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UNIVERSITE DE GENEVE
Département de Zoologie et Biologie Animale

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ECOLE POLYTECHNIQUE FEDERALE DE LAUSANNE
Laboratoire de Virologie et Génétique

FACULTE DES SCIENCES DE LA VIE
Professeur Didier TRONO

Lentivector-Mediated Transfer of Bmi-1 and Telomerase in Muscle Satellite Cells Yields a Duchenne Myoblast Cell Line with Long-Term Genotypic and Phenotypic Stability

THESE
présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention biologique

par

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de
Autigny (FR)

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intitulée :

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La Faculté des sciences, sur le préavis de Messieurs D. RUNGGER, professeur titulaire (Département de zoologie et biologie animale), D. TRONO, professeur ordinaire (Ecole polytechnique fédérale de Lausanne – Faculté des sciences de la vie – Lausanne, Suisse) co-directeurs de thèse, et J. LINGNER, professeur (Institut suisse de recherche expérimental sur le cancer – Epalinges, Suisse), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 10 mars 2006

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Le Doyen, Pierre SPIERER

Lentivector-Mediated Transfer of Bmi-1 and Telomerase in Muscle Satellite Cells Yields a Duchenne Myoblast Cell Line with Long-Term Genotypic and Phenotypic Stability

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CONTENTS

RESUME EN FRANCAIS

La Dystrophie Musculaire de Duchenne (DMD) est une des maladies les plus fréquentes : elle touche un garçon sur 3500. Cette maladie se caractérise par une dégénérescence progressive des muscles, si bien que les enfants qui en souffrent doivent utiliser une chaise roulante à partir d'une dizaine d'années et ils meurent en général entre 20 et 30 ans à la suite d'arrêt respiratoire ou cardiaque. Malgré le fait que cette maladie soit très répandue, aucune lignée cellulaire humaine n'était utilisée jusqu'à aujourd'hui. Nous avons voulu combler ce manque par l'immortalisation de cellules musculaires.

Afin d'immortaliser des myoblastes, nous avons utilisé des myoblastes issus de la culture de cellules satellites humaines provenant de biopsies de muscles. Les cellules satellites sont des cellules qui sont localisées soit directement sur les muscles, soit à proximité des muscles, dans la matrice extracellulaire. Ces cellules font partie de la lignée musculaire, si bien qu'elles peuvent fusionner entre elles et former des muscles ou réparer des muscles. Lorsque de telles cellules sont activées et prolifèrent, elles sont nommées myoblastes.

Nous avons utilisé des cellules humaines, afin d'obtenir des cellules modèles de la Dystrophie Musculaire de Duchenne, mais aussi afin de mieux connaître les événements menant à l'immortalisation de myoblastes. Or, il a été observé que les cellules animales, et particulièrement murines, sont beaucoup plus faciles à immortaliser que les cellules humaines. Cela vient du fait que les cellules humaines possèdent des mécanismes que les souris n'ont pas, et qui permettent plus de divisions cellulaires, tout en prévenant beaucoup de phénotypes cancéreux. En effet, les cellules murines se divisent beaucoup moins que les cellules humaines, 10^{11} mitoses contre 10^{16}. Cette différence de divisions permet aux humains d'avoir une taille plus grande, car la différence de taille entre les organismes humains et murins est comblée par un plus grand nombre de cellules et non par une plus grande taille des cellules. Or, il se trouve que les souris ont un risque de cancer de 30% à 1.5 ans et ce même risque de cancer est retrouvé dans des personnnes de 80 ans.

Une telle capacité de contrôler le cancer est possible chez l'homme, malgré un plus grand nombre de divisions cellulaires que chez la souris, grâce à différents mécanismes anti-cancéreux que la souris ne possède pas. Les cellules humaines, par exemple, régulent la

télomérase, afin d'empêcher que n'importe quelle cellule puisse se diviser indéfiniment. Cette enzyme a pour but de maintenir une longueur suffisante à la fin des chromosomes, les télomères. Si cette enzyme n'est pas activée chez l'humain, les télomères s'érodent au cours des divisions cellulaires, et une cellule ne pourra faire en général au plus qu'une cinquantaine de divisions cellulaires avant de mourir, dans un processus qui est nommé sénescence. Comme il existe plus de mécanismes cancéreux chez l'homme, il est compréhensible que davantage de barrières anti-cancéreuses doivent être dépassées avant d'obtenir une immortalisation avec des cellules humaines en comparaison des cellules murines.

Différentes méthodes peuvent permettre d'immortaliser des cellules dont notamment la mutagenèse chimique ou l'insertion d'oncogènes.

La mutagenèse chimique peut être très efficace afin d'obtenir les mutations conduisant à l'immortalisation de cellules. Toutefois, alors, les mutations induites conduisant à l'immortalisations ne sont pas connues. De même, on ne sait pas si des mutations qui sont inutiles pour l'immortalisation se sont produites. De telles mutations pourraient être négatives pour la qualité de la lignée cellulaire résultante car elles pourraient parfois avoir lieu dans des gènes importants pour la lignée en question ce qui fait que la lignée résultante pourrait avoir des phénotypes très différents de cellules primaires.

Sachant cela, nous avons choisi d'immortaliser les cellules par l'insertion d'oncogènes afin de connaître précisément les modifications apportées aux cellules primaires. Non seulement il est plus intéressant de travailler avec une lignée cellulaire dont on peut connaître précisément les modifications, mais, de plus, une telle approche permet de disséquer toutes les étapes menant à l'immortalisation.

Les gènes que nous avons utilisés afin d'immortaliser les myoblastes étaient TAg, Bmi-1, hTERT, Bcl-2. TAg est l'antigène large du virus simien SV40 et a déjà été utilisé précédemment pour ses vertus immortalisantes, notamment des cellules murines. Quant à Bmi-1, il a été montré récemment qu'il joue un rôle dans la sénescence des cellules, et quelques essais ont prouvé également ses propriétés immortalisantes, notamment pour les cellules endothéliales sinusoïdales du foie. La partie catalytique de l'enzyme télomérase (hTERT) a aussi été utilisée dans des essais d'immortalisation, surtout humains, afin

d'empêcher les télomères de ces cellules de s'éroder. Enfin, nous avons encore utilisé le gène Bcl-2 dans nos essais d'immortalisation car ce gène est connu pour avoir des effets anti-apoptotiques.

Après avoir choisi les gènes pouvant mener à une immortalisation, restait à choisir une méthode afin d'introduire ces gènes dans les myoblastes. Dans ce but, nous avons utilisé des vecteurs lentiviraux, dérivés du virus responsable du SIDA (Syndrome d'ImmunoDéficience Acquise), le VIH (Virus de l'Immunodéficience Humaine). De tels vecteurs lentiviraux sont très éloignés par divers aspects du VIH, car ils ont subi la délétion de la quasi-totalité du génome du VIH. Cela permet l'utilisation de tels vecteurs afin de transférer du matériel génétique, sans provoquer le SIDA. Dans le cas présent, nous avons utilisés de tels vecteurs car la capacité de leur capside était suffisante afin de contenir les gènes que nous voulions utiliser afin d'immortaliser les myoblastes. Toutefois, nous avons choisi ces vecteurs pour d'autres raisons: leur efficacité à insérer des gènes dans le génome des cellules et à produire souvent une bonne expression de ces gènes. A cet égard, nous avons d'abord vérifié que de tels vecteurs sont capables d'insérer des gènes dans des myoblastes à de très hautes efficacités. Nous avons aussi contrôlé que ces vecteurs peuvent permettre l'expression de transgènes à de hauts niveaux, ceci étant probablement possible grâce à une insertion dans les régions riches en gènes du génome de la cellule cible.

Différents mélanges de vecteurs encodant des transgènes ayant des qualités potentiellement oncogéniques furent utilisés dans les immortalisations. Ces mélanges de vecteurs étaient composés soit des vecteurs encodant Bmi-1, TAg ou hTERT seulement, soit des mélanges de vecteurs encodant Bmi-1+hTERT ou TAg + hTERT ou Bmi-1 + hTERT + Bcl-2. Il s'est avéré que seuls les mélanges de vecteurs Bmi-1 + hTERT ou TAg ou TAg + hTERT ou TAg + Bmi-1 + Bcl-2 + hTERT ont pu réellement conduire à l'immortalisation des myoblastes. Cela signifie que, dans nos expériences, les cocktails minimums d'oncogènes capables d'immortaliser les myoblastes furent TAg seul ou Bmi-1 + hTERT.

TAg a déjà été utilisé dans le passé pour l'immortalisation de différentes cellules, dont les myoblastes. Cependant, nous avons montré que les cellules qui étaient immortalisées avec TAg n'avaient pas un phénotype normal, elles étaient notamment beaucoup plus larges que les même cellules non immortalisées. Après analyse, il s'est avéré que ces cellules avaient un caryotype approchant la tétraploïdie alors que les cellules normales sont diploïdes. Nous

avons aussi pu constater que ces cellules avaient souvent subi des réarrangements chromosomiques, prenant la forme de translocations ou de cassures de chromosomes. Ces observations sont en accord avec d'autres articles décrivant les effets mutagènes que TAg peut avoir.

Au contraire de TAg, nous avons montré que bmi-1 et hTERT étaient capables non seulement de conduire à l'immortalisation de myoblastes, mais aussi de permettre à ce que la ligée cellulaire obtenue reste très proche des cellules primaires. Ceci a pu être montré notamment par une forme et un caryotype normaux de ces cellules. De plus, de telles cellules avaient une prolifération similaire aux cellules contrôles, ce qui n'était pas le cas des cellules immortalisées avec TAg. Enfin, nous avons montré que les cellules immortalisées avec bmi-1 et hTERT avaient la capacité d'arrêter leur prolifération en milieu de différenciation comme les cellules contrôles. Cela n'était pas le cas pour les cellules immortalisées avec TAg.

Malgré le fait que les cellules immortalisées avec TAg avaient beaucoup d'anomalies chromosomiques, nous avons noté que ces cellules n'étaient pas tumorigéniques après injections sous-cutanée dans des souris *nude.* Les souris *nude* ont comme caractéristiques d'être blanches, sans poils, et sans thymus. Ce phénotype est probablement dû à un défaut dans le développement de l'ectoderme. Comme ces souris n'ont quasiment pas de cellules T, elles ne peuvent pas empêcher la croissance des cellules tumorales. En conséquence, elles sont devenues un des modèles pour caractériser des cellules tumorales *in vivo*.

Même les cellules qui ont été immortalisées avec le plus d'oncogènes (TAg + Bmi-1 + hTERT + Bcl-2), n'ont pas été tumorigéniques après transplantation dans des souris *nude*. A l'opposé, l'injection sous-cutanée de cellules HeLa dans ces souris a conduit à de grandes tumeurs. Malgré de tels résultats, nous n'excluons pas que les lignées cellulaires que nous avons obtenues ne forment pas de tumeurs dans le long terme. En fait, nous pensons que les cellules qui contiennent TAg et qui ont été démontrées comme étant instables d'un point de vue génétique, peuvent être particulièrement aptes à produire des mutations qui pourraient permettre à ces cellules de devenir tumorigéniques. Quant aux autres lignées que nous avons produites et qui n'ont pas un caryotype instable, elles ont certainement moins la capacité de devenir tumorigéniques.

Un clone de myoblastes provenant d'un patient atteint de dystrophie musculaire de Duchenne a pu être utilisé dans ces immortalisations. Avec ce clone, nous avons pu obtenir une cellule immortalisée humaine contenant une mutation conduisant à la maladie de Duchenne. Jusqu'à maintenant, nous avons montré que cette lignée cellulaire a toutes les capacités de base des myoblastes primaires. Elle a un phénotype, un caryotype, et une prolifération qui sont normaux. De plus, cette nouvelle lignée est capable de proliférer et de fusionner normalement, jusqu'à 70% de fusion, comme les cellules contrôles. Cette lignée exprime encore Myf5 qui est un marqueur de surface caractéristique des myoblastes. Lorsqu'elle est placée en milieu de différentiation, cette lignée active aussi l'expression de Mef2, Desmin et Myogenin, ce qui est aussi caractéristique des myoblastes primaires placés en milieu de différentiation et qui deviennent des myotubes.

Par rapport aux myoblastes provenant de donneurs sains, toutefois, les cellules de Duchenne, que nous avons obtenues après immortalisation avec Bmi-1 et hTERT, ont une cinétique de fusion légèrement retardée dans le temps. Ceci peut être dû au fait que ces cellules ont une mutation de Duchenne, ou au fait qu'elles surexpriment Bmi-1 ou hTERT, ou encore à une différence clonale entre ces cellules. Pour savoir si le retard de fusion est dû à la mutation Duchenne ou à l'addition d'oncogènes ou à des mutations, il faudrait utiliser des cellules qui soient isogéniques par rapport à notre lignée Duchenne et qui contiennent ou non les oncogènes Bmi-1 ou hTERT. Même si nous avons pu obtenir une lignée Duchenne qui fusionne dans le long terme, tous les clones de myoblastes immortalisés avec bmi-1 et hTERT n'ont pas eu cette caractéristique. Ce fait pourrait être expliqué si certaines lignées subissent des mutations ou si différents types de myoblastes peuvent être immortalisés. Selon cette seconde hypothèse, il se pourrait donc que nous n'ayons pas toujours choisi les bons clones au début des essais d'immortalisation.

Même si nous n'avons pas observé d'aberrations chromosomiques dans les cellules immortalisées avec bmi-1, nous n'excluons pas que de telles cellules ont pu subir des mutations au niveau de quelques nucléotides provoquant des mutations. Il est en effet reconnu que les cellules peuvent subir soit des mutations visibles au niveau des chromosomes soit des mutations souvent issues d'instabilité de microsatellites, et celles-ci ne sont pas visibles dans les caryotypes. De telles mutations pourraient expliquer pourquoi nous n'avons obtenu que peu de clones qui fusionnent dans le long terme. Toutefois,

l'existence de différents types de cellules satellites et de myoblastes pourrait peut-être mieux expliquer ce fait. En effet, les cellules satellites sont connues pour leur hétérogénéité à différents niveaux. D'abord, il est connu que différentes populations de cellules satellites existent déjà *in vivo* et sont caractérisés par différentes expressions de marqueurs cellulaires. Ensuite, si un clone de ces cellules est cultivé *in vitro*, nous savons que de telles cellules conduisent aussi à une hétérogénéité de cellules dans un pétri. C'est ainsi que si un clone de cellules est soumis à un milieu de différentiation, seul environ 70% des cellules fusionnent. Quant aux 30% des cellules restantes, il est connu que seul un petit nombre d'entre elles peuvent se remettre à proliférer, si l'on revient en milieu de prolifération. Une telle hétérogénéité dans les myoblastes peut aussi expliquer pourquoi les clones que nous avons utilisés pour immortalisation n'ont pas pu tous produire des cellules avec des capacités de fusion dans le long terme.

Malgré l'obtention de clones ne fusionnant pas dans le long terme, nous avons pu obtenir une lignée cellulaire de myoblastes d'origine Duchenne, qui ressemblent beaucoup aux cellules primaires. En résumé, ces cellules ont donc un phénotype, un caryotype et surtout des capacités de différentiation normales qui sont très proches de celles des cellules primaires normales. Dans l'article décrivant l'obtention de cette lignée cellulaire, j'avais proposé que cette lignée cellulaire soit employée dans différents protocoles impliquant des myoblastes. Ce fut effectivement le cas, car cette lignée cellulaire nous a été demandée par différents laboratoires dans le monde. Parmi ceux-ci, nous avons fait une collaboration avec le groupe d'Antoine de Vries (Section de Thérapie Génique, Centre Médical de l'Université de Leiden, Leiden, Pays-Bas). Ce groupe travaille sur la thérapie génique de la Dystophie Musculaire de Duchenne. Cette collaboration leur a permis d'utiliser l'unique ou le quasi unique modèle humain disponible de la maladie de Duchenne. Quant à nous, cette collaboration nous a permis de profiter de leur expérience avec des vecteurs particulièrement adaptés pour corriger la Dystrophie musculaire. En effet, Manuel Goncalves, qui travaille dans ce groupe, a déjà élaboré de nouveaux vecteurs dont le but est de corriger cette maladie.

La Dystrophie Musculaire est provoquée par une mutation dans le gène humain le plus long connu : la dystrophine. Ce gène s'étend sur 2.5 Mbp, ce qui correspond à 0.1% du génome humain. On comprendra dès lors pourquoi la Dystrophie Musculaire est si

répandue : c'est parce qu'il est plus probable qu'un gène long soit muté par rapport à un gène court. Le fait que ce gène soit très long pose toutefois de grands problèmes dans sa correction telle qu'elle est envisagée dans les protocoles de thérapie génique. En effet, le cDNA de ce gène mesure 14 kb, ce qui est plus grand que la capacité de beaucoup de vecteurs, dont les vecteurs lentiviraux. C'est ainsi que les vecteurs lentiviraux ont été utilisés dans le but de corriger la Dystrophie Musculaire de Duchenne, seules des versions raccourcies de la dystrophine (appelées minidystrophines) ont été utilisées. Toutefois, le fait que ces versions soient ou non totalement fonctionnelles est un sujet de débat. Afin de ne pas entrer dans ce débat, nos collaborateurs ont au contraire opté pour la livraison d'une version complète de la dystrophine. Dans ce but, ils ont créé un nouveau vecteur qui est une version hybride de deux autres vecteurs connus. Des vecteurs dérivés des Adénovirus existent déjà, ils ont la capacité d'insérer dans des cellules de grandes quantités d'informations génétiques et donc de longs gènes. Par contre, ces vecteurs n'ont pas de capacités d'intégration. Au contraire, les vecteurs issus de Virus Adéno Associés (VAA) ne peuvent contenir que peu d'informations génétiques et donc que de courts gènes. Par contre, ces vecteurs ont la capacité intéressante de s'intégrer dans le locus *AAV1* du chromosome 19. Sachant cela, nos collaborateurs ont fait le design d'un nouveau vecteur combinant la capacité des Adénovirus de contenir beaucoup d'informations génétiques et la capacité des virus Adéno associés de s'intégrer dans le locus *AAV1*. Le vecteur résultant de cette manipulation est un mélange VAA-Adéno, et il a la capacité de contenir non seulement une version codante de la dystrophine, mais deux.

Un tel design VAA-Adéno a déjà été utilisé dans le passé et a pu conduire à la plus efficace correction de la Dystrophie Musculaire à ce jour. En effet, dans l'article relatant les résultats d'une telle manipulation, un vecteur codant deux unités d'expression de la dystrophine a permis jusqu'à 42% de correction de fibres du tibia antérieur de souris *mdx*.

Les cellules que nous avons obtenues précédemment ont été encore utilisées afin de démontrer la faisabilité de la correction de la dystrophine avec les vecteurs bi-Adéno-VAA. Avant de faire de telles expériences, la quantité de récepteurs CAR et CD46 a été contrôlée sur les myoblastes. Cela a été fait, car les vecteurs Adéno peuvent utiliser différents récepteurs, dont CAR et CD46 font partie, et si un type cellulaire a beaucoup d'un de ces récepteurs, il est plus judicieux d'utiliser un vecteur Adéno qui utilise le

récepteur en question, afin de rentrer au mieux dans ces cellules. Lors du contrôle de la quantité des récepteurs CAR et CD46, il a été démontré que les myoblastes contrôles mais aussi les myoblastes provenant de patients atteint de DMD et encore notre lignée cellulaire obtenue après immortalisation de cellules Duchenne avec Bmi-1 et hTERT, exprimaient tous à leur surface le récepteur CD46 et quasiment pas le récepteur CAR.

Prenant en compte ce dernier résultat, les vecteurs Adéno-VAA furent modifiés dans le but d'exprimer à leur surface une nouvelle fibre, la fibre 50 de l'Adénonovirus 50 (Ad50) qui lie CD46. Le but de cette modification était de créer des vecteurs qui pourraient s'attacher au mieux aux récepteurs CD46 présents sur les myoblastes et finalement de rentrer mieux dans ces cellules. Pour ce faire, le virus Adéno Aideur employé dans la production des vecteurs Adéno-VAA a été modifié afin d'arborer la fibre 50 du virus Ad50. Dès lors, le virus Aideur résultant fut nommé plox-Aideur-Adéno-fibre50. Il est à noter en passant que, dès le départ, tous les vecteur Adéno Aideurs qui ont employés ici ont été modifiés afin d'arborer de chaque côté de leur séquence d'encapsidation psi une séquence plox. Ces séquences plox permettent aux virus de *type sauvage* d'être en grande partie supprimés, car les cellules productrices Adéno-VAA expriment la recombinase Cre, qui permet l'excision de la séquence Ψ. L'élément Ψ étant la séquence clé pour l'encapsidation du génome viral, la séquence d'ADN codant pour le virus Adéno n'entrera pas dans la capside protéique virale, ce qui a pour effet d'empêcher la propagation du virus de *type sauvage*.

Les vecteurs résultants de toutes ces manipulations peuvent être nommés hc-bi-Adéno-AAV-fibre50. En effet, de tels vecteurs ont une haute capacité d'accueillir des transgènes si bien qu'ils permettent l'introduction de deux unités d'expression de la dystrophine (de là leur préfixe hc-bi) mais ils sont aussi composés à la fois du virus Adéno et de la capacité des vecteurs VAA de s'intégrer dans le locus AAV1 du chromosome 19. Enfin, de tels vecteurs ont été produits avec la fibre 50 à leur surface afin de permettre une entrée optimale dans les myoblastes, car ceux-ci expriment à de plus hauts niveaux le récepteur qui est associé à la fibre 50 : le CD46.

Il fut montré que les vecteurs hc-bi-Adéno-AAV-fibre50 pouvaient être produits aussi bien que les vecteurs n'étant pas composés avec la fibre 50. De plus, de tels vecteurs avec la fibre 50 pouvaient transduire des myoblastes beaucoup plus efficacement que les vecteurs avec la fibre 5. En fait, 30 fois moins de vecteurs ayant à leur surface la fibre 50 (qui lie

CD46) était nécessaire pour arriver au même niveau de transduction qu'avec les vecteurs arborant la fibre 5 (qui lie CAR). Tout cela a été possible, rappelons-le, parce qu'il y a sur les myoblastes plus de récepteurs CD46 pour la fibre 50, que de récepteurs CAR pour la fibre 5.

De tels vecteurs furent ensuite employés dans le but de corriger notre lignée cellulaire pour l'expression de la Dystrophine. Leur utilisation permit non seulement de produire la dystrophine, mais aussi la localisation normale du Beta-dystroglycan. En effet, après une mutation de type Duchenne, il est connu que le complexe associé à la dystrophine est détruit, ce qui mène à une localisation cytoplasmique. Au contraire, dans les myoblastes et muscles normaux, le béta-dystroglycan est localisé au plasmalemme, et après adjonction des vecteurs dystrophine sur notre lignée cellulaire, nous avons aussi pu observer la redistribution du beta-dystroglycan au plasmalemme.

Une des nouveautés de cet article est non seulement l'utilisation et le développement de nouveaux types de vecteurs basés sur les Adénovirus et les Virus Adéno Associés mais aussi la correction du phénotype Duchenne dans des cellules d'origine humaine. En effet, jusqu'ici, pour de tels travaux, en général, seules les cellules murines C2C12 ont été utilisées. Or, il est connu que le phenotype humain de la Dystrophie Musculaire de Duchenne est très différent par rapport à ce qui peut être vu dans les organismes murins. En effet, des souris avec une mutation dans la dystrophine sont souvent très similaires aux souris contrôles et elles n'ont des problèmes avec leurs muscles que tard dans leur vie. De telles différences ne sont pas encore totalement comprises mais proviennent au moins en partie de différences qu'il y a dans la régulation des cellules satellites humaines et murines. En sachant cela, il n'y aurait rien d'étonnant que la nouvelle lignée humaine obtenue ici ait la potentialité d'être beaucoup plus utile que des lignées murines dans les recherches sur la Dystrophie Musculaire de Duchenne.

Les articles présentés dans cette thèse montrent donc, d'abord, que l'on a pu obtenir une nouvelle lignée cellulaire Duchenne. Cette lignée a, en bref, un phénotype, un caryotype, et une différentiation qui sont normaux. Toutes ces caractéristiques font de la lignée Duchenne obtenue ici certainement la meilleure lignée humaine de myoblastes obtenue jusqu'ici. Ensuite, ils montrent que cette lignée a pu être utilisée afin d'étudier la Dystrophie musculaire de Duchennne mais aussi sa correction. A l'occasion de la

correction en dystrophine de notre lignée cellulaire de Duchenne, un nouveau type de vecteur a été obtenu ici.

L'obtention d'un nouveau modèle cellulaire de la maladie de Duchenne, pourra permettre un développement de la recherche dans le domaine de la Dystrophie Musculaire. L'obtention de ce modèle a aussi permis de mieux comprendre les étapes nécessaires à l'immortalisation des myoblastes. Quant à l'obtention du nouveau vecteur hc-bi-Adéno-AAV-fibre50, il pourra être utilisé dans d'autres protocoles de thérapie génique. De manière générale, la lignée cellulaire et le nouveau vecteur obtenu dans les articles présentés dans cette thèse permettront certainement le développement de la connaissance dans le domaine de la Dystrophie musculaire. Cela pourra être fait par nos groupes, mais aussi par d'autres, et je souhaite dès lors plein succès à tous ceux qui seraient tentés par cette approche.

D'autres possibilités peuvent encore découler de l'obtention de cette lignée de myoblastes humains. On peut penser qu'il pourrait être intéressant de fusionner de telles cellules, de les encapsuler, et de leur faire relâcher un produit thérapeutique. Par exemple, jusqu'à maintenant, les cellules murines C2C12 ont déjà été encapsulées dans le but de relâcher une molécule thérapeutique. Or, dans de telles expériences, il est connu que les cellules C2C12 subissaient toujours une mortalité importante dans les capsules, et cela pourrait être dû soit au fait que les cellules C2C12 prolifèrent encore dans la capsule soit au fait que beaucoup de cellules au centre de la capsule meurent car elles ne sont pas bien fusionnées aux autres. Les faits que notre lignée cellulaire puisse complètement arrêter de proliférer en milieu de différentiation et fusionne facilement pourraient suggérer que nos cellules peuvent être plus intéressantes à utiliser dans des encapsidations, car leurs mortalités pourraient être plus faible dans des capsules que de cellules C2C12. De plus, même si cela n'a pas été démontré, il se peut que les cellules C2C12, même encapsulées, relâchent un produit murin qui pourrait ne pas être bien toléré par l'homme, ou créer une réaction immunitaire. Au contraire, nos cellules ont l'avantage d'être humaines et très proches des cellules primaires, et donc, si elles sont injectées dans un hôte, elles pourraient être mieux tolérées par l'organisme et par le système immunitaire humain de l'hôte. Sachant cela, quelques essais de thérapies géniques non reliés au domaine des muscles pourraient être envisagés par exemple pour relâcher une molécule thérapeutique dans le sang. Toutefois,

sans attendre l'élaboration de telles expériences au résultat encore imprévisible, cette lignée est déjà utilisée par différents groupes dans différentes recherches académiques, et c'était bien là le but premier de sa fabrication.

ABSTRACT

Duchenne Muscular Dystrophy is a genetic muscle wasting disease touching 1 out of 3500 boys. However, the study of this disease was particularly difficult because no human cellular line with the features of these primary cells existed. During my thesis, I thus aimed at the fabrication of a human cellular model of this disease.

In this respect, myoblasts from muscle biopsies of normal or Duchenne patients were immonartalized. These cells could be immortalized with different cocktails of oncogenes, consisting of Bmi-1, Large T antigen, Bcl-2, and human telomerase (hTERT). For this purpose, lentiviral vectors were used because they have sufficient capacities for the transgenes we wanted to use, but also because they allowed successful incorporation and transcription of them in myoblasts. After characterisation of the resulting cell lines, it was observed that TAg-immortalized cells were bigger and rounder than their primary counterpart. TAg cell lines had also a karyotype in the tetraploid range, with numerous karyotipic anomalies, including translocations and chromosomal breaks. All these anomalies were not observed with cells immortalized with Bmi-1 and hTERT, which were shown to have a normal phenotype and a normal karyotype. A myoblast cell line with a Duchenne mutation could be established after immortalization with Bmi-1 and hTERT. This cell line had not only normal phenotypic karyotypic properties, but also a fusion very similar to normal primary cells. This cell line was also shown to express all the typical myogenic markers, once studied in proliferation or differentiation conditions.

This new cell line was then complemented with Dystrophin, to obtain a normal muscle cell line, with the same genetic background as our Duchenne cell line. This experiment was also the occasion to test the utility of our Duchenne cell line in the studies aiming to correct DMD, especially in gene therapy experiments. For this purpose, hich capacity vectors were used, which were combining the properties of the Adeno Associated Virus (AAV) to insert their genetic material into the *AAV1* locus of chromosome 19 and the properties of the Adeno virus to package large quantities of genetic material. At this occasion, the fibre on the surface of the vector was modified, to permit better entry into the myoblasts. This finally allowed the

complementation of our Duchenne cell line with Dystrophin, with correction of the DMD phenotype, as seen by visualization of Dystrophin in muscles, but also by correct relocalization of the beta-Dystroglycan complex at the plasmalemme.

ABBREVIATIONS

Ai6 apoptosis inhibitor-6

AML acute myelogenous leukaemia

Apc adenomatosis polyposis coli

AAV Adeno-Associated Virus

AAV1 Preferred integration locus of the AAV1 virus

Bmi-1 B lymphoma Mo-MLV insertion region

Brca breast cancer

CDK cyclin-dependent kinase

CDKI cyclin-dependent kinase inhibitor

CIN chromosomal instabilities neoplasias

CMV human cytomegalovirus immediate early promoter

CTLs cytotoxic T lymphocytes

Cx connexin

Cyc cyclin

DGC Dystoglycan complex

EGFP enhanced green fluorescent protein

FKHR Forkhead in human rhabdomyosarcoma

GH Growth hormone

GTPases guanosine triphosphatases

HIV Human Immunodeficiency Virus

HLA human leukocyte antigens

HSV1-TK thymidine kinase of herpes simplex virus type 1.

INK4 inhibitors of CDK4

IGF-I Insulin-like Growth Factor-I

IRES internal ribosomal entry site of the encephalomyocarditis virus

KIRs killer cell inhibitory receptors

KO knockout

LT Large T antigen

MAPK mitogen-activated protein kinase

MDM2 murine double minute 2

MIN microsatellite instabilities neoplasias

Mo-MLV Moloney Murine Leukemia Virus

mSP murine bone marrow side population

MyHC myosin heavy chain

Nf neurofibromatosis

NK natural killer

NOD/SCID non-obese diabetic/severe combined immunodeficient

NPM nucleophosmin

PAF-AH platelet-activating factor acetylhydrolase

PI3K phosphatidylinositol 3-kinase

PLD phospholipase D

PP2A phosphatase 2A

RAL–GEFs RAL guanine-nucleotide exchange factors

Rb retinoblastoma

RDC1 chemokine orphan receptor 1

RRE Rev-responsive element

SA splice acceptor

SCID severe combined immunodeficient

SD splice donor

SMT somatic mutation theory of carcinogenesis

SP side population

ST small T antigen

STASIS **ST**ress or **A**berrant **S**ignaling **I**nduced **S**enescence

SV40 Simian Virus 40

Terc telomerase RNA component

TJP tight junction protein

TOFT tissue organization field theory of carcinogenesis and metastasis

Ub Ubiquitin

VCAM vascular cell adhesion molecule

VLA very late antigen

VSV-G Vesicular stomatitis virus G protein

Ψ packaging signal

CHAPTER 1: INTRODUCTION

1.1 General concepts of senescence and oncogenesis

1.1.1 Senescence and immortalization

Normal somatic cells do not propagate indefinitely. Somatic cells can grow *in vitro* under appropriate culture conditions, but they have a limited doubling potential. After to have followed about 50 divisions (which often corresponds to less than one year in culture), most of the cell strains degenerate, and follow a process called senescence (Hayflick and Moorhead, 1961). However, it has been shown that the introduction of a limited number of viral-borne or tumor-derived genes was sufficient to render rodent cells immortal (Bishop, 1985). While the introduction of a single gene failed to immortalize these cells, two genes were sufficient to immortalize these cells, but also contributed to their transformation to a tumorigenic state (Land et al., 1983; Ruley, 1983). The transforming oncogenes were termed viral or tumor oncogenes, and they were found to have gain-of-function mutations compared to normal human genes, called proto-oncogenes. Many reports have subsequently identified numerous pairs of oncogenes that were capable to render rodent cells tumorigenic (Hunter, 1991). Nevertheless, despite the success of some pairs of oncogenes to immortalize and transform rodent cells, they often failed to immortalize human primary cells (Stevenson and Volsky, 1986). Such findings led to the proposal that a greater number of alterations are necessary to transform human cells than to transform murine cells.

1.1.2 Differences in senescence and oncogenesis between human and mouse cells

Apart from having a probable causal role in aging, the senescence mechanisms may suppress the development of cancer in mammals (Campisi et al., 2001; Reddel, 2000; Wright and Shay, 2001).

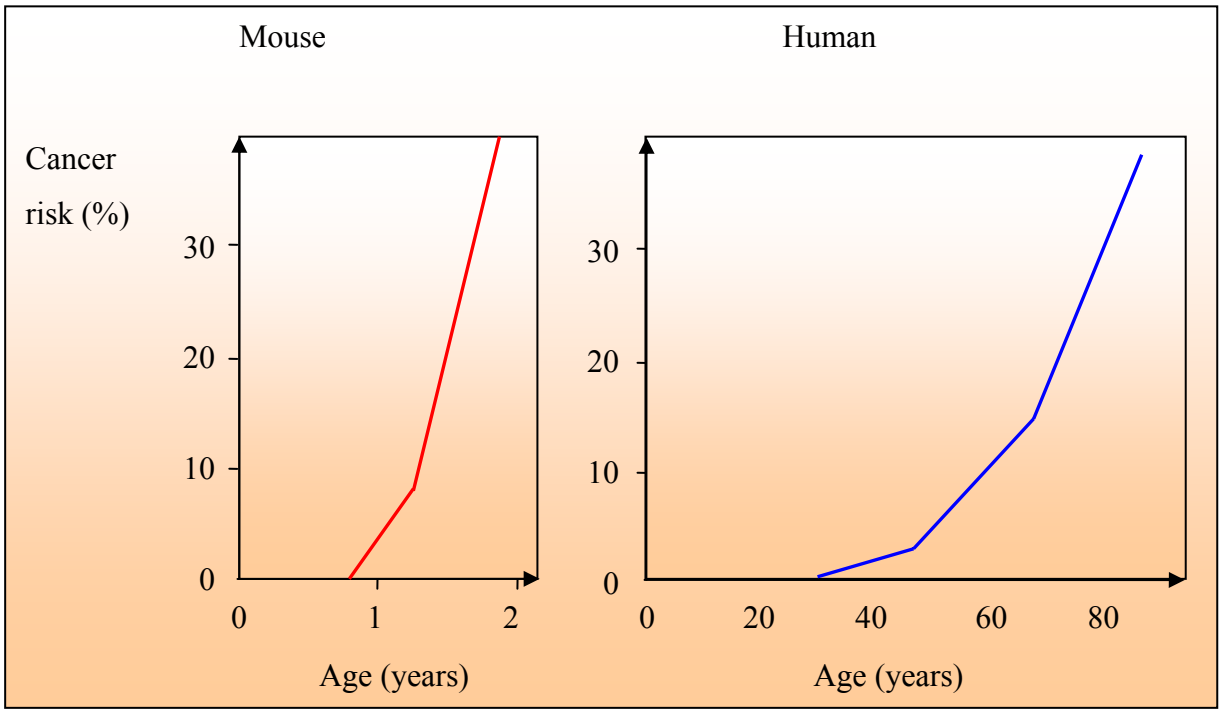

Figure 1. Cancer risks in mice and humans with age. Mice data are from (Pompei et al., 2001). Human data are from the American Cancer Society, Cancer Facts and Figures 2003. Figure redrawn from (Rangarajan and Weinberg, 2003).

The need for additional barriers to carcinogenesis in human certainly stems from the fact that human cells divide more than murine cells. Humans are about 3,000 times larger than mice, but they also have more cells, and live longer than mice. In fact, humans undergo about 10^5 more cell divisions in a lifetime (10^{16} divisions in humans versus 10^{11} mitosis in mice) (Rangarajan and Weinberg, 2003). Nevertheless, the risk to develop cancer during the life of mice or humans is comparable (Figure 1) (Rangarajan and Weinberg, 2003). However, humans live longer than mice, and this increase in lifespan with comparable cancer risks was possible since 80 million years after the development of new antineoplastic mechanisms (Hanahan and Weinberg, 2000).

Considering these facts, we can better understand why both similarities and discrepancies have been found between mouse and human carcinogenesis. This has been particularly

observed after the comparison of mouse knockout (KO) models and family inherited mutations in humans (Figure 2).

Tumour-suppresor gene	Spectrum of tumours in conventional mouse KO models	Spectrum of tumours in humans (loss of function)	Recent genetic modifications	Spectrum of tumours in modified KO models
Ink4a	Fibrosarcoma, lymphoma, squamous- cell carcinoma	Familial melanoma, sporadic pancreatic, brain tumours	Crossed with *Arf* $^{+/-}$ mice	Metastatic melanoma, sarcoma, carcinoma, lymphoma
Trp53	Osteosarcoma, lymphoma, soft-tissue sarcoma	Breast carcinoma, brain, sarcomas, leukaemia	Additional loss of *Terc*	Breast and other carcinomas
Apc (*Apc*^{MIN}, *Apc*$^{\Delta 716}$, *Apc*$^{\Delta 580}$)	Multiple polyps in small intestine	Polyps in colon progressing to carcinomas	Conditional colon-specific inactivation	Polyps in colon
Rb	Brain, pituitary	Retinoblastoma, osteosarcoma	Additional loss of *p107*	Retinoblastoma
Brca1	No tumour susceptibility	Breast, ovary	Conditionalmammary-specific inactivation of *Brca1*	Mammary tumours
Brca2	No tumour susceptibility	Breast, ovary	Conditional mammary-specific inactivation of *Brca2* and *Trp53*	Mammary tumours
Nf1	Pheochromocytoma, myeloid leukaemia	Neural-crest-derived benign neurofibroma and malignant fibrosarcoma	Additional loss of *Trp53*	Neural-crest-derived malignant glioblastoma
Nf2	Osteosarcoma, fibrosarcoma, lung adenocarcinoma, hepatocellular carcinoma	Schwannomas, meningiomas, ependymomas, gliomas	Schwann-cell precursor-specific ablation of *Nf2*	Schwannomas

Figure 2. Comparisons between mouse KO models and family inherited human alterations in tumor suppressor genes. *Apc*, adenomatosis polyposis coli; *Brca*, breast cancer; KO, knockout; *Nf*, neurofibromatosis; *Rb*, retinoblastoma; *Terc*, telomerase RNA component. Figure taken from (Rangarajan and Weinberg, 2003).

1.1.3 Are there senescence clocks?

Normal cells have a finite life span that seems to be counted, before they finally die. But if the life span is counted, what is the counting mechanism?

After the discovery that some nucleoproteins, named telomeres, shorten with every cycle of cell growth, the hypothesis that these structures could be the counting mechanism for cell senescence appeared. In such a model, progressive erosion of telomeres with cell divisions would trigger senescence, once a minimal threshold of telomere length is reached (Harley et al., 1990b). This mechanistic model was partially verified, because, at least in some cell types, ectopic expression of the catalytic unit of telomerase enzyme, hTERT (human telomerase reverse transcriptase), was sufficient to lead to extension of cellular life span (Bodnar et al., 1998a; Vaziri and Benchimol, 1998b).

Different telomerase expression levels may also explain differences observed in the immortalization of murine and human cells. Indeed, telomerase is expressed in most murine tissues (Prowse and Greider, 1995), but it is expressed only transiently and at low levels in normal human cells (Broccoli et al., 1995; Counter et al., 1995; Hiyama et al., 1995; Masutomi et al., 2003). However, additional differences still exist between the phenotypes produced by the loss of telomerase in humans and mice. While telomerase loss produces affects after about 50 cell divisions in most human cells types, the effect of telomerase loss in mice is only visible at the sixth generation, despite of continued telomere shortening. This discrepancy may be explained at least in part because there are less cellular divisions in mice. However, murine telomeres are also longer, they measure from 20 to 50 kb, and human telomeres sizes range between 8 and 15 kb. Then, both reduced lifetime of mice and longer telomere lengths may explain why it is only after six generations of telomerase knock-out in mice that abnormalities are observed. They include fusion of chromosomes, aneuploidy, and also sterility (Lee et al., 1998).

Additional observations suggested that telomere length is probably not the sole counting mechanism for cell life. Indeed, oxygen levels were found to have an effect on the lifespan of culture human fibroblasts. In fact, it was observed that when fibroblasts are propagated in 1-3% oxygen instead of 20% oxygen, they are able to undergo more divisions prior to

senescence. In the contrary, when oxygen levels are higher than 20%, their lifespan shortens (Chen et al., 1995; Packer and Fuehr, 1977; von Zglinicki et al., 1995). Other experiments have shown that senescence can also be induced by some forms of DNA damage (for example, oxidative stress), and by certain oncogenes (Campisi, 2001; Wright and Shay, 2002). This led to the conclusion that senescence can be induced by environmental stresses, and such phenomenon was variously termed premature senescence, culture shock, stress-induced senescence, or STASIS (**ST**ress or **A**berrant **S**ignaling **I**nduced **S**enescence) (Shay and Wright, 2004).

At this point, it was suggested that there is not only a telomerase-based clock in normal cells, but also a stress-based clock that also contributes to aging. Then, it would be the combined effect of these two clocks that would define the onset time of senescence.

Until now, at least these two clocks are thought to collaborate in defining the age of senescence of cells and in inducing of senescence, but other clocks may be possible. It could still be possible that only telomerase length is a real clock for senescence and that stress stimuli may have effects on telomeres, and finally on senescence. This is true to a certain extent at least in some cells have where telomeres have been shown to shorten more quickly in high oxygen environments (von Zglinicki et al., 1995). Nevertheless, senescence was also shown not to depend only on telomere lengths. Indeed, senescence can happen even with long telomeres, and such form of stress-related senescence can not be prevented by ectopic hTERT expression (Gorbunova et al., 2002; Wei et al., 1999).

These data, and other data that will be presented now, suggest that telomere length is not the only determinant of senescence. They also suggest that replicative senescence and stress-induced senescence are not as different as depicted until now: they use the same mechanisms and they generate one response program. At this point, the conceptual ideas of senescence clocks should be reexamined in view of the facts that pathways of replicative senescence and stress-induced senescence are mechanistically bound. The emerging picture is that different stimuli can activate senescence, and whether this occurs rapidly, or gradually, following a period of proliferation, is mainly determined by the combined levels of the stresses involved. Then, it seems that it is not one clock, two clocks, or more, that should be considered in the accomplishment of the senescence program, but, best, the integrated measure of different stresses.

1.1.4 Pathways toward senescence

Some genes have been shown to induce senescence, even in cells that are considered to be immortal, such as tumor cells (Figure 3) (Roninson, 2003). Such experiments, and others, that will be discussed now, have permitted to uncover the pathways leading to senescence.

Gene	Tumor cell line
p53	EJ bladder ca[a]; NSCLC H358 lung ca
p63	EJ bladder ca
p73	EJ bladder ca
RB	MDA-MB-468 breast ca; 5637 bladder ca; Saos-2 osteosarcoma
$p21^{Waf1/Cip1/Sdi1}$	HT1080 fibrosarcoma; EJ bladder carcinoma; H1299 lung ca
$p16^{Ink4A}$	U-1242 MG glioma; U251 glioma; U2-OS osteosarcoma
$P57^{Kip2}$	U343 MG-A, U87 MG, and U373 MG astrocytoma
$p15^{Ink4B}$	U251 glioma
E2 papillomavirus protein (inhibitor of E6 and E7)	HeLa, HT-3, and CaSki cervical ca
IGFBP-rP1	M12 prostate ca; MCF-7 breast ca
Activated RAF-1	LNCaP prostate ca
Activated MKK6	U2-OS osteosarcoma

Figure 3. Genes that induce senescence-like phenotype in tumor cells. [a] ca, carcinoma; NSCLC, non-small cell lung cancer. Figure taken from (Roninson, 2003).

Two main different pathways of senescence can be triggered and need to be bypassed for immortalization. These pathways of senescence can be $p16^{INK4a}$-retinoblastoma ($p16^{INK4a}$-Rb) dependant, or ARF-p53 dependant (Figure 4). These pathways have also a role in oncogenesis (Sherr, 2001).

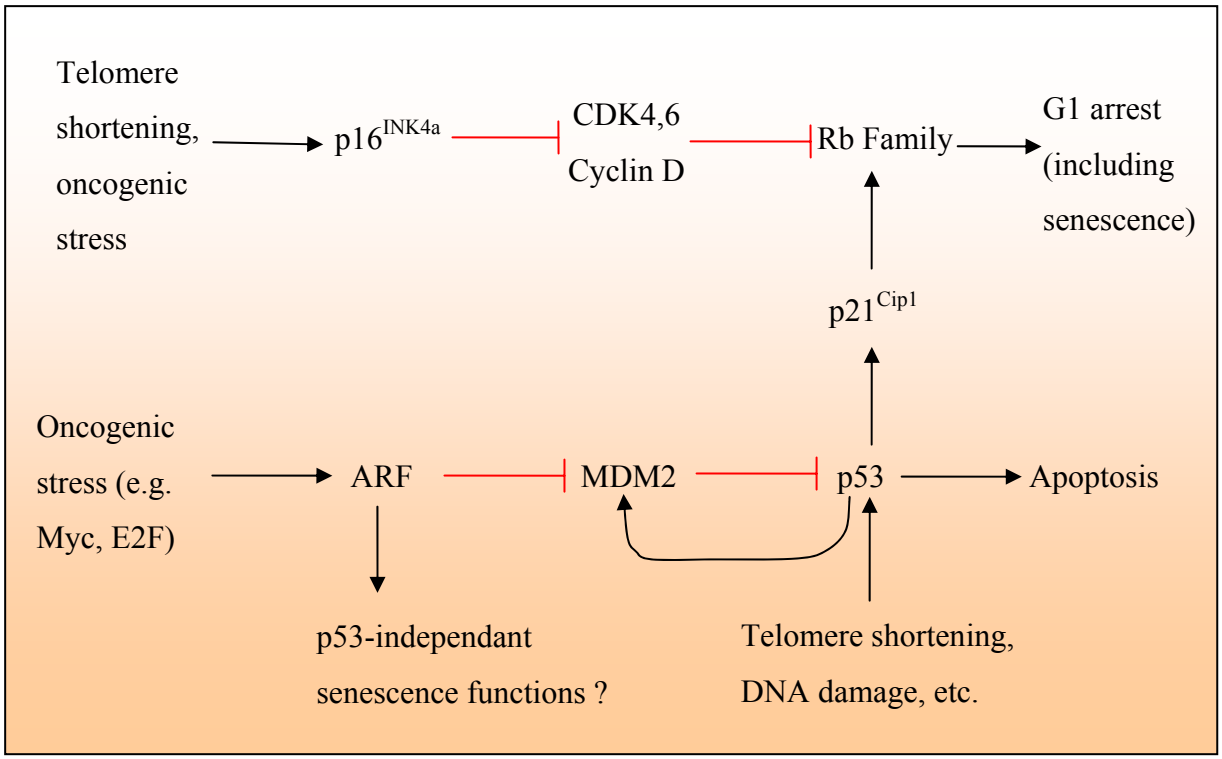

Figure 4. The $p16^{INK4a}$-Rb dependant, or ARF-p53 dependant pathways of senescence. Figure modified from (Drayton and Peters, 2002; Lowe and Sherr, 2003; Sharpless, 2004; Sherr, 2001; Sherr, 2004).

The tumor suppressors $p16^{INK4a}$ and ARF are implicated separately in the measure of lifespan. This was demonstrated by the fact that the lifespan of mice strains deprived of $p16^{INK4a}$ or ARF is shorter than wild-type animals but longer than the strains deprived of both (Kamijo et al., 1997; Serrano et al., 1996; Sharpless et al., 2001; Sharpless et al., 2004).

Nevertheless, disruptions of the $p16^{INK4a}$ and ARF pathways can lead to different phenotypes in different cell types, and in different organisms. Most MEFs acquire characteristic senescence-related morphologies by an ARF mechanism. In the contrary, murine lymphocytes, macrophages, astrocytes, and a variety of human cells, were reported to senesce *in vitro* by a $p16^{INK4a}$–dependent mechanism (reviewed by (Sharpless, 2004)).

Characterization of cancer cells showed inverse correlation between the presence of $p16^{INK4a}$ and wildtype RB, suggesting the existence of a link between $p16^{INK4a}$ and RB (Otterson et al.,

1994; Shapiro et al., 1995). Genetic observations also linked the tumor suppressor ARF to p53 (Chin et al., 1997; Kamijo et al., 1997; Pomerantz et al., 1998). The genetic linkage of pRB and p53 with senescence were verified by further experiments. They include the following ones:

- Inactivation of p53 extends the lifespan of human fibroblasts (Itahana et al., 2001), whereas MEFs from p53 null mice do not senesce (Harvey et al., 1993).
- MEFs with targeted disruption of all pRB family of proteins (pRb, p107, and p130) do not senesce (Dannenberg et al., 2000; Sage et al., 2000).
- Human cells that lose p53 and pRB functions are generally refractory to multiple senescence-inducing stimuli (Dimri et al., 2000; Serrano et al., 1997).

The $p16^{INK4a}$-Rb and of ARF-p53 senescence pathways will be presented more in detail now. Interestingly, these pathways begin either by $p16^{INK4a}$ or ARF activation, and it appeared that both these proteins are products of genes originating from the INK4a locus. Even though the $p16^{Ink4a}$ and $p14^{Arf}$ ($p19^{ARF}$ in mice) transcripts are initiated at different promoters, they share part of the same sequence, but in alternative reading frames (hence the name of Arf) (Figure 5). The reason why these two tumour-suppressor genes are so intimately linked is not known, but it is suspected that this chromosomal link may have a utility for the cells. As both these proteins are involved in interpreting responses to hyperproliferative signals and in modulating the Rb and p53 pathways, this chromosomal link could have a utility for these proteins to respond to similar signals. However, despite their intimate linkage, the two genes are independently positively and negatively regulated (Sherr, 2004). So, it is still not understood what selective advantage this linkage might have, if it has any. In fact, it seems particularly intriguing why two tumor suppressors are located in the same locus. This particularity makes that since some deletions in the INK4a-ARF locus can simultaneously compromise the functions of two tumor suppressors. This may seem to be a disadvantage for the cell, but if evolution has adopted this very peculiar conformation of tumor suppressor genes, it is probably for a good, but still unknown, reason.

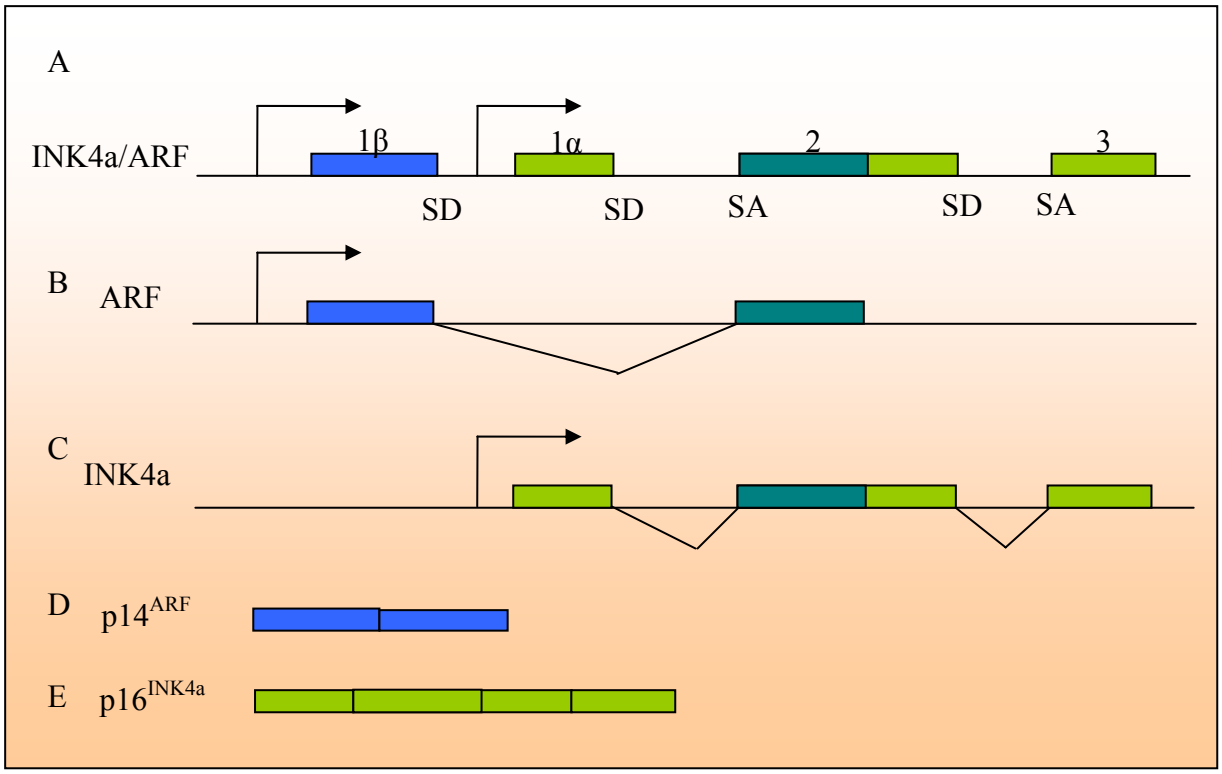

Figure 5. The INK4a/ARF locus. A The INK4a/ARF locus encodes two proteins : $p16^{Ink4a}$ and $p14^{Arf}$ ($p19^{ARF}$ in mice) by different promoters. (A) Overview of the INK4a/ARF locus, with the different promoters (arrows), exons (1β, 1α, 2 and 3), splice donors (SD), and splice acceptors (SA). 1β and 1α are alternative exons of ARF and $p16^{Ink4a}$, respectively. (B) ARF only. (C) INK4a only. The second exon of $p16^{Ink4a}$ is similar to the second exon of ARF, but in an alternative reading frame, so that ARF stop codon is not placed at the same position than that of INK4a. (D) (E) the resulting $p14^{ARF}$ and $p16^{INK4a}$ products. Figure adapted from (Collins and Sedivy, 2003; Hayward, 2003; Sharpless, 2004; Sherr, 2001).

1.1.5 The $p16^{INK4a}$-Rb pathway

The INK4 family comprises $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ ($p19^{INK4d}$ being distinct from murine $p19^{ARF}$). All these proteins are cyclin-dependent kinase inhibitors (CDKIs). The CDKIs inhibit the cyclin-dependent kinases that phosphorylate the related proteins p107 and

p130, leading to their inactivation, and to inhibition of cell cycle progression (Classon and Harlow, 2002). Implication of these proteins in tumorigenesis has been noted, but in different extents (Figure 6) (Sherr, 2001). Nevertheless, only INK4a and ARF, and not their related cell cycle inhibitors of the INK4 family, seem to have a major role in senescence. Moreover, only the expression of $p16^{INK4a}$ and ARF is increased in aged organs, once this expression is compared with the one found in young organs (Krishnamurthy et al., 2004).

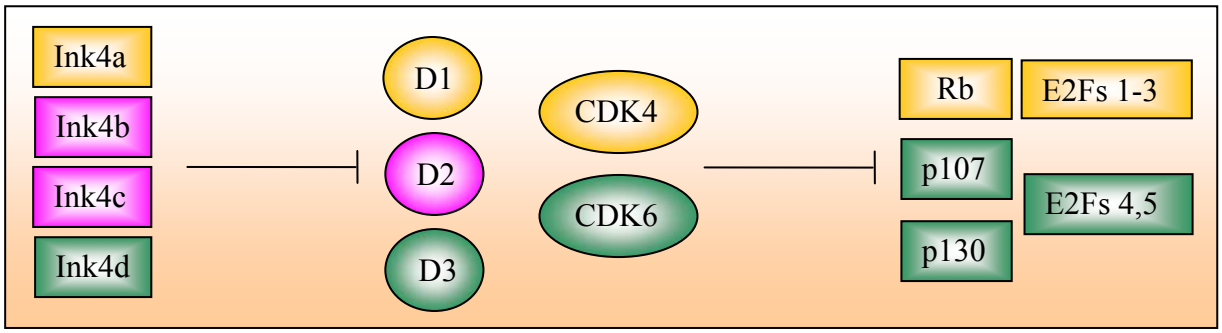

Figure 6. The ink4 family of CDKI. The four Ink4 proteins inhibit complexes of cyclins D1, D2 or D3 with either Cdk4 or Cdk6. These six cyclin D-dependent kinases complexes can inhibit the three Rb-family members that interact preferentially with different E2f subunits. Rb associates primarily with E2fs 1–3, and p107 and p130 preferentially forms complexes with E2fs 4 and 5. Yellow, pink and green shadings indicate the proteins that are often, less frequently or only rarely (if at all) involved in tumorigenesis. Figure redrawn from (Sherr, 2001).

The fact that INK4a-deficient human diploid fibroblasts were shown to be resistant to RAS-induced senescence, has led to the implication of $p16^{INK4a}$ in senescence (Brookes et al., 2002). Moreover, in mice, increased expression of $p16^{INK4a}$ by deficiency of the *SNF2*-like gene PASG was shown to cause premature aging (Sun et al., 2004). One further evidence suggesting that $p16^{INK4a}$ is implicated in senescence came from MEFs with a p16 binding deficient mutant of CDK4, that failed to senesce in culture (Rane et al., 2002). It was also shown that when rare human epithelial cells escape cell cycle arrest once placed in culture, they show epigenetic silencing of the INK4a gene (Drayton and Peters, 2002). However, it was also shown that this particular block could be avoided by alternate culture conditions. Indeed, co-culture of epithelial cells with feeder layers allowed them to behave more like fibroblasts (Ramirez et al., 2001).

Telomere shortening or telomere structure dysfunction is correlated with increased $p16^{INK4a}$ expression in senescing human fibroblasts (Bodnar et al., 1998b; Smogorzewska and de Lange, 2002). Despite this evidence, the signaling pathway linking uncapped telomeres to $p16^{INK4a}$ has not been completely elucidated. Additional events seem to regulate $p16^{INK4a}$ accumulation, but they are less well understood than that of ARF. Nonetheless, it is known that $p16^{INK4a}$ can be induced by certain forms of oncogene activation, such as by the RAS→RAF→MEK→ERK→Ets1/2 pathway (Zhu et al., 1998). Repressors such as Ets11 and Id are also known to attenuate $p16^{INK4a}$ expression (Alani et al., 2001; Huot et al., 2002; Ohtani et al., 2001). In the end, bmi-1, cbx-7 and tbx2 have also been reported to repress the transcription of $p16^{INK4a}$, but also of ARF (reviewed by (Sharpless, 2004)) (Figure 7).

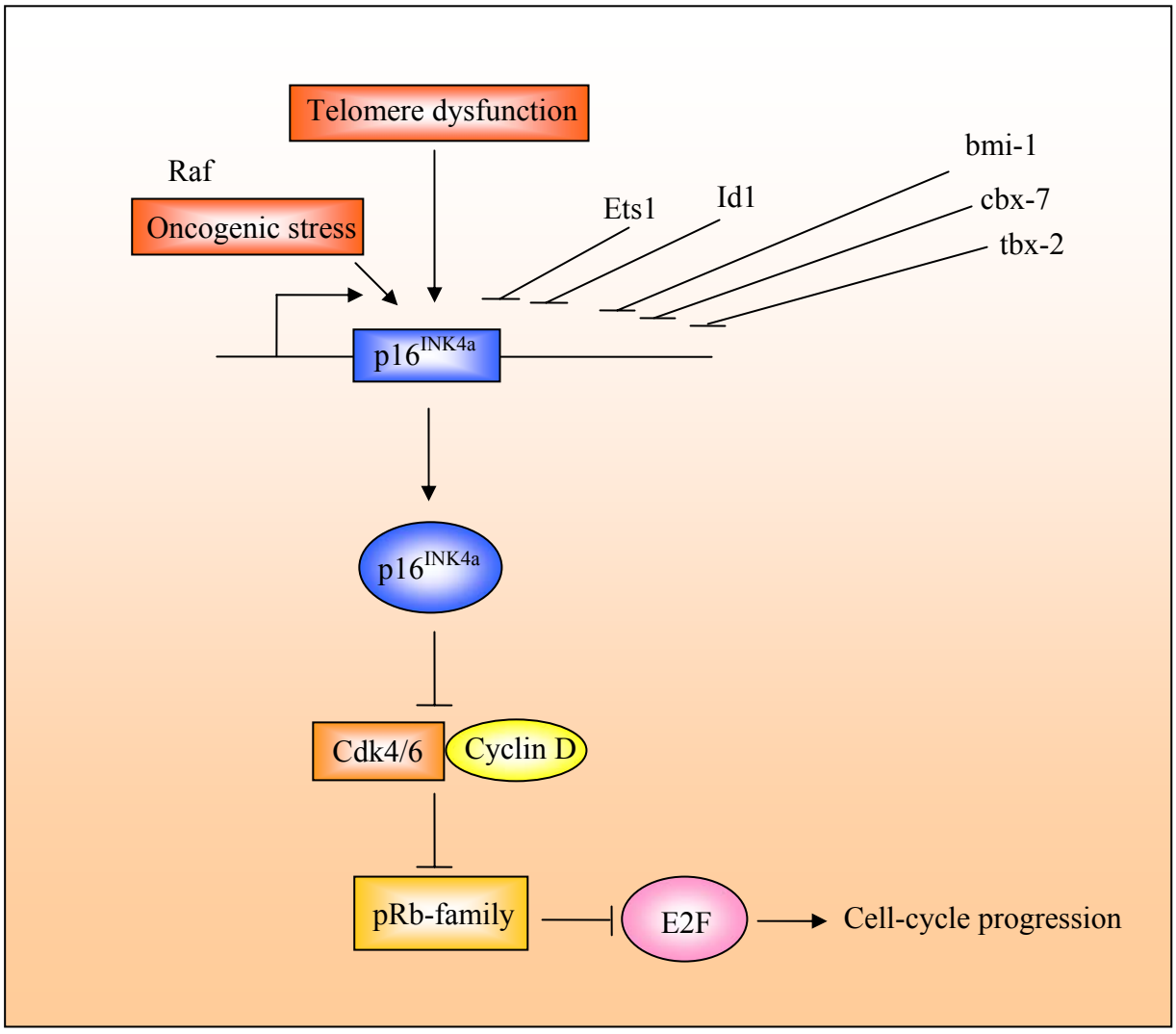

Figure 7. $p16^{INK4a}$ induction leads to cell cycle arrest. Telomere dysfunction or oncogenic stress can activate $p16^{INK4a}$. This prevents CDK4,6/Cyclin D complexes to phosphorylate

pRB-family of proteins, and leads to cell cycle arrest by pRB-family of proteins inhibition of E2F. Figure adapted from (Sharpless, 2004; Sherr, 2001).

The role of $p16^{INK4a}$ is to associate with CDK4/6 complexes, and to separate cyclin D from CDK4/6. Subsequently, Cyclin D is degraded and unactive CDK lead to pRb hypophophorylation and reactivation (Sherr, 2001). Normally, in late G1 of cycling cells, hyperphosphorylation of Rb disrupts its association with various E2F family members, which heterodimerize with DP proteins. This allows subsequent transcription of the E2F-DP target genes, which are necessary for DNA synthesis (Dyson, 1998; Nevins, 1998) (Figure 8). In the contrary, if there are high levels of $p16^{INK4a}$ in cells, hypophosphorylation of pRB leads to cell cycle arrest.

Modulation of expression of genes downstream of $p16^{INK4a}$ can have an effect on senescence and cell cycle, independently of $p16^{INK4a}$. For example, if E2F is overexpressed, it can lead quiescent cells into S phase (Johnson et al., 1993; Qin et al., 1994; Shan and Lee, 1994). In the contrary, pRb overexpression can lead to senescence, even in human immortal tumor cells (Xu et al., 1997).

Different factors can influence $p16^{INK4a}$ action on CDKs, such as the CDKIs $p21^{Cip1}$ and $p27^{Kip1}$. Their respective action is reviewed elsewhere (Sherr, 2001).

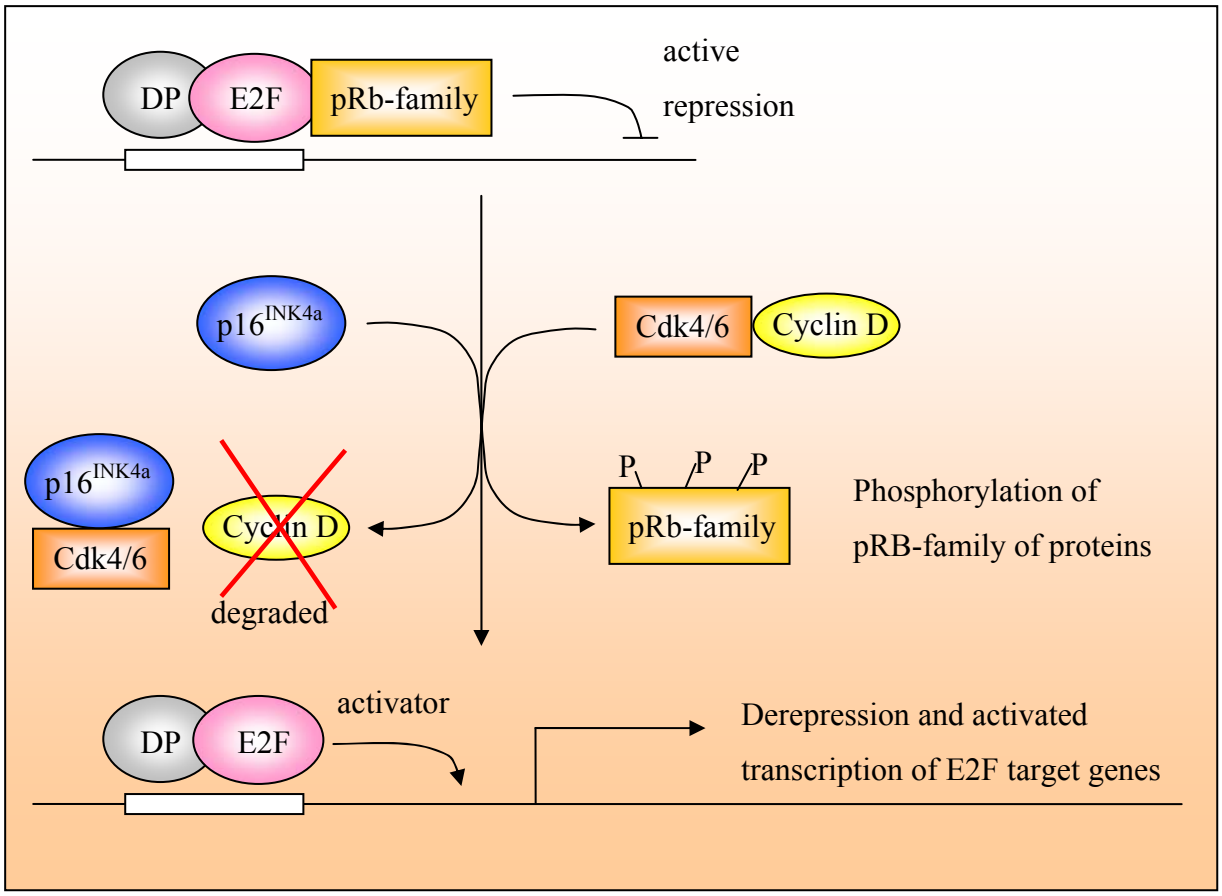

Figure 8. Transcription of E2F genes after pRb-family phosphorylation. In non-cyling cells, pRB-family of proteins are hypophosphorylated and repress transcription of E2F-DP (Malumbres and Barbacid, 2001). Once phosphorylated by Cyclin D-CDK4/6, proteins of the pRB-family dissociate from E2F, and permit transcription of E2F-DP target genes. Figure adapted from (Dyson, 1998).

1.1.6 The ARF-p53 pathway

Whereas mice lacking Arf develop normally but are highly tumor prone, MEFs from ARF null mice do not senesce and continue to proliferate much like established cell lines (Kamijo et al., 1997). The role of Arf in senescence seems to monitor latent oncogenic signals (Zindy et al., 2003). Among them, v-abl, c-myc, E1a, E2F or loss of RB have been demonstrated to induce ARF and thereby stabilize p53. In the contrary, the oncogenic transcription factors bmi-1, cbx-7, tbx-2, Twist and AML-ETO have been shown to repress $p14^{ARF}$ (Sharpless,

2004). Among them, bmi-1, cbx-7 and tbx2 also regulate the transcription of $p16^{INK4a}$ (Figure 9).

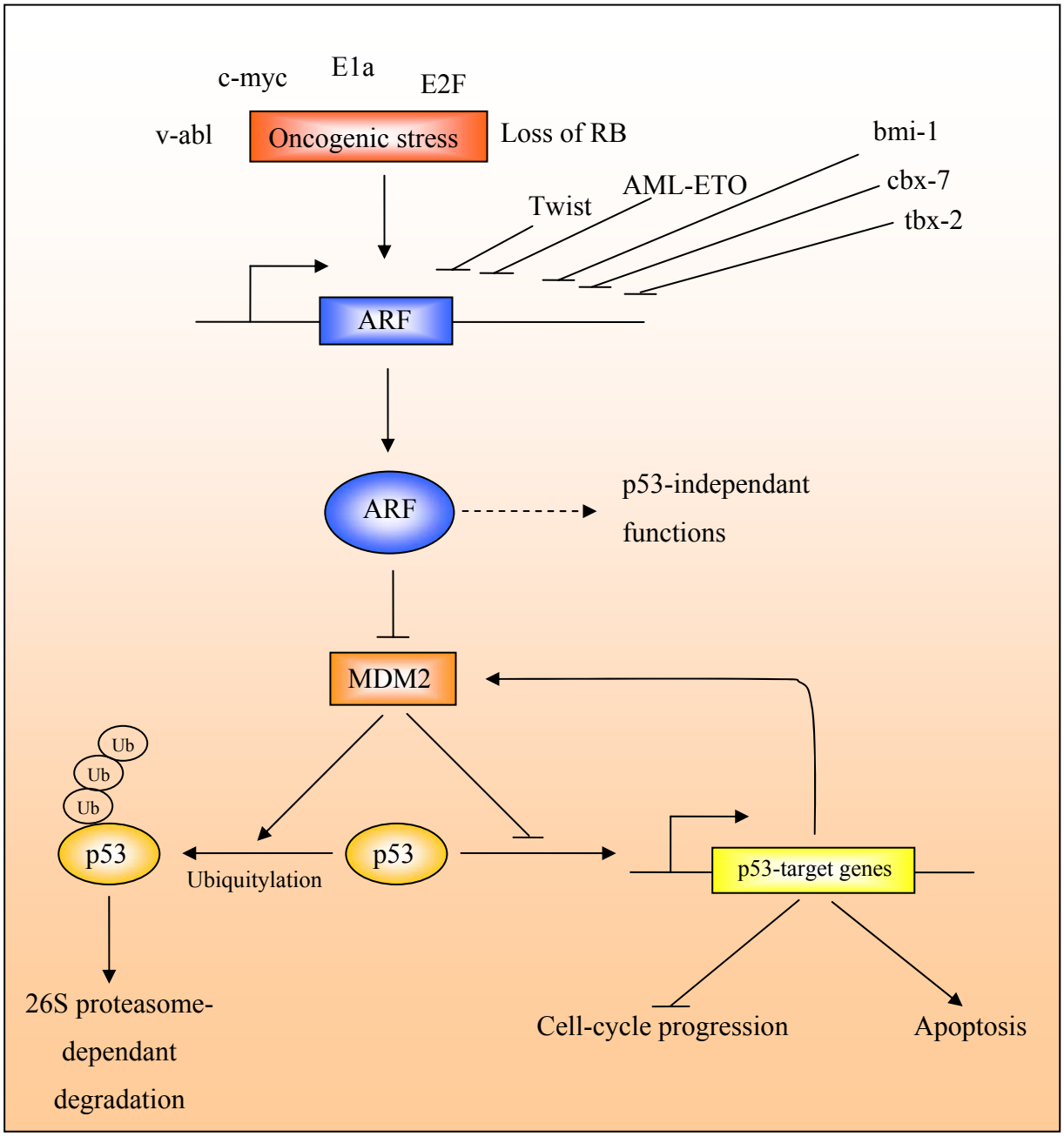

Figure 9. The ARF/p53 pathway. Under normal circumstances, the ubiquitin ligase MDM2 blocks p53 activity by targeting it for proteasomal degradation, and by directly blocking its transcriptional activity. The functions of MDM2 are auto-regulated by a negative feedback, because MDM2 is a p53-target gene. In case of strong proliferative signals, such as oncogene activation, ARF is transcribed, and negatively regulates MDM2. This leads to increased p53

activity, and cell-cycle arrest or apoptosis. Ub, Ubiquitin. Figure adapted from (Hipfner and Cohen, 2004; Sharpless, 2004).

ARF binds to and inhibits MDM2 (Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998). In turn, MDM2 is an E3 ubiquitin ligase that can target p53 for proteasomal degradation. MDM2 is also able to inhibit p53-mediated transcription. The MDM2-mediated repression of p53 is regulated by a negative feedback loop because MDM2 is a p53-target gene.

In addition to its stabilizing role of p53 by inhibiting Mdm2, ARF has also been reported to inhibit cell growth independently of p53, MDM2 and p21 (Korgaonkar et al., 2002). Apart its binding to Mdm2, ARF has also been reported to bind to E2F-1, topoisomerase I, MdmX, HIF-1α, PXF/pex19p and nucleophosmin (NPM)/B23 (Sharpless, 2004). Some of these interactions may explain p53-independent functions of ARF, and may also connect ARF not only to cell proliferation (through p53), but also to growth (through NPM and ribosomal processing).

The degradation of ARF has recently been shown to be Mdm2- and p53-independent, but depends on N-terminal polyubiquitination. Nevertheless, contrary to other ubiquitination processes, lysines are not required for ARF degradation (Kuo et al., 2004).

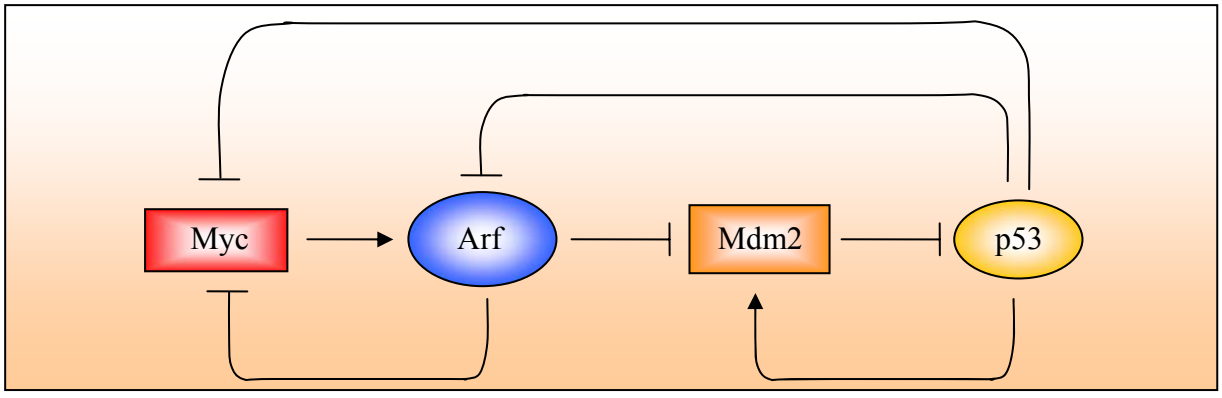

Figure 10. Multiple feedbacks in the Myc-Arf-Mdm2-p53 pathway. p53 negatively regulates Arf and Myc, but positively regulates Mdm2. ARF is an antagonist of Myc, but in a very specific manner on different target promoters. Only gene expression of Myc is impaired, but

not gene repression. This inhibits Myc's pro-proliferative, but not its apoptotic, functions. Figure modified from (Cleveland and Sherr, 2004).

Multiple regulations can affect the Arf-p53 pathway, and its activators. For example, evidences suggest that p53 negatively regulates Arf, by an unknown mechanism, and that p53 and Arf can negatively regulate Myc (Figure 10).

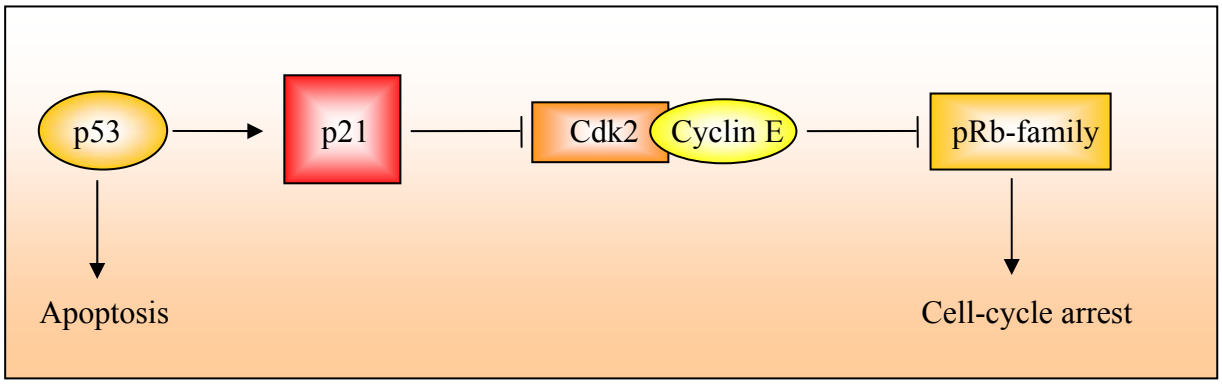

Figure 11. Link of p53 and pRb-family of proteins through p21. p53 induces p21, which inhibits different CDK complexes (Roninson, 2002). Figure modified from (Roninson, 2002; Sherr, 2004; Sherr and Roberts, 1999).

The role of p21 is to provide a link between p53 and pRB (Figure 11). The expression of p21 is high in senescent cells (Atadja et al., 1995; Itahana et al., 2001), and targeted disruption of p21 is sufficient to bypass senescence in human fibroblasts (Noda et al., 1994). Therefore, p21 may have a major role in induction of cellular senescence of human fibroblasts. In the contrary, MEFs from p21 null mice senesce normally (Pantoja and Serrano, 1999). This has led to the proposition that other factors downstream of the $p19^{ARF}$-p53 pathway may limit the growth of MEFs. At the molecular level, p21 (WAF1/CIP1) acts as a CDKI, but contrary to the INK4 CDKIs, p21WAF1 does not dissociate cyclin-CDK complexes. p21 associates with different cyclin-CDK complexes, and arrests cells in both G1 and G2/M stages of the cell cycle. The particular association of p21 with cyclin E and CDK2 provides a link between p53 and pRB. p21 is a p53 target, and through its association with Cyclin E and CDK2, it inactivates this complex, and maintains Rb in an hypophosphorylated form. This in turn permits the sequestration of E2F by Rb, and leads to cell cycle arrest, through activation of

E2F-DP target genes (Figure 12) (reviewed by (Roninson, 2002; Sherr, 2004; Sherr and Roberts, 1999)).

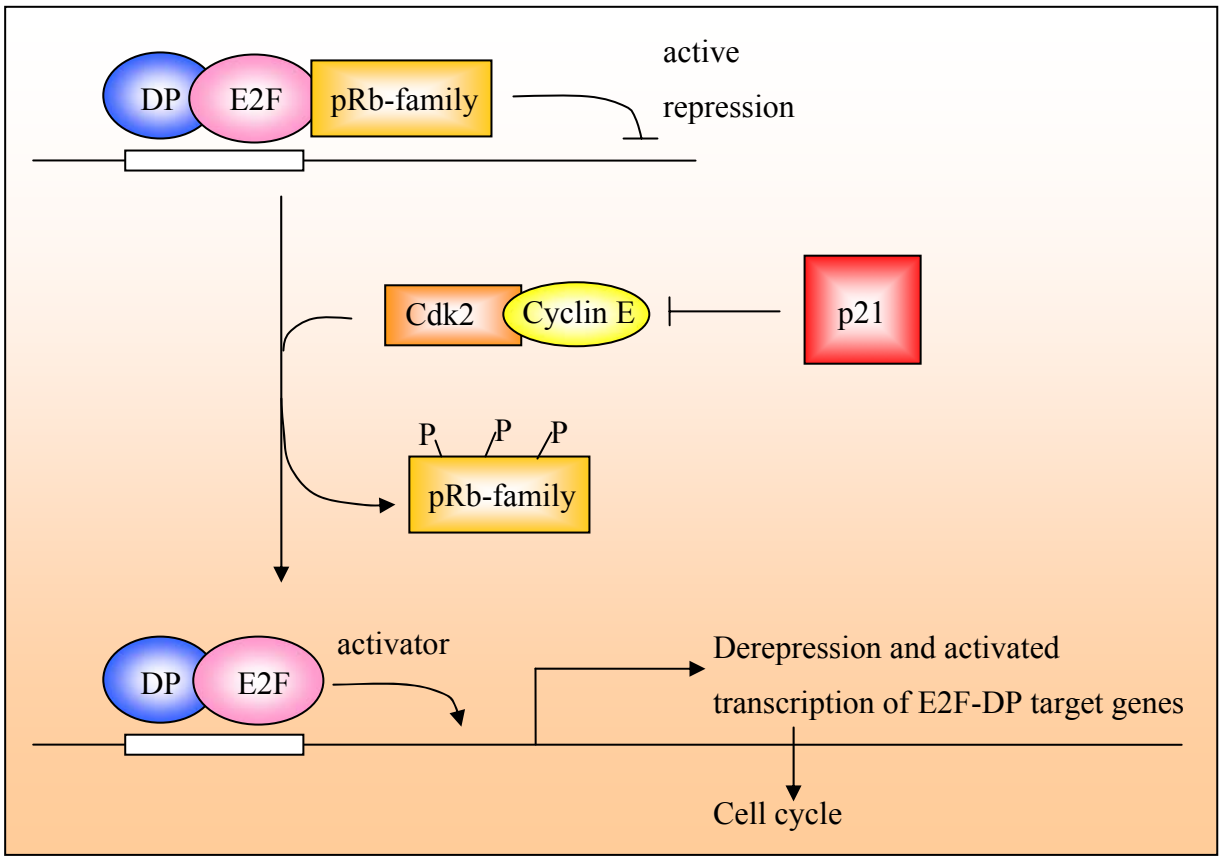

Figure 12. p21 can mediate cell cycle arrest. pRB can inhibit phosphorylation of pRB-family of proteins by interacting with cyclin/CDK complexes such as CDK2/Cyclin E, and this leads to cell cycle arrest. Figure adapted from (Dyson, 1998).

New findings suggest that p21 may have other properties, which may not be linked to cell-cycle. It may regulate apoptosis, and transcriptional activation (Andres, 2004). It has also been found recently that the involvement of p21 in senescence may be triggered by telomere shortening through a pathway involving ATM and p53, but not $p16^{INK4a}$ (Herbig et al., 2004).

1.1.7 Senescence, quiescence and apoptosis

Two cell statuses resemble senescence: quiescence and apoptosis, and they are all cell cycle arrest.

The major difference between senescence and quiescence is that the cell cycle arrest is irreversible (Beausejour et al., 2003; Dirac and Bernards, 2003; Gire and Wynford-Thomas, 1998; Sage et al., 2003). Distinct morphologic alterations are also associated with senescence, such as cellular flattening. Moreover, senescent cells also differ from quiescent cells at the molecular level. They have increased adherence, a loss of *c-fos* induction to serum stimulation, an increased expression of plasminogen activator inhibitor (PAI), the expression of senescence-associated ß-galactosidase activity (Campisi, 2001), and an unusual form of heterochromatin present in discrete nuclear foci, known as senescence-associated heterochromatic foci (SAHF) (Narita et al., 2003).

Apoptosis is more related to senescence than quiescence. For instance, different experiments have linked p53 not only to senescence, but also to apoptosis (Lowe et al., 1993; Yonish-Rouach et al., 1991). A role for $p16^{INK4a}$ in inducing apoptosis has also been suggested (Ausserlechner et al., 2001; Lagresle et al., 2002; Plath et al., 2000; Tamm et al., 2002). Moreover, cancer-related stimuli such as oncogene activation, DNA damage, and telomere shortening can not only induce senescence, but also apoptosis (Lowe and Sherr, 2003). Nevertheless, the molecular events at the basis of the decision-making between apoptosis and senescence are incompletely understood. They appear to depend on many variables including cell type, genetic context, and proliferation state.

1.1.8 Escape from senescence and crisis

Immortalization of murine cells is possible if they bypass a block of senescence, but immortalization of human cells is possible is possible only if they bypass a second block, crisis (Figure 13).

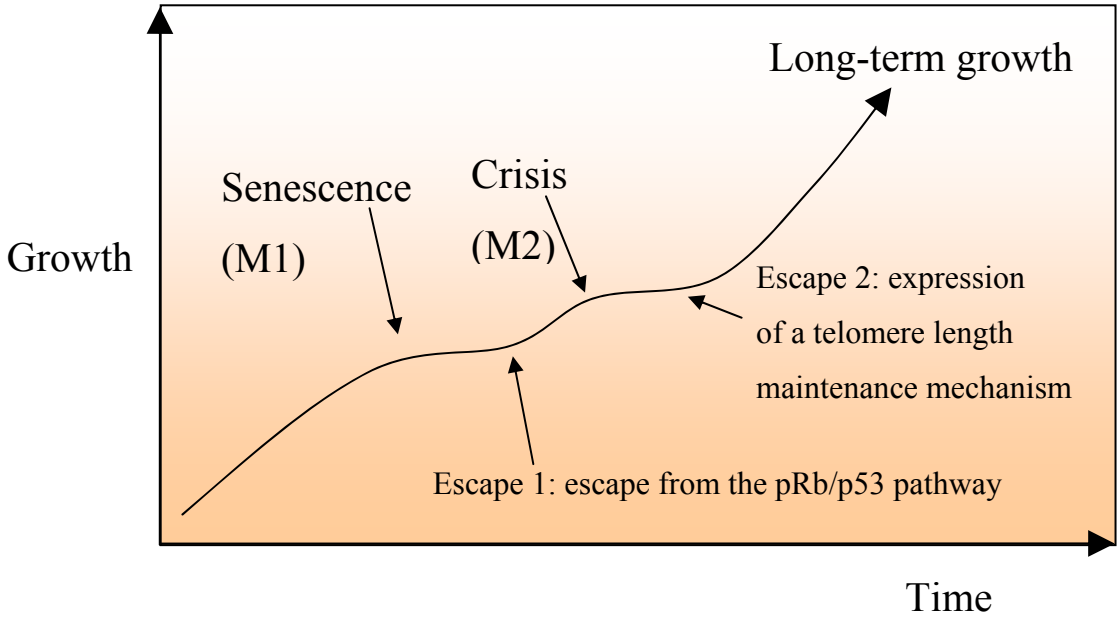

Figure 13. Immortalization and mortality stages. Normal cells have to bypass the two mortality stages M1 and M2 before to become immortal. Cells can escape the first mortality stage M1 called senescence if they bypass the pRb/p53 pathway by gene inactivation or by expression of an oncogene, and they can escape the second mortality stage M2 if they express a telomere length maintenance mechanism.

In normal human cells, cell division triggers telomere attrition (Harley et al., 1990a; Hastie et al., 1990). Critical telomere shortening causes replicative senescence, via activation of the p53 and pRb tumor suppressor pathways (Wright and Shay, 1992). Recent experiments suggest that replicative senescence is not determined by the shortest telomere, or by the mean length of all the telomeres, but rather by a specific group of chromosomes with the shortest telomeres (Zou et al., 2004). If the p53 and pRb pathways are inactivated by antisense mechanism (Hara et al., 1991) or by viral oncoproteins (Shay et al., 1991), cells can have increasing numbers of divisions before cell cycle arrest, but they finally enter into a crisis. This state is characterized by extensive telomere attrition that leads to chromosomal instability and finally to massive cell death (Counter et al., 1992a).

Rare events in cells subjected to crisis (1 in 10 million cells) lead to immortal cells that have acquired the ability to proliferate indefinitely. These cells often possess a telomerase activity

(Counter et al., 1992c). It is also at very low frequencies that virally transformed human cells escape crisis (Shay et al., 1991). In the contrary, crisis can be readily bypassed by telomerase expression (Bodnar et al., 1998b; Vaziri and Benchimol, 1998b) or by one or more mechanisms referred to as alternative lengthening of telomeres (ALT) (Bryan et al., 1997). TERT can stabilize telomere length and can lead to extension of life span (Bodnar et al., 1998b; Vaziri and Benchimol, 1998a). ALT is also a possible mechanism to maintain suitable chromosome lengths, but its mechanism of action is different from the hTERT mechanism. This mechanism is recombination-based in mammals (Roth et al., 1997). However, different ALT mechanisms exist in other organisms, such as a the retrotransposon-based ALT of *Drosophila melanogaster* (Biessmann et al., 1992). Nevertheless, even though both ALT and telomerase can extend telomere lenghths, they do it at different extents. ALT mechanisms lead to large increase in average telomere length, whereas telomerase activation can lead either to normal or to short telomeres (Figure 14).

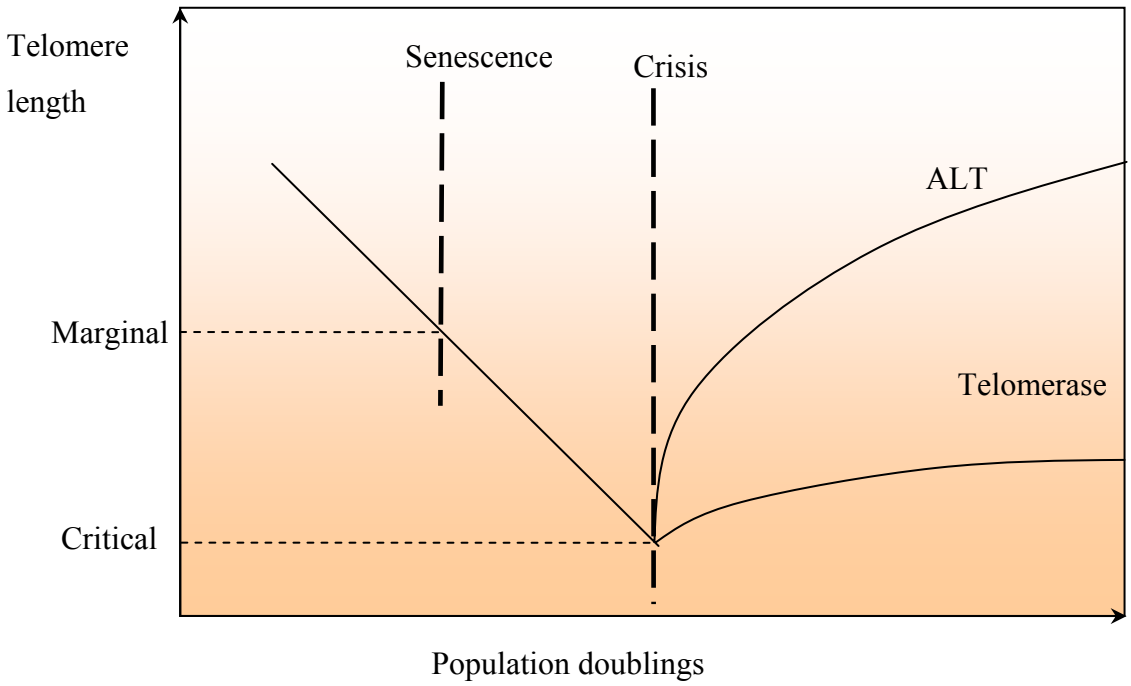

Figure 14. Telomere length maintenance and immortalization. When the telomere lengths of proliferating normal cells become too low, the cells undergo senescence. Cells can continue to proliferate by loss of function of key tumor suppressor genes, but the telomeres eventually

become critically short and the cells enter crisis. At this stage, activation of a telomere maintenance mechanism (telomerase or ALT) can permit cells bypass crisis and to become immortal. Telomerase activation often results in stably short telomeres, or in telomere lengthening and stabilization in the normal length range. In the contrary, ALT mechanisms result in a large increase in average telomere length, but there are very short and very long telomeres present within individual cells. Figure redrawn from (Reddel, 2003).

In immortal and malignant cells, senescence has been bypassed, and telomeres are maintained at stable lengths (Counter et al., 1994). Even when these cells have become immortal, continued expression of a telomere maintenance mechanism is still important. This was shown by the expression of a catalytically inactive mutant of hTERT in telomerase positive human cancer cell lines, that finally led to the inhibition of telomerase activity, to telomeric shortening and to widespread apopotosis (Hahn et al., 1999c; Zhang et al., 1999).

Nevertheless, in most cell types, upregulation of a telomerase maintenance mechanism is not sufficient to trigger immortalization. In fact, even though cells may harbor long telomeres, this is often not sufficient to trigger immortalization, except for some fibroblasts or for fetal hepatocytes (Blasco, 2002; Espejel and Blasco, 2002; Kiyono et al., 1998; Wege et al., 2003; Wei et al., 1999).

All these evidences suggest that both the block of the pRb/p53 pathways and the activation of a mechanism for telomere length maintenance are required to bypass senescence and crisis. When this is ensured, immortal cells can be obtained, and they can be non-tumorigenic. Nevertheless, additional modifications can render these cells tumorigenic. For instance, if cells express not only hTERT, but also SV40 small and large T antigen, and activated Ras, they become transformed (Hahn et al., 1999a).

1.1.9 Crisis and genetic instabilities

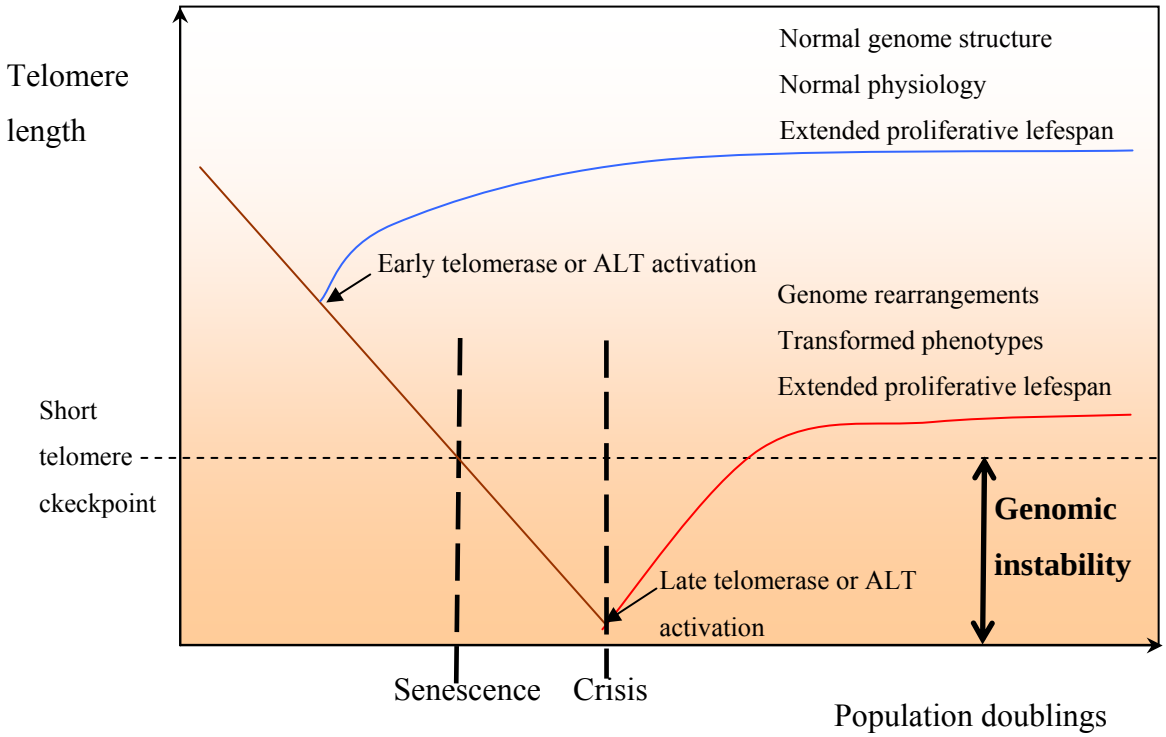

Figure 15. Genomic instabilities in cells bypassing senescence without activation of a telomere length maintenance mechanism. If cells bypass senescence without to express telomerase or ALT, telomeric shortenings lead to chromosomal instabilities. This source of mutations can permit to some rare cells (1 in 10 millions) (Radisky et al., 2002), to bypass crisis if they express a telomerase maintenance mechanism, such as telomerase or ALT. If cells express only at a late point a telomere length maintenance mechanism, they may be the subject of genome rearrangements, and this may lead to transformed phenotypes.

Even if cancer cells may be immortal, they may still have to evolve to acquire a large number of mutations, which will allow the acquisition of new cancerous properties (Loeb, 2001). Two different types of mutations can lead either to “microsatellite instabilities neoplasias” (MIN) or to “chromosomal instabilities neoplasias” (CIN). MIN are characterized by mutations in repetitive sequences, whereas CIN are characterized by chromosomal instabilities such as chromosomal breakages, translocations, and aneuploidy. The vast majority of human cancers are of the CIN type, but the molecular basis of CIN is less clear than that of MIN. CIN-type of

cancers are related to defective DNA mismatch repair (Lengauer et al., 1998). In the contrary, MIN cancers stem from defects in the mitotic checkpoint, from nonhomologous end-joining, and from DNA replication, as well as from increased oxidative stress and from telomere dysfunction (Bohr and Dianov, 1999; Elledge, 1996; O'Driscoll et al., 2001; O'Hagan et al., 2002; Shen et al., 2000). During crisis, it has been observed that telomere attrition leads to chromosomal instability and to massive cell death (Counter et al., 1992a). However, 1 in 10 millions cells escape crisis (Radisky et al., 2002), and these cells have often acquired mutations that have allowed upregulation of telomerase or of ALT. At this point, cells often also display a mutation-prone phenotype and the additional mutations that are provided from this point on are thought to be necessary for the cells to acquire complex tumorigenic phenotypes.

1.1.10 Somatic mutation theory of carcinogenesis

Different theories have been proposed for the origin of cancer cells (Soto and Sonnenschein, 2004). The prevailing theory for the last 50 years has been the somatic mutation theory of carcinogenesis (SMT). According to this theory, cancer cells are derived from a single somatic cell that has accumulated mutations over time. Two main evidences were supporting this theory. First, carcinogenic chemicals were mutagenic. Second, the oncogenes responsible for cancers were found to be mutated versions of the cellular proto-oncogenes. Another evidence supporting this theory was that DNA fragments from chemically transformed cells were able to transform recipient cells (Cooper, 1983).

Accordingly, two types of mutations were described to participate to carcinogenesis. They included the gain-of-functions of oncogenes, but also the loss-of-function of tumor suppressors. These gain- and loss-of-functions could define not only what events were necessary to transform cells, but also the new properties that cancer cells had to acquire before to become fully tumorigenic.

1.1.11 Properties of cancer cells

Hanahan and Weinberg have identified six “hallmark features” of metastatic cancer cells:

they generate their own mitogenic signals,

they overcome the growth-inhibitory signals,

they evade apoptosis,

they proliferate without limit,

they acquire vasculature,

they invade tissues,

they metastasize (Figure 15) (Hanahan and Weinberg, 2000).

It is often assumed that all tumor cells finally acquire all these properties, in a more or less determined order. However, it should be noted that (in rare cases) death can occur before cancer cells metastasize, because, for example, an organ important for survival has been too severely damaged. Moreover, discussions exist to know if these cancerous properties should be acquired in a strict order. The path toward tumorigenesis can be different in histologically similar cancers, and can be still more different in different forms of cancers (Hanahan and Weinberg, 2000). Nonetheless, in some cases, it is not simply mutations, but also their order that may be important to determine the propensity for neoplasia (Kinzler and Vogelstein, 1996).

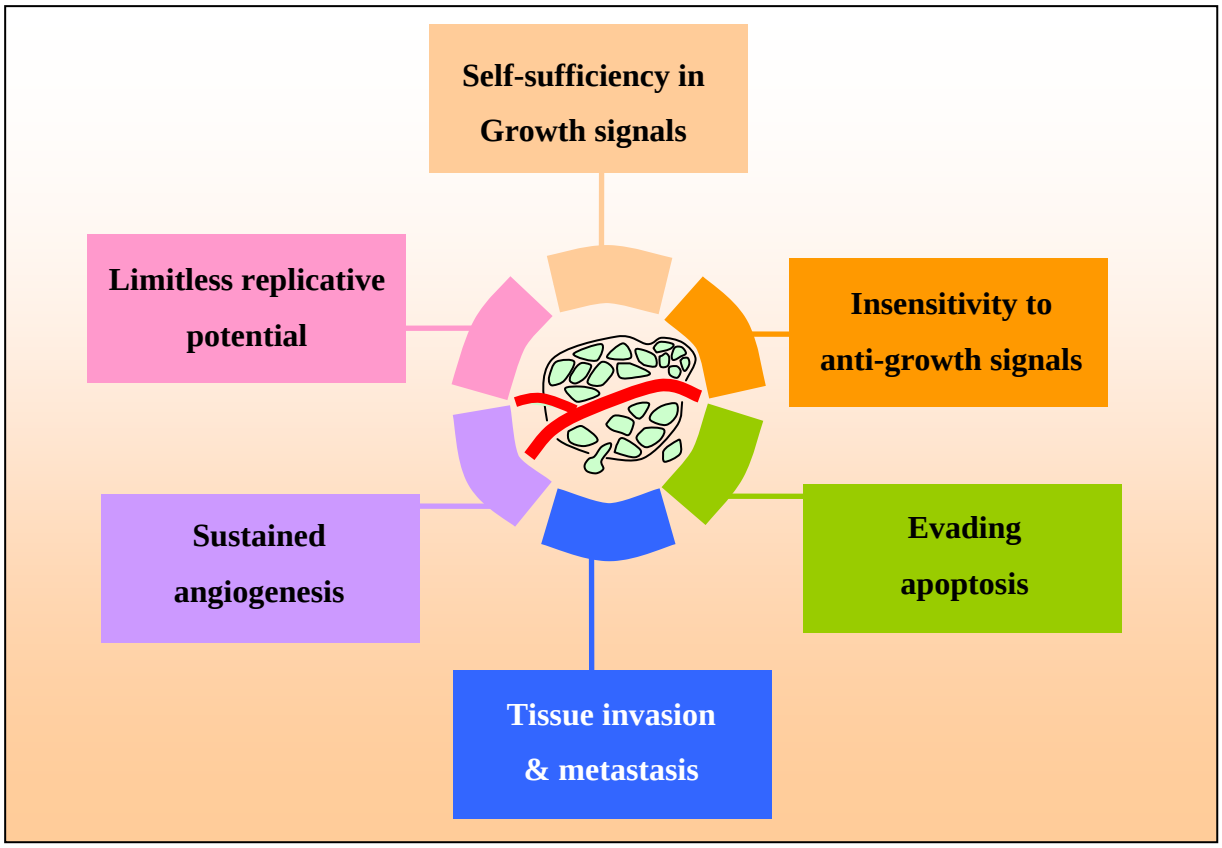

Figure 16. Different properties of cancer cells. See text for details.

1.1.12 How many mutations are required to trigger oncogenesis?

Mutations or deregulations of oncogenes or of tumor suppressors can both contribute to the acquisition of neoplasic phenotypes (DePinho and Jacks, 2001; Hakem and Mak, 2001; Knudson, 2001). In fact, dominantly acting oncogenes and recessive tumor suppressors are often mutated or lose a large portion of their genetic sequences in tumorigenesis (Sager, 1989). This implies that sequencing whole genomes of normal and cancer cells would permit the discovery of genes mutated in cancers, and would permit to know how many genes need to be mutated in cancers. Nevertheless, this method has two disadvantages. First, genome-sequencing of mutated cells is not yet feasible. Second, even if this method would exist, it would not be able to find the genes that contribute to cancer, but only the genes that are mutated. However, in cancers, dysfunction of genes may not always be caused only by

mutations but also by aberrant expression. In fact, a lot of genes important for carcinogenesis have already been shown to be differentially regulated in an epigenetic manner. For instance, tumor-suppressors have CpG islands of nucleotides in their promoter sequences that may be methylated and that may contribute to the expression of these genes (Baylin and Bestor, 2002). Usage of microarrays would be the tool of choice to determine the aberrantly functioning genes. Nevertheless, in this case, it may be difficult to find the genes relevant in carcinogenesis among a wide array of differentially expressed genes. For these reasons, the exact number of genes must be deregulated or mutated to induce tumorigenesis will probably not be defined soon, even though a lot of progress has already been done in this area. For the moment, it is assumed that at least four to six mutations might be required to reach tumorigenesis in humans, and fewer mutations seem to be required in mice (Hahn and Weinberg, 2002).

1.1.13 Pathways leading to tumorigenesis

Cancer cells have usually to become immortal, and then they often have to acquire additional features to become fully tumorigenic. Immortalization of human cells requires maintenance of a sufficient telomere length and inactivation of the retinoblastoma (pRB) and p53 tumor suppressor pathways. Maintenance of a sufficient telomere length is possible by activation of telomerase or of an alternative telomere length maintenance (ALT) mechanism. Inactivation of the pRB and p53 pathways is possible by large T antigen expression. At this stage, cells are immortal, but not yet tumorigenic. Until now, only few experiments were able to induce tumorigenesis of immortalized cells after addition of defined genetic elements. These experiments showed that if tumorigenesis was triggered, both the phosphatase 2A (PP2A) and the *H-RAS* pathways were perturbed. This was done by addition of small t antigen, and by *H-RAS* overexpression (Zhao et al., 2004) (Figure 17).

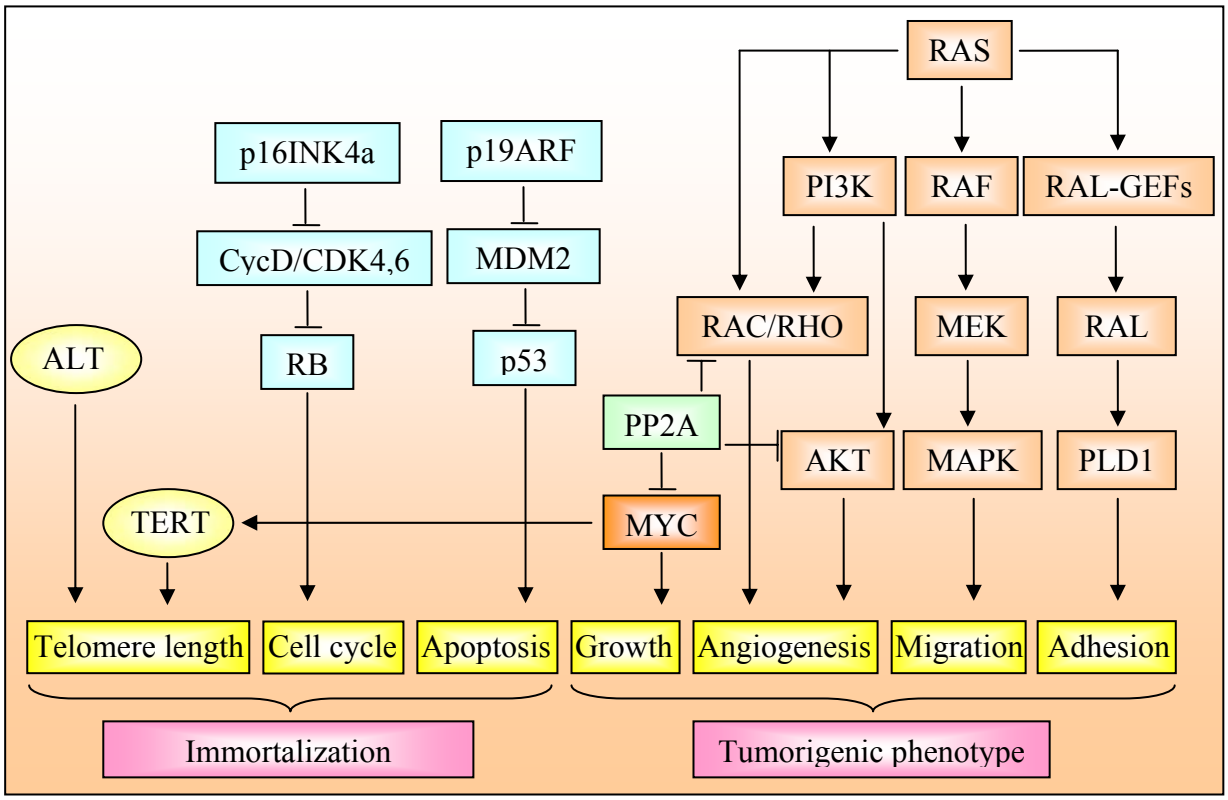

Figure 17. Pathways thought to be implicated in human cell transformation. This figure was modified from (Hahn and Weinberg, 2002; Zhao et al., 2004). More complicated pathways leading to tumorigenesis can be seen elsewhere (Hanahan and Weinberg, 2000).

The tumor suppressor PP2A is as a serine/threonine-specific phosphatase that regulates different signal transduction pathways, cell cycle progression, DNA replication, gene transcription and protein translation (Janssens et al., 2005). Its implication in tumorigenesis has been observed in various human cells where LT + ST + Ras + hTERT have been introduced. In such experiments, a mutant of ST unable to bind PP2A was also used. With this mutant, tumorigenesis was not observed (Hahn et al., 2002).

Even though it is known that the Ras pathway may be implicated in tumorigenesis, the precise protein(s) that are implicated in this phenomenon are not yet known. However, the anchorage-independence survival and growth of cancer cells seem to be mediated by PI3K, Stat3, and guanosine triphosphatases (GTPases) such as Cdc42 and Rac1 (Wang, 2004). Normally, cells have to adhere to their neighboring cells or to the extracellular matrix (ECM). If it is not the case, cells usually die or stop dividing. For instance, if epithelial cells lose their adhesion to their substratum, they undergo apoptosis. This phenomenon is known as “anoikis”. In the

contrary, if fibroblasts are deprived from their substratum adhesion, they only undergo cell cycle arrest. In any case, if cells can survive and grow, without to be anchorage-dependant, they have acquired one important property of cancers cells. The exact proteins allowing anchorange-independent growth are not known, but Rho and Cdc42 are candidates for obtention of this function. These proteins were initially known to regulate the formation of stress fibers and filopodia. Nevertheless, additional cellular functions have been subsequently discovered for these proteins, notably in cell growth, motility and transformation. This makes of these proteins interesting candidates for acquisition of properties related to anchorange-independent growth (Wang, 2004).

Other proteins have been involved not in the genesis of tumors such as the acquisition of anchorange-independent properties, but in metastasis tumor suppression. Then, it is not their induction that is thought to be important to induce tumorigenesis, as for Rho and Cdc42, but their downregulation. These proteins include KISS1, MKK4, BRMS1, CRSP3, TXNIP, Cadherins, SSeCKS, Nm23, KAI1 (CD82), TIMPs, RhoGDI2 and DRG-1. The roles that are currently proposed for these proteins in tumorigenesis are reviewed elsewhere (Shevde and Welch, 2003).

Until now, we have described mutations or modifications in cells that may trigger immortalization, and even tumorigenesis. Nevertheless, mutations in one single cell might not be sufficient to explain all cancers, because it is known that tumors may be heterogeneous in cell types. This has led to two proposals. Either cancer cells may originate from stem cells, or cancer cells may modulate other cell types in their environment.

1.1.14 Cancer, stem cells, and cancer stem cells

Stem cells have the capacity to self-renew indefinitely, but also to differentiate into the mature cells of the organ in which they reside. Some articles have suggested that these stem cells also have the capacity to transdifferentiate into other tissues. However, more recent studies have shown that this plasticity is the result of a rare fusion event between stem cells (or progenitor cells) and a cell of another organ (Vassilopoulos et al., 2003; Wagers et al., 2002; Wang et al.,

2003). The self-renewal capability of stem cells closely resembles the properties of at least some of the cancer cells, which are immortal. This similarity led to the proposal that at least some cancer cells might originate from stem cells. Cancer cells are nonetheless different from stem cells, because the proliferation of stem cells is tightly regulated (Morrison et al., 2002; Phillips et al., 2000; Phillips et al., 1992). Then, if cancer cells arise from stem cells, they would have to bypass these mechanisms of growth regulation. Further studies suggested that some cells in a cancer are derived from stem cells. For instance, only a minority of cancer cells have the capability to form a tumor once transplanted into another host, because only this subpopulation is able to proliferate extensively. This has been demonstrated with acute myelogenous leukaemia (AML), where only a minority of AML cells form tumors once transferred into NOD/SCID (non-obese diabetic/severe combined immunodeficient) mice (Lapidot et al., 1994). The tumor-forming cells closely resemble to normal haematopoietic stem cells (HSCs) in their cell-surface markers, in their multipotency and in their hierarchical self-renewal properties. This experiment put forward the concept of cancer stem cells, and it was suggested that leukemia stem cells either derive from HSCs or from more differentiated cells through acquisition of HSC properties.

In short, stem cells could be good candidates as cells of origin for cancer, because they already have the capacity for self-renewal and unlimited replication (Reya et al., 2001; Taipale and Beachy, 2001) that at least some cancer cells possess. The concept of cancer stem cells could also be interesting to explain why some cells can, once transplanted in NOD/SCID mice, reconstitute the heterogeneous surface markers that were present in the original cancer (Al-Hajj et al., 2003). Nevertheless, the general validity of the cancer stem cell concept has not been proven, and it is possible that cancers arise from other cells than stem cells. If cancer stem cells exist, they might also derive from more committed cells, and they might get stem cells properties by genetic or epigenetic changes that confer self-renewal ability (Passegue et al., 2003). This theory of cancer stem cells is interesting, that is why it has been presented here, but it is certainly not considered nowadays to be the main source of cancers. Moreover, now, experiments suggest that it is not only the genetic and epigenetic characteristic of a cell that counts for the explanation of its cancerous property, but also its context. This will be discussed now.

1.1.15 Context counts

It is often considered that the default state of a cell is quiescence. Then, the events leading to cancers would be mostly activation of dominant oncogenes in one cell. This view is notably supported by studies with oncogenes from Oncoretroviruses that have dominant effects on cells, and transform them toward a cancerous phenotype. This has led to the formulation of the somatic mutation theory of carcinogenesis (SMT), which considers that accumulation of mutations in one single renegade cell is the cause of cancer. However, one might also consider that the default state of a cell might be proliferation (Sonnenschein and Soto, 2005). Indeed, the basics concepts of natural evolution suggest that it would favor dividing cells: if cells are in a suitable environment, and if they have enough nutrients, they should multiply (Harris, 2004). Considering this, if cells are in a clement environment, with adequate nutrient supply, and if they do not multiply, these cells are not in “resting”, they are repressed. This conceptual view has some implications. First, it may suggest that the events leading to cancers would be mostly inactivation of recessive tumor suppressors. Second, it may suggest that the environment is important for the cell status of a cell. This would imply that mutations in one single cell may not be sufficient for carcinogenesis, independently of its environment. For this reason, this view was called the tissue organization field theory of carcinogenesis and metastasis (TOFT).

TOFT is verified in a large extent in heritable and acquired cancers, because they are mainly the results of gene inactivation, such as of Rb and p53. The context also seems to be important in other types of carcinogenesis, because, for example, teratocarcinoma cells can reverse their cancerous phenotype once placed into early embryos (Soto and Sonnenschein, 2004). This view is still supported by a new publication, which shows the importance of the stroma in the establishment of a cancer phenotype (Barclay et al., 2005). In this article, the same cell line can be cancerous or not, depending on the stromal cells used. Moreover, the cancer phenotype can be reversible if a new "normal" stroma is provided.

1.1.16 The cellular environment

Different signals from the environment of a cell can have an influence on its development in general, but also on the acquisition of carcinogenesis properties of this cell. For instance, stromal cells can affect the tumorigenesis of epithelial cells in a reciprocal way (Figure 18) (Radisky et al., 2001). More generally, environmental pressures, inflammation, immune activation, and acquired or inherited genetic mutations can influence tumorigenesis. Inflammation in general seems to trigger tumorigenesis, so that inhibition of cyclooxygenase-2 (COX2), an enzyme that is involved in the synthesis of prostaglandins, has an antitumorigenic activity (Prescott and Fitzpatrick, 2000). Fibrotic tissues, such as breast scars, have also been shown to predispose to cancers (Jacobs et al., 1999b). Such discoveries have led to the emergence of new therapies of hepatocarcinoma, where not only conventional chemotherapies are used, but where fibrosis is also targeted. Inflammatory conditions can trigger stromal fibroblasts to upregulate enzymes such as matrix metaloproteases (MMPs). In general, changes in the composition of the extracellular matrix (ECM) can lead to changes in gene expression, which notably lead to changes in cell shape and behavior (Roskelley et al., 1995). In fact, the differential composition of the basement membrane can lead not only to proliferation, but also to growth arrest and to apoptosis (Bissell and Radisky, 2001). Among the different changes that can affect the composition of the ECM, expression of stromelysin-1 (SL-1, also known as MMP-3) has been shown to be sufficient to lead to preneoplastic or even to neoplastic phenotypes (Thomasset et al., 1998).

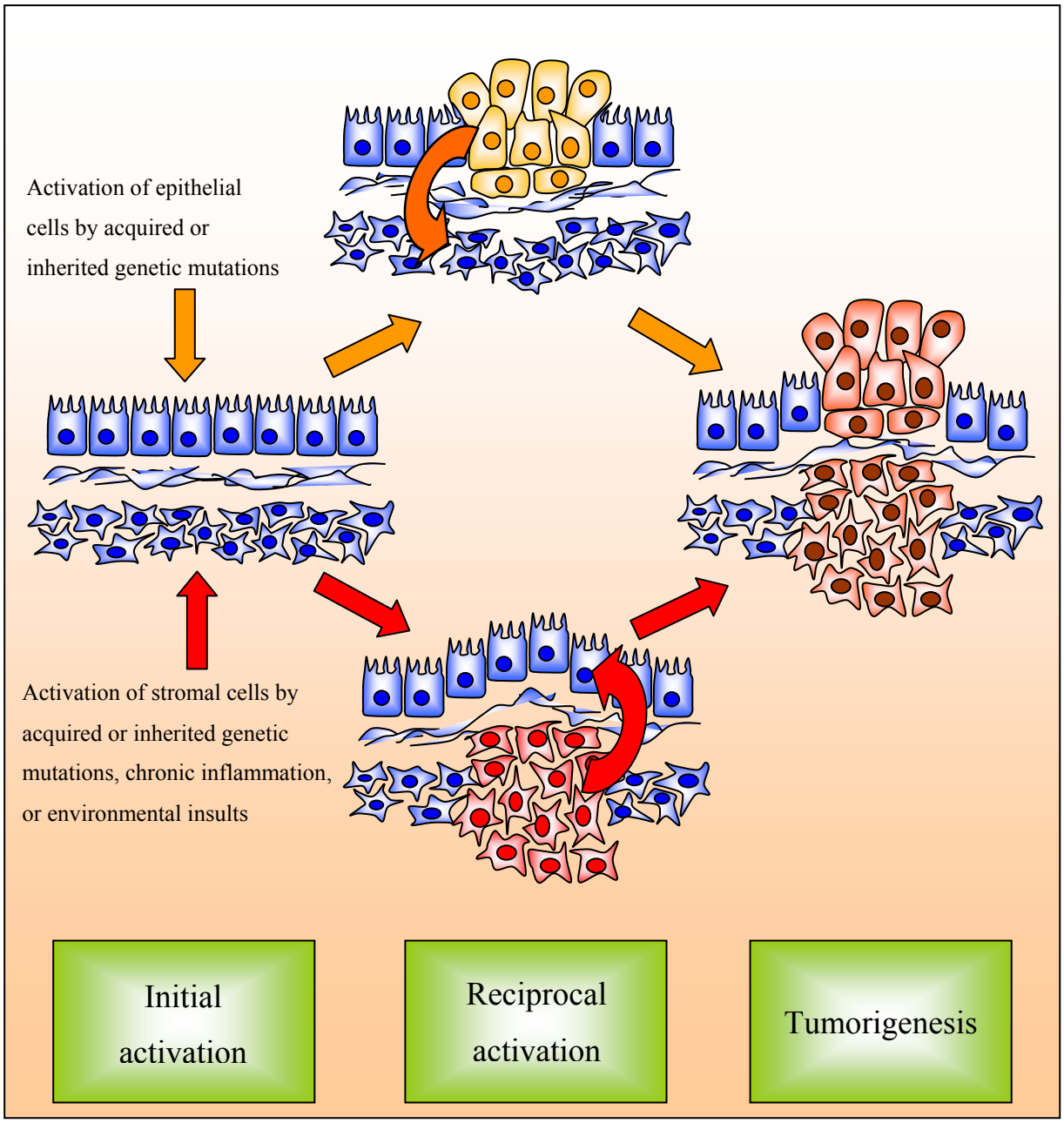

Figure 18. Model of reciprocal activation between stromal and epithelial cells. Genetic modifications or external stimuli such as chronic inflammation or environmental insults can activate epithelial or stromal cells. In turn, the neighboring cells are activated, and this finally might lead to tumorigenesis. Figure modified from (Radisky et al., 2001).

It is not only the extracellular matrix, but also the links between cells, which can be important in tumorigenesis. As a result, loss or alteration of some components implicated in cell-adhesion, such as E-cadherin, but not N-cadherin, can lead to tumorigenesis (Seidel et al., 2004). Moreover, differential expression of β-catenin, α-catenin, connexin 32 (Cx32) and Cx43 have also been shown to be important during tumor progression (Bissell and Radisky, 2001). Some myoepithelial proteins were even shown to suppress transformation of luminal epithelial cells *in vivo*. They include α-smooth muscle actin, Cytokeratin 5, α6-integrin, Caveolin-1, Connexin 43, Maspin, TIMP-1, Relaxin, and Activin (Bissell and Radisky, 2001). These proteins are produced by myoepithelial cells in the breast, and have been termed "class II tumor suppressors" (Sager, 1997) (Figure 19).

Protein	**Function**
α-smooth muscle actin	Cell structure
Cytokeratin 5	Cell structure
α6-integrin	ECM receptor
Caveolin-1	Cell-surface molecule
Connexin 43	Gap-junction component
Maspin	Protease inhibitor
TIMP-1	Protease inhibitor
Relaxin	Hormone
Activin	Hormone

Figure 19. The myoepithelial proteins that were shown to have tumor-suppressor activities. Figure taken from (Bissell and Radisky, 2001).

It has been proposed that reciprocal activation mechanisms occur between different cells in early tumors. As a result, this would activate some of the normal wound-healing responses, which might increase angiogenesis in a "vasculogenetic mimicry" (Bissell and Radisky, 2001).

Beside the importance of the ECM, of reciprocal activation of cells, and of wound healing responses in tumor formation, the immune system may also play role. Indeed, the immune system may not only limit tumor formation, but it may also be a tumor activator in some cases. For instance, NK cells can not only kill HLA-low cells, but it can also provide positive

signals for the progression of preneoplastic lesions, by producing MMPs, and degradation of ECM can further activates fibroblasts and angiogenesis (Bissell and Radisky, 2001).

1.1.17 Escape from the immune system

The immune system can to some extent limit or prevent tumor formation, and the T cells are main effectors for this immune surveillance. However, the innate immune system, which includes natural killer cells, macrophages, monocytes and mast cells, is also involved in immune response against tumors.

Cancer cells, in their development, can be more or less susceptible to attacks of the innate immune response. During the early stages of carcinogenesis, tumor cells are poor stimulators, or become resistant to the immune response. Malignant cells can escape to the action of cytotoxic T lymphocytes (CTLs) that recognize processed cancer peptides on human leukocyte antigens (HLA) class I by downregulating their immunogenicity (Algarra et al., 2004). Then, even though the primary tumor is HLA-positive, during development, HLA-low cells appear and are negatively immunoselected by T-lymphocyte antitumor responses. Nevertheless, the cells that escape to T lymphocytes harbor at their surface less HLA proteins, and this is recognized by killer cell inhibitory receptors (KIRs) of natural killer (NK) cells, which lead to NK-mediated killing of the HLA-low cells (Figure 20). However, the antitumorigenic killing of NK can also be escaped by cancer cells, for example if they upregulate only some HLA class I haplotypes, or if they lose the activatory NK receptors (Algarra et al., 2004).

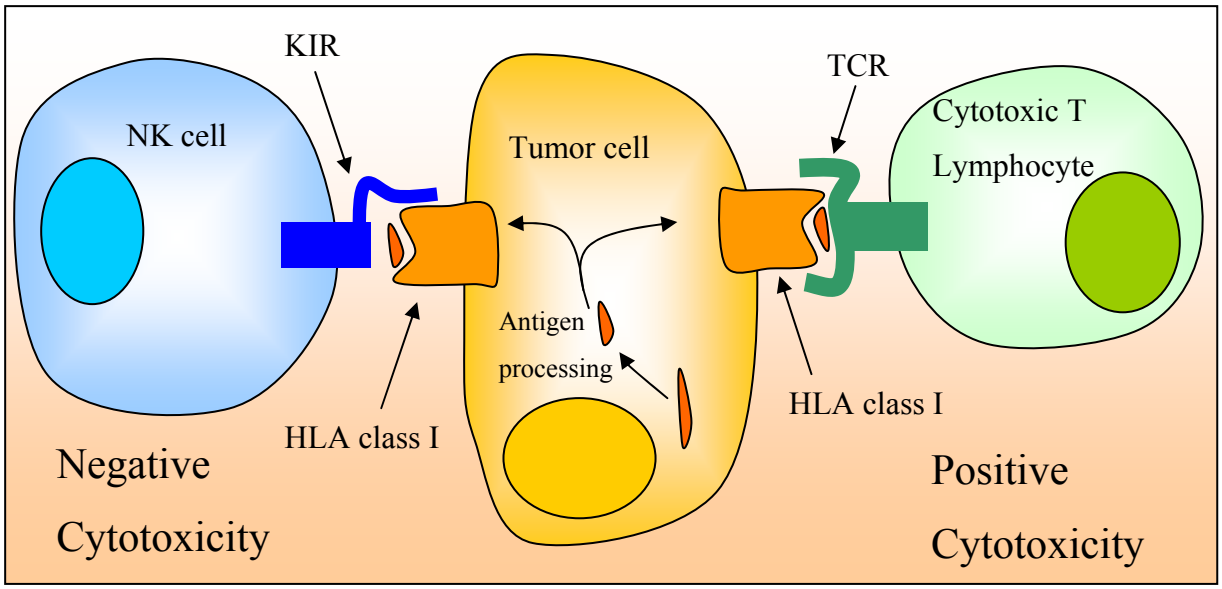

Figure 20. HLA class I interactions with T-lymphocytes and NK cells. Cancer peptides are presented on HLA class I and are recognized by cytotoxic T lymphocytes, which kill the transformed cells. Cells can escape from cytotoxic T lymphocytes by downregulating HLA class I complexes, but then NK cells recognize the lack of HLA class I cells and kill the potentially transformed, HLA-low, cells. Figure redrawn from (Garcia-Lora et al., 2003).

Tumors can evolve not only to escape the immune system; they can also modulate the immune system. For example, macrophage migration inhibitory factor (MIF) has been associated with tumor progression. It can be overexpressed in tumor cells, and might contribute not only to suppress immune surveillance, but also to suppress p53 function (Bissell and Radisky, 2001). At later stages, the tumors can impair the adaptive immune response by blocking the maturation and function of APCs. This leads to alteration of T-cell signal transduction and function (Rodriguez et al., 2003).

1.1.18 Immune senescence

Increase in cancer in elderly people may be explained by the multiple mutations that require tumorigenesis. However, with aging, there is also a decline in the T- and B-cell associated functions. This is termed immunosenescence (Hakim et al., 2004). These two phenomenons may both lead to increased tumorigenesis in the elderly, and they are difficult to dissociate

methodologically, but this topic has nevertheless become a new area of investigation. It has been shown that bone marrow stem cells renewal capacities decrease with age. Stem cells also mobilize less efficiently with age, and they commit less efficiently to lymphopoiesis. In the elder people, there is a decline in germinal center formation, diminished FDC function, diminished circulating memory B cells, lower T cell number, lower T cell functions, lower CD5+ B cells, and lower CD40 levels on B cells. Nevertheless, lower NK function is compensated by higher NK numbers, so that the total NK-cell toxicity remains stable. Despite this, both T-dependant and T-independent B-cell responses are diminished with aging. More importantly, there are alterations in T cell populations. There is a decline in naïve T cells, but there are also more memory cells and more effector cells with limited T-cell repertoire (Hakim et al., 2004).

1.1.19 Tests of carcinogenesis

Different *in vitro* and *in vivo* tests have been used to characterize and detect tumor cells.

In vitro tests include focus formation and colony formation assays, which can discriminate if cells still possess respectively, contact inhibition properties, or anchorage-dependence growth (Jacobsen et al., 2002).

For *in vivo* tests, different mouse strains have been used. The results of the tests for carcinogenesis largely depend on the genetic background of the mice used (Algarra et al., 2004). For example, chemically induced sarcomas produced in nude and SCID mice are more immunogenic than similar sarcoma cells induced in congenic immunocompetent mice. The classical model used for tumorigenesis is the athymic nude mouse, which is deprived of T lymphocytes (Rolstad, 2001). These mice have nonetheless NK and dendritic cells, and they may contribute to immune responses in the absence of mature T cells. Particularly, KIR-ligand mismatch in cell transplantation may trigger NK cell alloreactivity (Malmberg et al., 2005).

1.2 Genes used for immortalization

It would be too long to review all the genes that could be used for immortalization. For this reason, it was chosen to review only the genes that are of particular interest for us. They include telomerase, ALT, Bmi-1, SV40 Early region genes, and Bcl-2. These genes have the property to inhibit different proteins involved in the pathway toward senescence (Figure 21), but they also have additional functions, which will also be discussed now.

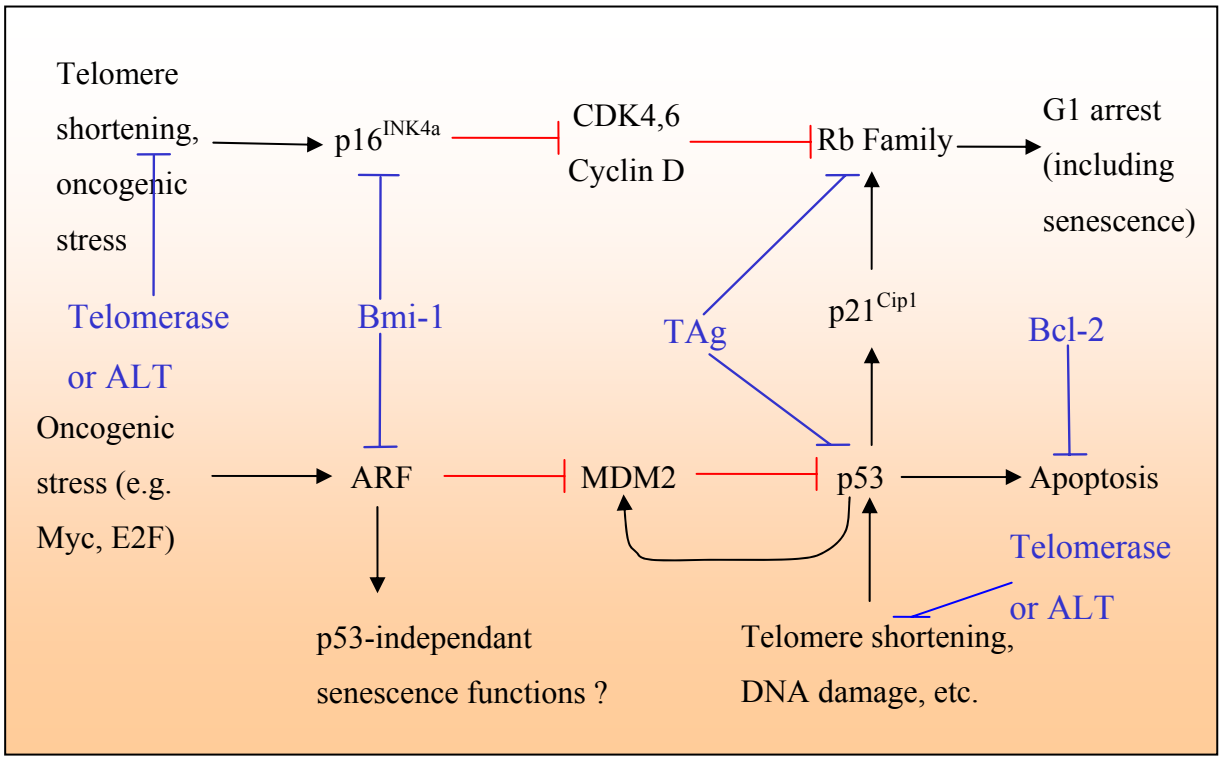

Figure 21. The Pathways depending upon $p16^{INK4a}$-Rb or ARF-p53, with various inhibitors of these pathways. Figure modified from (Drayton and Peters, 2002; Lowe and Sherr, 2003; Sharpless et al., 2004; Sherr, 2001; Sherr, 2004; Zhao et al., 2004).

1.2.1 Telomerase

More than sixty years ago, Barbara McClintock predicted from experiments in maize that telomeres are able to protect chromosomes from end-to-end fusion. From then on, a lot of progress has been done in the elucidation of the telomeric structures. Telomeres are composed of repeats of the hexanucleotide sequence TTAGGG at each chromosome end (Moyzis et al., 1988). The size of these repeated sequences is up to 15 kb of tandem repeats, but diminishes of 50-150 bp after each cell division (Harley et al., 1990a). The decrease in telomere may not always be constant for each cell division, depending on the environment of the cells. Stresses, and particularly hypoxia, induce increased shortenings of telomeres (von Zglinicki et al., 1995). As discussed earlier, telomere length has been taken as a marker of ageing because life-span can be extended by overexpression of telomerase (Bodnar et al., 1998a; Vaziri and Benchimol, 1998b). Nevertheless, telomere length is not sufficient *per se* to define ageing, because it has been shown that cells can senesce even though they harbor long telomeric sequences (Gorbunova et al., 2002; Wei et al., 1999).

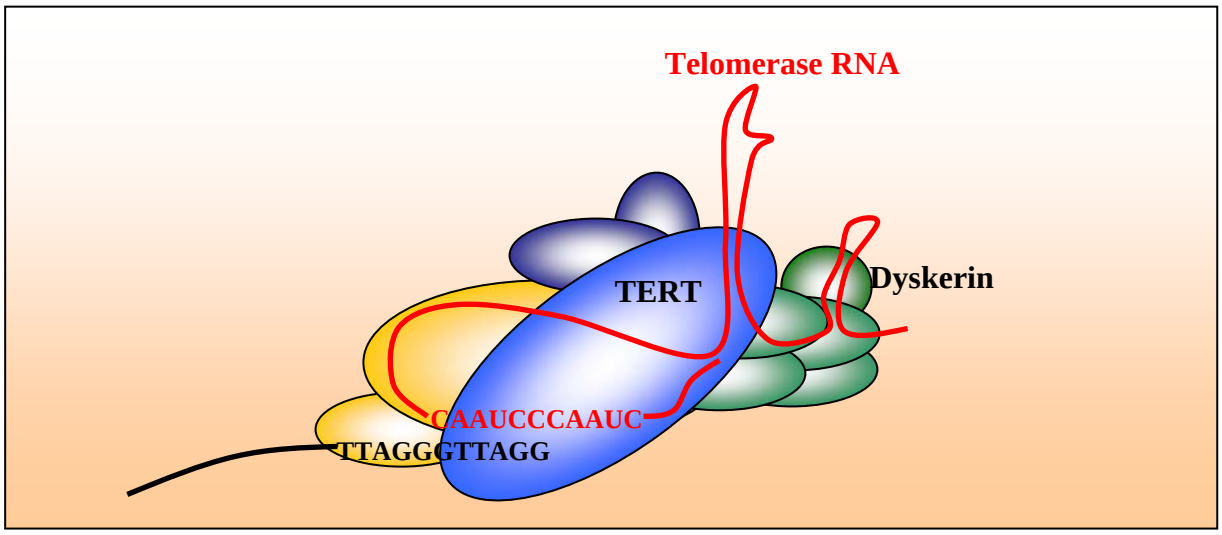

Figure 22. The human telomerase ribonucleoprotein complex. Telomerase RNA (red) must interact with H/ACA proteins (including dyskerin, green) to accumulate in cells as stable, processed functional RNA. TERT (blue) has reverse transcriptase properties, but probably other proteins must join the ribonucleoprotein complex to form a fully functional telomerase. Figure redrawn from (Wong and Collins, 2003).

Telomerase is a ribonucleic complex (Greider and Blackburn, 1985) composed of an RNA molecule, referred to as TR or TER, and a catalytic subunit, TERT. The RNA component of telomerase must interact with H/ACA proteins, like dyskerin, to be processed and to function properly (Figure 22). Telomerase is an enzyme that possesses reverse transcriptase activity, which can compensate the loss of telomeric sequences that occurs during DNA replication (Bryan and Cech, 1999).

Telomeres must be capped, so that the ends of chromosomes are not recognized as DNA breaks. Otherwise, telomeres are recognized by the DNA-repair machinery (Blackburn, 2001; Chan and Blackburn, 2002; Goytisolo and Blasco, 2002), often leading to cell-cycle arrest and/or apoptosis (De Lange, 2002; Goytisolo and Blasco, 2002). However, a "repair" can occur, it is triggered by the standard DNA repair machinery. However, this repair is not suitable for telomeres. After the DNA-damage responses, end-to-end chromosomes fusions happen, and this leads to loss of genomic integrity. Ku86, the protein which is essential for non-homologous end-joining (NHEJ) of DNA double strand breaks (DSBs), has been shown to mediate both end-to-end chromosome fusions and apoptosis that is triggered by critically short telomeres. This indicated that NHEJ proteins detect and signal the presence of non-capped telomeres (Espejel et al., 2002).

The functionality of telomeres does not only depend on a sufficient telomere length. Telomere capping requires a minimum length of TTAGGG repeats, the integrity of the 3'-overhang and functional telomere-binding proteins (De Lange, 2002; Espejel et al., 2002; Goytisolo and Blasco, 2002).

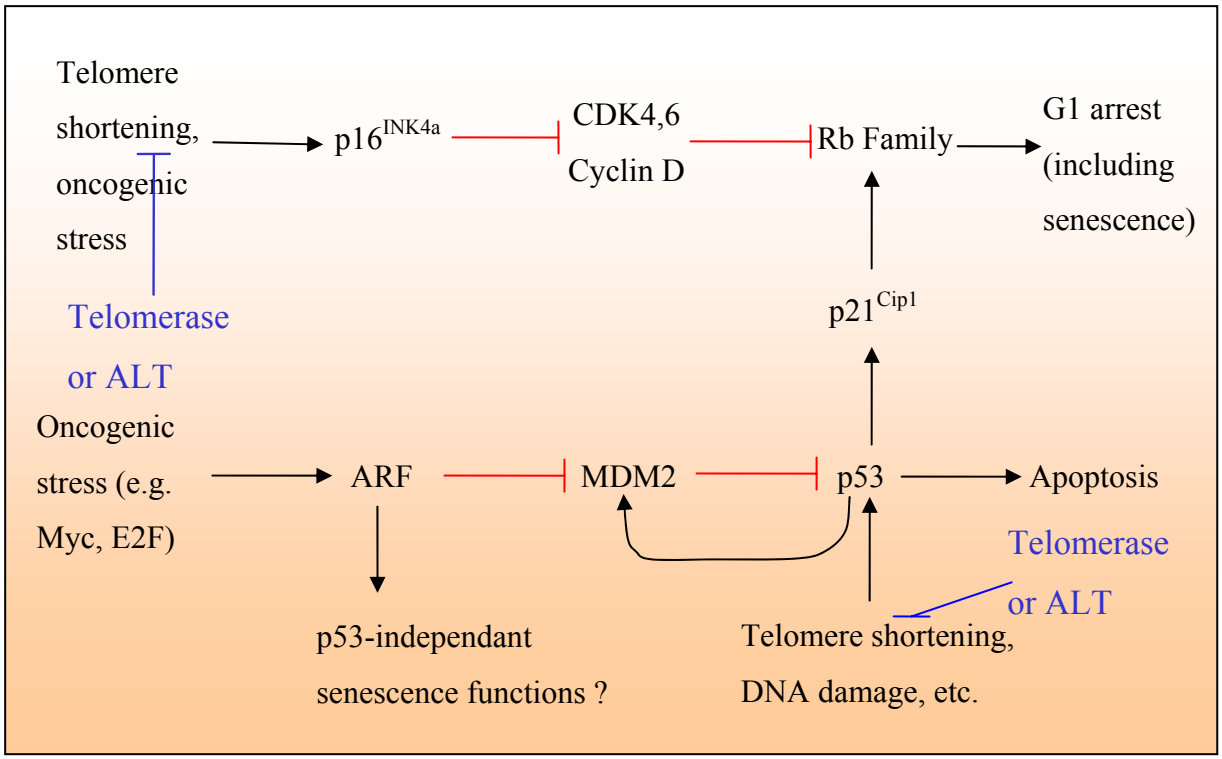

Figure 23. The Pathways depending upon $p16^{INK4a}$-Rb or ARF-p53, with telomerase or ALT actions. Figure modified from (Drayton and Peters, 2002; Lowe and Sherr, 2003; Sharpless et al., 2004; Sherr, 2001; Sherr, 2004; Zhao et al., 2004).

In some circumstances, if telomerase is expressed at a sufficient level in cells, it can be a sufficient condition for them to be or to become immortal. Normal cells, such as germ cells and a sub-population of adult stem cells, have telomeres maintained at a constant length, and are immortal (Harley et al., 1990a; Reyes et al., 2001). Some normal non-immortal cells can also be immortalized if telomerase is activated (Counter et al., 1992b), or if there is presence of an ALT mechanism (Bryan et al., 1995). Concerning cancers, 85% of them are telomerase-positive (Shay and Bacchetti, 1997), but additional features are also modified in these cells.

Telomerase has already been overexpressed at non-physiological levels, as shown in the telomerase-transgenic mice (Gonzalez-Suarez et al., 2001). In such a situation, there is no abnormal telomere lengthening, probably reflecting the existence of a regulated structure (T-loops) at the telomere (Blasco, 2002). If telomerase is upregulated in cells, the lengths of telomeres increase if they are too short, and survival signals from capping of telomeres are

triggered. This often allows the cells to become immortal, or to survive longer. In the contrary, if the regulation of the telomeres is inappropriate, such as in the case of NHEJ or TRF2 mutations, telomere fusions, cell cycle arrest and apoptosis can happen, even though the telomere length may be sufficient, and even though there may be telomerase activity (Blasco, 2002).

Senescence can be bypassed by hTERT overexpression, even in some human cells (Bodnar et al., 1998a). However, it has been reported that hTERT-immortalized fibroblasts can gradually acquire features associated with a transformed phenotype, so that safety concerns should be raised before hTERT-immortalized cells can be used for cell therapy (Milyavsky et al., 2003). Even though hTERT-mediated immortalization can escape genomic instability, in some cases, hTERT expression was not sufficient to induce immortalization. This suggests that, often, other mechanisms may exist (in addition to telomere length measure) to avoid that cells become immortal (Drayton and Peters, 2002; Yaswen and Stampfer, 2002). In cases of telomere-independent induced senescence, accumulation of $p16^{INK4a}$ was observed. This suggested that $p16^{INK4a}$ might be a sensor for telomere-independent induced senescence. However, $p16^{INK4a}$ accumulation was also suggested to be a result of inadequate growth conditions (Dickson et al., 2000; Herbert et al., 2002; Kiyono et al., 1998; Ramirez et al., 2001; Yaswen and Stampfer, 2002).

In addition to telomere maintenance, telomerase may have additional carcinogenesis properties (Blasco, 2002). Telomere shortening below a critical level results in end-to-end chromosome fusions, and dicentric chromosomes might generate the chromosome instability which seems to be important for the cancer cells. It may allow them to acquire new mutations that may induce in them new carcinogenetic properties (Artandi and DePinho, 2000; Gisselsson et al., 2001; Hackett et al., 2001). Moreover, telomerase might have pro-survival and anti-apoptotic activities to maintain cell survival and proliferation, independently of its telomerase enzymatic activity (Cao et al., 2002). It has already been observed that telomerase may contribute to tumorigenesis by a telomere length-independent mechanism, because the immortal ALT cell line GM847 could be transformed after the addition H-Ras and telomerase, but not after the expression of H-Ras alone (Stewart et al., 2002b).

Telomerase and telomere length has been implicated in different diseases, such as in HIV and in chronic hepatitis or liver cirrhoses (Wong and Collins, 2003). Telomere length implication in Duchenne's Muscular Dystrophy is also discussed (Oexle and Kohlschutter, 2001).

1.2.2 Alternative lengthening of telomeres (ALT)

Some cells are immortalized, but they are telomerase-negative. In such a case, until now, their telomere length was shown to be maintained through one or more mechanisms referred to as alternative lengthening of telomeres (ALT) (Bryan et al., 1997). In humans, ALT uses some telomeric sequences (or extrachromosomal telomeric DNA) as a copy template to elongate one particular telomere. This mechanism was demonstrated by inserting a DNA tag into a telomere of ALT cells, and in this experimental setting, the tag was copied on to other telomeres (Reddel, 2003). As a result, telomere length maintenance by TERT or by ALT are functionally two different mechanisms. The first mechanism is using a template in the enzyme telomerase to add sequences to the telomeres, while the second uses telomeric sequences from one telomere as a template to elongate another one. Consistent with this, ALT expressing cells are insensitive to inhibition of the hTERT function by the dominant-negative enzyme (Hahn et al., 1999b). In the contrary of telomerase positive cells, ALT cells have extremely heterogeneous telomere lengths, ranging from very short to extremely long (Bryan et al., 1997). This pattern of varying telomere sizes in one single cell is not seen in most normal human and murine cell types. Currently, no normal cell is known to express a normal counterpart of ALT, which would have the same activity as ALT in transformed or cancerous cells. It is only in telomerase-null mice that an ALT mechanism could be observed in some B cells (Herrera et al., 2000). Despite few human or murine cells seem to possess an ALT mechanism, some species of mammalian cells can have non-telomerase mechanisms for the conservation of their telomere length. For instance, mosquitoes (*Anopheles gambiae*) use a recombination-based ALT mechanism (Roth et al., 1997) and *Drosophila melanogaster* use a retrotransposon-based ALT (Biessmann et al., 1992) instead of telomerase.

Based on a limited number of published evidences, it seems that the expression of an ALT mechanism is present more often in cell lines and tumors of mesenchymal origin than in those of epithelial origin (Henson et al., 2002).

Some tumors have been shown to possess both ALT and telomerase activity (Bryan et al., 1997). *In vivo*, it is not known if this is the result of two processes of carcinogenesis. If it was the case, however, this would explain why there is sometimes within a tumor heterogeneity of ALT+ and telomerse+ cancer cells. However, these mechanisms may not exclude each other in a cell. Indeed, it has been shown *in vitro* that these two telomere length mechanisms (TMM) can coexist within the same tumor cells (Cerone et al., 2001; Grobelny et al., 2001; Perrem et al., 2001; Stewart et al., 2002a).

1.2.3 Bmi-1

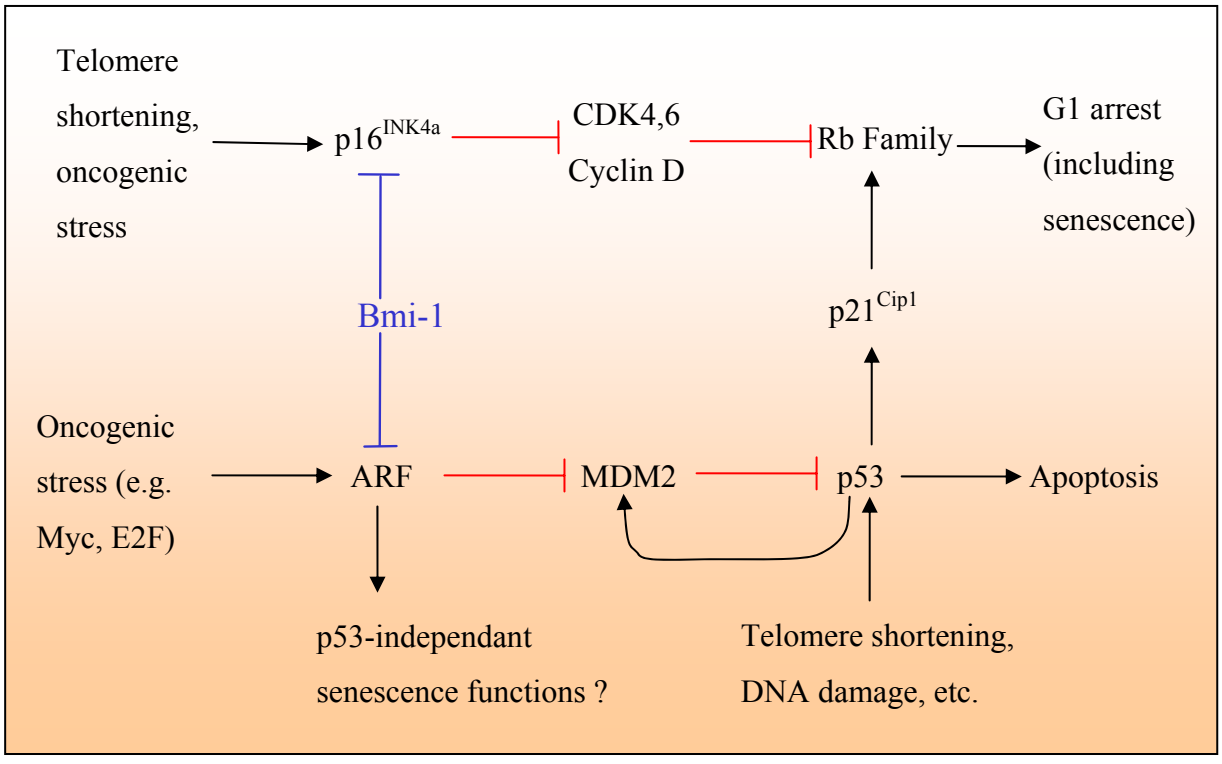

Figure 24. The Pathways depending upon $p16^{INK4a}$-Rb or ARF-p53, with Bmi-1 action on both pathways. Figure modified from (Drayton and Peters, 2002; Lowe and Sherr, 2003; Sharpless et al., 2004; Sherr, 2001; Sherr, 2004; Zhao et al., 2004).

Bmi-1 stands for B lymphoma Mo-MLV insertion region (van Lohuizen et al., 1991). The name of this gene derives from the manner it was discovered. It was observed that constitutive expression of the *myc* oncogene alone is not sufficient to cause B cell lymphoma. In an attempt to identify genes that cooperate with myc in B cell transformation, transgenic animals were used, in which the expression of myc was increased by placing an immunoglobulin heavy chain enhancer near myc (Eμ-*myc*). These animals were shown to develop lymphomagenesis only after a certain period of time, suggesting that other events are necessary to promote tumors (Adams et al., 1985; Cory and Adams, 1988). Nevertheless, these additional mutations are often small, and difficult to find in the genome, complicating the search for new genes cooperating in oncogenesis. In the contrary, retroviral insertion can permit to activate oncogenes, and this mutation can be easily found by classical inverse PCR methods. In the case of B cell transformation, one day old Eμ-*myc* transgenic mice were infected by 10^5 infectious units of Mo-MLV. This infection led to a dramatic acceleration of lymphomagenesis, and permitted to discover that in 35% of tumors, proviruses were integrated in the *bmi*-1 gene (van Lohuizen et al., 1991).

Bmi-1 is expressed in most organs, but higher levels can be detected in thymus, heart muscle, and brain. Relatively high levels of Bmi-1 were also found in testis, in embryonal stem cells, in placenta and in day 10.5 mouse embryos (van Lohuizen et al., 1991). This data pointed to a role of Bmi-1 in immortal cells, and indeed, Bmi-1 was subsequently shown to be necessary for self-renewing cell divisions of adult HSCs as well as adult peripheral and central nervous system neural stem cells (Molofsky et al., 2003; Park et al., 2003). It is also likely that Bmi-1 regulates the self-renewal of other types of somatic stem cells, because there is a broad range of phenotypic changes observed in *Bmi1*-deficient mice, including posterior transformation and neurological abnormalities (van der Lugt et al., 1994).

The main roles of Bmi-1 seem to be mediated mainly by its capacity to inhibit $p16^{INK4a}$ and $p19^{ARF}$, the two open-reading frame products of the INK4A-ARF tumor suppressor locus (Sharpless, 2004). Overexpression of Bmi-1 is associated with increases in the probability of neoplastic proliferation in lymphocytes (Alkema et al., 1997; Haupt et al., 1993). Bmi-1 may also play a key role in some cancers, including in mantle cell lymphomas, in B-cell non-Hodgkin lymphoma and in non-small cell lung cancer (Bea et al., 2001; van Kemenade et al., 2001; Vonlanthen et al., 2001). Bmi-1 has different effects in mouse or human fibroblasts. On

the first hand, in normal mouse embryonic fibroblasts (MEFs), overexpression of *Bmi-1* resulted in a proliferative advantage and extended MEF lifespan. After a slow growth period, *Bmi-1* overexpression even allowed immortalization of MEFs (Jacobs et al., 1999a). On the other hand, in WI-38 human fetal lung fibroblasts, Bmi-1 extended replicative lifespan but did not induce immortalization when overexpressed (Itahana et al., 2003). This discrepancy between human and rodent immortalization has often been explained by the fact that there is telomerase expression in most of the murine cells. Other data, in other systems, support the hypothesis that immortalization or infinite life span provided with *Bmi-1* overexpression is more likely if there is also telomerase expression.

There is evidence that Bmi-1 might upregulate telomerase expression in human mammary epithelial cells (MECs) and might play a role in the development of human breast cancer (Dimri et al., 2002). Mammary tissue can be explanted into tissue culture medium, and forms a heterogeneous population of MECs, termed preselection MECs. However, in vitro, only rare MECs are able to grow, they are termed postselection MECs. In these postselection MECs, the *p16Ink4a* gene is silenced, but the cells do not immortalize spontaneously, and they undergo replicative senescence though they have 30-40 additional doublings compared to preselection MECs (Brenner et al., 1998; Wong et al., 1999). In this system, postselection MECs but not preselection MECs can be immortalized by overexpression of either hTERT, human papillomavirus (HPV) E6, or Bmi-1. However, *Bmi-1* immortalization of postselection MECs was associated with higher hTERT expression (Dimri et al., 2002).

Bmi-1 does not only downregulate $p16^{Ink4a}$ and $p19^{Arf}$, and microarrays experiments have shown that it also regulates stem cell–associated genes, survival genes, antiproliferative genes, and genes associated with stem cell fate decisions (Figure 25).

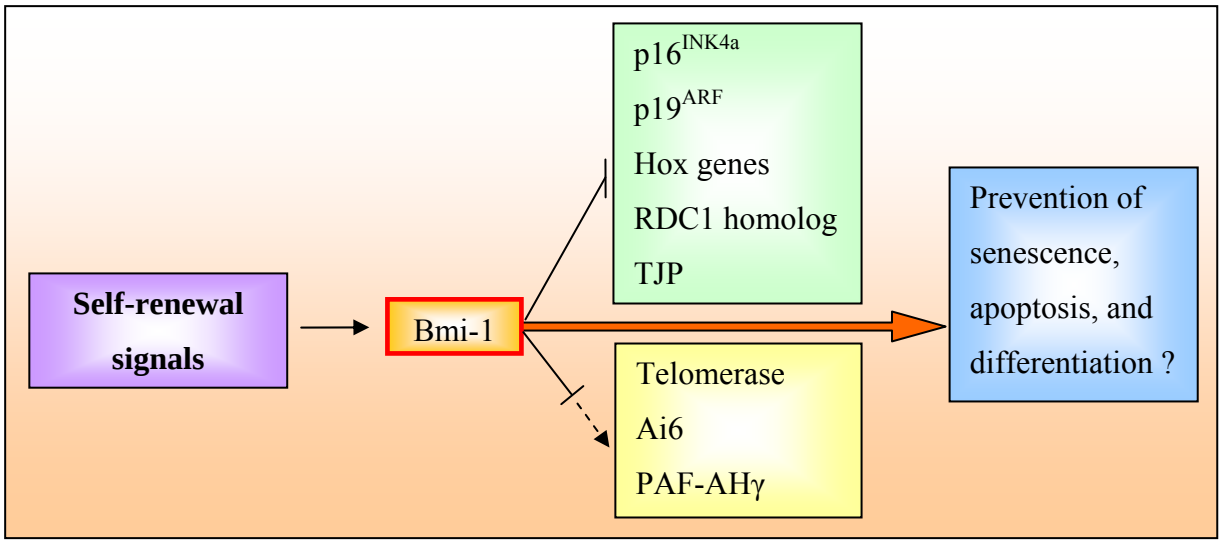

Figure 25. Postulated Bmi-1 targets. Self-renewal signals trigger stem cells to upregulate Bmi-1 levels. This inhibits p16Ink4a and p19Arf, but also Hox genes, chemokine orphan receptor 1 (RDC1) and tight junction protein (TJP). Bmi-1 also possibly activates, via indirect mechanisms, some genes including telomerase, apoptosis inhibitor-6 (Ai6), and platelet-activating factor acetylhydrolase (PAF-AHγ). These genes likely play a role in stem cell fate decisions including self-renewal and differentiation. Figure modified from (Park et al., 2004; Park et al., 2003).

The particular role of Bmi-1 in senescence has made of this gene a new candidate for immortalizing cells (Dimri et al., 2002; Jacobs et al., 1999a; Milyavsky et al., 2003; Salmon et al., 2000; Vonlanthen et al., 2001; Zeisig et al., 2004).

1.2.4 SV40 large T antigen

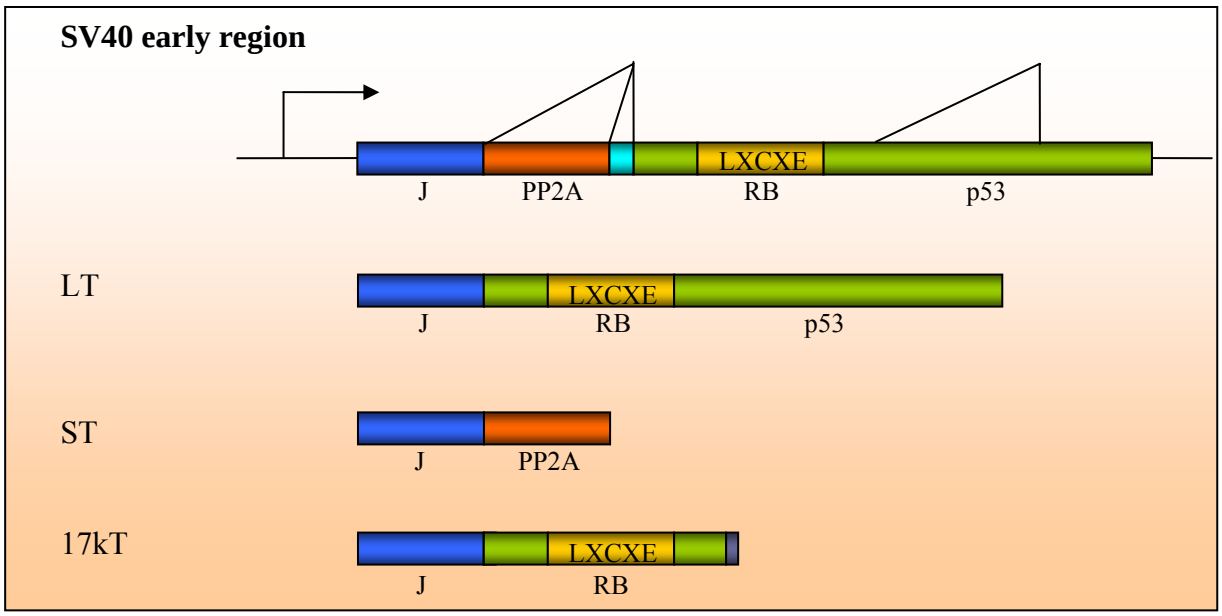

Figure 26. Production of SV40 Large T (LT), small T (ST) and 17kT antigens by SV40 early region. LT, ST and 17kT are produced by alternative splicings of the SV40 early region. All these proteins share the same 82 amino acids in their N-terminus, which has a DnaJ-related sequence. LT binds to the RB family members by its LXCXE sequence, and binds to p53 in two regions of its C-terminal part. ST binds to Phosphatase 2A (PP2A) by means of an intron in the LT-coding sequence. 17kT shares the amino-terminal part of LT, but a second splicing terminates this protein with three amino acids from an alternate reading frame (Boyapati et al., 2003). The resulting 17kT protein binds to RB, but not to p53 or PP2A (Rundell and Parakati, 2001).

Simian virus 40 (SV40) can ultimately transform cells with three proteins: large T (TAg or LT), small T (tAg or ST), and 17 kT antigens. All these proteins are produced by alternative splicing of SV40 early region (Figure 26). However, in practice, a lot of authors may talk about TAg, when they are using in fact the complete SV40 early region. Then, they are using LT, ST, and 17kT antigens. For this reason, it is often only when we read in detail the "Materials and Methods" of an article that we know what genes were effectively used. Despite this problem of nomenclature, it has been noticed that most of the effects due to SV40 early region are due to large T, but we are going to review the effects of all the proteins currently known in the SV40 early region. Already with the SV40 virus, the functions of the SV40 early regions are thought to be mainly accomplished with by large T antigen. In SV40,

the SV40 early region permits host cell transformation, higher cellular proliferation, and higher viral replication. This task is thought to be accomplished mainly by large T antigen, which disrupts the functions of both the Rb family members and of p53 (Figure 27).

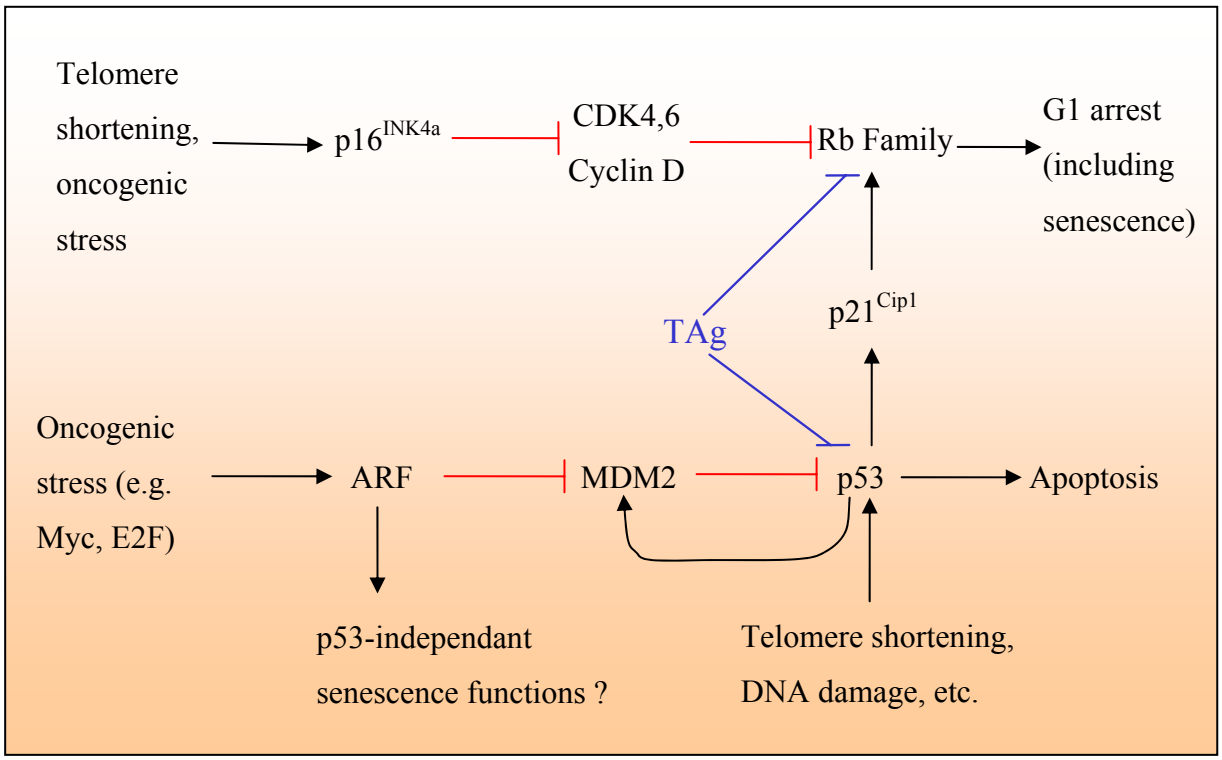

Figure 27. The main functions of TAg : Disruption of pRB and p53 functions. Figure modified from (Drayton and Peters, 2002; Lowe and Sherr, 2003; Sharpless et al., 2004; Sherr, 2001; Sherr, 2004; Zhao et al., 2004).

1.2.4.1 Large T domains

Large T has three main domains implicated in transformation (Figure 25). It has a bipartite carboxy-terminal region (amino acids 351-450 and 533-626), which binds to the tumor suppressor p53 (Kierstead and Tevethia, 1993; Peden et al., 1989; Srinivasan et al., 1989; Zhu et al., 1992), but also to CBP, p300, and the related p400 (Eckner et al., 1996; Lill et al., 1997). The 103-107 residues of Large T contain a LxCxE motif, which binds to the retinoblastoma proteins pRB, p107 and p130 (Peden et al., 1989; Srinivasan et al., 1989).

Finally, the J domain (residues 1-82) exhibits dnaJ domain function (Kelley and Georgopoulos, 1997) and binds to hsc70, a homologue of the heat shock protein hsp70 (Kierstead and Tevethia, 1993; Zhu et al., 1992).

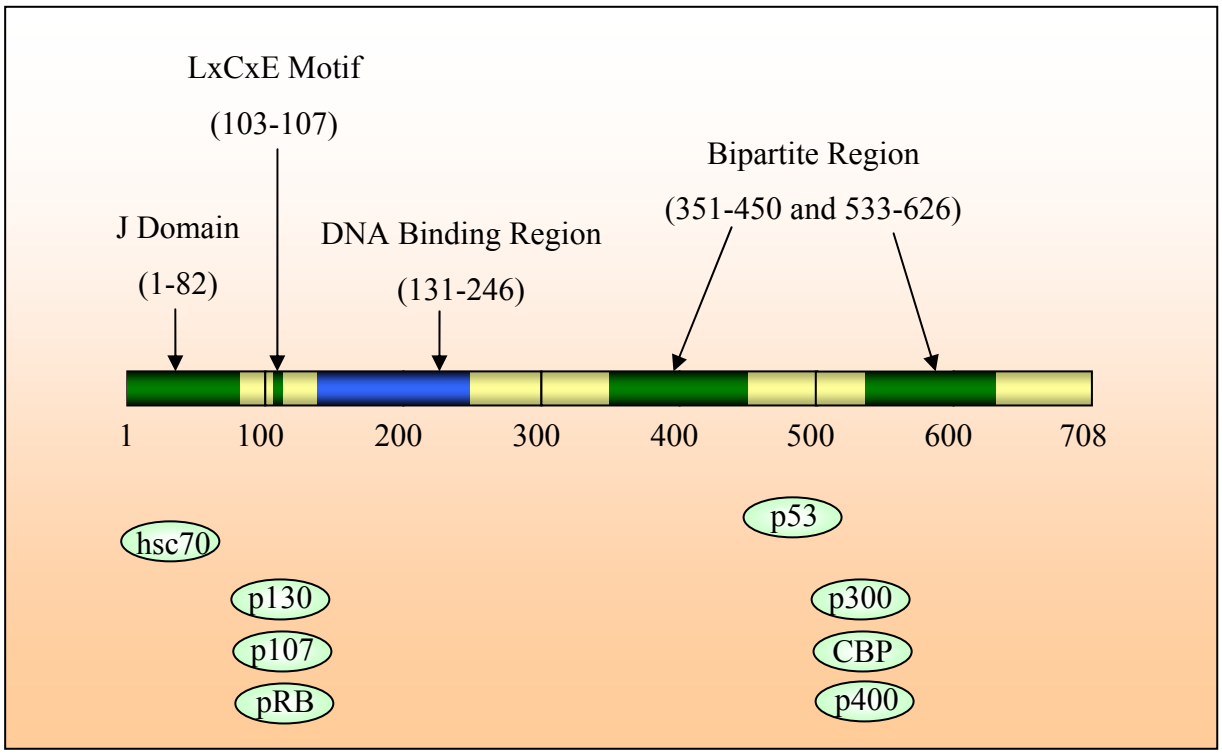

Figure 28. Domains of SV40 large T antigen. Three major domains are involved in transformation (green) : the J domain, the LxCxE motif, and the bipartite region. These domains respectively bind hsc70, Rb family members, or p53. Two regions may also contribute to full transformation of Tag : its DNA binding region, and its p300/CBP/p400 binding region. The latter appears to be located in the C-terminus of Tag but there are indications that certain N-terminal residues may also play a role in this binding.

Notably, the LxCxE motif of Large T has also been found in other oncoproteins such as adenovirus E1A, and the human papilloma virus (HPV) E7. In these viruses, this motif has also been found there to be important for binding to pRB and to pRB-related proteins p107 and p130 (DeCaprio et al., 1988; Dyson et al., 1989; Whyte et al., 1989).

1.2.4.2 TAg, p53 and pRB

The function of TAg in transformation is achieved mainly by the binding of p53 and pRB family of proteins. In humans, disruption of both p53 and pRB pathways are thought to be important for immortalization, whereas in mice, and especially in MEFs, disruption of only the p53 pathway by TAg can suffice for immortalization (Stubdal et al., 1997; Thompson et al., 1990; Zhu et al., 1992).

p53 was first discovered as a cellular protein associating with SV40 large T antigen. Additional experiments showed that p53 was a key protein in the mechanisms of cell cycle control (Friend et al., 1986; Ko and Prives, 1996). p53 acts at least in part as a transcription factor, and can lead to arrest in G1/S and G2 phases of the cell cycle. TAg can bind to p53 DNA binding domain, and this results in preventing p53-mediated transcription (Jiang et al., 1993; Mietz et al., 1992; Segawa et al., 1993). TAg can also increase the half-life and steady-state levels of p53 (Oren et al., 1981; Sladek et al., 2000). This is probably achieved by TAg binding of mdm2 (Brown et al., 1993; Henning et al., 1997; Zalvide et al., 1998), which normally facilitates the ubiquitination-dependant degradation of p53 and inactivates the transcriptional activity of p53. It is not currently understood why TAg needs to prevent p53 degradation by mdm2, since p53 sequestered by TAg is already transcriptionally inactive.

TAg J domain can suppress the repression of E2F activity by p130 and pRB (Avantaggiati et al., 1996). With hsc70 and ATP, TAg can dissociate E2F-4 from p130 (Friend et al., 1986), and can promote p130 degradation (Qin et al., 1992). TAg has also an effect in hypo-phosphorylating p107 and p130 (Hinds et al., 1992; Qin et al., 1992). All these effects lead to inactivations of the pRB proteins, and relieve their possible effect on blocks in G0/G1 or in S phase (Figure 29). Inactivations of p107 and p130 have been rarely found in human tumors, but these proteins may affect transformation, as seen in Rb-/- MEFs (Goodrich et al., 1991; Stiegler et al., 1998).

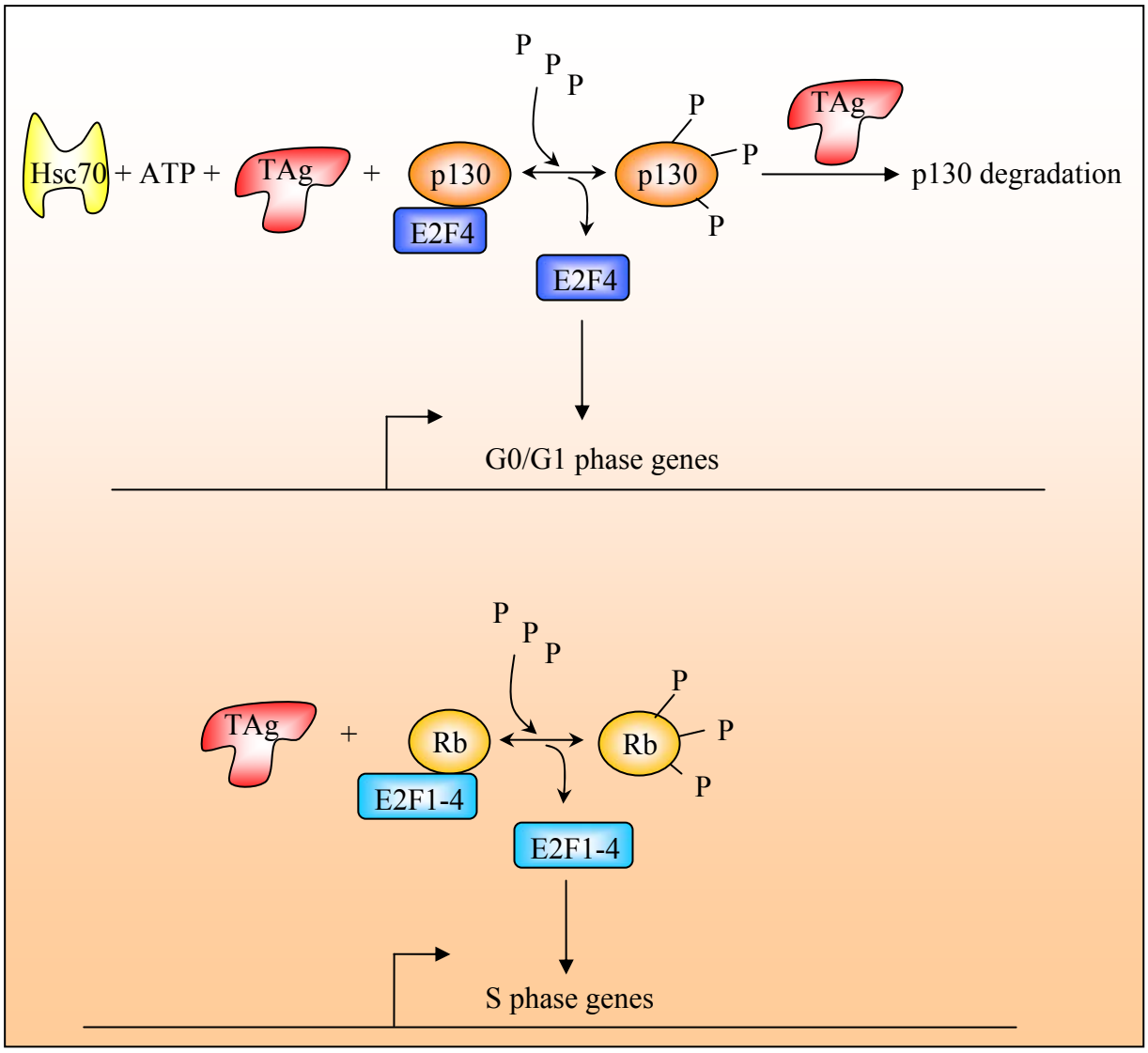

Figure 29. TAg interactions with RB family members. TAg leads to p130 elimination, possibly after p130 phosphorylation. This relieves E2F4 from p130 inhibition, and lead to transcription of G0/G1 phase genes. TAg can also interact with the hypophosphorylated form of Rb. This releases E2F1-4 transcription factor, which then transcribes S phase genes. Figure modified from (Ali and DeCaprio, 2001).

1.2.4.3 Other functions of TAg

TAg transformation capabilities are not only due to its binding to p53 and to pRb proteins. TAg contains a DNA-binding domain (residues 131-246), which is implicated in transcriptional transactivation, possibly of some cellular genes (DeCaprio et al., 1988). Moreover, TAg interacts with unphosphorylated form of p300 (Avantaggiati et al., 1996;

Eckner et al., 1996). This binding may have an effect in E2F-1-mediated transcription, because E2F1 transcriptional activity depends on its interaction with CBP (Trouche et al., 1996). Alternatively, this binding may have an effect in the inhibition of p53 degradation (Figure 30). New binding partners of TAg have recently been identified. They include p400, a member of the p300/CBP family, and a 193 kD protein with a bcl-2 homology (Ali and DeCaprio, 2001). In the end, a new domain in residues 525-541 of TAg has been identified to have antiapoptotic activities (Conzen et al., 1997).

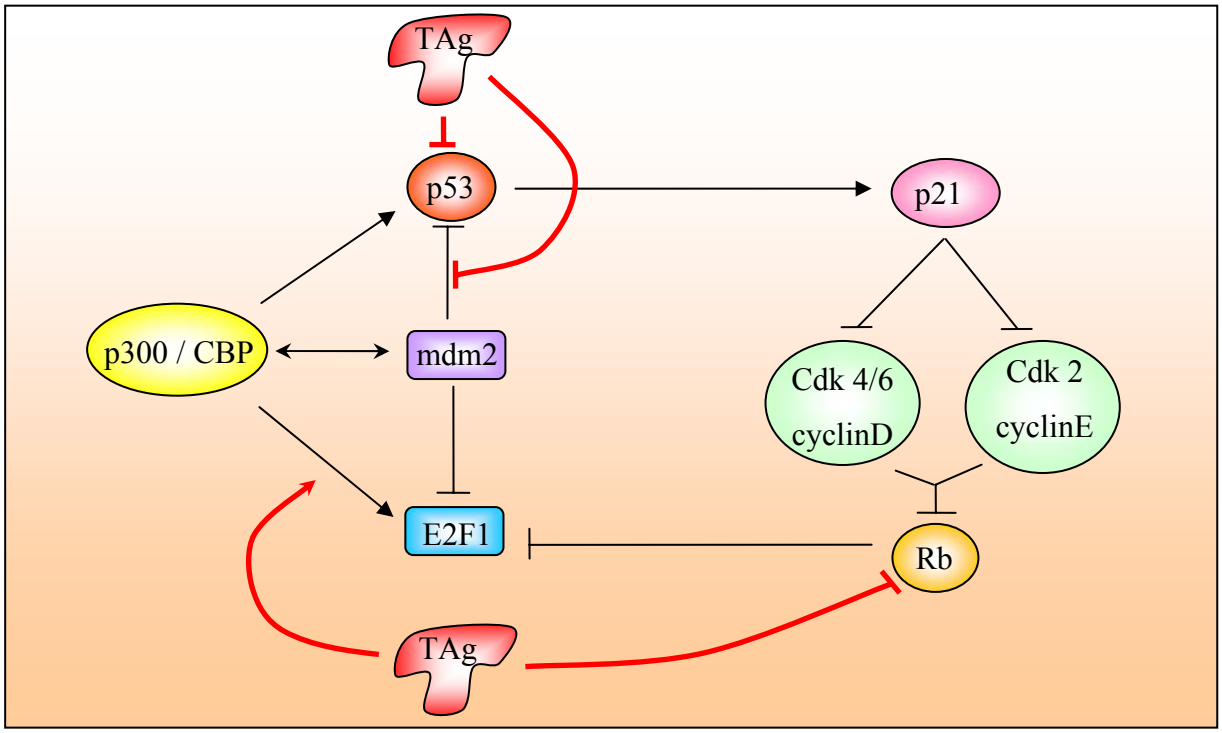

Figure 30. One model of TAg-mediated disruption of cell cycle checkpoints. TAg binds to p53, pRb and p300/CBP, and manipulates the activity of E2F1 through multiple routes. Figure modified from (Ali and DeCaprio, 2001).

1.2.4.4 Small t

Small t has the capability to bind to the catalytic core of protein phosphatase 2A (PP2A) and to inhibit its enzymatic activity toward most substrates (Yang et al., 1991). This interaction is important in small t-dependant transformation, including focus formation of HDF (Mungre et al., 1994; Porras et al., 1996). Small t presence, in addition with Large T, may be particularly

crucial for the transformation of quiescent cells, such as Chinese hamster lung cells, or murine lung and kidney cells (Choi et al., 1988; Cicala et al., 1992; Martin et al., 1979). These experiments and others have shown that the main purpose of small t is to transform the cells, in a c-myc dependent manner (Janssens et al., 2005).

Small t interaction with PP2A activates not only the Myc pathway, but also the MAPK pathway (Sontag et al., 1993), the Creb pathway (Wheat et al., 1994), the Rac/Rho pathway, and the Akt pathway (Figure 31).

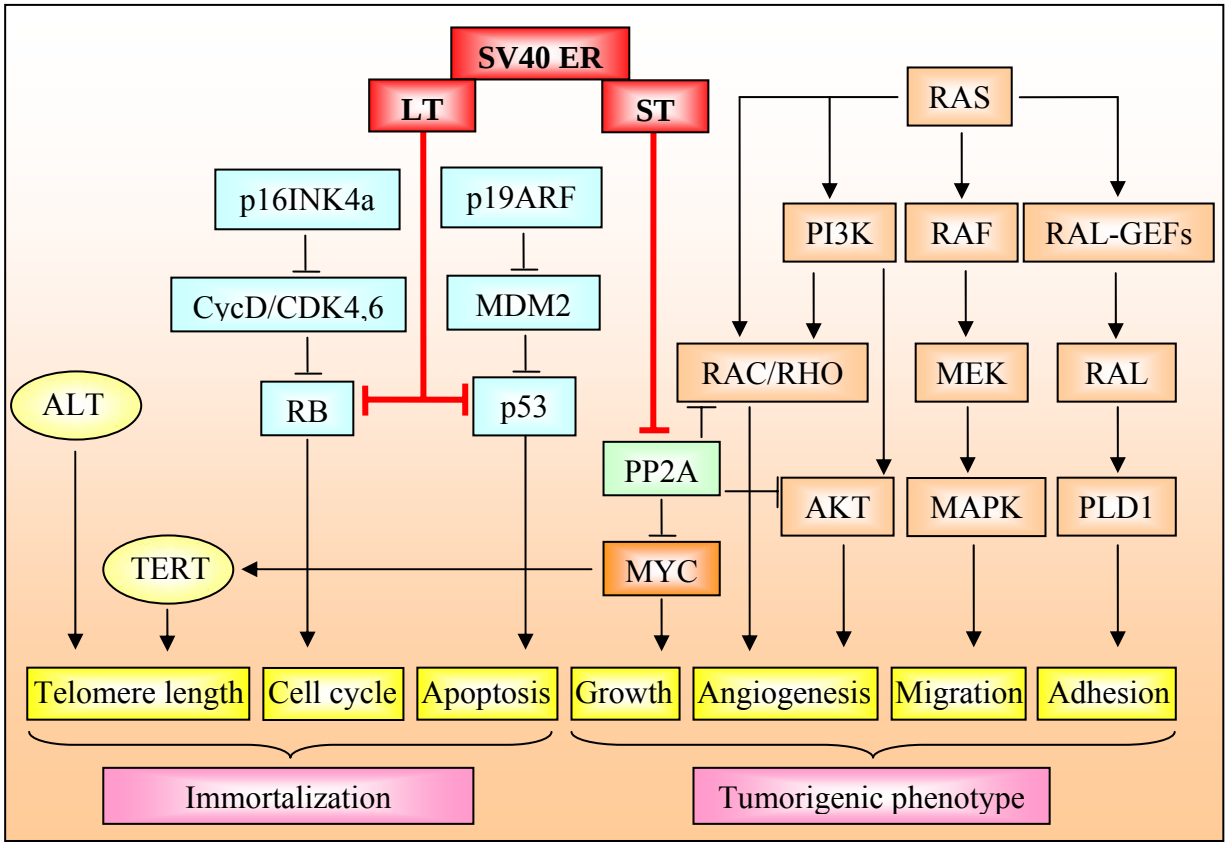

Figure 31. SV40 early regions (ER) proteins effects on cellular pathways. SV40 Large T antigen (LT) inactivates the pRB and p53 pathways, whereas small T antigen (ST) perturbs Phosphatase 2A (PP2A). This can lead some cell types to immortalization, and also to acquisition of tumorigenic phenotypes. Figure modified from (Zhao et al., 2004).

1.2.4.5 17kT

Until now, very few studies have been published about 17kT. It was shown that this protein has weak transforming activities in rat cells (Zerrahn et al., 1993). Similarly to Large T, it can reduce levels of pRb and of p130, and stimulate cell-cycle progression of quiescent fibroblasts (Boyapati et al., 2003).

1.2.4.6 Utility of TAg in immortalization

SV40 Large T, alone or in combination with small T, has been extensively used in a large number of immortalizations, sometimes with telomerase (Hahn et al., 1999a; Hahn et al., 2002; Rich et al., 2001; Yu et al., 2001).

Nevertheless, despite its utility in immortalization of a large variety of cells, TAg has some drawbacks. TAg can induce chromosomal aberrations, including aneupoidy, and translocations. These chromosomal aberrations are induced by large T antigen, but not by small t (Stewart and Bacchetti, 1991).

1.2.5 Bcl-2

Apoptosis can play different roles, which are related to carcinogenesis prevention. First, different stress signals such as oncogenic activation can lead to increased p53 activity and cell cycle arrest or apoptosis (Figure 32) (Hipfner and Cohen, 2004).

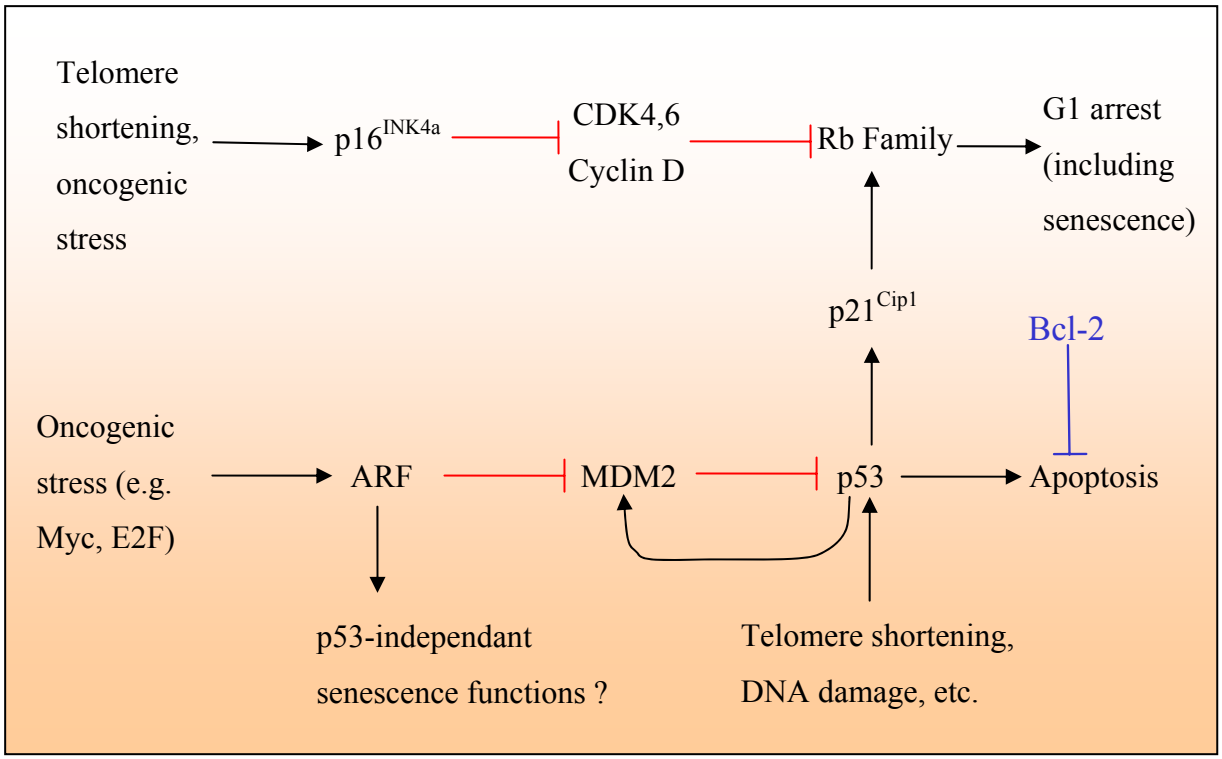

Figure 32. The Pathways depending upon $p16^{INK4a}$-Rb or ARF-p53, with Bcl-2 anti-apoptosis effects. Figure modified from (Drayton and Peters, 2002; Lowe and Sherr, 2003; Sharpless et al., 2004; Sherr, 2001; Sherr, 2004; Zhao et al., 2004).

Second, apoptosis can be triggered if cells lose their attachment to their substratum, a phenomenon known as anoikis (Figure 33).

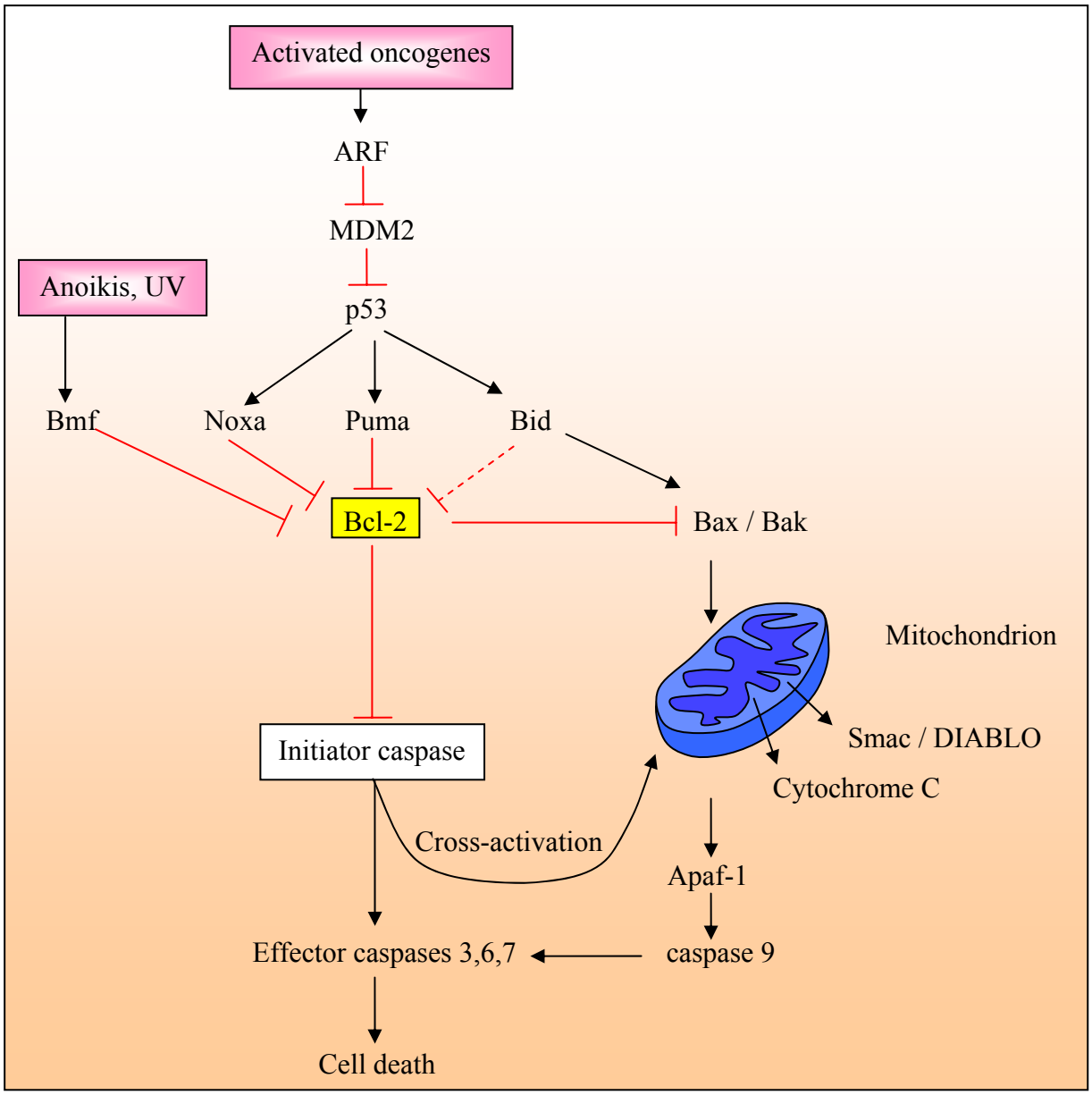

Figure 33. Speculative model for mammalian stress-induced apoptosis. Figure modified from (Cory et al., 2003; Heiser et al., 2004; Jacks and Weinberg, 2002).

The ARF-MDM2-p53-apoptosis pathway is a response to activated oncogenes. ARF responds to proliferation signals, and binds to MDM2. This inhibits p53 transcriptional silencing and degradation by MDM2. p53 activates target genes that promote cell-cycle arrest or apoptosis in response to cellular stresses such as oncogene activation. Anoikis can also trigger apoptosis, by Bmf activation, which suppresses Bcl-2 anti-apoptotic activities.

Cellular transformation and tumor progression has been associated with activation of anti-apoptotic pathways, both *in vitro*, and in some *in vivo* models (Jacks and Weinberg, 2002).

For all these reasons, Bcl-2 may be useful not only to immortalize cells, but also to induce transformation of cells.

1.3 Of the utility of cell lines

In the laboratory, it is useful to use cell lines with an infinite life span. These cells are often easy to cultivate, so that sufficient amounts of them can be obtained for nearly any experiments, and they allow the reproducibility of experiments. They also allow different researchers around the world and in different times to work with the same material. This then permits the comparison of different researches.

A lot of cell lines exist, for different cell types (e.g. for epithelial cells, hepathocytes, macrophages, T cells …). The first immortal cells to be obtained are called HeLa. These are human epithelial cells from a fatal cervical carcinoma transformed by Human Papilloma Virus 18 (HPV 18) (Gey et al., 1952; Scherer et al., 1953). These cells were obtained in 1951 from a 31 years old black woman living in Baltimore, Henrietta Lacks, whose name is now famous because of the utility that HeLa cells have had in laboratories around the world. This is reflected in the PubMed searches, where the word "HeLa" could retrieve more than 47000 entries in December 2005. However, despite the utility of HeLa cells as a cellular model, it has been shown that this cell line is considerably different from normal primary cells. Consistent with its cancerous origin, HeLa cells have different tumorigenic markers. They include a low level of p53, and an abnormal chromosome number (Belcher, 1971). Moreover, this cell line is tumorigenic in *nude* mice. Most of the cell lines also contain abnormal oncogenic and viral sequences when they are compared to the primary cells.

The cancerous origins of cell lines may be useful for some studies of cancer because both these types of cells may have common characteristics. Nevertheless, the cells lines are easy to cultivate and cancer cells are usually very difficult to put in culture (Masters, 2000). This is an illustration that cell lines are different from cancer cells. Moreover, even if a cell line is derived from a cancer, it may be different from the cancer it comes from. Indeed, an individual cancer cell line can be representative of only some of the cancer cells at the time biopsy was taken and they can still be the subject to additional genotypic instability during the time that they are cultivated *in vitro*. Despite these limitations, it remains that cell lines

originating from cancers have been largely used to study cancer but they could also allow the discovery a lot of molecular events that take place in normal cells.

Nevertheless, all cell lines do not originate from a cancer. Some of them were obtained in the laboratory, for instance with carcinogenic agents. The mutations caused by these agents can lead finally to immortalization of the cells. However, the cells obtained with this method can have an altered phenotype, once they are compared with the primary cells. This can be due either to unwanted mutations in genes which are unrelated to immortalization or this can be due to immortalization through a process resembling to tumorigenesis. However, when these cells are compared to cancer cells, they may have less tumorigenic properties. This may be the case, because the cell lines obtained *in vitro* did not have to escape from the immune system, and it is not the case for most of the cancer cell lines obtained *in vivo*.

Anyway, the multiple mutations that take place in chemical mutagenesis can often not be revealed, and this is one thing that researchers often would like to know. In fact, researchers would like to know with what kind of material they are working with. This is why cell lines with defined genetic elements were produced (Hanahan and Weinberg, 2000). For this purpose, either viral or oncogenic genes can be introduced into the primary cells, and can lead or not to immortalization. Such cell lines are certainly the cell lines of the future, because they allow researchers to know exactly with what kind of material they are working with.

The cell lines are largely used in biological research for a large number of studies. However, it is more and more envisaged that they could have other utilities, for example in gene therapy experiments. For instance, the mouse myoblast cell line C2C12 has already been genetically modified to release a therapeutic product, and this therapeutic cell line could then be encapsulated and implanted into a murine host (Cirone et al., 2004).

In the present days, it is probably too early to generalize the usage of cell lines for therapeutic purposes. To this end, new cell lines should probably be established. These cells should be as near as possible to the primary cultures, without to have a tumorigenic or altered phenotype. Such cell lines would also be advantageous for *in vitro* studies, because they would resemble more to the primary cells that researchers want to study. Moreover, if such cells could be created with defined genetic elements, it would be an advantage for researchers, because they could know exactly in what they differ from the primary cells they are derived from.

1.4 Lentiviral immortalization

We have presented until now the utility of cell lines, and the genes that could be useful to use for immortalization. However, we still need a vehicle to deliver the genes of interest into the genome of the host cells. For this purpose, we have chosen lentiviral vectors. These vectors have, first, the advantage to allow the successful delivery of genetic material into nondividing cells. Second, they have the advantage to allow high-level expression of the transgenes, because integration occurs in locations that are situated close to actively transcribed genes (Mitchell et al., 2004). Third, they permit the transfer of transgenes without transcription of viral proteins in host cells. Fourth, they allow the packaging of relatively long transgenes, which may cover most of the clinical applications. Fifth, and finally, progress in the design of lentiviral vectors have allowed the quasi-elimination of risks that replication-competent viruses may appear. All these properties have been illustrated in a number of studies (Naldini et al., 1996; Salmon et al., 2000; Zufferey et al., 1998; Zufferey et al., 1997). We are going now to review briefly the development of the lentiviral vectors, and we are going to describe the generation of lentiviral vectors that we have finally used.

The lentiviral vectors originate from a modified Human Immunodeficiency Virus (HIV). In fact, it has been shown that it is possible to exploit the properties of HIV to insert genes (also called transgenes) into the genome of non-dividing cells, thereby producing a lentiviral vector (Naldini et al., 1996). For this purpose, deletions in the HIV sequence were done. This allowed the production of HIV-derived vectors that were not harboring the usually HIV-related pathogenic effects. Different deletions were done in HIV, producing different generations of HIV-derived packaging systems.

In the second generation packaging system, the lentiviral vectors are produced from HIV deprived of the accessory but pathologic proteins Nef, Vif, Vpr and Vpu, but also of the HIV envelope. The safety of this system is also ensured by impairing HIV replication. This was done by splitting the components of the vector in three plasmids: the Packaging, the Transgene and the Envelope plasmids (Figure 34). The Packaging plasmid contains the genetic informations that are necessary for the construction of the capsid of the vector. This

plasmid also permits the productions of the proteins involved in the reverse transcription and integration processes. The Transgene plasmid provides the genetic material to be encapsidated into the vector. Finally, the Envelope plasmid provides the envelope that can give a host specificity of the vector. If the HIV envelope is replaced by Vesicular stomatitis virus G protein (VSV-G), the vector is called VSV-G-pseudotyped, and the type of the cells it can enter into (also called its tropism) considerably broadens (Naldini et al., 1996). In these plasmid constructs, only the Transgene plasmid was designed to contain the two determinants that could allow to its genetic information to be incorporated into the host genome. These two integration-related elements contained in the Transgene plasmid are Ψ, and the R U5 sequences. Ψ is the packaging signal that can permit incorporation of Transgene RNA into viral particles, and the R U5 sequence allows successful reverse transcription of the template RNA in the host. Further HIV deletions can be done in the Packaging plasmid. They include the deletions of Tat, Rev and of the rev responsive element (RRE), but removal of these sequences lead to lower vector titers (Gasmi et al., 1999; Pacchia et al., 2001).

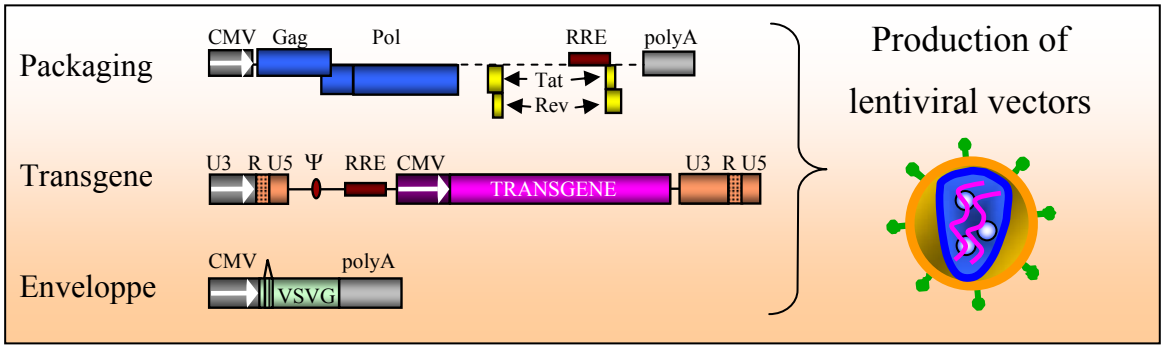

Figure 34. Plasmids used in transfections for production of lentivectors. Co-transfection of three plasmids are able to produce lentiviral vectors: packaging, transgene and envelope plasmids. See text for explanations. CMV: human cytomegalovirus immediate early promoter. Figure modified from (Zufferey et al., 1997).

The properties of the second generation lentiviral vectors were used to immortalize cells, with slight modifications (Salmon et al., 2000). For this purpose, a LoxP sequence was added in the R sequence at the 3' end of the transgene sequence. This allowed the insertion into the host genome of LoxP sites surrounding the transgene, and excision of the transgene cassette after Cre expression (Figure 35). At the same occasion, part of the HIV U3 sequence in the 3'

part of the Transgene plasmid was deleted, leading to a self inactivating lentiviral vector (SIN), which has the property not to give a functional viral promoter post reverse transcription (Dull et al., 1998; Miyoshi et al., 1998; Zufferey et al., 1998). After the immortalizing agent, an internal ribosomal entry site of the encephalomyocarditis virus (IRES) sequence was also inserted, with a following sequence from the thymidine kinase of herpes simplex virus type 1 (HSV1-TK). This ensured that the cells that would escape Cre-mediated excision could still be selectively killed after Acyclovir or Gancyclovir treatment (Salmon et al., 2000).

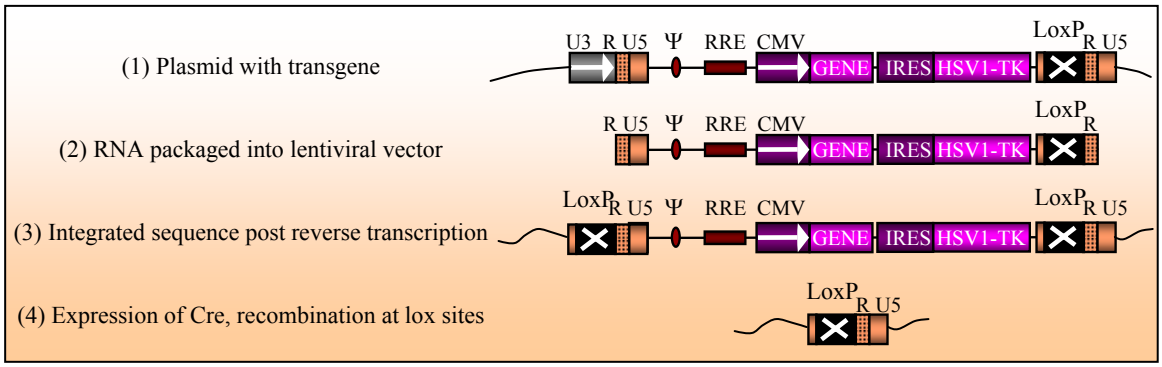

Figure 35. The lentiviral vector, the integrated transgene, and its excision. (1) Plasmids are transfected into 293T cells and lead to RNA species without the U3 part of the LTR. (2) RRE permits successful exit of the nucleus of non-spliced transcripts, while Ψ permits encapsidation of the transgenes into lentiviruses. (3) Once in the target cell, reverse transcription and integration lead to integration of the transgene in the genome, flanked by LoxP sites. (4) After expression of Cre, only the LoxP sites remain integrated in the genome, and R U5. Figure modified from (Salmon et al., 2000).

1.5 Why to immortalize human myoblasts?

Different cells can be immortalized for therapeutic purposes or for *in vitro* studies. We have chosen to immortalize myoblasts for two major reasons. First, before our work, no human cell line was reflecting the biology of real human myblasts. Second, we chose myoblasts as targets for immortalization because this cell type allows easily to study if resulting cells can still fully differentiate after immortalization, and immortalized cells were often reported to have problems of differentiation.

Numerous attempts have been done to immortalize human myoblasts (Corti et al., 2001; Di Donna et al., 2003; Miranda et al., 1983; Pincon-Raymond et al., 1991; Seigneurin-Venin et al., 2000; Simon et al., 1996; Tedesco et al., 1995; Webster et al., 1988). However, the resulting cell lines were often reported to behave very differently from the normal primary myoblasts. They often had tumorigenic properties or difficulties to differentiate. If immortalization was coupled with these properties, it was probably due to the methods of immortalization used in these articles, involving strong oncogenes or viral-derived genes.

Immortalizations with Ras were often reported to be coupled with induction of tumorigenesis (Hahn et al., 1999a; Kusakari et al., 2003) but also to modifications of cell cycle (Rajalingam et al., 2005). However, it is thought that only a tightly regulated cell cycle would allow the differentiation of a cell, even though this topic is still the subject of debate (Brown et al., 2003). At least, it is known that Ras and Bcl-2 may perturb the differentiation of memory B cells to antibody-producing B cells (Takahashi et al., 2005).

Viral genes can also, like oncogenes, lead to modifications of phenotypes and not only to immortalization. In fact, the purpose of the viral genes is not to give to researchers a good immortalized cell line but to modify the cells in a manner that the viruses they are encoded by can grow easier. We have previously discussed the immortalizations with Large T Antigen, which often led to aberrant karyotypes. In the specific case of myoblast immortalization, some viral proteins were already shown to perturb differentiation. For instance, Adenovirus E1A and T-Ag was shown to suppress myogenic differentiation of mouse myoblasts (Webster et

al., 1988). Moreover, T-Ag led to loss of contact inhibition, to anchorage-independent growth (Miranda et al., 1983), and to a strong tendency to apoptosis (Corti et al., 2001).

As, until now, most of the attempts to immortalize myoblasts were only half-successful, practically no human myoblast cell line with the basic properties of human primary myoblasts are used in laboratories. This means that human myoblasts have to be isolated from orthopedic surgery or diagnostic biopsies for different *in vitro* studies. Such primary cells have a limited replication potential, and repeated isolations of myoblasts must be performed. The satellite cells obtained from Duchenne patients are known to have even shorter lifespans and they can also be less easily obtained than normal primary myoblasts. Considering these facts, we wanted to attempt to immortalize human primary myoblasts, in such a way that these cells would keep their myogenic capacities after immortalization. We have done this, not only to obtain a human myoblast cell line which would be similar to primary cells, but also because myoblasts permit to study easily if the differentiation phenotype of these cells is the same after immortalization (Figure 36). For this purpose, we have used genes that might still allow differentiation of myoblasts after immortalization. They include *bmi-1*, *telomerase*, and *bcl-2*. We have already discussed previously the properties that these genes may have in immortalization processes. Now, we are going to talk more about myoblasts.

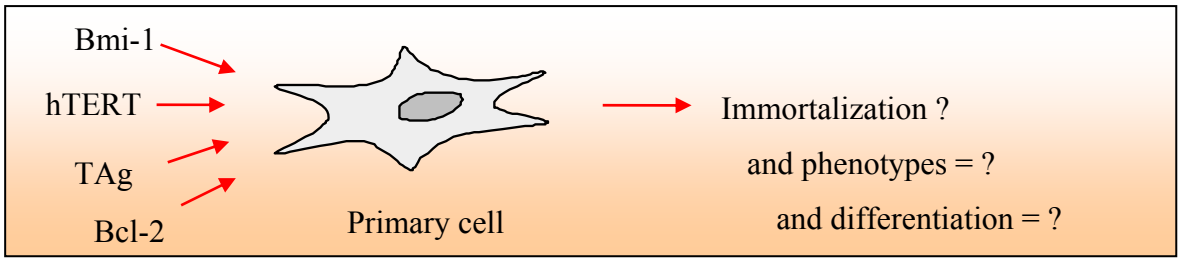

Figure 36. What happens after addition of oncogenes into primary cells? Are they immortalized? And what are their resulting phenotypes, also during differentiation?

1.6 Muscles and satellite cells: an overview

A number of specific reviews exist about the muscle, and because we can not discuss all the muscle-related issues, we will make an overview of the muscle physiology, particularly in our interest to immortalize control myoblasts, and myoblasts originating from patients suffering of Duchenne Muscular Dystrophy.

Muscles are derived from the mesodermal cells that are themselves derived from the somites. The myogenic precursor cells express the transcription factor Pax-3, the tyrosine kinase receptor c-Met, the transcriptional repressor msx1, and the transcription factor Lbx 1 but these precursors lack expression of the myogenic regulatory factors of the MyoD family (Hawke and Garry, 2001). Precursor cells are committed positively to the myogenic lineage by Noggin, Wnts, and Shh, while BMP4 has a negative effect (Figure 37). The committed cells finally upregulate MyoD and/or Myf5, the basic helix-loop-helix transcriptional activators of the myogenic regulatory factor family (MRF) and then can be termed myoblasts (Chargé and Rudnicki, 2004).

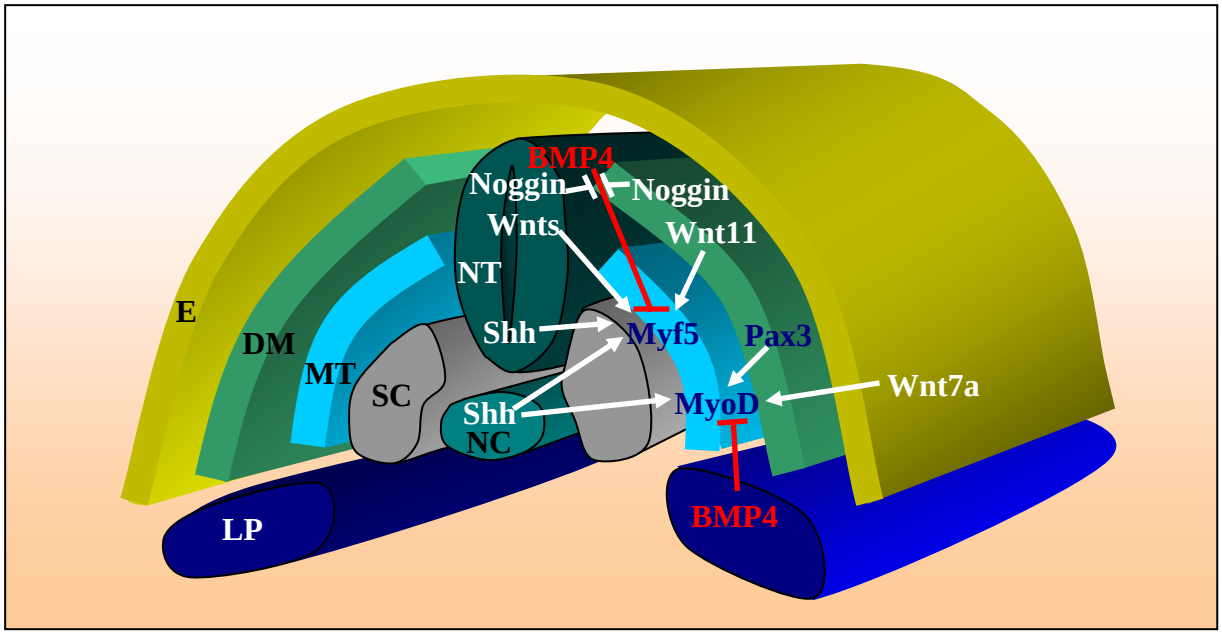

Figure 37. Signaling factors involved in embryonic skeletal muscle formation. *Wnts*, Sonic hedgehog (*Shh*) and *Noggin* induce (and BMP4 inhibits) the expression of the primary MRFs

(*Myf5* and *MyoD*) and commit the mesodermal somitic cells located in the dorsal part of the somite [dermomyotome (DM)] to the myogenic lineage. Commited myoblasts migrate laterally to form the myotome (MT), which eventually forms the skeletal musculature. *Pax3* promotes myogenesis in the lateral myotome. E, ectoderm; LP, lateral plate; SC, sclerotome; NC, notochord; NT, neural tube. Figure modified from (Chargé and Rudnicki, 2004).

Proliferating myoblasts withdraw from the cell cycle to become terminally differentiated myocytes that express the "late" MRFs, Myogenin and MRF4. These cells finally fuse to form a multinucleated syncytium, and mature into contracting muscle fibers. Only a subpopulation of myoblasts do not differentiate, and remain associated with the surface of the myofiber. They are the quiescent muscle satellite cells (Figure 38) (Chargé and Rudnicki, 2004). It is well established that the myogenic precursors derive from the multipotent mesodermal cells of the somite, but the origin of satellite cells is less well understood. It is currently proposed that satellite cells may originate from somitic or from endothelial cells. Nonetheless, it is known that Pax7 is responsible for the specification of progenitor cells to the satellite cell lineage. Quiescent and activated satellite cells both express *in vivo* Pax7 (Seale et al., 2000). Pax7 is closely related to Pax3, which is a key regulator of somatic myogenesis (Maroto et al., 1997; Tajbakhsh et al., 1997).

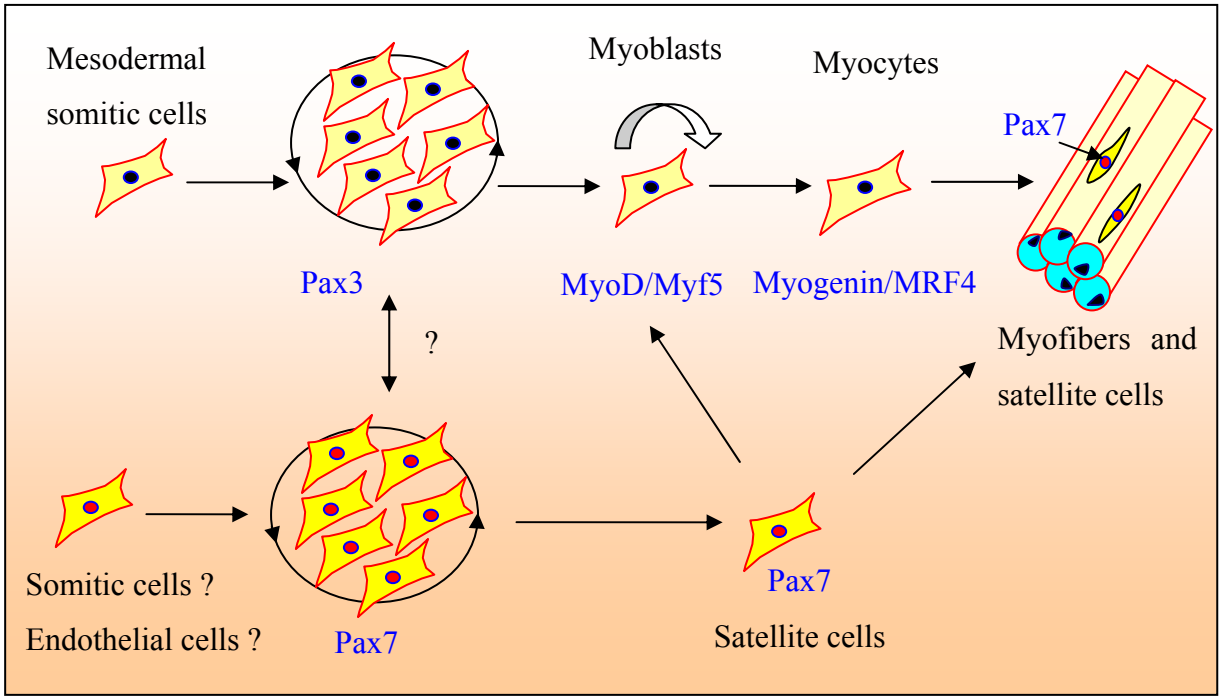

Figure 38. Pax3 expression in the lateral myotome promotes myogenesis. Pax3 expression in precursor cells contributes to myogenic cell expansion. Induction of MyoD and/or Myf5 commits cells to the myogenic lineage. After cell divisions, cells become quiescent myocytes, and upregulate the secondary MRFs (myogenin and MRF4). Finally, myocytes fuse and give rise to multinucleated myofibers. At a later embryogenic stage, some satellite cells give rise to myoblasts and promote fiber growth, while others remain associated with the myofibers in a quiescent undifferentiated state. It is known that Pax7 promotes the specification/expansion of the satellite cell population. The embryonic origin of the satellite cells still remains to be determined. Figure modified from (Chargé and Rudnicki, 2004).

The muscles are composed of a network of different myofibers, blood vessels, motor neuron, and extracellular tissue matrix. Muscle contraction is the result of a the sliding of a myosin-rich thick filament over an actin-rich thin filament after neuronal activation. Myofibers types can be either slow-contracting/fatigue resistant or fast-contracting/non-fatigue-resistant. The fast-contracting fibres are needed for movement, and the slow fibres are required for posture maintenance. The relative amount of slow type I myosin heavy chain isoform determines the type of muscle (Figure 39). If a muscle is rich in myofibers expressing the slow type I myosin heavy chain isoform, the muscle is defined as a slow contracting muscle (Kameda et al., 1993).

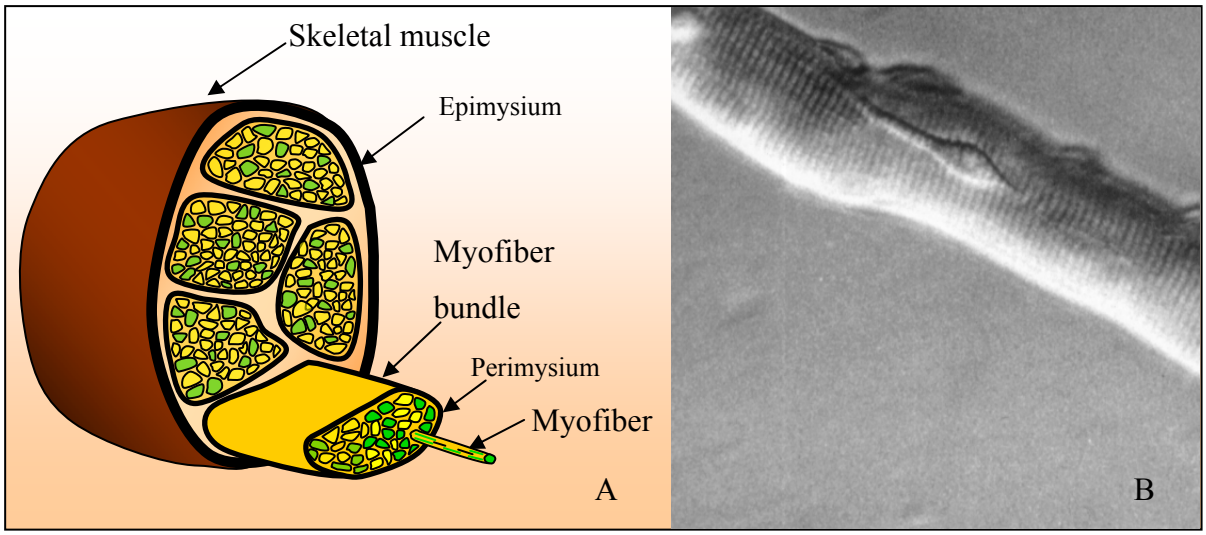

Figure 39. The muscle, the myofiber, and satellite cells. A. Schematic view of a skeletal muscle. A skeletal muscle is composed of myofibers, which are multinucleated syncitia with post-mitotic nuclei placed at the periphery. Here, a slow contracting (e.g. soleus and not plantaris) muscle is shown. This muscle is rich in myofibers expressing the slow type I myosin heavy chain isoform (green). Myofibers not expressing the slow type I myosin heavy chain isoform are shown in yellow. B. One satellite cell on a myofiber bundle. The schema of the skeletal muscle was modified from (Chargé and Rudnicki, 2004). The picture of the satellite cell placed on a myofiber was given by Laurent Bernheim.

The satellite cells are defined by their characteristic position surrounding individual fibers (hence their name). They are located close to the myofibers, between the sarcolemma and the basal lamina. These quiescent cells do not express myogenic regulatory factors of the MyoD or MEF2 families or other known markers of terminal differentiation. Satellite cells can be found on all muscles. Nonetheless, higher numbers of satellite cells are found surrounding the slow muscle fibers if they are compared with the fast fibers within the same muscle. Ageing is associated with decreases in satellite cell numbers in rodents, but not in humans (Jejurikar and Kuzon, 2003).

1.6.1 Fusion of myoblasts

Satellite cells can be activated for post-natal growth, for repair of skeletal muscle, or in reaction to hypertrophic stimuli. In such circumstances, these cells will proliferate, will express the myogenic markers MyoD and/or Myf5 and can be termed myoblasts (Figure 38) (Black and Olson, 1998; Chargé and Rudnicki, 2004). Myogenic cells differentiate and can fuse to existing damaged fibers for repair. This has been demonstrating by radiolabeling experiments of satellite cells. Once activated, satellite cells become myoblasts, which can fuse to one another to form new myofibers. This process is very similar as muscular embryogenesis. The fusion event is a process that follows an ordered set of cellular events: recognition, adhesion, alignment, and membrane union. Then, myoblast fusion is a highly ordered process. This is to avoid that myoblasts do not fuse without proper activation, but also

to avoir that myoblasts would fuse with any cell type, wich would be disastrous for an organism. This is why if myoblasts are able to fuse between them to form multinucleated syncitia, they do not fuse with other cell types (Okazaki and Holtzer, 1965).

Different molecules regulate mammalian myoblast fusion. They are membrane proteins, extracellular secreted factors, or intracellular proteins (Figure 40). Until now, the events of fusion have been mainly studied *in vitro*, and relatively little is known about the process of myoblast fusion *in vivo*. This is largely due to the difficulties encountered when studying fusion in animals.

Recent experiments suggest that Kir2.1 channel activation is a required key early event that initiates myogenesis by turning on myogenin and MEF2 transcription factors via a hyperpolarization-activated Ca^{2+}-dependent pathway (Konig et al., 2004).

Calcium, calmodulin and $PGF_{2\alpha}$ act upstream of NFAT to induce skeletal muscle growth (Horsley and Pavlath, 2004). IL-4 is a crucial factor in muscle growth and controls the fusion of myoblasts with nascent myotubes in a pathway downstream of NFATC2 (Horsley et al., 2003).

Cell surface proteins have also been identified to be important for myoblast fusion. Particularly, myoblast fusion can be inhibited when myoblasts are treated with trypsin (Knudsen and Horwitz, 1977). Monoclonal antibodies can also be raised against fusing myoblasts (Greve and Gottlieb, 1982). Further studies have identified integrins as important cell surface proteins for fusion. They may influence alignment or fusion of secondary myoblasts with primary myotube, as seen with antibodies raised against the integrin VLA-4 or its counterreceptor VCAM-1 that inhibit myoblast fusion (Rosen et al., 1992).

Other molecules have been implicated in fusion. They include GRP94, ADAM12, calpain and caveolin-3. However, their role still remains unclear (Horsley and Pavlath, 2004). Among them, calpain likely leads to destabilization of the plasma membrane before membrane fusion (Horsley and Pavlath, 2004).

Molecule	Proposed function/activity
Membrane proteins	
ADAM12 (meltrin α)	Metalloprotease
Caveolin-3	
VLA-4 (α4 integrin)	Alignment via interaction with VCAM-1
VCAM-1	Alignment via interaction with VLA-4
β1 integrin	Extracellular matrix receptor
Inward rectifying K^+ channels	Induction of intracellular Ca^{++} elevations
T-type Ca^{++} channels	Induction of Ca^{++} influx
GRP94	Interactions on the cell surface
Extracellular/secreted factors	
Ca^{++}	Regulation of extracellular protein activity
Cathepsin B	Protease
IL-4	Recruitement of myoblast fusion
PGE1	
PGF2α	Activation of NFATC2 pathway
Intracellular proteins	
Calmodulin	Cytoskelettal rearrangements, activation of signaling pathways
Calpain	Protease destabilisation of sarcolemma
FKHR	Regulation of gene expression
Calpastatin	Inhibition of calpain
NFATC2	Regulation of gene expression in nascent myotube

Figure 40. Molecules that regulate mammalian myoblast fusion. FKHR: Forkhead in human rhabdomyosarcoma, VCAM: vascular cell adhesion molecule, VLA: very late antigen. Figure taken from (Horsley and Pavlath, 2004).

The early events differentiation events lead to activation of myoblasts transcription factors myogenin and MEF2, and myoblast terminal differentiation is characterized by upregulation of MRF4 (Figure 41) (Black and Olson, 1998; Chargé and Rudnicki, 2004; Cornelison and Wold, 1997).

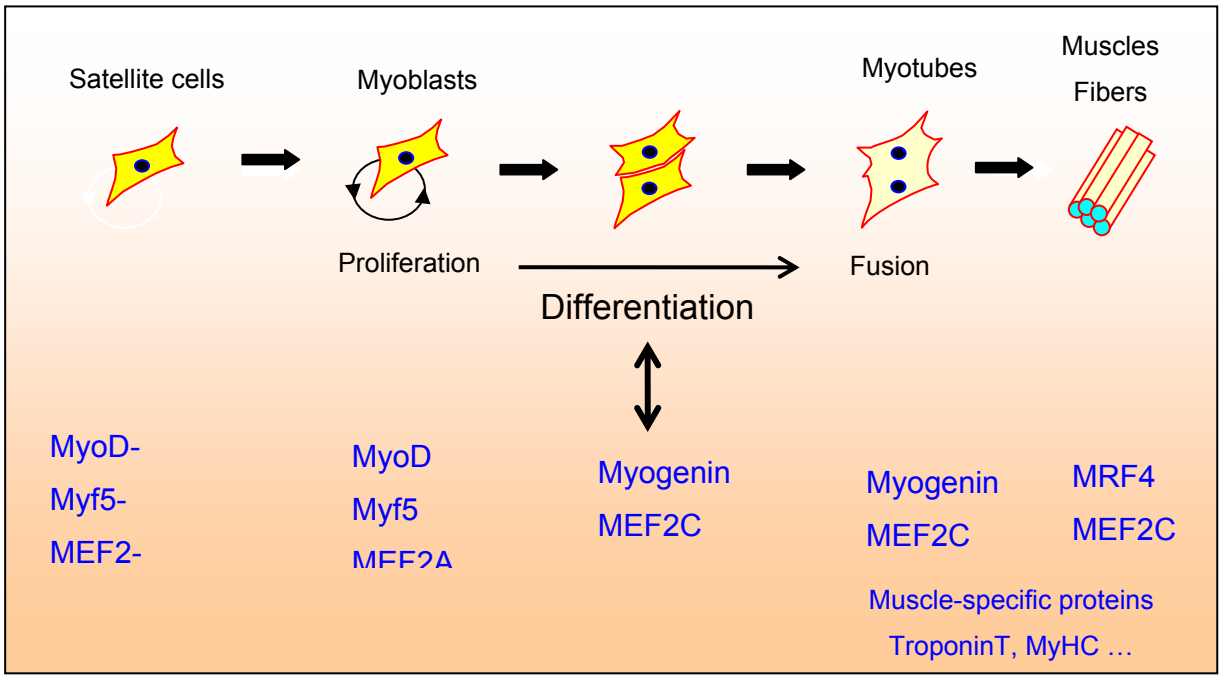

Figure 41. Myoblast differentiation. Activated satellite cells are called myoblasts. They express MEF2A, and MyoD and/or Myf5. Differentiation and fusion leads to MEF2C and myogenin expression. Finally, muscle fibers express MRF4 and MEF2C, but also the muscle-specific proteins, such as Troponin T and MyHC. Figure elaborated with (Black and Olson, 1998; Chargé and Rudnicki, 2004; Cornelison and Wold, 1997).

1.6.2 Satellite cell markers and heterogeneity

Satellite cells are not only defined by their specific location on the myofibers. They also possess small heterochromatic nuclei compared to those of adjacent myofibers, and different markers, whose expression may vary during activation of the cell (Figure 42).

Molecular Markers	Satellite Cell Expression		Experimental Protocol
	Quiescent	Proliferating	
Cell surface			
M-cadherin	+/-	+	In vivo/in vitro
Syndecan-3	+	+	In vivo/in vitro
Syndecan-4	+	+	In vivo/in vitro
c-*met*	+	+	In vivo/in vitro
VCAM-1	+	+	In vivo
NCAM	+	+	In vivo
Glycoprotein Leu-19	+	+	In vivo/in vitro
CD34	+/-	+/-	In vitro
Cytoskeletal			
Desmin	-	+	In vivo/in vitro
Transcription factors			
Pax7	+	+	In vivo/in vitro
Myf5	+/-	+	In vivo/in vitro
MyoD	-	+	In vivo/in vitro
MNF	+	+	In vivo/in vitro
MSTN	+	-/+	In vitro/in vivo
IRF-2	+	+	In vivo
Msx1	+	-	In vitro

Figure 42. satellite cell markers. MSTN, myostatin; VCAM-1, vascular cell adhesion molecule-1; NCAM, neural cell adhesion molecule; MNF, myocyte nuclear factor; IRF-2, interferon regulatory factor-2. Figure taken from (Chargé and Rudnicki, 2004).

Analysis of satellite cell markers is complicated by the fact that these markers may vary during different phases of cell activation, and from cell to cell. Indeed, single-cell analyses of myogenic markers in satellite cells by multiplex RT-PCR have shown heterogeneities in this population (Cornelison and Wold, 1997). In this study, during the early time point of satellite cell activation, it was shown that either MyoD or myf5 were expressed. Then, myoblasts expressed both myf-5 and MyoD simultaneously. After cell cycle arrest, myogenin was expressed with MyoD and myf5. Ultimately, many cells expressed all four MRFs simultaneously (Figure 43). It has been suggested that MyoD and myogenin may be located in fast-twitch and slow-twitch adult myofibers, respectively, but a RT-PCR study on these fibers was not able to show such biases (Cornelison and Wold, 1997; Hawke and Garry, 2001).

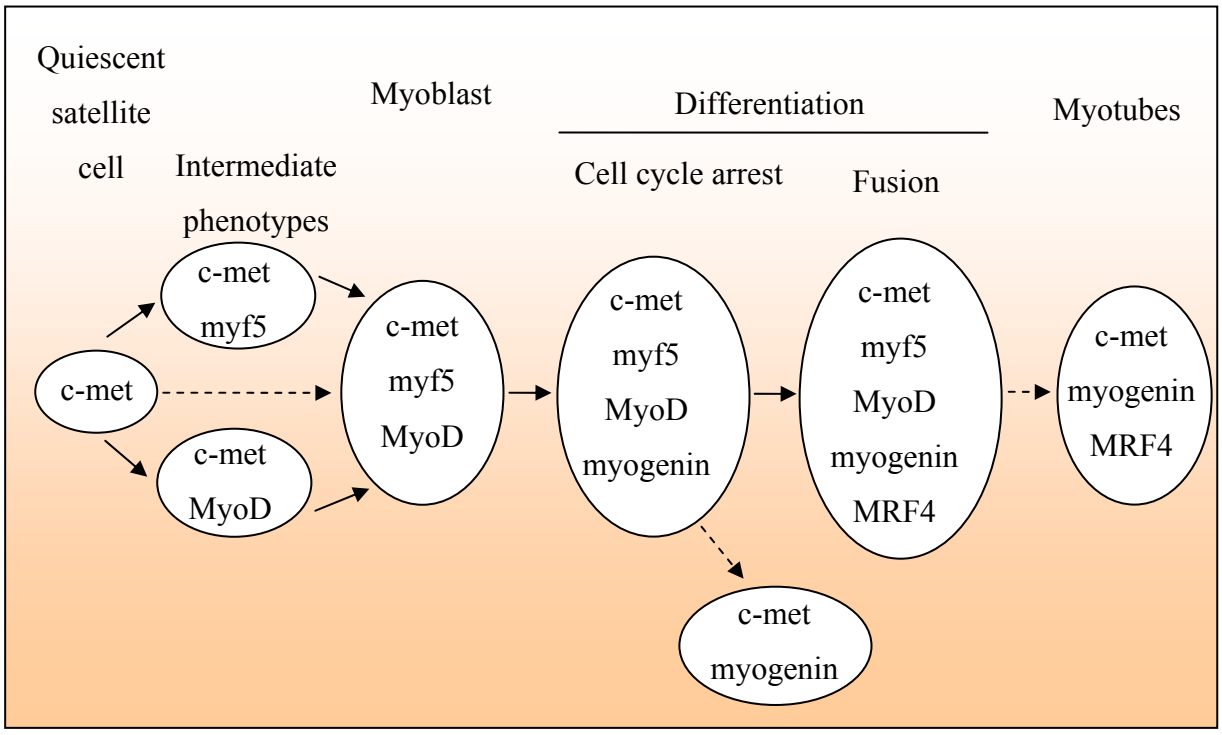

Figure 43. Model of MRF coexpression during satellite cell activation. Figure redrawn from (Cornelison and Wold, 1997).

As seen with their differential expression of markers, different populations of satellite cells may exist. Differences in satellite cell population may also stem from their differential origin.

Discussions exist to know if satellite cells may derive from somitic or from endothelial cells (Chargé and Rudnicki, 2004). It has also been suggested that satellite cells might also derive in some circumstances from mesoangioblasts, which are vessel-associated stem cells, from neural stem cells, and from bone marrow cells (Chen and Goldhamer, 2003). Even in uninjured skeletal muscle, some satellite cells express the hematopoietic marker CD45. This specific subset of satellite cells have been shown to be poorly myogenic compared to CD45-negative cells. In the contrary, the CD45-positive subpopulation of cells has been shown to contribute to neo-vascularization in regenerating muscle, and the CD45-negative population does not seem to have this property (Chen and Goldhamer, 2003).

Some experiments also suggest that some myotubes might dedifferentiate to become satellite cells when placed in special circumstances. At least, it has been shown that terminally differentiated myotubes from C2C12 cells are capable to dedifferentiate and to form myoblasts when exposed to the urodele homeobox-containing transcriptional repressor Msx1 (Odelberg et al., 2000).

A subpopulation of satellite cells, termed side population (SP) cells can be identified using the DNA-binding dye Hoechst 33342 and dual-wavelength flow cytometric analysis (Hawke and Garry, 2001). These cells have the differential ability to export the Hoechst dye, and can adopt alternative fates in permissive environments. Only 100 SP cells are sufficient to reconstitute the entire bone marrow of a lethally irradiated mouse (Jackson et al., 1999). SP cells can also be isolated from the bone marrow of a normal mouse, and transplanted into an irradiated mdx mouse bone marrow. In such experiments, these cells have been shown to be able to reconstitute the bone marrow of mdx mice, but they could also be recruited from the bone marrow and participate in muscle repair (Gussoni et al., 1999). Some satellite cells can also differentiate into hematopoietic cells, adipocytes, chondrocytes, cardiomyocytes, and smooth muscle cells (Peng and Huard, 2004).

It is not only SP cells from the muscle that can contribute to the bone marrow reconstitution. Some experiments also suggest that bone marrow cells can also transdifferentiate to muscle cells, albeit at a low rate. In the murine bone marrow side population (mSP), most cells (92%) express Sca-1, whereas only 16% express CD45 consisting of both Sca-1+ (9.2%) and Sca-1– (6.8%). Both CD45+ and CD45– mSP cells can undergo myogenic conversion when

cocultured with myoblasts, but the myogenic potential of CD45- muscle-derived cells is higher after intramuscular injection (McKinney-Freeman et al., 2002).

However, even though circulating stem cells can regenerate skeletal muscle, some evidences indicate that resident stem cells are the cells primarily responsible for the regeneration or repair of local tissues (Polesskaya et al., 2003).

The satellite cell population is not only heterogenous. It has also been shown that satellite cells follow asymmetric division in vitro. For instance, Numb, a plasma membrane-associated cytoplasmic protein, is asymmetrically segregated within dividing satellite cells (Conboy and Rando, 2002). The asymmetric division of satellite cells has been mainly shown *in vitro* but it may be also important *in vivo* to ensure that a significant number of muscle stem cells remain on myofibers, even after some satellite cells have fused for the repair of muscles (Baroffio et al., 1996). Despite these properties, there is a decrease in satellite cell numbers in rodents with ageing. In the contrary, human satellite celle numbers do not decrease with age (Jejurikar and Kuzon, 2003).

1.6.3 Factors modulating satellite cell activity

Different factors can modulate satellite cell activity. These factors have been extensively studied either alone or in combination, with satellite cells isolated from animals. For this purpose, satellite cells have often been isoltated from young animals, because satellite cells are about 6 times more abundant in young animals (Hawke and Garry, 2001). However, these studies are to be put in the perspective that older satellite cells do not react as younger ones (Tatsumi et al., 1998). These factors can be grouped into two main subcategories: the growth-factors molecules and the cytokines. The growth-factors molecules include FGF, HGF, IGF-I, IGF-II, TGF-β, $PDGF^{AA/AB}$, $PDGF^{BB}$ and EGF. The cytokines are IL-6 and LIF. All these molecules often have chemotactic properties, and positive effects on proliferation, but their effect on differentiation may vary (Figure 44). However, this list of factors playing a role in satellite cell activity is not exhaustive. For instance, testosterone and nitric oxide (NO) may

also play a role in satellite cell activity, as well as crushed muscle or platelet-derived extract (Hawke and Garry, 2001).

Factors affecting satellite cell activity

Growth factor	Chemotactic Ability	Proliferation	Differentiation
FGF	↑/NE	↑	↓
HGF	↑	↑	↓
IGF-I		↑	↑
IGF-II		↑	
TGF-β	↑	↓/↑	↓/↑
$PDGF^{AA/AB}$	NE	NE	
$PDGF^{BB}$	↑/NE	↑	
EGF	NE	↑*	
LIF	↑	↑/NE	
IL-6		↑	

Figure 44. Factors affecting satellite cell activity. A number of factors affect satellite cell proliferation, differentiation, and chemotaxis. NE, no effect. FBF, HGF, IGF, TGF, PDGF, and EGF, fibroblast, hepatocyte, insulin-like, transforming, platelet-derived, and endothelial growth factor, respectively. LIF, leukemia-inhibitory factor. * Stimulation of satellite cell proliferation in the presence of serum but no effect in serum-free medium. ↑ and ↓, Increase or decrease in effect, respectively. Figure taken from (Hawke and Garry, 2001).

In vivo, it is thought that the activity of a satellite cell can be modulated in response to myotraumatic events, but also by the vasculature, the immune responses, the motor neurons, the surrounding satellite cells, and by growth factors, cytokines, hormones and autocrine factors (Figure 45).

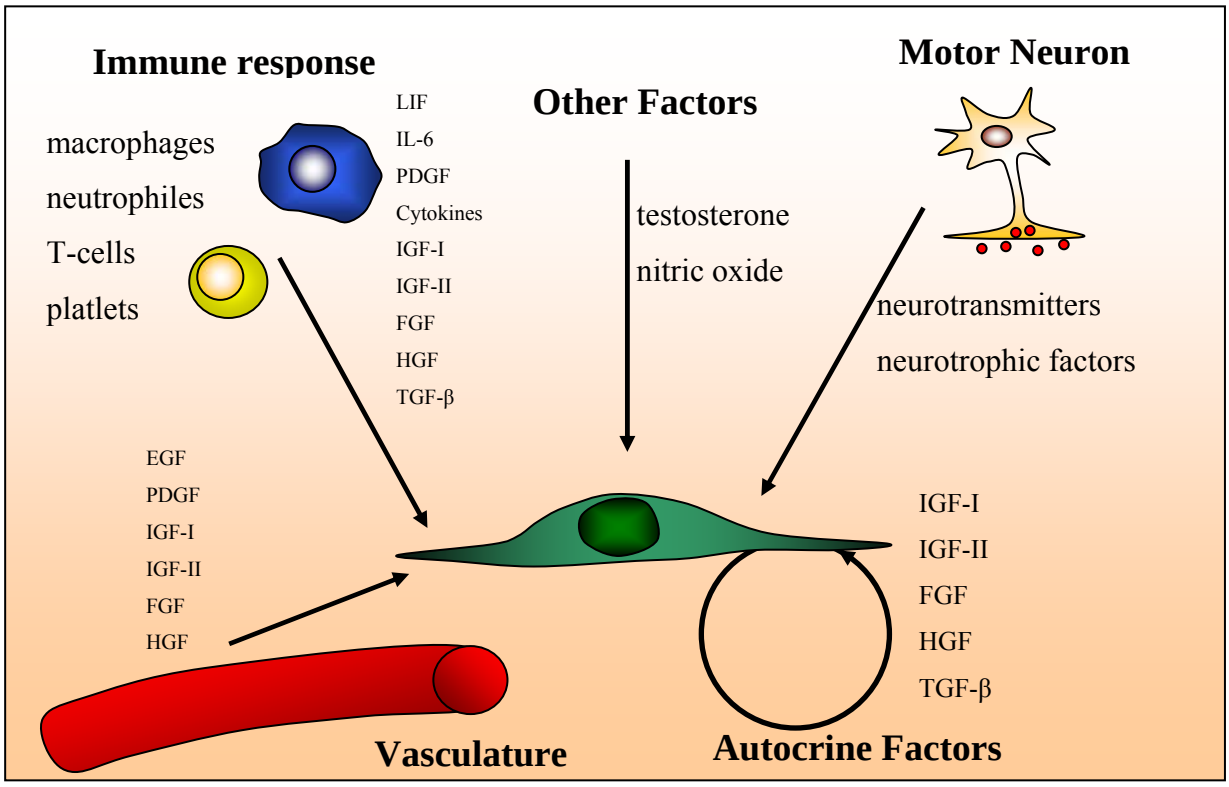

Figure 45. Extracellular factors affecting satellite cell activity. Figure redrawn from (Hawke and Garry, 2001).

1.6.4 Muscle regeneration

A large variety of mouse strains exist to study muscle regeneration. However, regeneration differs between the varying mice strains that can be used (Figure 46). Even though these differences are not well understood, it seems that the genetic background may play a role. The differential expression of Pax7 isoforms, MyoD and FGF have been particularly correlated with efficiency of repair (Grounds, 1987; Grounds and McGeachie, 1989; Irintchev and Wernig, 1987; Mitchell et al., 1992; Roberts et al., 1997). However, targeted mutations of additional muscle genes have also been shown to be able to affect muscle regeneration in mice (Figure 46). They are reviewed elsewhere (Chargé and Rudnicki, 2004). One specific mutation is more interesting for us; it is mutations in the Dystrophin gene, causing Duchenne

Muscular Dystrophies in humans. We are going now to talk more in detail about this disease, among the other muscular dystrophies.

Targeted Mutation	Adult Muscle Phenotype	Muscle Regeneration Phenotype	Satellite Cell Phenotype (In Vitro)
	Transcription factors		
MyoD$^{-/-}$	Minor alterations	Impaired	Increased proliferation Delayed differentiation
Pax7$^{-/-}$	Growth deficit, satellite cells absent	Deficient	No satellite cell
Slug$^{-/-}$	Fairly normal	Impaired	ND
MNF$^{-/-}$	Growth deficit	Impaired	Decreased proliferation Normal differentiation
	DGC components		
MCK (Cre)-dystroglycan (LoxP)	Increased degeneration without fibrosis or fat replacement, muscle mass increase	Efficient	ND
Dystrophin$^{-/-}$ (*mdx*)	Increased degeneration with fibrosis and fat replacement	Impaired in older mice	Normal proliferation
-Sarcoglycan$^{-/-}$	Increased degeneration with fibrosis and fat replacement	Impaired in older mice	ND
	Growth factors		
FGF-6$^{-/-}$	Fairly normal	Normal or Impaired	ND
LIF$^{-/-}$	Normal	Impaired	ND
MSTN$^{-/-}$	Muscle mass increase (hyperplasia and hypertrophy)	Improved	ND
	Others		
M-cadherin$^{-/-}$	Normal	Normal	ND
Desmin$^{-/-}$	Some degeneration	Delayed	ND

Figure 46. Targeted germline mutations in mice affecting muscle degeneration/regeneration process. MNF, myocyte nuclear factor; MCK, muscle creatine kinase; FGF, fibroblast growth factor; LIF, leukemia inhibitory factor; MSTN, myostatin; ND, not determined. Figure taken from (Chargé and Rudnicki, 2004).

1.7 Muscular dystrophies

1.7.1 General definition of muscular dystrophies

Muscular dystrophy describes a group of severe and progressive muscle-wasting diseases of genetic origin. These diseases are classified clinically in six groups by the distributions of muscle weaknesses (Figure 47) (Emery, 2002). However, similar locations of muscle weaknesses do not always reflect the same gene being affected. Gene mapping experiments have led to the identification of a number of genes being implicated in a particular clinically-defined muscle dystrophy. Although clinically similar diseases may be found, the mutated genes that are associated with these diseases may be located on different chromosomes (Figure 48) (Dalkilic and Kunkel, 2003; Emery, 2000). Particularly, limb-girdle muscular dystrophy is extremely heterogeneous in the genes that can be implicated (Laval and Bushby, 2004).

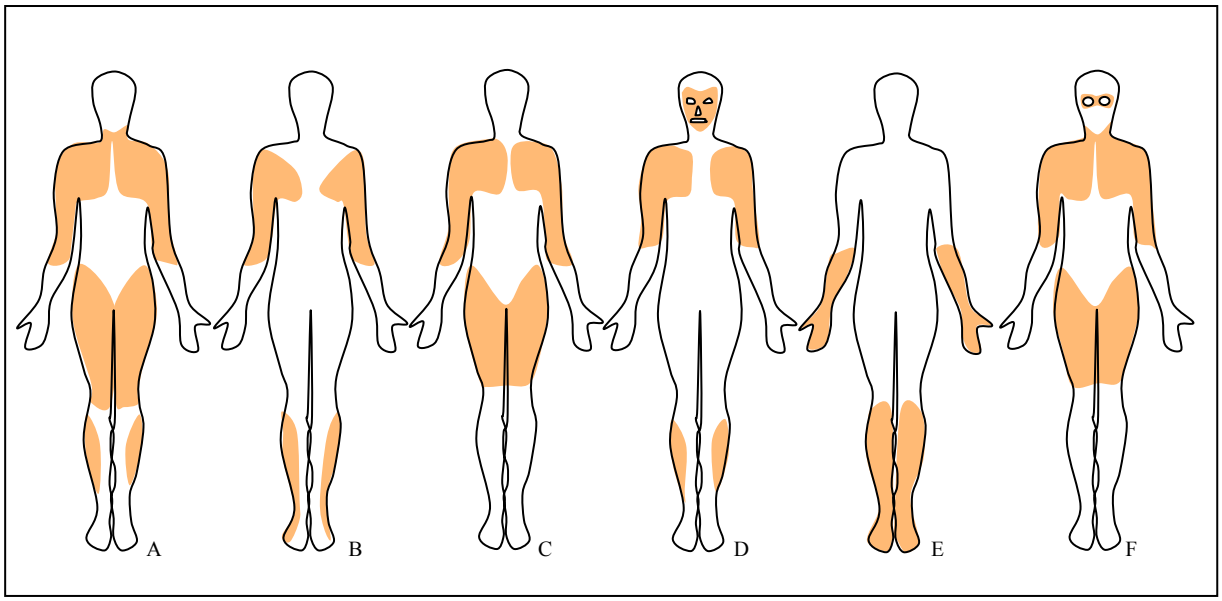

Figure 47. Distribution of predominant muscle weakness in different types of dystrophy, A, Duchenne-type and Becker-type; B, Emery-Dreifuss; C, limb-girdle; D, facioscapulohumeral; E, distal, F, oculopharyngeal. Shaded=affected areas. (Reproduced from *BMJ* 1998; **317:** 991–95).

Taken together, the muscular dystrophies are mapped until now to 29 different loci and they can give rise to 34 distinct disorders. Their mode of inheritance can also differ: they can be recessively X-linked, autosomal dominant, or autosomal recessive (Figure 48) (Dalkilic and Kunkel, 2003; Emery, 2000).

Disease	*Mode of inheritance*	*Gene locus*	*Gene product*
Duchenne/Becker muscular dystrophy	XR	Xp21	Dystrophin
Emery-Dreifuss muscular dystrophy	XR	Xq28	Emerin
Emery-Dreifuss muscular dystrophy	AD/AR	1q11	Lamin A/C
Limb-girdle muscular dystrophy			
LGMD 1A	AD	5q22–q34	Myotilin
LGMD 1B	AD	1q11–21	Lamin A/C
LGMD 1C	AD	3p25	Caveolin-3
LGMD 1D	AD	6q22	
LGMD 1E	AD	7q	
LGMD 2A	AR	15q15	Calpain-3
LGMD 2B	AR	2p13	Dysferlin
LGMD 2C	AR	13q12	γ-Sarcoglycan
LGMD 2D	AR	17q12–q21	α-Sarcoglycan
LGMD 2E	AR	4q12	β-Sarcoglycan
LGMD 2F	AR	5q33–q34	δ-Sarcoglycan
LGMD 2G	AR	17q11–q12	Telethonin (TCAP)
LGMD 2H	AR	9q3–q34	TRIM32
LGMD 2I	AR	19q13.3	Fukutin-related protein
LGMD 2J	AR	2q	Titin
Congenital muscular dystrophy (CMD)			
CMD 1A	AR	6q22	Lamimin α2
CMD 1B	AR	1q	
CMD 1C	AR	19q	Fukutin related protein
Fukuyama CMD	AR	9q31–q33	Fukutin
α7 integrin congenital myopathy	AR	12q13 a7	Integrin
Muscle-eye–brain (MEB) disease	AR	1p32–p34	O-mannose β -1,2-N-acetyl-glucosaminyl transferase
Walker-Warburg syndrome	AR	9q	O-mannosyltransferase 1
Rigid spine CMD	AR	1p35–36	Selenoprotein N1
Bethlem myopathy/Ullrich syndrome	AD	21q22	Collagen VI α1
	AD	21q22	Collagen VI α2
	AD	2q37	Collagen VI α3
Distal muscular dystrophy			
Miyoshi myopathy	AR	2p13	Dysferlin
Tibial MD	AD	2q	Titin
	AD	14q	
Facioscapulohumeral muscular dystrophy	AD	4q35	
Oculopharyngeal muscular dystrophy	AD	14q11.2–q13	Poly A binding protein 2

Figure 48. Muscular dystrophies and gene locations.*AD, autosomal dominant; AR, autosomal recessive; LGMD, limb-girdle musclar dystrophy; XD, X-linked dominant; XR, X-linked recessive. *This table includes disorders which have been mapped to a chromosome. Figure modified from (Dalkilic and Kunkel, 2003; Emery, 2000).

Despite all the heterogeneities mentioned, most of these diseases seem to have one common determinant: they seem to be caused by loss of proteins involved in the structure of muscles. These structural proteins can be part of the linkage between the extracellular matrix and the actin cytoskeleton, or they can be located in the sarcomeric and in the nuclear structures (Figure 49) (Dalkilic and Kunkel, 2003; Emery, 2000). These structures may also be connected between them (Blake and Martin-Rendon, 2002).

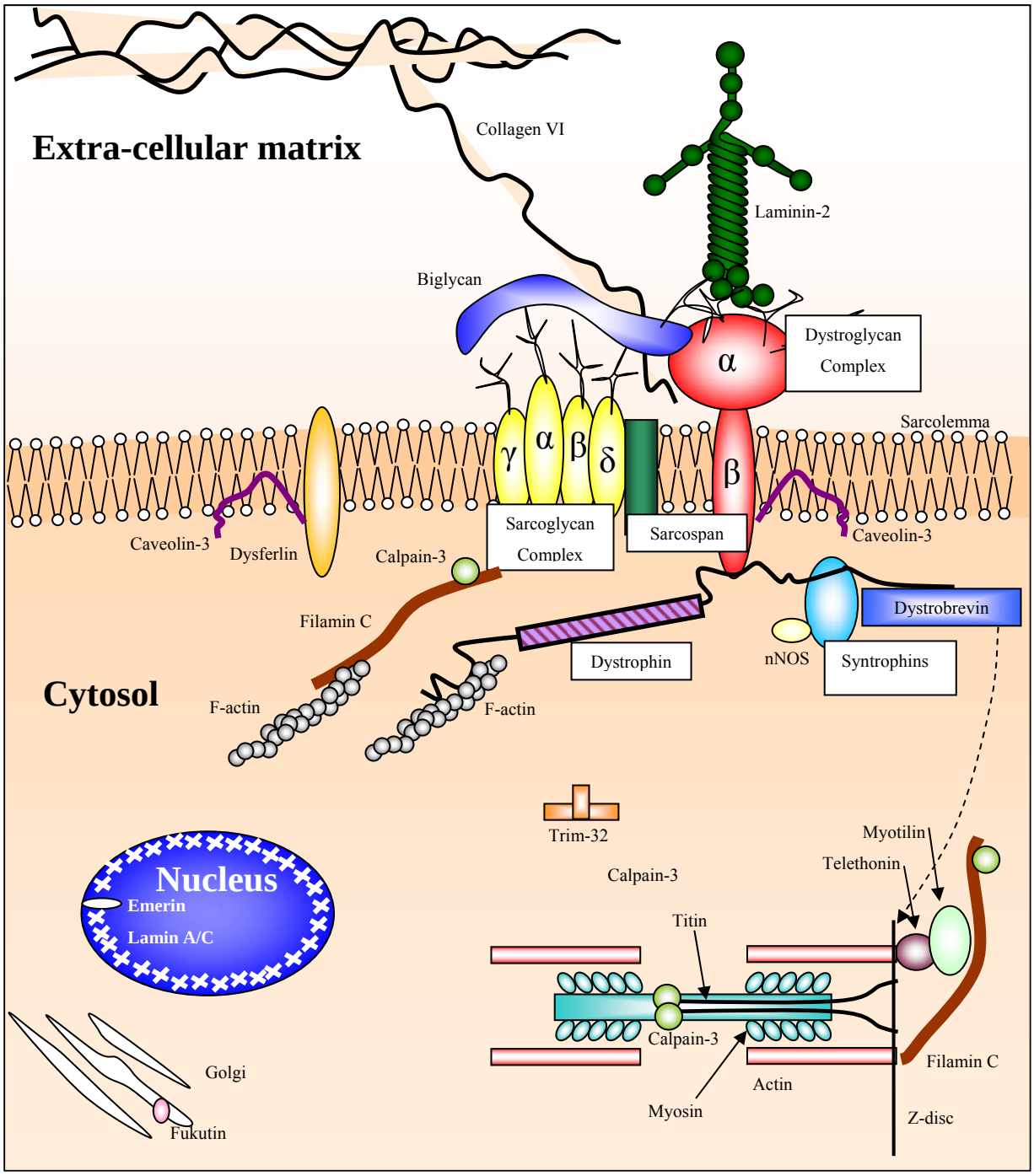

Figure 49. Schematic view of the location of the proteins currently known to be involved in muscular dystrophies. Figure inspired from (Dalkilic and Kunkel, 2003; Emery, 2000).

We have mentioned the existence of a large number of muscular dystrophies discovered until now, but we will not talk extensively about all them. They are reviewed elsewhere (Dalkilic and Kunkel, 2003; Emery, 2000). We are going now to talk more in detail about one of these dystrophies, Duchenne muscular dystrophy, its animal models, and the therapeutic strategies currently aimed at the correction or at the improvement of the clinical features observed in this disease.

1.7.2. Duchenne Muscular Dystrophy (DMD)

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disorder affecting 1 in 3.500 live males at birth. About a third of cases occur as de novo mutations, in the dystrophin gene. Dystrophin is encoded by a 2.5 Mbp gene, which represents 0.1% of the genome, but only about 1% of this gene codes for the 86 exons of the Dystrophin protein (Muntoni et al., 2003). The fully processed transcript is only 14 kbp, and produces a 427 kDa protein, composed of 3685 amino acid residues. The large size of this gene contributes significantly to its high rates of mutations. Most *dys* mutations in DMD patients are large deletions or insertions that result in downstream codon reading frameshifts (58%) or small frameshift rearragements or point mutations (41%), and the remaining mutations are the results of duplication events. DMD is usually diagnosed at 4-5 years of age, on the basis of gait abnormalities. DMD patients experience severe and progressive loss of muscle mass and function. Typically, wheelchair use is necessary by the age of 8-10. When they are teenagers, patients are wheelchair bound. In the late teens or early twenties, the DMD patients usually die, because muscle degeneration occurs to such an extent that it finally leads to respiratory or cardiac failure. Nevertheless, with some specific mutations, some patients can have milder phenotypes of disease and can live longer. Then, these patients are often referred as to suffer from the milder Becker muscular dystrophy (BMD) (Muntoni et al., 2003). Mutations in the dystrophin can also cause another disease. It consists of the cardiomyopathy seen in DMD and in BMD, but without significant muscle disease. Then, this myopathy is referred to as X-linked dilated cardiomyopathy (XLDCM) (Lapidos et al., 2004).

1.7.3 Dystrophin

Dystrophin is a major component of the dystrophin-glycoprotein complex (DGC). Its function is to link the myofiber cytoskeleton to the extracellular matrix. Once this complex is disrupted, injury occurs during muscular contractions, and sarcolemmal damage finally leads to myofiber necrosis. Dystrophin has 4 domains: the N-terminal and parts of the helical-rod domain bind to actin whereas the C-terminal and a cysteine-rich domain distal to the rod

domain form complexes with syntrophin and beta-dystroglycan, respectively (Figure 50) (Dalkilic and Kunkel, 2003; Emery, 2000). In myogenic cells, Dystrophin provides a crucial structural link between the extracellular matrix and the intracellular cytoskeleton. Dystrophin mutations occur in DMD and BMD, and cause in both diseases sarcolemmal instability, which leads to destabilization of the sarcolemmal dystrophin-associated complex, but a wide range of severity phenotypes are possible both in DMD and BMD. The genetic mutations that cause DMD or BMD are not fully understood, but it seems that the correct expression of the C-terminal part of dystrophin, and particularly the cysteine-rich domain (that binds β-dystroglycan), often allowed by exon skipping or by non frame-shift mutations, is often leading to a partially functional dystrophin protein and to milder symptoms (Muntoni et al., 2003; Tidball and Wehling-Henricks, 2004). Nevertheless, exceptions exist to this rule (Muntoni et al., 2003). Some rare dystrophin mutations can also cause neither DMD nor BMD, but an almost exclusive cardiac disease in X-linked dilated cardiomyopathy. This rare disorder is caused by a cluster of mutations towards the 5′ end of the gene, or by missense mutations that disrupt dystophin preferentially or exclusively in the cardiac muscle (Muntoni et al., 2003). Patients with mutations in the dystrophin gene have not only muscle problems, their mutations were also associated with lower IQ results (mean IQ of 82) and retina problems (reduction of the b-wave amplitude in the scotopic electroretinography) (Muntoni et al., 2003).

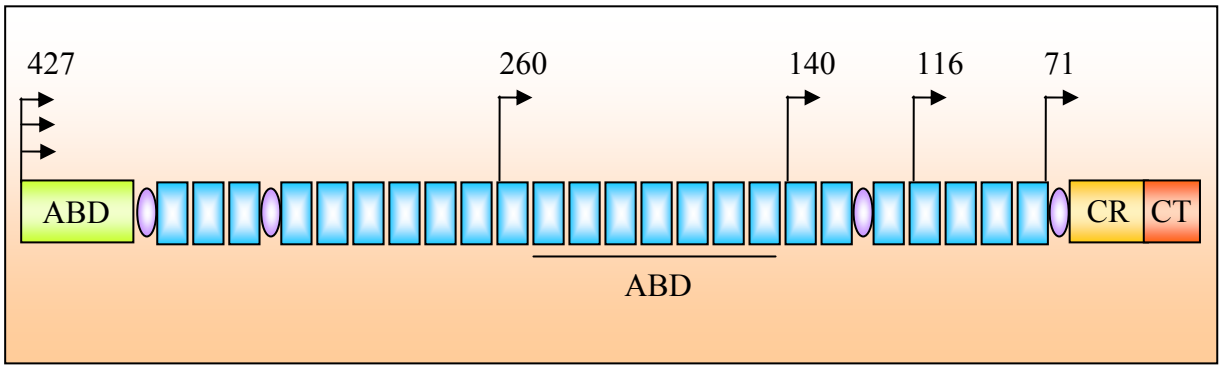

Figure 50. Schematic regions of *dystrophin* gene. 3 different promoters permit the expression of the 14-kb cDNA dystrophin in cardiac muscle, skeletal muscle, and the brain. This full-length 427 kDa form of Dystrophin includes a classical actin binding domain (ABD) at its N-terminus, and rod region composed of 24 spectrin repeats (blue square). The rod regions are

separated by 4 regions (ovals) to create flexible hinge regions in the rod. A second actin binding domain has been characterized in the rod region in spectrin repeats 11 through 17. The carboxy-terminus of dystrophin includes a cysteine-rich (CR) and carboxy-terminal (CT) domains that interact with the remainder of the DGC. Thre is existence of 4 promoters within the *dystophin* gene, which drive the expression of dystrophin short forms. These forms are devoid of the actin binding domain, and are differentially expressed. Dp260 is highly expressed in the retina, whereas Dp140 are expressed in the nervous system, and Dp71 is widely expressed in nearly all cell types.

1.7.4 Dystrophin deficiency and muscle degeneration

Skeletal muscle regeneration in adults is thought to occur thank to the myogenic satellite cells that are located in close association with mature muscle fibers. Under normal circumstances, satellite cells are quiescent, but in response to stress, they are activated, prompting multiple rounds of proliferation. Some satellite cells fuse with one another to form myotubes, whereas others serve as bridges between myofibers, aiding in their regeneration (Chargé and Rudnicki, 2004). In normal human muscles, the number of quiescent satellite cells essentially remains stable within adults over multiple cycles of injury and regeneration. However, the absolute number of satellite cells can decrease in case of pathologic situations, such as with Duchenne muscular dystrophy and with chronic muscle denervation, but also at the onset of advanced age or after atrophy secondary to deconditioning or immobilization. In dystrophic muscles, loss of dystrophin results in a mechanically weaker membrane that is more easily damaged during muscle contraction. In such a situation, there is probably an extensive satellite cell activation to repair damaged muscle, as seen with the rate of telomere loss in DMD patients that is 14 times greater than that observed in controls. Consequently, the proliferative potential of satellite cells decrease with age. For instance, it has been shown that a 9-year-old dystrophic patient has a satellite cell proliferative potential of one-third of age-matched controls (Jejurikar and Kuzon, 2003). After different cycles of degeneration-regeneration, regeneration gradually fails. This leads to degeneration of skeletal muscle fibers and connective tissue replacement. Degeneration is probably due in part to satellite cell depletion

and their impaired proliferation, which lowers their ability to form new myofibers, or repair existing ones. As a result, in the individual, skeletal muscle contraction significantly worsens. It is currently not fully understood why there is satellite cell depletion and why they have diminished proliferation potential. However, it is assumed that senescence and apoptosis may play a role to explain this fact. (Jejurikar and Kuzon, 2003). Particularly, the host environment within dystrophic skeletal muscle may contribute to the impaired proliferative potential of satellite cells. In dystrophic patients, IGF may not be available to satellite cells because fibroblasts release increasing amounts of IGF binding proteins, and this may lead to senescence. Moreover, apoptosis may play a role in diminishing satellite cell numbers because DNA fragmentation has been seen in *mdx* mice, before senescence is observed (Jejurikar and Kuzon, 2003).

1.7.5 Therapeutic strategies for DMD

Gene correction of DMD muscles is probably a method of choice to correct DMD, as well as transplatations of stem cells or muscle precursor cells, but these approaches currently raise several issues.

Gene correction can be done either by introducing a functional dystrophin gene into the muscles of DMD patients, or by correcting the existing gene. Dystrophin can be added into muscles by viraly derived vectors, but its large size (14 kb) has hampered the introduction of the full gene into small viral capsids. Large adenoviral capsids can be used, but then the vectors are too large to cross the extracellular matrix that surrounds mature myofibres (van Deutekom and van Ommen, 2003). To circumvent this problem, it is only recently that special viral constructs have been designed to carry the full dystrophin cDNA (Goncalves et al., 2005). Nevertheless, it has been shown that dystrophin size can be shrunk and can still lead to a functional protein (Scott et al., 2002). Such engineered proteins have been put into viral vectors and have been able to correct some of the *mdx*-associated phenotypes (Harper et al., 2002). Others have attempted to correct dystrophin by overexpressing utrophin, which is a shorter homolog. In fact, utrophin is 80% identical to dystrophin in its cysteine-rich and C-

terminal domains, and these domains are knowon to bind to the dystrophin associated protein complex (DAPC), including the dystroglycans and syntrophins (Perkins and Davies, 2002). Replacement of dystrophin by utrophin can be interesting, because it has the benefit of not eliciting an immune response against a foreign gene. In this respect, it is better to replace dystrophin deficiency by utrophin than to try to correct DMD by adjunction of dystrophin, because this method has the risk that Dystrophin would be recognized as a foreign protein by the immune system of the patient. Other approaches have been attempted to correct muscular dystrophies, such as oligonucleotide-mediated therapy (Rando, 2002). In this case, the dystrophin gene is corrected by chimeric RNA/DNA oligonucleotides ("chimeraplasts") that repair point mutations.

All these approaches are nonetheless problematic to use in patients, because it is difficult for a viral vector to target all the muscles that can be afflicted in DMD patients. For this reason, other approaches have been used. For instance, stem or precursor cells isolated from DMD patients can be corrected *ex vivo* and transplanted back into DMD patients (Gregorevic and Chamberlain, 2003). Nevertheless, also with such a method, it is not a small task to allow correction of all muscles associated with DMD disease. Indeed, it has been shown that cell transplantation experiments often led to poor survival of the myoblasts immediately post transplantation (van Deutekom and van Ommen, 2003).

The use of viral vectors either in *in vivo* or *ex vivo* approaches should consider the integration of the foreign DNA. Particularly, the integration site of the transgene should not induce bystanding harmful proto-oncogenes, and for that, careful choice of viral vector should be made (Check, 2005).

Considering all the potential problems that are still associated with gene therapy of DMD, until now, only conventional drug treatments are clinically done with DMD patients. Such treatments do not correct the cause of DMD, but they attempt only to correct some of the different negative effects associated with this disease, and they are often associated with severe side-effects, as it will be discussed now.

Anabolic steroids are one of classes of drugs that have been used in hope to have a beneficial effect in the muscle mass of DMD patients. Nevertheless, their use was found to produce only minimal improvements in muscle functions, and was accompanied by androgenic side-effects

(they include priapism, acne, and growth of pubic hair). However, more recent studies have identified oxandrolone as an anabolic steroid candidate for DMD treatment, because it may protect muscle mass with minor androgenic side-effects (Tidball and Wehling-Henricks, 2004).

Growth hormones (GH) were used in the objective to maintain muscle mass and function of DMD patients, but they often produced opposite catabolic effects. These results suggested that GH inhibitors may reduced pathology of DMD, but the clinical trials that have been done subsequently have produced only slight improvements in muscle function (Cittadini et al., 2003). Nonetheless, Insulin-like Growth Factor-I (IGF-I) could be a candidate to protect DMD muscles, because it has been shown to be a potent anabolic agent that can increase muscle mass (Gregorevic et al., 2002). In *mdx* mice, this agent was also shown to reduce exercise-induced weakness (De Luca et al., 2003). However, despite these advantages, IGF-1 should be used with caution, because it has been shown to increase the proliferation and metastasis of cancer cells (Chan et al., 1998).

Beneficial effects could be produced by leukemia inhibitory factor (LIF). LIF is a potent mitogen, especially for undifferentiated cells, such as embryonic stem cells, hematopoietic stem cells and germ cells, but also for muscle cells (Barnard et al., 1994). Consequently, LIF treatment has therapeutic potential, but before this treatment is applied, side-effects of this drug should be better known.

Inhibition of calcium-dependent proteases has been suggested after it was noted that there was an influx of extracellular calcium into dystrophin-deficient muscles. β_2-adrenergic agonists can increase calpastatin expression, which is an inhibitor of the calcium-dependent calpain proteases. These agonists were used in DMD boys and were shown to improve muscle function, but no significant difference was seen in muscle function tests, such as in the ability to climb stairs (Spencer et al., 1997).

The inhibition of the ubiquitin/proteasome system using MG-132 protease inhibitor has been shown to diminish pathology in mdx mice. This inhibitor has been shown to increase localization of the N-terminal portion of the Dystrophin protein at the membrane, by a mechanism that is still not completely understood (Bonuccelli et al., 2003).

Decreasing the inflammatory responses has also been attempted in DMD, because it has been shown that depletions of $CD4^+$ or $CD8^+$ T cells or macrophages significantly reduced the pathology in mdx mice (Spencer et al., 2001). Accordingly, immunosuppressants such as the glucocorticoid prednisone were shown to maintain muscle strength and function, and delayed disease progression (Yilmaz et al., 2004). However, prednisone was accompanied with side-effects such as weight gain, suppression of growth, bone demineralization, cushingoid appearance, hirsutism, and behavioral changes that often prompted cessation of treatment. Nondaily dosing may reduce these adverse side-effects. However, additional research is needed to demonstrate the mode of action of prednisone. Indeed, even though prednisone is known as an immunosupressive drug, its benefic action in DMD may not be related to immunosuppressive effects. This is suggested by the fact the immunosuppressant azathioprine has not been shown to have the similar effects on DMD patients (Griggs et al., 1993). However, it is true that azathioprine is not an immunosuppressant *per se*. In fact, it is a purine analog that inhibits DNA synthesis. Therefore, the beneficial mode of action of prednisone in DMD may still be related to immunosuppressive effects. Nevertheless, it is known that prednisone has additional effects beneficial effects on DMD muscles, including the modulations of calcium homeostasis (Metzinger et al., 1995), of protein metabolism (Rifai et al., 1995), and of cell survival (Sklar and Brown, 1991).

Chinese herbal medecine has a beneficial effect in DMD therapy, and dhis effet is probably glucorticoid-dependent (Courdier-Fruh et al., 2003). However, such medecine may be potentially harmful in some cases, particularly if the glucocorticoid content is not measured in different herbal preparations.

For the treatment of DMD, modulation of the immune system has also been attempted with TNF-α or with nitric oxide. The rationale to treat DMD patients with NO stems from the fact DMD muscle release neuronal-type NO synthase (nNOS), the protein responsible to synthesize NO in skeletal muscle (Brenman et al., 1995). However, even though normalization of nitric oxide (NO) production in mdx muscles had benefic effects (Wehling et al., 2001), this approach has the risk of adverse side-effects, because NO plays a role in different physiologic systems that are unrelated to its function in muscles.

Myostatin, a negative regulator of muscle mass, could be a possibility to try to cure DMD. Indeed, anti-myostatin injections have already been shown to produce desirable effects in mdx

muscles (Bogdanovich et al., 2002). Nevertheless, further experiments are required in this field before clinical trials are attempted in humans.

Consequently, currently, no efficient treatment (either by gene therapy by chemicals) exists for DMD, and there is still need of a lot of research on this disease and on putative therapies.

1.7.6 A dual high capacity hybrid Adeno/AAV vector

Recently, an interesting new vector has been designed for the transduction of dual expression units for the full-length form of dystrophin (Goncalves et al., 2005). It is a dual high capacity hybrid Adeno/AAV (hcAd/AAV) vector. This vector has the advantage to combine the capacity of AAV to integrate into the *AAVS1* locus, and the Adeno property to package large genes. It is constituted of the the Adeno capsids and packaging signal, but also of the AAV sequence required for integration in the *AAVS1* locus. A further addition in such vectors of the AAV ITR in the 3' of their sequence also allowed the formation of dual genomes with tail-to-tail organization (Goncalves et al., 2005).

The resulting vectors are constituted of the 5'Ad ITR, the 3'AAV ITR, and of the p5IEE (AAV integration efficiency element), which is necessary for integration in the *AAVS1* locus (Philpott et al., 2002a; Philpott et al., 2002b). All these elements can be located in a plasmid, and production of dual hcAd/AAV hybrid vectros can be done if this plasmid is transfected in producer cells, with a Helper Adeno virus (Ad.floxedψ). With two plox sites around the Adeno packaging signal ψ, the encapsidation of the helper Adenovirus Ad.floxedψ can be largely prevented, if their producer cells express Cre. In the contrary, dual Ad/AAV genomes can be packaged into Adenovirus capsids, because they contain the Adeno packaging signal ψ in their 5'AdenoITR.

The figure 51 summarizes the production of dual hcAd/AAV vectors, after transfection of Mss I-digested transgene plasmid in Cre-expressing cells, and after Helper Ad.floxedψ infection.

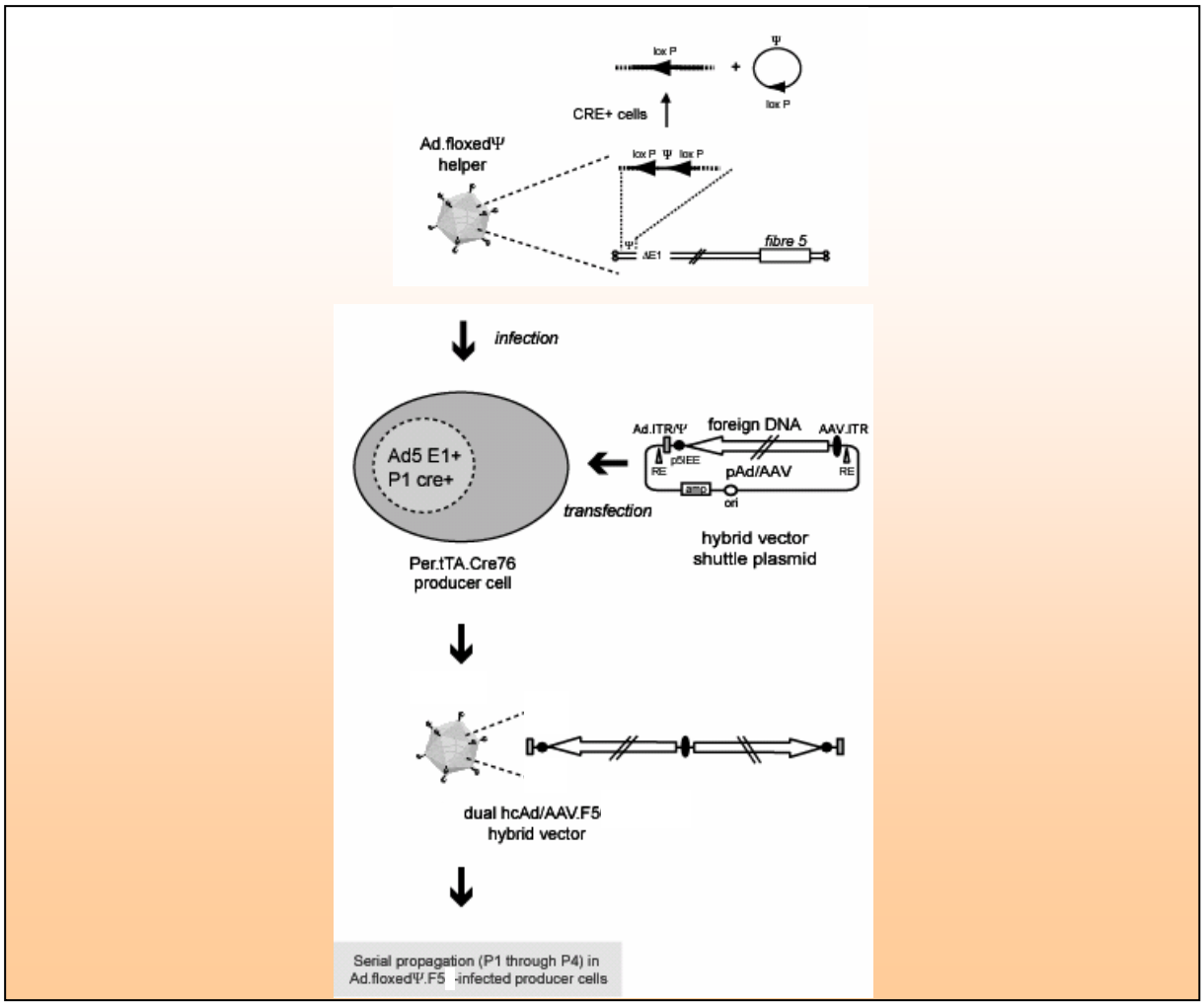

Figure 51. Production of dual hcAd/AAV. Figure adapted from manuscript of (Goncalves et al., 2005).

The resulting vectors can integrate in chromosome 19 if Rep proteins are expressed in transduced cells (Goncalves et al., 2005). For this purpose, the AAV Rep78 and Rep68 proteins are able to interact with the p5IEE elements of incoming vectors and with the *AAV1* sequence of the host, and finally trigger integration in the *AAV1* locus (Chiorini et al., 1994; McCarty et al., 1994).

Two dystrophin cDNAs have already been introduced with CAG promoter into Adeno capsids, in a so-called dual high capacity adenovirus (hcAd) vectors. This allowed the highest expression until now of the full-length dystrophin in *mdx* mice (Gilbert et al., 2003; Gilbert et al., 2002). This is why our collaborators also used the same method. Another interst in

making expression units composed of two dystrophin cDNAs is their hability to be packaged in a good manner. Indeed, the maximal size of packaging of Adeno genome is ~36 kb but packaging of small fragments of DNA are not efficient and lead to genomic rearrangement. Experiments have concluded that genomes with 75% of the Adeno size (27.7 kb) are efficiently packaged (Parks and Graham, 1997). As said earlier, such expression units have allowed the transduction results of Dystrophin expression in fibers up to now. In fact, it allowed transduction of up to 42% of fibers in the tibialis anterior of mdx neonatal mice (Gilbert et al., 2003).

The hcAd/AAV vectors package dual tail-to-tail expression units. This is done by using one of the properties of AAV replication, which can result in tail-to-tail tandems, and not head-to-head dimers (Figure 52).

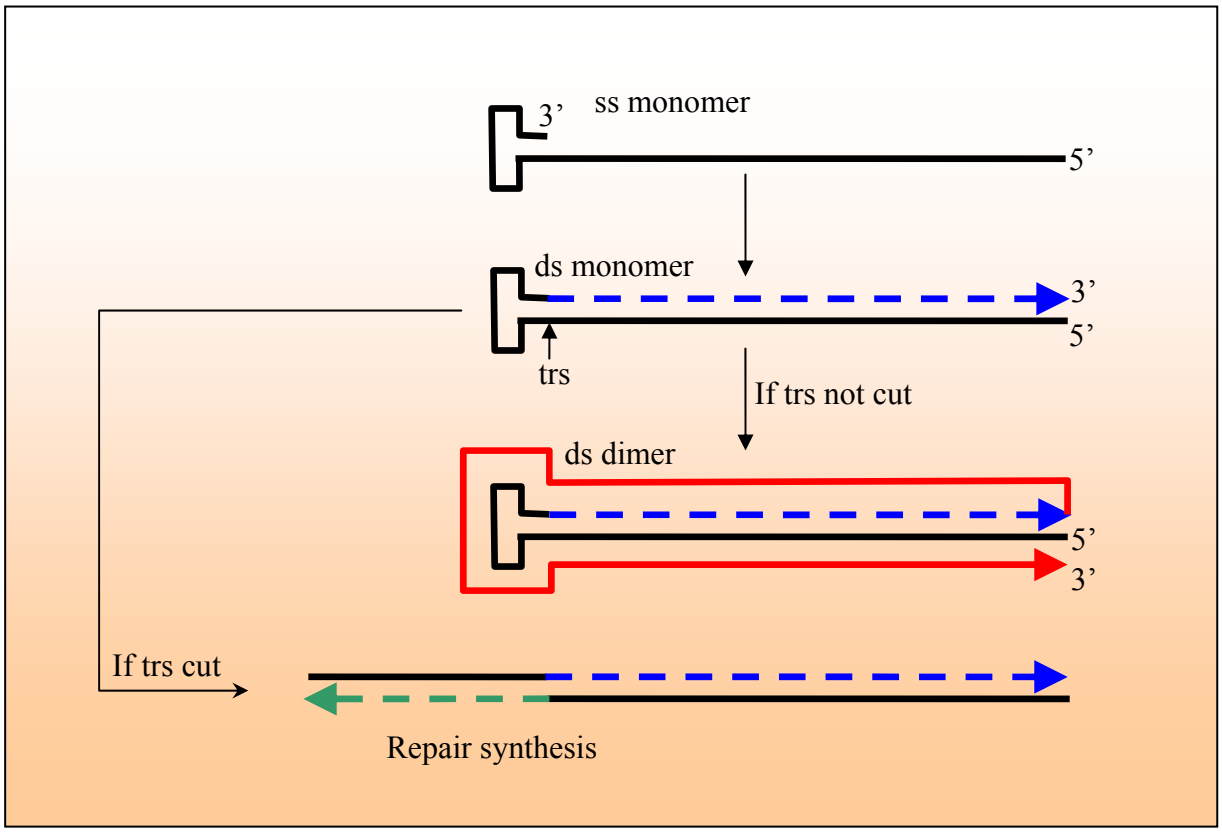

Figure 52. Model of AAV DNA replication. The 3'ITR of can form a hairpin structure and primes the synthesis of a complementary strand, leading to a ds monomer. If there is no cut at the terminal resolution site (trs), the 3'ITR AAV can once again form a hairpin structure, and prime synthesis, leading to a ds dimer, but also even to longer intermediates. Alternatively, if

is cut at the trs position, repair synthesis can occur, leading to a unit-length monomer duplex. Figure modified from (Hong et al., 1994).

The property of AAV to form long intermediates has been exploited in hcAd/AAV vectors, because they contain not only the 5' Ad ITR, but also the 3'AAV ITR. After transfrection of the shuttle plasmid, there is formation of dsmonomers. Then, two pathways have been postulated to lead to the formation of dual genomes in hcAd/AAV vectors (Figure 53).

The first pathway postulates that Holliday-like structures of AAV ITR are resolved by Rep78 and Rep68 or by cellular proteins to lead to dimer molecules that can be then propagated by replication from their Ad ITR.

The second pathway postulates that the 3'AAV ITR hairpin structure allows the priming for the formation of dimers, after strand displacement. Then, the 5' Ad ITR provides an origin of replication for the Ad DNA polymerase complex.

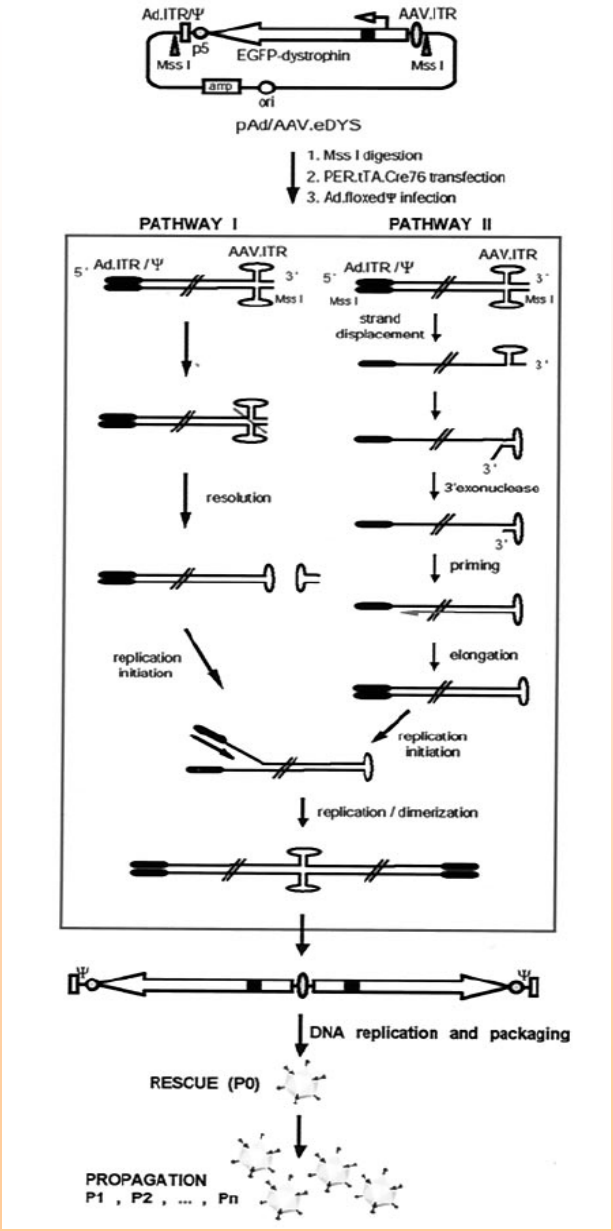

Figure 53. Tail-to-tail packaging of dual hcAd/AAV hybrid vectors. See text for details. Figure taken from (Goncalves et al., 2005).

1.7.7 Models of DMD, and their limitations

Different animal models of Duchenne Muscular Dystrophy already exist. Among them, there are notably mice models, dog models, feline models, but also non-mammalian models, such

as the zebrafish model and the C. elegans model. Despite the utility of animal models, the extent to which these models reproduce the DMD pathology seen in humans is variable.

The *mdx* mouse model of dystrophin deficiency is one of the most used models, because it has a small size and it is genetically and biochemically similar to human DMD. Nevertheless, in mice, null mutation of dystrophin causes a progressive pathology that begins at an old age, so that dystrophin-deficient mice live a nearly normal lifespan. On the contrary, in humans, null mutations produce a disease in early life, and evolution of pathology greatly reduces life expectancy.

The mild phenotype of dystrophin deficiency in mice has been attributed to expression in these animals of the homologous protein utrophin. For this reason, mice which are null for both dystrophin and utrophin (*mdx/utrn*-/-) have been generated. These mice have a more severe phenotype, comparable to DMD. Particularly, in contrast to *mdx*, *mdx/utrn*-/- mice exhibit growth retardation, weight loss, spinal curvature and joint contractures, weight loss, spinal curvature and joint contractures, early diaphragmatic pathology and premature death (Collins and Morgan, 2003). However, these *mdx/utrn*-/- mice are still not similar with DMD pathology seen in humans, mainly because of their size, and because the regulation of satellite cell number and activity is different in mice. For instance, rodent muscles have decreasing satellite cell population with increasing age, but not human skeletal muscles (Jejurikar and Kuzon, 2003). To circumvent these problems, other models have been used.

Spontaneous mutations of dystrophin, leading to X-linked muscular dystrophy, have been detected in several breeds of the domestic dog, including the Golden Retrivever, the Rottweiler, the German short-haired pointer, and a beagle model. Among these models, the golden retriever dystrophic (GRMD) dog model has been most extensively studied and best characterized. This model seems to be one of the nearest pathological counterpart of human DMD. Nevertheless, this model is not the most used one, because dogs are not ideal laboratory animals. Colonies of dogs are difficult to maintain, their genome is not manipulable, and they are highly sentient and emotive. In the cat model, dystrophin deficiency produces hypertrophic feline muscular dystrophy (HFMD), rather than atrophy and fibrosis seen in both DMD and GRMD. For this reason, this model is not widely used. However, this model has additional disadvantages, particularly because researchers do not

wish to practice experiments on a large, sentient and emotive animal, for economic and ethic reasons.

Both Zebrafish and *C. elegans* express a dystrophin homologue that interacts with other DGC proteins. Both these models have been also used, because they have a low complexity, and because they harbor less individual-related variations. *C. elegans* has also the property to be easily genetically manipulable. Nevertheless, the biology of these animals and their size, that are not similar than that of humans, have prevented their large use in study of DMD. Nevertheless, these models may be valuable tools for specific experiments.

Currently, *in vitro* models of DMD consist mainly of cells isolated from *mdx* mice, even though we have already discussed that the mdx cells do not completely reflect the biology of humans. No *in vitro* human model of DMD is largely used, because they do not exist, or because the few existing ones are thought not to be similar to DMD.

In an attempt to create such an *in vitro* human model for DMD, we have attempted to immortalize human myoblasts, either normal or of DMD origin.

CHAPTER 2: FIRST STUDY:

Lentiviral Immortalization of Myoblasts

2.1 GENERAL INTRODUCTION FOR THE FIRST STUDY

"Lentivector-Mediated Transfer of Bmi-1 and Telomerase in Muscle Satellite Cells Yields a Duchenne Myoblast Cell Line with Long-Term Genotypic and Phenotypic Stability"

HUMAN GENE THERAPY 14:1525–1533 (November 1, 2003)

Here, we are going to summarize the most important facts of this introduction in regard to our particular task of immortalization of myoblast. We are going also to discuss here why we have chosen to immortalize myoblasts, and why we have used some particular methods to reach this goal.

Normal primary cells do not have an infinite lifespan. Usually, they can divide about 30-50 times and they finally die after a crisis phase where they usually stop proliferating. Only rare cells (~1 in 10 million) can escape crisis and become immortal, but they often are associated with aberrant phenotypes. These facts imply that for getting large amounts of cells of a particular cell type, multiple isolations of primary cells have to be done, often from different donnors. However, for some goals, isolation of cells derived from different donors is not possible. This is the case if we want to work with a specific individual or to work with a specific genetic background. Then, cell lines have to be created, so that they should be as close as possible to their corresponding primary cells.

The human cellular models are more sought than murine ones, because researchers often want to pursue fundamental biological human studies and medical studies of human cells. Nevertheless, there are still very few human cell lines, whose properties are similar to primary cells. It is for such reasons that we have aimed to create a human cell line.

Until now, a lot of the existing cell lines are derived from murine cells because murine cells are more easily immortalized than human cells. Nevertheless, these murine cell lines have often been reported to be genetically and phenotypically distinct from the normal primary cells, particularly from human primary cells. However, biomedical research often needs to work with models that are as close as possible to normal human cells. Fundamental researchers often like to get cells that are genetically and phenotypically as similar as possible to primary human cells. Because most cell human or murine lines have cancerous origin, they may not be in some circumstances the most relevant models. Indeed, these cancerous cell lines often have poorly known biochemical and genetic alterations, and they may therefore in some circumstances not be similar with some of the properties of the related primary cells. This is why we aimed to create a cell line with defined genetic elements, which would be able to trigger immortalization, but not tumorigenesis, of human cells.

At this point, we could have immortalized different human cell types. We have chosen to immortalize human primary myoblasts, for two major reasons. First, Duchenne Muscular Dystrophy (DMD) is one of the most devastating diseases: it touches 1 out of 3500 boys. A lot of basic research is still probably needed to find a cure to this neuromuscular disease, and having a proper human cellular model would be useful for this task. The second reason why we have chosen to immortalize myoblast was because we wanted to know if the immortalized cells could still differentiate. Indeed, after immortalization of different cell types, the resulting cells are often said to be very similar to their primary counterparts because they possess the same surface markers. Nevertheless, presence of some surface markers on a cell does not always mean that this cell is completely similar to primary cells. It does not mean, either, that this cell is still able to follow the specific tasks of a primary cell. This has been particularly obvious with cells that can differentiate: even if these cells were immortalized and were thought to be normal, they often had lowered or impaired differentiation properties. Among the cell types that can differentiate, myoblasts have a phenotype of differentiation that is easy to observe: they have the ability to fuse between them and to form multinucleated myotubes. With such visible phenomenon of differentiation, myoblasts become an interesting paradigm to test in a simple manner if immortalization still allows differentiation. Indeed, even if immortalization of myoblasts has already been attempted, their authors often recognized that the resulting cells had lower fusogenic properties compared to primary myoblasts.

If immortalized myoblasts were often reported to have low fusogenic properties, it was certainly due to the use of virus-derived immortalizing genes, because the functions of such genes are known to lead to immortalization but also to alter cellular functions, and to have mutagenic properties. For instance, TAg is known to perturb different pathways, but also to alter karyotypes, leading to translocations, and aneuploidy, with chromosome numbers often in the diploid range. TAg is a protein of the SV40 virus whose properties are to act in different pathways, especially with its two major different splicings referred to as Large T and small t. In this respect, Large T was reported to inhibit at least the p53 and RB pathways, whereas small t was reported to act on Phosphatase 2A. The action of these proteins on these anti-tumorigenic mechanisms has already been reported to allow immortalization of numerous cell types. Other non-viral methods have been used for immortalization, with strong oncogenes, or with chemical mutagenesis. However, these alternatives often led to massive genetic changes, which could even often not be mapped.

To circumvent these problems, we have used another approach. More precisely, we have attempted to find a method to immortalize myoblasts with defined and minimal genetic elements. This alternative way of immortalization would allow uncovering precisely what genes are useful for immortalization of myoblasts. This method would also be more appropriate to lead to cells with minor genetic and biochemical modifications.

For this purpose, what have used Telomerase, Bcl-2 and Bmi-1. These proteins were chosen, because they were already suspected to play a role in immortalization. In fact, they had already been described to have either anti-senescence or anti-apoptosis effects. We are going now to summarize briefly their known function(s).

First, Telomerase (hTERT) is the catalytic component of the human telomerase. This enzyme is able to replace the chromosome end repeats, which are eroded after multiple rounds of cellular divisions. Second, Bcl-2 has anti-apoptotic properties. Third, Bmi-1 is an oncogene regulating the tumor suppressors $p16^{INK4a}$ and $p19^{ARF}$. Both these tumor suppressors originate from the same chromosomal locus, and they have been implicated in the senescence process.

At this point, we still had to define what vector we should use to deliver the immortalizing genes. We have chosen here to use the HIV-derived lentiviral vectors, because they have sufficiently large capacities to carry the genes of interest, but also because they can easily

transduce a small number of cells. This property was important in our case because some myoblasts with altered phenotypes are present only in small amounts. Another reason to use lentivectors was because of their integrational properties. Indeed, lentiviral vectors allow their transgenes to be inserted in the genome of host cells in open chromatin regions. This allows high level expression of the transgenes, in our case of the genes used for immortalization. All transgenes inserted into myoblasts were surrounded with plox sequences, so that these sequences could be removed after expression of Cre recombinase.

We would like to mention some of the previous attempts to immortalize human myoblasts. In the past, TAg has already been used together with telomerase, for the immortalization of human myoblasts. Nevertheless, as mentioned earlier, TAg immortalizations are known to be highly mutagenic, and to lead to karyotypes in the diploid range. Shortly before the publication of our results, it was also shown that telomerase alone was not sufficient to immortalize human myoblasts. We have confirmed these results, and we could demonstrate an alternative possibility for the immortalization of myoblasts. Here, we could show that Bmi-1 + Telomerase also allow immortalization of myoblasts. This finally led to a Duchenne immortalized cell line, with normal phenotypic and fusogenic properties. We are going now to present these results, which were published in the journal Human Gene Therapy.

2.2 RESULTS OF THE FIRST STUDY :

Cudré-Mauroux et al. 2003 Hum. Gene Ther. 14 :1525-1533

Lentivector-mediated expression of Bmi-1 and telomerase in muscle satellite cells yields a Duchenne myoblast cell line with full differentiation potential and long-term genotypic and phenotypic stability

Running title:
Conditionally immortalized DMD myoblasts

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ABSTRACT

Conditionally immortalized human cells are valuable substrates for basic biological studies, as well as for the production of specific proteins and for the creation of bioartificial organs. We previously demonstrated that the lentivector-mediated transduction of immortalizing genes into human primary cells is an efficient method for obtaining such cell lines. Here, we used human muscle satellite cells as model targets to examine the impact of the transduced genes on the genotypic and phenotypic characteristics of the immortalized cells. The most commonly used immortalizing gene, the SV40 large T antigen (T-Ag), was extremely efficient at inducing the continuous growth of primary myoblasts, but the resulting cells rapidly accumulated major chromosomal aberrations and exhibited profound phenotypic changes. In contrast, the constitutive expression of telomerase and Bmi-1 in satellite cells from a control individual and from a patient suffering from Duchenne muscular dystrophy yielded cell lines that remained diploid and conserved their growth factor-dependence for proliferation. Furthermore, the Duchenne-derived cell line exhibited all the phenotypic characteristics of its primary parent, including an ability to differentiate fully into myotubes when placed in proper culture conditions. It should constitute a useful reagent for a wide range of studies aimed at this disease.

OVERVIEW SUMMARY

The aim of this study was to generate conditionally immortalized human muscle cell lines. For this, we transduced satellite cells with lentiviral vectors expressing various immortalizing genes. We found that T-Ag is extremely efficient at immortalizing muscle cells, but induces a high degree of genetic instability that rapidly leads to chromosomal aberrations and profound phenotypic changes. In contrast, the constitutive expression of telomerase and Bmi-1 yielded myoblast cell lines with long term genotypic and phenotypic stability. A Duchenne cell line was obtained, which fully conserved the fusogenic ability of primary myoblasts. This cell line will be a useful cellular model for studies focusing on this form of muscular dystrophy. More broadly, this work provides important information on approaches aimed at generating phenotypically relevant cell lines from primary human tissue.

INTRODUCTION

The controllable expansion of human primary cells, be they early progenitors or differentiated effectors, represents an attractive means of producing large quantities of cells for experimental or therapeutic purposes. Through a process known as ex vivo immortalization, primary cells can be induced to grow indefinitely in vitro by genetic manipulation. Cell lines thereby obtained can be expanded, cloned, extensively characterized both in their immortalized and "de-immortalized" (i.e. after removal / silencing of the immortalizing genes) states, and used for analyses that range from basic physiology to proteomics, for the production of specific proteins and, in selected cases, for transplantation.

Classically, genes encoding for at least two types of proteins have been used to immortalize human cells. First, proteins that trigger entry into the cell cycle, e.g. growth promoters or cell cycle inducers. Prototypes of this category are the SV40 large T antigen, which acts through the binding of Rb and p53 (Ali and DeCaprio, 2001), and Bmi-1, which downregulates the p16 and p19Arf tumor suppressor genes encoded by the *ink4a* locus (Jacobs *et al.*, 1999). Second, anti-senescence factors such as telomerase (Greider and Blackburn, 1987), which prevents the progressive shortening of telomeres that occurs as the number of cell divisions augments.

We previously demonstrated that lentiviral vectors are convenient tools to introduce immortalizing genes into human primary cells, owing to their ability to mediate the stable integration and long term expression of transgenes in a wide variety of targets irrespective of their proliferative status (Salmon *et al.*, 2000). Furthermore, lentiviral vectors containing *loxP* sites into the vector long terminal repeats (LTR) allow for the Cre recombinase-mediated excision of the provirus, hence for the efficient de-immortalization of the expanded cell populations (Salmon *et al.*, 2000).

Here, we applied this technique to the immortalization of human primary myoblasts from either a control individual or a patient suffering from Duchenne muscular dystrophy (DMD). This disease results from mutations in the gene coding for dystrophin, a component of the dystrophin-glycoprotein multimolecular complex (DGC), which connects the cytoskeletal actin with the transmembrane protein β-dystroglycan and thereby plays an essential role in the function of skeletal muscles (Spence *et al.*, 2002). Molecularly, the muscular dystrophies are a heterogeneous group of disorders, some of which result from abnormalities in various components of the DGC. DMD is the most common type of muscular dystrophy affecting approximately 1 out of 3500 males. Patients with DMD present with a childhood onset progressive loss of muscle function and generally die by their early twenties as a result of either respiratory or cardiac failure (Emery, 2002).

Although naturally occurring or genetically engineered mouse models can be used for studying some aspects of the muscular dystrophies, there is a shortage of reagents for molecular analyses conducted at the cellular level, in areas ranging from physiology to gene therapy (Allamand and Campbell, 2000).

Myoblasts were previously immortalized by expressing proteins derived from adenovirus or polyomavirus SV40. However, immortalization often coincided with differentiation defects or other anomalies. Adenovirus E1A and SV40 large T antigen (T-Ag) suppressed myogenic differentiation of mouse myoblasts (Webster *et al.*, 1988). Moreover, T-Ag led to loss of contact inhibition, to anchorage independent growth (Miranda *et al.*, 1983), and to a strong tendency to apoptosis (Corti *et al.*, 2001). To circumvent these problems, T-Ag expression was restricted to undifferentiated cells (Pincon-Raymond *et al.*, 1991; Corti *et al.*, 2001) or used in mutated versions. Expression of a cytoplasmic form of T-Ag was reported to be fully compatible with the terminal differentiation of C2 cells, despite enabling their growth in semisolid medium (Tedesco *et al.*, 1995). The continuous growth of normal and Duchenne

human muscle cells was obtained with a temperature sensitive Large T antigen (Simon *et al.*, 1996), and Duchenne myoblasts were immortalized by co-expressing SV40 Large T and telomerase (Seigneurin-Venin *et al.*, 2000). The long-term genotypic and phenotypic stability of these cells, however, remains unestablished.

The present study tested different combinations of genes to immortalize primary human myoblasts. Our results indicate that, while T-Ag is extremely efficient at achieving this goal, it induces a high degree of genetic instability, which rapidly leads to chromosomal aberrations and profound phenotypic changes. In contrast, the constitutive expression of telomerase and Bmi-1 yielded myoblast cell lines devoid of these problems. Furthermore, a Duchenne muscle cell line was obtained, which fully and stably conserved the phenotypic characteristics of primary myoblasts.

MATERIALS AND METHODS

Lentiviral vector production. LoxP-containing HIV-derived lentiviral vectors expressing the enhanced green fluorescent protein (GFP), SV 40 T-Ag, Bmi-1, human telomerase (hTERT), Bcl-2 and a nuclear localization signal-tagged form of the Cre recombinase (nls-Cre) from an internal human cytomegalovirus (CMV) immediate early promoter were previously described (Salmon *et al.*, 2000). Vector stocks were produced by transient transfection of 293T cells (Zufferey *et al.*, 1997), concentrated by ultracentrifugation and resuspended in X-VIVO medium (BioWhittaker). The GFP and nls-Cre vectors were titered on HeLa and HeLa-4.5 cells, respectively. HeLa-4.5 is a HeLa-derived clone that contains a single copy of a loxP-flanked, GFP-expressing lentivector provirus. Stocks of vectors expressing the immortalizing transgenes were evaluated by reverse transcriptase (RT) assay (Aiken and Trono, 1995).

Culture and transduction of primary myoblasts. Samples of human skeletal muscle were obtained during corrective orthopedic surgery or diagnostic biopsies, in accordance with the guidelines of the ethical commitee of Geneva University Hospital. Myoblast clonal cultures were prepared from satellite cells following dissociation of the muscle biopsies by manual cloning using a micropipette under a microscope. Cells were seeded individually in growth medium consisting of Ham's F10 (Gibco) supplemented with 15% fetal calf serum, bovine serum albumin (0.5 mg/ml), fetuin (0.5 mg/ml), EGF (10 ng/ml), dexamethasone (0.39 µg/ml), insulin (0.18 mg/ml), creatine (1 mM), pyruvate (100 µg/ml) and uridine (50 µg/ml) (Baroffio *et al.*, 1995). Differentiation medium consisted of DMEM (Gibco) supplemented with bovine serum albumin (0.5 mg/ml), EGF (10 ng/ml), creatine (1mM), pyruvate (100 ng/ml) and uridine (50 µg/ml) (Baroffio *et al.*, 1995). For transduction, 30% confluent

cultures of myoblasts were exposed to lentiviral vectors at a multiplicity of infection (MOI) of 0.5 to 1 (as determined on HeLa cells) for the GFP vector, and of 0.5 to 3 (as determined on HeLa-4.5 cells) for the nls-Cre vector. For the immortalizing vectors, amounts of particles with an RT content equal to that of an MOI 1 of GFP vector were used.

Protein analyses. Cells were either directly harvested in lysis buffer (1% triton X-100, 1mM aprotinin and phenylmethylsulphonyl –PMSF- in PBS) before pelleting nuclei by centrifugaion, or resuspended in one volume of TNE buffer (Tris pH 7.5 50 mM, NaCl 150 mM, EDTA 1 mM) and one volume of Laemmli Sample Buffer (BIORAD) before boiling for 3 min. Extracts containing 10 µg of protein (as determined with the BCA Protein assay, Pierce) were subjected to SDS-PAGE before transfer to PVDF transfer membranes (Polyscreen ; NEN Life Science). Membranes were blocked with T-TBS containing 5% non-fat milk and hybridized with the following primary antibodies: mouse monoclonal antibodies against SV40 large T antigen (Santa Cruz), Myogenin (clone F5D obtained from W.E. Wright), Desmin (clone D33, DAKO) or α-Tubulin (clone DM1A, Sigma), and rabbit polyclonal antibodies against human p16, MEF2 or Myf5 (Santa Cruz). After incubation for one hour with HRP-conjugated goat anti-mouse or rabbit antibodies (BIORAD), blots were extensively washed with T-TBS, and processed using ECL reagents and MP Hyperfilms (Amersham Pharmacia Biotech).

PCR amplifications. DNA was extracted using the DNeasy kit (Qiagen) and PCR amplifications were performed with the primers fwdCMV (5'-ATTTCCAAGTCTCCACCCCATT-3') and with revHBCL2 (5'-GCGGGATGCGGCTGGAT-3') or with the primers fwdCMV and revBMI1 (5'-TAGGACAATACTTGCTGGTCTCCA-3').

Karyotypic analyses. Cells plated at 1/3 confluence 72 hours before analysis were processed with the Synchroset kit according to the manufacturer's instructions. For each of the 12 karyotypes analyzed, at least 3 different metaphase spreads were examined.

Fusion assays. For assaying the fusogenic ability of primary myoblasts and cells immortalized with Bmi-1 and hTERT, differentiation medium was substituted to growth medium at confluence. For T-Ag-expressing cells (which kept proliferating in differentiation medium), various levels of confluence (from 20 to 80%) were tested, and in some cases 0.3 mM aphidicolin (Sigma) was added to arrest the cells. At the time of analysis, nuclei were stained with 1 μg/ml Hoechst 33342 for 10 min, cells were washed twice with PBS, and examined with a CCD camera mounted on an inverted microscope (Nikon Eclipse TE 30). The fusion index was determined every 8 hrs according to the following formula: F.I. = number of nuclei in fused myoblasts / total number of nuclei. For each time point, a mean of about 300 nuclei in three randomly chosen fields were counted.

Measurements of cell growth kinetics. The number of population doublings at each passage was defined as log N/log 2, where N is the number of cells harvested at confluence divided by the number of cells initially seeded. For analyzing growth in differentitiation medium, cells were periodically detached with Trypsin, counted, and replated using the trypsin inhibitor type I-S from Soybean (Sigma) to permit re-attachment in the absence of serum.

Cell cycle analyses. Cells were plated so as to be 50% confluent 3 days later at the time of analysis. Hoechst 33342 dye was added into the medium at a concentration of 1 μg/ml, the cells were incubated at 37° C for 5 to 10 min, washed with PBS, detached with trypsin, fixed with paraformaldehyde 3.7%, and examined by FACS, using the CellQuest program for data analysis. For cell cycle analysis in DM, subconfluent cells were placed in DM, detached with trypsin, incubated in a buffer containing 0.03% saponin (Sigma), and stained with 20 μM 7-amino-actinomycin D (7AAD) (Sigma). The cells were incubated at room temperature for 30

min, fixed with paraformaldehyde 3.7%, and examined by FACS, using the CellQuest program for data analysis.

Immunofluorescence. After 3 days in differentiation medium, cells were washed with PBS, fixed for 10 min with MeOH at –20 °C, washed 5 times with PBS, and incubated in PBS containing 1% BSA for 40 min at room temparature. Dystrophin-specific antibodies NCL-Dys1, NCL-Dys2, NCL-Dys3 (Novocastra, obtained from G. Pizzolato) were added at a dilution of 1:2, 1:100 and 1:40, respectively. After 40 min, cells were washed and anti-IgG Alexa-conjugated antibody was added at a dilution of 1:400 in PBS BSA 1% at room temperature; 30 min later, cells were washed and examineed with a Zeiss Axiocam epifluorescence microscope.

RESULTS

Lentivector-mediated conditional immortalization of human myoblasts. When satellite cells isolated from human muscle biopsies were exposed to a lentiviral vector expressing the green fluorescent protein (GFP) from the CMV promoter at a MOI of 1, levels of transduction of about 80% were observed, indicating a degree of sensitivity slightly higher than that of the HeLa cell standard (not illustrated). DNAs encoding the SV40 T-Ag, Bmi-1, telomerase (hTERT) or Bcl-2 were then introduced by lentivector-mediated transduction into satellite cells obtained from a 13 year-old boy, either alone or in combinations. Only cells engineered to express T-Ag, T-Ag + hTERT, Bmi-1 + hTERT, or T-Ag + Bmi-1 + hTERT + Bcl-2 escaped the crisis that occurred in control myoblasts at about 45 population doublings after transduction, corresponding to approximately 65 population doublings after initial isolation (Fig. 1). These cells have now been maintained in culture for more than two years, or more than 250 passages, and can thus be considered as immortalized. When examined by light microscopy, T-Ag-containing cells exhibited a rounder shape and a lower tendency to align regularly on the plate, compared to control or Bmi-1/hTERT immortalized myoblasts (Fig. 2).The lentiviral vectors used in these experiments results in integrants flanked by *loxP* sites, and as previously described for similarly immortalized liver endothelial cells (Salmon *et al.*, 2000), addition of Cre recombinase led to the rapid growth arrest of these cells (not illustrated). This demonstrated that their continuous proliferation was strictly dependent upon the presence of the immortalizing transgenes. From each population, at least 2 clones were randomly selected for further study. Expression of the appropriate exogenous proteins was verified by Western blotting with antibodies against T-Ag, p16 (a protein downregulated by Bmi-1), or by TRAP assay (which measures telomerase activity) (Fig. 3 and data not shown). Surprisingly, the highest TRAP score was found in cells tranduced with a vector expressing

T-Ag but in which no exogenous hTERT had been introduced. Although it explained the robust and long-term growth of these cells, it suggested that some intercurrent event had occurred following T-Ag transduction.

Karyotypic analyses. Six months after transduction, karyotypic analyses were performed on randomly chosen clones. Seven out of seven T-Ag-containing clones (2 T-Ag only, 3 T-Ag + hTERT, 2 T-Ag + hTERT + Bmi-1 + Bcl-2) exhibited major karyotypic anomalies, including aneuploidy as well as chromosomal translocations and fragmentations, often in a near-triploid or -tetraploid background. One of these clones was analyzed twice, at six and seven months post-immortalization. While marked chromosomal aberrations were already detected the first time, numerous additional karyotypic changes had accumulated a month later. In contrast, five out of five clones immortalized with Bmi-1 and telomerase stably conserved the diploidy of control primary cells (Fig. 4).

Cell cycle analyses. T-Ag expression was associated with an increased proportion of the cells residing in the G_2/M phase of the cycle (Fig. 5). When Bmi-1/hTERT immortalized myoblasts were placed in growth factor-depleted differentiation medium, they stopped dividing and accumulated in the G_0/G_1 phase of the cycle like non-immortalized primary myoblasts. Once re-incubated in growth medium, they immediately resumed their proliferation (Fig. 6). In contrast, T-Ag-containing cells still underwent a few divisions in differentiation medium, before exhibiting a high rate of death (not illustrated).

In vivo tumorigenicity. The subcutaneous injection of 10^6 cells from two randomly chosen clones from each of the immortalized myoblasts populations did not lead to tumor development in nude mice after 11 weeks, while mice injected in parallel with HeLa cervical carcinoma cells exhibited prominent tumors in less than 4 weeks. The myoblast cell lines were thus not tumorigenic in this model, even when containing marked chromosomal abnormalities.

Immortalization of DMD myoblasts. Satellite cells purified from a muscle biopsy obtained from a seven year-old patient with Duchenne muscular dystrophy were transduced with lentiviral vectors expressing Bmi-1 or telomerase, alone or in combination. Only cells having received both vectors became immortalized, consistent with the results obtained with normal myoblasts. One clone was selected for further studies. The presence of the Bmi-1 and TERT transgenes was verified, and the karyotypic analyses revealed no abnormality (not illustrated). A light microscopic study of these cells demonstrated morphological features very close to that of control myoblasts, while indirect immunofluorescence confirmed their lack of dystrophin expression (Fig. 7).

Differentiation ability of immortalized myoblasts. Control, non-immortalized myoblasts rapidly fused and formed myotubes when exposed to differentiation medium. Similarly, the DMD myoblast cell line also stopped growing and exhibited extensive fusion activity in differentiation medium, albeit with kinetics that were slightly slower (Fig. 8). This correlated with a pattern of gene expression typical of myotube differentiation, with a downregulation of Myf5, and an upregulation of Mef2, Myogenin, and desmin (Fig. 9). In contrast, little fusion and no significant switch in the expression of muscle-specific genes were observed when clones obtained from wild-type muscle were placed in differentiation medium. This differentiation block was present irrespectively of the immortalizing cocktail used, and whether or not the cells were "de-immortalized" with Cre (Fig. 9).

DISCUSSION

In this study, the lentivector-mediated expression of SV-40 T-Ag, with or without TERT, and Bmi-1 + TERT in human muscle satellite cells yielded continuously growing human muscle cell lines. In contrast, TERT or Bcl-2, either alone or in combination, or Bmi-1 alone, were not sufficient to immortalize human myoblasts. T-Ag-containing or the Bmi-1/TERT immortalizing regimens had remarkably distinct genotypic and phenotypic consequences. Most strikingly, the introduction of SV40 large T into human muscle satellite cells led, over time, to a great degree of chromosomal instability. This correlated with the acquisition of novel phenotypic characteristics such as cell rounding, increased rates of progression into the G_2/M phase of the cycle, and serum- and growth factor-independent proliferation. We have noted a similar degree of phenotypic drift in human keratinocytes, retinal epithelial cells and endothelial cells transduced with T-Ag-expressing lentiviral vectors (unpublished results), suggesting that this viral protein can induce genetic instability in a wide variety of targets. Here, the high levels of activity exhibited by T-Ag-expressing cells in the TRAP assay in the absence of exogenous TERT likely reflected the T-Ag-mediated induction of endogenous telomerase (Foddis *et al.*, 2002; Yuan *et al.*, 2002).

The immortalizing capacity of T-Ag is generally attributed to its inhibitory association with p53, which relieves the cell cycle G_1 checkpoint (Zhu *et al.*, 1991). However, p53 also controls DNA repair and recombination in several ways, for instance by interfering with the RAD51 pathway (Saintigny and Lopez, 2002) and by associating with 53BP1, a transcriptional co-activator that participates in the formation of nuclear foci accumulating at the sites of double strand breaks (Schultz *et al.*, 2000). Relatedly, DNA repair by the

MRE11/RAD50/nibrin pathway is disturbed in the presence of T-Ag, which may lead to increased mutation rates (Lanson *et al.*, 2000; Digweed *et al.*, 2002). Noteworthy, in the present work, T-Ag-induced chromosomal changes were not prevented by co-expression of the catalytic subunit of telomerase, suggesting that this genetic instability was not simply a response to telomere attrition (Maser and DePinho, 2002).

In sharp contrast, we found that cells immortalized by expression of Bmi-1 and telomerase did not exhibit such chromosomal instability. Bmi-1 is a polycomb-group oncoprotein that acts as a negative regulator of the *ink4a-ARF* locus, which encodes the tumor suppressors p16 and p19arf (Jacobs *et al.*, 1999). p16 blocks cell cycle progression by inhibiting cyclin D-dependent kinases and thereby prevents the phosphorylation of Rb (Serrano *et al.*, 1993), whereas p19arf prevents the degradation and inactivation of p53 by binding to Mdm2 (Pomerantz *et al.*, 1998; Weber *et al.*, 1999). *bmi-1* $^{-}$/- mice suffer from severe proliferation defects in both the hematopoietic system and brain, while Bmi-1 overproduction leads to lymphomas (Alkema *et al.*, 1997). In vitro, overexpression of Bmi-1 in MEFs was found to delay senescence and facilitate immortalization (Jacobs *et al.*, 1999).

Here, Bmi-1 alone could not immortalize human myoblasts, but in conjunction with temolerase it yielded continuously growing cell lines that exhibited three interesting properties. First, these cells displayed morphological features very close to those of primary myoblasts. Second, they were devoid of visible chromosomal aberrations and their genome apparently remained stable over a long period of time, suggesting that, in contrast to T-Ag, Bmi-1 is not by itself highly mutagenic. Third, the proliferation and arrest of Bmi-1/hTERT-immortalized cells remained fully controllable by addition or withdrawal of growth factors.

Using the Bmi-1/TERT immortalizing cocktail, we obtained a Duchenne myoblast cell line that conserved many characteristics of primary muscle satellite cells, in particular a full ability to differentiate into myotubes. In contrast, the wild-type myoblast immortalized clones

obtained in this study exhibited minimal differentiation ability, even though the starting material, a satellite cell clone isolated from the muscle biopsy of a 13 year old boy, could be induced to differentiate normally. The reasons for this limitation are unknown. Expression of Bmi-1 or TERT per se could not account for the differentiation block, since immortalized DMD cells expressing these genes readily formed myotubes. Furthermore, the fusogenic potential of the wild-type immortalized cells was not recovered after Cre-mediated excision of the immortalizing genes. It could therefore be that subtle mutations may have occurred in these cells, inactivating some critical master regulator of differentiation. Alternatively, muscle satellite cells may be heterogeneous in their ability to yield fusogenic myoblasts, and precursors with low fusion ability may be easier to immortalize. Future studies should address these possibilities, in particular by generating muscle satellite cell lines from a larger number of clones and from different donors. This will help determine whether the hereby-described immortalization protocol could be used broadly to obtain muscle cell lines from individuals suffering from other types of genetic disorders.

It remains that the hereby described DMD myoblast cell line will facilitate a wide range of studies directed at muscular dystrophy, whether they focus on the physiopathology of this disease, on the testing of drugs to improve the function or survival of dystrophin-defective muscle cells, or on the evaluation of genetic approaches for the treatment of this devastating illness.

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FIGURE LEGENDS

FIG. 1. Proliferation curves of control and lentivector-transduced myoblasts.

Satellite cells isolated from a human muscle biopsy were transduced with the indicated lentiviral vectors and their growth was monitored by measuring population doublings. The four regimens indicated on the upper left side yielded immortalized cell lines.

FIG. 2. Phase contrast microscopy:

Cells immortalized with Bmi-1 + hTERT are elongated and regularly aligned, whereas T-Ag-containing cells are rounder and grow in disorderly fashion. Magnification 40x. Results are representative of more than ten clones from each population.

FIG. 3. Detection of immortalizing agents.

(A) Western blotting of T-Ag and of p16 (downregulated by Bmi-1). Two representative clones are shown from each immortalized cell population.

(B) PCR assays to detect Bcl-2 and Bmi-1 transgenes in the clones containing T-Ag + Bmi-1 + Bcl-2 + hTERT.

(C) TRAP assay to detect telomerase activity.

Numbers correspond to cell clones indicated in lower right part.

MB = myoblasts; H.I. = heat inactivated.

FIG. 4. Karyotypic analyses.

While control primary myoblasts and (Bmi-1 + hTERT)-immortalized cells exhibit similarly diploid genomes, T-Ag-containing cells accumulate chromosomal abnormalities. The karyotype of cells immortalized with Bmi-1 + T-Ag + hTERT + Bcl-2 was similarly perturbed (not shown).

FIG. 5. Cell cycle analyses.

The DNA content of cells was determined by Hoechst staining and FACS analysis. The percentages indicate the fraction of cells in G_2/M for each population. The G_o/G_1 peak was set at the same value for all clones, even though T-Ag-containing clones had higher ploidies. Results are representative of more than three clones from each population.

FIG. 6. Proliferation in differentiation medium.

(A) Proliferation of (Bmi-1 + hTERT)-immortalized clones is externally controllable. Cultures were consecutively incubated in growth and differentiation medium (GM and DM, respectively) as indicated, and cell numbers determined by counting. Results representative of more than 10 individual clones tested.

(B) Cell cycle status of cells incubated for 7 days in differentiation medium, evaluated by staining their DNA with 7AAD followed by FACS analysis. The G_o/G_1 peak was set at the same value for all clones, even though T-Ag clones had higher ploidies. The percentages indicate the fraction of cells in G_2/M for each population.

FIG. 7. Dystrophin expression in control but not in the DMD immortalized myoblasts. Phase contrast microscopy (A-C, G-I) and indirect immunofluorescence (D-F, J-L) of control

primary myoblasts (A-F) and (Bmi-1 + TERT)-immortalized DMD muscle cells (G-L), using antibodies binding to the rod domain (Dys1), the N-terminus (Dys2) or the C-terminus (Dys3) of human dystrophin.

FIG. 8. Fusogenic potential of control and immortalized DMD myoblasts.

Differentiation medium was substituted to growth medium when cells reached confluence. Fusion indexes were calculated in three randomly chosen microscopic fields by dividing the number of nuclei in fused cells by the total number of nuclei.

FIG. 9. Differentiation-associated gene expression in myoblast cell lines.

Extracts from Duchenne (left) or wild-type (middle and right) myoblasts clones immortalized with indicated cocktails were subjected to Western blotting with indicated antibodies after incubation in growth medium (GM), in differentiation medium for 4 days without (DM) or with (Cre) prior de-immortalization with an adeno-Cre vector. Only the Duchenne clone exhibits the downregulation of Myf5 and induction of Mef2, Myogenin and Desmin that are typical of myotube differentiation.

FIGURES

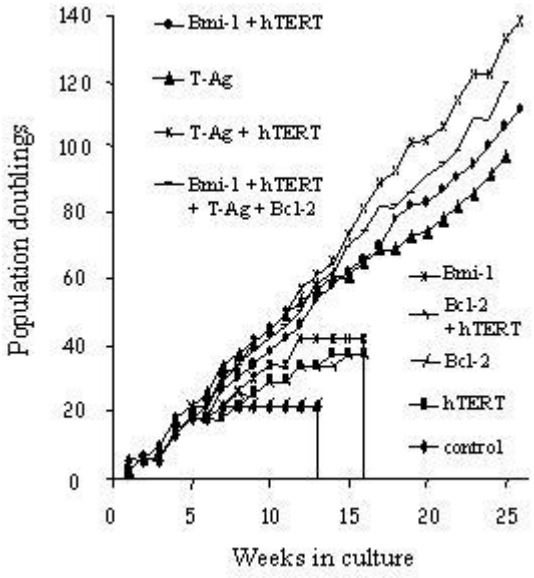

FIG. 1

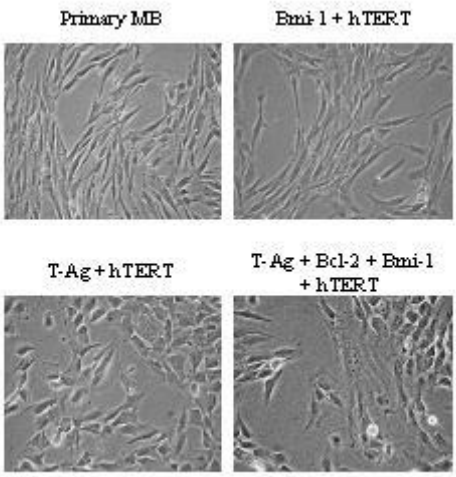

FIG. 2

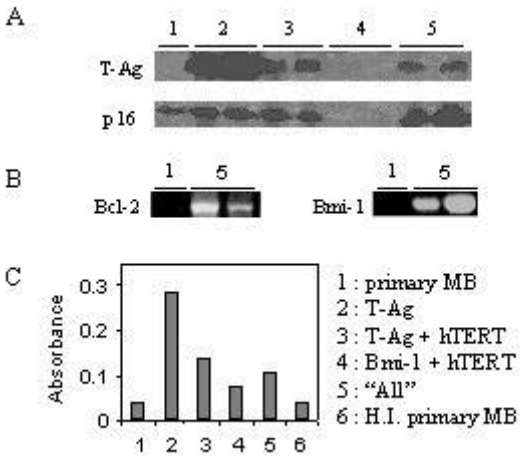
A
1
2
3
4
5
T-Ag
p16
B
1
5
Bcl-2
1
5
Bmi-1
C
Absorbance
0.3
0.2
0.1
0
1 2 3 4 5 6
1 : primary MB
2 : T-Ag
3 : T-Ag + hTERT
4 : Bmi-1 + hTERT
5 : "All"
6 : H.I. primary MB

FIG. 3

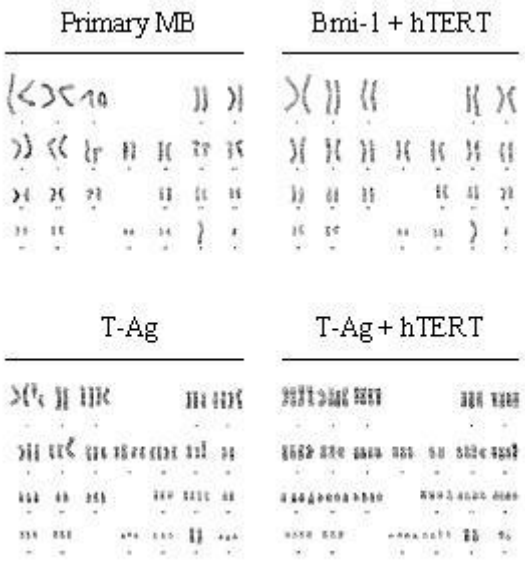

FIG. 4

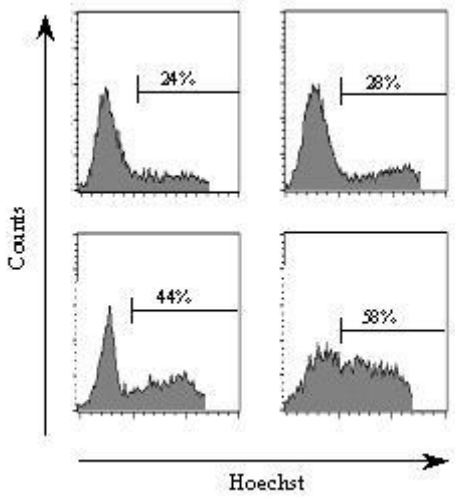

FIG. 5

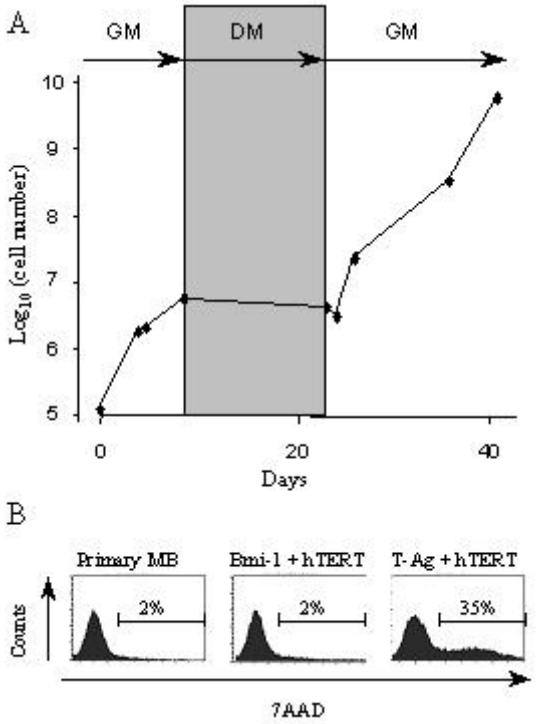
A
GM
DM
GM
10
9
8
7
6
5
Log10 (cell number)
0
20
40
Days
B
Primary MB
Bmi-1 + hTERT
T-Ag + hTERT
2%
2%
35%
Counts
7AAD

FIG. 6

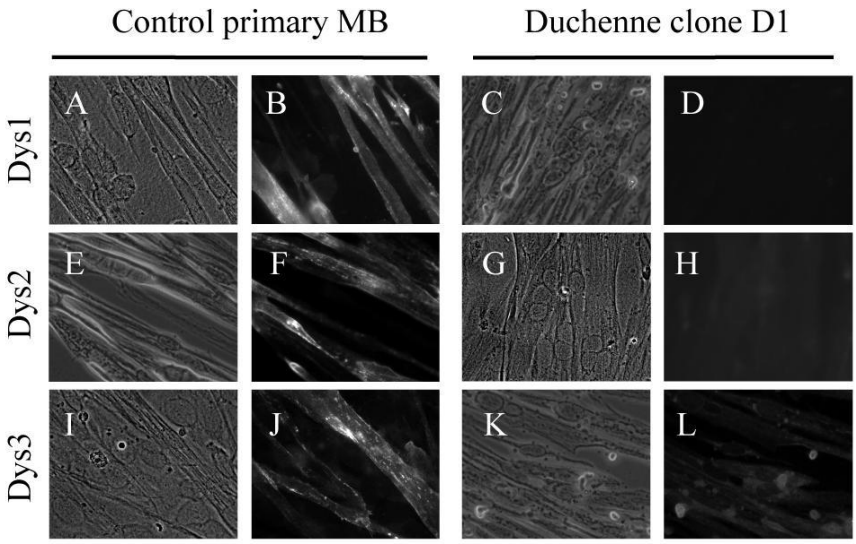
Control primary MB
Duchenne clone D1
Dys1
Dys2
Dys3
A
B
C
D
E
F
G
H
I
J
K
L

FIG. 7

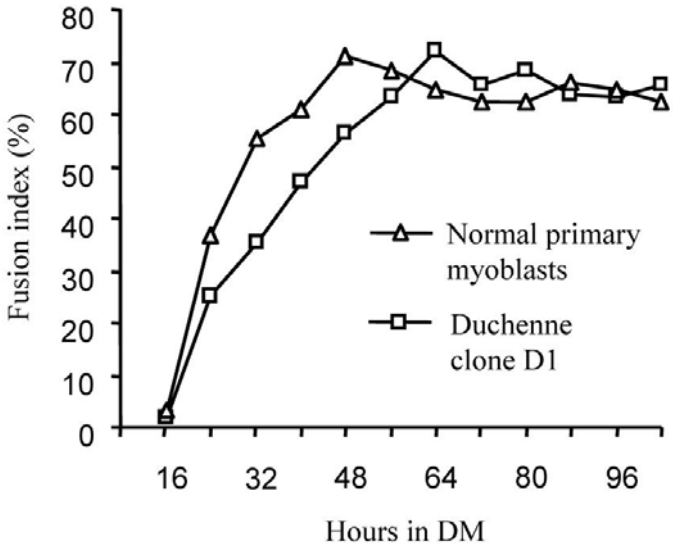
80
70
60
50
40
30
20
10
0
Fusion index (%)
16
32
48
64
80
96
Hours in DM
Normal primary myoblasts
Duchenne clone D1

FIG. 8

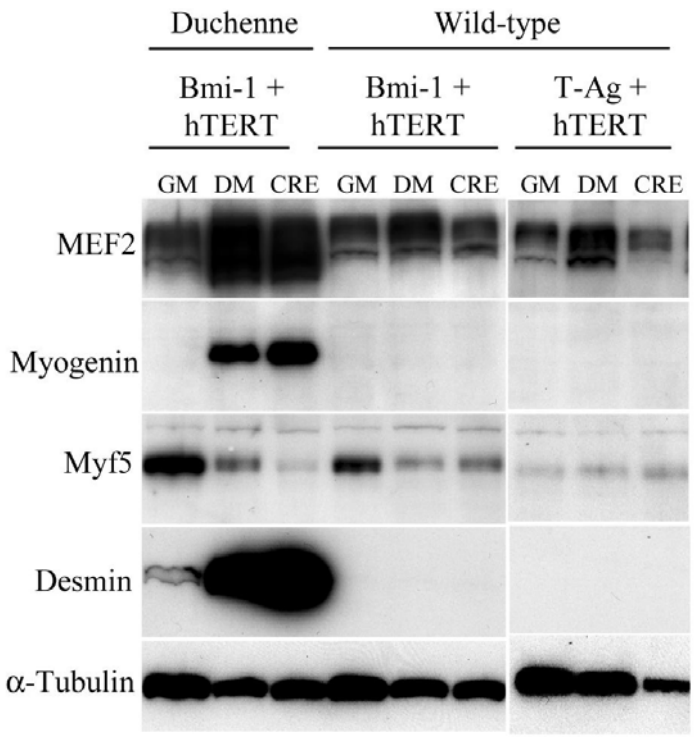
Duchenne
Wild-type
Bmi-1 + hTERT
Bmi-1 + hTERT
T-Ag + hTERT
GM DM CRE GM DM CRE GM DM CRE
MEF2
Myogenin
Myf5
Desmin
α-Tubulin

FIG. 9

2.3 CONCLUSION OF THE FIRST STUDY

In this article, we have demonstrated the feasability of lentiviral immortalization of myoblasts with bmi-1 and hTERT. This finally resulted in a new human cell line originating from a Duchenne patient, with long-term normal genotypic and phenotypic properties.

We have used combinations of lentivectors coding for TAg, Bmi-1, hTERT and Bcl-2 for the immortalization of human primary myoblasts. Different combinations of transgenes allowed immortalization of human primary myoblasts. They include *bmi-1* + *hTERT*, *TAg*, *TAg* + *hTERT* and *bmi-1* + *TAg* + *Bcl-2* + *hTERT*. Thus, the minimal cocktails of transgenes leading to immortalization were either *TAg* alone, or *bmi-1* + *hTERT*. Nonetheless, the immortalization with Bmi-1 and hTERT were shown to be respectful of the phenotype of primary cells than TAg-containing immortalization. Indeed, cells immortalized with TAg or TAg + hTERT or with Bmi-1 + TAg + Bcl-2 + hTERT had larger cell shape, increased cell proliferation, unusual growth in differentiation medium, and altered differentiation in differentiation medium, once these cells were compared to the normal primary cells. This was not the case for cells immortalized with Bmi-1 and hTERT, which were shown to have normal cell shape and proliferation in growth medium and also normal growth arrest and differentiation when placed in differentiation medium. Moreover, and in the contrary to TAg-containing cells, the cells immortalized with Bmi-1 and hTERT are karyotipically stable. All the cell lines that were tested here were non-tumorigenic after subcutaneous injection into *nude* mice. Even the TAg-immortalized cells were non-tumorigenic, although ongoing genetic and phenotypic changes were shown to occur in these cells. Nevertheless, it is probable that the karyotipic instability of TAg-immortalized cells might trigger sufficient mutations to lead to tumorigenesis, particularly if the cells are placed in a propper selective environment.

All these results suggest that the cells immortalized with Bmi-1 + hTERT may be used as cell lines, or for encapsidation purposes, because they are very similar to the primary cells.

However, most of the immortalized myoblasts were shown to have low and decreasing fusogenic capacities with time. We found also the evidence that both fusing and non-fusing clones existed. Even though this may seem intriguing, this fact might be explained from the

choice of the satellite cells we have been picked up in the beginning or our experiments, but also by the differentiation that these cells may have *in vitro*.

The satellite cell pool in Duchenne patients may be very different than the satellite cell pools in normal patients. Then, we may have picked in Duchenne patient the rare satellite cells that can be immortalized and can still retain long-term fusogenic properties. Different long-term fusogenic properties might also be derived from the heterogeneity of satellite cell population. Indeed, it is know that satellite cells are a heterogeneous population of cells, differing notably in their surface markers. This population is notably composed of side-populations cells and stem cells, and they may differ in their proliferative and differerentiation capacities, both *in vitro* and *in vivo* (Bachrach et al., 2004; Jankowski and Huard, 2004).

The *in vivo* differences of the satellite cell population still not reflect the extent of differentiation properties of this cell population. Indeed, it has been noticed that a single clone of satellite cells can lead *in vitro* to different myoblast types. For instance, it is well known that the clonal culture of one single satellite cell can lead *in vitro* to a heterogeneous population of cells, with 70% of differentiation-prone cells. Among the non-fusing myoblasts, some small cells can resume proliferation and can lead once again to fusing and non-fusing myoblasts. This notably suggests that there is *in vitro* the presence of stem cells among the population of non-fusing myoblasts (Baroffio et al., 1996).

The pre-cited evidences show that there is not only heterogenity of satellite cell pool, but also heterogenity of cells obtained from one single satellite cell. This may explain why we did not obtain a clone of normal myobasts immortalized with Bmi-1 and hTERT with long-term fusogenic properties. Then, probably that a large number of isolation procedures of satellite cells from normal donnors and different immortalizations procedures may help in the obtention of a normal clone of myoblast. However, this clone would not be isogenic compared to our obtained Duchenne clone.

A Duchenne cell line could be obtained in this study. It was shown to be phenotypically very similar to the normal primary myoblasts. Particularly, it has normal cell shape and proliferation properties compared to normal primary myoblasts. This cell line is also able to stop proliferating, to align, and to fuse, once placed in differentiation medium. Moreover, the fusion index of this cell line is about 70%, which is similar to the one found in normal primary cells. After more than one year in culture, the fusion index did not decrease, and the cells were not observed to acquire aberrant properties, in contrary to the TAg-immortalized cells. It was also verified that this cell line was not expressing Dystrophin. The obtained cell line was further analysed for expression of surface markers. It was shown that this cell line was expressing Myf5, one marker associated with normal myoblasts. These cells was also able to upregulate the differentiation markers that are associated with differentiation, including Mef2, Desmin and Myogenin. Only one difference could be noted between this cell line and normal primary myobasts: a slightly delayed speed of differentiation. Indeed, wheras normal maximum fusion was reached after 48 hours of differentiation, similar differentiaion of the Duchenne cell line was only observed after 64 hours. Delayed differentiation might be attributed either to the Duchenne phenotype, or to the immortalization itself. We believe it might be associated with the Duchenne phenotype, but only a control cell line with the same genetic background could prove this point. For this, a new cell line with normal genetic background had to be generated.

For *in vitro* studies, the obtention of a new cell line containing dystrophin, and isogenic compared to our Duchenne cell is interesting. This is why, in the next article, the introduction into our Duchenne cell line of Dystrophin was pursued. The purpose of this work was not only to obtain a control for our Duchenne cell line, but also to find one method to correct Dystrophin deficiency in these cells. This was done as one of the first steps before thinking to correct Dystophin deficiency in Duchenne patients.

CHAPTER 3: SECOND STUDY:

Complementation of Duchenne Muscular Dystrophy Muscle Cells by Dual High-Capacity Hybrid Viral Vectors

3.1 GENERAL INTRODUCTION FOR THE SECOND STUDY

After obtention of a new human Duchenne cell line, it was possible to study the correction of the human Duchenne Muscular Dystrophy *in vitro*. So, we aimed to generate dystrophin-positive cell lines, with the same genetic background as our previously described human Duchenne cell line.

Three main limitations have to be overcome to allow gene therapy in muscles. First, a convenient vehicle has to be chosen to carry the transgene. Second, the genetic material has to enter into muscle-derived cells. Finally, the transgene has to be able to be expressed in the long-term.

Different methods of delivery of dystrophin cDNA have been used in attempts to correct DMD. For instance, injection of plasmids has already been attempted even in one clinical trial (Romero et al., 2004). However, such method is thought to be difficult to use for gene therapy purposes throughout a whole body. Indeed, it might be difficult for a genetic material to be directed to all the muscles that might suffer from DMD. This is why gene therapy of DMD is often attempted on precursor cells, which then may divide and fuse with existing fibers. However, it is not sufficient to be able to target such cells. For instance, Adeno vectors can transduce both dividing and non-dividing cells, but they do not allow integration into the host genome, and usually remain episomal, like most plasmids. This was not convenient for correction of progenitor muscular cells, because the episomal transgene is not duplicated after serial divisions of these cells, and becomes probably diluted in the resulting population of cells (Moisset et al., 1998). In fact, only the vectors that can integrate transgenes are thought to be successful candidates for attempts of DMD therapy. For this purpose, the vectors derived from the Adeno Associated Virus, but also the lentiviral vectors, have been methods of choice.

AAV and Lentiviruses have the property to insert genes into the genome of cells, which is thought to be important for the long-term expression of a gene in dividing cells, specially in

immunocompetent animals (Greelish et al., 1999; Harper et al., 2002). However, these two viruses have different integration methods. Lentiviruses integrate in gene-rich regions of the host genome, and AAVs integrate into the chromosome 19. Lentiviral integration in gene-rich regions allows high level expression of a transgene. In the contrary, AAV is also is able to insert its genome into a specified locus of chromosome 19, *AAVS1* (Kotin et al., 1991). This feature would be particularly interesting to express a transgene without eventuality to cause severe damage to the host genome.

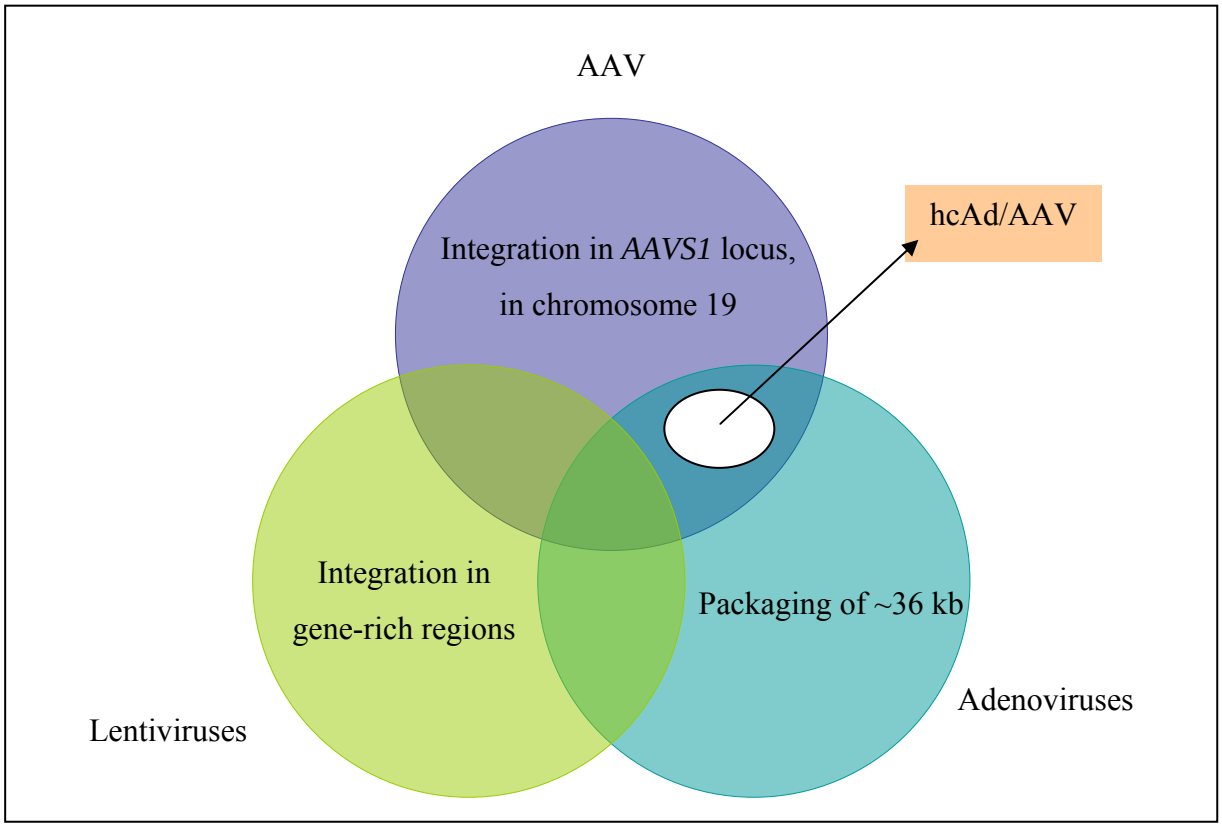

Figure 54 “Advantages” of different viruses, and fabrication of the hybrid vector hcAd/AAV.

Despite its property to integrate in a very specific location, AAV has a limited packaging capacity. This is also the case for lentiviruses, but not for Adenoviruses. Nevertheless, for correction of DMD, a sufficient packaging size is needed. Dystrophin is the largest gene known in nature, spanning over 2.4 Mb in the human genome. Its cDNA measures 14 kb, and very few viral-derived vehicles allow the delivery of such a large size of genetic informations. For instance, despite their utility in other protocols lentiviral vectors do not allow the

packaging of large cDNAs. This is why only truncated versions of the dytrophin cDNA (called microdystrophins) can be packaged into lentiviral vectors (Bachrach et al., 2004). However, it is suspected that the microdystrophins are not able to restore muscle strength, even though they may stop ongoing muscle injury (Harper et al., 2002). To circumvent these problems, a hybrid vector was designed, with the property of AAV to integrate in *AAVS1*, and the property of Adenovirus to have high packaging capacities (Figure 54). This vector was also designed to have the capacity to package two *dystrophin* expression units. The resulting hybdid vector was named dual high capacity Adenovirus/Adeno-Associated Virus (dual hcAd/AAV) vector. In target cells, additional expression of Rep proteins was shown to lead to insertion of the expression units of this vector into the *AAV1* locus of chromosome 19 (Goncalves et al., 2005). In the following article, a further modification has been done in the fiber used by this vector, to permit better entry into muscular cells. For this purpose, the vector was modified to harbor part of the Ad 50 fibers, and not the conventional Ad5 fibers. This finally allowed complementation of our Duchenne cell line with *dystrophin* cDNA and led to correction of the DMD phenotype *in vitro*, as shown by restoration of the Dystrophin-associated β-Dystroglycan Complex.

3.2 RESULTS OF THE SECOND STUDY

Efficient Genetic Complementation of Duchenne Muscular Dystrophy Muscle Cells by Retargeted Dual High-Capacity Hybrid Viral Vectors Encoding Full-Length Dystrophin

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Duchenne muscular dystrophy (DMD), a progressive muscle-wasting disease, is caused by mutations in the *dystrophin* gene, making it amenable to gene- or cell-based therapies. Another possible treatment entails the combination of both principles by transplantation of autologous myogenic cells after their genetic complementation. This approach requires efficient and stable transduction of these cells with recombinant *dystrophin* genes. Recently, we generated a dual high-capacity (hc) adenovirus (Ad)–adeno-associated virus (AAV) hybrid vector that can be used to deliver two full-length human dystrophin-encoding modules into target cells. Furthermore, we showed that hybrid vector transduction of human cells containing AAV Rep proteins leads to the insertion of foreign DNA into the *AAVS1* locus on chromosome 19. Here, we focused on improving hybrid vector entry into muscle cells from DMD patients. After having verified that these cells barely express the Coxsackie B and Ad receptor (CAR), which constitutes the primary attachment molecule for Ad serotype 5 (Ad5) fibers, we equipped dual hcAd/AAV hybrid vector particles with Ad serotype 50 fiber domains to achieve CAR-independent uptake. These retargeted vectors much more efficiently complemented the genetic defect of dystrophin-defective myoblasts and myotubes than their isogenic counterparts with conventional Ad5 fibers. Importantly, the accumulation of β-dystroglycan along the membranes of vector-treated DMD myotubes pointed to the proper assembly of dystrophin-associated glycoprotein complexes.

Key words: high-capacity; hybrid vector; adenovirus; adeno-associated virus; retargeting; human muscle cells; duchenne muscular dystrophy; full-length dystrophin; CAR; CD46

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a recessive genetic disorder caused by mutations in the *circa* 2.5-megabase X-linked *dystrophin* gene. Knowledge about the precise roles of the various proteins encoded by *dystrophin* is incomplete. However, it is recognized that the 3,685-amino acid isoform of the dystrophin protein (Dp427m) plays a key structural function in muscle fibers by connecting the intracellular cytoskeleton to the extracellular matrix via its association with a cluster of proteins in the sarcolemma called the dystrophin-associated glycoprotein complex (DGC) (Blake et al., 2002 [1]). In dystrophin-defective muscle tissue the absence of this linkage leads to sarcolemmal disruption followed by necrosis of muscle fibers. The damaged multinucleated fibers of DMD patients are initially repaired by fusion of myoblasts derived from resident muscle-committed progenitor cells called satellite cells. After exhaustion of these progenitors, functional muscle is substituted by adipose and connective tissues. This muscle wasting process is irreversible and inevitably ends in early death. Although the development of a therapy for DMD is fiercely pursued, there is currently no efficacious treatment available to prevent the disease or halt its progression.

A possible route to DMD therapy consists of combining gene- and cell-based approaches via the *ex vivo* introduction of corrective genetic information into dystrophin-defective myogenic cells followed by their autologous transplantation. The application of the patient's own cells will reduce the risk of implant rejection and may preclude the need for stringent immune suppression regimens. Experiments aiming at stable phenotypic rescue of dystrophin-defective myogenic cells e*x vivo* used herpes simplex virus amplicon vectors in combination with fluorescence-activated cell sorting and G418 selection of transduced mouse myoblasts (Bujold et al., 2002 [2]) or lentivirus vectors to insert foreign DNA in so-called side population cells isolated from murine muscle (Bachrach et al., 2004 [3]). This resulted in

stable transduction of target cells, most likely through non-targeted host cell DNA integration, with a fully functional full-length dystrophin-coding sequence (Bujold et al., 2002 [2]) or with a truncated version of it (Bachrach et al., 2004 [3]). A non-viral procedure based on electroporation of myogenic cells with plasmids encoding a dystrophin fusion protein and the ϕC31 integrase followed by G418 selection has also been explored (Quenneville et al., 2004 [4]). Although in the latter study site-specific chromosomal insertion of the full-length dystrophin-coding sequence was demonstrated, the cell mortality was high and the absolute transduction efficiency was low. Further improvements in gene delivery systems are warranted to achieve, simultaneously, efficient transduction of myogenic cells and targeted chromosomal integration of the full-length dystrophin-coding sequence to reduce the risk of insertional oncogenesis. Accordingly, we are developing a so-called dual high-capacity (hc) adenovirus (Ad)–adeno-associated virus (AAV) hybrid vector that can deliver two full-length human dystrophin-encoding expression units and can achieve maintenance of the foreign genetic information in the host cell by targeted integration into its chromosomal DNA (Gonçalves et al., 2005 [5]).

In this study, we sought to increase the efficiency of dual hcAd/AAV hybrid vector entry into muscle cells from DMD patients to improve the extent of phenotypic correction. We found that the primary cellular attachment molecule for Ad serotype 5 (Ad5), the Coxsackie B and Ad receptor (CAR) (Bergelson et al., 1997 [6]; Tomko et al., 1997 [7]), is barely detectable on the surface of DMD myoblasts, whereas CD46, recently shown to be a receptor for the CAR-independent species B Ads (Segerman et al., 2003 [8]; Gaggar et al., 2003 [9]), is abundantly expressed at the plasma membrane of these cells. Moreover, the cell surface amounts of CAR and CD46 in DMD myoblasts correlated very well with the transducibility of these cells by early region 1 (E1)-deleted Ad5 vectors carrying either Ad5 fibers or chimeric fibers with shaft and knob domains from the species B Ad serotype 50

(Ad50). These results provided a strong rationale to endow dual hcAd/AAV hybrid vectors encoding full-length human dystrophin with the same fiber chimeras. Hence, a new helper Ad5 vector encoding fibers consisting of basal shaft sequences from Ad5 and distal shaft and knob motifs from Ad50 was constructed. This new recombinant helper Ad was subsequently used to generate dual hcAd/AAV hybrid vector particles carrying the chimeric fibers. The rescue and propagation of these dual hcAd/AAV hybrid vector particles was as good as that of isogenic particles displaying conventional Ad5 fibers. Importantly, the tropism-modified dual hcAd/AAV hybrid vectors were far more efficient at complementing the genetic defect in DMD muscle cells than their isogenic, Ad5 fiber-bearing counterparts. Transduction of DMD muscle cells with retargeted dual hcAd/AAV hybrid vectors encoding full-length human dystrophin caused redistribution of β-dystroglycan, a key component of the DGC, to its normal location along the plasma membrane.

RESULTS

Surface Expression Levels of CAR and CD46 on DMD Myoblasts

For mice it has been shown that *CAR* expression is down regulated in the course of skeletal muscle cell maturation (Nalbantoglu et al., 1999 [10]). This phenomenon may, at least partially, explain the inefficient Ad5-mediated gene transfer into adult muscle cells both *in vitro* and *in vivo*. Indeed, Ad5 vectors much more efficiently transduced mature skeletal muscle fibers of CAR transgenic mice than those of non-transgenic control animals (Nalbantoglu et al., 2001 [11]). Ad vectors containing fiber motifs that bind receptors other than CAR have the potential to effectively by-pass the CAR-dependent entry route. Ad serotypes from species B do not bind CAR but, alternatively, can utilize CD46 as a receptor (Segerman et al., 2003 [8]; Gaggar et al., 2003 [9]). Therefore, cells that express CD46 on their surface can be regarded as potential targets for genetic modification by Ad5 vectors carrying fiber domains from species B Ads.

To evaluate plasma membrane expression of CAR and CD46 in myoblasts isolated from DMD patients, primary and *TERT*- and *Bmi*-1-immortalized cells (Cudré-Mauroux et al., 2003 [12]) were labeled with CAR- and CD46-specific monoclonal antibodies (MAbs) and subjected to flow cytometrical analyses. Human epithelial cervical carcinoma cells (HeLa) served as positive controls for anti-CAR and anti-CD46 immunoreactivity. The results depicted in Fig. 1 clearly reveal that CAR is barely detectable on the surface of dystrophin-defective human myoblasts as shown by the virtually complete overlap of the histograms of cell cultures incubated with the CAR-specific MAb and of cells labeled with the control MAb. On the contrary, DMD myoblasts incubated with the anti-CD46 MAb yielded histograms with a strong rightward shift as compared to those corresponding to control cultures, demonstrating

that the large majority of these cells have significant amounts of CD46 on their surface (Fig. 1).

Gene Transfer to DMD Muscle Cells by Replication-Defective Ad5 Vectors Displaying Ad50 Fiber Domains is Superior to that by Conventional Ad5 Vectors

Subsequently, we investigated whether a direct correlation exists between CAR and CD46 surface levels and the permissiveness of DMD myoblasts to E1-deleted Ad5 vectors carrying fiber shaft and knob domains from either CAR-dependent (i.e., Ad5) or CAR–independent (i.e., Ad50) serotypes. To this end, these cells were transduced with the unmodified Ad5 vector or with the fiber-modified Ad5F50 vector at different multiplicities of infection (MOIs). Both vector types harbored the same *EGFP* expression unit to monitor and accurately quantify relative gene transfer activities. Both direct fluorescence microscopy (Fig. 2A) and flow cytometry (Fig. 2B) were performed. Fluorescence microscopy revealed that gene delivery into DMD myoblasts by the fiber-modified Ad5F50 vectors is much more efficient than that achieved by conventional Ad5 vectors (Fig. 2A). Flow cytometry data are presented both as the frequency of transgene-expressing cells (Fig. 2B, upper graph) and as the average amount of EGFP per cell (Fig. 2B, lower graph). The latter parameter is expressed in terms of mean fluorescence intensity (MFI) values. The flow cytometrical analyses showed that at 10 infectious units (IU) per cell of Ad5F50 vector, virtually all cells expressed the transgene (Fig. 2B, upper graph). When the same vector was applied at 1 and 3 IU per cell *circa* 40 and 80% of the myoblasts became EGFP-positive, respectively. Approximately 30-fold higher dosages were necessary to achieve similar frequencies of *EGFP*-expressing cells with the control Ad5 vector (Fig. 2B, upper graph). Moreover, at every MOI, the loads of reporter protein per cell were consistently higher in cells infected with Ad5F50 than those in Ad5 vector-transduced cells. In fact, there was a clear vector dose-related increase in MFI values

in cells incubated with Ad5F50 but much less so in cells exposed to the Ad5 vector (Fig. 2B, lower graph). This indicates that few DNA copies of the latter vector reached the nuclei of DMD myoblasts and suggests that Ad5 vector particles enter these cells through low-affinity interactions with non-specific attachment molecules. Finally, the chimeric fiber-bearing Ad5F50 vector also clearly outperformed the Ad5 vector in inducing foreign DNA expression in differentiated DMD myotubes (Fig. 2C, compare the second with the fourth row). These data are in accordance with previous human myoblast transduction experiments using a luciferase-encoding E1-deleted Ad vector carrying Ad50 fiber domains (Havenga et al., 2002 [13]). In addition, they demonstrate for the first time the superiority of this type of vectors over unmodified Ad5 vectors in transducing differentiated human myotubes.

Generation of Dual hcAd/AAV Hybrid Vectors Displaying Ad50 Fiber Domains is as Efficient as that of Ad5 Fiber-Bearing Dual hcAd/AAV Hybrid Vectors

The previous data provided a strong rationale to alter the tropism of full-length human dystrophin-encoding dual hcAd/AAV hybrid vectors by exchanging the Ad5 fiber domains for those of Ad50. To this end, we constructed the E1-deleted Ad helper vector Ad.floxedΨ.F50 to produce dual hcAd/AAV hybrid vectors with Ad50 fiber domains (dual hcAd/AAV.F50 vectors) instead of conventional Ad5 fibers (Fig. 3A). Three types of hybrid vector shuttle plasmids were used in combination with either Ad.floxedΨ.F50 or the standard E1-deleted helper Ad vector Ad.floxedΨ according to the scheme shown in Fig. 3B. The pAd/AAV.EGFP shuttle construct (Fig. 3B) was used to monitor and compare the rescue and propagation profiles of hybrid vector particles using each type of helper Ad vector. Preparations of the EGFP-encoding dual hcAd/AAV.F50 and dual hcAd/AAV vectors had, after four passages, titres of 1.2×10^9 and 1.6×10^9 HeLa cell-transducing units (HTU) per ml,

respectively, as determined by endpoint titration using flow cytometry as readout. We thus conclude that dual hcAd/AAV.F50 hybrid vectors can be produced with similar efficiencies as their isogenic counterparts carrying Ad5 fibers.

Structure of Genomes Packaged in Conventional and Fiber-Modified Dual hcAd/AAV Hybrid Vector Particles

Previous experiments showed that dual hcAd/AAV hybrid vector genomes consist of tail-to-tail dimers. Experimental evidence pointed to a dimerization process that involves the progression of the Ad DNA replication complex through the AAV inverted terminal repeat (ITR) hairpin structure as opposed to intermolecular recombination between two hybrid vector monomers at the AAV ITRs (Gonçalves et al., 2005 [5]). The use of Ad.floxedΨ.F50 as helper was expected to change neither this process nor the structure of the packaged hybrid vector DNA. Nonetheless, we decided to confirm this postulate and to investigate the stability of dual hcAd/AAV.F50 hybrid vector genomes upon serial propagation. To this end, producer cells were transfected with the hybrid vector shuttle plasmid pAd/AAV.eDYS and subsequently infected with either Ad.floxedΨ.F50 or Ad.floxedΨ according to the scheme depicted in Fig. 3A. After four rounds of propagation, DNA was extracted from purified hybrid vector particles, digested with *Hinc*II and subjected to Southern blot analysis with a mixture of two vector-specific probes. The autoradiograph shown in Fig. 4A demonstrates that, regardless of the recombinant helper Ad used, packaged hybrid vector DNA consists of tail-to-tail dimers (Fig. 4B). This structural organization together with the absence of any other DNA forms confirm previous experiments (Gonçalves et al., 2005 [5]) and show that hybrid vector genomes propagated with the aid of Ad.floxedΨ.F50 are as stable as those amplified with the support of Ad.floxedΨ.

Transfer of the Full-Length Human Dystrophin-Coding Sequence into DMD Myoblasts by Dual hcAd/AAV Hybrid Vectors Displaying Ad50 Fiber Domains is Superior to that by Conventional Dual hcAd/AAV Hybrid Vectors

Next, we evaluated the gene transfer levels in DMD myoblasts transduced with unmodified or fiber-modified dual hcAd/AAV.F50 hybrid vectors. To this end, we made use of hybrid vectors encoding a fusion product between EGFP and the full-length human dystrophin protein (eDYS). This combination allowed straightforward and accurate quantification of recombinant *dystrophin* transfer and expression in target cells. At 1, 3 and 10 HTU per cell, the average percentages of dystrophin-positive DMD myoblasts were 112-, 54- and 18-fold higher for the tropism-modified vectors than for the control vectors (Fig. 5). These results show that the highly efficient transduction of human myoblasts by fiber-modified E1-deleted Ad vectors carrying reporter genes can be extended to dual hcAd/AAV.F50 hybrid vectors encoding therapeutic proteins.

Genetic Complementation of DMD Muscle Cells by Dual hcAd/AAV Hybrid Vectors Displaying Ad50 Fiber Domains

To test the capacity of dual hcAd/AAV.F50 hybrid vectors to deliver to and express in DMD muscle cells the full-length human dystrophin-coding sequence, two stocks of dual hcAd/AAV.F50 hybrid vectors encoding both DsRed and the human dystrophin protein were generated with the aid of Ad.floxedΨ.F50 (Fig. 3A). These vector preparations had titers of

1.4×10^9 and 3.2×10^9 HTU per ml as determined by limiting dilution assays and DsRed-based flow cytometry performed 48 h post-infection. Fiber-modified hybrid vectors expressing two different transgenes can thus be produced equally well as those encoding a single gene product.

Next, DMD myoblasts were incubated with the two-gene-containing fiber-modified hybrid vector particles at an MOI of 30 HTU per cell. Mock-transduced cultures served as a control in these experiments. The DMD myoblasts were either kept under growth conditions or were induced to differentiate into myotubes. Direct fluorescence microscopy and immunofluorescence staining of mock-infected myoblasts (Figs. 6A, B and C) and myotubes (Figs. 6G, H and I) showed neither DsRed- nor dystrophin-specific signals, respectively. The same assays applied to cells exposed to the dual hcAd/AAV.F50 hybrid vector revealed robust and widespread synthesis of DsRed and dystrophin proteins in myotubes (Figs. 6K, L, M, N, O and P) as well as in their precursor cells (Figs. 6D, E and F). Although in the majority of cells and syncytia both signals could be simultaneously detected, their intracellular distribution differed (Figs. 6F, M and P). DsRed permeated the nuclei and was more homogeneously distributed throughout the cytoplasm than dystrophin. In fact, accumulation of dystrophy along the plasma membrane of DMD myotubes was observed indicating correct localization of the protein (see arrowheads in Figs. 6N, O and P and in the corresponding insets). Importantly, neither the vector itself nor the encoded products blocked myotube differentiation since both mock- and vector-transduced DMD myoblasts could form large multinucleated myotubes as evidenced by staining with the nuclear dye Hoechst 33342 and a skeletal myosin heavy chain (skMHC) type II-specific MAb (Fig. 6J). Taking all data together, we conclude that moderate MOIs of dual hcAd/AAV.F50 hybrid vectors suffice to effectively complement the genetic defect of myoblasts and myotubes from DMD patients. The ability of dual hcAd/AAV.F50 hybrid vectors to also direct synthesis of full-length

human dystrophin in differentiated, non-dividing, dystrophin-defective muscle cells was clearly established after adding the two-gene-encoding dual hcAd/AAV hybrid vector at an MOI of 30 HTU per cell to DMD myotube cultures and performing dystrophin immunocytochemistry (Figs. 6Q, R, S, T, U and V).

The absence of dystrophin prevents the proper assembly of the DGC at the sarcolemma of muscle cells (Blake et al., 2002 [1]). An essential element of a functional membrane-associated DGC is the type I transmembrane protein β-dystroglycan. We therefore asked whether recombinant dystrophin synthesis directed by dual hcAd/AAV.F50 hybrid vectors resulted in rescue of DGC assembly by examining the distribution of β-dystroglycan in mock-infected (Figs. 7A, C, E and G) and vector-treated (Figs. 7B, D, F and H) DMD myotubes. Immunofluorescence microscopy revealed that in mock-infected DMD myotubes the β-dystroglycan staining was rather weak and diffuse (Figs. 7 C and G) whereas in genetically modified DMD myotubes it was stronger and more structured (Figs. 7D and H). Indeed, in vector- but not in mock-transduced DMD myotubes β-dystroglycan-specific staining lining the plasma membrane could clearly be discerned suggesting normal DGC formation (see arrowheads in Fig. 7H).

DISCUSSION

To guide the development of dystrophin-encoding dual hcAd/AAV hybrid vectors for the effective genetic complementation of DMD myoblasts, we analyzed the expression of attachment receptors for human Ads on the surface of these therapeutically relevant muscle precursor cells. The plasma membranes of DMD myoblasts were found to contain hardly any CAR and significant amounts of CD46, which can serve as receptors for Ad5 and Ad50, respectively (Bergelson et al., 1997 [6]; Tomko et al., 1997 [7]; Segerman et al., 2003 [8]; Gaggar et al., 2003 [9]). These data were consistent with the outcome of transduction experiments of DMD myoblasts using E1-deleted Ad5 vectors displaying unmodified fibers or chimeric fibers carrying shaft and knob sequences from Ad50. Dystrophin-defective myotubes were also much more efficiently transduced by the fiber-modified than by conventional Ad5 vectors. These results were subsequently translated into a context involving dual hcAd/AAV hybrid vectors encoding the 427-kDa muscle-specific isoform of the human dystrophin protein. Flow cytometry and immunofluorescence microscopy revealed that a tropism-modified version of dual hcAd/AAV hybrid vectors corrected very efficiently the genetic defect of DMD muscle cells resulting in the clustering of both the recombinant dystrophin and the endogenous β-dystroglycan proteins along the plasma membrane of myotubes. The relocation of β-dystroglycan to the periphery of myotubes is in accordance with previous results obtained in humans where the presence of the complete dystrophin protein was shown to be important for stable membrane integration of this DGC component (Helliwell et al., 1994 [14]).

An important aspect of the present study is the use of human instead of rodent muscle cells. So far, the vast majority of studies on Ad-based gene transfer to skeletal muscle precursor cells deployed surrogate murine systems such as those based on the mouse myoblast cell line C2C12 and on primary myoblasts isolated from the DMD mouse model *mdx*. Although these systems are undoubtedly useful they cannot accurately mimic human cell-specific parameters. For instance, the molecular composition and expression levels of cellular receptors and co-receptors engaged by *Adenoviridae* members may differ between human and rodent cells.

Early region-deleted Ad vectors are of limited use for DMD gene therapy because they cannot accommodate the full-length human dystrophin-coding sequence and encode immunogenic viral gene products. For these reasons, attention has been directed to hcAd vectors, which do not encode viral gene products and can induce the synthesis of the complete, and thus fully functional, dystrophin protein (Kochanek et al., 2001 [15]). However, hitherto hcAd vectors have mostly been based on Ad5, which uses CAR to enter cells. Hence, these vectors do not transduce muscle cells efficiently. Redirecting hcAd vectors to alternative cell surface molecules is an attractive approach to by-pass the CAR dependency. By using capsid-modified helper Ad vectors carrying an RGD peptide or a polyhistidine stretch in the HI loop of the fiber knob, Biermann and coworkers altered the tropism of hcAd vectors (Biermann et al., 2004 [16]). More recently, Bramson and colleagues generated hcAd particles incorporating RGD or polylysine sequences into the same fiber loop (Bramson et al., 2004 [17]). These authors demonstrated that vectors with the polylysine-modified knobs yielded 12- and 21-fold higher reporter gene activities in mouse C2C12 myoblasts and myotubes, respectively, than control vectors. The effect of the latter fiber modification on the gene transfer activity in skeletal muscle was less pronounced. Another genetic method to alter the tropism of hcAd vectors consists of endowing them with fibers derived from alternative

Ad serotypes. Using hcAd vectors displaying Ad serotype 35 fiber domains, Balamotis and colleagues showed efficient transduction of human cancer and blood cell types with reporter genes (Balamotis et al., 2004 [18]). To by-pass the CAR dependency, we provided dual hcAd/AAV hybrid vector particles with Ad50 fiber domains. In side-by-side rescue and propagation experiments, fiber-modified and control vectors encoding different heterologous DNA combinations including recombinant *dystrophin* expression units reached comparable titers. Moreover, we demonstrated that two-gene-encoding dual hcAd/AAV hybrid vectors particles can be produced as good as single-gene-encoding versions. This feature can be exploited to, for instance, combine full-length human dystrophin synthesis with expression of myogenic or cell survival genes to enhance muscle regeneration or preserve myoblast survival after transplantation, respectively.

The very efficient genetic complementation of dystrophin-defective human muscle cells by tropism-modified dual hcAd/AAV hybrid vectors encoding the complete human dystrophin protein constitute an important step towards an autologous cell therapy for DMD. Potentially useful target cells for DMD cell therapy are satellite cells and their myoblast progeny (Skuk and Tremblay, 2003 [19]) but also recently isolated and characterized stem cells that have the capacity to differentiate into or fuse with muscle cells (Cossu and Sampaolesi, 2004 [20]). Stable transduction of these myogenic cells is best accomplished through targeted host DNA integration to minimize positional effects on therapeutic gene expression and to dispel the emergence of insertional oncogenesis. Conventional hcAd vectors do not fulfill this requirement. Dual hcAd/AAV hybrid vectors, on the other hand, can stably transduce human cells through AAV Rep-mediated site-specific insertion of foreign DNA into the host cell genome (Gonçalves et al., 2005 [5]). Ongoing research in our laboratory focuses on the development of methods to introduce and express in cells with myogenic potential AAV Rep activities in a transient manner.

MATERIALS AND METHODS

Recombinant DNA. The construction of the hybrid vector shuttle plasmids pAd/AAV.EGFP and pAd/AAV.eDYS has been detailed elsewhere (Gonçalves et al., 2005 [5]). The DsRed- and human dystrophin-encoding construct pAd/AAV.DsRed.DYS was made by insertion of a human *UbiC* promoter fragment into the unique *NruI* site of the hybrid vector shuttle plasmid pAd/AAV.eDYS (Gonçalves et al., 2005 [5]). The *NruI* recognition sequence precedes the *dystrophin* ORF and lies immediately downstream of the simian virus 40 pA of the *DsRed* expression unit. The *UbiC* promoter fragment was amplified from plasmid pUbCR#1 (Nenoi et al., 1996 [21]) (a kind gift from M. Nenoi) by polymerase chain reaction using the Platinum Taq DNA polymerase High Fidelity enzyme mixture (Invitrogen) together with primers hUbiCR (5'-CGTAAGCTTGTCTAACAAAAAAGCCAAAAACGG-3') and M13F (5'-GACGTTGTAAAACGACGGCCAGT-3'). The amplified DNA was digested with *Hind*III (Fermentas) and blunt-ended with the large fragment of *Escherichia coli* DNA polymerase I (Fermentas) and the resulting 2,2-kb product was ligated to *Nru*I-linearized and shrimp alkaline phosphatase-treated pAd/AAV.DsRed.

Cells. HeLa (American Type Culture Collection) and 911 (Fallaux et al., 1996 [22]) cells were kept in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 5% fetal bovine serum (FBS) (Invitrogen). For the production of dual hcAd/AAV hybrid vectors we used Per.tTA.Cre76 cells. These Ad5 *E1*- and bacteriophage P1 *cre*-expressing cells were cultured as detailed before (Gonçalves et al., 2002 [23]). Cultures of primary DMD myoblasts were kindly provided by P.A. 't Hoen. The growth and differentiation conditions for the *TERT*- and *BmiI*-immortalized DMD myoblasts have been described elsewhere (Cudré-Mauroux et al., 2003 [12]). These cells have been shown to maintain a diploid

karyotype and the ability to form myotubes upon extensive subculturing (Cudré-Mauroux et al., 2003 [12]). Importantly, cultures of *TERT*- and *BmiI*-immortalized DMD myoblasts are devoid of fibroblasts. In contrast, primary myoblast cultures are often contaminated with fibroblasts, which tend to outgrow the muscle progenitor cells and may confound interpretation of gene transfer experiments. Their human origin together with the features mentioned above makes cultures of the immortalized dystrophin-defective myoblasts an excellent *ex vivo* model to test strategies aiming at the rescue of dystrophin synthesis.

Immunophenotyping. The expression of CAR and CD46 on the surface of HeLa cells and of DMD myoblasts was determined by flow cytometry. Aliquots of each cell type were incubated with MAbs directed against CAR (Hsu et al., 1988 [24]) or CD46 (Becton Dickinson). The CAR-specific MAb is of the IgG1 isotype and was isolated from cell line RmcB, whereas the anti-CD46 antibody is of the IgG2a isotype and was derived from hybridoma E4.3. The CD46-specific MAb was coupled to the fluorescein isothiocyanate (FITC) fluorochrome, allowing direct detection. The CAR-specific MAb was detected with a secondary FITC-conjugated goat anti-mouse antibody (Becton Dickinson). This secondary antibody was added to the cells immediately after the addition of the primary CAR-specific MAb. As controls for non-specific binding, we used either fluorescently labeled isotype-matched MAbs specific for a non-human antigen (negative control for CD46 staining) (Becton Dickinson) or incubated the cells exclusively with the FITC-labeled secondary antibody (negative control for CAR staining). After a 30-min incubation at 4°C the cells were washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (Sigma-Aldrich) and were analyzed directly using a FACSort flow cytometer (Becton

Dickinson). Typically, 10,000 events were acquired and the data were stored in list mode files and processed using CellQuest Software (Becton Dickinson).

Replication-defective Ad vectors with the E1 deleted. The production and titration of the E1-deleted Ad vectors Ad5 and Ad5F50 have been described previously (Knaän-Shanzer et al., 2001 [25]). The number of virus particles (VP) per ml was determined using the spectrophotometric method described by Maizel and colleagues (Maizel et al., 1968 [26]). The number of IU per ml was determined by $TCID_{50}$ assays using the E1-complementing 911 cells (Fallaux et al., 1996 [22]). The VP/IU ratios of the Ad5 and Ad5F50 vector stocks were 11 and 5, respectively. Details about the generation and characterization of the helper Ad vector Ad.floxedΨ have been specified elsewhere (Gonçalves et al. 2002 [23]). The fiber-modified recombinant helper Ad Ad.floxedΨ.F50 was produced in the same manner has Ad.floxedΨ except that the shuttle plasmid pAd.floxedΨ (Gonçalves et al., 2002 [23]) was transfected together with cosmid pWE/Ad.Afl-rITR.pac.RFib50 instead of pWE/Ad.Afl-rITR.pac.RFib5 (Knaän-Shanzer et al., 2001 [25]).

Production of dual hcAd/AAV hybrid vectors. Dual hcAd/AAV hybrid vector stocks were obtained as previously described (Gonçalves et al., 2005 [5]). Titres were determined through limiting dilutions on HeLa indicator cells followed by flow cytometry (Gonçalves et al., 2005 [5]).

Vector DNA extraction and Southern blot analysis. The extraction, purification and Southern blot analysis of dual hcAd/AAV hybrid vector DNA have been specified elsewhere (Gonçalves et al., 2005 [5]).

Transduction conditions. Gene transfer into DMD myoblasts by the EGFP-encoding Ad5 and A5F50 vectors was tested as follows. One hundred thousand immortalized DMD myoblasts were seeded in triplicate in wells of 24-well plates (Greiner). The next day, the cells were incubated for 3 h at 37°C either with Ad5 or Ad5F50 vector particles at 1, 3, 10, 30 and 100 IU per cell. Both direct fluorescence microscopy and flow cytometry were performed at 48 h post-infection. Gene transfer into DMD myoblasts by dual hcAd/AAV hybrid vectors was carried out as follows. Immortalized DMD myoblasts were seeded at a density of 3.3×10^5 cells per well of 6-well plates (Greiner). After overnight incubation in a humidified air-5% CO_2 atmosphere, the cells were transduced with the DsRed- and dystrophin-encoding dual hcAd/AAV.F50 hybrid vector at 30 HTU per cell for 3 h at 37°C. The next day, infected and mock-infected cultures were either exposed to myotube differentiation medium and cultured in a 10% CO_2 atmosphere or were maintained in normal growth medium in a 5% CO_2 atmosphere. Direct fluorescence microscopy (to visualize DsRed) and immunofluorescence microscopy following staining with dystrophin- or skMHC type II-specific MAbs were performed at 6 days post-infection.

DMD myotube formation was induced by culturing 1.2×10^6 immortalized DMD myoblasts in wells of 6-well plates (Greiner) for 5 to 6 days in differentiation medium in a 10% CO_2 atmosphere. The resulting DMD myotube cultures were exposed for 3 h to Ad5, Ad5F50 or the dual hcAd/AAV.F50 hybrid vector encoding both DsRed and dystrophin. The Ad5 and

Ad5F50 vectors were applied at an MOI of 100 IU per cell, whereas the dual hcAd/AAV.F50 hybrid vector was used at an MOI of 30 HTU per cell. These dosages were calculated on the basis of the number of myoblasts present immediately prior to the start of the differentiation process. Mock-transduced cultures provided negative controls for (immuno)fluorescence microscopy.

Dystrophin, β-dystroglycan and skMHC type II immunostaining. Dystrophin was detected using NCL-DYS2 (Novocastra), a carboxy terminus-specific MAb of the IgG1 isotype together with the Alexa Fluor 488 goat anti-mouse IgG1 secondary antibody (Molecular Probes). These primary and secondary antibodies were diluted 40- and 500-fold, respectively, in PBS containing 10 mM glycine and 5% FBS. The presence of fast-twitch skMHC was investigated with the aid of the IgG1 molecules secreted by hybridoma MY-32 (Sigma) in combination with the Alexa Fluor 568 goat anti-mouse IgG1 secondary antibody (Molecular Probes). These primary and secondary antibodies were used at dilutions of 1:200 and 1:500, respectively. The β-dystroglycan immunofluorescence assays were performed with a 50-fold dilution of the IgG1 MAb NCL-b-DG (Novocastra) and the aforementioned Alexa Fluor 488 antibody. Except for the use of other antibodies, a reduction in the concentration of Triton-X to 0.1% (vol/vol) and the fixation with 10% formalin for 30 min, the protocol of Gonçalves et al. (2005) [5] was followed.

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LEGENDS

FIG. 1. Analysis of the expression of CAR and CD46 on the surface of DMD myoblasts. Presented are histograms of flow cytometry data from HeLa cells (upper panels) and from representative primary (middle panels) and immortalized (lower panels) DMD myoblasts stained with antibodies directed against CAR (left panels) and CD46 (right panels). The black lines represent background fluorescence of samples incubated with control antibodies.

FIG. 2. Comparative analysis of the transduction of (A, B) DMD myoblasts and (C) myotubes by Ad5 or Ad5F50 vectors encoding EGFP using (A, C) direct fluorescence microscopy and (B) flow cytometry. The flow cytometry data are presented as the frequency of EGFP-positive cells (upper graph) and their MFI values (lower graph). Target cells were incubated with each vector for 3 h at the indicated dosages and analyzed at 48 h post-infection.

FIG. 3. Strategy used to retarget dual hcAd/AAV hybrid vectors. (A) The genome of the E1-deleted (ΔE1) helper Ad vector Ad.floxedΨ.F50 differs from the Ad.floxedΨ DNA in that the Ad5 sequence encoding most of the shaft and the complete knob domain is substituted by homologous sequences derived from Ad50. In both helper Ad vectors, the packaging elements (Ψ) are framed by a direct repeat of bacteriophage P1 loxP sites (solid triangles). This construction allows negative selection against the assembly of helper Ad particles in bacteriophage P1 cre-expressing cells. In hybrid vector shuttle plasmids, the eukaryotic foreign DNA is flanked at one end by the 5' Ad5 sequence encompassing the Ad origin of DNA replication and packaging signal (shaded box designated Ad.ITR/Ψ) plus the AAV

integration efficiency element (solid circle labeled p5IEE) and at the other end by an AAV ITR (solid oval designated AAV.ITR). The *β-lactamase* (amp) and the prokaryotic origin of replication (ori) are also indicated. Rescue of the hybrid vector DNA into Ad capsids is initiated by releasing it from the plasmid backbone at the indicated restriction enzyme recognition sites (RE) and transfecting the digestion products into Per.tTA.Cre76 cells. These cells are subsequently infected with Ad.floxedΨ.F50 to supply in trans all Ad DNA replication activities and Ad structural proteins (among these the chimeric fiber proteins) needed for the amplification and packaging of the dual hcAd/AAV hybrid vector DNA, respectively. Replication- and packaging-competent tail-to-tail dimers are assembled from input monomers due to Ad-dependent DNA replication in concert with AAV ITR-mediated dimerization (Gonçalves et al., 2005 [5]). The resulting hybrid vector particles display on their surface Ad50 fiber domains and can be amplified by serial propagation in Ad.floxedΨF50-infected producer cells. (B) In the present study, hybrid vector shuttle plasmids containing three different combinations of foreign DNA were used. Construct pAd/AAV.EGFP has an expression unit consisting of the human elongation factor 1α (EF1α) gene promoter (open box with broken arrow), the *EGFP* open reading frame (ORF) (solid box) and the human growth hormone gene polyadenylation signal (pA) (arrowhead). In this construct the human dystrophin-coding sequence is used as stuffer DNA (open box). Plasmid pAd/AAV.eDYS contains an expression module that encodes a fusion product (eDYS) between the EGFP (solid box) and full-length human dystrophin (open box). This sequence is framed by the EF1α gene promoter (open box with broken arrow) and the rabbit β-globin gene pA (arrowhead). Construct pAd/AAV.DsRed.DYS harbors two transcription units. The first of these units consist of the EF1α gene promoter (open box with broken arrow), the *DsRed* ORF (shaded box) and the simian virus 40 pA (arrowhead). The second expression

cassette is composed of the human *ubiquitin C* (*UbiC*) promoter (solid box with broken arrow), the full-length human dystrophin-coding sequence (open box) and the rabbit β-globin gene pA (arrowhead).

FIG. 4. (A) Structural characterization of dual hcAd/AAV hybrid vector genomes packaged in Ad5 capsids (F5) or in Ad5 capsids with fiber shaft and knob sequences derived from Ad50 (F50). DNA extracted from purified hybrid vector particles obtained after four rounds of propagation on Per.tTA.Cre76 cells infected with Ad.floxedΨ (right lane) or with Ad.floxedΨ.F50 (middle lane) was digested with *Hinc*II, subjected to agarose gel electrophoresis and analyzed by Southern blot hybridisation with a mixture of probes 6563 and 2832. Lane M, GeneRuler DNA Ladder Mix molecular weight marker (Fermentas). (B) *Hinc*II restriction map of dimeric hybrid vector molecules with a tail-to-tail genomic organization. Solid bars represent DNA probes 6563 and 2832 drawn in relation to their target DNA sequences. Numerals correspond to restriction DNA fragment sizes in kilobases.

FIG. 5. Comparative analysis of the transduction of DMD myoblasts by unmodified (F5) or fiber-modified (F50) dual hcAd/AAV hybrid vectors encoding eDYS. Target cells were incubated for 3 h with the specified vector dosages. At 72 h post-infection, the percentages of dystrophin-positive cells were determined by flow cytometry using the amino-terminal EGFP tag of eDYS for detection. Mock-infected myoblasts were used to establish background fluorescence.

FIG. 6. Genetic complementation of DMD muscle cells by fiber-modified dual hcAd/AAV hybrid vectors. DMD myoblasts were either mock-infected (A, B, C) or incubated with a dual hcAd/AAV.F50 hybrid vector coding for DsRed and dystrophin at 30 HTU per cell (D, E, F).

Direct (A, D) and indirect (B, E) fluorescence microscopy were performed after culturing the myoblasts for 6 days in growth medium. DsRed- and dystrophin-specific signals were also merged (C, F). DMD myotubes differentiated from mock-infected (G, H, I, J) or from dual hcAd/AAV.F50 hybrid vector-transduced myoblasts (K, L, M, N, O, P) were analyzed at 6 days post-infection by direct fluorescence microscopy (G, K, N) and by immunofluorescence microscopy following staining with MAbs directed against dystrophin (H, L, O) or skMHC type II (J). In panels I, M and P, the DsRed- and dystrophin-specific signals were merged. Differentiated DMD myotubes were also directly transduced with the two-gene-containing dual hcAd/AAV.F50 hybrid vector at 30 HTU per cell and analyzed by direct (Q, T) and indirect (R, U) fluorescence microscopy at 32 h post-infection. DsRed- and dystrophin-specific signals were also merged (S, V). The nuclei in each of the DMD myotube cultures were stained with the blue-emitting fluorochrome Hoechst 33342.

FIG. 7. Rescue of β-dystroglycan membrane association in DMD myotubes following transduction with fiber-modified dual hcAd/AAV hybrid vectors coding for DsRed and full-length human dystrophin. Differentiated DMD myotubes were either mock-infected (A, C, E and G) or transduced with the two-gene-containing dual hcAd/AAV.F50 hybrid vector at 30 HTU per cell (B, D, F and H) and analyzed at 32 h post-infection by direct fluorescence microscopy (A, B, E and F) and by immunofluorescence microscopy using a MAb specific for β-dystroglycan (C, D, G and H). The arrowheads in panel H point to β-dystroglycan accumulation along the plasma membrane of hybrid vector-treated DMD myotubes. Nuclei were stained with the blue-emitting fluorochrome Hoechst 33342.

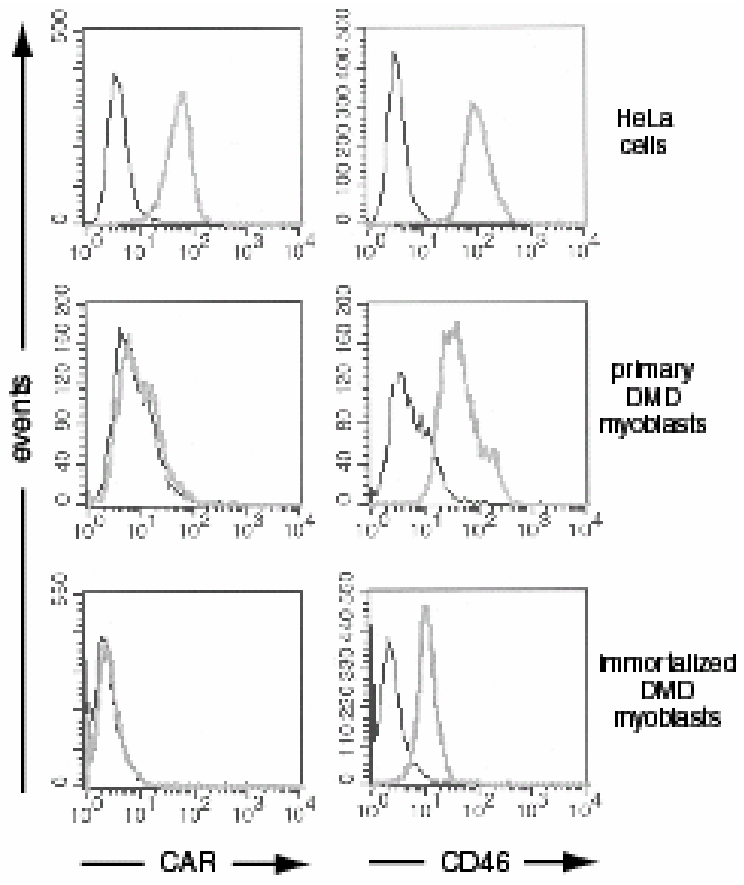
events
HeLa cells
primary DMD myoblasts
Immortalized DMD myoblasts
CAR
CD46

FIG. 1

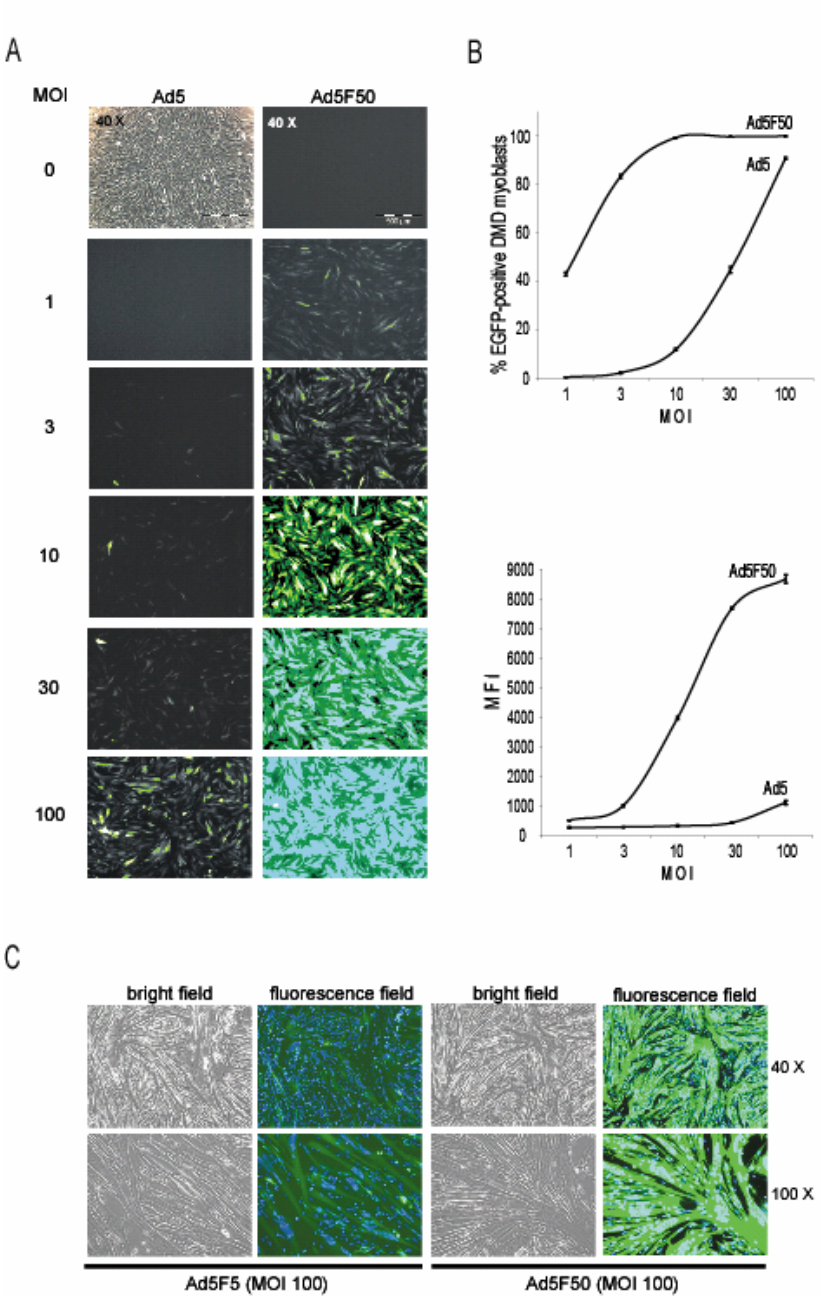
A
MOI
Ad5
Ad5F50
40 X
0
1
3
10
30
100
B
% EGFP-positive DMD myoblasts
Ad5F50
Ad5
MOI
MFI
C
bright field
fluorescence field
bright field
fluorescence field
40 X
100 X
Ad5F5 (MOI 100)
Ad5F50 (MOI 100)

FIG. 2

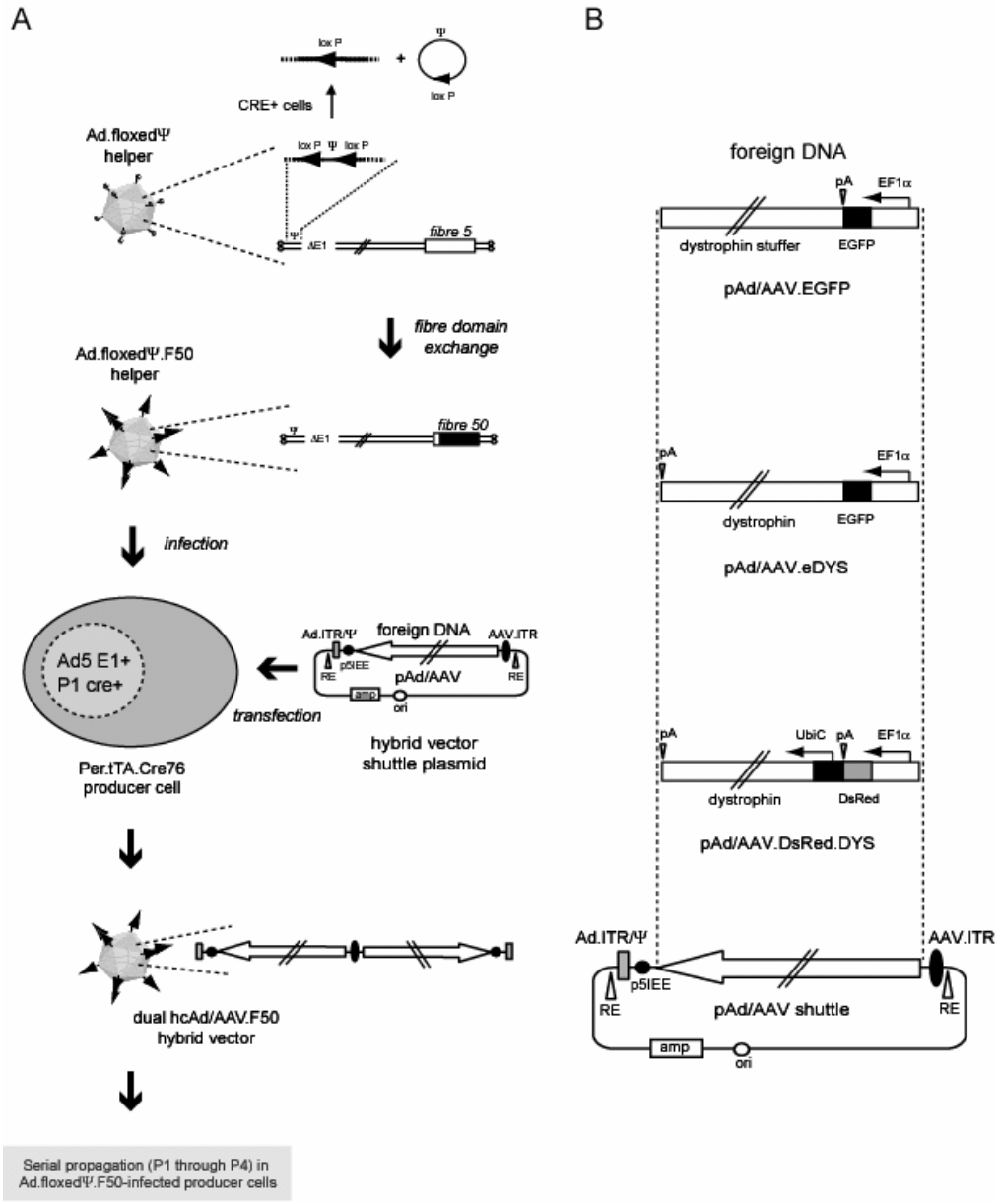
A
lox P
Ψ
lox P
CRE+ cells
Ad.floxedΨ
helper
lox P Ψ lox P
ΔE1
fibre 5
fibre domain
exchange
Ad.floxedΨ.F50
helper
Ψ
ΔE1
fibre 50
infection
Ad5 E1+
P1 cre+
Ad.ITR/Ψ
foreign DNA
AAV.ITR
p5IEE
RE
pAd/AAV
RE
amp
ori
transfection
hybrid vector
shuttle plasmid
Per.tTA.Cre76
producer cell
dual hcAd/AAV.F50
hybrid vector
Serial propagation (P1 through P4) in
Ad.floxedΨ.F50-infected producer cells
B
foreign DNA
pA
EF1α
dystrophin stuffer
EGFP
pAd/AAV.EGFP
pA
EF1α
dystrophin
EGFP
pAd/AAV.eDYS
pA
UbiC
pA
EF1α
dystrophin
DsRed
pAd/AAV.DsRed.DYS
Ad.ITR/Ψ
AAV.ITR
p5IEE
RE
pAd/AAV shuttle
RE
amp
ori

FIG. 3

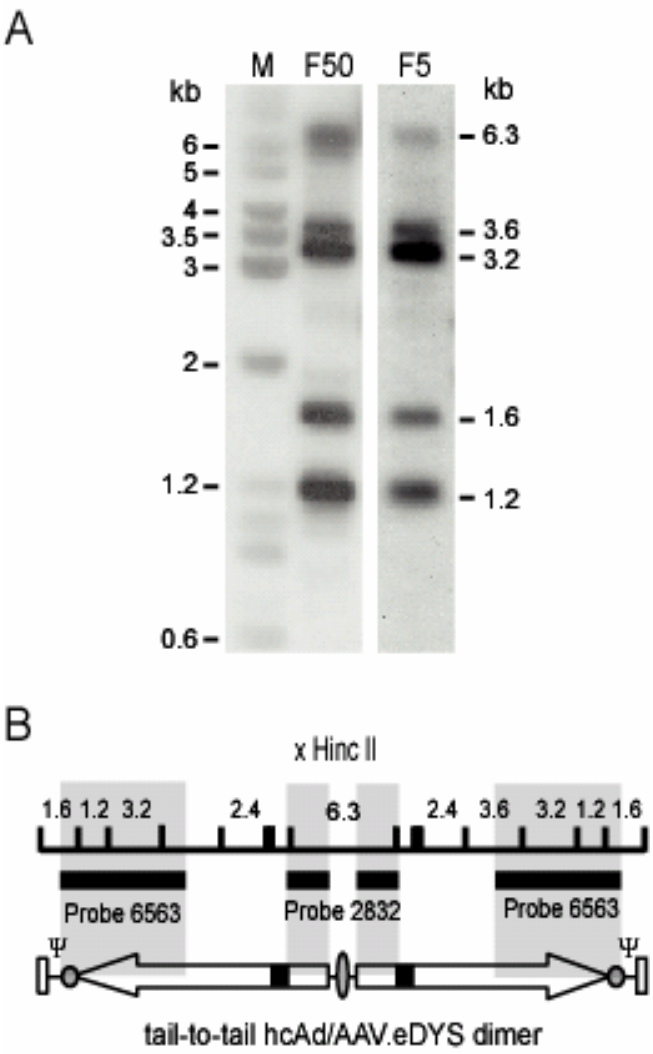
A
M
F50
F5
kb
kb
6
5
4
3.5
3
2
1.2
0.6
6.3
3.6
3.2
1.6
1.2
B
x Hinc II
1.6 1.2 3.2
2.4
6.3
2.4
3.6
3.2 1.2 1.6
Probe 6563
Probe 2832
Probe 6563
Ψ
Ψ
tail-to-tail hcAd/AAV.eDYS dimer

FIG. 4

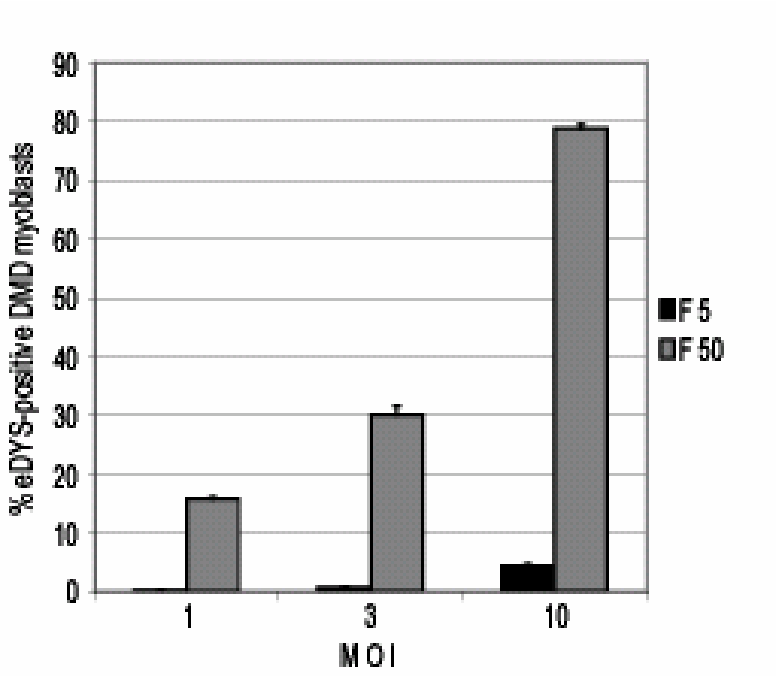
% eDYS-positive DMD myoblasts
90
80
70
60
50
40
30
20
10
0
1
3
10
MOI
F 5
F 50

FIG. 5

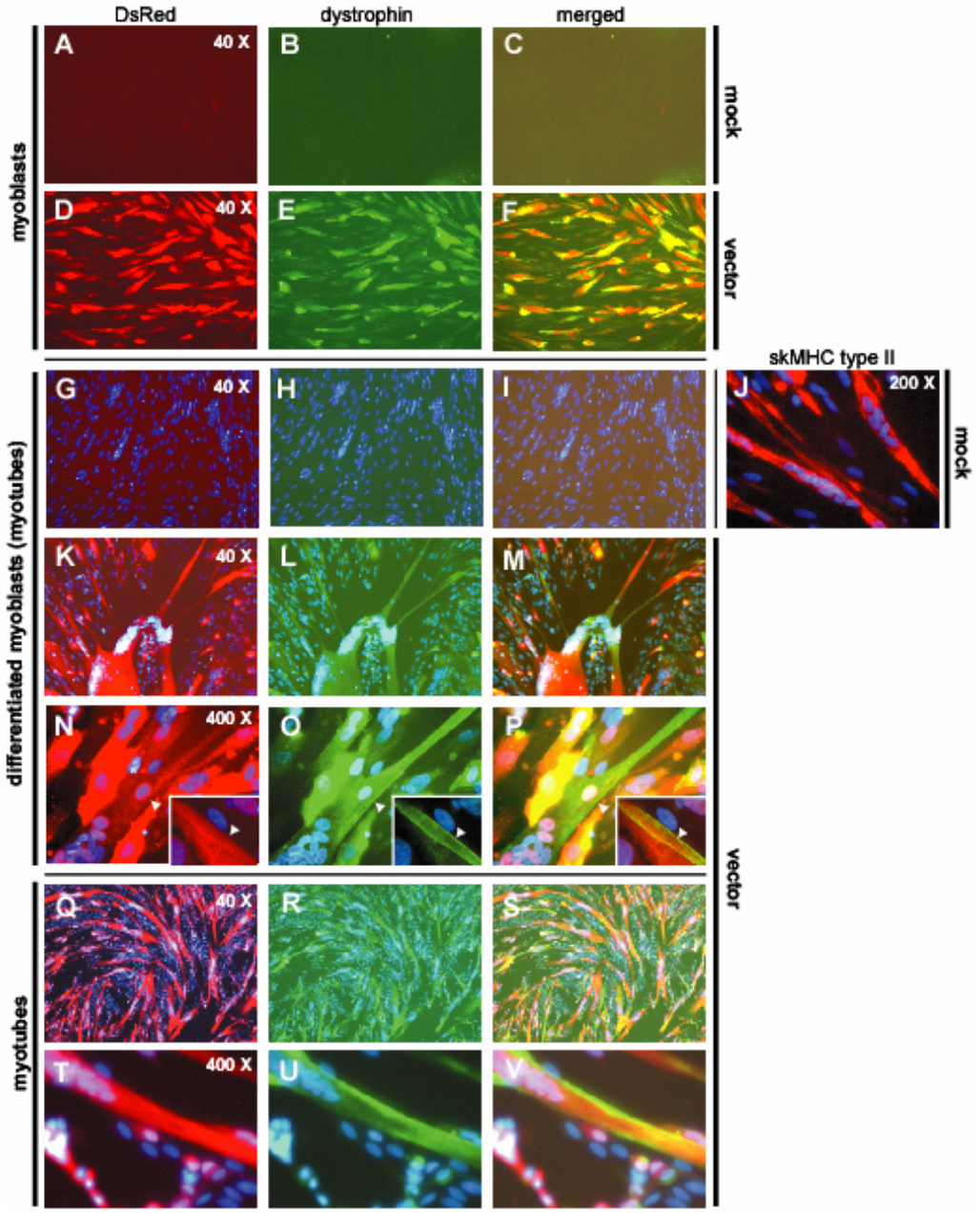
DsRed
dystrophin
merged
myoblasts
A
40 X
B
C
mock
D
40 X
E
F
vector
skMHC type II
differentiated myoblasts (myotubes)
G
40 X
H
I
J
200 X
mock
K
40 X
L
M
N
400 X
O
P
vector
myotubes
Q
40 X
R
S
T
400 X
U
V

FIG. 6

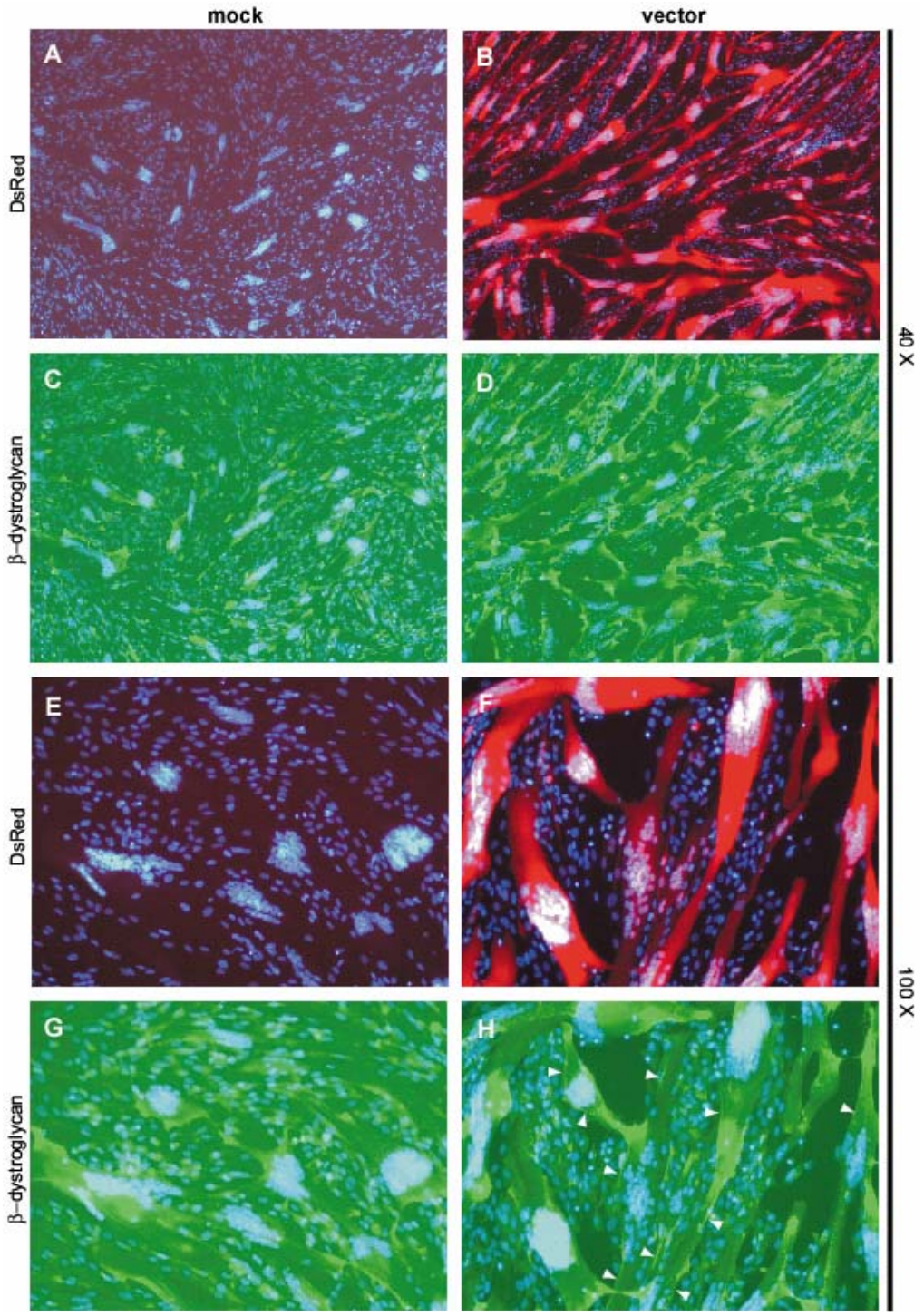
mock
vector
A
B
DsRed
C
D
β-dystroglycan
40 X
E
F
DsRed
G
H
β-dystroglycan
100 X

FIG. 7

3.3 CONCLUSION OF THE SECOND STUDY

In an attempt to improve delivery of dual hc Ad/AAV vectors in DMD muscle cells, surface labelings of these cells were done with antibodies directed against CAR and CD46, which are, respectively, receptors for the Ad5 and Ad50 fibers. It was found that CAR is poorly expressed at the surface of DMD muscle cell. In the contrary, CD46 is well expressed on DMD muscle cells. Similar observations could be done with human DMD myotubes. Consequently, the envelope of the dual hc Ad/AAV vectors were modified to bind to CD46. This was done by modifying the Helper Adenovirus used for production of the vectors. Briefly, the sequences of the Ad5 Heloper Adenovirus were replaced by those of the Ad50 Virus, which are able to bind to CD46.

The newly designed Ad50 fibers dual hc Ad/AAV vector could be produced at similar efficiency as the former Ad5 fibers dual hc Ad/AAV vectors, and it was used for complementation of dystrophin in our human DMD cell line. This resulted in a high transduction of these cells, at rates highly superior of that of the former control dual hc Ad/AAV vector. This suggested that high transduction levels can be done in myoblasts with Adeno vectors surrounded of fibre 50 proteins. The resulting complemented DMD muscle cells were characterized. It was controlled that dystrophin was present in these cells, but not in mock-transfected cultures. When the complemented cells were induced for differentiation, not only Dystrophin could be seen in myotubes, but also modifications in the Dystroglycan Complex were observed. Particularly, after complementation of dystrophin, DMD myotubes were found to have membrane-positioning of β-dystroglycan. In the contrary, the staining of uncomplemented DMD muscles for β-dystroglycan was weak and diffuse. This suggested that Dystrophin cDNAs brought by dual hc Ad/AAV vectors were able to correct the phenotype of DMD *in vitro*. Indeed, sufficient amounts of Dystrophin are known to contribute to the formation of a proper DGC complex at the sarcolemma of muscle cells (Blake et al., 2002).

These experiments proved the value of the newly designed Ad50 fibers dual hc Ad/AAV particles to correct our DMD immortalized cell line with Dystrophin. As a result, these

experiments also showed the value of our Duchenne cell line for the study of the *in vitro* correction of the DMD phenotype. Until now, most of the time, only the murine cell line C2C12 was used for studies of DMD. However, it is known that muscle cells from human or mice have different regulation mechanisms. Then, our human Duchenne cell line permits a new way to study DMD. Particularly, it allows studying DMD in a human system, without to have to extract cells from human Duchenne muscle biopsies.

Thus, this article has demonstrated the potentiality of our Duchenne cell line for cell studies, particularly for studies aiming at the correction of the human Duchenne phenotype. It has also demonstrated that fibre 50 dual hc Ad/AAV vectors may be a tool of choice for correction of DMD. However, additional experiments are needed to prove the utility of these vectors in other systems, and particularly *in vivo*. Before that, ongoing research in the laboratory of our collaborators is aiming at expressing AAV Rep proteins in a transient manner in host cells. This would allow to have specific integration of the transgenes in the *AAV1* locus, but without to express the Rep proteins when they are not needed.

CHAPTER 4: GENERAL CONCLUSIONS

Aiming at the fabrication of a model for Duchenne Muscular Dystrophy, the articles presented in this thesis have shown, first, the generation of a new human DMD cell line which has normal phenotypic properties, and second, complementation of this cell line with Dystrophin, to obtain a normal muscle cell line with the same genetic background as our Duchenne cell line.

We could obtain a DMD cell line after addition of different oncogenes into human primary DMD myoblasts. Lentiviral vectors were used for these immortalization purposes. We have used these vectors because they have sufficient packaging capacities and because they have integrational properties that allow long term expression of a transgene in dividing cells. After addition of different cocktails of lentivectors containing the transgenes TAg, Bmi-1, hTERT and Bcl-2, we found that myoblasts immortalized with Bmi-1 and hTERT were superior to other immortalized cells. Not only these cells had normal shapes and proliferation capacities as compared to the primary myoblasts, they also had normal growth arrest and differentiation once placed in differentiation medium. Additional experiments have verified that these cells had a normal karyotype, and this was not the case for cells immortalized with TAg, or with TAg + hTERT. None of the cell lines tested here was shown to be tumorigenic in nude mice. This does not mean, however, that these cell lines may not acquire carcinogenic properties. Particularly, we think that the unstable karyotype of TAg-immortalized cells might be particularly able to trigger the mutations that are necessary to lead to carcinogenesis. In the contrary, it has been shown that Bmi-1 + hTERT immortalized cells have stable karyotypes. This may suggest that these cells have normal anti-tumorigenic properties, and, therefore, these cells may be largely protected from the acquisition and perpetuation of mutations leading to tumorigenesis.

In our study, a Duchenne cell line was obtained, after immortalization with Bmi-1 and telomerase. The resulting cell line was able to fuse at normal rates, and has been observed to conserve the normal phenotypic properties of myoblasts even after more than one year in

culture. We believe that this cell line will be useful for sudies aiming to correct DMD in gene therapy purposes, but also to test various drugs for DMD.

In the second article, we show that the DMD immortalized cell line could be complemented with Dystrophin, to lead to a new human normal muscle cell line, with the same genetic background as our Duchenne cell line. For this purpose, an existing dual high capacity Ad/AAV vector was used. Lentiviral vectors and AAV vectors alone are not able to carry the full-length cDNA of dystrophin, which is the longest human gene currently known. However, the AAV capacities to integrate in the *AAV1* locus of chromosme 19 could be exploited here, as well as the Adenovirus vector property to package very large transgenes. So, a dual hc Ad/AAV vector was used. This vector was further modified to target a receptor abundantly found on myoblast cells. Indeed, the existing dual hc Ad/AAV were dependint upon their Ad5 fibre for entry, and then dependant upon presence of CAR receptors on target cells. However, it was shown that CAR receptors are not abundantly distributed on myoblast cells, but CD46 receptors were. For this reason, the Adeno Helper virus used to produce dual hc Ad/AAV vectors were modified to be surrounded by the part of the Ad50 fibre which can bind to CD46. The resulting pseudotyped vector was shown to be as efficiently produced as the control vector from which it originated.

The dual Dys hc Ad/AAV vectror was used for transduction of our Duchenne cell line that was immortalized with Bmi-1 and hTERT. This led to correction of the DMD phenotype *in vitro*, with correct expression of dystrophin at the plasma membrane, in the contrary of control mock-transduced DMD myotubes. Because relocalization of beta-dystroglycan was also observed at the plasma membrane, this suggests that our DMD cell line could be complemented with Dystrophin to allow formation of a functional DGC complex.

Currently, our collaborators aim to express Rep proteins in a transient manner in myotubes, to still ensure that the transgenes are introduced into the *AAV1* locus, but also to prevent the cells from the danger associated with the ongoing expression of these viral genes. However, even if Rep transient expression if achieved, this might not be sufficient to ensure that no mistaken integration occurs outside of the *AAV1* locus. For this reason, we are going now to talk about the long-term perspectives linked to find a cure to DMD. We are also going to talk more broadly about immortalization purposes.

4.1 Long-term perspectives

After the immortalization of myoblats with Bmi-1 and hTERT, new cell lines have been established in our laboratory with these same transgenes, but all cell types could not be immortalized with this regimen of oncogenes. Therefore, still additional research should be pursued before we are able to immortalize all cell types. Immortalization of additional cell types will enable not only to obtain new cell lines, but also cell lines with therapeutic purposes. For instance, obtention of a hepatic cell line is of importance to help to cure liver-related diseases. Moreover, obtention of pancreatic beta cells would be useful in attempts to cure Type I diabetes. However, obtention of immortalized cells does not mean that all diseases can be easily corrected.

For instance, if a Duchenne phenotype has been corrected *in vitro*, some people might think that there is only one step to cross to correct the DMD phenotype in patients. However, *in vivo* correction of DMD is still a long-term goal. Moreover, even though immortalized cells could be used for this purpose, it could be better and easier to use other types of cells, which could be used with less genetic modifications.

This is why, until now, *in vivo* correction of DMD is envisaged through transplantation of cells that can finally express dystrophin. These cells may be stem cells, satellite cells or progenitor muscular cells. Nonetheless, the problem, until now, is that the transplantated cells have poor survival capacities and only a small fraction of them cells contribute to muscle regeneration. These limitations have to be overcome before to envisage *in vivo* correction of DMD in patients.

Before any clinical trials are begun with gene therapy, different possible adverse effects should be considered. They relate to the correction of the muscle cells before transplantation. Indeed, it is often viral-derived vectors that are used to correct in the long-term genetic defects, in our case Dystrophin deficiency. Nevertheless, these vectors may integrate in

regions where there are tumor suppressors or oncogenes, which may knock down some tumor suppressors or enhance the expression of oncogenes.

In the second article presented here, a strategy has been proposed to insert dystrophin into the *AAV1* locus of chromosome 19. However, it is probable that this technique can not avoid insertional mutagenesis because of mistakenly integrations outside of the *AAV1* locus. Thus, this adverse possibility remains to be investigated.

To ensure proper integration, it may be an interesting to try to achieve a targeted integration in the exact region to be corrected. This has already been attempted with rAAV, but at the moment only 1 targeted integration occurs for 10 non-targeted integrations (Chamberlain et al., 2004). Adenoviral vectors have also been recently used for this purpose, but they are also not yet very efficient for targeted integration (Ohbayashi et al., 2005).

Despite the current difficulty to reach targeted integration, it is desirable that such a method is made available. Indeed, this may be a very adequate mechanism for gene therapy purposes. However, random transgene integration in different areas of the genome may also be beneficial. However, it remains to be defined where exactly the integrations are not detrimental for the host. Once this is known, it will be able to test (by PCR methods) all the integration events in a gene therapy experiment, before to transplant the modified cells into a host. In fact, it is probable that in the long-term, only cells with properly defined insertional events will be suitable for transplantation into hosts. Until this is achieved, before to begin a gene therapy experiment, it should be at least ensured that the risk associated with gene therapy purposes should be lower than the risk associated with the disease.

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