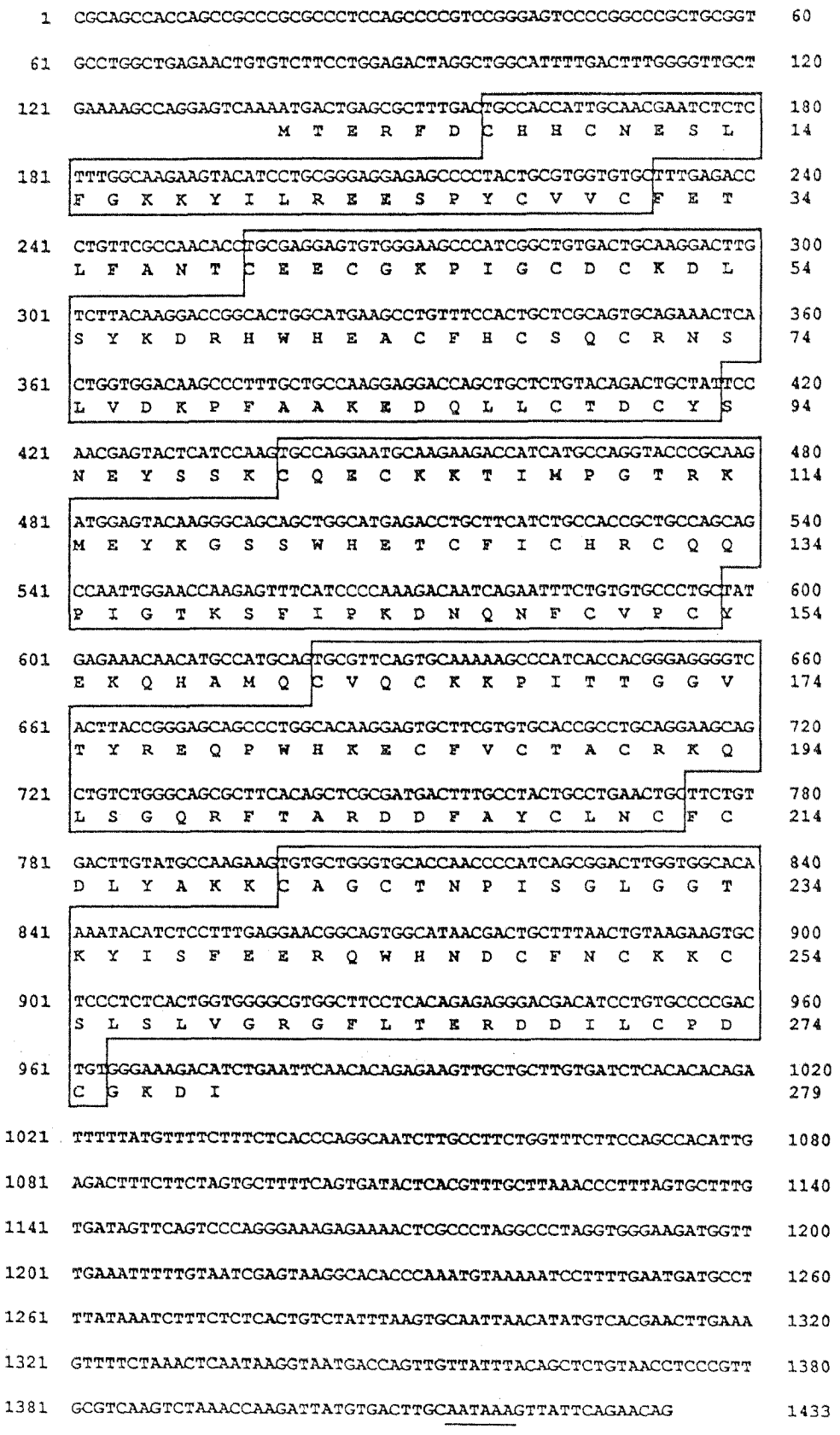


FIG. 1. Nucleotide sequence and deduced amino acid sequence of DRAL cDNA. The deduced amino acid sequence reveals the presence of four complete LIM domains and a half LIM domain at the amino terminus (boxed). The 5′ end of the cDNA was determined in a RACE experiment. Clone A33-35, which was used for hybridization of the cDNA library, comprises nucleotides 64–451 of this cDNA. The putative polyadenylation signal is underlined. These sequence data are available from EMBL/GenBank under accession number L42176.

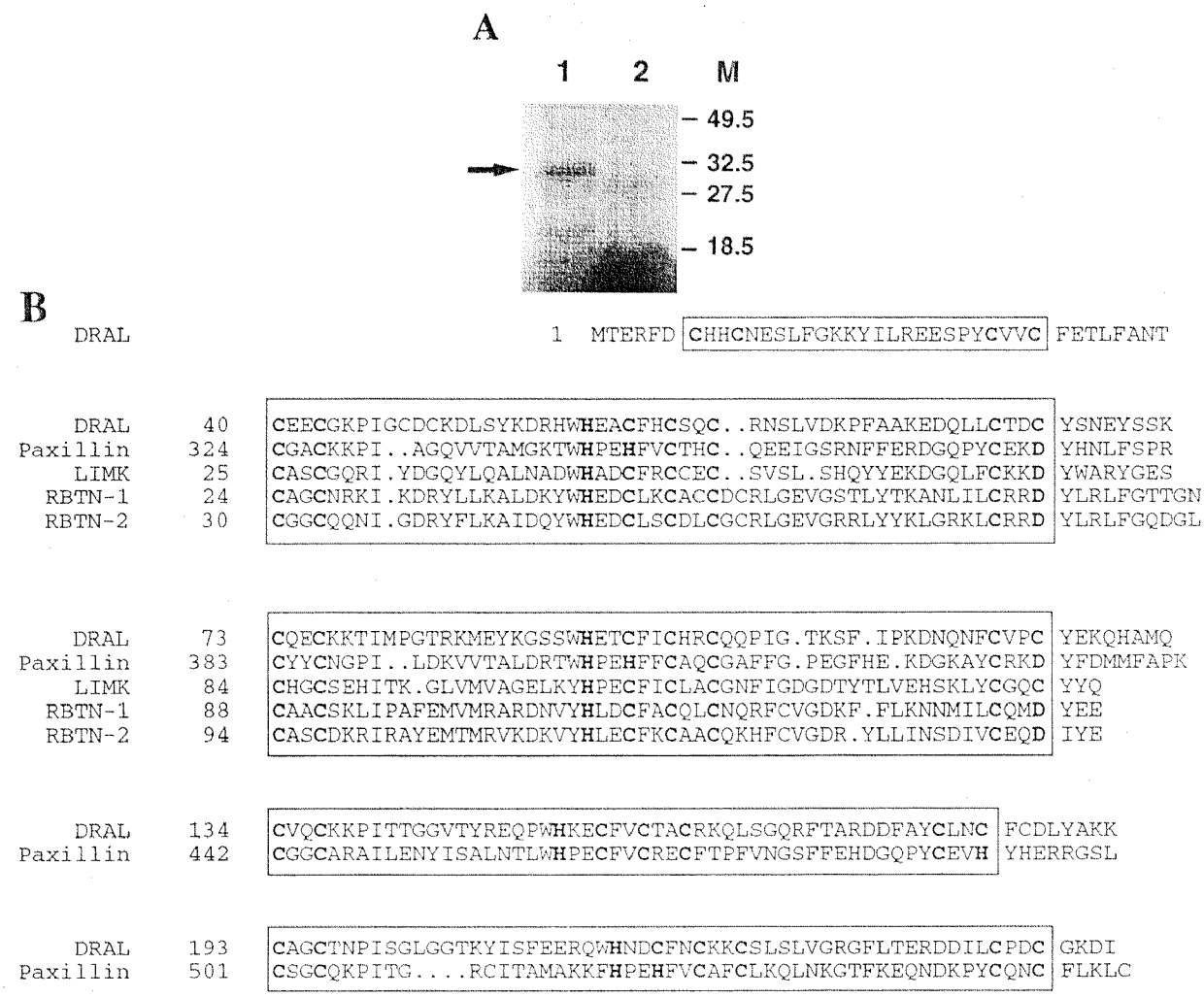


FIG. 2. *In vitro* translation and sequence alignments. A. *In vitro* translation of DRAL cDNA was carried out in the presence of [^{35}S]methionine. The protein products were separated on a 10% NaDodSO$_4$ polyacrylamide gel and visualized by autoradiography. In lane 1, DRAL cDNA was added to the lysate, giving rise to a main protein product of about 30 kD (arrow). As a negative control, in lane 2, no cDNA was added to the lysate. Molecular mass markers (M) are also shown (kD). B. Sequence alignments of the LIM-domains (boxed) of DRAL with other cloned human LIM-domain proteins. The putative metal-binding amino acid residues that are conserved between family members are shown in bold.

other cloned human LIM domain proteins are shown (Fig. 2B). All cysteine, histidine, and aspartate residues required for the formation of the two zinc fingers in each domain are strictly conserved within the amino acid sequence. Interestingly, the length of the spacer region between the LIM domains is conserved in DRAL, being always 8 amino acids. This feature has also been observed in Paxillin and LIMK (Mizuno *et al.*, 1994; Turner and Miller, 1994).

Conservation of DRAL among species and chromosome localization

To answer the question of whether only one gene codes for DRAL and if it is conserved among species, we performed zoo-blot analysis. Genomic DNA isolated from human, pig, mouse, rat, chicken, *Drosophila,* and macaco was hybridized with a 5′-end human probe covering the region between hp 1–150 of DRAL cDNA. The results in Fig. 3 show that DRAL is conserved in all species tested except in *Drosophila,* suggesting that the gene is specific for vertebrates. Furthermore, in all species tested but human and chicken, a single band was detected, implying the presence of a single gene. The presence of multiple bands in human and chicken is most likely due to cross hybridization, since the intensity of one band is stronger than the others. This suggests the existence of a single gene in human and chicken as well.

The localization of the DRAL gene in the human genome was investigated by *in situ* hybridization on metaphase spreads. In the 100 metaphases cells examined, 36.7% of the silver grains were located on chromosome 2; 86.8% of them mapped to the q12–q14 region of the long arm of chromosome 2. The single chromosomal locus supports the notion that a single gene encodes DRAL. No alteration of this particular chromosomal region associated with RMS has been described.

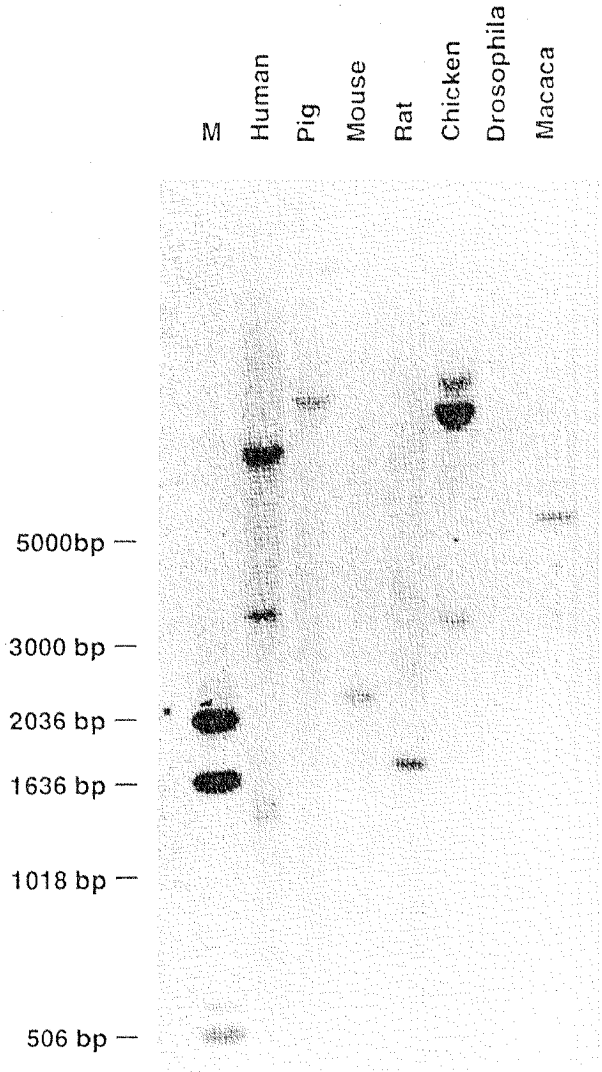


FIG. 3. Zoo blot analysis of DRAL. Ten micrograms genomic DNA from the indicated species was digested by *Hind* III and loaded on each lane. A 5′-end fragment of DRAL (150 bp) was labeled by random priming with ^{32}P and used for hybridization. Molecular weight markers are indicated (lane M).

Tissue distribution of DRAL

Tissue distribution of DRAL was examined on Northern blots containing mRNAs isolated from various adult human tissues (Fig. 4). DRAL was expressed at high levels in heart and ovary. Skeletal muscle, prostate, testis, small intestine, and colon expressed lower levels of DRAL, whereas in other tissues, its expression was barely or not at all detectable. The high expression in heart suggests that DRAL may have a function in the specification or maintenance of the terminal differentiated phenotype in heart muscle cells. Because DRAL cDNA has been isolated from a human primary myoblast library and its transcripts are present at high levels in these cells, we expected skeletal muscle to express comparable high levels of DRAL mRNA. The fact that in skeletal muscle its transcripts are expressed at relatively low levels suggests that DRAL might be a protein whose high expression is needed during embryogenesis for correct muscle development, but is sufficient in low amount to maintain the terminally differentiated skeletal muscle phenotype.

Expression of DRAL in tumor cells

To study whether the differential expression of DRAL was limited to the cells used for subtractive hybridization or was a more general phenomenon extendable to other types of cells, we carried out Northern blots containing RNAs derived from normal and tumorigenic cells (Fig. 5A). In the RMS cell line RD, DRAL transcripts could not be detected, and the other RMS lines Rh1, Rh18, and Rh30 expressed only low levels of DRAL mRNA. The human primary myoblasts A33 and B6M express DRAL transcripts at similar levels throughout differentiation. In human primary lung fibroblasts the level of mRNA was slightly reduced when compared to the myoblasts. Interestingly, DRAL was also expressed in the benign breast cancer cell line HBL100, whereas it is down regulated in the aggressive, metastatic MDA 231 cells (Fig. 5A). From these results, we conclude that the down-regulation of DRAL is not confined to RD RMS cells, but is a more general feature observed in several RMS (both embryonal and alveolar) and other unrelated tumor cells.

Subcellular localization of DRAL

To gain more insight into the possible function of DRAL, we attempted to determine its subcellular localization. To this end an antibody was raised against a glutathione-*S*-transferase fusion peptide comprising amino acids 2–279 of DRAL (Fig. 6A) and tested on Western blots containing recombinant DRAL, A33 primary myoblasts, and RD RMS cell extracts as well as extracts derived from human left and right ventricles (Fig. 6B,

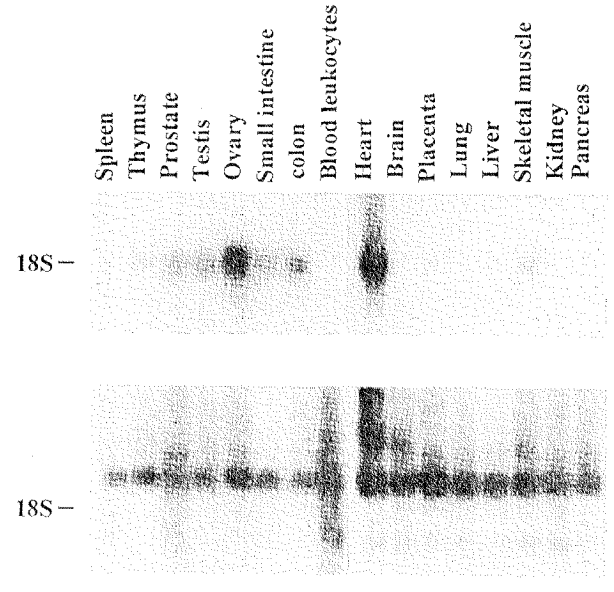


FIG. 4. Expression of DRAL in adult human tissue. Human multiple tissue Northern blots (Clontech Laboratories, Palo Alto, CA) containing 2 μg of poly $(A)^+$RNA isolated from the indicated tissues were used for hybridization. The insert from clone A33-35 was labeled by random priming with ^{32}P and used as a probe for hybridization. The positions of the molecular weight marker are indicated. Rehybridization with a β-actin probe is shown as a control for loading of RNA.

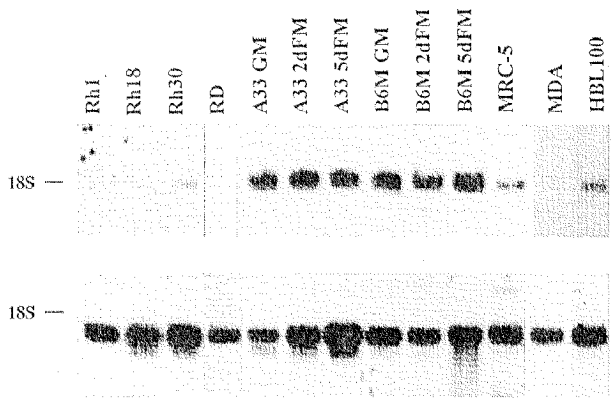


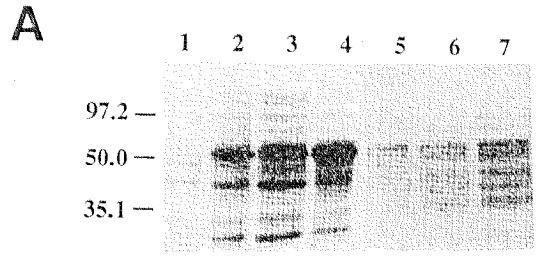
FIG. 5. Expression of DRAL in tumor cells. Northern blot analysis of DRAL transcripts. Each lane contains 5 μg of total RNA of the indicated cells. RD, Rh1, Rh18, and Rh30 are human rhabdomyosarcoma cell lines. B6M/7 and A33/9 are human primary myoblasts isolated from a 6-month-old boy (gastrocnemius) and from a 33-week-old boy (quadriceps), respectively. GM indicates that the cells were cultivated in growth medium. 2dFM and 5dFM indicate that the cells were grown in fusion medium for 2 days or 5 days, respectively. The blot was rehybridized with a β-actin probe as a control for RNA quantities.

lanes 1–5, respectively). A33 myoblasts and the human left and right ventricles (lanes 2, 4, and 5) were found to contain DRAL, whereas RD cells (lane 3) did not show any expression of the protein, confirming the results obtained with Northern blots (Figs. 4 and 5). The fact that recombinant DRAL, after cleavage with endoprotease factor Xa, (lane 1) is slightly smaller than the endogenous protein might be the consequence of lacking post-translational modifications or of some proteolytic degradation of DRAL in the bacterial cells. The origin of the upper band migrating at 64 kD is not clear. It might be due to cross-reactivity of the anti-DRAL antibody.

Subsequently, subcellular localization of DRAL was examined by immunofluorescence studies on A33 myoblasts. Although the signal was weak, a predominant nuclear staining was recognizable (data not shown). To confirm this result, DRAL cDNA was transiently transfected into NIH-3T3 fibroblasts and into RD and Rh30 RMS cells under the control of a CMV promoter element (pSCT-DRAL). In both NIH-3T3 and RD cells, a predominant nuclear staining was observed in transfected cells only (Fig. 7A–F). The nuclear staining is present in both methanol- (Fig. 7A,B,D,E) and Triton X100- (Fig. 7C,F) treated cells, indicating that it is not an experimental artifact due to the permeabilization method. In Rh30 cells, DRAL is distributed uniformly between nucleus and cytoplasm (Fig. 7G–I). As negative control, cells transfected with DRAL cDNA in antisense orientation were stained with the antibody. In addition, DRAL-transfected cells were stained with preimmune serum. In both cases, no signal was seen in the immunofluorescence, establishing the specificity of the reaction observed with immune serum (data not shown). In addition, we did not observe association of DRAL with cytoskeletal structures in these experiments.

DISCUSSION

Here we describe the subtractive cloning of DRAL, a novel cDNA encoding a LIM-domain protein with four complete LIM domains and an amino-terminal truncated domain corresponding to the second part of the consensus. The absence of other known structural elements classifies DRAL to the LIM-only proteins (Sanchez-Garcia and Rabbits, 1994; Dawid *et al.*, 1995). Other proteins containing multiple LIM domains have already been described, but DRAL and hic-5 are the only two



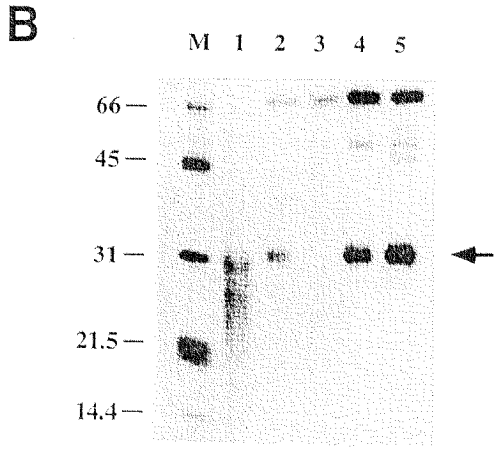


FIG. 6. Western blot analysis of DRAL. A. Expression and purification of GST-DRAL. Transformed bacteria were grown in medium containing either no (lane 1) or 0.1 m*M* IPTG (lanes 2–4) and analyzed for expression of GST-DRAL on a 10% $NaDodSO_4$ polyacrylamide gel. Lane 2, crude lysate of induced BL21-LysS cells; lanes 3 and 4, supernatant and pellet, respectively; lanes 5–7, elution fractions of GST-DRAL from glutathione agarose. Protein was extracted from 500 ml of induced bacterial culture and mixed with glutathione agarose beads. Three elution steps with 500 μl of solution containing 15 m*M* glutathione were accomplished. Forty microliters of each fraction was loaded on a 10% $NaDodSO_4$ gel and stained with Coomassie Blue. B. Western blot analysis of DRAL. Recombinant DRAL after cleavage with factor X (lane 1) and 10-μl extracts from A33 primary myoblasts, RD cells, human heart left ventricle, and human heart right ventricle (lanes 2–5 respectively) were transferred to PVDF membrane and reacted with DRAL antiserum. The position of DRAL is indicated by an arrow.

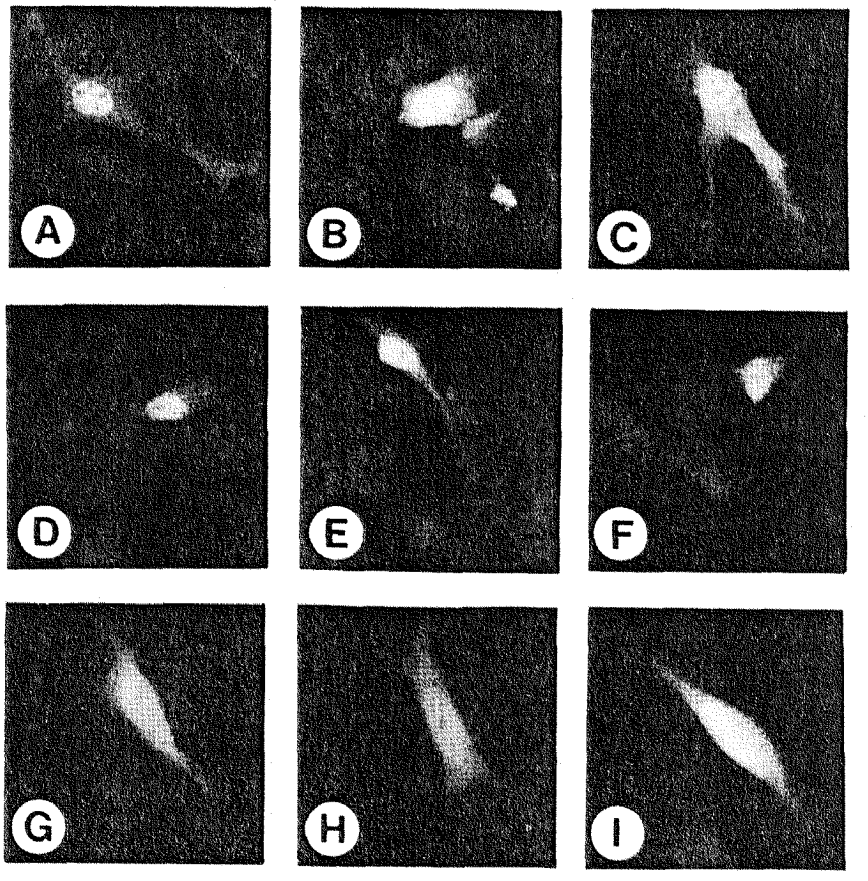


FIG. 7. Subcellular localization of DRAL. NIH-3T3 (A,B,C), RD (D,E,F), and RH30 cells (G,H,I) were transiently transfected with DRAL cDNA cloned into a CMV-driven expression vector. Transfected cells were stained with antiserum recognizing DRAL. In A, B, D, E, G, and H, the cells were permeabilized with 100% methanol. In C, F, and I, the cells were permeabilized with 0.5% Triton X100.

genes encoding a protein with an incomplete LIM domain. Similar to DRAL, three domains of hic-5 are complete with two zinc binding sites; the fourth is a half-domain with only one zinc-binding site (Shibanuma *et al.*, 1994). The function of this half-domain is at the moment not known.

As expected, Northern blot analysis showed that DRAL is expressed in human primary myoblasts and down-regulated in tumor cells. Interestingly, down-regulation of DRAL occurs in both embryonal and alveolar rhabdomyosarcoma subtypes, supporting the hypothesis of a general mechanism affecting DRAL gene expression in tumor cells. Other LIM domain proteins are down-regulated in transformed cells. Expression of the ril protein has been shown to be abolished in *ras*-tranformed mouse fibroblast and to be reinduced in phenotypic revertants (Kiess *et al.*, 1995). However, the function of ril in normal cells and the elucidation of its role in the process of malignant transformation and phenotypic reversion is not clear. The transforming growth factor-β (TGF-β)-inducible hic-5 gene is also associated with cellular growth control and transformation. Its expression is repressed in *ras*-transformed mouse cell lines and in many human tumor-derived cell lines. In addition, hic-5 shows some cytostatic effect on cellular growth, which is probably associated with cellular senescence and terminal differentiation (Shibanuma *et al.*, 1994). Another protein that seems to be responsible for the promotion of differentiation is MLP (Arber *et al.*, 1994). Its expression is enriched in striated muscles and occurs concomitantly with terminal muscle differentiation. Interestingly, MLP is also highly expressed in the heart, suggesting that LIM domain proteins in general might be particularly important factors for the specification or maintenance of the heart phenotype.

Gene expression can be affected by chromosome alterations leading to gene rearrangement or to complete deletions. To test the possibility that down-regulation of DRAL in rhabdomyosarcoma might be the consequence of genomic alterations, we performed Southern blot analysis with RD RMS cells and normal human genomic DNA. The experiment demonstrated that the gene coding for DRAL is not deleted in RMS cells (data not shown). However, we were not able to answer the question about gene rearrangements. In fact, already the two normal human samples we tested together with the RD genomic DNA showed different patterns, and therefore we cannot exclude the presence of a polymorphism in the DRAL gene. To clarify this problem, it is necessary to analyze several samples from normal individuals and compare them with samples of RMS origin. In addition, it would be very helpful to know the genomic sequence of DRAL to directly observe whether the gene is rearranged in RMS cells.

Dameron *et al.* (1994) showed that the expression of thrombospondin-1, an inhibitor of angiogenesis (Rastinejad *et al.*, 1989; Tolsma *et al.*, 1993), is regulated by the tumorsuppressor gene p53. Because RD RMS cells have a mutated p53 gene (Stratton *et al.*, 1990; Felix *et al.*, 1992), experiments are currently being performed to verify if expression of DRAL could be restored by introduction of wild-type p53 into these cells. RD cells are also known to contain mutated forms of the N-*ras* and K-*ras* oncogenes (Stratton *et al.*, 1989). These mutated oncogenes are able to inhibit myogenic differentiation, thus preventing the expression of muscle-specific genes (Gosset *et al.*, 1988; Sternberg *et al.*, 1989), and might therefore also be responsible for the down-regulation of DRAL. This mechanism has already been shown for another LIM domain protein, ril, which is down-regulated in H-*ras*-transformed fibroblasts and restored in phenotypic revertants (Kiess *et al.*, 1995).

Subcellular localization of DRAL was studied by immunofluorescence in A33 primary myoblasts and in different cell lines transfected with DRAL cDNA. In the A33 primary myoblasts, NIH-3T3 cells, and RD cells, DRAL was predominantly present in the nucleus, whereas in C2C12 and RH30 cells DRAL was localized in nucleus as well as in cytoplasm. This is strikingly similar to the distribution observed for RBTN-2 (Neale *et al.*, 1995) and for metallothionein-1 (Danielson *et al.*, 1982; Young *et al.*, 1991). It has been argued that the LIM domains may be involved in protein–protein interactions (Sanchez-Garcia and Rabbits, 1994; Dawid *et al.*, 1995), and thus the specific function of DRAL may depend upon subcellular sequestration through interaction with different co-factors. Moreover, the subcellular distribution of DRAL is similar to the one observed with MLP, which accumulates both in the nucleus and along actin-containing filaments in the cytoplasm (Arber and Caroni, 1996). This, together with the similar distribution in heart, supports the hypothesis that DRAL and MLP might have similar functions in muscle development.

Of particular interest is the association of LIM-domain proteins with members of the helix–loop–helix family of proteins. It was shown that hamster Lmx-1 is functionally linked to a basic helix–loop–helix protein in a way that seems to be dependent on the LIM domains (German *et al.*, 1992). In addition, RBTN-2 and the basic helix–loop–helix protein tal1 are co-expressed (Warren *et al.*, 1994) and form a complex with each other in erythroid cells, which seems to be crucial for normal erythroid development (Valge-Archer, 1994). In muscle cells, several helix–loop–helix proteins like the muscle-specific regulatory factors (Ludolph and Konieczny, 1995) and the immunoglobulin enhancer-binding factors E12 and E47 (Murre *et al.*, 1989) orchestrate the program of tissue-specific gene transcription, leading to myogenic differentiation. Hence, it might be possible that DRAL interacts with these helix–loop–helix proteins, and thereby influences the regulatory pathway leading to normal muscle cell differentiation.

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5.3 DRAL is a p53 responsive gene whose four and a half LIM domain protein product can induce apoptosis

(submitted)

DRAL is a p53 responsive gene whose four and a half LIM domain protein product can induce apoptosis

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Running title: DRAL, a p53 inducible LIM domain protein

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Abstract

DRAL is a four-and-half LIM domain protein identified because of its differential expression between normal human myoblasts and the malignant counterparts, rhabdomyosarcoma cells. In the current study, we demonstrate that transcription of DRAL can be stimulated by wt p53, since transient expression of functional p53 in rhabdomyosarcoma cells as well as stimulation of endogenous p53 by ionizing radiation in wild type cells enhances DRAL mRNA levels. In support of these observations, five potential p53 target sites could be identified in the promoter region of the human DRAL gene. To obtain insight into the possible functions of DRAL, ectopic expression experiments were performed. Interestingly, DRAL expression efficiently triggered apoptosis in three cell lines of different origin to the extent that no cells could be generated which stably overexpressed the protein. However, transient transfection experiments allowed for the localization of DRAL to different cellular compartments, namely cytoplasm, nucleus, focal contacts, as well as Z-discs in cardiac myofibrils. These data suggest that downregulation of DRAL might be involved in tumor development. Furthermore, DRAL expression might be important for heart function.

Introduction

Development of cancer results from multiple genetic events occurring in a single cell and culminating in the inactivation and/or malfunction of several key proteins. Among these, the tumor suppressor gene p53 is the most frequently mutated gene in human cancer (El-Deiry, 1998; Harris, 1996). p53 plays a central role in cells by inducing or repressing transcription of a multitude of target genes. Transcriptional regulation of target genes by p53 requires the presence of two copies of the 10 bp motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0 to 13 bp (El-Deiry, et al., 1992). This tandem motif can be located either in the 5' region, in introns, or the 3' regions of the target genes. Although it has been suggested that there may be several hundred p53 inducible genes (Tokino, et al., 1994), only about 50 have been identified and described to date. These can be subdivided into several specific groups according to their function, such as involvement in G1 arrest, DNA repair, G2/M arrest, and apoptosis. Among the target genes which participate in the apoptotic pathway (reviewed in Amundson, et al., 1998) are proteins like BAX, IGF-BP3, FAS, DR5, PAG608, and TRID (Buckbinder, et al., 1995; Israeli, et al., 1997; Miyashita and Reed, 1995; Owen-Schaub, et al., 1995; Sheikh, et al., 1998; Sheikh, et al., 1999; Wu, et al., 1997) which are induced, whereas Bcl-2, MAP4 (a microtubule stabilizing protein), and IGFI-R levels are repressed by p53 (Miyashita, et al., 1994; Murphy, et al., 1996; Prisco, et al., 1997). However, the sole absence of one target gene is not sufficient to block the functions of p53. Recently, a number of additional p53 inducible genes involved in apoptosis or growth arrest have been identified by the SAGE technique (Polyak, et al., 1997). Since the inducible genes exhibited variable kinetics of induction, p53 might act not only on multiple target genes, but also at different time points. Whereas most of the above mentioned target genes are ubiquitously expressed, there must also exist p53 inducible genes which act in a cell type dependent manner, as shown for example, for the muscle specific phosphoglycerate mutase gene which is induced by p53 specifically in muscle cells (Ruiz-Lozano, et al., 1999).

Our previous studies focused on the identification of potential new tumor suppressor genes from a pediatric sarcoma tumor model. For this purpose we performed a subtractive cDNA library screening between normal cultured myoblasts and the human embryonal rhabdomyosarcoma (RMS) cell line RD (Genini, et al., 1996). 48 genes were identified as being downregulated in RD cells, 19 of which were coding for unknown genes. One of these genes was subsequently characterized as a novel LIM-domain

protein and named DRAL (down-regulated in rhabdomyosarcoma LIM protein, now also called SLIM3, or FHL-2) (Genini, et al., 1997).

The LIM domain is a cysteine-rich domain of approximately 50 amino acids which folds into two specialized zinc fingers and is involved primarily in protein/protein interactions (for reviews see Dawid, et al., 1998; Sanchez-Garcia and Rabbitts, 1994; Schmeichel and Beckerle, 1994). DRAL was the first four and a half LIM domain (FHL) protein discovered. Now four additional proteins have been assigned to this subfamily, namely SLIM1 (FHL1) and SLIM2 (FHL3) both mainly expressed in skeletal muscle (Greene, et al., 1999; Morgan, et al., 1995), and ACT and FHL4 with highest expression in testis (Fimia, et al., 1999; Morgan and Madgwick, 1999). All these proteins range in size from 31 to 35 kD. Thus far, none of these FHL proteins have been implicated in cancer development and not much is known about their function. However, SLIM1 can interact with the mammalian homologue of the *Drosophila* transcription factor suppressor of hairless (Taniguchi, et al., 1998) and ACT binds to and stimulates the cAMP responsive element modulator CREM. Hence, proteins of the four and a half LIM domain subclass might directly be involved in modulation of transcription. The homologous structure of these five LIM-only proteins suggests that they might have similar functions, but are restricted to different tissues or developmental stages.

Here, DRAL was identified as a p53 responsive gene. Given the potential unique role of DRAL in tumor biology and to obtain insight into possible functions of this protein, we investigated the effects of ectopic DRAL expression and determined its intracellular localization in a range of cell types.

Materials and methods

Cell lines

All cell lines were grown in DME supplemented with 10% fetal bovine serum (Life Technologies, Basel, Switzerland), except for primary myoblasts which were cultured in F12 medium with 15% fetal bovine serum; both media contained 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Basel, Switzerland). The non-muscular cells were maintained in 5% CO_2, muscle cells in 10% CO_2 at 37°C. The human embryonal RMS cell line RD, NIH 3T3 mouse fibroblasts, and COS-1 african green monkey kidney cells were obtained from American Type Culture Collection (Rockville, MD). RD-tsp53 (RD cells expressing a temperature sensitive p53 mutant, amino acid 135 Ala to Val) and RD-Neo cells (vector alone) were generated as described (De Giovanni, et al., 1998). Neonatal rat cardiomyocytes were isolated and maintained as described (Auerbach, et al., 1999).

Northern blot analysis

Total RNA was extracted from different cells by guanidinium-isothiocyanate lysis followed by centrifugation through a 5.7M caesium chloride cushion. It was then separated on a 1% agarose gel in the presence of 2.2M formaldehyde and transferred to Nytran nylon membranes by capillary transfer. Alternatively, a commercially available human RNA Master Blot™ was used (Clontech, Basel, Switzerland). Equal loading of the blots was confirmed by hybridization with β-actin or ubiquitin, respectively. Probes (inserts of the clones A33-35 (nucleotides 64-451 of the DRAL cDNA), A33-89, A33-124, and EST clone 470149 ($p21^{WAF1}$) were generated by random priming (Prime-a-gene, Promega, Madison, WI) with α^{32}P-dATP (NEN, Boston, MA) and used for hybridization at 68°C with QuickHyb Hybridization Solution (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. The membranes were exposed to Kodak X-ray films with intensifying screens at -70°C.

Ionizing radiation (IR) treatment

Primary human myoblasts and RD cells were plated on 10 cm dishes 24 h prior to treatment, for each time point in duplicate. After exposure to 10 Gy of IR (from a ^{137}Cs source) cells were fed with fresh medium and cultured until harvesting.

Cloning of the human DRAL promoter

A human P1 library was screened by PCR (GenomeSystems Inc.) using the DRAL specific primers DRAL-FOR2 5'-ACCCGCAAGATGGAGTA-3' and DRAL-REV3 5'-GCAGGGCACACAGAAATTCTG-3' under the following cycling conditions: 15 s at 94°C, 30 s at 56°C, and 60 s at 72°C for 30 cycles. Southern blot analysis was carried out either using nt 64 to nt 150 of the DRAL cDNA or a 2.5 kb PstI subfragment of the P1 clone as probes with QuickHyb solution according to the manufacturer's procedures (Stratagene). Probes were random primed as described above. Subcloning into pUC18 and sequencing was done according to standard procedures. Putative transcription factor binding sites in the promoter region were identified by computer programs (WebSignalScanProgram 4.05 (TFD database) and MatInspector 2.2).

Plasmid constructions

Two constructs tagging the C-terminal and N-terminal end of the DRAL coding region with the FLAG epitope DYKDDDDK (DRAL-CF and DRAL-NF, respectively) were constructed by PCR amplification from full-length human cDNA using the Expand™ High Fidelity PCR System (Roche, Switzerland) and the following primers which were designed to encode a BamH I (5' end) or Xba I (3' end) restriction site and the FLAG epitope: for DRAL-CF (C-terminally FLAG-tagged DRAL): 5'-CGGGATCCGCCACCATGACTGAGCGCTTTGACTGC-3' and 5'-GCTCTAGATCACTTGTCATCGTCGTCCTTGTAGTCGATGTCTTTCCCACA GTC-3'; and for DRAL-NF (N-terminally FLAG-tagged DRAL): 5'-CGGGATCCGCCACCATGGACTACAAGGACGACGATGACAAGACTGAGCG CTTTGACTGC-3' and 5'-GCTCTAGATCAGATGTCTTTCCCACA-3'. Amplified fragments were digested and ligated into pcDNA3 (Invitrogen, Carlsbad, CA). All constructs were verified by sequencing prior to use.

Transfection and immunofluorescence

RD, NIH 3T3, and COS-1 cells were plated one day prior to transfection on 35 mm dishes (Falcon). For transfections 5 µg of DNA (DRAL, DRAL-CF, DRAL-NF, or pFLAG-CMV-2-BAP as control plasmid (Kodak)) and 5 µl lipofectAMINE™ (Life Technologies, Basel, Switzerland) for each dish were added to the cells in unsupplemented DME. Medium was changed six to eight hours post transfection. Cells were fixed with 3.7% formaldehyde, permeabilized with methanol and then stained with

the anti-FLAG primary monoclonal antibody M2 (Sigma, diluted 1:300), followed by a Cy3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, diluted 1:200). DNA was visualized by Hoechst staining. Cells were viewed through a Zeiss fluorescence microscope and transfected cells were quantified by photographing three representative regions of each plate. Every picture showed 280 to 500 cells, the exact cell number was obtained by counting the Hoechst-stained nuclei. DRAL positive cells were counted as apoptotic when they were round-shaped and displayed condensed nuclei. Three independent experiments were carried out.

Neonatal rat cardiomyocytes were transfected as described (Auerbach, et al., 1999) and stained with the anti-FLAG antibody M2 (see above) and a polyclonal anti-chicken heart myosin binding protein-C antibody (Bähler, et al., 1985) followed by secondary antibodies, FITC-coupled anti-mouse (Cappel, West Chester, PA) and Cy3-coupled anti-rabbit (Jackson, West Grove, PA).

Confocal Microscopy

Cell lines were cultured in 35 mm plastic dishes (Falcon) or on fibronectin coated glass coverslips in 24-well plates. Immunofluorescence labeling was done as described above, except that DNA was labeled using Pico Green (Molecular Probes, Inc., diluted 1:200). Stainings were visualized with a confocal laser scanning microscope consisting of a Leica inverted microscope (DM IRB/E) equipped with an argon/krypton mixed gas laser. Image processing was done on a Silicon Graphics workstation using the software "Imaris" (Bitplane AG, Zürich, Switzerland and (Messerli, et al., 1993))

Results

Identification of DRAL as a p53 inducible gene

Previously, we applied a subtractive cloning procedure to identify molecular changes occurring during progression from normal myoblasts to rhabdomyosarcoma (Genini, et al., 1997). From this experiment, a list of 48 different genes downregulated in the human RMS cell line RD was compiled, among them 19 unknown genes. Since RD cells have a mutation in the p53 tumor suppressor gene (Felix, et al., 1992), we hypothesized that downregulation of some of these genes might be due to lack of wt p53. Indeed, analyzing the cDNAs obtained by the subtractive screen already identified one known p53 target gene, namely thrombospondin-1 (A33-186) (Dameron, et al., 1994). Hence, taking advantage of a RD-tsp53 cell line, which stably expresses a temperature sensitive p53 cDNA ($p53^{Val137}$) that is inactive at 37°C and assumes wild type conformation at 32°C, all unknown genes were screened for possible p53 dependent expression. As control, RD-Neo (vector alone) cells were used. As expected, by dropping the temperature to 32°C, expression of thrompospondin-1 was induced in the RD-tsp53 cells but not in the RD-Neo cells (Fig. 1A). As a further control, enhanced mRNA levels of the p53 target gene $p21^{WAF1}$ were detected in the same experimental series. Analyzing the expression of clones from the subtractive screen then revealed that most clones tested from our library were not induced such as A33-124, or induced solely by dropping the culture temperature to 32°C like A33-89, suggesting that expression of the respective gene might be influenced by stress. In contrast, expression of the previously characterized cDNA coding for DRAL (A33/35) could be substantially stimulated in a p53 dependent manner (Fig. 1A), analogous to the known p53 targets $p21^{WAF1}$ and thrombospondin-1.

To confirm and characterize these initial findings in more detail, gene expression was analyzed in a time dependent manner by extracting RNA at eight different time points after temperature shift (from 2 to 48 h). Northern blot analysis revealed an increase of DRAL mRNA levels specifically in the RD-tsp53 cell line and not in RD-neo cells (Fig. 1B). The increase was highest after 24 h, similar to $p21^{WAF1}$ used as control. Hence, induction of wt p53 in the RD cell line led to the selection of a potential new p53 inducible gene, DRAL, from the initially screened cDNAs.

To determine whether DRAL can also be induced upon activation of endogenous p53, DRAL expression was studied in primary human myoblasts (wt p53) and RD cells (mut p53) following exposure to γ-irradiation, a potent means to activate endogenous p53 (Fig. 1C). As expected, exposure of the cells to 10 Gy of ionizing radiation (IR) resulted

in an increase of $p21^{WAF1}$ and a more modest elevation of DRAL RNA levels in myoblasts, but not in RD cells. The apparent slight increase at the 2h time point is due to a loading effect as revealed by the β-actin loading control. Therefore, maximal increase in RNA levels was seen 24 hours after IR. These data suggest that transcription of endogenous DRAL can be stimulated by irradiation in a p53 dependent manner.

Cloning and partial characterization of the DRAL gene and its 5' upstream region

To gain some insight into the regulation of DRAL expression on the molecular level, the promoter region of the human DRAL gene was cloned. Screening of a human P1 library by PCR using DRAL specific primers (amplifying nt 472 to nt 597 of the DRAL cDNA) resulted in one positive clone, P1:21280. To ensure that no recombination event occurred in the P1 clone, Southern blot analysis was performed with either human genomic DNA or the P1 clone digested with two different restriction enzymes and using a 2.5 kb PstI fragment from the P1 clone as a probe. Since both DNAs revealed the same hybridization pattern, any gross recombination in the relevant region of the P1 clone can be excluded (Fig. 2B). The 7.8 kb HindIII fragment identified in this Southern blot analysis was then further analyzed in detail and its sequence determined completely (available under accession number AF211174). The fragment contained 1.5 kb of upstream sequence and exon 1(63 bp) and exon 2 (51 bp), separated by a 2276 bp intron of the DRAL gene (Fig. 2A). The sequence of this intron is flanked by consensus splice donor and acceptor sites. In the upstream region, a TATA box is present 72 bp upstream of the start of the cDNA (which was isolated by RACE experiments). Therefore, it is likely that the start of transcription is located further upstream and the cDNA extends another 40-50 bp. Additionally, two GC rich sequences (GC-boxes) were identified at positions -132 and -166 (relative to the start of the cDNA). A search for putative sequence elements able to bind upstream regulatory transcription factors within the promoter, identified several sites including one for myocyte enhancer binding factor 2 (MEF-2) at position -890, five E-boxes at positions -480, -508, -708, -878, and -1274 as well as five binding sites for the homeobox protein NKX2.5 at positions -417, -636, -815, -868, and -1426 (only the first four sites for both are indicated in Fig. 2A). The presence of multiple E-boxes might explain expression of DRAL in normal muscle tissue. Additionally, the five NKX2.5 sites are likely to contribute to the strong expression of DRAL in cardiac tissue (see below). Intriguingly, five putative p53 binding sites were identified within the 7.8 kb fragment and designated as p53 I to p53 V

(Fig. 2C). The sites p53 I and p53 II are located in the 5' region whereas the sites p53 III, IV and V are located in intron 2.

In summary, cloning and characterization of the DRAL promoter region identified five potential p53 target sites, further supporting the observed p53 dependent transcriptional activation of the DRAL gene.

Expression pattern of DRAL in normal tissues

To obtain a broad view of DRAL expression in human tissues, we analyzed its mRNA expression pattern in 50 human adult and fetal tissues. The results confirmed previous observations (Genini, et al., 1997) in that DRAL expression was highest in fetal and adult heart followed by ovary (Fig. 3). Equivalent levels of expression were further detected in placenta, uterus, mammary gland, and adrenal gland. In skeletal muscle, colon, bladder, prostate, stomach, trachea, testis, small intestine, thyroid gland, and kidney DRAL mRNA was still clearly detectable. No significant difference in DRAL expression was evident between fetal tissues and their adult counterparts (brain, heart, kidney, liver, spleen, thymus, and lung), suggesting that the average RNA levels are not altered during the developmental period analyzed. Furthermore, it appears that DRAL is only marginally expressed in the brain. The small signal observed for *E. coli* DNA indicates the possibility that related sequences are present in *E. coli*. The predominant expression of DRAL in heart is also reflected by examination of the origin of EST sequences present in the databases, since nearly half of the ESTs coding for DRAL originate from heart cDNA libraries. Hence, the tissue origin of the ESTs correlates with the pattern of DRAL expression seen in the Northern blot analysis. Although DRAL is predominantly expressed in human heart, lower levels of transcription are observed in a wide range of tissues.

Ectopic expression of DRAL induces apoptosis

To gain insight into possible functions of DRAL, an expression vector carrying the DRAL cDNA was transfected into human rhabdomyosarcoma (RD), transformed monkey kidney (COS-1), and normal mouse fibroblast (NIH 3T3) cells. After selection with neomycin only a small number of clones was obtained whereas empty vector readily generated several hundred colonies from a parallel transfection. Surprisingly, none of the clones stably expressed DRAL protein (data not shown). To analyze the possible reason for this phenomenon, transient transfection experiments were

performed in the same cells. Since DRAL consists only of LIM domains, the previously raised polyclonal antibody might also interact with other LIM domain proteins. To eliminate this possibility, two plasmids were constructed producing epitope tagged DRAL protein, namely DRAL-CF (C-terminally FLAG-tagged DRAL) and DRAL-NF (N-terminally FLAG-tagged DRAL). As control in these experiments, the pFLAG-CMV-2-BAP plasmid (FLAG-tagged bacterial alkaline phosphatase) was used. Expression of FLAG-tagged proteins was analyzed by indirect immunofluorescence staining together with Hoechst DNA staining at different time points after transfection. In all cells highest FLAG-DRAL expression was detected after 32 h. Interestingly, the number of FLAG-DRAL positive cells rapidly declined afterwards. At the same time labeled remnants were observed in the culture which were reminiscent of apoptotic bodies (Fig. 4). Neither decline in expressing cells nor cell remnants were observed with FLAG-BAP, indicating that the effects observed are specific for DRAL. In addition, no difference was observed between DRAL-CF and DRAL-NF constructs. To unambigously identify apoptotic cells, simultaneous stainings were performed with either pico green labeled DNA (Fig. 5) or annexin V (data not shown). These experiments confirmed that FLAG-DRAL expressing cells indeed underwent apoptosis. A quantitative summary of the results is shown in Figure 6. A dramatic increase in the number of apoptotic cells (70% - 90% after 72h depending on cell type) was paralleled by a rapid decline in the number of transfected cells in transfections with both FLAG-DRAL constructs. In contrast, expression of pFLAG-CMV-2-BAP resulted in a constant number of transfected cells over the observation period and no specific increase in apoptotic cells.

We conclude that ectopic expression of DRAL specifically induced apoptosis in all cell types analyzed, including human rhabdomyosarcoma cells. This notion is consistent with a possible role for DRAL as a tumor suppressor molecule.

Subcellular localization of DRAL

To obtain some hints at potential protein/protein interactions which might be functionally relevant, the subcellular localization of DRAL was studied after transient transfection of FLAG-DRAL into the same cell types used before (RD, COS-1 and NIH 3T3). The previously reported predominant nuclear staining of DRAL (Genini, et al., 1997) was confirmed in RD and NIH 3T3, to a lesser extent in COS-1 cells (Fig. 7). In addition, in some cells uniform staining was observed (Fig. 7D, G), sometimes even with

exclusion of the nucleus (Fig. 7H, I). Interestingly, DRAL was also detected in the cellular periphery where spreading occurs, most likely resembling focal contact staining, in all three cell lines (Fig. 7A, B, C). Indeed, this conclusion could be confirmed by colocalization of DRAL with vinculin (data not shown). No difference was detected in these experiments in regard to the position of the FLAG epitope on either side of DRAL.

Since highest expression of DRAL was found in fetal and adult heart, we also wished to examine the subcellular localization of DRAL in cardiomyocytes. To this end neonatal rat cardiomyocytes were transiently transfected with either FLAG-DRAL (Fig. 8) or the FLAG-BAP plasmid (data not shown). Interestingly, in addition to the already described localization of DRAL in the nucleus and at the cell membrane, a distinct cross-striated pattern was observed. Double staining of the same cells with the myosin binding protein-C and subsequent overlapping of the two staining patterns revealed that DRAL localizes specifically to the Z-discs and, to a lesser extent, to the M-band of myofibrils (Fig. 8C and F, small insert). In cardiomyocytes transfected with the FLAG-BAP plasmid only completely diffuse labeling could be observed, indicating that the localization is specific for DRAL (data not shown).

In these experiments, DRAL could be localized specifically in the nucleus, the focal contacts and the Z-discs as well as the M-band of heart myofibrils. Presumably, DRAL will have a different protein interaction partner at each of these locations.

Discussion

This study identifies a member of the FHL subfamily of LIM-only proteins, DRAL, as a novel target gene for the tumor suppressor protein p53. Intriguingly, DRAL protein was capable of efficiently triggering apoptosis in a wide range of cell types upon ectopic expression.

We provide three different experimental criteria suggesting that DRAL might be a direct transcriptional target of p53. First, induction of wild type p53 in rhabdomyosarcoma cells through a temperature sensitive p53 allele specifically increased transcription of endogenous DRAL, in a manner comparable to known p53 target genes like $p21^{WAF1}$ and thrombospondin-1. Second, exposure of primary myoblasts to γ-irradiation induced an increase in DRAL mRNA that paralleled the increase in $p21^{WAF1}$ mRNA. No increase was seen in cells which express mutated p53, like rhabdomyosarcoma cells. The moderate induction of DRAL observed in the irradiation experiments might be due to the specific dose of radiation used, which also failed to induce apoptosis in these cells (data not shown). Finally, analysis of the genomic structure of DRAL revealed five potential consensus p53 binding sites.

In Northern blot experiments, DRAL expression was predominantly found in fetal and adult heart. The five putative NKX2.5 binding sites identified in the promoter region are in concurrence with this expression pattern. In fact the homeobox gene Nkx2.5 represents the earliest known marker of the cardiac lineage in vertebrates and its expression is maintained throughout the developing and adult heart (Schwartz and Olson, 1999). Additionally, a MEF-2 binding site was found in the DRAL promoter. MEF-2 factors are known coregulators for myogenic basic helix loop helix proteins and play a pivotal role in determination and differentiation of skeletal and cardiac muscle cells (Black and Olson, 1998). Finally, five E boxes which can be found in the control regions of many genes specifically expressed in skeletal muscle, are likely to contribute to expression of DRAL in skeletal muscle.

Since transcription of DRAL can be induced by wild type p53, the next question was if DRAL would participate in any of the known functions of p53 like growth arrest or apoptosis. To test this notion, ectopic expression of DRAL was achieved in a range of cell types including human rhabdomyosarcoma cells. Interestingly, this resulted in efficient induction of apoptosis in all cell types analyzed. Indeed, we were unable to generate stable cell lines expressing DRAL. Even rhabdomyosarcoma cells ectopically expressing Bcl-2 (to protect cells from apoptosis) failed to produce DRAL expressing

clones (results not shown). Therefore, DRAL might participate in the apoptotic response following activation of p53. Very recently, another LIM-only protein was described to induce apoptosis in myoblasts upon ectopic expression, namely the paxillin homologue Hic-5 (Hu, et al., 1999) which is a LIM-only protein containing four LIM domains. Hic-5 is also known to bind to cell adhesion kinase β which can induce apoptosis in fibroblasts (Xiong and Parsons, 1997) and probably other cell types. Remarkably, like DRAL, Hic-5 levels are downregulated in transformed cells compared to normal cells (Shibanuma, et al., 1994). Since a number of other LIM proteins are downregulated in transformed cells, such as Ril (Kiess, et al., 1995) and Zyxin, (Schenker and Trueb, 1998) DRAL might not be the only LIM domain protein to play a role in tumor development.

To begin to investigate the mechanisms by which ectopic expression of DRAL can induce apoptosis, its intracellular localization was determined. Staining of RD-tsp53 cells with the polyclonal DRAL antibody at the permissive temperature provided the first evidence for localization of DRAL at focal contacts (data not shown). To avoid potential cross reactions of the antibody with other LIM domain proteins, FLAG-tagged protein was expressed for all subsequent localization studies. Using this approach, DRAL was found in focal contacts, the cytoplasm, nucleus and the Z-discs of myofibrils. Focal contacts are known to establish a transmembrane linkage between the actin cytoskeleton and the extracellular matrix and are implicated in a number of signaling pathways. Some of the proteins at these specialized sites (e.g. focal adhesion kinase, paxillin) serve as scaffolding molecules and can act as signaling centers by providing docking sites for other proteins (Burridge and Chrzanowska-Wodnicka, 1996). Intriguingly, several of these scaffolding molecules are characterized LIM domain proteins, such as Hic-5, paxillin, PINCH, and zyxin, (Beckerle, 1997; Hagmann, et al., 1998; Salgia, et al., 1995; Tu, et al., 1999). Hence, DRAL might fulfil a similar function at this site.

On the other hand, nuclear localization of DRAL might also be responsible or contribute to its pro-apoptotic function. Interestingly, dual localization in focal contacts and the nucleus is known for a number of other protein, e.g. zyxin, Hic-5 and β-catenin, a multi-functional protein activated by the Wnt signalling pathway (for recent review see Miller, et al., 1999). Whether DRAL localization could be influenced by external signals in a manner analogous to β-catenin is currently not known.

Since high levels of DRAL have been found in fetal and adult heart, it was important to identify its subcellular localization in this tissue. There, DRAL protein was additionally detected in association with cardiac myofibrils, both at the Z-discs and in the M-band. The Z-disc localization is shared with a second LIM domain protein, MLP (Arber, et al., 1997), whose expression seems to be very important for the structural organization of myofibrils, since mice deficient in this protein show a disrupted cytoarchitecture leading to dilated cardiomyopathy and finally heart failure. A possible function of DRAL in this respect will have to be investigated. MLP also shares nuclear expression with DRAL, but only MLP is associated with the actin filaments. Hence, the two protein might have some overlapping, but not completely redundant functions.

Although the knowledge of the function of FHL proteins is still very limited, they may act either as scaffolding molecules to link several proteins and thus activating distinct signaling pathways or as modulators of transcription by binding to transcription factors or both. The possible role of DRAL as a p53 dependent class II tumor suppressor molecule might involve one of these mechanisms.

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Abbreviations used in this paper:

BAP	bacterial alkaline phosphatase
FHL	four and a half LIM domain
RMS	rhabdomyosarcoma
DRAL	down-regulated in rhabdomyosarcoma LIM protein
DRAL-CF	C-terminally FLAG-tagged DRAL
DRAL-NF	N-terminally FLAG-tagged DRAL
MEF-2	myocyte enhancer binding factor 2

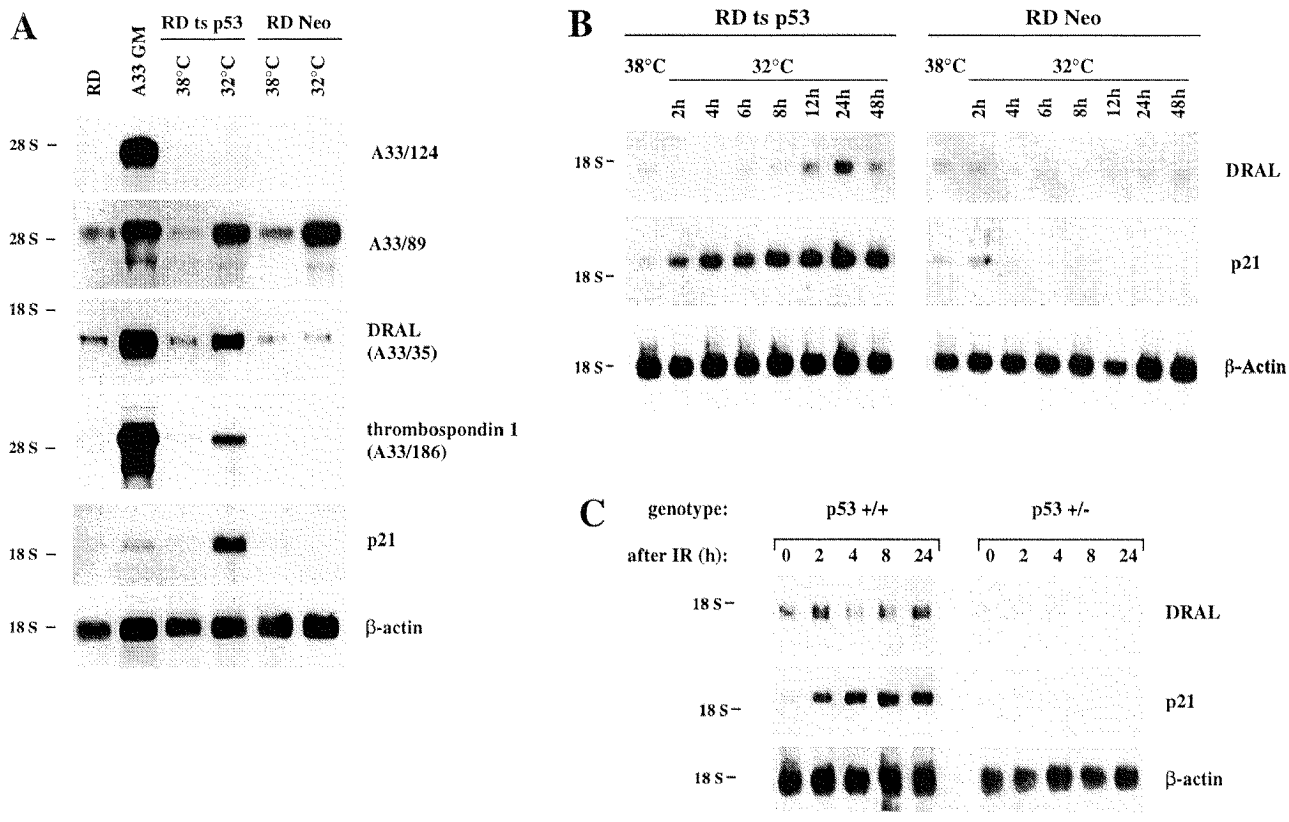


Fig. 1 A) Screening for p53 inducible genes. 5 µg total RNA from the indicated human cell lines was loaded in each lane and hybridized with the labeled cDNAs A33/124, A33/89, A33/35, A33/186, $p21^{WAF1}$ and β-actin. RD are rhabdomyosarcoma cells, A33 GM are primary myoblasts kept in growth medium, RD-tsp53 are RD cells expressing a temperature sensitive p53 mutant, and RD-Neo are RD cells expressing the vector alone. The mutant p53 protein is inactive at 38°C, but dropping the temperature to 32°C leads to wild type p53 protein functions. RNA was extracted 1 day after temperature shift. B) Time course analyses of p53 target gene induction. Total RNA was extracted from RD-tsp53 or RD-Neo cells either grown at 38°C or 32°C for the indicated time. 3 µg total RNA was loaded in each lane and hybridized with the labeled cDNAs coding for DRAL, $p21^{WAF1}$ and β-actin. C) Induction of DRAL expression after γ-irradiation. Normal human myoblasts (p53+/+) and RD rhabdomyosarcoma cells (p53+/–) were treated with 10 Gy, and afterwards fed with fresh medium. Total RNA was isolated at the time points indicated and subjected to Northern blot analysis (3 µg/lane). Inserts from the indicated cDNAs were isolated, labeled by random priming, and used for hybridization. The blot was also probed for β-actin as a control for equal loading and transfer.

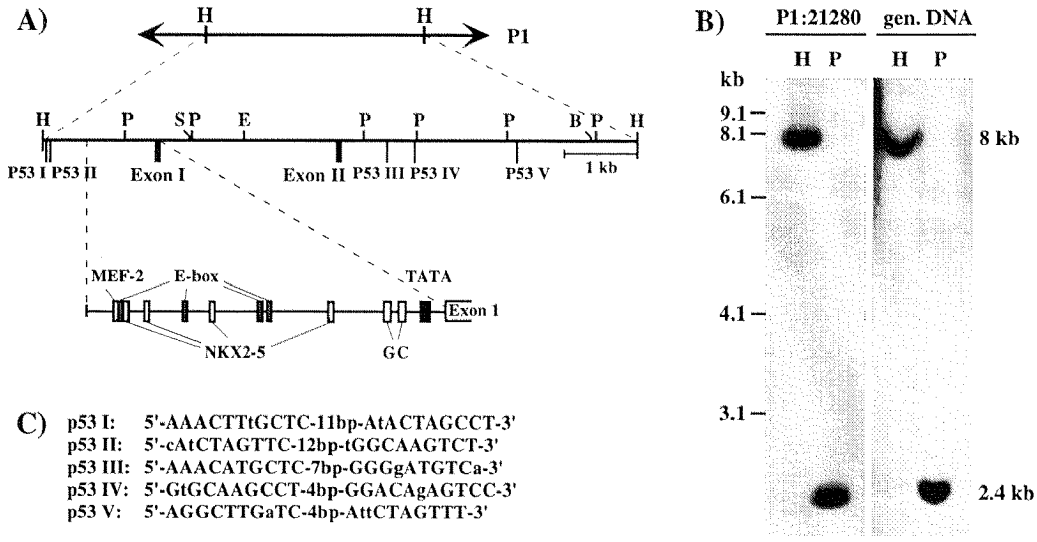
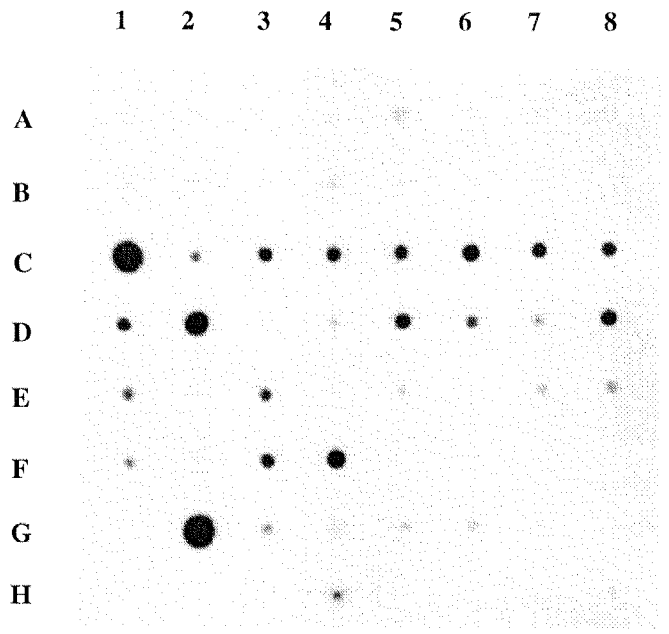


Fig. 2 Partial genomic organization of the human DRAL gene. A) The 7.8 kb HindIII fragment of the P1 clone 21280 contains the first two non-coding exons (exon 1: nt 1 to nt 63, exon 2: nt 64 to nt 114 of the DRAL cDNA). Letters correspond to the restriction sites H = HindIII, P = PstI, S = SstI, E = EcoRI, and B = BamHI. Putative transcription factor binding sites in the promoter region are indicated. The five putative p53 binding sites within the 7.8 kb subclone are designated as sites p53 I - p53 V, sequences are listed separately in C. B) Southern blot analysis using either human genomic DNA or the P1:21280 clone digested with HindIII (H) and PstI (P). The blot was hybridized with the 2.5 kb PstI fragment of the 7.8 kb subclone containing exon II. The sequence of the 7.8 kb HindIII fragment is available from Genbank under accession number AF211174.



	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	sub-thalamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	*kidney*	*liver*	small intestine	*spleen*	*thymus*	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	
H	yeast total RNA 100 ng	yeast tRNA 100 ng	*E. coli* rRNA 100 ng	*E. coli* DNA 100 ng	Poly r(A) 100 ng	human C_0t1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

Fig. 3 Expression pattern of DRAL in normal human fetal and adult tissues. A commercially available human RNA dot blot containing normalized quantities of mRNA per dot was hybridized with a labeled DRAL cDNA probe. The tissues used for mRNA extraction are indicated in the lower panel.

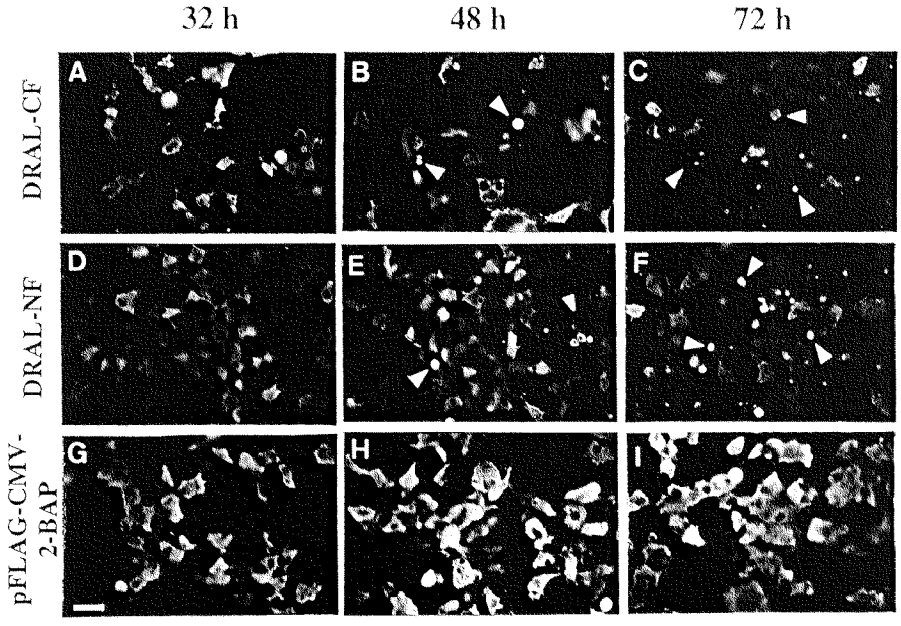


Fig. 4 Time course of DRAL expression in COS-1 cells. Cells were transfected with either DRAL-CF (A-C), DRAL-NF (D-F), or the control plasmid pFLAG-CMV-2-BAP (G-I). After 32, 48, and 72 hours cells were fixed and stained with the monoclonal anti FLAG antibody. Pictures were taken from representative areas. The scale bar line is valid for all the pictures and indicates 100μm. Arrows point to antibody labeled remnants, which were absent in the transfections with the control pFLAG-CMV-2-BAP construct.

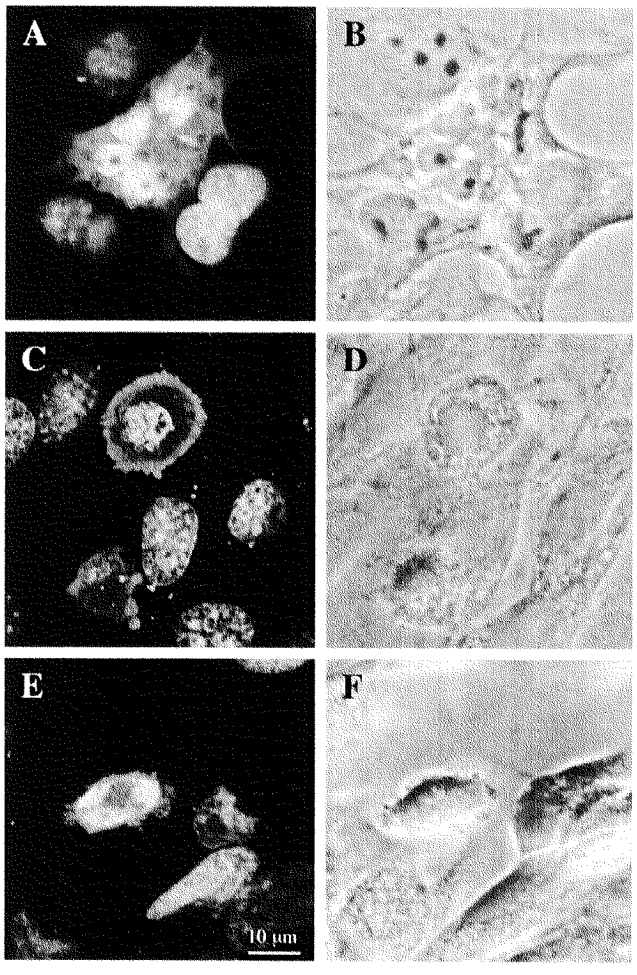


Fig. 5 Induction of apoptosis by ectopic expression of DRAL. COS-1 (A, B), NIH 3T3 (C, D), and RD (E, F) cells were transiently transfected with FLAG-tagged DRAL. A, C, and E are composite confocal microscopy images of DRAL (red) and DNA (green) staining. B, D, and F represent the corresponding phase contrast picture. The scale bar is the same for each picture, and is 10 μm.

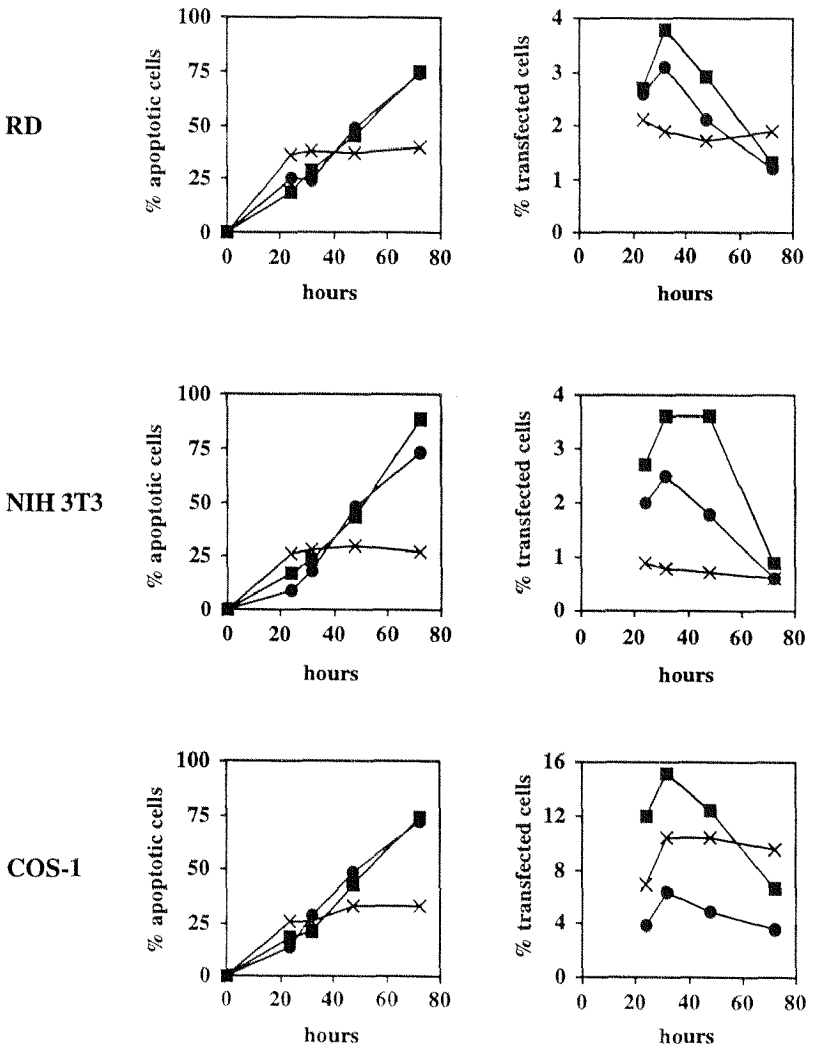


Fig. 6 Quantitative analysis of the events following ectopic expression of DRAL. COS-1, RD, and NIH 3T3 cells were transfected with DRAL-CF (circle), DRAL-NF (square), or the control plasmid pFLAG-CMV-2-BAP (cross). After 24, 32, 48, and 72 hours, cells were fixed and stained for FLAG and Hoechst. Data are shown as mean values representing three independent transfection experiments. % apoptotic cells refers to rounded FLAG positive cells displaying nuclear condensation and fragmentation in percent of the total number of FLAG positive cells. In the right hand panels, transfected cells were counted as FLAG positive cells and indicated as percentage of total cell number during time course.

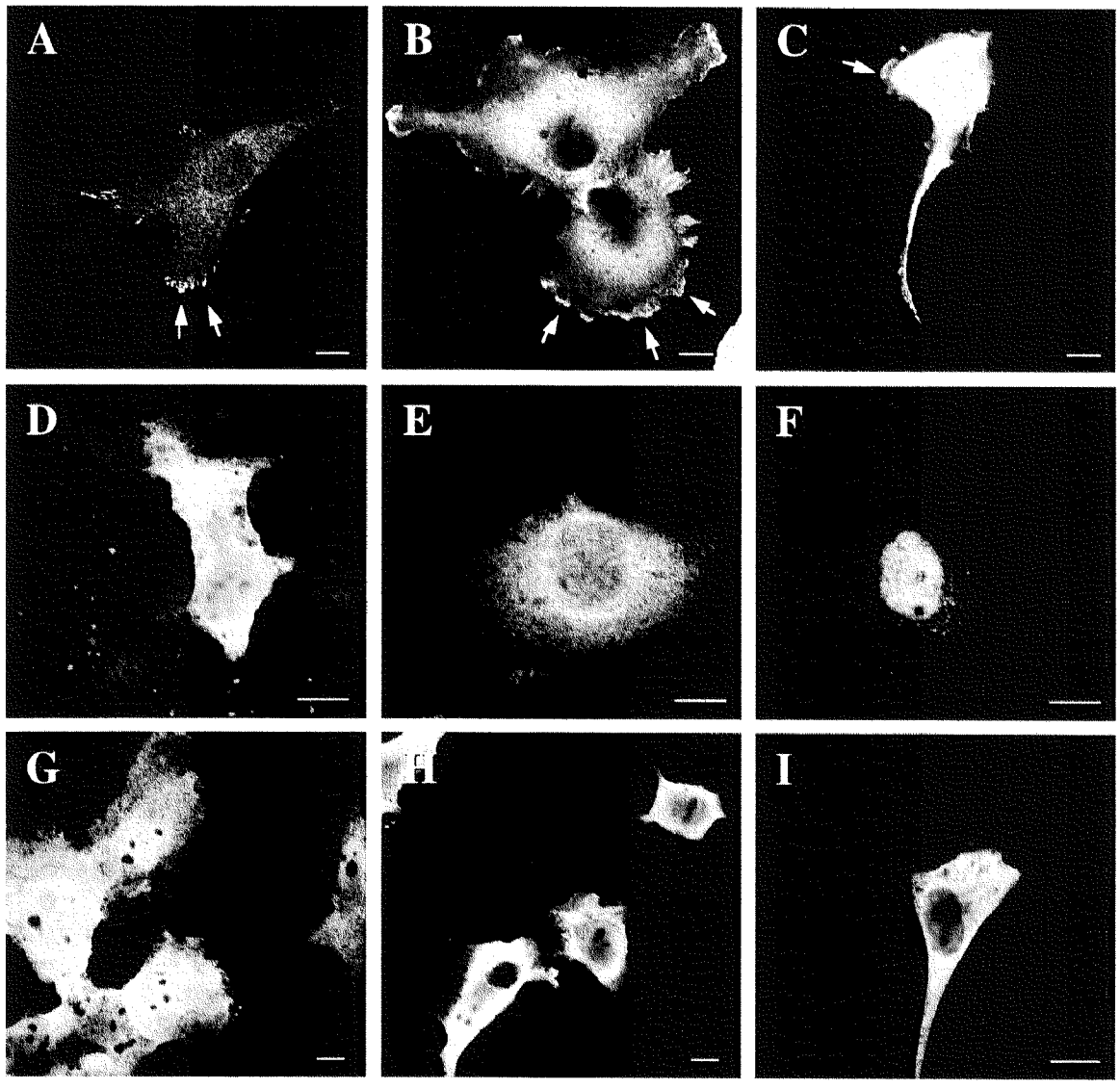


Fig. 7 Subcellular localization of DRAL. NIH 3T3 (A, I), COS-1 (B, G, H), and RD (C-F) cells were cultured on fibronectin coated cover slips or plastic dishes and transiently transfected with FLAG-DRAL. Cells were immunostained with anti-FLAG monoclonal antibody M2 and Cy3-conjugated goat anti-mouse polyclonal antibody and analyzed by confocal fluorescence microscopy. The arrows point to the focal contacts. The scale bars indicate 10 μm.

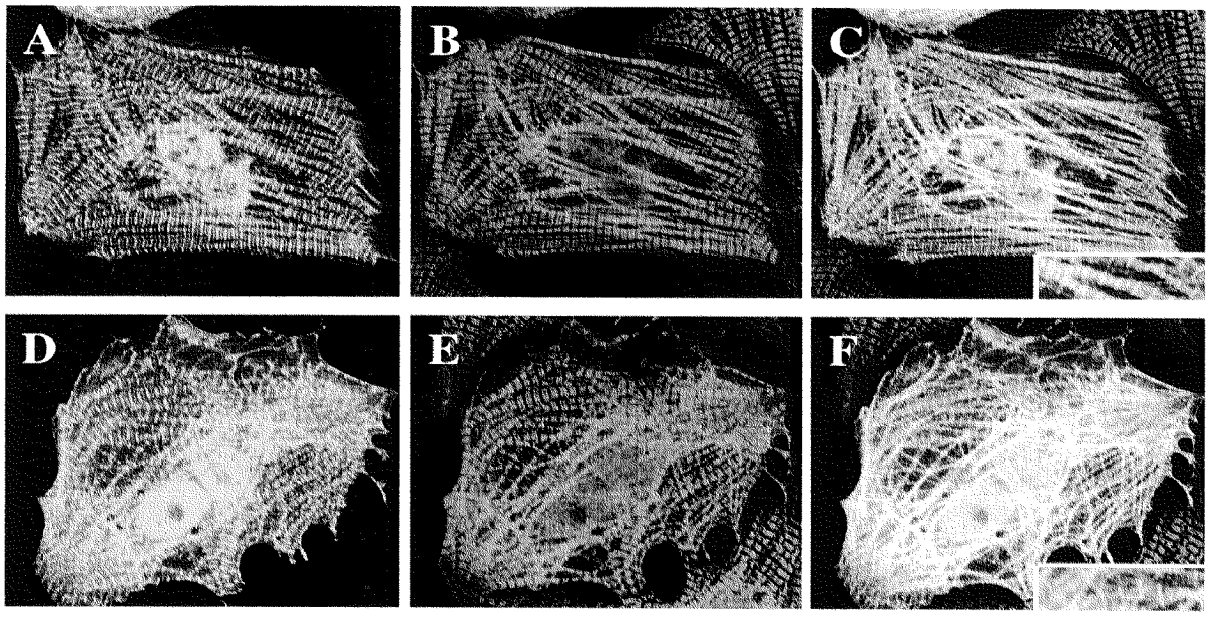


Fig. 8 Subcellular localization of DRAL in cardiomyocytes. Neonatal rat cardiomyocytes were transiently transfected with FLAG-DRAL (DRAL-NF in A-C, DRAL-CF in D-F) and immunostained with anti-FLAG monoclonal antibody (A, D) or anti-myosin binding protein-C antibody (B, E). C and F represent superposition of A and B or D and E, respectively. The insert shows an enlargement of myofibrils. Analysis was carried out with a confocal fluorescence microscope using "Imaris" software.

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5.4 Molecular features of a human rhabdomyosarcoma cell line with spontaneous metastatic progression

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Molecular features of a human rhabdomyosarcoma cell line with spontaneous metastatic progression

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Summary A novel human cell line was established from a primary botryoid rhabdomyosarcoma. Reverse transcription polymerase chain reaction investigations of this cell line, called RUCH-2, demonstrated expression of the regulatory factors PAX3, Myf3 and Myf5. After 3.5 months in culture, cells underwent a crisis after which Myf3 and Myf5 could no longer be detected, whereas PAX3 expression remained constant over the entire period. Karyotype analysis revealed breakpoints in regions similar to previously described alterations in primary rhabdomyosarcoma tumour samples. Interestingly, cells progressed to a metastatic phenotype, as observed by enhanced invasiveness in vitro and tumour growth in nude mice in vivo. On the molecular level, microarray analysis before and after progression identified extensive changes in the composition of the extracellular matrix. As expected, down-regulation of tissue inhibitors of metalloproteinases and up-regulation of matrix metalloproteinases were observed. Extensive down-regulation of several death receptors of the tumour necrosis factor family suggests that these cells might have an altered response to appropriate apoptotic stimuli. The RUCH-2 cell line represents a cellular model to study multistep tumorigenesis in human rhabdomyosarcoma, allowing molecular comparison of tumorigenic versus metastatic cancer cells. © 2000 Cancer Research Campaign

Keywords: rhabdomyosarcoma; myogenic transcription factors; PAX; metastasis; cytogenetic alteration

Rhabdomyosarcoma (RMS), a solid tumour of skeletal muscle origin, is the most common soft tissue tumour in children, accounting for approximately 5–8% of all paediatric neoplasias (Pappo, 1995). Histologically, it can be divided into four categories: embryonal, botryoid, alveolar, or pleomorphic rhabdomyosarcoma (Newton et al, 1995). The embryonal type (eRMS) is more prevalent and accounts for 50–60% of all cases. It has a more favourable prognosis affecting predominantly children under 15 years of age in the head-and-neck region. It is characterized by frequent loss of heterozygosity on chromosome 11p15.5 (Scrable et al, 1987), expression of myogenic regulatory factors (Tonin et al, 1991) and deregulated expression of PAX3 and/or PAX7 (Schäfer et al, 1994). Botryoid RMS, a subgroup of the embryonal type with usually the best prognosis, is almost exclusively found in young children. Its frequency is about 4–8% of all RMS. The majority of this RMS type is found in mucosa-lined hollow organs, such as the nasal cavity, nasopharynx, bile duct, urinary bladder and vagina. Alveolar RMS (aRMS), an aggressive tumour occurring in children and young adults, is the second most common type of RMS (18–20%) and is characterized by one of the following specific translocations: t(1;13)(p36;q14) PAX7/FKHR or t(2;13)(q35;q14) PAX3/FKHR (Barr et al, 1993, 1998; Galili et al, 1993; Shapiro et al, 1993; Davis et al, 1994).

Unlike other tissue types, muscle development can serve as paradigm for understanding the molecular basis of cell lineage establishment and differentiation. The current picture of myogenesis is based on the isolation and characterization of mainly two families of transcriptional regulators, the muscle regulatory factors (MRF, including Myf3 (MyoD), Myf4 (myogenin), Myf5 and Myf6 (MRF4)) and the myocyte enhancer factor 2 (MEF2) family (Ludolph and Konieczny, 1995). Since differentiation and proliferation are mutually exclusive events, muscle cells can also serve as a model to investigate cell cycle control. In contrast to normal myoblasts, RMS cells are blocked in the differentiation process and have lost growth control.

To facilitate the study of multistage carcinogenesis it is necessary to establish cell cultures that can be used as tumour models in vitro. Although few RMS cell lines are available, each line has its specific advantages and disadvantages. The most common used culture model is the RD cell line that dates from 1969. Like this cell line, many of the RMS cell lines were derived from relapse tumours or metastasis, which might not fully reflect the properties of the original RMS tumour and does not allow to study tumour progression in vitro. One additional problem is an uncertainty regarding the origin of some of the lines available. This is especially true when cells were established from eRMS, have no specific translocations, and lost in some cases expression of the characteristic myogenic regulators like Myf3 (MyoD). Then the generally accepted interpretation would suggest that the original tumour was misdiagnosed and the cells represent a different tumour, e.g. Ewing's sarcoma or primitive neuroectodermal tumour (PNET).

To obtain additional insight into RMS tumour development, we started an initiative to isolate and establish additional well characterized cell lines of RMS origin. Our interest is to find and investigate additional factors which might influence the balance between growth and differentiation in RMS cells, allowing the molecular characterization of the mechanisms implicated in multistage development of this neoplasm. In this work, we describe the isolation of a new human cell line derived from a primary botryoid RMS, and its evolution during time in culture.

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MATERIALS AND METHODS

Clinical history

The patient was a 15.5-month old white girl. The tumour was diagnosed unequivocally as a botryoid rhabdomyosarcoma in the vagina, belonging to group III of the Intergroup RMS Study Surgical-Pathological Grouping Classification. The immunohistochemistry showed strong staining with antibodies against vimentin, desmin, α-muscle actin and some weak myoglobin. A tumour biopsy was taken before any treatment and used to establish the RUCH-2 cell line. The patient was subsequently treated with standard chemotherapy and is still in remission.

Mice

Nude mice were maintained under spf (specific pathogens-free) conditions in the animal facility of the University of Zurich (Central Biological Laboratory). Experiments were conducted according to the Swiss ordinance for animal experiments.

Establishment of the RUCH-2 cell line

Primary tumour fragments from the biopsy were injected subcutaneously (s.c.) into 4- to 6-weeks old female ICR *nu/nu* mice. The xenograft was removed after two passages and minced under aseptic conditions. The tissue pieces were seeded into a 100-mm culture dish containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units ml^{-1} penicillin 100 μg ml^{-1} streptomycin, and maintained in an incubator at 37°C and 10% carbon dioxide (CO_2). The culture medium was changed three times a week. After reaching confluence, tumour cells were passaged 1–3 times a week using trypsin solution. RUCH-2 cells were routinely monitored for mycoplasma and murine cell contamination by fluorescent staining with Hoechst 33258, and found to be free of both.

Cell lines

Human cell lines were cultured in DMEM supplemented with 10% FBS and 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in 10% CO_2 and 37°C. The embryonal RMS cell line RD was obtained from ATCC (Rockville, MD, USA) (McAllister et al, 1969). The embryonal RMS Rh1 and the alveolar RMS Rh30 cell lines were a generous gift from Dr PJ Houghton (St Jude Children's Research Hospital, Memphis, TN, USA). The alveolar RMS cell line RC2 was kindly provided by Dr C De Giovanni (Istituto di Cancerologia, Bologna, Italy) (Nanni et al, 1986).

Chromosomal studies

Metaphase preparations were obtained according to standard cytogenetic methods or for the tumour biopsy as previously described (Betts et al, 1997). Along with the original tumour, the RUCH-2 cell line was karyotyped at passages 3, 20, 32, 46, 62, 84 and 104. Cytogenetic analysis and interpretation were made according to ISCN (1995).

Tumorigenicity of cell lines in vivo

To evaluate the tumorigenic capacity of the RUCH-2 cell line, cells at passage 30 were injected s.c. into the dorsal region of three females C57Bl/6 *nu/nu* mice (5–7 weeks old). Each mouse received 100-μl cell suspension containing 5×10^6 viable cells as determined by trypan blue exclusion. As control an additional mouse was injected with the same amount of FUCH-1 cells (normal human fibroblast cell line of the same patient at passage 6) and two further mice were injected with the same amount of RD cells. The experiment was repeated at passage 67 of the RUCH-2 cell line, whereby ICR *nu/nu* mice (6–8 weeks old) were used. Control FUCH-1 and RD cells grew similarly in both mouse strains.

Invasion assay

The Biocoat® Matrigel® Invasion Chamber was used to perform invasion assays (Becton Dickinson Labware, Lot 906658). The chambers, placed in a 24-well plate, were rehydrated with 250 μl DMEM for 2 h at room temperature. Single-cell suspensions of different passages of the RUCH-2 cell line were obtained with 5 mM EDTA/PBS (phosphate-buffered saline). The cells were resuspended in 0.1% bovine serum albumin (BSA)/DMEM to a final volume of 1×10^5 cells ml^{-1}. Then, 750 μl 5% fetal bovine serum (FBS)/DMEM (antibiotica-free) was added to the wells as a chemoattractant and 500 μl of cell suspension was added to each chamber. After 22 h non-invasive cells were removed from the upper surface of the membrane with a cotton swab. The chambers were then fixed with ice-cold methanol for 10 min and stained with crystal violet. The membranes were removed, placed on coverslips and invasive cells were counted under the microscope.

RT-PCR analyses

Reverse transcription polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR System Kit (Promega) in a 25 μl reaction volume with 100 ng total RNA. As positive and negative controls, RNA from the established rhabdomyosarcoma cell lines RD, Rh1, Rh30, or RC2 were used. Total RNA was extracted using guanidinium-isothiocyanate lysis followed by centrifugation through a 5.7 M caesium chloride (CsCl) cushion. All reactions started with reverse transcription at 48°C for 45 min, followed by a 2-min initial denaturation step at 94°C, and 40 cycles at 94°C for 30 s, 60 or 68°C for 1 min, 68°C for 1 min, with a final extension at 68°C for 7 min on a PCR system (Perkin-Elmer Cetus). The 68°C annealing temperature was used only for the Myf-3 amplification. The FKHR, PAX3 and PAX7 products were generated in 0.5 mM magnesium sulphate ($MgSO_4$); the PAX3PAX7/FKHR, Myf-3, and Myf-5 in 1.0 mM $MgSO_4$. The following primers were used to amplify a 324-bp long fragment of FKHR [*FKHR-F5* 5′-GCAGATCTACGAGTGGATGG-3′ and *FKHR-F3* 5′-AACTGTGATCCAGGGCTGTC-3′] (Galili et al, 1993), a 349-bp fragment of PAX3 [*PAX3-5′* 5′-GCACTGTACACCAAAGCACG-3′ and *PAX3-REV* 5′-TAGGTGGGTGGACAGTAGGA-3′], a 414-bp fragment of PAX7 [*PAX7-3* 5′-GGCGTAAGCAGGCAGGAG-3′ and *PAX7-4* 5′-GCAGCGGGGAGATGGAGA-3′], a 868-bp resp. 862-bp fragment for the translocations PAX3/FKHR resp. PAX7/FKHR [*PAX3PAX7* 5′-CCAAACACAGCATCGACG-3′ (this primer anneals to both PAX3 and PAX7) (Davis et al, 1994) and *FKHR-F3*], a 159-bp fragment of Myf-3 [*MYF3-FOR* 5′-CTGTGGGCCTGCAAGGCGTGCAAG-3′ and *MYF3-REV* 5′-CACCTTGGGCAACCGCTGGTTTGG-3′] (Anand et al, 1994), and finally a 612-bp fragment of Myf-5 [*MYF5-FOR1* 5′-AGCCTGCAAGAGGAAGTCC-3′ and

MYF5-REV 5′-AGCCTTCTTCGTCCTGTGTA-3′]. One-third of the RT-PCR products were separated by agarose gel electrophoresis.

Hybridization of arrays

Atlas™ Human Cancer cDNA Expression Arrays were purchased from Clontech (Lot 8090622). mRNAs (RUCH-2 at passage 11 and passage 63) were obtained by isolation of total RNA (CsCl-centrifugation), followed by treatment with RQ1 RNAase-free DNase I (Promega). Thereafter poly-A^+ RNA was isolated with a mRNA isolation kit (Boehringer Mannheim), finishing with a phenol:chloroform:isoamylalcohol (25:24:1) extraction and ethanol precipitation. ^{32}P-labelled cDNA probes were prepared from 1 μg of mRNA. Both probes, with a final concentration of 2×10^6 cpm ml^{-1}, were hybridized to separate arrays according to the user manual. The membranes were exposed to Kodak X-ray films with intensifying screens at – 70°C for different time points. After hybridization, the results were analysed by autoradiography, normalizing the blots according to the transcriptional abundance of the housekeeping genes included on the array (ubiquitin, GAPDH, α-tubulin, β-actin, 23-kDa highly basic protein, ribosomal protein S9). As criteria for changes in gene expression, the intensity difference had to be at least twofold, whereas a more than fourfold difference was designated as strong alteration.

Northern blot analyses

Total RNA from different passages of RUCH-2 cells was obtained as previously described under RT-PCR analyses. Five micrograms of total RNA were used per lane. The probes (inserts coding for cyclin A (EST clone 2394165), p21 (EST clone 470149), MMP-3 (EST clone 2113735) and the human TIMP-3, Myf3 and Myf5) were generated by random priming (Prime-a-gene, Promega) with α^{32}P-dATP and used for hybridization at 68°C with QuickHyb Hybridization Solution (Stratagene) according to the manufacturer's instructions. The membrane was exposed to Kodak X-ray films and was reprobed thereafter.

RESULTS

Isolation of the cell line

To establish a cell line from the tumour specimen, mice were subcutaneously (s.c.) injected with minced tumour pieces. After 5 weeks, the tumour was resected and placed in culture. In parallel, some tumour material was injected again into a nude mouse. In culture, morphologically distinct cells grew rapidly out of the tumour pieces; however, the homogenous cell population finally resulting after several passages was identified as human fibroblasts by morphology, karyotype analysis, as well as RT-PCR experiments for myogenic markers (see below). Therefore this cell line was called FUCH-1 (for *F*ibroblast of *U*niversity *C*hildren's *H*ospital).

After the second xenograft reached 5 mm in diameter (3 months in total), it was minced and placed in culture. This time no human fibroblasts could be detected by microscopic examination. The cells appeared mostly elongated and spindle-shaped with a few multinucleated cells detectable. Molecular characterization clearly established that these cells are derived from the original tumour (see below). Compared to other RMS cell lines these cells are rather large. The resulting cell line was called RUCH-2

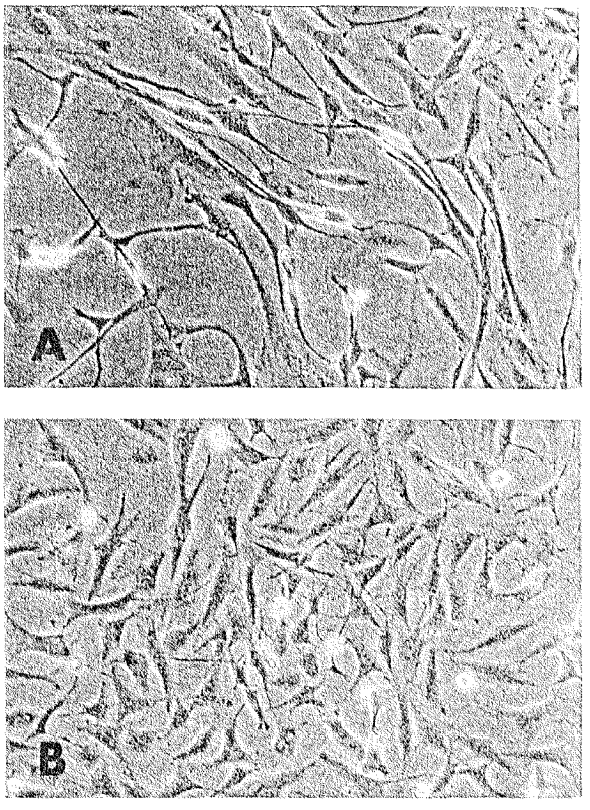

Figure 1 Morphology of RUCH-2 cells. (**A**) Phase contrast picture of low passage (P22) and (**B**) high passage (P63) RUCH-2 cells (100 × magnification)

(*R*habdomyosarcoma of *U*niversity *C*hildren's *H*ospital) and is in continuous culture since 16 July 1997. As expected, the cells underwent a crisis in culture around passage 32–38 (~ 3.5 months in culture). They became extremely flat and almost stopped dividing. Then, they started to proliferate again which was accompanied by phenotypic changes to polygonal homogenous cells, resembling myogenic precursor cells (Figure 1). These morphological observations were later confirmed on the molecular level (see below).

The RUCH-2 cells were split 1:2 every 65–72 h until passage 30 (P30), and every 48 h after P47. Interestingly, cells below P30 synthesized extensive extracellular matrix as evidenced by a gelatinous appearance of the culture supernatant, which disappeared after P38. These results demonstrate that we could successfully establish a new cell line derived from a botryoid rhabdomyosarcoma.

Karyotype analyses of RUCH-2 cells

To better characterize and monitor possible changes in the RUCH-2 karyotype, cells were analysed at passage 3, 20, 32, 46, 62, 84 and 104 and compared with the original tumour. Cytogenetic investigation of the original tumour revealed a karyotype of:

54~57,X,–X,+7,+inv(7)(q11q32),+8,+8,+12,+13,+13,+19,+20, +21,+r,+0~2mar[cp6].

In comparison, initial analysis of the cell line (at P3) demonstrated a karyotype of:

55,X,–X,+7,+inv(7)(q11q32),+8,+8,+12,+13,+13,+19,+20,+21 /55,idem,del(20)(q13)[cp40].

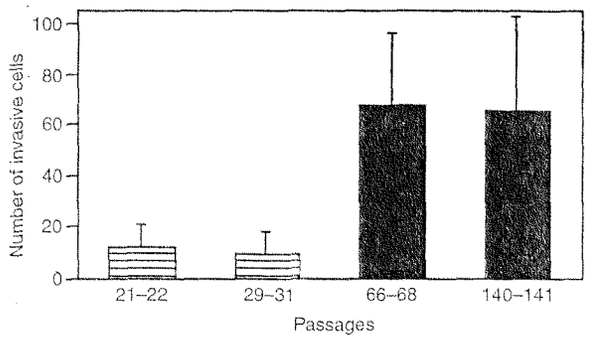

Figure 2 In vitro invasiveness of RUCH-2 cells. Cells at different passages were analysed for their invasive behaviour. Low passage cells, P21–22 and P29–31 (striped bars), were compared with higher passages, P66–68 and P140–141 (black bars). The number of cells which invaded the matrigel was determined after staining with crystal violet and counting under the microscope. Data represent mean values ± s.e.m of three independent experiments

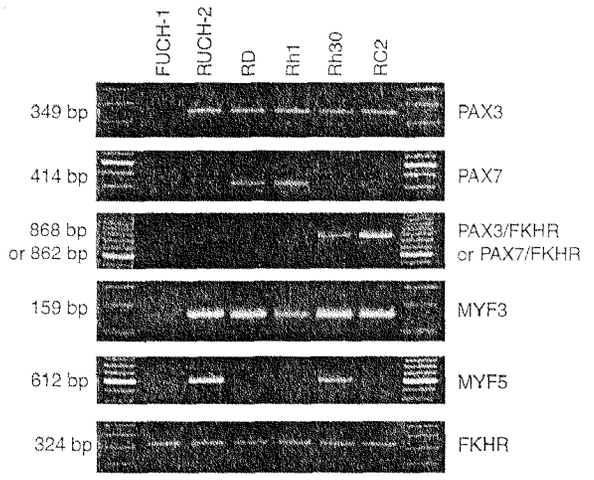

Figure 3 Expression of Pax genes and members of the Myf gene family in human cell lines. One hundred nanograms of total RNA per RT-PCR reaction were used. All cell lines (RUCH-2, RD, Rh1, Rh30, and RC2) are RMS, except FUCH-1, which is a fibroblast line. FKHR was amplified as an internal control for RNA quality. RT-PCR products were electrophoresed in 1–2.5% agarose gels, stained with ethidium bromide. A 100-bp ladder was used as marker (strong band = 600 bp). The expected length of the PCR products are indicated at the left

Hence, this karyotype was essentially identical to the one of the original tumour. Further analysis at passages 20 and 32 displayed a similar karyotype without any major changes. Hyperdiploidy, as seen here, with few structural aberrations appears to constitute a distinct karyotype subgroup in RMS (Wang-Wuu et al, 1988). A different, earlier, study of ten eRMS tumour samples showed gain of the whole or a large part of various chromosomes, notably chromosomes 2 (60% of cases), 7 (50%), 8 (60%), 12 (60%), 13 (60%), 17 (40%), 18 (40%) and 19 (40%) (Weber-Hall et al, 1996). Hence, the original tumour and the cells up to P32 reflect many of these described alterations by displaying a gain of chromosomes 7, 8, 12, 13 and 19.

Interestingly, marked changes in the karyotype were observed starting from P46. Initially, we detected large cell to cell variation with the simplest clone being near triploid and containing multiple additional rearrangements. Also present was a distinct polyploid population and some metaphases with double minutes (dmin). Cells with dmin continued to be present at P62, but were no longer detected at P84. The final analysis at P104 showed a karyotype of:

67,X,add(X)(p11),–X,add(1)(q12)x2,del(1)(p34),+del(1)(p21),–2,t(3;12)(q21;p13),–6,–9,–9,add(9)(p12),–10,der(11)t(1;11)(p22;q22),add(12)(q13),der(12)t(3;12)(q21;p13),–13,–14,add(16)(q12),–17,add(18)(q23),der(18)t(?17;18)(q21;q23),+20,+20,–21,–22,– 22,+4~5mar[cp50].

The above karyotype showed at this point only limited cell to cell variation anymore. However, with the exception of the add(X)(p11) and the del(1)(q12), all major structural rearrangements were already present at P46. In contrast certain rearrangements identified at P46 were lost thereafter, such as a del(2)(p22), add(9)(q34) and add(17)(p11). These might have been apparent precursors for the subsequent loss of the entire chromosomes. Among the abnormalities acquired between P32 and P46 those of del(1p), t(3q), –9, add(9p) and add(18q23) have been previously reported and/or observed in primary RMS samples karyotyped at our hospital (data not shown). Hence, the RUCH-2 karyotype at late passage (P104) had karyotypic similarities to primary metastasis or relapse RMS tumours. Based on these results the changes of RUCH-2 cells observed during evolution in culture show some interesting parallels to normal RMS tumour progression.

Induction of metastasis in late passage RUCH-2 cells

To examine their tumorigenic potential, 5×10^6 RUCH-2 cells at early and late passages were injected s.c. into nude mice. The control cell line FUCH-1, normal human fibroblasts (judged by karyotyping) of the same patient, did not lead to tumour growth even after 3.5 months of observation. In contrast RUCH-2 cells at P30 induced, in two out of three mice, tumours of 4–6 mm in diameter after 3.5 months, which is about a quarter of the size of tumours induced by the established RMS cell line RD. In none of the animals obvious metastases were observed.

Next, we used RUCH-2 cells at P67. Whereas tumours grew to the same size as cells at P30 in all three mice, one animal had to be sacrificed after ~2.5 months because of metastasis. After dissection numerous huge metastases were observed in the liver and the spine. Both, the xenographed tumours and the liver metastases were confirmed to be of RMS origin by immunohistochemical investigations demonstrating desmin, myoglobin and α-skeletal actin staining (data not shown), whereby the metastasis showed more intense staining. A similar result was obtained with a second animal, whereas only a small tumour grew in a third animal. In contrast, no metastases were detected in mice injected with RD cells. These results suggest that RUCH-2 cells progressed in culture to acquire a metastatic phenotype.

In vitro invasivity of the RUCH-2 cell line

The notion that metastases were observed in two out of three mice injected with late passage RUCH-2 cells suggested that the cells gained metastatic properties including the ability to invade extracellular matrix. To confirm this suggestion in vitro, modified Boyden chambers were used to measure the ability of the cells to invade extracellular matrix. At P21–31, 8.7 to 11.5 cells invaded the matrigel, in contrast to P66–141 where 65.5 to 67.7 cells were counted (see Materials and Methods) (Figure 2). Hence, late passage cells were significantly (eightfold) more invasive than cells at early passages supporting the observation that they acquired a more invasive and metastatic phenotype.

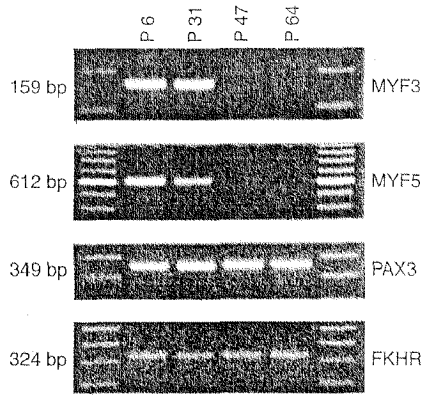

Figure 4 Expression of myogenic regulators at different passages of RUCH-2 cells. RT-PCR reactions were performed with RNA isolated from cells at different passages. FKHR was amplified as an internal control for RNA quality. RT-PCR products were loaded on agarose gels and stained with ethidium bromide. As marker a 100-bp ladder was used (strong band = 600 bp). The expected length of the PCR products are indicated at the left

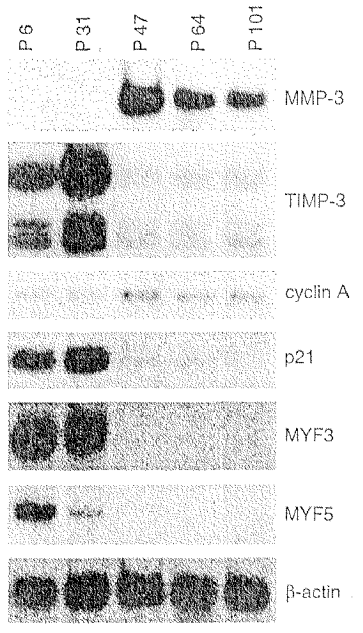

Figure 5 Northern blot analyses of RUCH-2 cells at different passages. The indicated genes (MMP-3, TIMP-3, cyclin A, p21, MYF3, MYF5) were analysed sequentially on the same blot. TIMP-3 showed a downregulation for all three splice variants. The amount of total RNA was confirmed with a β-actin probe

Molecular characterization of RUCH-2

To get insight into possible molecular changes accompanying the observed biological behaviour of RUCH-2 cells, we first analysed the expression pattern of regulatory proteins known to be involved in muscle differentiation. RT-PCR analyses were performed to characterize possible changes in the expression of PAX transcriptional regulators and myogenic determination factors (Myf). As controls, cell lines were used which provide either positive or negative controls for every RT-PCR reaction. FKHR was amplified as a control for RNA quality. As shown in Figure 3, similar FKHR levels could be detected in all samples, indicating integrity of the RNAs. RT-PCR results further showed that RUCH-2 cells express PAX3, similar to other RMS cell lines (RD, Rh1, Rh30 and RC2). Only the corresponding fibroblast cell line did not express PAX3 confirming the aberrant expression of PAX3 in RMS. Neither translocation products (PAX3/FKHR, PAX7/FKHR) nor PAX7 expression could be detected in RUCH-2 cells. Myf3 expression is assumed to be characteristic for RMS tumours and cell lines (Clark et al, 1991) and was also detected in RUCH-2 (Figure 3, lane 2). In addition, Myf5 is expressed in RD and RUCH-2 cells, whereas Myf4 was detected in RD, RC2, and very weakly in Rh30 but not in RUCH-2 and Rh1 cells (data not shown). Since both myogenic markers Myf3 and Myf5 were expressed in RUCH-2 cells at P3, the myogenic origin of the RUCH-2 cell line could be confirmed on the molecular level.

To monitor possible progression in culture, RT-PCR investigations were repeated at passage 6, 31, 47, and 64 (Figure 4). Interestingly, Myf3 and Myf5 transcripts were no longer detected after P31 (results confirmed by Northern blot analyses, see Figure 5), whereas FKHR and PAX3 expression remained constant. Hence, the appearance of a metastatic population was associated with changes in the karyotype as well as alterations in the expression levels of myogenic regulatory factors, Myf3 and Myf5, whose expression was down-regulated in late passage RUCH-2 cells.

Differential gene expression in early and late passage RUCH-2 cells

To monitor global changes in expression pattern of genes likely to be involved in tumour progression, expression of 588 cancer and metastasis-related genes were simultaneously examined using cDNA microarray hybridization. Gene expression was monitored using ^{33}P-labelled cDNA generated from RNA isolated from cells at P11 and P63. Ninety-two genes displayed altered expression levels between non-metastatic and metastatic cells, representing almost every sixth gene investigated. Of these 92 genes, 64 were down-regulated in high passage RUCH-2 cells, 17 of them strongly. In contrast, 28 investigated cDNAs were up-regulated including five strong ones. To facilitate the identification of potentially important genes and because of the large number of alterations observed, three functional categories were selected and examined in more detail, namely apoptosis-related genes, proteases and protease inhibitors, and cell cycle regulators. Genes from these categories showing altered expression are listed in Table 1. The complete list of the 92 differentially expressed genes in all categories can be viewed at our homepage: *http://www.unizh.ch/kispi/clinchem_html/schafer.html*. To independently confirm the alterations observed in the microarray, additional Northern blot analyses were performed using several different passages of RUCH-2 RNA (Figure 5). These experiments revealed that the expression of genes analysed from different functional groups strictly correlated with expression data from the microarray study: up-regulation of MMP-3 and, to a lesser extent, cyclin A, down-regulation of TIMP-3 and p21. Additionally, loss of expression of myogenic markers, as seen in RT-PCR experiments (Figure 3), could be confirmed quantitatively (Myf3, Myf5).

Looking at the differentially expressed genes in Table 1, extensive changes in the composition of the extracellular matrix were observed. Furthermore, as could probably be expected, this was accompanied by down-regulation of tissue inhibitors of metalloproteinases (TIMP) and up-regulation of some matrix metalloproteinases (MMP). Genes of particular interest might include MMP-14 and TIMP-2, since coexpression of both genes during

Table 1 Differential gene expression (RUCH-2 P63 compared to RUCH-2 P11)

	Gene	
Functional category[a]	Down-regulated	Up-regulated
Apoptosis-related	caspase-2	caspase-4 + caspase-5
	TNFR1	Akt-1
	A1[c]	
	Bag-1[c]	
	DR3[c]	
	CD27[c]	
Proteases and inhibitors	MMP-14	MMP-3[c]
	TIMP-1	MMP-8[c]
	TIMP-2	PAI-2[c]
	TIMP-3	
	EMMPRIN	
	t-PA	
	LRP	
Cell cycle regulators[b]	cyclin D1	Cyclin A
	cyclin G1	Cyclin B1
	p21[c]	

[a]Only selected categories and [b]genes, entire list presented on our homepage: *http://www.unizh.ch/kispi/clinchem_html/schafer.html*; [c]strong alterations.

muscle development has been reported (Apte et al, 1997). In addition, the extracellular matrix metalloproteinase inducer (EMMPRIN), expressed on the cell surface, was down-regulated. Its absence in the vertical growth phase and in metastatic lesions of melanomas suggests that other factors are involved in tissue degradation during later stages of tumour progression in malignant melanoma (van den Oord et al, 1997). Furthermore, strong up-regulation of plasminogen activator inhibitor 2 (PAI-2) was coordinated with a decreased expression of tissue-type plasminogen activator (t-PA). Indeed low t-PA and high PAI-2 levels have been correlated with poor overall survival in a number of primary tumours of different origin (Andreasen et al, 1997).

Interestingly, we also observed strong down-regulation of death receptors belonging to the tumour necrosis factor and nerve growth factor superfamily, namely DR3, CD27 and TNFR1. This suggests a possible hampered response of high passage RUCH-2 cells to appropriate apoptotic stimuli. In support of this, the signalling kinase Akt-1, a strong mediator of cell survival, is overexpressed as well in late stage RUCH-2 cells. However, contrasting this potential progression towards apoptosis resistance, two anti-apoptotic molecules, bag-1 and A1 (bfl-1) were also down-regulated. Hence, not single alterations but a complex coordinated up- and down-regulation of molecules involved in regulation of apoptosis was observed, which might shift the balance of the cellular response into one particular direction. This can also be seen when analysing genes involved in cell cycle regulation. Most prominently, cyclin A and cyclin B1 were up-regulated (stimulating progression through the cell cycle), whereas cyclin D1 as well as cyclin G1 were down-regulated (slowing progression). Furthermore, a striking down-regulation is seen for the cell cycle inhibitor p21 (again stimulating progression). Here, as a net result from these complex alterations, the proliferation rate of the cell population increases.

In summary, we identified a large number of genes whose expression is altered during RUCH-2 progression. Some of these might play a predominant role in tumour progression towards the metastatic phenotype that can now be further evaluated in additional investigations, involving primary tumour samples.

DISCUSSION

In this work we describe the isolation and molecular characterization of a new human cell line derived from a botryoid RMS. The key feature of this new cell line is its availability at low, non-metastatic and high, metastatic, passages following a spontaneous progression in vitro. This provides an interesting cellular model, since cells could initially be established from the original tumour not under treatment at time of biopsy.

Unlike other cell culture models of metastasis which are mostly based on single clone selection, progression observed in the RUCH-2 cell line occurred spontaneously in the population. We provide here three different experimental criteria demonstrating this evolution: changes in morphology and enhanced growth rate, gain of invasiveness through matrigel in vitro and, finally, de novo appearance of metastases in a nude mouse model in vivo.

How does tumour progression in culture relate to in vivo progression? Genome instability and resulting selection of subpopulation underlie progression in vitro as well as in vivo. However, because of culture conditions, different subpopulations might be selected. To assess this notion frequent examinations of the tumour cell karyotype were carried out. These cytogenetic investigations revealed that the karyotype in early passages was very similar to the original tumour. However, in later passages, a number of chromosomal gains and rearrangements were observed. Strikingly, several of the breakpoints which were newly observed, namely 1p21, 3q21, 12p13, 12q13 and 17q21 seem to be non-random, since they were also reported in primary RMS tumour samples (Whang-Peng et al, 1992; Kullendorff et al, 1998). This also includes a rather rare breakpoint at 18q23. Although comparison between in vitro and in vivo progression is clearly very limited, RUCH-2 cells represent, to our knowledge, the only progression model for human RMS available and hence provide a starting point for the identification of novel genes involved in rhabdomyosarcoma progression.

To begin to investigate possible molecular alterations in progression, we first performed RT-PCR experiments to study the expression levels of selected known regulatory molecules. While RUCH-2 cells at all passages expressed PAX3, the expression of the myogenic regulators Myf3 and Myf5 was restricted to early passages and completely disappeared later on as demonstrated by RT-PCR and quantitative Northern blot analysis. These results have implications for other tumour cell lines which are thought to be of RMS origin, since doubts have been expressed over their origin based on the missing expression of the myogenic regulatory proteins. Examples of such publicly available cell lines are A204 and Hs729. Our results suggest that they should not be excluded as having RMS origin, especially when they have been cultivated for an extensive period. On the other hand, analysis of Myf genes in primary RMS tumours revealed that only 18 out of 20 samples displayed Myf gene expression (Clark et al, 1991). Interestingly, a second RMS cell line established in our group, RUCH-3, which was derived from a relapse embryonal RMS, did never express Myf3 and Myf5, but PAX3 (the patient died a short time after excision). Hence, lack of Myf3 might be confined to more advanced tumour stages.

To provide a more comprehensive analysis of the molecular changes involved in metastatic progression, we then applied the cDNA microarray technique to compare expression of 588 known tumour-associated genes from late with early passage cells. The microarray hybridization most likely reflects true changes in expression level, because the alteration of selected genes like TIMP-3, MMP-3, cyclin A and $p21^{WAF1}$ could be confirmed with quantitative Northern blot analyses of RUCH-2 cells at various passages (Figure 5). As expected, a number of alterations (either up- or down-regulation) were observed in the composition of the extracellular matrix, including enhanced expression of proteinases. However, other alterations might provide novel insight into RMS development. Some of the most striking changes were observed in the category of apoptosis-related genes, where the down-regulation of three receptors of the TNF receptor family, namely TNFR1, DR3 and CD27, was very prominent. So far, neither the expression nor the role of these death receptors has been investigated in skeletal muscle cells. Down-regulation of death receptors has, however, been correlated with the development of other cancers, e.g. TNFR1 in non-small-cell lung cancer (Wyllie, 1997; Tran et al, 1998). Furthermore, an enhanced resistance of metastatic cancer cells to programmed cell death has been described for several neoplasms (Glinsky et al, 1997; Takaoka et al, 1997). Hence, these receptors could play an important role in RMS development as well. Interestingly, TNFR1 and CD27 are both located at 12p13, a locus where rearrangements have been observed in high passage RUCH-2 cells, raising the possibility that their expression might be influenced by the translocation event.

A more extensive use of DNA microarray technology should undoubtedly allow the identification of additional changes important for RMS progression in the near future. Evidently, all changes will have to be verified in additional RMS cells and, ideally, biopsy samples from primary tumours as well as metastases from the same patient. Nevertheless, the RUCH-2 cell line provides a starting point for the identification of progression associated genes in RMS, genes which might be important for other tumours as well.

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5.5 PAX3 is expressed in human melanomas and contributes to tumor cell survival

(submitted)

PAX3 is expressed in human melanomas and contributes to tumor cell survival

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ABSTRACT

Deregulated expression of the transcription factor PAX3 was previously observed in several tumors like rhabdomyosarcoma and Ewing's sarcoma. During development, PAX3 is expressed in pluripotent neural crest cells, precursors of melanocytes. It is therefore conceivable that melanomas, tumors derived mostly from cutaneous intra-epidermal melanocytes, might reexpress PAX3 during their de-differentiation process. Indeed, we detected expression of PAX3 in the majority of the melanomas analyzed both by RT-PCR and in situ hybridization. Furthermore, downregulation of PAX3 expression achieved through a specific antisense oligonucleotide-based treatment resulted in over 70% death rate specifically in PAX3 positive melanomas. These experiments suggest that PAX3 is expressed in a high percentage of melanoma cells which appear to become dependent on expression of this transcription factor.

INTRODUCTION

PAX proteins are developmentally expressed transcription factors that play a fundamental role in the establishment of cell lineages (1). Their importance has been underscored by several loss-of-function mutations that usually lead to a lack of the specific structures or organs where a PAX protein is normally expressed (2). For example, PAX3 is expressed during normal development in specific areas of the neural tube, sensory organs and the dermomyotome. A naturally occurring PAX3 loss-of-function mutation in mice (*Splotch Sp*) exhibits severe pigmentation defects and fails to establish hypaxial skeletal muscle cells (3). Homozygous affected mice die shortly after birth. Mutations in the human PAX3 gene cause Waardenburg syndrome (WS) which is characterized by pigmentation abnormalities and hearing impairment due to absence of melanocytes (4, 5).

Melanoma is a tumor with increasing incidence (6). Advanced disease is associated with a poor prognosis and responds very poorly to treatment modalities including chemotherapy and immunotherapy (7, 8). Despite the availability of a fair number of marker genes/proteins (e.g. melan A/MART-1, MAGE-3, tyrosinase) there is a need to identify proteins involved in the conversion from benign to malignant disease and in tumor cell survival. First, these factors should have diagnostic or prognostic implications and second might also be functionally important for tumor development.

Apart from the normal physiological role during development, several PAX genes are reexpressed in malignant neoplasms (9), e.g. PAX3 in rhabdomyosarcoma and in Ewing's sarcoma. Since PAX3 is expressed during development in proliferating melanocyte precursor cells, this gene might be reexpressed in melanomas, analogous to the situation in rhabdomyosarcoma. In this report, we demonstrate that PAX3 is indeed present in the majority of melanomas and has a survival function in these tumor cells.

MATERIAL AND METHODS

Cell culture

A total of 35 primary cultures established from melanoma lesions of patients in different stages and 5 melanoma cell lines were cultured as monolayer at 37°C and 5% CO_2 in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 µg/ml) as previously described (10).

Analyses of RNA transcripts

Total RNA extraction and RT-PCR reactions were performed as previously described (11). RNA integrity was checked by amplification of the ubiquitous FKHR cDNA. RT-PCR was also performed to monitor presence of the melanoma associated antigen MAGE-3, MelanA and tyrosinase mRNA as previously described (10, 12).

In situ hybridization

The phPAX3-sp6 plasmid containing 152 bp of human PAX3 (13) was linearized with EcoRI in order to synthesize biotin-14-CTP labeled antisense RNA (MAXIscript™, Ambion Inc.). In situ hybridization of paraffin sections was performed as detailed in the instruction manuals of the mRNAlocator-Hyb™ and mRNAlocator-biotin™ kit (Ambion Inc.). Briefly, sections were hybridized overnight at 55°C and the detection reaction was performed for 6 h at 37°C.

Oligonucleotide (ODN) Incubation

ODN treatment was performed as reported earlier (14). The ODNs were synthesized as phosphothioate molecules by Microsynth (Microsynth GmbH, Balgach, Switzerland), purified over a reverse phase column and resuspended in TE.

Apoptosis assay

An annexinV-FITC Kit (BenderMedSystems, Vienna, Austria) was used to assess apoptotic cells. 65 h after ODN treatment cells were washed with DMEM and stained for 10 min at room temperature with annexinV-FITC according to the manufacturer's instructions.

RESULTS

PAX3 expression in melanoma cell lines

To test initially if PAX3 mRNA is expressed in melanomas, several cell lines were screened by RT-PCR. Indeed, three of the five investigated cell lines were found to be positive for PAX3 expression, namely MEL15, G-361, and UKRV-Mel2 (Fig. 1A). No PAX3 expression was detected in the other two melanoma derived cell lines (A365, Küng-A375). Hence, 60% of the investigated melanoma cell lines expressed PAX3 mRNA.

PAX3 expression in cultured melanomas

Since expression of PAX3 was found only in some of the melanoma cell lines analyzed, the next question was whether PAX3 expression might depend on a given tumor stage or is a property of established cell lines only. To answer this, cultured primary melanomas established from different stages and locations from a cohort of patients were analyzed (10). From 35 melanoma cultures analyzed totally, 27 were found to express PAX3 representing 77% of all investigated tumors (Table 1). Highest prevalence of PAX3 expression was found in stage III tumors, where 11/12 (92%) of all cultures analyzed were PAX3 positive. In stage I, 9/11 (82%) and in stage II 7/12 (58%) melanoma cultures expressed detectable levels of PAX3.

To investigate whether PAX3 expression changes with time of tumor progression and to test whether a correlation exist with other melanoma markers, cultured melanomas established from the same patients at different disease stages, were analyzed (Table 2). In four out of eight patients analyzed, an increase in PAX3 expression was found in more advanced tumors (patient II, III, V, VII), whereas three patients had no alterations in the levels of PAX3 expression (patient IV, VI, VIII). Finally, one patient lost PAX3 expression in a more advanced stage (patient I). However, there was no correlation either with expression of the melanoma specific antigens melan-A, MAGE-3 nor with tyrosinase. Hence, PAX3 expression identifies a distinct subset of melanomas.

PAX3 expression in tumor sections

To ensure that expression of PAX3 in short-time melanoma cultures does accurately reflect expression in the tumor tissue, in situ hybridization of paraffin tissue sections with a PAX3 specific antisense probe was performed. Sections from the original tumors of the same biopsy were used to establish melanoma cultures from a total of 10 different

lesions were hybridized. Six of these were found to be positive for PAX3 (950728, 961127, 960306, 960819, 961209, 980513) whereas four tumors showed no PAX3 expression (950504, 961205, 970917, 980229). Hence, the in situ hybridization data correlate perfectly with the RT-PCR results of the cultured melanomas (Table 1). Examples of a PAX3 positive (980513) and a PAX3 negative (970917) metastasis are shown in Fig. 2A and B. Intriguingly, PAX3 expression was confined to malignant metastases and not observed in the surrounding normal tissue (Fig. 2C). In PAX3 negative tumors, only melanin deposits can be seen and tumor cells are clearly negative (Fig. 2D). When tumor and normal skin are present in the same sections and tumor cells are clearly positive (Fig. 2E), epidermis including melanocytes is negative (Fig. 2F). Hence PAX3 expression is restricted to malignant melanomas already at stage I and their metastases and not detected in normal skin melanocytes.

Survival of melanoma cells depends on PAX3 expression

We previously described that PAX3 expression in rhabdomyosarcoma (RMS), a childhood tumor derived from myogenic precursor cells (13, 15) provides a critical survival function (14). Therefore, we hypothesized that expression of PAX3 in melanomas might also affect cell survival. To test this, different melanoma cell lines were treated with oligonucleotides designed to downregulate PAX3 expression and cell survival was monitored by counting after trypan blue exclusion. To determine liposome and oligonucleotide toxicity, cells were incubated with lipofectin alone (Fig. 1B, black bar) and with missense oligonucleotide (MS-ODN) (Fig. 1B, hatched bar). Incubating PAX3 positive cells with specific antisense oligonucleotides (AS-ODN) resulted in dramatically reduced cell viability (down to 28-32%, Fig. 1B, open bars). In contrast AS-ODN treatment of PAX3 negative cells had no effect on cell viability compared to lipofectin treated cells. The cellular morphology observed during ODN treatment of the PAX3 positive melanoma culture 980928 and the PAX3 negative cell line Küng-A375 is illustrated in Figure 1C. AS-ODN treated 980928 cells show nuclear condensation, rounding up and finally detachment from the culture dish, indicating an apoptotic process. To assess this observation on the molecular level, the same cells were stained for annexinV after incubation with either MS-ODN or AS-ODN (Fig. 1D). 65 h after ODN incubation, the few 980928 cells still remaining after AS-ODN treatment stained to 30% (29/96) positive for annexinV, whereas incubation with MS-ODN did not yield in annexinV positive cells. In contrast, in the PAX3 negative cell line A365 neither

incubation with AS-ODN nor MS-ODN resulted in any annexinV stained cells (Fig. 1D). These results demonstrate that treatment of PAX3 expressing melanomas with AS-ODN targeting PAX3 results in reduction of viability via an apoptotic pathway.

DISCUSSION

PAX3 appears to play an essential role in the establishment of melanogenic and myogenic cell lineages, as suggested by the severe phenotypes in mice and in humans with reduced functional PAX3 protein. Since PAX3 is implicated in the pediatric tumor rhabdomyosarcoma both because of the occurrence of a specific translocation involving PAX as well as its overexpression (9), we speculated that reexpression of PAX3 might also occur in melanoma.

Indeed, in this study we demonstrate by sensitive and specific RT-PCR analysis in cultured melanomas as well as by in situ hybridization on tissue sections that PAX3 is expressed in a substantial fraction of melanomas. Importantly, PAX3 positive and negative cultured melanomas could be unambiguously distinguished by RT-PCR and confirmed by in situ hybridization on the corresponding tissue sections. Whereas PAX3 was confined to malignant melanomas and not detected in normal skin by in situ hybridization, sensitive RT-PCR methods were also able to generate a signal in normal skin (data not shown). Therefore sensitive RT-PCR should be used cautiously when analyzing biopsy specimens.

One evident interpretation of these results might be that PAX3 expression simply indicates progressive dedifferentiation of melanocytes. However, some interesting parallels exist between myogenesis and melanogenesis. PAX3 seems to be necessary for the development of both lineages and in each case activates tissue specific transcription factors of the basic helix-loop-helix class which are important in cell determination and differentiation (myoD family members in muscle and MITF in melanoblasts) (16, 17). How the activity of PAX3 is regulated towards either one or the other lineage is at present unknown.

In tumor cells expression of developmentally regulated genes occurs fairly often. However, it is unclear if the reexpressed genes play any role in the development or maintenance of the tumorigenic phenotype. Therefore an antisense strategy to

investigate the functional role of PAX3 expression in melanomas was applied. Indeed, PAX3 expressing melanoma cell lines and cultures were susceptible to AS-ODN treatment and showed reduced viability compared to control cells. Since dying cells could readily be stained with annexinV and show clear nuclear condensation we conclude that these cells die by apoptosis. The effect is specific since PAX3 negative cells did not respond to AS-ODN treatment nor did cells treated with control MS-ODN. These experiments suggest that PAX3 has a conserved anti-apoptotic function in both melanoma and rhabdomyosarcoma.

The PAX3 dependent survival pathway is not yet fully understood. Since PAX3 is a transcription factor, either activation or repression of target genes might be involved. Recently, several direct targets for PAX3 have been identified. The best studied PAX3 target gene is the proto-oncogene c-met, which encodes the tyrosine kinase receptor for hepatocyte growth factor/scatter factor (HGF/SF). The importance of the c-met/HGF signalling pathway for tumor development and cell survival is well documented and indeed strong c-met expression is found in both melanomas (18) and rhabdomyosarcomas (19). In addition, transgenic mice ectopically expressing HGF/SF develop malignant melanomas, which were shown to overexpress both HGF/SF and c-met (20). Hence, stimulation of c-met expression by PAX3 might be an important pathway in melanoma development. Additional PAX3 target genes that might play a role in cell survival include another member of the tyrosine kinase receptor family, the insulin-like growth factor receptor 1 (IGFR-1) (Murmann et al. submitted) as well as the anti-apoptotic survival gene bcl-x_L (Margue et al. submitted).

Expression of PAX3 in the majority of melanomas might participate in their development and/or maintenance since cell survival appears to dependent on PAX3 expression. PAX3 might therefore represent a possible novel target for therapeutic molecular interventions in melanomas.

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Table 1: Expression of PAX3 in melanomas

Patient	Stage, TNM[a]	PAX3 RT-PCR[b]	PAX3 in situ[c]
950504	I, T3N0M0	-	-
950803	I, T2N0M0	++	
951010	I, T3N0M0	++	
951019	I, T3N0M0	++	
960514	I, T4N0M0	++	
960704	I, T1N0M0	++	
960724	I, T1N0M0	++	
961008	I, T1N0M0	±	
961121	I, T4N0M0	++	
961122	I, T1N0M0	++	
961205	I, T3N0M0	-	-
941209	II, TxN2aM0	-	
950322	II, TxN2M0	++	
950526	II, TxN2aM0	-	
950728	II, TxN2aM0	++	++
950822	II, TxN2aM0	++	
960104	II, TxN2bM0	++	
960312	II, TxN1M0	-	
961127	II, TxN2bM0	++	++
970325	II, TxN1M0	++	
970917	II, T4N2aM0	-	-
980229	II, TxN2aM0	-	-
980325	II, TxN2aM0	±	
960306	III, TxNxM1a	++	++
960618	III, TxNxM1a	++	
960819	III, TxNxM1a	++	++
960924	III, TxNxM1a	++	
961209	III, TxNxM1b	++	++
970604	III, TxNxM1a	++	
970703	III, TxNxM1b	±	
970723	III, TxNxM1b	++	
980409	III, TxNxM1b	++	
980513	III, TxNxM1b	++	++
980924	III, TxNxM1b	-	
980928	III, TxNxM1b	++	

I: patients with primary melanoma, II: patients with locoregional lymphnode metastases, III: patients with remote metastases,
[a] TNM classification according to UICC, - no PAX3 expression, ± just detectable PAX3 signal, ++ PAX3 expression, [b] cultured melanomas, [c] tissue sections

Table 2: PAX3 expression in melanomas of the same patient

Patient	Melanoma culture	Stage, TNM[a]	PAX3	MAGE-3[b]	MelanA[b]	Tyrosinase[b]
I	961121	I, T4N0M0	++	+	+	+
	970917	II, T4N2aM0	-	n.d.	n.d.	n.d.
II	941209	II, TxN2aM0	-	-	-	-[c]
	950526	II, TxN2aM0	-	+	-	+
	950822	II, TxN2aM0	++	+	+	+
III	960312	II, TxN1M0	-	-	-	-
	970325	II, TxN1M0	++	-	-	-
IV	980229	II, TxN2aM0	-	-[c]	-[c]	+[d]
	980924	III, TxNxM1b	-	-	+	-[d]
V	980325	II, TxN2aM0	±	+	-[c]	+[d]
	980928	III, TxNxM1b	++	+[c]	+[c]	-[c]
VI	950322	II, TxN2M0	++	-	-	+
	961209	III, TxNxM1b	++	-[c]	-[c]	-[c]
VII	970703	III, TxNxM1b	±	-[c]	-[c]	+[c]
	970723	III, TxNxM1b	++	-[c]	+[c]	-[c]
VIII	980409	III, TxNxM1b	++	+[c]	-	+
	980513	III, TxNxM1b	++	+[d]	+[d]	+[d]

Samples between the rows-separation are from the same patient
I: patients with primary melanoma; II: patients with locoregional lymphnode metastases,
III: patients with remote metastases, [a]TNM classification according to UICC
n.d. not done, - no PAX3 expression, ± just detectable PAX3 signal, ++ PAX3 expression
[b]expression was analyzed by RT-PCR, or [c]FACS, or [d]immunohistochemistry

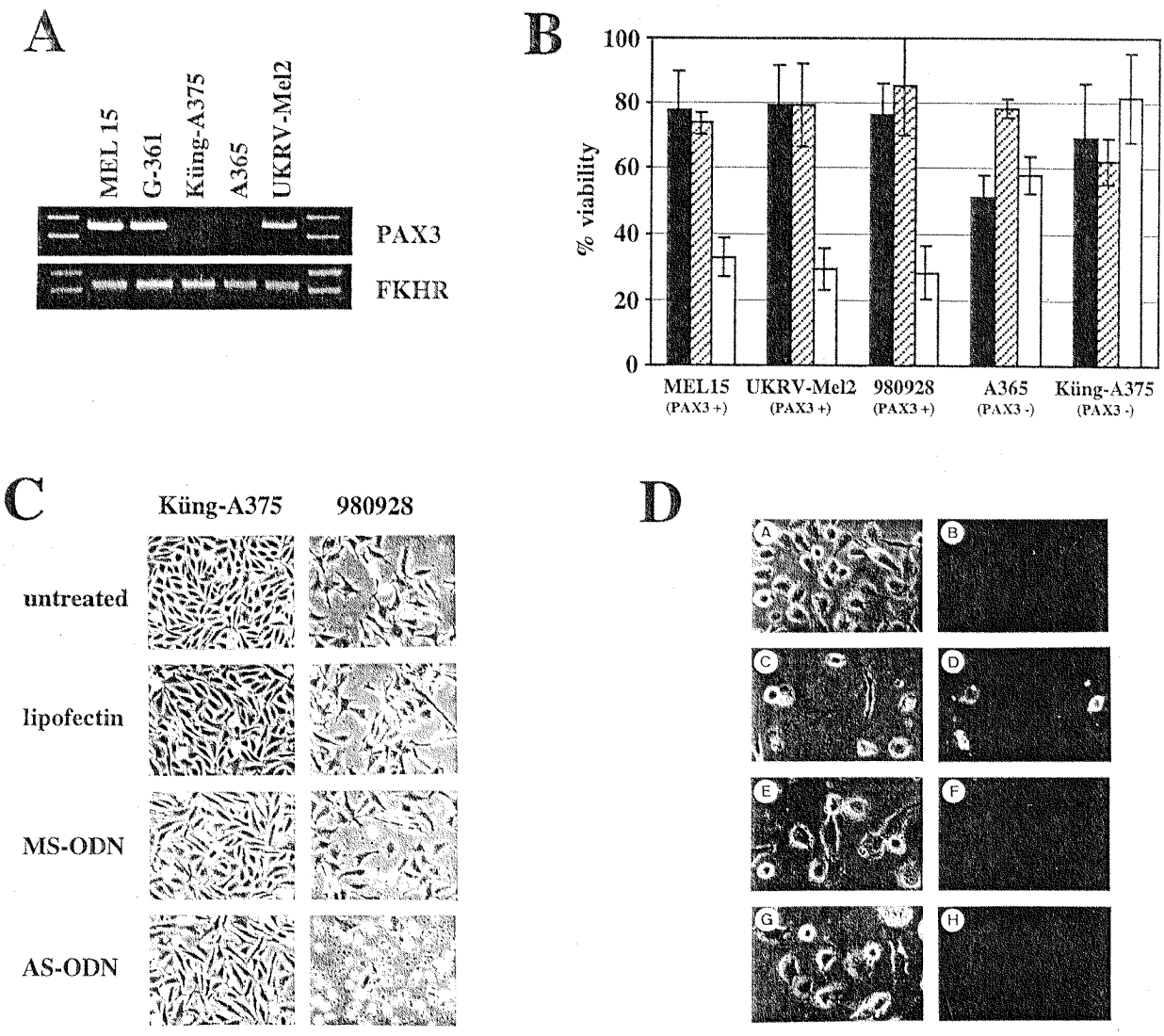

Figure 1 A) Expression of PAX3 in human melanoma cell lines. 100 ng total RNA for each RT-PCR reaction was used. FKHR was amplified as an internal control for RNA quality. RT-PCR products were loaded on 2% agarose gels and stained with ethidium bromide. As marker a 100-bp ladder was used. B) Viability of melanoma cell cultures treated with lipofectin (black bar), missense ODN (hatched bar) and antisense ODN (open bar). Cells were treated with 1 μM ODN and 10 μg lipofectin or with lipofectin alone and counted after 72 h. At least three independent experiments were carried out for each treatment, each experiment as a triplicate. C) Morphology of treated melanoma cells, 72 h after start of the ODN treatment. Küng A375 and 980928 melanoma cells were treated with either lipofectin alone or in combination with 1μM missense oligonucleotides (MS-ODN) or antisense oligonucleotides (AS-ODN). D) Detection of apoptosis after antisense ODN treatment. AnnexinV-FITC labeling of 980928 (A-D) and A365 (E-H) cells 65 h after incubation with 1μM of either AS-ODN (C, D, G, H) or MS-ODN (A, B, E, F). A, C, E and G are phase contrast pictures.

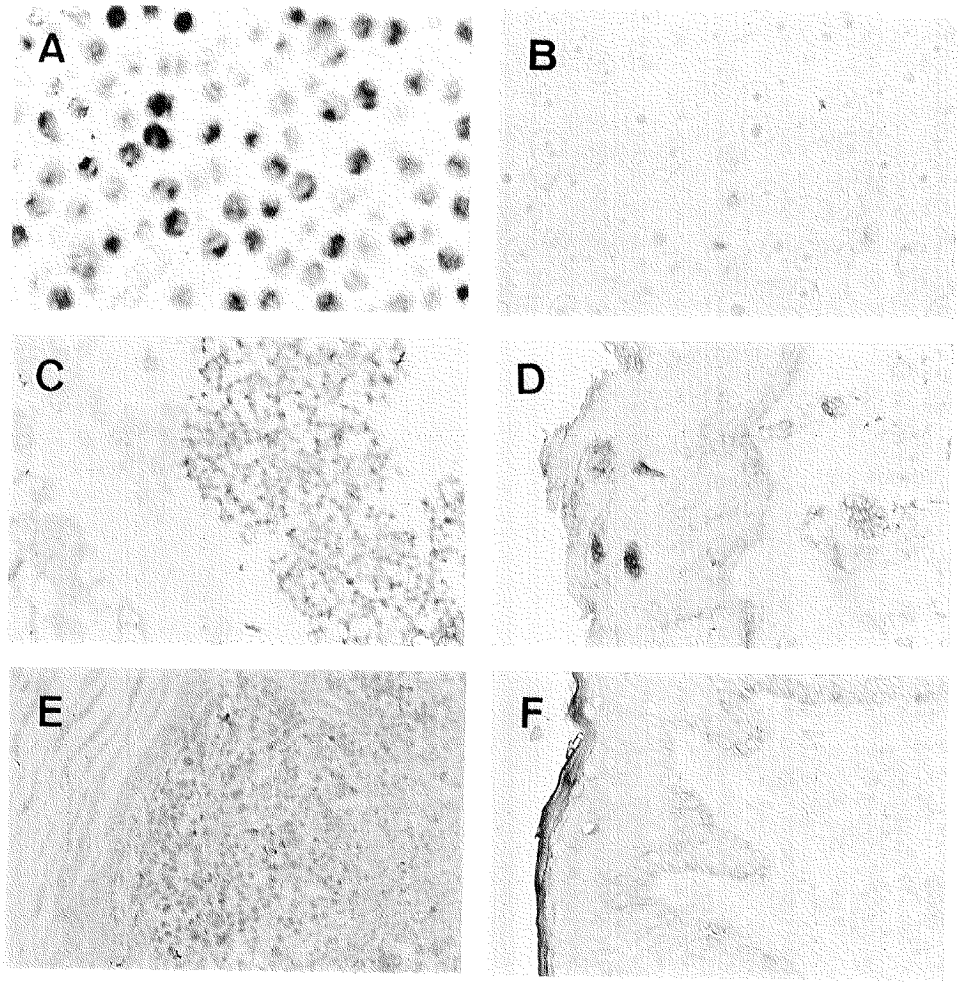

Figure 2 Detection of PAX3 expression in melanomas. Tissue sections from malignant metastasis (A, B, C and E, samples 980513, 970917, 950728, and 960819 respectively), primary tumor (D, samples 961205) were hybridized with a biotinylated PAX3 specific antisense riboprobe. F represent a different partial section of sample 960819. Positive reaction is indicated by the blue stain, brown stain is caused by melanin deposits. Magnifications are A: 25x, B, D: 100x, C: 200x, and E, F: 400x.

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6. Additional results, general conclusions and outlook

In this work we have applied a subtractive cloning procedure between myoblasts and RMS designated to isolate potential tumor suppressor genes. We established and characterized the RMS cell line RUCH-2 which showed spontaneous metastatic progression. Finally, we cloned and identified DRAL as a new p53 target gene, and discovered the aberrant expression of PAX3 and its inhibitory function of apoptosis in melanoma.

6.1 The subtractive library

First, we identified 48 clones downregulated or absent in the RMS cell line RD as compared with cultured myoblasts. These clones contain inserts of 300-600 bp. Initially, 29 of the identified clones were coding for known genes and 19 were coding for unknown genes (see section 5.1). In the meantime progression in large world wide sequencing projects with the goal of obtaining the complete sequence of the human genome, was made publicly available as expressed sequence tags (EST). Therefore, an update of the results since the publication of our initial analysis in regard to the clones of the subtractive screening will be given.

The latest reevaluation of our unknown genes in the Genebank/EMBL database, October 18th 1999, resulted in only 8 unknown clones, whereby the clones A33/106 and A33/217 are coding for the same gene because both are homologous to the human EST KIAA1063. These two clones are separated by approximately 1100 bp on this 5223 bp long EST, whereby the 5' sequence of this gene is missing. The remaining putative 7 genes are listed in table 4. This table summarizes the information available on the still unknown genes, indicating genes with the closest homology, and the differential expression pattern. To evaluate a potential more general role for these genes in cancer, their differential expression (absence in tumor) was not only analyzed for RMS, but also for additional neoplasms. First, the expression of the clones was compared in 4 RMS

Table 4. Differentially expressed clones with unknown sequence

Clone number A33/ ...	Closest homology	% identity/bp[a]	Accession number[b]	Consistent diff. expressed in RMS	Consistent diff. expressed in fibrosarcoma[c]	Consistent diff. expressed in breast cancer[d]
89	mouse EST uj38h12.yl	83%/211	AI528012	yes	no	yes
106	human EST KIAA1063	99%/358	AB028986	partial	n.d.	n.d.
124	human EST cn14g07.xl	99%/345	AI752290	yes	yes	yes
187	human EST tg37d02.xl	97%/236	AI418596	yes	no	yes
217	human EST KIAA1063	99%/355	AB028986	yes	n.d.	n.d.
246	none			partial	n.d.	n.d.
277	human DNA clone 329A5	99%/330	Z97832	yes	yes	no
284	human EST wv36e11.x1	98%/309	AI951552	yes	no	yes

[a] % of sequence identity between the differentially expressed clones and Genbank/EMBL cDNAs within the length of the region of identity
[b] Genbank/EMBL sequence database accession number
[c] more than 2 fold downregulated in the human fibrosarcoma cell line HT1080 compared with the cell line MRC-5
[d] more than 2 fold downregulated in the human the breast carcinoma cell line T47D compared with breast cell line HBL100
yes: clone only expressed in normal cells, except residual expression in tumorigenic cells
partial: clone expressed in normal cells, and maximal 2 of 5 RMS cells
no: clone expressed in normal cells and more than 3 RMS cells, or less than 2 fold expression difference in the other tumors
n.d.: not done

cell lines (RD, Rh1, Rh18, Rh30) and one primary RMS tumor to different primary myoblasts (Tab. 4). As indicated in table 4, the clones A33/89, A33/124, A33/187,A33/217, A33/277, and A33/284 were differentially expressed. The last 2 clones A33/106 and A33/246 are just partially differentially expressed, meaning that 2 of the 5 RMS cells had similar levels of expression to normal muscle cells. To assess the possible differential expression in other tumor types, we compared the expression of our clones in different pairs of normal/transformed cells, namely in the fibroblast cell line MRC-5 and the human fibrosarcoma cell line HT1080, as well as in the human breast cell line HBL100 and the human breast carcinoma cell line T47D. In fibrosarcoma only the unknown clones A33/124 and A33/277 were differentially expressed, whereas clones A33/89, A33/124, A33/187, and A33/284 were differentially expressed in breast cancer. Some genes were also analyzed which are now known, being A33/35 (DRAL) (see section 5.2) with altered expression in RMS and breast cancer, A33/108 (integrin α11) which was differentially expressed in only RMS, and finally A33/193 (developmentally regulated endothelial cell locus 1 (Del1)) which was differentially expressed in all 3 tumor types.

6.1.1 Unknown clones of the subtractive library

Clone A33/124

The clone A33/124 might be a candidate gene to clone and analyze, because of its consistent differential expression in the investigated tumors. Northern blot investigations revealed highest expression of A33/124 in testis (with occurrence of a second shorter band in this tissue, possibly due to alternative splicing), lower but consistent amounts in placenta, brain, heart, lung, ovary, and small intestine. The A33/124 clone has a length of only 430 bp, but the Northern blot analysis indicate a predominant 6.5 kb transcript. To further investigate this clone, we ordered at the beginning of 1997 2 EST clones, which were homologues to A33/124: EST ze62h10 (hereafter called 837-D20) and EST zf64a05. Both were approximately 3.5 kb long,

whereby we chose to sequence the 837-D20 clone. The sequence contains ALU sites at 1050-1350 bp of this clone. No open reading frame could be found, but several polyadenylation signals are present (first at 940 bp), therefore it likely represents the 3' end of the cDNA. Two additional EST clones (AI752290, AI752769), which have high homology to A33/124 and have an open reading frame, are in the database since June of this year. These two ESTs are oriented in the opposite direction and might contain the missing 5' part of this gene, however no data is available about their lengths. Nevertheless, they might represent the missing part of this unknown gene and should be ordered, and analyzed in more detail.

Clone A33/277

Since October 15th 1999 human DNA sequence corresponding to clone A33/277 is in the database. This DNA clone is 117 kb long and is located on human chromosome 6p21.1-21.33. It is likely that the whole genomic sequence of this unknown cDNA is on this clone, because first matches with A33/277 are at bp 35771. Further, the clone A33/277 matches in two fragments 180 bp (60 aa) and 189 bp (63 aa) on the genomic DNA, separated by 417 bp (likely representing an intron). Careful analyses could reveal the whole coding sequence of this gene as well as its genomic structure. If the putative cDNA sequence could be obtained, the actual cDNA sequence might be amplified by RT-PCR or by PCR screening of 96-well plate cDNA libraries. The genomic structure could be ascertained by a similar PCR approach and/or Southern blot analyses. It is already known to be localized on chromosome 6p21.1-21.33 and this could be verified and perhaps more precisely defined by FISH analysis (facility is present in our hospital). Further, the expression pattern of this gene in normal tissues could be assessed by hybridization of human Northern blots already present in our lab.

6.1.2 Identification of additional p53 inducible genes?

Another approach we used to narrow down the possibly interesting clones, was to take advantage of a RD subline which expresses a temperature sensitive p53 gene, and a

control cell line expressing just the empty plasmid. By lowering the temperature to 32°C the p53 gene is activated and fulfills wt p53 functions. Several clones of our subtractive library were analyzed for their potential p53 dependency at the transcriptional level. The results are listed in table 5.

Table 5. Clones induced by p53

yes	no	induced through lowering of the temperature
A33/35 (DRAL) A33/186 (thrompospondin 1)	A33/12 (osteoblast specifc factor 2) A33/51 (talin) A33/67 (testican) A33/90 (βigH3) A33/124 A33/175 (IGF binding protein 5) A33/187 A33/193 (Del1) A33/248 (G(i) protein α2 subunit) A33/277 A33/284	A33/22 (22 kdSMP) A33/89

As expected, p53 activated the transcription of thrombospondin 1 as well as of two additionally well known p53 targets bax and p21, which were used as an internal control. Interestingly, the previously cloned gene DRAL (A33/35) showed a p53 dependent induction at the transcriptional level (see section 5.3). The clones A33/12, A33/51, A33/67, A33/124, A33/175, A33/187, A33/193, A33/248, A33/277, and A33/284 showed no p53 dependent transcription. Finally, two analyzed clones, A33/22 and A33/89, were induced in both cell lines by dropping the temperature to 32°C, a result that indicate that they might be stress inducible genes. Hence, no additional clones could be identified which respond to p53 activation.

6.1.3 Known clones of the subtractive library

All the known differentially expressed clones and their function derived from our subtractive hybridization procedure are listed in table 6, according to their intracellular localization.

Table 6. Differentially expressed clones with known sequence

Cellular localization	Name	Proposed function	Clone Nr. A33/...	Consistent differentially expressed
Extracellular	Aggrecan	Extracellular matrix	214, 232, 273, 274	n.d.
	Breast epithelial antigen BA46	Extracellular matrix	199	partial
	Collagen I, α 1	Extracellular matrix	10, 92, 131, 174, 258	n.d.
	Collagen I, α 2	Extracellular matrix	28, 33, 211, 224, 298	n.d.
	Collagen V, α 1	Extracellular matrix	66	n.d.
	Collagen VI, α 2	Extracellular matrix	80	n.d.
	Collagen VI, α 3	Extracellular matrix	9	n.d.
	CYR 61	Growth regulator	210	partial
	Dell	Inhibition of angiogenesis	193	yes
	Fibrillin 1	Extracellular matrix	16, 46, 76, 158, 166, 205, 209, 294	n.d.
	Fibronectin	Extracellular matrix	2, 36, 55, 113, 170, 192, 194, 230	n.d.
	IGF binding protein 5	Modulator of IGF and IGF-receptor interactions	175	partial
	Lysyl oxidase	Cross-linking of collagen molecules	4, 34	n.d.
	Plasminogen activator inhibitor 1	Protease inhibitor	45, 49, 101, 244	yes
	Testican	Cell adhesion	67	yes
	Tissue inhibitor for metalloproteinase 3	Protease inhibitor	19	partial
	TGF-ß induced gene product 3 (ßigH3)	Mediator of TGFß 1 effects?	90, 159	yes
	Thrombospondin 1	Inhibition of angiogenesis	32, 151, 186	yes
	Thrombospondin 2	Inhibition of angiogenesis	60	yes
Cell membrane	Integrin α5	Receptor for fibronectin	26, 192	partial
	Integrin α11	Cell adhesion	21, 108	yes
	Jagged 1 (HJ1)	Ligand for vertebrate notch 1	111	partial
	LDL receptor related protein	Receptor	120	no
	Osteoblast specific factor 2	Adhesion molecule ?	12, 213	partial
	PDGF receptor	Receptor	102, 136	no
	SCN8A	Sodium channel	292	n.d.
	Tyrosine kinase receptor	Receptor	84, 265	no
Cytoplasm	Cytoskeletal γ-actin	Cytoskeleton	132	n.d.
	Filamin	Actin binding	77, 125, 250, 252	n.d.
	G(i) protein α 2 subunit	Adenylate cyclase inhibiting GTP binding protein	248	partial
	MKK3	Signal transduction	64	n.d.
	Nonmuscle myosin heavy chain	Cytoskeleton	109, 302	n.d.
	RhoC	GTPase	163	no
	22 kd smooth muscle protein	Ca^{2+} binding protein	22, 53, 247	yes
	Talin	Actin binding	51	partial
multiple	DRAL	?	35	yes
?	SIH002, CGI-151	?	58	partial
	Prostacyclin synthase (PTGIS)	?	129	yes
Nucleus	CDEI binding protein	Function in the cell cycle?	279	partial

partial: the clone is expressed in normal cells and in maximal 2 of 5 RMS cells.
yes: the clone is expressed in all normal cells and not in any of the RMS cells
no: the clone is expressed in all normal cells and in more than 3 RMS cells.
n.d.: not done

A plenitude of different genes were listed in table 6; three especially interesting genes were selected for further analyses.

DRAL (A33/35)

In this work, we demonstrated that expression of DRAL can be induced by p53. However, the more challenging aspect is to gain more information on the function of this interesting protein.

DRAL is a member of the four and a half LIM domain family. Given the large sequence homology and equal structure of the five known FHL proteins so far, it is likely that they possess similar function in different tissues and under different conditions, e.g. depending on intracellular signaling. In analogy to other LIM domain proteins, it is also feasible that they interact with several binding partners as indicated by their different intracellular localization. Two possible roles might be generally attributed to the FHL proteins: they either act as scaffolding molecules by binding several proteins and thus permitting distinct signaling, or they displace other LIM domain proteins thereby inhibiting their actions.

Intriguingly, at least one alternative splice variant SLIMMER (KyoT2) was found for SLIM1 (Brown, et al. 1999; Taniguchi, et al. 1998). Only this variant with an altered C-terminus was shown to interact with the transcription factor RBP-J, and not the classical variant SLIM1. Additionally, competitive experiments demonstrated that RBP-J binds alternatively Notch1 or KyoT2 individually, indicating that KyoT2 counteracts the Notch1 signaling pathway. Until now no alternative splice variants have been identified for the other FHL proteins. Therefore, it would be very interesting to investigate whether splice variants of DRAL exist. The clarification of the complete genomic sequence of DRAL or at least the 3' end will contribute to elucidate the question about the possible existence of a similar alternative splice variant. We already have a P1 clone with at least the first half of the DRAL gene, but based on its size it is likely that the 3' end is also contained on this P1 clone.

The findings that DRAL is localized in different intracellular compartments (nucleus, cytoplasm, Z-disc, focal adhesions) raise the question if a given LIM domain might be responsible for a given localization. To answer this question several deletion constructs of DRAL containing the LIM domains as single copies or in combination should be tested for its subcellular localizations. It is likely, that the partner proteins to which DRAL binds are responsible for the different localization within the cell.

The next question is the identification of DRAL interaction partner(s). Different experimental approaches can be taken to tackle this question: one possibility is to use a yeast two-hybrid system using DRAL as bait to search for binding partners (experiments in progress). A different approach would be to use affinity columns (with bound DRAL or only domains) or perform immunoprecipitations with DRAL antibody and use a panel of antibodies to assess the binding partner(s). These last two approaches require some idea of the nature of the binding partner(s) and should be used to verify any clone from the two hybrid screen, or candidate proteins based on DRAL localization, e.g. in the focal adhesions, where also other LIM domain protein are known to be localized (e.g. zyxin, vinculin) and possibly interact with DRAL.

Further, by designing and constructing a selectable, inducible GFP-tagged DRAL construct a possible intracellular shuttling mechanism of DRAL as described for zyxin could be investigated (Nix and Beckerle 1997).

Finally, since DRAL is downregulated in neoplastic tissues, perhaps its absence could be used as a tumor marker to serve as a specific prognostic tool. This requires a specific antibody which only recognizes DRAL and no other LIM domain proteins. It is not yet available, but in order to obtain a specific antibody several peptide antibodies could be generated and investigated in regard to their specificity.

TGF-β induced gene product 3 (A33/90)

TGF-β induced gene product 3 (βigH3) was also selected for more detailed analysis. We studied the effect of ectopic expression of βigH3 in the RD cell line. Because of its potential tumor suppressor function an inhibitory effect on cell proliferation could be

expected. To overcome this potential problem we decided to use a tetracycline inducible system (Clontech Inc. (Gossen and Bujard 1992)) to be able to grow enough cells for the subsequent experiments. This system allows the inhibition of expression of the gene of interest, in our case βigH3, in the presence of tetracycline. In practical terms, two plasmids must be expressed, one coding for the tetracycline induced repressor and one plasmid coding for the gene of interest. After selection we obtained two clones, called RD βigH3 4 and RD βigH3 7 as well as the RD cell line with only the first tetracycline plasmid (RD control). However, the system was not optimal, since both cell clones had a dramatically reduced doubling time, for clone 4 even more pronounced, despite the presence of tetracycline in the medium (nowadays better systems exist to regulate gene expression, e.g. one plasmid tetracycline inducible (or repressable) system, or the ponasterone A (an ecdysone analog) inducible control of gene expression system). Further experiments demonstrated that in the presence of tetracycline they expressed some βigH3 protein confirming the leakage of the tetracycline system, thereby resulting in the reduced growth of the cells. Next, we wanted to investigate whether the effect observed in vitro would be the same in nude mice experiments showing reduced tumor growth. 9 weeks after the subcutaneous injection of cells (2 animals with RD βigH3 4, 3 animals with RD βigH3 7, 3 animals with RD control) an average tumor mass (TM) of 1,5 cm^3 was observed for the RD control mice, the RD βigH3 4 animals showed a TM of 0,02 cm^3, and the RD βigH3 7 animals a TM of 0,25 cm^3. All animals except the RD βigH3 4 animals were euthanized at that time point. The RD βigH3 4 animals were kept until week 12, when the average TM reached 0,45 cm^3. These in vivo data correlate with the previously obtained in vitro data, revealing a reduced cell growth by ectopic expression of βigH3.

A similar tumor growth suppression has been reported by ectopic expression of βigH3 in CHO cells (Skonier, et al. 1994).

Del 1 (A33/193)

The full-length sequence of the clone A33/193, Del1, has been published (Hidai, et al. 1998) while we were in the process of isolating the full-length cDNA of this clone. The gene encodes two major transcripts, which have 30 bp difference, and a minor transcript. We also observed the major transcripts with or without the 30 bp by PCR experiments, but not the minor transcript. Additionally we cloned a different 3' end (not completely sequenced) which more likely corresponds to the observed 4.7 kb mRNA. We detected the highest level of expression of Del1 in brain, followed by small intestine, colon, heart, ovary, prostate, testis, and lung using commercially available Northern blots (Clontech) prepared from several human tissues. Interestingly, this secreted matrix protein was shown by Hidai et al. to inhibit the formation of vascular-like structure in an in vitro angiogenesis assay. Further, a new report demonstrate that Del1 induces integrin signaling and anti-angiogenesis by ligation of $\alpha V\beta 3$ with recombinant protein (Penta, et al. 1999). These data suggest a possible role of Del 1 in cancer. In fact downregulation of Del 1, as we demonstrated in 3 tumor cell lines, at least at the transcriptional level, might be necessary for massive angiogenesis, a typical feature of tumors. Unfortunately, no experimental evidence proving the role of Del 1 in tumorigenesis is available yet. Hence, investigation of Del1 protein expression (no data exist of possible translational regulation) in different tumor types would furnish a better understanding of the possible role of Del1 in tumorigenesis. A further approach to assess the role of Del1 in tumorigenesis would be to analyze the effect of its reexpression in RD, HT1080, and T47D in cell culture. In parallel, one could investigate whether the 3 splice variants have the same functions. Possibly, no direct effect might be seen in cell culture, but injection of Del1 expressing cells in nude mice should demonstrate possible alterations in angiogenic behavior and perhaps tumorigenesis. Because all three cell lines are known to be tumorigenic in mice, less vascularized tumors are expected to grow resulting in reduced tumor growth due to less nutrient support.

Hence, we identified a multitude of molecules which play or might play an important role in cancer. Interestingly, a similar subtractive hybridization procedure was

performed between human fibroblasts and their SV40 transformed counterpart resulting in a similar list of clones coding for 24 proteins (Schenker and Trueb 1998). Quite remarkable is the large similarity of their identified proteins with the proteins of our subtractive list. Identical are: fibronectin, βigH3, collagen VI α2, collagen VI α3, 22 kDa smooth muscle protein, and IGF-BP5. It is therefore likely, that some of these proteins play an essential role in tumorigenesis in a more general way.

6.2 Proteins involved in RMS formation and progression

RMS is a rare tumor and only limited amounts of material derived from primary tumors are available. Although the survival rate for RMS patients has increased in the last few years, the remaining sequels should be taken in better consideration and not be ignored. Further, to facilitate the research of such rare neoplasms the handling of tumor materials should be perfectly organized and collected in a common center.

Hence, to identify and better characterize the genes involved in RMS formation, we took advantage of the spontaneous metastatic progression in our established botryoid RMS cell line RUCH-2, to investigate the changes occurring at the molecular level. The use of cDNA microarray containing 588 cancer related genes allowed us to identify 92 genes with over 2 fold altered expression levels in this cell line.

How might the metastatic progression of the cell line be explained and related to normal tumor progression?

Botryoid RMS usually has the best prognosis and metastasize rarely. This might be related to earlier detection of the botryoid RMS than the others RMS due to its localization near the skin and because it is simple to resect. The observed experimental metastatic progression might be due to different conditions in vitro compared to the in vivo situation, such as selection pressure and lower passage number most likely in the patient.

It would be interesting to determine whether early passages of RUCH-2 cells (e.g. P3) would again result in a similar metastatic and tumorigenic line. Will these cells enter a crisis and if so at the same time point? Do the same chromosomal changes occur? Which genes are abnormally expressed?

A different approach to determine the role of specific genes in the metastatic phenotype, would be to analyze whether metastatic features can be reverted upon ectopic expression of the downregulated genes in late passage RUCH-2 cells, or induced by overexpression of upregulated genes.

Hence, we possess two lists of genes which might play a crucial role in RMS tumor formation, one depicting the changes between RMS cell and normal myoblasts, the other reflecting changes between the tumorigenic and the tumorigenic and metastatic tumor status.

How might we determine the importance and possible correlation of these genes with tumor formation and progression?

First, both lists should be combined and spotted on novel custom arrays. Afterwards, the observed alterations in gene expression could be more widely investigated in the RMS cell lines, and most importantly in primary RMS tumor samples. This approach could help to answer the following question: Are some of these changes specific to one type of RMS (e.g. alveolar, embryonal)? Can one of these genes or a combination of several genes be taken as a prognostic marker to better define the tumor stage, and allow a more appropriate tumor treatment? Are these markers able to distinguish between Ewing's sarcoma and RMS?

6.3 Role and expression of PAX3, PAX7, PAX3/FKHR, and PAX7/FKHR in tumors

6.3.1 Tumor markers: diagnostic implications

The aberrant expression of PAX3, PAX7, and the two translocation products PAX3/FKHR and PAX7/FKHR were claimed to be specific for RMS. More recently, expression of PAX3 and PAX7 was found also in Ewing's sarcoma (Schulte, et al. 1997).

To ascertain and facilitate the classification of these neoplasms, we established specific one tube RT-PCR reactions (PAX3, PAX7, PAX3/FKHR, PAX7/FKHR, EWS/Fli-1, Myf3, Myf4, Myf5, Myf6, FKHR, and GAPDH) to permit a simple and rapid investigation of a given tumor (using as little material as possible). Using the established RT-PCR reactions we are able to distinguish between eRMS, aRMS, and Ewing's sarcoma and use it as a diagnostic tool. The occurrence of the PAX3/FKHR and PAX7/FKHR translocations was until now not found in any tumor other than aRMS. The PAX7/FKHR translocation is only detectable by RT-PCR or FISH analysis, however not by karyotyping the tumor, because this translocation is only present on double minutes.

Handling of the tumors would be easier if RNA did not have to be extracted and protein could be directly detected, but until now no PAX antibodies exist that are able to detect endogenous PAX3 or PAX7 levels. Therefore an effort should be undertaken to generate sensitive and specific antibodies. To generate antibodies solely detecting the translocations will be difficult. It is crucial to identify these translocations with no doubt because aRMS metastasize early in disease and have the worst prognosis. Therefore the use of RT-PCR to assess the translocations PAX3/FKHR and PAX7/FKHR is most likely the best option today.

Further, we demonstrated PAX3 but not PAX7 expression in melanomas. To ascertain the expression of PAX3 in a range of tumor types, additional neoplasms should be analyzed.

6.3.2 Survival factors: therapeutic implications

By using an anti-sense oligonucleotide (AS-ODN) approach we demonstrated that PAX3 plays an important anti-apoptotic function in tumor cells.

Interestingly, downregulation of bcl-2 levels in melanomas by AS-ODN directed against bcl-2 combined with cytotoxic treatment, was shown to induce apoptosis in cell culture as well as in mice experiments (Jansen, et al. 1998). Now, the first clinical trials using this AS-ODN are performed and the preliminary data are quite promising.

Hence, due to the common tumor specific expression of PAX3 and its anti-apoptotic feature, PAX3 might represent an ideal target for tumor designed treatment. Additionally, several PAX3 target genes have been described (e.g. c-met, MITF, TRP-1) each of them being implicated in different pathways of cellular function. Therefore, the use of AS-ODN targeting PAX3 blocks several pathway simultaneously, probably resulting in a more efficient induction of apoptosis than by just targeting one protein, e.g. c-met. Consequently, the efficacy of our AS-ODN directed against PAX3 should be evaluated in animal experiments. Subcutaneous injection of RMS, Ewing's sarcoma, and/or melanoma cells results after several weeks in growth of a localized and vascularized tumor. Treatment of the mice with AS-ODN, alone or in combination with potent cytostatica should provide an appropriate model for the in vivo efficacy of the AS-ODN approach targeting PAX3. This treatment should result in specific induction of apoptosis in the tumor cells. Further, the metastatic cell line RUCH-2 could be used as a model for metastasis to evaluate if growth of metastasis could be influenced by AS-ODN treatment as well.

In parallel, one should investigate more carefully the action of PAX3 and search for additional target genes. PAX3 and PAX3/FKHR have common target genes (e.g. c-met) (Epstein, et al. 1996; Ginsberg, et al. 1998), but there are also target genes which are specifically activated by either PAX3 or PAX3/FKHR (e.g. PDGFRα is only a target of PAX3/FKHR and not PAX3) (Epstein, et al. 1998). Hence, as our AS-ODN

downregulates PAX3 as well as PAX3/FKHR protein levels, the downstream actions of both proteins are blocked regardless of their target specificity.

The identification of PAX3 and/or PAX3/FKHR target genes by arrays is now ongoing in our lab.

6.4 Identification of additional molecular mechanisms involved in RMS formation

The histologic appearance of aRMS (alveolar like spaces) has never been directly correlated with the presence of either one of the translocation products, PAX3/FKHR or PAX7/FKHR. Intriguingly, we analyzed primary RMS samples, classified as aRMS by histology, and found that they did not always expressed one of these translocation products (unpublished results). These results are confirmed by a study where 85% of aRMS had a specific translocation (Barr, et al. 1995). Interestingly, SF/HGF the ligand for c-met is known to stimulate cellular motility and functions as a morphoregulatory agent. The PAX3 target gene c-met, is a pleiotrophic receptor capable of stimulating mitogenesis, motility, invasive growth and induce formation of alveolar like structures. It has been shown that during limb muscle development Pax3 modulates the expression of c-met (Epstein, et al. 1996). Subsequently, ectopic application of SF/HGF protein induces emigration of myogenic cells at interlimb level in vivo (Brand-Saberi, et al. 1996). In addition, transgenic mice overexpressing SF/HGF develop several neoplasms, including RMS (Takayama, et al. 1997). Therefore, strong activation of the c-met receptor might result in a similar phenotype with alveolar like spaces without having an aRMS specific translocation. Hence, ectopic expression of either PAX3/FKHR, PAX7/FKHR, PAX3, PAX7, c-met, SF/HGF, and c-met and SF/HGF simultaneously in primary myoblasts would perhaps clarify the morphological features of aRMS. Because normal myoblasts can undergo just a limited number of cell divisions in culture, no direct effect of these genes might be seen and the use of immortalized myoblasts might be necessary. Recently, the group of Weinberg demonstrated that it is possible to immortalize human fibroblasts by ectopic expression of the telomerase catalytic subunit

(hTERT) (Hahn, et al. 1999). Further, by adding distinct oncogenes they could generate human tumor cells. To analyze more carefully the oncogenic potential of PAX genes and both translocation products our group just started to express hTERT in myoblasts which will hopefully also be immortalized by this gene.

An additional interesting report is that mice heterozygous for patched overexpress Gli1 and develop RMS with high incidence (Hahn, et al. 1998). Patched is the receptor for sonic hedgehog and is associated with the transmembrane protein smoothend. Gli proteins are large transcription factors that bind DNA in a sequence specific manner. One target gene of Gli1 is HNF-3β (Lee, et al. 1997). The careful investigation of this pathway could clarify some supplementary aspects of RMS tumor formation.

Even more interesting is a just recently published paper detecting a repressor of PAX3 transactivation, hDaxx. This protein probably represses the transactivation of all class II and class III PAX proteins, which correlates with the PAX proteins expressed in cancer (Hollenbach, et al. 1999). Nothing is known about the expression of hDaxx in tumors. Therefore, screening of several tumors known to express PAX3 (e.g. RMS, Ewing's sarcoma, melanomas) for hDaxx expression in comparison with their normal counterparts could reveal some interesting links.

In conclusion, this research might contribute to the knowledge about the basic biology underlying tumor cell development and help to design new approaches for cancer treatment.

6.5 Future experiments

In this work several different aspects of cancer biology were investigated. While as mentioned some interesting results could be obtained, these stimulated at the same time new questions and novel ideas emerged.

How and with what priorities would I proceed?
In the preceding pages I brought up numerous possibilities for further projects and some of the most interesting ones are already ongoing in our lab, like the identification of potential new PAX3 and/or PAX3/FKHR target genes, the establishment of human immortalized myoblasts by hTERT expression to assess the function of PAX genes and both aRMS specific translocations, and the screening for potential DRAL interaction partners with a yeast-two hybrid system.
To determine which LIM domain of DRAL interacts with the different candidate binding partners, I would in the meantime design and create different deletion constructs containing each LIM domain separately and also truncation variants of DRAL. These constructs could also be used to investigate which LIM domain is responsible for a given subcellular localization.

Interestingly, analysis of the list containing the clones of our subtractive library (table 6) revealed that several proteins contain a Arg-Gly-Asp (RGD) motif. In the last few years it was shown that the RGD motif plays a pivotal role in integrin mediated cell adhesive interactions with extracellular matrix and plasma proteins. However, integrins are also exploited as receptors by pathogens (e.g. bacteria, viruses) for cell binding and entry. This pathway into the cell is now being exploited as delivery system for gene therapy (Hart 1999). Further, it was recently demonstrated that RGD containing peptides are able to induce apoptosis by direct activation of caspase-3 without any requirement for integrin mediated cell clustering or signals (Buckley, et al. 1999). Additionally, using antibodies targeting the RGD motif results in inhibition of cell adhesion. Until now the aspect of absence of RGD containing proteins within cancer cells, resulting in enhanced

motility of cancer cells and perhaps insensitivity to apoptosis, needs to be better investigated. Therefore, I would ascertain the data obtained for βigH3 and try to reverse in vitro its effect, namely reduced cell growth, by adding antibodies targeting the RGD motif. Del 1 represents an even more attractive protein, because it is likely to be involved in angiogenesis, possesses a RGD motif, and is downregulated in all tumors we analyzed in comparison to normal cells. The role of Del 1 as a potential tumor suppressor gene should be analyzed in this regard in detail.

But before starting a new project, I would first spend some time to investigate the DNA sequence that has recently become available and represents the genomic structure of the clone A33/277, since previous Northern blot experiments showed a consistent absence of expression of this gene in the investigated RMS cell lines and in the fibrosarcoma cell line HT1080, indicating a potentially more wide involvement of this gene in tumorigenesis. If this DNA sequence has a start site and a polyadenylation site located at a convenient distance from the sequence we already know, it is likely that the whole gene is on this chromosomal fragment and the putative cDNA sequence as well as the genomic organization of this gene could be solved. If this is not the case, the next step might be to screen the database regularly to look for potential homologous EST clones.

In parallel some hints towards a possible involvement of hDaxx in cancer could be easily obtained, at least at the transcriptional level, using available RNAs already used to investigate the expression of PAX3, PAX7, PAX3/FKHR, and PAX7/FKHR by RT-PCR. Northern blot analysis of RNAs of RMS, Ewing's sarcoma, and melanoma cell lines, as well as myoblasts and muscle tissue could be used to investigate the expression of hDaxx in neoplastic and normal tissues. The levels of hDaxx could be directly correlated with the RT-PCR data.

The same Northern blots containing the RMS cell lines could be further rehybridized with patched, Gli-1, Gli-3, and smoothend to investigate their expression and role in health and disease.

Next, the list of genes involved in RMS formation and progression we obtained should be analyzed in tumor samples and metastasis by use of custom made arrays. The only problem at this point is the non-availability of the requested samples, but perhaps the penury of tumor samples will be solved in a few years and the investigation could be fulfilled, or at least one could try to collect appropriate samples.

Finally, we wondered if other PAX members than PAX3 and PAX7 have a similar anti-apoptotic function in tumor cells. Therefore, tumors were searched in which PAX gene expression has been reported, like brain tumors. Surprisingly, not the expected PAX mRNA could be amplified, but instead PAX3 expression was found. The experiments will be completed with functional data with the goal of writing another publication.

In summary, the efforts undertaken to identify and characterize genes involved in RMS formation and progression as potential diagnostic and/or prognostic factors should be continued and enlarged. Second, the occurrence and role of PAX genes in cancer should be better assessed, especially in regard to their use as possible theurapeutic targets. Finally, the identification of binding partners of DRAL might help to elucidate some of the functions of this protein.

7. Literature

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8. CURRICULUM VITAE

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Education

1995-2000	Ph.D. Thesis work at the Swiss Federal Institute of Technology (ETH) Zurich, performed in the University Children's Hospital of Zurich, Department of Pediatrics, Division of Clinical Chemistry & Biochemistry in Zurich, Switzerland
1993-1995	Research at the University Hospital Zurich, Department of Surgery, Traumasurgery in Zurich
1988-1993	Studies in Natural Sciences, Biology, at the Swiss Federal Institute of Technology (ETH) in Zurich
1984-1988	High school (Gymnasium, Maturität Typus C), at the Kantonsschule Rämibühl in Zurich
1982-1984	Secondary School (Sekundarschule) in Egg
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