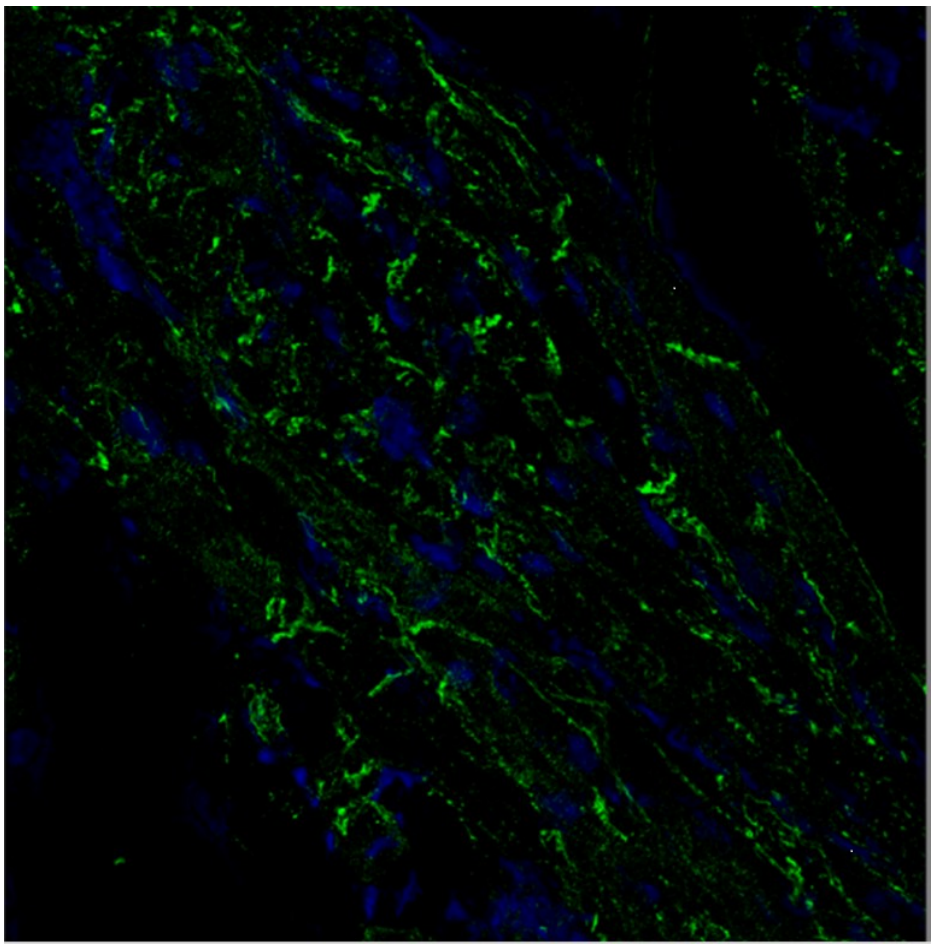
UNIVERSITY OF CRETE
DEPARTMENT OF MEDICINE

BIOMEDICAL RESEARCH FOUNDATION
ACADEMY OF ATHENS

GRADUATE PROGRAM IN THE
MOLECULAR BASIS OF HUMAN DISEASES

STUDY OF KERATIN 8/18 INTERMEDIATE FILAMENT NETWORK AND ITS RELATION TO CARDIOPROTECTION



MASTER THESIS

KONSTANTINOS FRAGKIADAKIS

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ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
ΙΑΤΡΙΚΗ ΣΧΟΛΗ

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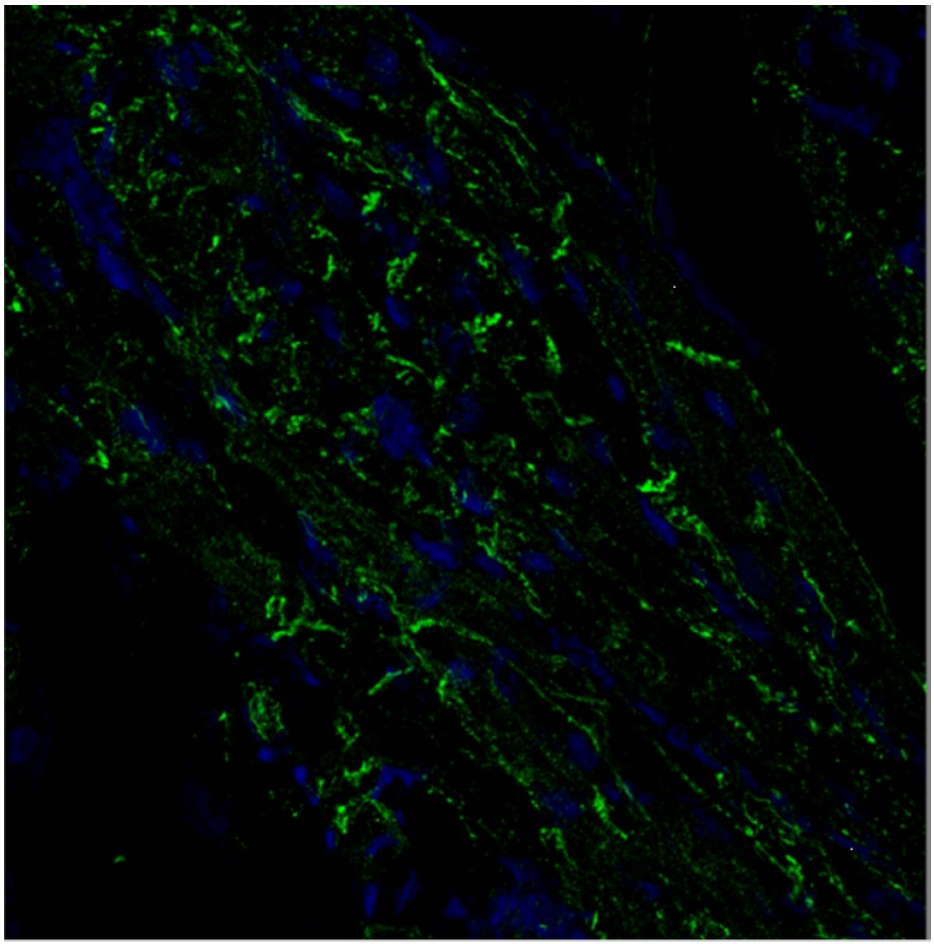

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ΚΩΝΣΤΑΝΤΙΝΟΣ ΦΡΑΓΚΙΑΔΑΚΗΣ

ΗΡΑΚΛΕΙΟ, ΜΑΡΤΙΟΣ 2014

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Επιβλέπων: Γιασεμή Καπετανάκη

Μέλη τριμελούς επιτροπής:

1) Γιασεμή Καπετανάκη , Ερευνήτρια Α' , Κέντρο Βασικής Έρευνας I.ΙΒ.Ε.Α.Α
2) Δέσποινα Σανούδου , Επίκουρη Καθηγήτρια Φαρμακολογίας, Ιατρική Σχολή ΕΚΠΑ
3) Δημήτρης Καρδάσης , Καθηγητής Βιοχημείας , Ιατρική Σχολή Κρήτης

<<Η ολοκλήρωση της εργασίας αυτής έγινε στο πλαίσιο της υλοποίησης του μεταπτυχιακού προγράμματος το οποίο συγχρηματοδοτήθηκε μέσω της Πράξης <<Πρόγραμμα χορήγησης υποτροφιών ΙΚΥ με διαδικασία εξατομικευμένης αξιολόγησης ακαδ. Έτους 2012-2013>> από πόρους του Ε.Π <<Εκπαίδευση και Δια Βίου Μάθηση>> του ευρωπαικού Κοινωνικού Ταμείου (ΕΚΤ) και του ΕΣΠΑ (2007-2013)>>

Ευχαριστίες

Με την ολοκλήρωση της μεταπτυχιακής μου εργασίας θα ήθελα να ευχαριστήσω κάποιους ανθρώπους που βρέθηκαν κοντά μου τα τελευταία δύο χρόνια. Πρώτα από όλους θα ήθελα να ευχαριστήσω τους καθηγητές μου, που μου μετέδωσαν τις γνώσεις τους, και κυρίως την καθηγήτρια μου Κα Γιασεμή Καπετανάκη που με εμπιστεύτηκε και με φιλοξένησε στο εργαστήριο της. Εν συνεχεία θα ήθελα να ευχαριστήσω τον Κο Δημήτρη Μπούμπα, τον Κο Δημήτρη Καρδάση και την Κα Δέσποινα Σανούδου για την στηριξή και την εμπιστοσύνη που μου έδειξαν κατά την διάρκεια του μεταπτυχιακού. Δεν θα μπορούσα να μην αναφερθώ στα μέλη του εργαστηρίου της Βασικής Έρευνας, πρώτα από όλους το Σταμάτη Παπαθανασίου που με βοήθησε σημαντικά, το Κο Μανόλη Μαυροειδή, το Κο Στέλιο Ψαρρά, την τεχνικό Κα Ιωάννα Κοσταβασίλη, τη Μαίρη Τσίκιτη, την Αντιγόνη Διοκμετζίδου, τη Ζωή Γαλατά, την Έλσα Τσούπρη, το Γιάννη Βατσέλλα, τη Θεοδοσία Δημοπούλου, τη Σοφία Νικούλη, τη Μάρω, τη Λορέντζα που με βοήθησαν με τις συμβουλές τους είτε υπήρχαν είτε δεν υπήρχαν δυσκολίες αλλά και δημιουργώντας μία όμορφη ατμόσφαιρα στο εργαστήριο. Θα ήθελα ακόμη να ευχαριστήσω όλα τα παιδιά του μεταπτυχιακού για τις όμορφες ώρες που περάσαμε μαζί. Στο σημείο αυτό δεν μπορώ να μην ευχαριστήσω την γραμματέα του προγράμματος Κα Μαίρη Αδαμάκη που ήτανε δίπλα μας όποτε το χρειαστήκαμε όλα τα παιδιά του μεταπτυχιακού. Δεν θα μπορούσα να μην αναφερθώ στους γονείς μου Μανόλη και Μαρία και στον αδελφό μου Δημήτρη που με υποστηρίζουν σθεναρά. Τέλος, θέλω να ευχαριστήσω τη Δήμητρα, το Δημήτρη, την Αργυρώ και το Μανόλη που είναι δίπλα μου.

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Περίληψη

Παρά τη σημαντική ευαισθητοποίηση του κοινού και των τεχνολογικών εξελίξεων που ευνοούν την έγκαιρη διάγνωση και τις άμεσες θεραπευτικές παρεμβάσεις για την καρδιαγγειακή νόσο, η καρδιακή ανεπάρκεια παραμένει ένα άλυτο πρόβλημα. Με τον επιπολασμό της να αφορά στο 2% των ενηλίκων και να αυξάνεται με την πρόοδο της ηλικίας, και με την θνητότητάς της παράλληλα να αυξάνεται, η προσπάθεια κατανόησης της παθοφυσιολογίας της αποτελεί απαραίτητο βήμα. Αν και η σημερινή φαρμακευτική αγωγή της καρδιακής ανεπάρκειας (που στηρίζεται στην αναστολή του αδρενεργικού συστήματος καθώς και του άξονα ρενίνης-αγγειοτενσίνης-αλδοστερόνης) συνέβαλλε σημαντικά στη βελτίωση της συμπτωματολογίας και της επιβίωσης, δεν ελάττωσε τη πρόοδο της νόσου. Επομένως, η μελέτη παράλληλων σηματοδοτικών μονοπατιών και η ανεύρεση σημαντικών κόμβικών σημείων στη παθοφυσιολογία του μυοκαρδιοκυττάρου δύναται να αυξήσει την αποτελεσματικότητα της θεραπείας και την συνολική επιβίωση. Πρόσφατες μελέτες αναδεικνύουν το ρόλο του μυοκαρδιακού κυτταροσκελετού σημαντικό τόσο στη φυσιολογία όσο και στην παθοφυσιολογία του μυοκαρδιοκυττάρου. Ιδιαίτερη αναφορά έχει πραγματοποιηθεί σχετικά με το δίκτυο των ενδιαμέσων ινιδίων που εκτός απο το μηχανικό του ρόλο στη διατήρηση της δομικής ακεραιότητας του κυττάρου έχει και συντονιστικό ρόλο, ρυθμίζοντας ενδοκυττάριες λειτουργίες. Η δεσμίνη, η μυοειδική πρωτεΐνη των ενδιαμέσων ινιδίων, δημιουργεί ένα εκτενές δίκτυο που συνδέει τη συσταλτή συσκευή των μυοκυττάρων με την κυτταροπλασματική μεμβράνη στο επίπεδο των εμβόλιμων δίσκων για τα καρδιομυοκύτταρα, τον πυρήνα και διάφορα μεμβρανώδη κυτταρικά οργανίδια. Πρόσφατα στο πλαίσιο διερεύνησης του μηχανισμού καρδιοπροστασίας που παρέχει ο κυτταροσκελετός της δεσμίνης, παρατηρήθηκε ότι η αποδιοργάνωση του κυτταροσκελετού είναι μια βασική διαδικασία για την εξέλιξη του μονοπατιού της απόπτωσης. Συγκεκριμένα, η υπερέκφραση του παράγοντα νέκρωσης των όγκων (TNF-α) στο μυοκάρδιο ποντικών, μοντέλων καρδιακής ανέπάρκειας (MHCsTNF-α), οδηγεί σε απομάκρυνση της δεσμίνης από τους εμβόλιμους δίσκους με διατάραξη της αρχιτεκτονικής τους και συσσώρευση της με τη μορφή ενδοκυττάριων συσσωματωμάτων. Εν συνεχεία, ανευρέθηκε αναπάντεχα ότι η έλλειψη της δεσμίνης στο μυοκάρδιο που υπερεκφράζει TNFa (TNF-α desm-/-) διορθώνει την παθολογία του μυοκαρδίου που δε φερει δεσμίνη αποτρέποντας την καρδιακή ανεπάρκεια. Είναι γνωστό ότι η έλλειψη δεσμίνης στο μυοκάρδιο (desm-/-) οδηγεί σε μιτοχονδριακή δυσλειτουργία, κυτταρικό θάνατο μυοκαρδιοκυττάρων, ίνωση, ασβεστίωση και καρδιακή ανεπάρκεια. Πρόσφατα, μελέτη του TNF-α desm-/- μυοκαρδίου ανέδειξε έκτοπη έκφραση των κερατινών 8/18. Οι κερατίνες 8/18 ανήκουν στην οικογένεια των ενδιαμέσων ινιδίων όπως η δεσμίνη, αλλά εκφράζονται κυρίως σε επιθηλιακά κύτταρα. Δεδομένου ότι οι κερατίνες 8/18 είναι πρωτείνες των ενδιαμέσων ινιδίων (μέρος του κυτταροσκελετού) και χαρακτηρίζονται από σπουδαίες ιδιότητες όπως η δεσμίνη, ρυθμίζοντας τη δομική ακεραιότητα του κυττάρου, τη λειτουργία των μιτοχονδρίων καθώς και το μονοπάτι της απόπτωσης, η μελέτη τους είναι σημαντική. Ο στόχος της συγκεκριμένης μελέτης είναι η κατασκευή φορέων έκφρασης ποντικίσιων κερατινών 8/18 , μελέτη του ενδιαμέσου δικτύου και των χαρακτηριστικών τους σε ινοβλάστες καθώς και τέλος συσχέτισή τους με την προστασία του μυοκαρδίου. Στη συγκεκριμένη μελέτη παρατηρήθηκε ότι η κερατίνη 18 σταθεροποιεί την έκφραση της κερατίνης 8 και το αντίστροφο. Η έκτοπη έκφραση της κερατίνης 8 μαζί με την κερατίνη 18 οδηγεί στο σχηματισμό ενός ινώδους δικτύου που περιβάλλει το πυρήνα, διατρέχει το κυτταρόπλασμα και φτάνει στη περιφέρεια. Επιπλέον, η έκτοπη έκφραση του ενδιαμέσου δικτύου κερατινών στους ινοβλάστες δεν αλληλεπιδρά με το φυσικό ενδιάμεσο δίκτυο της βιμεντίνης. Καταλήγωντας, η μελέτη της έκφρασης του ενδιαμέσου δικτύου των κερατινών στο πάσχον μυοκάρδιο ίσως αναδείξει καρδιοπροστατευτικούς μηχανισμούς.

Abstract

Despite considerable public awareness and technological advances that foster early diagnosis and aggressive therapeutic interventions for cardiovascular disease, heart failure, a critical unsolved problem. With its prevalence to regard 2% of adults and increasing in the elderly, its mortality to increase , understanding its pathophysiology is necessary. Although current drug therapy of heart failure (dependent on adrenergic system and renin-angiotensin-aldosterone axis blockage) improved symptoms and increased overall survival, the progressive nature of heart failure has not been overcome yet. Consequently, studying parallel signaling pathways and identifying important nodal points in the pathophysiology of cardiomyocyte may increase the effectiveness of treatment and overall survival. Recent studies demonstrate that the role of cardiac cytoskeleton is important both in physiology and pathophysiology of cardiomyocyte. Particular reference has been made on the network of intermediate filament that besides the mechanical role in maintaining cellular integrity, it plays a coordinating role in regulating intracellular functions. Desmin, the muscle specific intermediate filament protein, forms a three dimensional scaffold which links the contractile apparatus to the plasma membrane intercalated disks (IDs), the nucleus and also other membranous cellular organelles. Recently under investigation of the mechanism of cardioprotection provided by the desmin cytoskelleton, it was identified that disruption of the cytoskeleton is a key event in apoptotic cell death pathways. Specifically, overexpression of tumor necrosis factor (TNF-α) in the myocardium of heart failure mice models (MHCsTNF-α) leads to removal of desmin from the intercalated disks (IDs) with destruction of their architecture and accumulating into intracellular aggregates. Thereafter, it was found unexpectedly that desmin deficiency in overexpressing TNFa myocardium (TNF-α desm-/-) rescues desmin null myocardium pathology, preventing heart failure. It is known that desmin deficiency in myocardium (desm-/-) results in mitochondrial dysfunction , cardiomyocyte death , fibrosis, calcification and heart failure. Recently, study of TNF-α desm-/- myocardium revealed ectopic expression of keratin 8/18 in myocardium. Although, K8/18 belong to the family of intermediate filament as desmin, they are expressed in simple epithelial cells. Since keratins 8/18 are intermediate filament proteins (part of the cytoskeleton) and are characterized by crucial properties like desmin such as regulating cellular integrity, mitochondrial function as well as apoptosis, their investigation is important. The objective of this study is to construct expression vectors of mouse keratins 8/18, study their intermediate network and their characteristics in fibroblasts as well as their association with cardioprotection. In this study it was observed that the keratin 18 stabilizes the expression of keratin 8 and vice versa. Ectopic expression of keratin 8 together with keratin 18 leads to the formation of a filamentous network that surrounds the nucleus, spans the cytoplasm and reaches the periphery. Furthermore, ectopic expression of keratin intermediate network in fibroblasts does not interfere with the natural intermediate filament network of vimentin. In coclusion, the study of the expression of keratin intermediate network in the failing myocardium may reveal cardioprotective mechanisms.

1. Introduction

1.1. Heart failure

Despite considerable public awareness and technological advances that foster early diagnosis and aggressive therapeutic interventions for cardiovascular disease, heart failure, a pathophysiologic state in which blood delivery is insufficient for tissue requirements, remains a critical unsolved problem.Heart failure happens after an adverse defect on cardiac structure or fuction that results in oxygen delivery failure to peripheral tissues despite normal filling pressures[1]. Heart failure is presented with shortness of breath , leg swelling and exercise intolerance. It is more often in elderly with a prevalence of 10% over 70years [2] and it is associated with high 5-year mortality rate since approximately a 60% of elderly would die within 5year [3] . Major cause is coronary artery disease while minor causes are viral infections (myocarditis) and cardiomyopathies such as dilated cardiomyopathy.

1.2. Pathophysiology of Heart failure

Heart failure is a progressive disorder that is initiated after an "index event" which reduces cardiac pumping capacity either by damaging cardiac muscle or disrupting myocardium to generate force. This index event may have an abrupt onset, as in the case of a myocardial infarction, it may have a gradual or insidious onset, as in the case hemodynamic pressure or volume overloading, or it may be hereditary, as in the case of genetic cardiomyopathies. In most cases , patients will remain asymptomatic as a result of compensatory mechanisms with activation of adrenergic , renin-angiotensin or cytokine system. However, after some time, sustained action cause secondary damage, left ventricle remodeling and HF.

Left ventricle remodeling includes alterations in myocyte biology with fetal myosin heavy chain gene expression , b-adrenergic desensitization , cytoskeletal protein changes as well as myocardial changes with myocyte loss, matrix degradation and replacement fibrosis that lead to LV geometry changes with wall dilation and thinning. Therefore, the close relationship between the development of contractile dysfunction and cardiac remodeling is responsible for disease progression in primary or secondary dilated cardiomyopathy (DCM) [4] . Additionaly, aging of cardiomyocytes and cardiac interstitial cells (fibroblasts , endothelial cells) is also related with the progression of heart failure [5] .

A broad range of molecular pathways are thought to be involved in the pathophysiology of heart failure (Fig. 1). Particularly, cell-surface receptors are activated by the binding of a ligand or by a mechanical stimulus, and this induces the activation of stress-response protein kinases and phosphatases such as cyclic-AMP-dependent protein kinase (PKA), protein kinase C(PKC), protein kinase D (PKD), MAPKs, CAMKII and calcineurin (Fig. 1). These enzymes, in turn, stimulate transcription factors, which target multiple genes resulting in a change in the cellular structure, size, shape and molecular regulation of the heart, often collectively referred to as cardiac remodelling. Although early attempts aimed at finding a common pathway that could be manipulated to rescue pathological cardiac remodelling , the identification of complex parallel signalling cascades made it complicated . Nonetheless, there seem to be crucial signalling 'nodes' (points at which several pathways converge) — for example, glycogen synthase kinase 3β (GSK3β) and histone deacetylases (HDACs) — and remodelling signals that can be modulated by administering small molecules and reducing the progression of heart failure [6] .

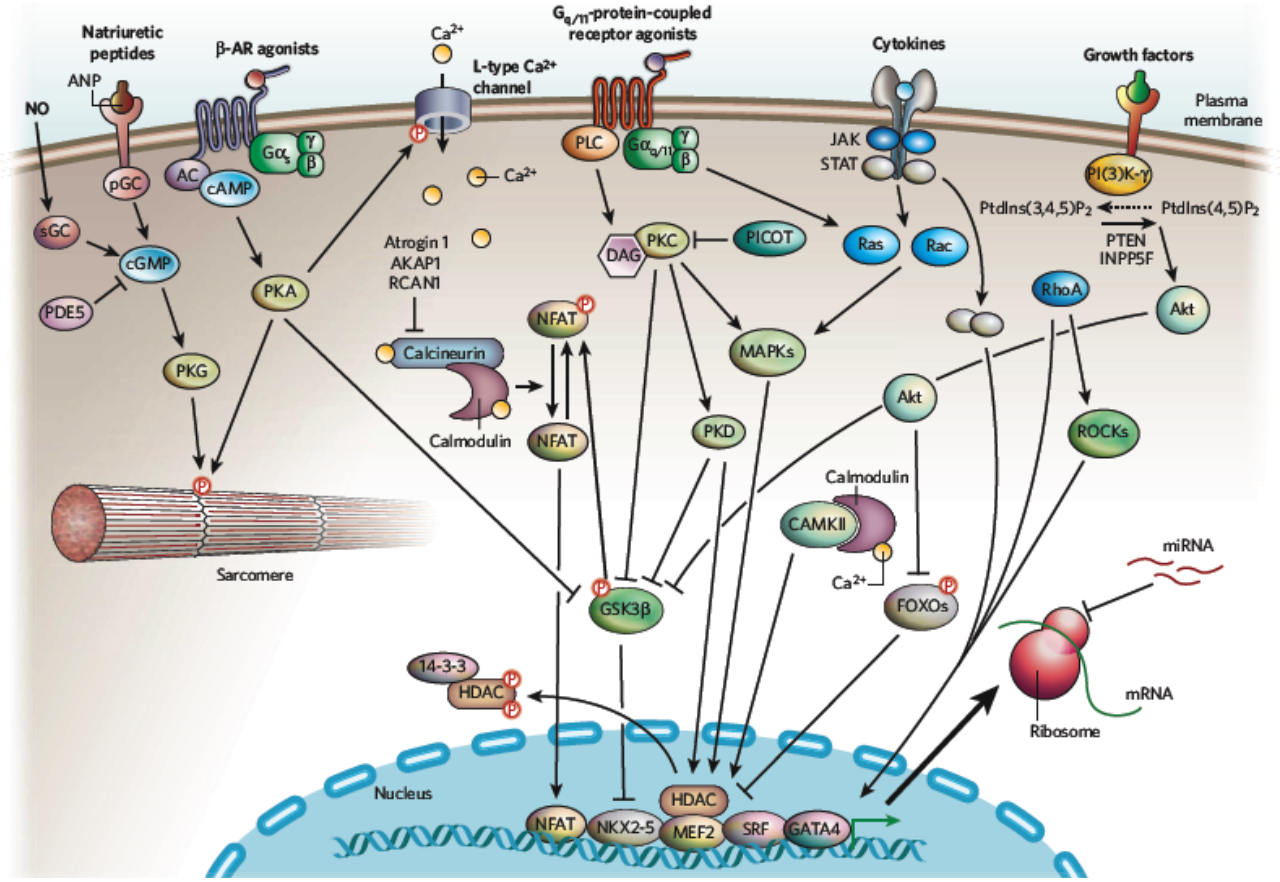

Figure 1: Cardiomyocyte signalling pathways involved in the pathophysiology of heart failure.

1.3. Cardiac Cytoskeleton

Among those molecular pathways which are involed in the pathophysiology of heart failure, the cardiac filament network (cytoskeleton) plays a crucial role.Cardiac muscle cells are highly organized in a characteristic striated appearance which allows high efficiency in force generation, transmission and movement. In addition to the force generating contractile apparatus, composed primarily of interdigitating actin and myosin filaments, striated muscle contains at least three cytoskeletal systems. The intrasarcomeric cytoskeleton, located within the myobrillar lattice itself and composed of titin and nebulin, the extrasarcomeric cytoplasmic filaments composed mainly of intermediate filaments (IFs), and the membrane skeleton[7],[8]. The IF containing lattices interconnect the myofibrils to each other, through the Z-discs, and the entire contractile apparatus to the sarcolemma cytoskeleton, several cytoplasmic organelles and the nucleus. This allows the formation of a continuous network connecting the extracellular with the nuclear matrix, that could be involved in diverse functions including cell integrity, coordination of cellular functions, force transmission and mechanochemical signaling, both within a single cardiomyocyte as well as throughout the entire heart [9] .

1.4. Intermediate Filament Proteins

The intermediate filament (IF) cytoskeleton consists of heterogeneous protein subunits which share the ability to polymerize into 10-nm rope-like structures.All IF proteins share a tripartite domain structure consisting of a highly conserved α-helical central rod domain flanked by

variable N-terminal head and C-terminal tail domains. The central rod domain represents a major driving force during the self-assembly of all IF proteins. In addition to contributing to assembly, the end domains are important sites of regulation and interaction with other cellular elements [10] (Figure 2).

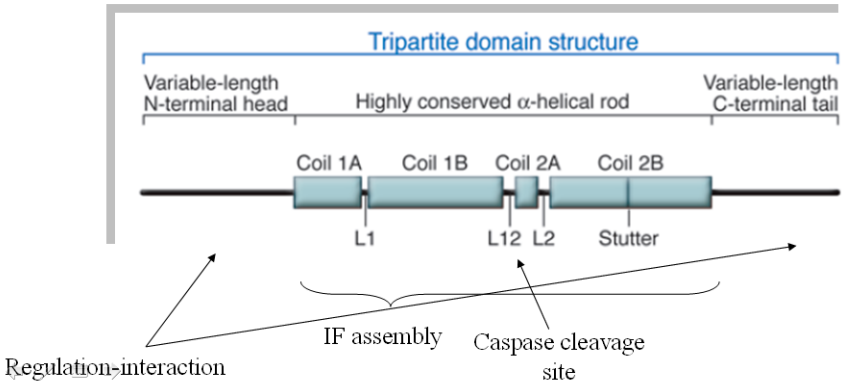

Figure 2: Intermediate filament protein structure.

Unlike microtubules and microfilaments, which are build from ubiquitously expressed subunits, the different members of the IF superfamily show tissue-specific and developmentally regulated expression. Thus, epithelial cells express keratines, neuronal cells express nestin, the neurofilament triplet proteins (NFL, NFM and NFH), peripherin and a-internexin; glial cells express the glial fibrillary acidic protein (GFAP); mesenchymal cells, such as blood, fibroblasts and endothelial cells, express vimentin and some synemin; lens cells express vimentin, filensin and phakinin. In addition to the tissue specific cytoplasmic IFs, all cells express B-type nuclear lamins (B1, B2, or B3) and all differentiated cells express A-type nuclear lamins (A and C) [11]

1.5. Cardiomyocyte IF protein network

The IF cytoskeleton of mature cardiac muscle is composed predominantly of the muscle specific IF protein desmin[12],[13]. The desmin IF lattice surrounds the Z-discs, interconnects them together and links the entire contractile apparatus to different membranous compartments and organelles including the costameres of the sarcolemma, desmosomes of the intercalated discs, mitochondria and nucleus.This allows the formation of a continuous network that connects extracellular matrix and adjacent cardiomyocytes with the nucleus (Figure 3). The crucial role of desmin in the healthy and failing heart is indicated by genetic association of desmin missense mutations with idiopathic dilated cardiomyopathy in humans. Furthermore, in gene targeting studies , ablation of desmin expression leads to heart chamber dilation, extensive myocyte cell death and heart failure [14], [15].The mechanism by which the absence or mutation of desmin leads to dilated cardiomyopathy and heart failure is under investigation.

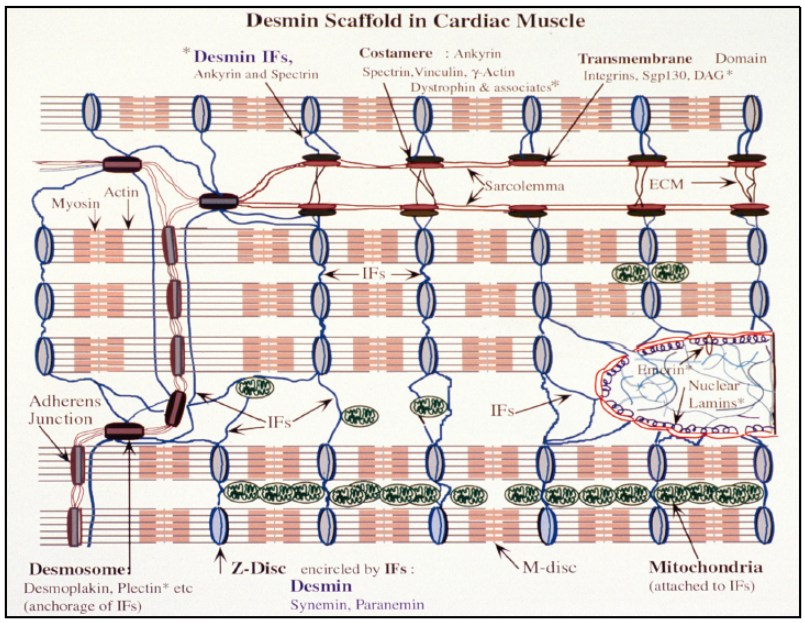

Figure 3: Schematic representation of desmin IF scaffold in cardiac muscle.

Nowadays, while genetic engineering is continuously growing, genetically modified mouse models of heart failure are useful tools to delineate the pathophysiology of cardiac decompensation as well as the physiology of heart muscle. After Levine et al. identified that TNF-α (tumour necrosis factor alpha) a pluripotent proinflammatory cytokine is overexpressed in patients with end stage heart failure [16.] , Mann et al. identified that cardiac specific overexpression of TNF-α in transgenic mice (aMHCsTNF-α mouse model) lead to dilated cardiomyopathy, LV dysfunction and heart failure [17] , [18]. TNF-α provokes cardiomyocyte apoptosis and LV wall thinning after activation of extrinsic and intrinsic apoptotic pathways that converge on effector caspases [19] which may cleave several intracellular substrates. Recently, Panagopoulou et al. identified that desmin is a substrate since TNF-α-dependent caspase activation induces desmin cleavage in aMHCsTNF-α mice.This cleavage provokes intercalated disk re-organization and formation of desmin aggregates in cardiomyocytes. Therefore, TNFa induces mislocalization of proteins that are found in intercalated disks which are specific sarcolemmal structures that maintain the proper connection and communication between cardiomyocytes [20].

The crucial role of muscle-specific IF protein desmin in cardiomyocyte biology is indicated in desmin knock-out mice. In desmin null mouse, desmin gene is disrupted and thus gene expression is ablated. The deficiency of desmin IF protein in cardiac muscle leads to cardiomyocyte loss within the first 2-3weeks of age , then, inflammation , replacement fibrosis and calcification are presented with an initial (within 6months) cardiac hypertrophy which finally progresses to dilative cardiomyopathy [15]. Although desmin null cardiomyocytes are characterized by myofibril disruption and IDs impairment ,the primary cause of desmin null cardiomyopathy is mitochondrial abnormalities [21].

Recently, in the context of revealing more the role of desmin in the TNF-α-induced heart failure and its relation with the apoptotic pathway, Panagopoulou et al. constructed a mouse model

that over-expresses TNF-α in heart and lacks desmin. According to the first observations , in the heart of TNF-α desmin-/- mice there is not increase of apoptotic cell death as it was expected but great improvement of desmin null pathologic phenotype. Particularly, TNF-α over-expression reduced LV remodeling , cardiac calcification, myofibrilllar disruption and mitochondrial defects found in desm-/- [20] *(Papathanasiou et al. unpublished data)*. Although, there are a lot of possible explanations of this different action of TNF-α in desmin-/- myocardium, one might be that the absence of desmin as well as its aggregates in cardiomyocytes reduce the myocardial stress and their impaired function. Moreover, TNF-α overexpression in the absence of desmin might be related with the expression of cardioprotective genes that improve TNF-α or desmin-/- pathology. Therefore, investigating the role of TNF-α in desm-/- myocardium could unravel possible cardioprotective mechanisms.

Recently, Papathanasiou et al. (*unpublished data)* identified that keratins 8 and 18, simple epithelium-specific , members of IF family are expressed in TNF-α desm-/- myocardium.

1.6. Keratin Family

Keratin 8 and 18 proteins belong to the keratin intermediate filament protein family. There are 54 genes in human genome that encode keratins.. They are divided into type I and type II keratins (Figure 4). Type I keratis (except K18) are clustered in chr.17 while type II in chr.12 [22].Type I proteins tend to be smaller (40-64 kDa) and more acidic (pI ~4.7-6.1) compared to the larger (52-68 kDa) and basic-neutral (pI ~5.4-8.4) type II proteins [23, 24, 25]. keratins have common structure (a-helical central rod domain flanked by non helical head and tail domains, see Figure 2) as the other members of IF family. In contrast to the other members of IF family, Keratin IF assembly obligatorily begins with the formation of type I-type II heterodimers [23, 26, 27]. This requirement underlies the pairwise transcriptional regulation of keratin genes. Moreover, it was identified that, single keratin proteins deviating from equimolar type I/type II amounts are rapidly degraded [28]. They form stable long , unbranched filament -10nm in diameter and they braid the nucleus, span through the cytoplasm, and are attached to the cytoplasmic plaques of the typical epithelial cell-cell junctions , the desmosomes [29] . In addition, regulation of keratin gene expression depends upon both the type and stage of differentiation in epithelia [26, 25] .

Table 1 The new human keratin nomenclature (Schweizer et al. 2006)

	Type I		Type II	
Keratin types	new name	former name	new name	former name
Epithelial keratins	K9	K9	K1	K1
	K10	K10	K2	K2
	K12	K12	K3	K3
	K13	K13	K4	K4
	K14	K14	K5	K5
	K15	K15	K6a	K6a
	K16	K16	K6b	K6b
	K17	K17	K6c	K6e/h
	K18	K18	K7	K7
	K19	K19	K8	K8
	K20	K20	K76	K2p
	K23*	K23	K77	K1b
	K24*	K24	K78*	K5b
			K79*	K6l
			K80*	Kb20
Hair follicle-specific epithelial keratins (root sheath)	K25	K25irs1	K71	K6irs1
	K26	K25irs2	K72	K6irs2
	K27	K25irs3	K73	K6irs3
	K28	K25irs4	K74	K6irs4
			K75	K6hf
Hair keratins	K31	Ha1	K81	Hb1
	K32	Ha2	K82	Hb2
	K33a	Ha3-I	K83	Hb3
	K33b	Ha3-II	K84	Hb4
	K34	Ha4	K85	Hb5
	K35	Ha5	K86	Hb6
	K36	Ha6		
	K37	Ha7		
	K38	Ha8		
	K39	Ka35		
	K40	Ka36		

*Expression pattern still unknown, only gene information available

For K1–K20 (in gray), the numbering of the original catalog (Moll et al. 1982b, 1990) has been maintained. The respective gene names (“*KRT*”) by the human genome consortium utilize the same numbers, e.g. “*KRT20*”

Figure 4: Keratin Family

Besides the variety of epithelial (soft or cyto-) keratins , hairs and nails are built up from a distinct subfamily of hard and trichophytic keratins (“hair keratins”) [30, 31] .The keratins associated with hair are uniquely specialized for stability reinforced by extensive extracellular disulfide bridging. The specialized keratins of the outer epidermis (K1, K2, K9, K10) are distinguished by tail domains greatly enriched in glycine and by the lack of caspase cleavage sites within the type I extracellular keratins (K9, K10). These keratins contribute to the structural integrity essential for the barrier function of the skin . Intracellular epithelial keratins such as K5 and K14 crossbridge desmosomal junctions and thus provide structural continuity with the edges of epithelial cells. Individual keratins bind to the desmoplakin component of desmosomes with varying affinity and structural requirements [32] .

Keratins play a major role in cellular integrity , mechanical stability either in single or not epithelial tissues.Beside their mechanical role (hard principles) , new “soft” principles have been identified. Keratins are involved in cell signaling, regulating the availability of other abundant cellular proteins, trafficking proteins in polarized epithelial and act like stress proteins [33] (Figure 5).

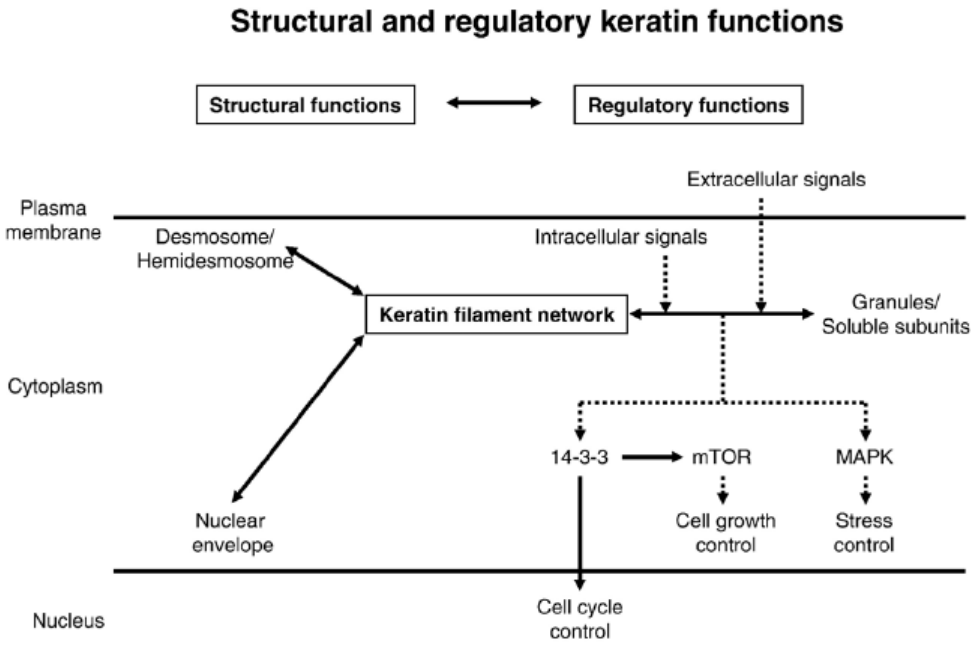

Figure 5: Structural versus regulatory functions of keratins [34].

1.7. Intermediate Filaments as stress-proteins

Whether injury is inflicted upon skin, muscle, central nervous system, liver, or a number of other tissues, rapid changes are elicited in regards to the transcription of IF genes and/or regulation of IF proteins themselves [35] **.** Environmental or internal stresses (see Figure 6) initiate stress signaling cascades, which activate the stress response and transcriptional machineries that induce the expression of the classical stress-induced HSP (heat-shock protein) genes. Stress also induces the transcription and translation of constitutive/resident IF genes (green circles), non-resident IF genes (blue circles) and the nuclear lamin IFs (orange). The increased expression of IFs provides pools for new filament assembly (not shown), binding to IF-associated proteins (IFAPs such as HSPs; red triangles). IF posttranslational modifications (e.g. phosphorylation (P)), serve important functions in IF dynamics, binding to IFAPs and targeting for degradation initially or after recovery from stress. Exposure to chronic stress, in the appropriate context, leads to inclusion body formation, which also includes HSPs and other chaperones. The mechanisms by which these IF alterations in response to stress impart a protective role include: (i) providing a mechanical support basally and during recovery from injury; (ii) serving as a scaffold that binds a variety of proteins including kinases, adaptor proteins such as 14-3-3, and HSPs such as HSP70. This may be part of multi-protein complexes that include protein kinase C, K8 and HSP70; (iii) serving as anti-apoptotic proteins; (iv) regulating organelle functions; and (v) providing a phosphate buffer or sponge to contain unwarranted phosphorylation of proapoptotic proteins that are activated by phosphorylation [36] [37].

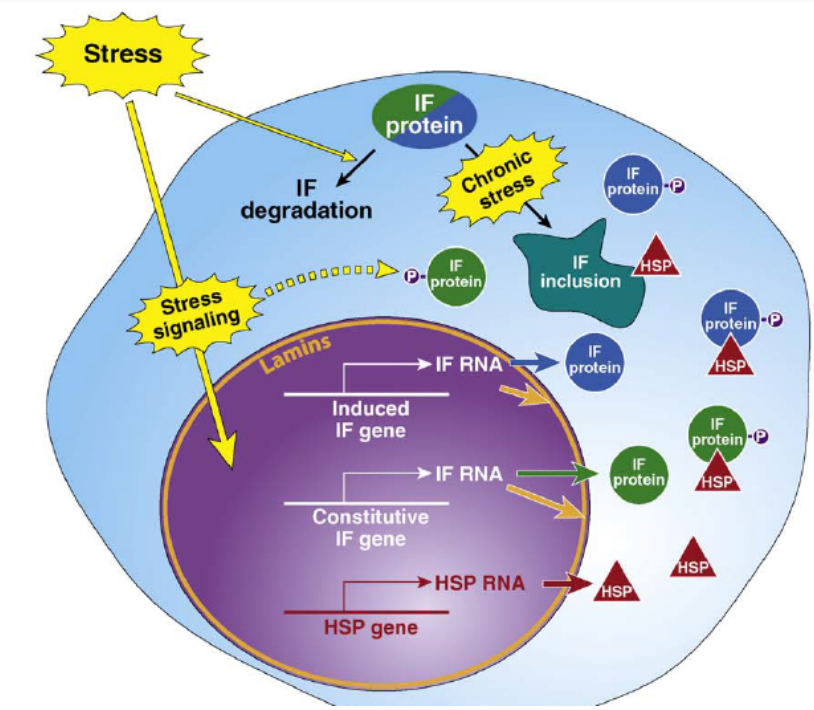

Figure 6: Specific stresses upregulate IFs and HSPs.

1.8. Keratin 8 and 18

Keratin 8 and 18 (K8/18) are the major components of the IFs of simple or single layered epithelia found in the gastrointestinal tract, liver, exocrine pancreas, and mammary gland, from which many carcinomas arise [37] . K18 is the only type I keratin found within the type II keratin multigene locus found on human chromosome 12. The other type I keratins are found on chromosome 17. The genes for K8 and K18 are adjacent to each other and at the distal end of the Krt type I locus. In mus musculus K8 & 18 genes are located in chromosome 15.They are the first keratins that appear in embryogenesis as early as in the pre-implantation embryo as well as the oldest keratins in phylogenesis [38] .It should be noted that K8 and K18 are not strictly epithelium-specific since expression of K8 and K18 may occur in rare mesenchymal cells (more frequently in fetal stages) such as certain smooth muscle cells and fibroblastic reticulum cells of lymph nodes as well as various mesenchymal tumors including rhabdo- and leiomyosarcomas [39 , 40] where they are co-expressed with other intermediate filament types, notably vimentin and desmin. Already the tissue distribution of K8/K18—mainly in internal epithelia—suggests that structural and mechanical functions are not their key roles, although their absence or dysfunction may be associated with hepatocyte and trophoblast fragility [34] .

1.9. Keratin 8 or 18 deficiency

Genetic targeting techniques using K8 or K18 knock-out mice models have revealed distinct regulatory functions of these keratins [34] . They play a role in protecting the placental barrier function [41] and protecting cells—in particular liver cells—from apoptosis [42] , against stress, and from injury

[43], possibly by functioning as a phosphate "sponge" for stress-activated kinases [44] . Interestingly, K8 and K18 may play a role in the regulation of the cell cycle, whereby phosphorylation of these keratins and binding of 14-3-3 adaptor proteins seem to be involved [45, 46, 47, 34]. In human pathology, defects in K8 and K18 may predispose to liver diseases, in particular cryptogenic liver cirrhosis [48] , as well as to chronic pancreatitis and in inflammatory bowel disease [49] . Altered K8 and K18 proteins, together with several stress proteins, in particular ubiquitin and p62, constitute the hyaline protein aggregates of hepatocytes of several (e.g. alcoholic) liver diseases now known as (MB) Mallory–Denk bodies [50] . Moreover, keratin 8 and 18 expression attenuates TNFa-mediated apoptosis through association with TRADD and regulates c-FLIP and ERK1/2 anti-apoptotic pathway [51, 52] . Furthermore, they regulate mitochondrial shape and function in hepatocytes [53] . Finally, keratin 18 overexpression inhibits MB formation [54] .

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1.10. DNA cloning

Discovery of the structure of DNA in 1953 and subsequent elucidation of how DNA directs synthesis of RNA, which then directs assembly of proteins—the so-called central dogma—were monumental achievements marking the early days of molecular biology. However, the simplified representation of the central dogma as DNA → RNA → protein does not reflect the role of proteins in the synthesis of nucleic acids. Moreover, proteins are largely responsible for regulating gene expression, the entire process whereby the information encoded in DNA is decoded into the proteins that characterize various cell types.

Techniques for DNA cloning have opened incredible opportunities to identify or study the genes involved in almost every known biological process . DNA cloning is the basis for other related technologies, such as gene therapy and genetic engineering of organisms. DNA cloning also made it possible to do genome sequencing. DNA cloning is a technique for isolating and reproducing a large numbers of identical DNA fragments. By introducing recombinant DNA into host cells the foreign DNA is reproduced along with the host cells.

Cloning of DNA molecules from genome can be achieved by two different approaches: cell based cloning or using polymerase chain reaction . For both these methods the use of restriction enzymes plays an important part. Restriction enzymes recognize a specific sequence on a DNA strand and cleave the DNA by catalyzing breaks in specific phosphodiester bonds [55] . The cleavage is on both strands of the DNA so that a double stranded break is made . This cleavage can give two types of ends, depending upon the specific restriction enzyme. Some restriction enzymes make sticky ends, with protruding single strands, which form hydrogen-bonded base pair with complementary sticky ends of other DNA fragments cut with the same enzyme. Other restriction enzymes make blunt ends; cleave both strands of DNA at the opposing phosphodiester bonds, leaving no unpaired bases on either end . Any blunt end can be joined to any other blunt end regardless of how the blunt end was generated.

The restriction enzymes were discovered in bacteria in the late 1960s [55] . Bacteria use restriction enzymes as protection against intruding DNA from other organisms, such as viruses and other bacterial cells. There are three classes of restriction enzymes, indicated I, II and III. Type I and III cleave the recognition sequence unspecific and at random sites. Type II restriction enzymes, the type most used within the gene technology, is specific and cleave the DNA within the recognition sequence itself . The DNA sequence recognized by Type II restriction enzymes is symmetric and usually palindromic. The DNA sequence is between 4 and 8 bp in length, with most restriction enzymes recognizing 4 or 6 bp. When the plasmid vector and the DNA fragments to be cloned are

cut with the same restriction enzymes, the DNA fragments can be ligated into the plasmid vector and form a circular recombinant DNA molecule. During the ligation reaction DNA ligase form phosphodiester bonds between the 3'hydroxyl and the 5' phosphate ends of the nucleic acid molecule [56] . Cell based DNA cloning involves separating a specific gene or DNA fragment from a larger chromosome using restriction enzymes. In order to clone a gene, its DNA sequence needs a carrier that can take it into the cell.

There are many different kinds of vectors and most of them are isolated from larger plasmids that occur naturally in bacterial cells. Plasmid vectors are small circular molecules of double-stranded DNA that can replicate separately from the host chromosome. Generally, a cloning vector contains three elements: a cloning site where the foreign DNA fragment can be inserted, a gene for antibiotics resistance and a replication origin to allow the plasmid to be replicated in the host cell.

When cloning DNA segments amplified by PCR into plasmid vectors there are different ways to do this. One possibility is to introduce specific PCR primers modified at their 5' ends so that they contain a suitable recognition site for restriction enzymes. By doing this the amplified DNA fragment of interest will carry restriction sites at its termini that can be used for further cloning into plasmid vectors. The vector and the amplified DNA fragment can thus be cleaved with appropriate restriction enzymes and ligated together [57] . Another method is TA cloning [58] , a much more efficient cloning strategy than blunt-end ligation and useful when compatible restriction sites are not available for subcloning DNA fragments from one vector into another. By using *Taq* polymerase, single deoxyadenosine is added to the 3' ends of the PCR fragments. This can be exploited by cloning the PCR product into linearized T vector, fitted with single overhanging 3' deoxythymidine residues at each of its 3'termini. PCR fragments that carry unpaired deoxyadenosin residues at their 3'termini can easily be cloned into vectors with single unpaired deoxythymidine residues at its 3'termini [59] , a fact that several commercial companies have taken advantage of .

The recombinant DNA generated by PCR or cell based cloning is then transformed into suitable host cells and reproduced along with the host cell DNA. Bacteria are most often used as host cells, and strains of Escherichia coli (E. coli) cells are the most common cell to use because they are easy to transform with DNA plasmid and its metabolism are well understood . To be able to take up foreign DNA, the bacteria cells need to be made competent. This is often achieved by treating them with divalent cations under cold conditions.For E. coli transformation with plasmid the DNA needs assistance to pass through the cell membranes and to reach the site where it can be expressed and replicated.

The plasmids can be introduced chemical or by electroporation . Chemical transformation involves a short heat shock of the solution to induce the cells to take up the DNA. In electroporation a brief electrical pulse is applied to the solution containing the cells and the DNA fragments to be inserted. In both cases the intention is to simplify the penetration through the cell membranes. When recombinant DNA is transferred into bacterial cells the recombinant DNA replicate independently of the cells genome. The host cells transformed by recombinant DNA are grown in culture and as the bacterium grows, the new recombinant DNA molecule is copied by DNA replication and, as the cell divides, the number of cells carrying the recombinant molecule increases.

To distinguish bacteria transformed by recombinant plasmids from bacteria that have no recombinant plasmid different methods have been developed. One way to do this selection is to use vectors carrying genes for antibiotic resistance. Most plasmid vectors contain antibiotic resistance gene that will change the antibiotic resistant of the bacteria when a fragment is transformed. This allows selection for recombinant plasmids because only those bacteria that have been successfully transformed with the desired recombinant plasmid molecules will grow on agar plates in the presence of that bacterium.

To select for clones that have insert in their vectors a so-called blue-white screening can be

done.This is a non-destructive histochemical procedure to detect β-galactosidase activity in transformed bacteria . However, this requires the use of plasmid vectors that carry unique restriction sites that serve as a marker with a marker gene. When DNA fragments are incorporated these marker genes are disrupted . Using α-complementation (blue-white selection), the β-galactosidase enzyme will not be produced when the lacZ gene in the plasmid vector is disrupted, but a plasmid without an insert will still produce β-galactosidase because the lacZ gene is still intact . β-Galactosidase cleaves a synthetic sugar, X-gal, that is similar to lactose, into two sugar components, galactose and glucose , one of which is blue [60] . Therefore, colonies containing plasmid vectors without insert will turn blue; on the other hand colonies containing plasmid vectors with insert will remain white .

Instead of blue-white selection, in this study, restriction digestions were performed to select the transformed clones. To identify clones carrying the recombinant plasmid, with the desired DNA insert, colonies are picked and grown in culture containing the appropriate antibiotic. The plasmid DNA is extracted by the standard minipreparation technique and analysed by restriction digest. The size of the insert or the orientation of the insert can be verified by restriction analysis of minipreps of vector DNA. After digesting the DNA, the samples are run on a agarose gel and the band sizes formed are compared with DNA fragments of known size. To confirm that the cloned DNA fragments are correct sequencing need to be done. This is especially important when the cloned DNA fragment is generated by PCR, since the polymerase can make mistakes, incorporating wrong nucleotides.

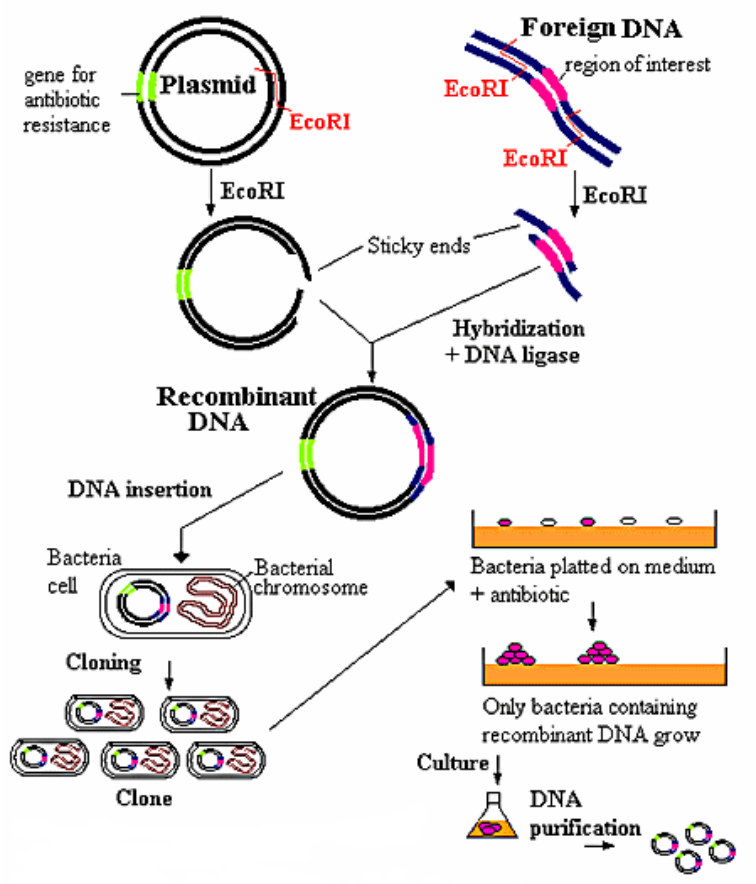


Figure 7: Basic principles of DNA cloning .

1.11. Gene-transfer strategies

Gene transfer to animal cells can be achieved essentially via three routes. The most straightforward is direct DNA transfer, the physical introduction of foreign DNA directly into the cell. For example, in cultured cells this can be done by microinjection, whereas for cells *in vivo* direct transfer is often achieved by bombardment with tiny DNA-coated metal particles. The second route is termed transfection, and this encompasses a number of techniques, some chemical and some physical, which can be used to persuade cells to take up DNA from their surroundings. The third is to package the DNA inside an animal virus, since viruses have evolved mechanisms to naturally infect cells and introduce their own nucleic acid. The transfer of foreign DNA into a cell by this route is termed transduction. Whichever route is chosen, the result is transformation, i.e. a change of the recipient cell's genotype caused by the acquired foreign DNA, the transgene. Transformation can be transient or stable, depending on how long the foreign DNA persists in the cell.

DNA-mediated transformation of animal cells occurs in two stages, the first involving the introduction of DNA into the cell (the transfection stage) and the second involving its incorporation into the genome (the integration stage). Transfection is much more efficient than integration; hence a large proportion of transfected cells never integrate the foreign DNA they contain. The DNA is maintained in the nucleus in an extrachromosomal state and, assuming it does not contain an origin of replication that functions in the host cell, it persists for just a short time (1-2days) before it is diluted and degraded. This is known as transient transformation (the term transient transfection is also used), reflecting the fact that the properties of the cell are changed by the introduced transgene, but only for a short duration. In a small proportion of transfected cells, the DNA will integrate into the genome, forming a new genetic locus that will be inherited by all clonal descendants. This is known as stable transformation, and results in the formation of a 'cell line' carrying and expressing the transgene. Since integration is such an inefficient process, the rare stably transformed cells must be isolated from the large background of non-transformed and transiently transformed cells by selection.

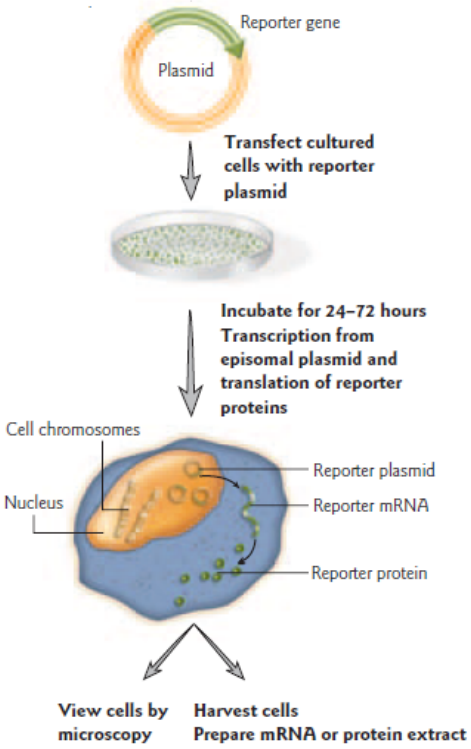


Figure 8 : Transient transfection of a reporter gene

1.12. Polyethylenimine (PEI) transfection

Mammalian cells take up exogenous DNA with various techniques such as the DNA/calcium phosphate coprecipitate method or phospholipid mediated endocytosis with liposomes or lipofection as well as electroporation.Recently, the use of polymers as transfection agents confers several advantages, due to their ease of preparation, purification and chemical modification as well as their enormous stability. The cationic polymer polyethylenimine (PEI) has been widely used for non-viral transfection *in vitro* and *in vivo* and has an advantage over other polycations in that it combines strong DNA compaction capacity with an intrinsic endosomolytic activity. It facilitates the tight compaction of plasmid DNA into small and positively charged complexes, which are readily internalized via non-specific adsorptive endocytosis by a large variety of cells *in vitro*.PEI has emerged as a potent candidate for gene transfer, even though the use of PEI-derived gene delivery vehicles is still limited by a relatively low transfection efficiency and short duration of gene expression [61] compared to viral transfection systems, as well as cytotoxic effects [62] . PEI provides effective DNA binding and protection, combined with a high endosomolytic competence and nuclear localization, which all contribute to the superior transfection efficacy of the corresponding DNA vectors compared to another agents [63] .

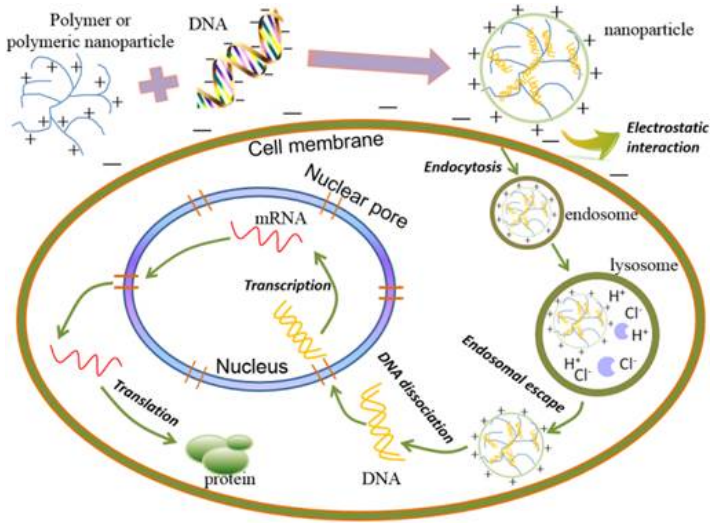


Figure 9: Polyethylenimine-mediated gene delivery

1.13. Adenoviral vectors and adenovirus construction system

Recombinant E1-deleted vectors derived from human adenovirus serotype 5 (Ad5) are highly efficient for *in vitro* and *in vivo* gene transfer into a variety of mammalian cells and tissues and have been used in functional and gene therapy studies , vaccination , and, lately, the

introduction of cDNA libraries into cell-based assay systems for gene discovery . Numerous methods for their construction have been described . These vectors also offer the advantage of high levels of transient transgene expression and relative ease of construction, propagation, and purification to high-titer stable virus [64] . Therefore, they are considered to be particularly suited as vector systems for functional genomics and cDNA expression cloning.

The principles of adenovirus construction system involve a donor virus (helper DNA) that contains genes essential for viral replication and a donor plasmid (vector DNA) that has the transgene as well as non-coding viral *cis*-acting elements that include a packaging domain. Separation of viral genes and *cis*-acting sequences during production is an important factor determining the efficiency and safety of a vector system preventing their reconstitution by recombination into productive viral particles. Recently, Hillgenberg et al. described a high-efficiency system for the construction of adenovirus vectors and its application to the generation of representative adenovirus-based cDNA expression libraries [65] . According to the assay described in Hillgenberg et al., construction of adenoviral particles that express keratin 8 and 18 IF proteins could be performed for infecting cardiomyocytes.Although preparation of recombinant adenovirus is complex and time consuming , adenoviruses have high infectivity, broad host range and gene transfer is not dependent on active cell division providing several advantages over plasmid DNA- and retroviral vector–mediated gene transfer into eukaryotic cells [66] and particularly cardiomyocytes.

In this study , adenoviral shuttle vector , pAdCMVloxP was used. It includes the adenoviral 5 'ITR, a packaging signal of the adenoviral genome,the cytomegalovirus immediate early enhancer/promoter, a multiple cloning site, a single loxP sequence on 3' end of the transgene along with SV40 poly(A) signal [67] . Except from being used as a donor plasmid for adenovirus contstrction, it can be used an adenoviral vector for transient transgene expression in mammalian cell lines.

1.14. Hypothesis

Since keratin 8 and 18 (K8-18) are ectopically expressed in the TNF-α desmin-/- cardiac muscle in which the pathological phenotype of desmin's deficiency is absent , K8-18 expression may have a cardioprotective role.

1.15. Aim of the study

Among all molecular pathways involved in the pathophysiology of heart failure, the cardiac cytoskeleton and particularly the IF protein network plays a crucial role. Beside their mechanical role in cardiomyocyte, IF proteins have also a regulatory role in cell signalling, cell growth and stress control as well as organelle function such as mitochondria. Therefore, investigating the role of keratin 8 and 18 IF network in TNFa desmin-/- cardiomyocytes may reveal the mechanisms that participate in cardioprotection.

The main goal of this study is to investigate the potential cardioprotective role of keratin 8/18 IF network *in vitro*. Particularly, whether K8/18 IF network compensates desmin's deficiency. Since cardiomyocytes do not express keratin 8-18 under normal conditions , cloning of murine keratin 8 and 18 cDNA should be initially performed. Therefore, specific aims of the study are:

1) construction of K8 and 18 plasmid adenoviral vectors
2) expression analysis in NIH 3T3 cell line (non-expressing K8/18 cell line)
3) construction of adenoviruses expressing K8 or K18
4) Infection of desmin-/- cardiomyocytes and performance of cell death assay with H202

Because of time-limitation, this study will involve the construction of K8 and K18 adenoviral vectors and the expression analysis experiments of keratin 8 and 18 IF proteins in NIH 3T3 cell line. The expreriments will study the expression of K8 or K18 or both after :

1) single transfection with K8 adenoviral plasmid-vector (pAd-*Krt8*)
2) single transfection with K18 adenoviral plasmid vector (pAd-*Krt18*)
3) double transfection with pAd-*Krt8* and pAd-*Krt18*

with immunofluorescence technique. The properties of Keratin 8/18 IF proteins will be studied along with the ability to form filamentous network in a non-expressing keratin cell line. Furthermore, keratin and vimentin (native IF of NIH) filamentous network interaction will be studied.

2. Materials and Methods

2.1. RNA electrophoresis

Reagents and instruments requirements

- Liver total RNA
- 37% Formaldehyde (12,3M)
- Ethidium Bromide 100mg/ml
- Formamide
- Glycerol
- Bromophenol Blue
- 10x MOPS buffer
  - 200mM MOPS
  - 50mM CH3COONa
  - 10mM EDTA
  - pH=7 (adjust with NaOH)
  - Store at RT in a dark place
- Agarose gel with 37% Formaldehyde
  - Agarose (1-1,2%)
  - 1x MOPS buffer
  - 0,7% Formaldehyde
  - Ethidium Bromide (EthBr) ,100mg/ml
  - autoclave, distilled water
- Running Buffer
  - 1x MOPS buffer
  - Formaldehyde 0,74%
  - autoclave, distilled water (RNase-free)
- RNA sample Loading Buffer
  - 4mM EDTA
  - 2,66% Formaldehyde
  - 50% Glycerol
  - 30% Formamide
  - 2,5x MOPS buffer
  - bromophenol blue
  - autoclave, distilled water
- Electrophoresis Machine (agarose tank)
- Ultraviolet Radiation Machine

Method

After RNA electrophoresis buffers were prepared, 1,2% agarose gel with formaldehyde is set up. 1,2 gr agarose in 75ml autoclaved, distilled water is heated until dissolved and then cooled to 60oC. Then, 10ml 10x MOPS , 1,8ml Formaldehyde 37% (12,3M) and 1ul EthBr (100mg/ml) are added. The gel is poured in a tank and a comb is used that will form wells large enough to accommodate at least 25 µl. The gel after congealment, is assembled in the electrophoresesis tank and enough running buffer is added to cover the gel by a few millimeters and then the comb is removed. Afterwards , 1,5ug from each RNA sample is diluted in 1x RNA loading buffer, then ,

heated (RNA denaturation) at 65oC for 3-5mins, chilled on ice (4oC) and finally, it is loaded in agarose wells. Electrophoresis is accomplished at 6-10V/cm until the bromophenol blue (the faster-migrating dye) has migrated at least 2-3 cm into the gel, or as far as 2/3 the length of the gel (duration: 20-30mins). Finally, the gel is visualized on a UV transilluminator.

2.2. Primer Pair Design

Primer Pair design is essential for several applications as PCR, DNA sequencing and hybridization.Polymerase chain reaction is widely held as one of the most important inventions of the 20th century in molecular biology. Small amount of genetic material can now be amplified with a DNA polymerase and a primer pair in order to identify and/or manipulate DNA.

The ideal primer pair have the following characteristics. The optimal length of each primer is generally 18-22 bases , long enough for specificity and short enough to bind easily at annealing temperature.The primer's GC content which determines primer melting temperature (Tm) should be between 50-60% .Tm is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates duplex stability. A primer with Tm>65oC has a tendency for secondary annealing. The optimal range of primer Tm is 58-62oC and Tm difference between primer pair should be 2 units. In addition , it is important to design primers that are specific for the desired PCR product. Primer-BLAST, an online software, was developed at NCBI to help users make primers that are specific to the input PCR template. It supports PCR primers design and then, submits them to BLAST search against user-selected database. The blast results are then automatically analyzed to avoid primer pairs that can cause amplification of targets other than the input template. Moreover, it is recommended to avoid 3 or more Gs Cs at the 3' primer end , mismatches at the 3'end, complementary sequences within the primer as well as primer-dimer formation by complementarity at the 3'ends of primer pairs. Finally, the potition range of its primer on input template and the amplicon length depend on the experimental goals.

2.2.1 Primer reconstitution

Reagents

- primer pellet
- autoclave, distilled water

Method

Using the nanomole quantity – to reconstitute to a given concentration , convert nmole to umole and then divide the desired concentration, usually 100umole/L (100uM).

2.3. Reverse Transcription

Reagents and Instruments Requirements

- total RNA (in our case from liver)
- Random primers or gene-specific antisense primer
- dNTP mix (10mM each dATP,dGTP,dCTP and dTTP at neutral pH)

- Autoclave, distilled water
- 5x First Strand Buffer
  - 250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM MgCl2
- 0,1M DTT
- RNase OUT Recombinant Ribonuclease Inhibitor (40u/ul)
- Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT)
- E.Coli RNase H
- PCR machine
- Ice
- nuclease-free microcentrifuge tubes

Method

Reverse transcription is a method used frequently for expression analysis experiments after quantitive PCR as well as molecular cloning applications. Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase is a recombinant DNA polymerase that synthesizes a complementary DNA strand (cDNA) from single stranded RNA, DNA or an RNA:DNA hybrid. It lacks DNA endonuclease activity and has lower RNAse H activity compared to another reverse transcription enzymes. The size of cDNA formed may be up to 7kb and its optimal activity is at 37oC.

For the first strand cDNA synthesis using M-MLV reverse transcriptase (M-MLV RT), it is usually used a 20ul reaction volume for 1ng-5ug of total RNA. In each nuclease-free microcentrifuge tube, 2ug total RNA (from liver) and sterile , distilled water were added so that final volume would be 10ul. Afterwards, a second mix solution was prepared with dNTPs and with random primers.Random primers are DNA hexamers that anneal on the template and are essential for priming by M-MLV RT. Particularly, 0,09ul random primers (0.3ug), 0,5ul 10mM dNTPs and sterile, distilled water were added to a final volume of 2ul. Previous solutions were always on ice. Then, total RNA solution and random primers with dNTPs mix were blended, then, heated to 65oC (improves RNA denaturation and primer annealing) for 5min and quick chilled on ice for 2min .Afterwards, 4ul of 5x First Strand Buffer, 2ul 0,1M DTT and 1ul RNAse Out recombinant ribonuclease inhibitor (40u/ul) were added to each previous RNA mix and then, heated to 37oC for 2min. In the continuity, 1ul M-MLV reverse transcriptase was added , then, each mix was heated to 37oC for 50min (reverse transcription).

The cDNA can now be used as a template for amplification PCR. However, amplification of PCR targets >1kb may require the removal of RNA complementary to the cDNA. In order to remove RNA complementary to the cDNA, 1ul of E.Coli RNase H was finally added and then, the solution was incubated at 37oC for 20min. Finally , it was stored to freeze storage at -20oC

2.4. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reagents

– with Taq DNA polymerase
- ssDNA(cDNA) or dsDNA
- 10x PCR buffer [200mM Tris-HCl (pH 8.4), 500mM KCl
- 50mM MgCl2
- 10mM dNTP Mix (10mM ATP, 10mM TTP, 10mM GTP, 10mM CTP)
- Forward primer , 10uM

- Reverse primer, 10uM
- Taq Polymerase (5u/ul)
- autoclaved , distilled water

– PCR HF Supermix
- ssDNA (cDNA) or dsDNA
- PCR SuperMIx High Fidelity
  - 66mM Tris-SO4 (pH 9,1 at 25oC) ,
  - 19,8mM (NH4)2SO4 ,
  - 2,2mM MgSO4,
  - 220uM dNTPs,
  - Stabilizers
  - 22U/ml DNA polymerase mixture
    - Taq DNA polymerase
    - Proofreading Pyrococcus species GB-D polymerase

– Q5 HF DNA polymerase
- ssDNA (cDNA) or dsDNA
- 5x Q5 reaction bufferr
- 10mM dNTPs
- 10uM Forward primer
- 10uM Reverse primer
- Q5 HF DNA polymerase
- Autoclaved, distilled (Nuclease-free) water

– Cycling Conditions
– Instruments
- PCR machine: thermal cycling

Method

The polymerase chain reaction (PCR) is a powerful method for fast in vitro enzymatic amplifications of specific DNA sequences. Each PCR is likely to require specific optimization in order to achieve specificity, fidelity and efficiency for the template/primer pairs chosen.

In addition , according to the experimental procedure , different DNA polymerases may be used. In case of molecular cloning , the fidelity of PCR is essential . DNA Polymerase is important for the fidelity of the PCR products. Except from the tradditional Taq polymerase, there are another polymerases and mixtures with higher fidelity than Taq. A PCR High fidelity Supermix with Taq and the proofreading Pyrococcus species GB-D polymerase provides six times higher fidelity than Taq polymerase. Additionaly, a Q5 high fidelity polymerase has a 50 fold lower error rate than Taq polymerase and 6 fold lower than Pyrococcus furiosus(Pfu) DNA polymerase.

AII of the reaction components were mixed in together in a 0.5-mL PCR tube in any sequence except for the DNA polymerase, which was added last. All the components were also right before PCR cycling. However, it is not necessary , the PCR was set up on ice.

The optimal conditions of the PCR reactions and thermal cyclings for our specific cDNA targets, keratin 8 and 18 as well as for b-actin , the positive control are indicated below:

- **<u>Reaction and Cycling Conditions</u>**

- **b-actin PCR**

Add:	**µl**
cDNA template after RT with random primers	**1**
PCR Buffer 10x , Invitrogen	5
MgCL2 50mM , Invitrogen	1,75
dNTPs 25mM	0,4
b-actin FW , 10µM	1,25
b-actin RV, 10µM	1,25
Taq Polymerase Invitrogen	0,4
ddH20	38,95
Final Volume	50

Step	Temp	Time	Note
1	94oC	5min	
2	94oC	30sec	
3	60oC	30sec	
4	72oC	30sec	repeat steps 2-4 for 29 cycles
5	72 oC	3min	
6	4 oC	indefinite	hold

- Keratin 8 (*KRT8*) PCR
- **Keratin 18 (*KRT18*) PCR**

KRT8 RT-PCR	
	µl/reaction
cDNA	2
KRT8 FW 10µM	1
KRT8 RV 10µM	1
PCR SuperMix High Fidelity	45
ddH2O	1
Vf	50
KRT18 RT-PCR	
	µl/reaction
cDNA	2
KRT18 FW 10µM	1
KRT18 RV 10µM	1
PCR SuperMix High Fidelity	45
ddH2O	1
Vf	50

PCR program:	Step	Temp	Time	Note
KRT8-18	1	94°C	2min	
	2	94°C	30 sec	
	3	60oC	30 sec	
	4	72°C	1min 45sec	repeat steps 2-4 for **30 cycles**
	5	72°C	10 min	
	6	4oC	10min	hold

- **Keratin 8 (KRT8) PCR**

Reagents	ul/reaction
total cDNA (liver)	0,5
5x Q5 reaction buffer	5
dNTPs, 10mM	0,5
KRT8 FW, 10uM	1,25
KRT8 RV, 10uM	1,25
Q5 HF DNA polymerase	0,25
ddH20	16,25
Vf	25

Cycling Conditions

Step	Temperature	Time	Note
1	98oC	30sec	
2	98oC	15sec	
3	61oC	30sec	
4	72oC	1.45min	repeat steps 2-4 for 24times
5	72oC	2min	
6	4oC	indefinite	hold

Steps

- 1-2 : Denaturation
- 3 : Annealing
- 4 : Extension

FW : Forward primer
RV : Reverse primer

2.5. DNA electrophoresis

Reagents

- DNA samples
- Loading Dye
  - 6x Orange G
- DNA ladder (e.x 1kb)
- Agarose Gel (x %, w/v)
  - 1x TAE (ml)
  - Agarose (gr)
  - Ethidium Bromide (5ul/100ml agarose gel)
- Running Buffer
  - 1x TAE
  - distilled water
- Instruments
  - Gilson Pippetes/ pippete tips
  - Gel tanks/ comb
  - Electrophoresis Tank and Machine
  - Ultraviolet Radiation Machine

Method

Gel electrophoresis separates DNA molecules according to size.According to the expected DNA band sizes the percentage of agarose in gel differentiates (see figure below) . To prepare 1,5% agarose gel, 1,5gr agarose in 100ml running buffer 1x TAE are heated until dissolved and then, cooled to 60oC. After the addition of 5ul EthBr (100mg/ml) per 100ml agarose gel, gel is poured on tank with comb. When gel is cooled and harden , remove comb from gel, and place it inside an electrophoresis tank filled with 1x running buffer enough to cover the gel. Afterwards, an amount (it depends on the case) of DNA samples with 1x Orange G loading dye is loaded into each well. Electrophoresis is accomplished at 6-10V/cm until the orange G dye (the faster-migrating dye) has migrated at least 2-3 cm into the gel, or as far as 2/3 the length of the gel (duration: 20-30mins).Negatively charged molecules as DNA or RNA are run on the positive end of electrophoresis tank. Finally, the gel is visualized on a UV transilluminator. The sizes of the bands in the unknown samples can be calibrated by comparison to size markers (for example, 1kb DNA ladder) that have been run also (ex. in the leftmost lane) in the gel.

TABLE 5-2 Range of Separation of DNA Fragments through Different Types of Agaroses

	Size Range of DNA Fragments Resolved by Various Types of Agaroses			
Agarose (%)	Standard	High Gel Strength	Low Gelling/Melting Temperature	Low Gelling/Melting Temperature Low Viscosity
0.3				
0.5	700 bp to 25 kb			
0.8	500 bp to 15 kb	800 bp to 10 kb	800 bp to 10 kb	
1.0	250 bp to 12 kb	400 bp to 8 kb	400 bp to 8 kb	
1.2	150 bp to 6 kb	300 bp to 7 kb	300 bp to 7 kb	
1.5	80 bp to 4 kb	200 bp to 4 kb	200 bp to 4 kb	
2.0		100 bp to 3 kb	100 bp to 3 kb	
3.0			500 bp to 1 kb	500 bp to 1 kb
4.0				100 bp to 500 bp
6.0				10 bp to 100 bp

2.5.1. Preparation of DNA or RNA loading Dye

Bromophenol Blue

- **Reagents**
  - 30% (v/v) glycerol
  - 0,25% (w/v) bromophenol blue
  - 0,25% (w/v) xylene cyanol FF
  - sterile, distilled water
  - Store at 4oC or RT

Orange G

- **Reagents**
  - 30% (v/v) glycerol [or Sucrose(40%), Ficoll (15%)]
  - 0,25% (w/v) Orange G
  - sterile, distilled water
  - Store at 4oC or RT

Method

In 50ml sterile distilled water , 25gr Orange G are dissolved and then 30ml of glycerol are added . Finally, sterile distilled water is added to reach a final volume of 100ml. Bromophenol Blue loading Dye is formed like orange G.

2.6. Gel extraction Protocol (QIAGEN)

Reagents

- DNA Samples
- Loading Dye
- 1% Agarose Gel
- Running buffer
  - 1x TAE
- QIAquick gel extracion buffers
  - Buffer QG
  - Buffer PE with Ethanol
- Isopropanol
- Autoclaved , distilled water

Instruments

- balance
- Water-bath
- Clean Sharp Scalpel
- QIAquick spin columns
- Centrifuge machine
- Freeze Storage (-20oC)

Method

In the beginning , cleaning of electrophoresis tank , preparation of fresh 1x TAE running buffer and 1% agarose gel with 5ug EthBr / 100ml were set up. DNA sample with its loading dye is loaded to the gel along with the DNA ladder. After sample (according to the loading dye) is run enough in the gel at the electrophoresis tank (5-6V/cm), the gel is visualized with a low frequency UV transilluminator. Then, the DNA fragment is excised from the agarose gel with a clean, sharp scalpel. The gel slice is weighed in a 1,5ml microcentrifuge tube and 3 volumes of buffer QG to 1 volume of gel (100mg of gel slice corresponds approximately to 100ul). Then , the tube is incubated at 50oC for 10min and every 2-3min during the incubation the solution is mixed by vortexing. If the color of the mixture is orange or violet, 10 µl of 3 M sodium acetate pH=5 are added, and mixed. After the gel slice is completely dissolved , 1gel volume of isopropanol is added to the sample and mixed. The sample is applied to a QIAquick spin column with a 2ml collection tube and is centrifuged for 1min. After DNA is bound in the column , flow-through is discarded and the column is placed again in the same collection tube. Then, 0,5ml of QG buffer is added and is centrifuged for 1min. The flow-through is discarded again. Then, in order to wash bound DNA, 0,75ml of PE (with ethanol) buffer is added to the column , is centrifuged for 1min and the flow-through is discarded. Column is centrifuged for an additional 1min and residual ethanol from Buffer PE is discarded. QIAquick column was let with open lid for 1 min. Then, column is placed into a clean 1,5ml microcentrifuge tube. 10ul of ddH20 are added to the centre of the membrane and the column is let stand for 1min. Then, centrifuge for 1min is applied and DNA is eluted. DNA solution is stored to freeze storage at -20oC. All previous centrifugations were at 13000rpm.

2.7. Nucleic Acid Quantitation

Reagents

- Nucleic Acid (DNA or RNA)
- Autoclave, distilled water

Instruments

- Spectrophotometer
- Quartz tube

Method

Nucleic Acid concentration may be quatitated by the absorbance at 260nm (OD_{260}nm or A260) through a spectrophotometer. Particularly, DNA sample is diluted 1:500 with sterile, distilled water and transferred to a quartz tube. Then, the concentration is measured using the following formula :

C= OD_{260nm} * dilution factor (500) * 50 µg/ml .

Nucleic Acid Species	Concentration for 1 A260 Unit (µg/ml)
dsDNA	50
ssDNA (oligonucleotides)	33
ssRNA	40

2.8. Luria-Bertani (LB) medium and agar plates

Reagents

- Tryptone
- Yeast Extract
- NaCl
- NaOH for pH adjustment
- deionized water
- Agar (15gr/L) , 1,5%, w/v
- 1000x Antibiotic (ex. 50mg/ml kanamycin or 100mg/ml ampicillin, etc)

Instruments

- Autoclave
- Fire machine
- 100mm plates
- bottles

Method

The Luria-Bertani (LB) medium contains 1% tryptone, 1% NaCl and 0,5% Yeast Extract. The pH of the solution should be from 7 to 7,4 and optimally at 7,2 as it is essential for cell growth.Particularly, for 1liter LB, 10gr Tryptone, 10gr NaCl and 5gr Yeast Extract are dissolved to 950ml deionized water. The pH of the soultion is adjusted to 7,2 with the addition of NaOH and the final volume is bringed up to 1 liter. Then, it is autoclaved on liquid cycle for 3hrs. The sollution is allowed to cool to 55oC. If it is needed a antibiotic is added, either kanamycin or ampicillin to a final concentration of 50 and 100ug/ml , respectively. Finally, LB is stored at room temperature while if antibiotic is added at 4oC.

For the preparation of LB agar plates, LB medium is prepared as above, but before autoclaving 15gr agar per liter are added. The mix is autoclaved again in liquid cycle and is allowed to cool to 55oC. Immediately, an antibiotic, either kanamycin or ampicillin is added to a final concentration of 50 or 100ug per ml, respectively. Then, before the solution starts to congeal , it is poured into 10cm plates and is let harden over-nigh on bench before storing at 4oC in the dark.

2.9. TA Cloning Reaction-Ligation

Reagents

- pCRTMII-TOPOR vector (4,0 kb)
- PCR products with 3'-A overhangs
- Salt solution
- 1,2M NaCl
- 0,06M MgCl2
- Autoclave, distilled water

Equipments

- Cold storage

Method

As mentioned previously, PCR High fidelity supermix has a mixture of Taq DNA polymerase and the proofreading Pyrococcus species GB-D polymerase. *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. Particularly, this mix provides higher fidelity than single Taq polymerese and a majority of PCR products with 3-adenine overhang.

The plasmid, pCRTMII-TOPOR vector is supplied by Invitrogen linearized with single 3' deoxythymidine (T) residues. This molecular state is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved (by topoisomerase I) strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the PCR product, reversing the reaction and releasing topoisomerase I [68] . Generally, 5'ends of primer are provided dephoshorylated and thus 5'ends of PCR products are also dephosphorylated. Therefore, the interaction between the linearized vector and the PCR product results in their efficient ligation and formation of a circular plasmid along with the release of topoisomerase I. With this TOPO vector, DNA ligase is no needed.

For TOPO Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM MgCl2 in the TOPO® Cloning reaction increases the number of colonies over time. Moreover, per cloning reaction 10ng of TOPO vector (10ng/ul) are used. The amount of insert is calculated with the following formula :

insert(ng) = vector (ng) x size of insert (kb) x 1/size of vector(kb) x molar ratio insert/vector

For an efficient ligation the molar ratio of insert to vector is 3:1.

According to the size of TOPO vector which is 4kb and that of the PCR products keratin 8 (c*K8*) and 18 (c*K18*) which is 1,6kb and 1,35kb respectively, the amount of its component is calculated for the TA cloning reaction. Particularly, 10ng of TOPO vector are mixed with 12ng of c*K8* along with 1ul salt solution (1.2 M NaCl; 0.06 M MgCl2) and autoclaved, distilled water to a final volume of 6ul. The solution was gently mixed and incubated for 20min at room temperature (25oC). Then , it was placed on ice and transformation of chemically competent E.coli (DH5A) cells was followed. If not , mix may be stored at freeze storage (-20oC). c*K18* and TOPO vector cloning reaction was performed with the same way like c*K8*. In this case, 10ng of c*K18* were mixed with 10ng vector.

2.10. Transformation of chemically competent E.Coli (DH5A) cells

Reagents

- DH5A chemically competent cells
- Plasmid
- LB
- LB agar plates with antibiotic

Instruments

- Water-bath
- Incubator

Method

In the beginning, chemical competent DH5A cells are removed from -80oC freezer and

thawed on ice for 10min. Then, the plasmid is added into a tube with 100ul competent cells , mixed gently and placed on ice for 30min. An amount of 1-10ng plasmid is usually enough for a transformation. Then, the tube is removed to a water-bath at 42oC and heat-shock is performed to the DH5A cells for 30-45sec. The tube is immediately removed on ice for 1min. 900ul LB medium are added and the mix is incubated in an horizontal shaker at 37oC for 1 hour. During the incubation, transformed cells would express the antibiotic resistance gene. Finally, the 1/10 and the 9/10 of the solution are spread carefully under sterile conditions using fire to pre-warmed agar plates with antibiotic (kanamycin or ampicillin) and are incubated over-night (12-16hrs) at 37oC. The transformed DH5A cells would form colonies on the plate.

2.11. Mini or Midi or Max Liquid Culture

Reagents

- Pick with tips
- LB medium with
  - antibiotic (kanamycin or ampicillin)

Instruments

- Shaking Incubator

Method

After the formation of colonies on selective plates , the growth of cells may be performed in liquid cultures depending on the experimental procedures. According to the volume of liquid culture , there are mini (5ml) , midi (50-100ml) and max (200-250ml) cultures. For mini-cultures , a colony is picked with a tip and is inoculated in 5ml LB medium antibiotic (kanamycin or ampicillin) to a final concentration of 100ug/ml. In case of midi and max liquid cultures, pre-inoculation step is performed where transformed cells are grown in 1-2ml LB medium with antibiotic and then , depending on the final volume, 1/1000 from the pre-inoculum volume is added. In all cases, the liquid cultures are incubated at 37oC for 12-16 hrs until they are blurred.

2.12. DNA preparation

2.12. 1. Mini DNA preparation (mini-prep)

Alkaline Preps

- Reagents
  - Mini-culture
  - Solution P1
    - 50mM Glucose
    - 10mM EDTA, pH=8
    - 25mM Tris, pH=8
    - Autoclaved, distilled water
  - Solution P2
    - 0,2N NaOH
    - 1% SDS
    - Autoclaved distilled water
  - Solution P3 (4oC)

- 15% Acetic acid glacial
- 3M CH3COOK
- Autoclaved, distilled water

- Chorophorm : Phenol : Isoamyl , 24:25:1
  - Phenol
  - Chlorophorm : isoamyl alcohol (24:1)
- Ethanol (100% and 70%)
- Autoclaved, distilled water
- TE buffer (10 mM Tris·Cl. 1 mM EDTA, pH 8)
- RNase A

Instruments

- Centrifuge Machine (Cold and RT)
- Cold Storage

Method

In the beginning, after the solutions P 1-3 are set up, 2-5ml from the mini-culture are centrifuged at 3000rpm , at 4oC for 10min. The supernatant is removed and the cell pellet is resuspended using 150ul of solution P1. After vortexing and pipeting, the mix is transferred to new 1,5ml centrifuge tube and is let for 2min at room temperature. Then, 300ul of solution P2 are added, mixed carefully by inverting the tube 5-6 times and the mix is placed on ice for 5min. Afterwards, 225ul of solution P3 are added , mixed as previously and the mix is also placed on ice for 5min. After cell lysis reaction is finished, the mix is centrifuged at 13000rpm at 4oC for 30min and the supernatant is transferred to new 1,5ml microcentrifuge tube. 600ul phenol and chlorophorm isoamyl (CPI) solution is added and mixed with mild vortex. Then, the solution is cetrifuged at 13000rpm for 5min at room temperature. Upper phase (-500ul) is transferred to new 1,5ml tube and 1200ul of room temperature (RT) 100% Ethanol are added. The solution is mixed well and let at RT for 2min. Then, it is centrifuged at RT for 5min and supernatant is removed. Tube is drained while keeping DNA pellet inside.1ml of 70% Ethanol is added and mixed by brief vortex in order to wash DNA pellet. It is centrifuged at 13000rpm for 5min at room temperature. Supernatant is discarded and the tube is drained enough leaving at room temperature with open lid (or using vaccum centrifuge for 5min) for 15min so that DNA pellet is not enough dry. According to the size of DNA pellet, it is resuspended in 30-100ul ddH20 or TE (Tris-EDTA) with RNase A to a final concetration of 200ug/ul (ex. 2ul of 10mg/ml RNase per 100ul ddH20). Mini-DNA preps may be stored at -20oC.

2.12.1.1 Plasmid DNA preparation with mini-columns

Reagents

- Nucleospin Plasmid Isolation kit buffers
  - Resuspension Buffer A1
  - Buffer A2 (SDS/alkaline lysis)
  - Buffer A3 (inactivation)
  - Buffer A4+Ethanol
- Autoclave, distilled water

Instruments

- nucleospin mini-columns
- Centrifuge Machine

Method

1-5ml mini-culture is centrifuged at 3500rpm for 10min and supernatant is discarded. Cell pellet is resuspended with 250ul buffer A1. Then, 250ul buffer A2 are added , mixed gently by inverting 6-8 times and the mix is left at room temperature for 5min or until lysate appears clear. Alkaline lysis is then, inactivated with the addition of 300ul buffer A3 and mixing by inverting 6-8 times. It is recommended not to vortex the mix in order to avoid sheering of genomic DNA in the solution. Afterwards, the lysate is centrifuged at 13000rpm for 5min at room temperature. If supernatant is not clear , centrifugation should be repeated. Then, supernatant is transferred to mini-columns. It is centrifuged at 11000g for 1min at RT and DNA is bound on column while flow-through is discarded. The column is washed by adding 600ul A4 with Ethanol and centrifugation for 1min at 11000g , discarding flow-through. The column is centrifuged for 2min at 11000g to discard excess ethanol and dry the membrane of mini-column. The DNA is eluted by adding 50ul ddH20 to the centre of the membrane and centrifugation for 1min at 11000g. The DNA sample may be stored at freeze storage (-20oC).

2.12.2 DNA midi preparation (QIAGEN)

Reagents

- midi prep buffers (QIAGEN)
  - RES Buffer with RNase A (resuspension)
  - Lysis Buffer
  - EQU buffer (equilibration)
  - NEU buffer (neutralization)
  - Wash buffer
  - Elution Buffer
- Isopropanol
- Ethanol (100% , 70%)
- Autoclaved, distilled water or TE (Tris-EDTA)

Instruments

- Midi-prep columns
- Column filters

Method

An over-night 100ml (midi) culture is centrifuged at 3000rpm for 10min at 4oC. The supernatant is discarded and the cell pellet is resuspended with 8ml RES buffer with RNase A. After the addition of 8ml lysis buffer, the solution is mixed gently by inverting the tube 5times and is incubated at RT for exactly 5min to avoid degradation of plasmid DNA or liberation of chromosomal DNA. Then, 8ml of NEU buffer are added and immediatelly mixed by inverting 10-15times so that lysis reaction is inactivated. The column is equilibrated with the addition of 12ml EQU buffer onto the rim of column filter and allowing to empty by gravity flow. After inverting the tube with the lysate 3times , it is applied to the equilibrated column which is allowed to empty by gravity. The column filter is discarded and the column is washed with the addition of 8ml WASH buffer. Then, column is placed on new sterile 50ml centrifuge tube and DNA is eluted with the

addition of 5ml ELU buffer. Afterwards, 3,5ml RT isopropanol is added for plasmid DNA precipitation. The solution is mixed with vortex and it is centrifuged at 15000g for 30min at 4oC. Then, supernatant is discarded and DNA pellet is washed with 2ml 70% ethanol after centrifugation at 15000g at RT for 5min. Supernatant is discarded and DNA pellet is let to dry at RT. DNA pellet is reconstituted with 100ul ddH2O or TE. DNA solution may be stored at -20oC freeze storage.

2.13. PCR product purification protocol (QIAGEN)

Reagents

- Sample
  - 100bp-10kb ss or ds DNA
- Buffers
  - PB (although is confidential, it contains guanidine hydrochloride and isopropanol)
  - PE with Ethanol
- Autoclave, distilled water

Instruments

- QIAquick spin column (<10ug/column)
- Collection tube
- Centrifuge machine

Method

Using PCR purification kit , 100bp to 10kb ss or ds DNA may be purified from a solution using mini-columns while all the rest elements such as buffers, enzymes are removed. In the beginning , 5volumes of buffer PB are added to 1volume of the PCR sample and mixed. Then, the sample is applied to a spin column in a provided 2ml collection tube. It is centrifuged at 13000 rpm for 1min at RT so DNA is bound on membrane. The flow-through is discarded and 0,75ml buffer PE are applied and centrifuged again with the same speed and time to wash the column. The flow-through is discarded again and spin column is centrifuged again as previously to remove excess ethanol. Then, column is allowed to dry with open lid for 2min at RT. Finally, 50ul ddH2O are added to the centre of membrane and let it stand for 2min. With centrifugation with the same conditions as previously, DNA is eluted. It may be used either immediately for expreriment or stored at freeze storage (-20oC).

2.14. DNA Digestion with restriction enzymes

Design

- NEB cutter online software
- DNA sequence
  - plasmid vector
  - insert
- Identify Restriction maps

Reagents

- DNA

- 10x NEB buffer
- 100x BSA (optional)
- Enzyme (units / volume)
- Autoclaved, distilled water

Instruments

- Water-Bath

Method

DNA Digestion is used frequently in molecular cloning either to identify recombinant plasmids or use DNA fragments for cloning techniques. The amount of DNA that is cutted depends on the application. Diagnostic digestions typically involve ~500ng of DNA, while molecular cloning often requires 1-3µg of DNA. The total reaction volume usually varies from 10-50µL depending on application and is largely determined by the volume of DNA to be cut. Generally, the amount of enzyme used is 1-5units/ug DNA and should be added by the end. By definition, one unit of enzyme will cut 1ug of DNA in a 50 µL reaction in 1 hour The enzymes also need a digestion buffer and optionally bovine serum albumin for an efficient digestion. Additionally, the maximum volume that an enzyme can be used is 1/10 of the total reaction volume. Digestion buffers provided by New England Biolabs (NEB) are known as NEB1, 2, 3 or 4 buffers and are also provided as 10x buffers. According to NEB , each enzyme can act optimally under certain NEB buffers along with BSA or not.Besides the buffer that comes with the enzyme, buffers from other company can be used , too as long as the contents are the same. For example, 1ug DNA are digested in 20ul total reaction. 2ul 10x NEB buffer , 0,2ul 100x BSA if recommended , 0,5ul (10u/ul) enzyme and autoclaved, distilled water to reach 20ul final volume are added. Then, the solution is gently mixed by pippeting and is incubated at the optimal temperature of the enzyme for 1-3hrs in water-bath. Finally, 2-5ul of the digested sample , as well as the uncut DNA along with the loading dye and a DNA ladder are run on appropriate (w/v) agarose gel to identify the result and a proportional experimental procedure is applied.

2.15. DNA sequencing

Reagents

- DNA samples
  - Plasmid or
  - PCR product
- Primers
  - Universal (Forward, reverse)
  - Own primer

Instruments

- Sequencing machine
- By VBC Genomics

Method

DNA sequencing is essential in molecular cloning. It is based on Sanger method. The automated method uses 4 fluorescently labeled terminationg nucleotides present in a single reaction. The fragments from PCR with a labeled primer are seperated by electrophoresis in a single vertical

gel and as migrating fragments pass through the scanning laser , they fluorescence. A fluorescent detector records the color order of the passing bands and that order is translated into sequence date by a computer.

This method is performed by VBC Genomics. DNA samples should be diluted either in ddH20 or TE (Tris-EDTA). The concentration of a plasmid DNA should be at least 80ng/ul and the amount per reaction should be 500ng. In contrast, the concentration of PCR product should be at least 14fml/ul and the amount for each reaction 80-100fmol. It is recommended to send 3 times more amount for each reaction in case that repeat is needed. Additionally, for the sequencing reaction could be used either universal or own designed primers. The concentration of the primer is like in PCR , 10mM and usually an amount of 20ul/ reaction from own primer is sent.

The sequencing results may be analyzed through a multi-align online software where the sequencing result from each reaction is aligned with known DNA sequence.

2.16. DNA Polymerase I, Large (Klenow) Fragment reaction

Reagents

- DNA with sticky ends
- NEB buffer 1-4 or T4 DNA ligase buffer
- dNTPs 1mM
- Klenow fragment (5u/ul)
- EDTA, pH=8

Instruments

- PCR machine

Method

DNA polymerase I , Large Fragment (Klenow) catalyzes 3' overhang removal along with 5' overhang fill in and results in formation of blunt ends to polynucleotides. DNA should be dissolved in 1x NEB buffer 1-4 or T4 DNA ligase reaction buffer supplemented with 33uM dNTPs. Then, 1unit Klenow per 1ug DNA is added and the solution is incubated at 25oC in PCR machine for 15min. The reaction is stopped by adding EDTA to cf=10mM and heating at 75oC for 20min in PCR machine. DNA may be purified either with gel extraction or using QIAGEN mini-column. DNA sample may be stored at freeze storage (-20oC).

2.17. Dephosphorylation with Antarctic Phosphatase

Reagents

- Digested either vector or DNA
- 10x antarctic phosphatase buffer
- Antarctic phosphatase (5u/ul)
- Autoclaved, distilled water

Equipments

- Water-bath

Method

Dephosphorylation is sometimes necessary to avoid self-ligation of the vector. DNA dephosphorylation may be achieved by antarctic phosphatase (AP) which can be heat inactivated. The protocol that is usually followed is : 1/10 volume of 10X Antarctic Phosphatase Reaction Buffer is added to 1– 5 μg of DNA cut with any restriction endonuclease in any buffer. Then, 1 μl of Antarctic Phosphatase (5 units) is added and mixed. The mix is incubated for 15 minutes at 37°C for 5′ extensions or blunt-ends, 60 minutes for 3′ extensions. Generally, 30min are enough for the reaction. Finally, heat inactivation of enzyme for 5 minutes at 65°C is performed.

2.18. T4 DNA Ligase

Reagents

- Linearized duplex DNA or RNA
- 10x T4 DNA ligase buffer
- T4 DNA ligase
- Autoclaved, distilled water

Equipments

- Water-bath

Method

T4 DNA ligase catalyzes the formation of phosphodiester bond between juxtaposed 5'phosphate and 3'OH termini in duplex DNA or RNA. Moreover, with the replication of the plasmid in bacterial cells the continuity would be established for the rest. The final volume of the reaction usually varies from 5-10ul (the lower the better). Ligation is commonly used in molecular cloning and introduction of an insert to a vector. Particularly, insert and vector are combined with molar ratio 3 to1 and final concentration 10-11 ng/ul in the ligation reaction. In general , the amount of insert in ngs is calculated with the following formula:

ngs insert= (ng vector x kb size of insert / kb size of vector) x molar ratio insert to vector

According to that we can define insert/vector ratio in ngs as well as the exact amount of each in the reaction with final volume 5ul and final concentration 10-11ng both of them per ul. Then, 1/10 of reaction volume of 10x T4 DNA ligase buffer along with unit/ul T4 DNA ligase The mix is incubated at 16oC , over-night.

Ligation reaction may be also performed at room temperature (20-25oC). It is usually used 1ul T4 DNA ligase per reaction for 10min when there are sticky ends while for 2hours when there are blunt ends. Then, the product of ligation reaction is used in transforming competent bacterial cells.

2.19. Glycerol Stock of bacterial cultures

Reagents

- Mini-culture
- Autoclaved 80% glycerol

Instruments & materials

- 2ml micro-centrifuge tubes

- Liquid Nitrogen
- Fire
- Cold storage (-80oC)

Method

Making permanent stocks is an important part of doing molecular cloning. All the strains, clones and mutants that are made in lab should be stocked as glycerol stocks as well as DNA solution in order to be able to recover them.

In the beginning , an overnight culture of the bacterial strain that needs to be stocked is grown up. If bacterium carries a plasmid , the culture is grown up with the appropriate antibiotic (as mentioned in another chapter). Then, 1ml of autoclaved , 80% glycerol is added with sterile conditions into 4ml of culture and mixed.The freezing vials, two for each strain are labelled with the name of bacterial strain, plasmid in the strain , person stocking and date. Afterwards, 1,8ml of each culture are aliquoted into two pre-labelled and pre-chilled freezing vials. Tubes are placed either to liquid nitrogen or dry ice-ethanol bath. Once the cultures are frozen, the tubes are placed in -80oC freeze storage.

2.20. T4 PN-Kinase reaction

Reagents

- 5’-dephosphorylated nucleotides
  - PCR product
- 10x T4 PNK buffer
- 100mM ATP
- PNK enzyme (10u/ul)
- Autoclaved, distilled water

Instruments

- PCR machine

Method

T4 Polynucleotide (PN) kinase catalyzes the transfer and exchange of Pi from γ-position of ATP to 5-OH terminal of polynucleotides. Usually, the 5-ends of PCR products are dephosphorylated because 5' primer end is provided hydroxylated and not phosphorylated. In ligation reactions , T4 DNA ligase catalyzes the formation of phosphodiester bond between juxtaposed 5'phosphate and 3'OH termini in duplex DNA. Therefore, a blunt ligation between a PCR product and a dephoshorylated vector requires 5'end phosphorylation of the PCR product with T4 PN kinase.For non-radioactive phosphorylation ,up to 300pmol is recommended (or the total amount of purified PCR product) to be used in 50ul reaction containing 1x T4 Polynucleotide kinase buffer , 1mM ATP and 10 units of T4 PNK.The mix is incubated at 37oC for 30min. The product may be purified either with PCR purification kit or gel extraction and used for ligation or stored at freeze storage (-20oC).

2.21. Cell culture

Aseptic Enviroment Preparation

After molecular cloning and construction of expression plasmid vectors with the desired DNA insert are performed, expression analysis is generally performed in a cell line. Cell culture is an essential part in molecular biology. All cell cultures must be undertaken in microbiological safety cabinet using aseptic technique to ensure sterility. Hood sah should be close to proper position so laminar flow is maintained. Cluttering in hood must be avoided. Additionally all media, supplement and reagents should be sterile to avoid microbial growth. It is recommended to use pre-autoclaved, DNAse/RNAse-free pippete filter tips, autoclaved glass 9" pasteur pipettes as well as 70% Ethanol for surface cleaning.

2.22. Thawing a cell line

Materials

- Dulbecco's Modified Eagle's Medium (DMEM)
- Fetal bovine serum (FBS)
- Penicillin-Streptomycin (P-S)
- cryovial of a cell line

Instruments

- Incubator 37oC, 5% CO2
- Centrifuge Machine

Method

In the beginning , preparation of complete growth medium is performed. Each cell line requires specific media and supplements to grow. It is usually used Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS) and an antibiotic such as penicillin along with streptomycin (P-S). Particularly, in our experimental procedure 3T3 mouse embryonic fibroblasts would be used. According to ATCC, complete growth medium of 3T3 MEFs is DMEM , supplemented with FBS and PS to a final concentration of 10% and 1% , respectively.

To begin with, cryovial is thawed by gentle agitation in a 37oC water bath with the lid above the water. Cell suspension is transferred from the vial to a 15ml sterile centrifuge tube with 9ml complete growth medium. Then, it is centrifuged at 600rpm for 5min to remove the cryopreservative DMSO. DMSO is cytotoxic above 4oC. Supernatant is discarded and cell pellet is resuspended with 1ml complete medium. Cell suspension is added to a new flask or plate containing prewarmed complete growth medium and it is incubated in a suitable incubator at 37oC, 95% air and 5% CO2. Culture medium should be changed every 2-3 days and when cell culture reach 80% confluency, a sub-cultivation ratio of 1:5 or 1:10 is recommended. In addition, growth medium should be always be prewarmed at 37oC to diminish cold-induced stress on cell line.

Except from the growth medium, culture conditions depends on the cell line and it is generally recommended to check manufactorer's details for its requirements.

2.23. Freezing a cell line

Materials

- DMEM or Phosphate Buffer Saline (PBS)
- Trypsin
- Complete growth medium
- FBS
- DMSO

Instruments

- Centrifuge Machine
- Cryovials

Method

Adherent cultured cells are washed with 1x Phosphate Buffer Solution (PBS) or DMEM. Trypsin is added for 2min at RT or lesser at 37oC and cell deattach from flask surface. Trypsinization is inactivated by the addition of FBS with contains trypsin inhibitors. Cells are harvested with pipetting and transferred to a 15ml centrifuge tube. Cell suspension is centrifuged and supenatant with trypsin is discarded. Cell pellet is resuspended with complete growth medium (1ml). Then , FBS and DMSO are added to a final concentration of 50% and 5% ,respectively. Then, the solution is aliquoted to cryovials (1-1,5ml/vial) so that each will contain 2-3 x 10^6 cells per vial. Cryovials should be labelled with the cell type , number, passage and date. It is recommended to immediately transfer the vial on ice and to freeze storage at -80oC. For long term storage , vial is removed to liquid N2 freeze storage at -130oC (usually, after one day).

2.24. Sub-culturing adherent cultured cells

Materials

- PBS or DMEM
- complete growth medium.
- Trypsin

Instruments

- flasks or well plates
- centrifuge machine
- incubator

Method

When a cell line reaches aproximatelly 80% confluency in a flask or plate, sub-culturing (splitting) is needed. For example, a 80% confluent T-75 flask is washed with 1x PBS or DMEM. 1,5-2ml trypsin are added immediatelly and flask is incubated at RT for 2min. Trypsinization is inactivated by addition of 8-8,5ml complete growth medium, then , cells are harvested by pipetting and transferred to a 15ml centrifuge tube. After centrifugation at 600g for 5min at RT , supernatant is discarded, cell pellet is resuspended with 1ml complete growth medium. Then, 4ml complete medium are added and sub-cultivation with a ratio 1:5 or 1:10 (1ml from 5ml) is performed to a new T-75 flask with 10ml prewarmed complete growth medium. Spread cells to all the surface by shaking the flask to all the directions. If plating a specific number of cells per well to well plates is

needed fo experiment, haemocytometer is used to count cell number/ml. Finally,the flask or well plate is incubated in a suitable incubator, usually, at 37oC and 5%CO2.

2.25. Cell counting using a heamocytometer

Materials

- cell suspension

Instruments

- heamocytometer

Method

In the beginning, heamocytometer is cleaned with 70% Ethanol and affix the coverslip using gentle pressure. Cell suspension to be counted is well mixed by gentle pipetting. Using a 200ul Gilson pipette, a heamocytometer chamber at the edge is filled with the cell suspension so that is drawn under the coverslip by capillary action. According to the grid lines of the haemocytometer and using a 10X inverted microscope , cell counting at 5 squares (4 corners and 1 center) is performed. The number of cell per ml is determined by the following formula:
cells/ml = average count per square x 10^4

2.26. Transfection with Polyethylenimine (PEI)

Materials

- expression recombinant DNA vectors
- Polyethylenimine (PEI)
- DMEM
- DMEM/4%FBS (partial growth medium)
- complete growth medium

Instruments

- well plate with cultured cells
- coverslips (optional)
- incubator

Method

Polyethylenimine is used as transfection reagent with DNA. Negative charged DNA molecules are neutralized with polyethilimine and those particles are easier to approach cell membrane and DNA is introduced to cells.

To begin with, 2-3hrs before the transfection, complete growth medium is changed to DMEM/4% FBS/no antibiotic (D4) from a 6well plate with cultured cells at 60% confuency per well. The confluency varies according to the experimental procedure. In addition , it is known that starvation increases endocytosis and transfection efficiency. Thus, a deprivation phase with D4 before transfection is recommended. Then, 2ug DNA are added in 200ul DMEM at a 1,5ml centrifuge tube and incubated at RT for 5min. Afterwards, 3x (DNA) ug PEI are added (6ug) , mixed with agitation and the solution is incubated at RT for 15min so that DNA and PEI particles are formed. Then, D4 is removed and transfection mix is added to each well. 1,5ml to 2ml DMEM are added per well so the surface is covered enough. Then, cultured cells are incubated at 37oC, 5%CO2 for 5-6hrs. Transfection mix is changed with complete growth medium and cultured cells

are incubated again for 20-24hrs according to the experiment in a suitable incubator (37oC, 5%CO2). If double transfection is performed , 2ug from each DNA are mixed (4ug total) and 3x4= 12ug PEI are also added with the same manner as above.

According to the experimental procedure, coverslips are sterilized and added before sub-cultivation of cells in well plates. Transfection is performed as above. It is essential that the well surface is always covered with enough medium so cells not to dry.

2.27. Immunofluorescence

Materials

- 1x phosphate buffer saline
- Methanol/ Ethanol (70:30)
- PBS/0,5% Triton X-100 (PBS-0,5T)
- PBS/0,1% Triton X-100 (PBS-0,1T)
- PBS/0,1% Triton X-100 /5% Bovine Serum Albumin (BSA)
- PBS/0,1% Triton X-100/2,5% BSA (PBS-0,1T-2,5%BSA)
- Primary Antibodies
- Secondary Antibodies conjugated with fluophore
- DAPI
- mounting immunofluorescence buffer

Instruments

- PAP-PEN
- coverslips

Method

Antibodies are an important tool for demonstrating both the presence and the subcellular localization of an antigen. Cell staining may be used to determine subcellular localization of an antigen as well as concentration of an antigen especially by an image analyzer.

To begin with , adherent cultured cells either on well or on coverslip are washed with 1x PBS twice and fixed with pre-cooled Methanol/Ethanol (70:30) for 12min at -20oC. Methanol fixation is harsh and can cause loss of cells. Then, fixed cells are washed with 1xPBS twice and permeabilized with PBS-0,5T for 5min , (shaking at 25rpm.) Afterwards, permeabilized cells are washed with PBS-0,1T twice . If cultured cells are on well surface, PAP-PEN is used to form circles so immunostaining at specific areas is performed. If cells are on coverslip , it is removed from well and is placed on histology slide so cell surface is above (PAP-PEN is not needed). Then, blocking is performed with PBS-0,1T-5%BSA for 1hr to avoid unspecific binding of antibodies in the continuity. Afterwards, it follows incubation with primary antibody diluted in PBS-0,1T-2,5%BSA for 1,5-2hrs at RT. Primary antibody dilution is usually 1:50 or 1:100 , however, it is recommened to check manufactorer's details. If more than one primary antibody is used , they should be from different host. Then, it follows wash with PBST-0,1T twice and 10min washes with PBST-0,1 in case of well plates. From this time point , light exposure should be minimized by covering the slides or plates with aluminium foil because fluorophore-conjugated secondary antibody is light sensitive.Then,it follows incubation with secondary Antibody diluted in PBS-0,1T-2,5%BSA for 1-1,5hr. Secondary antibody dilution is usually 1:1000, however, manufactorer's details should be checked. If more than one secondary antibody is used , each one should recognize different host of its primary antibody and the fluorophores should have also differential excitation and emision

lengths. It follows then, wash exactly as previously with PBS-0,1T and afterwards with PBS twice. Then, nuclei are stained with DAPI (1ug DAPI per 10ml PBS) for 4min. DAPI is also photosensitive. After washing again with the same manner with PBS this time , the slides or the well plate is allowed to dry. Then, immunofluorescence mount solution is placed on cells. In case of well plates coverslips are placed upon cells. Instead, cells on coverslips are turned over on slides. Corners are sealed with nail polish to prevent coverslips from moving. Finally, they are stored at 4oC protected from light. Slides can be analyzed right away or later on inverted or upright fluorescence microscope in case of well plate or slides respectively.

In general, all steps can be performed at RT unless otherwise stated mentioned.Additionaly, when well plates are used , rinses and washes are done with 2-3ml without shaking unless a time is specified. If pap-pen is used to immunostain specific areas , shaking is not needed.

2.28. Fluorescence Microscopy

Perhaps the most versatile and powerful technique for localizing and quantifying proteins within a cell by light microscopy is fluorescent staining of cells and observation by fluorescence microscopy. A chemical is said to be fluorescent if it absorbs light at one wavelength (the excitation wavelength) and emits light (fluoresces) at a specific and longer wavelength. Most fluorescent dyes, or flurochromes**,** emit visible light, but some (such as Cy5 and Cy7) emit infrared light. In modern fluorescence microscopes, only fluorescent light emitted by the sample is used to form an image; light of the exciting wavelength induces the fluorescence but is then not allowed to pass the filters placed between the objective lens and the eye or camera.The common chemical dyes just mentioned stain nucleic acids or broad classes of proteins. However, investigators often want to detect the presence and location of specific proteins. A widely used method for this purpose employs specific antibodies covalently linked to flurochromes. Commonly used flurochromes include rhodamine and Texas red, which emit red light; Cy3, which emits orange light; and fluorescein*,* which emits green light. These flurochromes can be chemically coupled to purified antibodies specific for almost any desired macromolecule. When a flurochrome–antibody complex is added to a permeabilized cell or tissue section, the complex will bind to the corresponding antigens, which then light up when illuminated by the exciting wavelength, a technique called immunfluorescence microscopy. Staining a specimen with two or three dyes that fluoresce at different wavelengths allows multiple proteins to be localized within a cell.Immunofluoresence can be used in combination with other, non-antibody methods of fluorescent staining, for example, use of DAPI to label DNA.

There are two classes of immunofluorescence techniques, primary (or direct) and secondary (or indirect).Primary, or direct, immunofluorescence uses a single antibody that is chemically linked to a fluorophore. The antibody recognizes the target molecule and binds to it, and the fluorophore it carries can be detected via microscopy. Although direct immunofluorescence is faster and reduces background signal by avoiding antibody cross-reactivity or non-specificity, it has less sensitivity. Secondary, or indirect, immunofluorescence uses two antibodies; the unlabeled first (primary) antibody specifically binds the target molecule, and the secondary antibody, which carries the fluorophore, recognises the primary antibody and binds to it. Multiple secondary antibodies can bind a single primary antibody. Although, this protocol is more complex and time consuming than the direct protocol above, it provides signal amplification by increasing the number of fluorophore molecules per antigen and allows more flexibility because a variety of different secondary antibodies and detection techniques can be used for a given primary antibody.

Monoclonal Primary Antibodies

1) a-keratin 8 (Troma-I,rat)
2) a-Keratin 18 (N16, sc-31700,goat)
3) a-vimentin (goat)

Polyclonal Secondary antibodies

1) a-rat , Alexa 488nm (green fluorophore)
2) a-goat, Alexa 594nm (red fluorophore)

3. Results

3.1 K8 and K18 gene locus, cDNA and protein

Keratin 8 and 18 (Krt8, krt18) are the major intermediate filament proteins in simple epithelium of gastrointestinal tract, liver, exocrine pancreas as well as mammary gland [37]. Murine keratin 8 and 18 genes are located in chromosome 15 (Figure 10). Krt8 mRNA is 1805bp in length and keratin 8 protein has 490 amino acids (aa) with a molecular weight of 55kDa (Figure 11-12). While krt8 obligate heteropolymer partner, krt18 has a mRNA of 1400bp in length. Krt18 protein has 423 aa and a molecular weight of 45kDa (Figure 13-14).

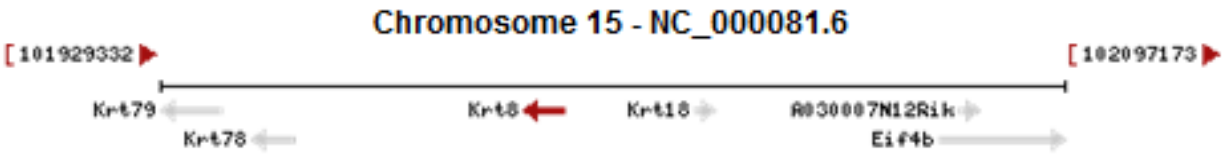


Figure 10: Gene locus of keratin 8 and 18

```
ORIGIN
        1 ataaagggcc ttcgctgggc gctgtcccac cgtctagaag cagctgctta gctcgctctc
       61 gaacctccgt cttcagctca ctgccttcgc tccagacttc accatgtcca tcagggtgac
      121 tcagaaatcc tacaagatgt ccacctccgg tccccgggcc ttcagcagcc gctcgttcac
      181 gagtggaccc ggtgccccga tcagctcttc cagcttttcc cgggtgggca gcagcagcag
      241 cagcttccgg ggaagcatgg gcaccggcgt gggtctgggc ggctttggcg gggctggtgt
      301 cgggggcatc acagccgtca cggtgaacca gagcctgttg agccccttga agctggaggt
      361 ggaccccaac atccaggctg tgcgcactca ggagaaggag cagattaaat ccctgaacaa
      421 caagttcgcc tccttcattg acaaggtgcg cttcctggag cagcagaaca agatgctgga
      481 gaccaagtgg agcctgttgc agcagcagaa gacgtcgagg agcaacatgg acaacatgtt
      541 tgagagctac atcaacaacc tccgccggca gctggaagcc ctgggccagg agaagctgaa
      601 gctggaggct gagcttggca acatgcaggg cctggtggag gacttcaaga ataagtatga
      661 ggatgagatc aacaagcgta cagagatgga gaatgaattt gtcctcatca agaaggatgt
      721 ggacgaagca tacatgaaca aggtggaact agagtcccgc ctggaaggac tgaccgacga
      781 gatcaacttc ctccggcaga tccatgaaga ggagatccgt gagttgcagt ctcagatctc
      841 agacacgtct gtggtgctgt ctatggacaa cagccgctcc ctggacatgg atggcatcat
      901 cgctgaagtt cgtgcccagt acgaggacat tgccaatcgc agccgagctg aggctgaaac
      961 catgtaccag attaagtatg aggaattgca gaccctggct gggaagcacg gggatgatct
     1021 gcgccgcaca aagacagaga tctccgagat gaaccgcaac atcaaccgcc tgcaggcgga
     1081 gattgaagcc ctcaaaggcc agagggcatc gttggaggca gccatcgctg atgctgagca
     1141 gcgtggggag atggccatta aggatgccca gaccaagctg gctgagctgg aggctgccct
     1201 gcaacgggcc aagcaggaca tggccaggca gctgcgcgag taccaggagc ttatgaacgt
     1261 caagctggcc ctggacatcg agatcaccac ctaccgcaag ctgctggagg gggaggagag
     1321 caggctggag tctgggatgc agaacatgag cattcatacg aagaccacca gcggctactc
     1381 aggaggactg agttcatcct atgggggact cactagccct ggcttcagct acggaatgag
     1441 ctccttccag cccggcttcg gttctgccgg gggatccaac actttcagcc gcaccaccaa
     1501 ggctgtggtt gtgaagaaga ttgaaacccg agatgggaag ctggtgtccg agtcttctga
     1561 tgtcgtgtcc aagtgaatgg ccactgaagt ccttgccagc ctgagctcct gcagctgctc
     1621 agggctcacg gggagacagc tgtatggcag agtgcaggga actagagacc cgtccgagga
     1681 tcagccctaa tcctctggcc aaccttagga ggaatttcct atctgggata tcccaatggc
     1741 ttctgcctcc catccaaacc caattcagtt gtatttttcta aaataaagcc tcagctggct
     1801 ctgtc
//
```

Figure 11: .**Mus musculus keratin 8 linear mRNA,** 1805 bp , (NCBI Reference Sequence: NM_031170.2), coding sequence is marked.

```
ORIGIN
        1 msirvtqksy kmstsgpraf ssrsftsgpg arissssfsr vgssssfrg smgtgvglgg
       61 fggagvggit avtvnqslls plklevdpni qavrtqekeq ikslnnkfas fidkvrfleq
      121 qnkmletkws llqqqktsrs nmdnmfesyi nnlrrqleal gqeklkleae lgnmqglved
      181 fknkyedein krtemenefv likkdvdeay mnkvelesrl egltdeinfl rqiheeeire
      241 lqsqisdtsv vlsmdnsrsl dmdgiiaevr aqyedianrs raeaetmyqi kyeelqtlag
      301 khgddlrrtk teisemnrni nrlqaeieal kgqrasleaa iadaeqrgem aikdaqtkla
      361 eleaalqrak qdmarqlrey qelmnvklal dieittyrkl legeesrles gmqnmsihtk
      421 ttsgysggls ssyggltspg fsygmssfqp gfgsaggsnt fsrttkavvv kkietrdgkl
      481 vsessdvvsk
```

Figure 12: **Mus musculus (type II) keratin 8 protein** , 490aa , (NP_112447.2)

```
ORIGIN
        1 ctgccaccct ccgcggcgga actcctgttc tggtctctcg cttcgctctc ctctccagac
       61 aagatgagct tcacaactcg ctccaccacc ttctccacca actaccggtc cctgggctct
      121 gtgcgaactc ccagccagcg ggtccggcct gccagcagcg cagccagcgt ctatgcaggt
      181 gctgggggct ccgggtcccg gatatccgtg tcccgctctg tctggggtgg ctctgtgggg
      241 tccgcaggcc tggcgggaat gggtggaatc cagaccgaga aggagaccat gcaagacctg
      301 aacgatcgcc tggccagcta cctagacaag gtgaagagcc tggaaactga gaacaggaga
      361 ctggagagca aaatccggga acatctggag aagaaggggc cccagggcgt cagagactgg
      421 ggccactact tcaagatcat cgaagacctg agggctcaga tctttgcgaa ttctgtggac
      481 aatgcccgca tcgtcttgca gatcgacaat gcccgccttg ccgccgatga ctttagagtc
      541 aagtatgaga cagaactagc catgcgccag tctgtggaga gcgacatcca tggactccgc
      601 aaggtggtag atgacaccaa catcacaagg ctgcagctgg agacagaaat cgaggcactc
      661 aaggaagaac ttctgttcat gaagaagaat catgaagagg aagtccaagg tctggaagcc
      721 cagattgcca gctctggatt gactgtggaa gtggatgccc ccaaatctca ggacctcagc
      781 aagatcatgg cggacatccg cgcccagtat gaagcgctgg ctcagaagaa ccgcgaggaa
      841 ctggacaagt actggtctca gcagattgag gagagtacca cagttgtcac caccaagtct
      901 gccgaaatca gggacgctga gaccacactc acggagctga gacgcaccct ccagaccttg
      961 gagattgact tggactccat gaaaaaccag aacatcaact tggagaacag cctcggggat
     1021 gtggaggccc gatacaaggc acagatggag cagctcaatg gggtccttct gcatctggag
     1081 tcagagctgg cacaaactcg ggcagagggc cagcgccagg cccaggaata tgaagccctc
     1141 ttgaacatca aggtgaagct tgaggcagag attgccacct accgccgctt gctggaggat
     1201 ggagaagatt tcagtctcaa cgatgccctg gactccagca actccatgca aactgtgcag
     1261 aagacaacta cccgtaagat cgtggatggc agagtggtgt ccgagactaa tgacaccaga
     1321 gttctgaggc actgaggcag agaaggaggg aacccctggg aactgaggga ccaataaaag
     1381 ttgagagctc actggacatc
//
```

Figure 13: **Mus musculus keratin 18 mRNA**, 1400 bp , (NCBI Reference Sequence: NM_010664.2), coding sequence is marked.

```
ORIGIN
        1 msfttrsttf stnyrslgsv rtpsqrvrpa ssaasvyaga ggsgsrisvs rsvwggsvgs
       61 aglagmggiq teketmqdln drlasyldkv ksletenrrl eskirehlek kgpqgvrdwg
      121 hyfkiiedlr aqifansvdn arivlqidna rlaaddfrvk yetelamrqs vesdihglrk
      181 vvddtnitrl qleteiealk eellfmkknh eeevqgleaq iassgltvev dapksqdlsk
      241 imadiraqye alaqknreel dkywsqqiee sttvvttksa eirdaettlt elrrtlqtle
      301 idldsmknqn inlenslgdv earykaqmeq lngvllhles elaqtraegq rqaqeyeall
      361 nikvkleaei atyrrlledg edfslndald ssnsmqtvqk tttrkivdgr vvsetndtrv
      421 lrh
```

Figure 14: **Mus musculus (type I) keratin 18 protein,** 423aa, (NP_034794.2)

3.2. Cloning keratin 8 or 18 to plasmid vectors

Keratin 8 and 18 are the major intermediate filament proteins in hepatocytes. In order to isolate and clone these keratins to plasmid vectors, reverse transcription and polymerase chain reaction with specific primers are required.

3.2.1. K8 and K18 Primer Pairs

According to the nucleotide sequence of each keratin mRNA , a specific primer pair was designed depending on the online software Primer-BLAST by NCBI . Each primer pair was designed with that manner so that the PCR product would contain all the open reading frame (ORF) along with parts of the 5' and 3' untranslated regions (UTRs). Particularly, primer pair characteristics for each keratin as well as the annealing site of forward and reverse primer on mRNA sequence are indicated below (Figures 15-16). PCR product for *Krt8* would have 1599bp length while that of *Krt18*, 1337bp.

***Krt8* primer pair**

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ACCTCCGTCTTCAGCTCACT	Plus	20	63	82	60.54	55.00	4.00	1.00
Reverse primer	TTCCCTGCACTCTGCCATAC	Minus	20	1661	1642	59.75	55.00	4.00	1.00
Product length	1599								

***Krt18* primer pair**

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTTCGCTCTCCTCTCCAGACA	Plus	21	41	61	60.95	57.14	4.00	2.00
Reverse primer	TTATTGGTCCCTCAGTTCCCAG	Minus	22	1377	1356	59.42	50.00	3.00	1.00
Product length	1337								

Figure 15: Characteristics of keratin 8 and 18 primer pairs. PCR product for *Krt8* has 1599bp length while for *Krt18* has 1337bp. Each pair is specific for the respective keratin.

Murine cytokeratin 8 , mRNA

ATAAAGGGCCTTCGCTGGGCGCTGTCCCACCGTCTAGAAGCAGCTGCTTAGCTCGCTCTCGAACCTCCGT
CTTCAGCTCACTGCCTTCGCTCCAGACTTCACCATGTCCATCAGGGTGACTCAGAAATCCTACAAGATGT
CCACCTCCGGTCCCCGGGCCTTCAGCAGCCGCTCGTTCACGAGTGGACCCGGTGCCCGCATCAGCTCTTC
CAGCTTTTCCCGGGTGGGCAGCAGCAGCAGCAGCTTCCGGGGAAGCATGGGCACCGGCGTGGGTCTGGGC
GGCTTTGGCGGGGCTGGTGTCGGGGGCATCACAGCCGTCACGGTGAACCAGAGCCTGTTGAGCCCCTTGA
AGCTGGAGGTGGACCCCAACATCCAGGCTGTGCGCACTCAGGAGAAGGAGCAGATTAAATCCCTGAACAA
CAAGTTCGCCTCCTTCATTGACAAGGTGCGCTTCCTGGAGCAGCAGAACAAGATGCTGGAGACCAAGTGG
AGCCTGTTGCAGCAGCAGAAGACGTCGAGGAGCAACATGGACAACATGTTTGAGAGCTACATCAACAACC
TCCGCCGGCAGCTGGAAGCCCTGGGCCAGGAGAAGCTGAAGCTGGAGGCTGAGCTTGGCAACATGCAGGG
CCTGGTGGAGGACTTCAAGAATAAGTATGAGGATGAGATCAACAAGCGTACAGAGATGGAGAATGAATTT
GTCCTCATCAAGAAGGATGTGGACGAAGCATACATGAACAAGGTGGAACTAGAGTCCCGCCTGGAAGGAC
TGACCGACGAGATCAACTTCCTCCGGCAGATCCATGAAGAGGAGATCCGTGAGTTGCAGTCTCAGATCTC
AGACACGTCTGTGGTGCTGTCTATGGACAACAGCCGCTCCCTGGACATGGATGGCATCATCGCTGAAGTT
CGTGCCCAGTACGAGGACATTGCCAATCGCAGCCGAGCTGAGGCTGAAACCATGTACCAGATTAAGTATG
AGGAATTGCAGACCCTGGCTGGGAAGCACGGGGATGATCTGCGCCGCACAAAGACAGAGATCTCCGAGAT
GAACCGCAACATCAACCGCCTGCAGGCGGAGATTGAAGCCCTCAAAGGCCAGAGGGCATCGTTGGAGGCA
GCCATCGCTGATGCTGAGCAGCGTGGGGAGATGGCCATTAAGGATGCCCAGACCAAGCTGGCTGAGCTGG
AGGCTGCCCTGCAACGGGCCAAGCAGGACATGGCCAGGCAGCTGCGCGAGTACCAGGAGCTTATGAACGT
CAAGCTGGCCCTGGACATCGAGATCACCACCTACCGCAAGCTGCTGGAGGGGGAGGAGAGCAGGCTGGAG
TCTGGGATGCAGAACATGAGCATTCATACGAAGACCACCAGCGGCTACTCAGGAGGACTGAGTTCATCCT
ATGGGGGACTCACTAGCCCTGGCTTCAGCTACGGAATGAGCTCCTTCCAGCCCGGCTTCGGTTCTGCCGG
GGGATCCAACACTTTCAGCCGCACCACCAAGGCTGTGGTTGTGAAGAAGATTGAAACCCGAGATGGGAAG
CTGGTGTCCGAGTCTTCTGATGTCGTGTCCAAGTGAATGGCCACTGAAGTCCTTGCCAGCCTGAGCTCCT
GCAGCTGCTCAGGGCTCACGGGGAGACAGCTGTATGGCAGAGTGCAGGGAACTAGAGACCCGTCCGAGGA
TCAGCCCTAATCCTCTGGCCAACCTTAGGAGGAATTTCCTATCTGGGATATCCCAATGGCTTCTGCCTCC
CATCCAAACCCAATTCAGTTGTATTTTCTAAAATAAAGCCTCAGCTGGCTCTGTC

Murine cytokeratin 18 , mRNA

CTGCCACCCTCCGCGGCGGAACTCCTGTTCTGGTCTCTCGCTTCGCTCTCCTCTCCAGACAAGATGAGCT
TCACAACTCGCTCCACCACCTTCTCCACCAACTACCGGTCCCTGGGCTCTGTGCGAACTCCCAGCCAGCG
GGTCCGGCCTGCCAGCAGCGCAGCCAGCGTCTATGCAGGTGCTGGGGGCTCCGGGTCCCGGATATCCGTG
TCCCGCTCTGTCTGGGGTGGCTCTGTGGGGTCCGCAGGCCTGGCGGGAATGGGTGGAATCCAGACCGAGA
AGGAGACCATGCAAGACCTGAACGATCGCCTGGCCAGCTACCTAGACAAGGTGAAGAGCCTGGAAACTGA
GAACAGGAGACTGGAGAGCAAAATCCGGGAACATCTGGAGAAGAAGGGGCCCCAGGGCGTCAGAGACTGG
GGCCACTACTTCAAGATCATCGAAGACCTGAGGGCTCAGATCTTTGCGAATTCTGTGGACAATGCCCGCA
TCGTCTTGCAGATCGACAATGCCCGCCTTGCCGCCGATGACTTTAGAGTCAAGTATGAGACAGAACTAGC
CATGCGCCAGTCTGTGGAGAGCGACATCCATGGACTCCGCAAGGTGGTAGATGACACCAACATCACAAGG
CTGCAGCTGGAGACAGAAATCGAGGCACTCAAGGAAGAACTTCTGTTCATGAAGAAGAATCATGAAGAGG
AAGTCCAAGGTCTGGAAGCCCAGATTGCCAGCTCTGGATTGACTGTGGAAGTGGATGCCCCCAAATCTCA
GGACCTCAGCAAGATCATGGCGGACATCCGCGCCCAGTATGAAGCGCTGGCTCAGAAGAACCGCGAGGAA
CTGGACAAGTACTGGTCTCAGCAGATTGAGGAGAGTACCACAGTTGTCACCACCAAGTCTGCCGAAATCA
GGGACGCTGAGACCACACTCACGGAGCTGAGACGCACCCTCCAGACCTTGGAGATTGACTTGGACTCCAT
GAAAAACCAGAACATCAACTTGGAGAACAGCCTCGGGGATGTGGAGGCCCGATACAAGGCACAGATGGAG
CAGCTCAATGGGGTCCTTCTGCATCTGGAGTCAGAGCTGGCACAAACTCGGGCAGAGGGCCAGCGCCAGG
CCCAGGAATATGAAGCCCTCTTGAACATCAAGGTGAAGCTTGAGGCAGAGATTGCCACCTACCGCCGCTT
GCTGGAGGATGGAGAAGATTTCAGTCTCAACGATGCCCTGGACTCCAGCAACTCCATGCAAACTGTGCAG
AAGACAACTACCCGTAAGATCGTGGATGGCAGAGTGGTGTCCGAGACTAATGACACCAGAGTTCTGAGGC
ACTGAGGCAGAGAAGGAGGGAACCCCTGGGAACTGAGGGACCAATAAAAGTTGAGAGCTCACTGGACATC

Figure 16: Murine keratin 8 and 18, mRNA sequence and primer pair annealing sites. Annealing sites of forward and reverse primers are indicated with yellow colour. The starting codon is coloured red while terminating codon blue. PCR products contain ORF and parts of 5',3' UTRs.

3.2.2. Liver total RNA electrophoresis

Murine liver total RNA had been isolated and stored at -80oC freeze storage. Before reverse transcription was applied, electorphoresis of the liver total RNA samples had beed performed to determine their integrity. Electrophoresis to an 1,2% agarose gel with formaldehyde (denaturing agent) was performed for two samples A and B (Figure 17).

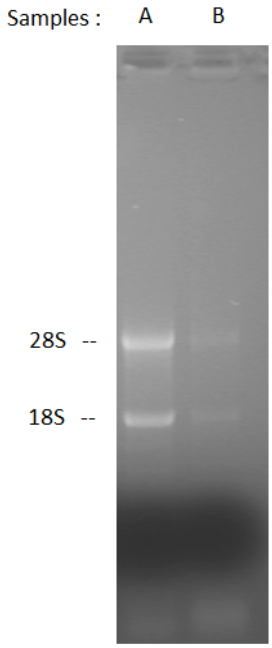


Figure 17: RNA electrophoresis for sample A and B. Sample A had sharp 28S and 18S rRNA bands with a 2:1 intensity ratio which are characteristics of an intact total eykaryotic RNA.Sample B RNA was partially degraded.

As it is generally known an intact total eykaryotic RNA forms two bands on a denaturing gel when it is electrophorised. These bands correspond to the 28S and 18S rRNA and the intensity of the first one should be 2 times more than the second. According to that, total liver RNA of sample A was intact and was used as a template for reverse transcription.

3.2.3. Reverse Transcription-PCR

Then, reverse transcription and polymerase chain reaction were used to identify and multiply our desired products of *Krt8* and *Krt18*. First Strand cDNA synthesis was performed using M-MLV reverse transcriptase along with random primers .

Then, reverse-transcription PCR was performed either with *Krt8* or *Krt18* primer pair. Polymerase chain reactions were performed with polymerases that could determine the high fidelity of each PCR product for molecular cloning techniques. The PCR for *Krt8* was performed with Q5 high fidelity polymerase while for *Krt18* a PCR high fidelity Supermix was used including Taq and the proofreading Pyrococcus species GB-D polymerase. A PCR High fidelity Supermix provides six times higher fidelity than Taq polymerase while a Q5 HF polymerase has a 50 fold lower error rate than Taq polymerase. In order to identify that reverse-transcription was successful , control reaction was also applied using instead, b-actin primer pair . Optimized PCR protocol and cycling conditions were described on Chapter 2.2-4. After electrophoresis of PCR products was finished, it was observed that reverse-transcription was efficient since the 0,5Kb b-actin band was present. Except

from that, PCR for *Krt8* and *Krt18* resulted in a 1,6kb and 1,4kb products, respectively, that according to the primer-blast program with the designed primer-pairs correspond to the expected products lengths (Figure 18) .

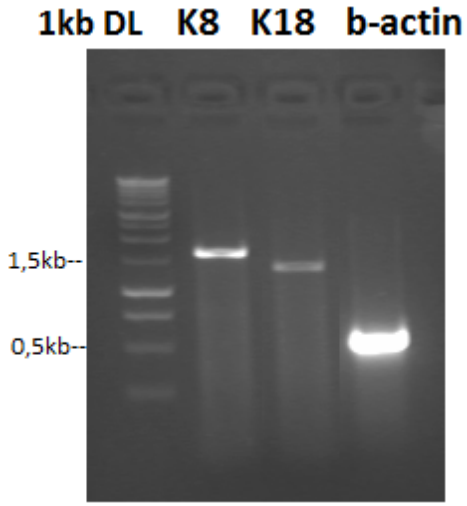


Figure 18 : **RT-PCR for *Krt8* and *Krt18***.
PCR products for *Krt8* and *Krt18* are approximately 1,6Kb and 1,4Kb in length, respectively. Control b-actin band is 0,5kb.

Since RT-PCR was successful, PCR was repeated 4times for each keratin to increase the amount of product. Afterwards , according to the gel extraction protocol of QIAGEN , the expected DNA products were extracted and purified from the agarose gel (Figure 19). DNA concentration of the PCR products was quantitated through spectrophotometer.

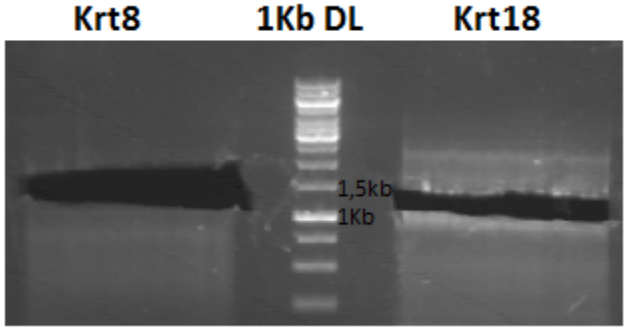


Figure 19 : Gel Extraction of *Krt8* and *Krt18* PCR products

Although PCR HF supermix provides lower fidelity than Q5 HF polymerase, it produces products that have mixed blunt and 3-A' ends, however , the majority of them have a 3'-A overhang. In contrast Q5 HF polymerase produces blunt-end products. According to that , *Krt8* blunt-end PCR products would be subcloned to pBluescript vector with blunt-end cloning while *Krt18* PCR products with 3'A overhang would be subcloned to pCRII-TOPO vector with TA cloning.

3.2.4. Subcloning *Krt8* to pBluescriptII SK(+) vector

Krt8 PCR product was produced by Q5 HF DNA polymerase to determine high fidelity for molecular cloning techniques. Q5 HF DNA polymerase is a thermostable DNA polymerease with 3' → 5' exonuclease activity , fused to a processivity-enhancing domain to support robust DNA amplification. It has a 50 fold lower error rate than Taq polymerase and produces blunt-ends products.

Krt8 PCR product was subcloned to pBluescriptII SK(+) vector with blunt-end cloning. pBluescriptII SK(+) plasmid vector contains a origin of replication, an ampicillin-resistance gene as well as a multiple cloning site with a variable number of different restriction sites along with annealing sites for universal primers used for sequencing (Figure 20).

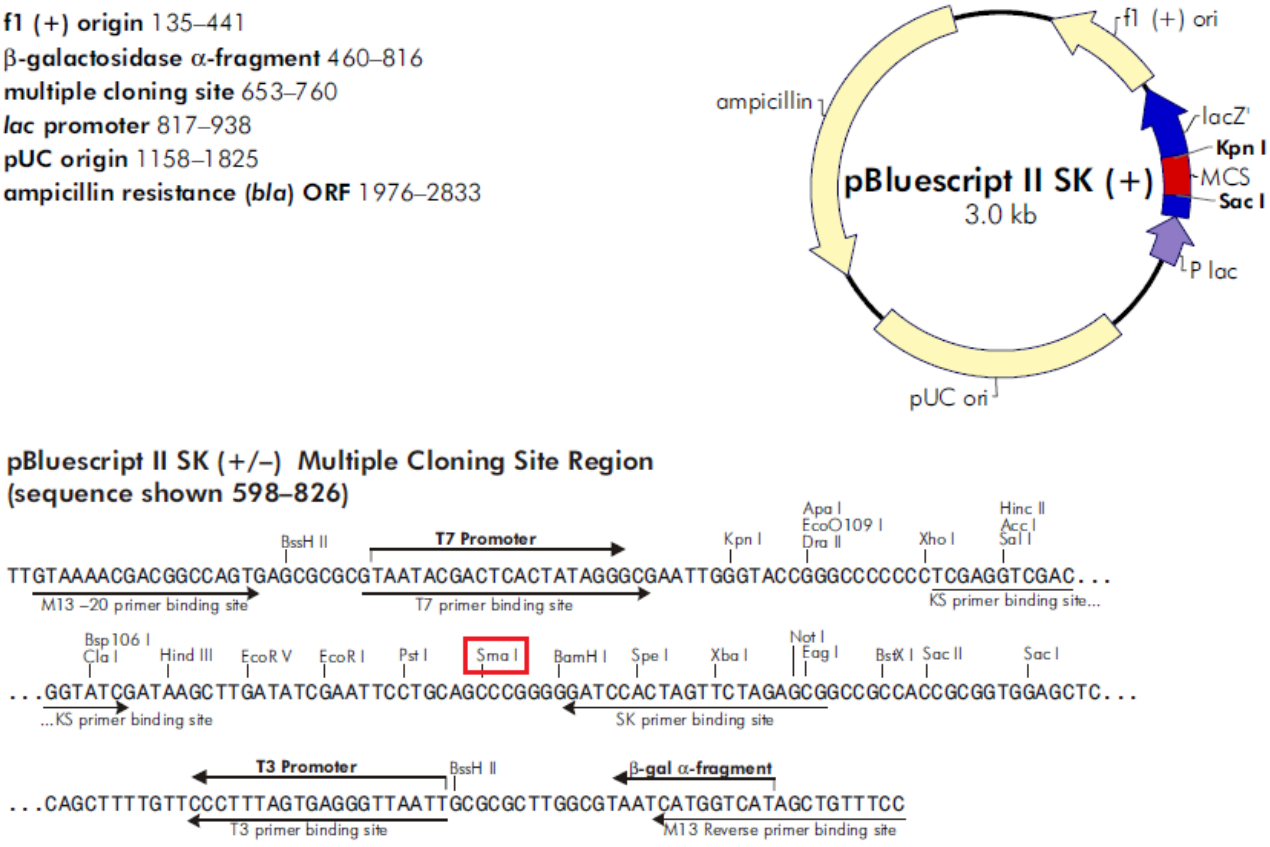


Figure 20: pBluescriptII SK(+) vector and the sequence surrounding the SmaI cloning site

In the beginning, (3ug) pBluescript vector was digested with SmaI which is a restriction enzyme that leaves blunt ends CCC/GGG .
GGG/CCC

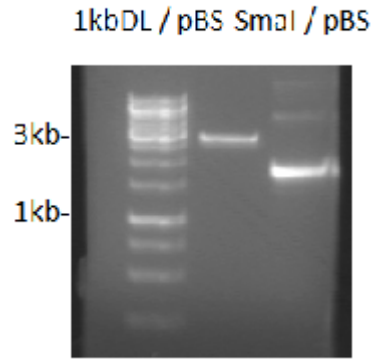


Figure 21: pBlusecriptII vector digested with SmaI.
pBS digested with SmaI has a 3kb band while undigested pBS has a different pattern with a lower supercoiled band and higher polymer bands.

Then, it was dephosphorylated by antarctic phosphatase in order to inhibit self-ligation . Afterwards, *Krt8* PCR product was treated with T4 polynucleotide kinase (PNK) in order to phosphorylate the 5'-hydroxyl ends of the product . This 5'end phosphorylation of the PCR product was necessary for T4 DNA ligase to catalyze the formation of phosphodiester bond between juxtaposed 5'phosphate and 3'OH termini . *Krt8* PCR product phosphorylation and pBS vector dephosphorylation reactions were inactivated with electrophoresis and after phosphorylated *Krt8* PCR product and dephosphorylated pBS vector were extracted from the gel (Figure 22) , ligation with T4 DNA ligase was performed.

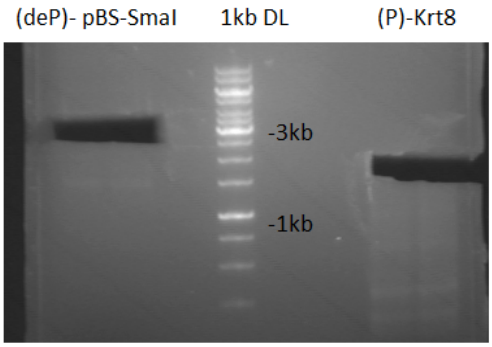


Figure 22: Gel extraction of dephosphorylated pBluescript vector and phoshorylated *Krt8* PCR product

The result of ligation reaction would form a circular plasmid with *Krt8* PCR product inside the pBluescript vector (pBS-*Krt8*).

After ligation reaction was performed, the presence of a variable number of restriction sites surrounding SmaI cloning site provided a number of sites for subcloning. Additionally, the presence of universal primers annealing sites surrounding SmaI cloning site provided a direct potential for sequencing.

In the continuity, pBS-*K8* was used for transformation of chemically competent DH5A cells. Then, a number of colonies was isolated from the selective plates (with ampicillin) and grown in liquid mini-cultures containing ampicillin (see material and methods). Afterwards, isolation of pBS-*Krt8* from each colony was performed using DNA preparation protocol (pBS-*Krt8* mini-preps).

In the continuity, the insertion as well as the orientation of *Krt18* PCR product inside the pBluescriptII vector was determined with identifying digestions of pBS-*Krt8* mini-preps. pBS-*Krt8*

mini-preps were digested with BamHI restriction enzyme. BamHI is a single cutter of *Krt8* PCR product (on 1410 site) as well as a single cutter of pBS vector since the restriction sites of multiple cloning site (MCS) are unique (Figures 23 and 20).

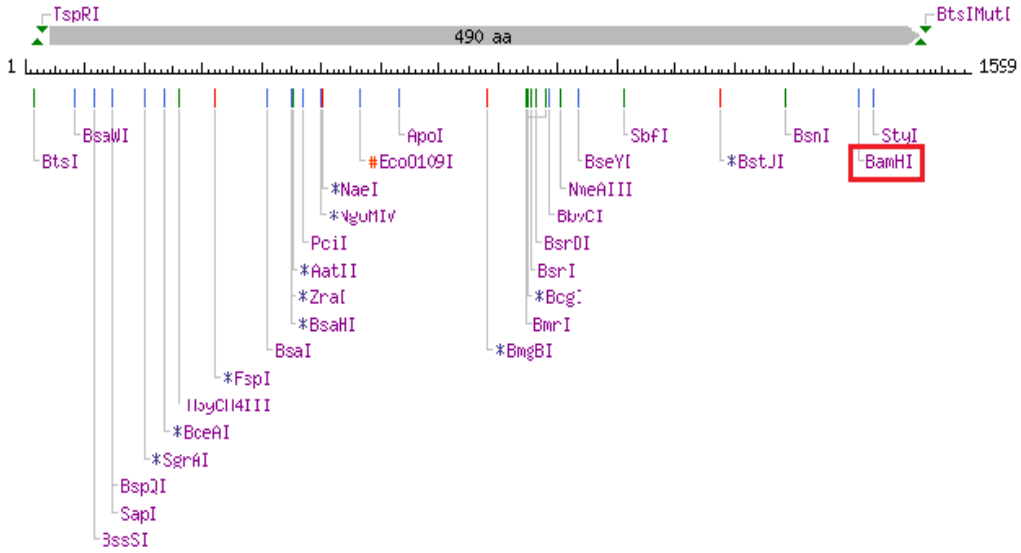


Figure 23: *Krt8* single enzyme cutters. BamHI cuts on 1410 site of *Krt8*.

The length of pBluescript vector is 3 Kb while *Krt8* PCR product has 1,6 Kb length. Therefore, pBS-*Krt8* length is approximately 4,6 Kb. Restriction digestion with BamHI would result in specific DNA fragments depending on insertion and PCR product orientation (Figure 24).

Restriction Enzymes	**Plasmid DNA (Kb)**	**PCR product orientation**	**Digestion DNA Fragments (kb)**
BamHI	pBS-*Krt8* (4,6Kb)	5' → 3'	4,4 + 0,2
	pBS-*Krt8* (4,6Kb)	3' → 5'	1,4 + 3,2
	Self ligated vector (3Kb)	No insert	3

Figure 24: BamHI restriction digestion of pBS-*Krt8* mini-preps

Restriction digestions with BamHI were performed for approximately 35 clones because it would be difficult to isolate the desired clone from a blunt-end ligation reaction. However, a number of these clones had the *Krt8* PCR product with the desired 5'→3' orientation (Figure 25).Particularly, the 5th ,8th,10th and 32th colonies had a an approximately 5kb band along with a 0,2kb DNA band (Figure 25).

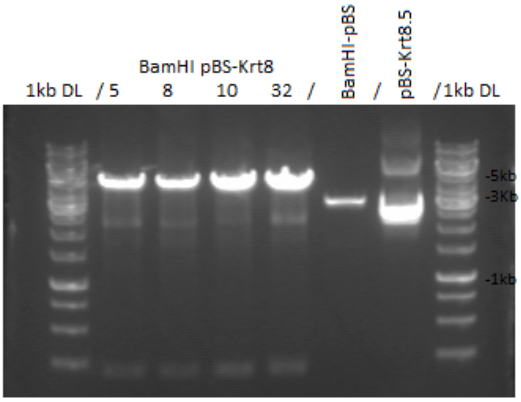


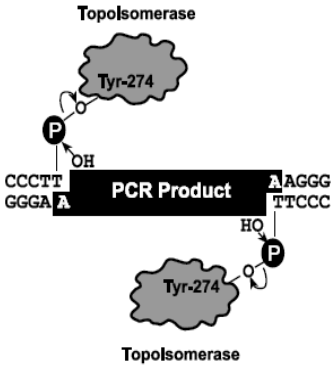
Figure 25: Positive pBS-*Krt8* clones digested with BamHI. Digestions of 5,8,10,32th clones result in a 4,5kb+ 0,2kb bands. Undigested pBS-*K8* and BamHI-pBS are the control samples.

After positive pBS-*Krt8* mini-preps were idientified , they were quantitated and an amount of them was sent for sequencing by VBC genomics (Go to Sequencing Results Chapter).

3.2.5. Subcloning *Krt18* to pCRII-TOPO vector

As far as *Krt18* subloning to pCRII-TOPO vector is concerned, the plasmid, pCRII-TOPO vector is supplied linearized with single 3'thymidine (T) overhangs for TA cloning. Topoisomerase I from *Vaccinia* virus is covalently bound to the 3-T' overhangs of the vector with a phospho-tyrosyl bond [68] . The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the PCR product which corresponds to the 5' hydroxyl primer end, reversing the reaction and releasing topoisomerase (Figure 26).

Figure 26: Topoisomerase I covalently bound to linearized pCRII-TOPO vector



Depending on TA cloning technique , purified *Krt18* PCR product was mixed with pCRII-TOPO vector. The result of this reaction would form a circular plasmid with *Krt18* PCR product inside the multiple cloning site of pCRII-TOPO vector (pTP-*Krt18*). The sequence surrounding the TOPO cloning site provides a variable number of restriction sites for subloning as well as annealing sites of universal primers for sequencing (Figure 27). Additionally, it provides antibiotic-resistance genes for selection and an origin of replication. Galactosidase selection assay was not used.

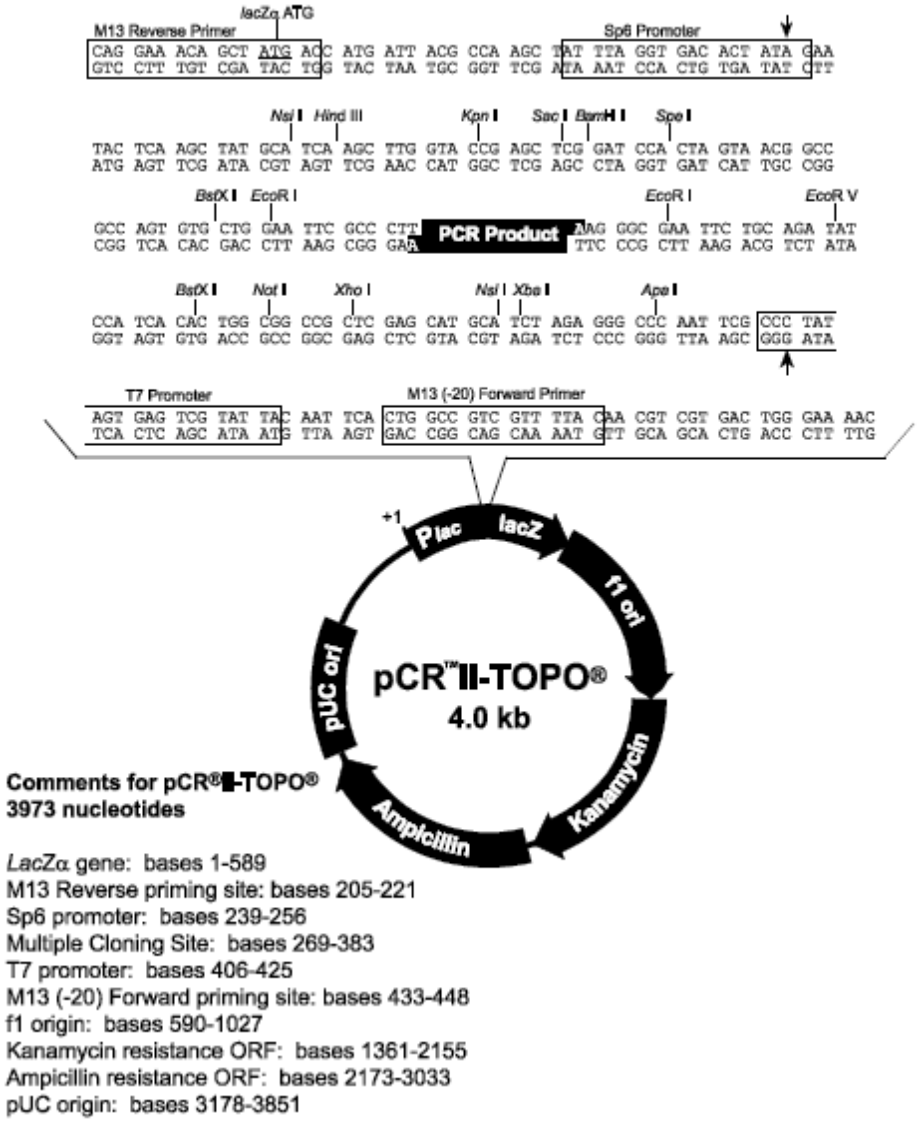


Figure 27: pCRII-TOPO vector and the sequence surrounding the TOPO cloning site.

Plasmid *Krt18* (pTP-*Krt18*) was used for transformation of chemically competent DH5A cells . Then, a number of colonies was isolated from the selective plates (with antibiotic) and grown in liquid mini-cultures containing antibiotic . Afterwards, isolation of p*Krt18* from each colony was performed using DNA preparation protocol (pTP-*Krt18* mini-preps.

In the continuity, the insertion as well as the orientation of *Krt18* PCR product inside the TOPO vector was determined with identifying digestions of pTP-*Krt18* mini-preps. pTP-*Krt18* mini-preps were digested with HindIII restriction enzyme. HindIII is a single cutter of *Krt18* PCR product (on 1116 site) as well as a single cutter of TOPO vector since the restriction sites of multiple cloning site (MCS) are unique (Figure 28 and 27**)**.

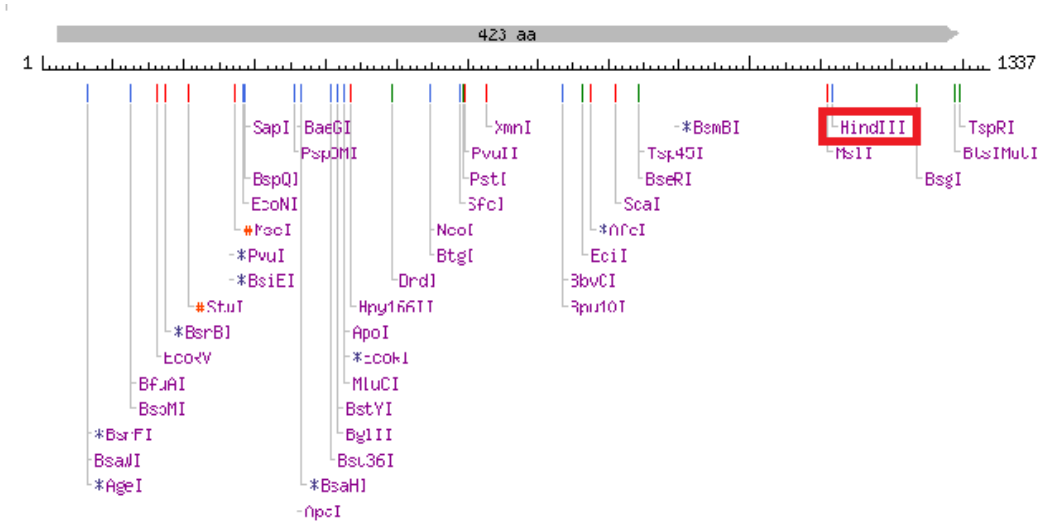


Figure 28: *Krt18* single cutters. HindIII cuts at 1116 site (NEB cutter)

The length of TOPO vector is 4 Kb while *Krt18* PCR product has 1,35 Kb length. Therefore, pTP-*Krt18* length is approximately 5,4 Kb. Restriction digestion with HindIII would result in specific DNA fragments depending on insertion and PCR product orientation (Figure 29).

Restriction Enzymes	**Plasmid DNA (Kb)**	**PCR product orientation**	**Digestion DNA Fragments (kb)**
HindIII	pTP-*Krt18* (5,4Kb)	5' → 3'	1,2 + 4,2
	pTP-*Krt18* (5,4Kb)	3' → 5'	0,3 + 5,1
	Self ligated vector (4Kb)	No insert	4

Figure 29: HindIII restriction digestion of pTP-*Krt18* mini-preps

According to the previous , identifying digestions for p*Krt18* mini-preps were performed with HindIII restriction enzyme .Electrophoresis to 1,5% agarose gel revealed that the 1st colony contains the plasmid with *Krt18* PCR product along with the desired orientation 5' → 3'. The digestion of 1st clone with HindIII results in 2 DNA bands , a 4kb and a 1kb bands that correspond to the expected ones (Figure 30). Finally, plasmid *Krt18* from the 1st colony (pTP-*Krt18*.1) was quantitated and an amount of it was sent for sequencing by VBC genomics.

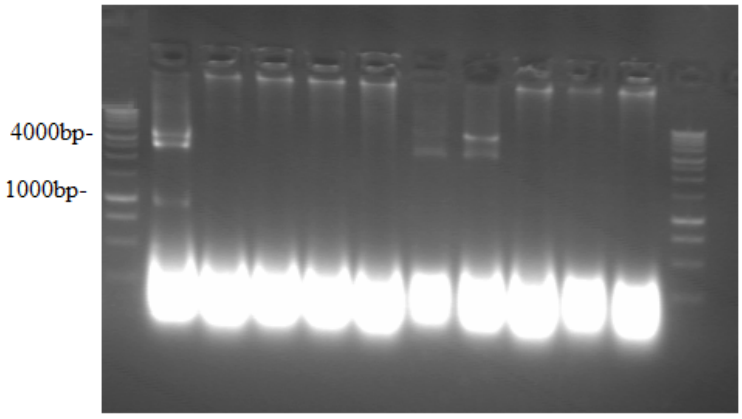


Figure 30: Identifying digestions of pTP-*Krt18* mini-preps with HindIII. Despite the RNA backround at the bottom of the gel , 1st clone has a 4Kb and a 1Kb DNA bands.

3.3. Sequencing of murine *Krt8* and *Krt18*

pBS-*Krt8* contains murine *Krt8* PCR product while pTP-*Krt18* contains the murine *Krt18* PCR product. Clones with the desired orientation of the insert such as the 5th pBS-*Krt8* clone (pBS-*krt8*.5) and the 1st pTP-*Krt18* clone (pTP-*Krt18*.1) were sent for sequencing. Sequencing results by VBC genomics were analyzed using the online Multi-align software (Figure 31-35).

3.3.1. Sequencing m*Krt8* from pBS-*Krt8*.5

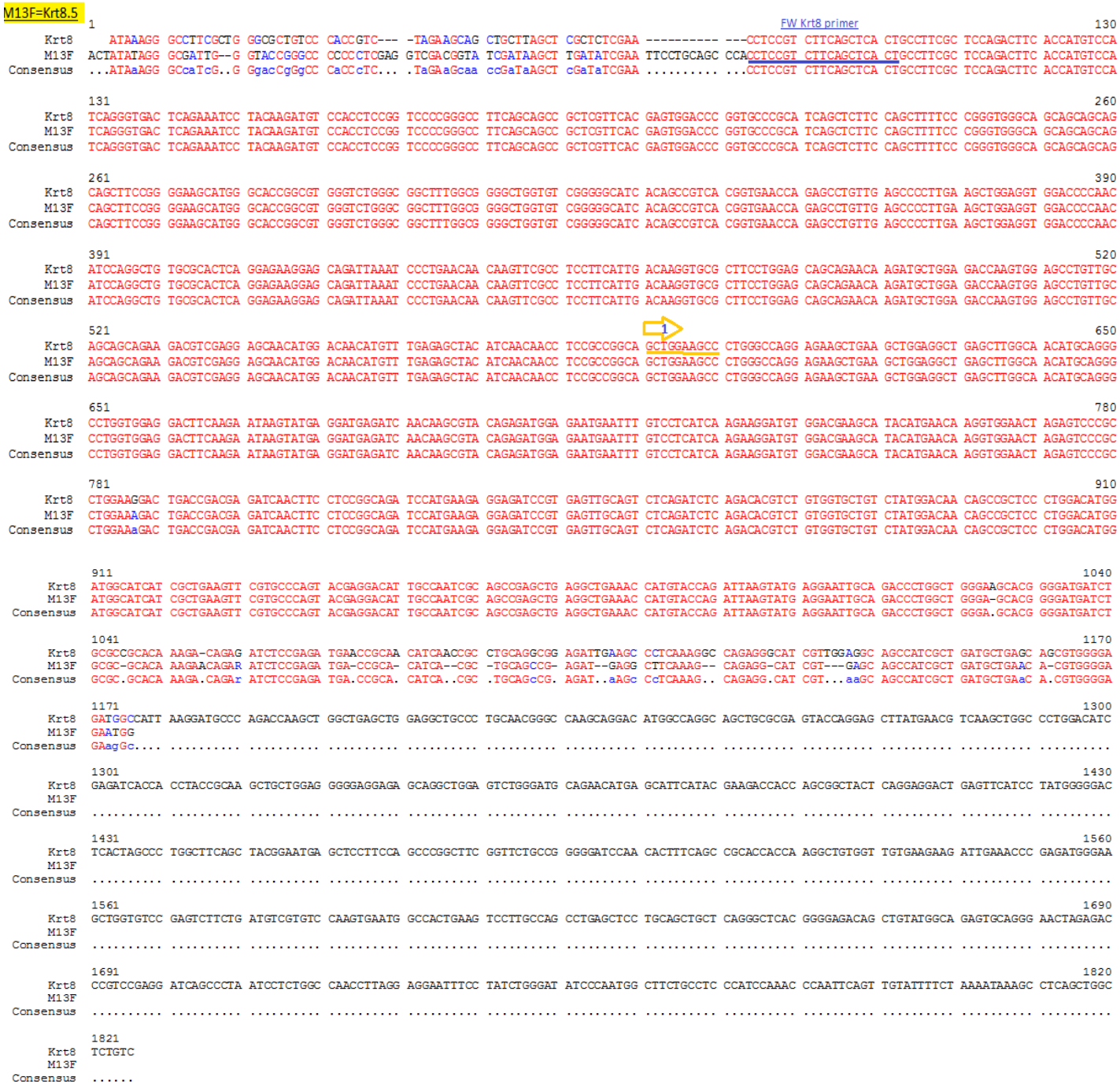


Figure 31: Forward sequencing of *Krt8* in pBS-*Krt8*.5.

There are no mutations from the start (*Krt8* FW primer) until the 1st yellow arrow. Continue to middle sequencing.

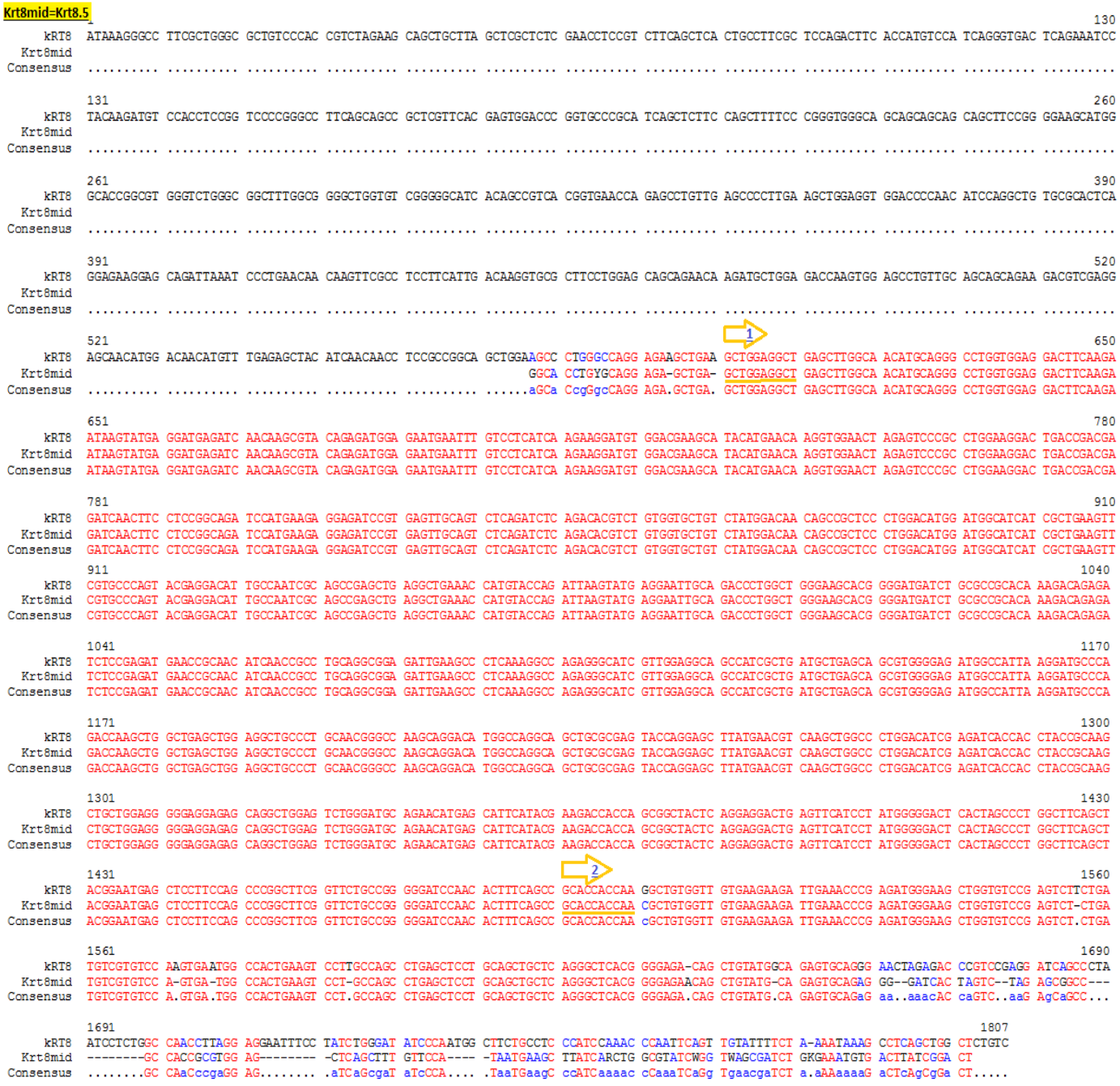


Figure 32: Sequencing of the middle part of *Krt8* in pBS-*Krt8*.5.
No mutations are identified between the 1st and the 2nd yellow arrow. Continue to the reverse sequencing.

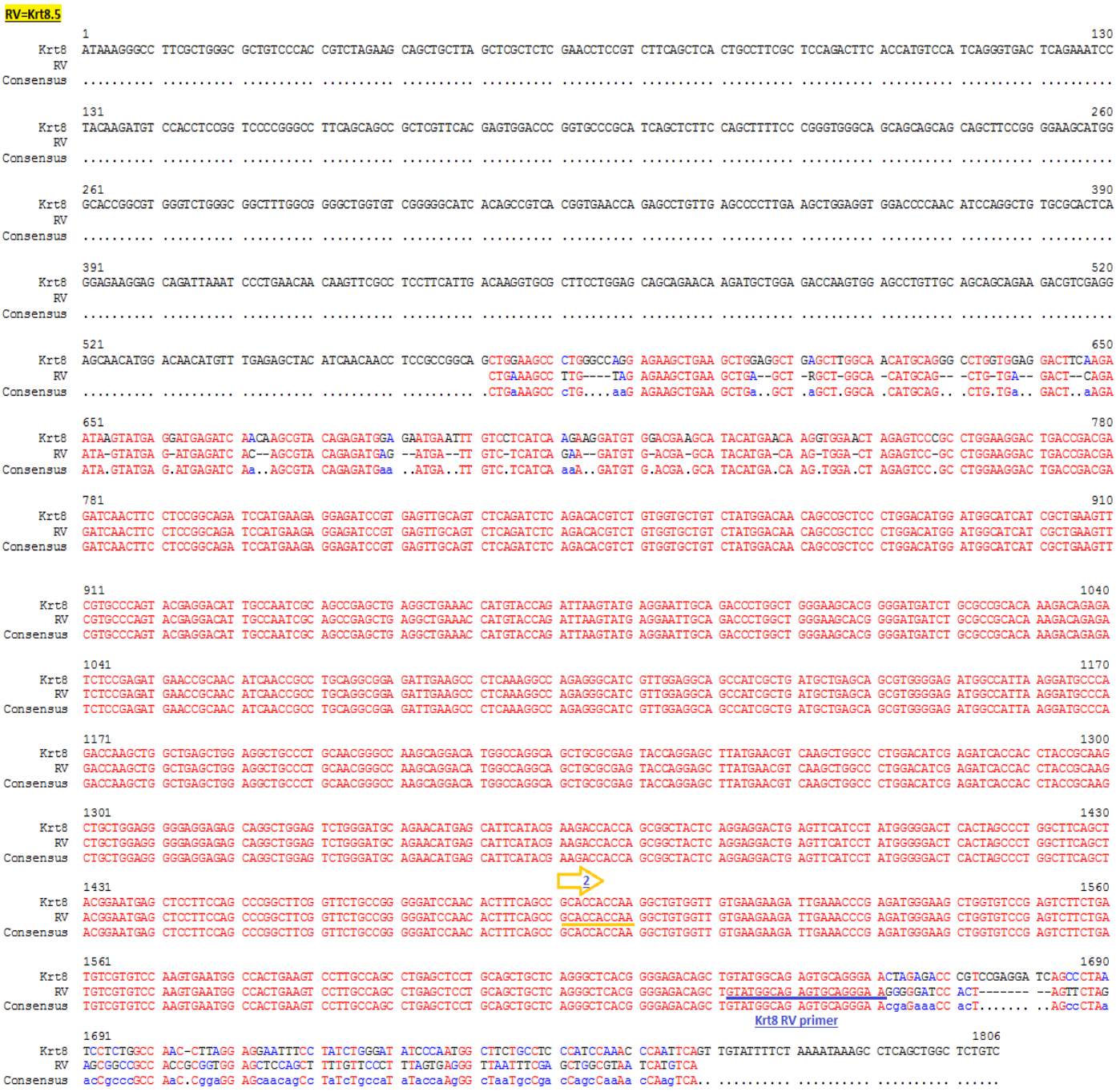


Figure 18: Reverse sequencing of *Krt8* in pBS-*Krt8*.5.
No mutations were identified between the end (*Krt8* RV primer) and the 2nd yellow arrow.

3.3.2 Sequencing of m*Krt18* from pTP-*Krt18*.1

Figure 34: Forward sequencing of *Krt18* PCR product in pTP-*Krt18*.1 .
No mutations were found from the start (FW *Krt8* primer sequence) until 870bp position (995).

Figure 35: Reverse sequencing of *Krt18* PCR product in pTP-*Krt18*.1.
No mutations were found from the end (RV *Krt18* primer) back to 870bp position (911).There is also an overlap between the FW and RV sequencing (from the yellow to the blue line) where no mutations were identified.

In conclusion, m*Krt8* and m*Krt18* subloned in pBS-*krt8*.5 and pTP-*Krt18*.1 were sequenced using the universal primers of the vectors along with a designed primer for the middle part of *Krt8*. Depending on the results , no mutations were identified (Figure 31-35). Among each sequencing there were ,also, overlapping areas that confirmed these results.In the continuity, the previous positive clones were stocked as glycerol stocks. Since, the fidelity of *Krt8* and *Krt18* PCR products were determined, subcloning those products to expression vector (pAdCMVLoxP vector) was initiated and performed.

3.4. Subcloning *Krt8* and *Krt18* to adenoviral expression vectors

m*Krt8* and m*Krt18* cDNAs encode keratin 8 and keratin18 , respectively. Keratin 8 and 18 are intermediate filament proteins found in simple epithelium as previously described [37] . In order to study the cellular effect of keratin 8 and 18 intermediate filament network formation in various cell lines, expression of those cDNAs is necessary. For this purpose, subcloning of *Krt8* and *Krt18* cDNAs to expression vectors was performed.

Expression vectors are usually a plasmid or virus designed for protein expression in cells. The vector is used to introduce a specific gene into a target cell, and can commandeer the cell's mechanism for protein synthesis to produce the protein encoded by the gene. The plasmid is engineered to contain regulatory sequences that act as enhancer and promoter regions and lead to efficient transcription of the gene carried on the expression vector. Vectors are transfected into the cells and the DNA may be integrated into the genome by homologous recombination in the case of stable transfection, or the cells may be transiently transfected. Examples of mammalian expression vectors include the adenoviral vectors, the pSV and the pCMV series of vectors. The promoters for cytomegalovirus (CMV) and SV40 are commonly used in mammalian expression vectors to drive protein expression.

In our expreriment procedures pAdApt LoxP CMV TbGH(+) plasmid (pAdMCSloxP) will be used. It is a adenoviral shuttle vector that can propagate either in prokaryocytes or mammalian cells. pAdMCSloxP includes the adenoviral 5 'ITR, a packaging signal from 0–1 map unit (mu) of the adenoviral genome, a multiple cloning site, a single loxP sequence 3' of the transgene, and 9.2–16.1 mu. The pAdCMVloxP plasmid was constructed by the insertion of cytomegalovirus immediate early enhancer/promoter (CMV promoter) and SV40 poly(A) signal into pAdMCSloxP [67] (Figure 36).

The pAdCMVLoxP vector provides the ability to transfect transiently a mammalian cell line for expression analysis.However, the main reason was that it may be also used as a donor plasmid of *Krt8* or *Krt18* for the construction of recombinant adenoviruses after the homologous recombination with AdlantisI donor virus, kindly provided by Hillgenberg M et al. [65] .

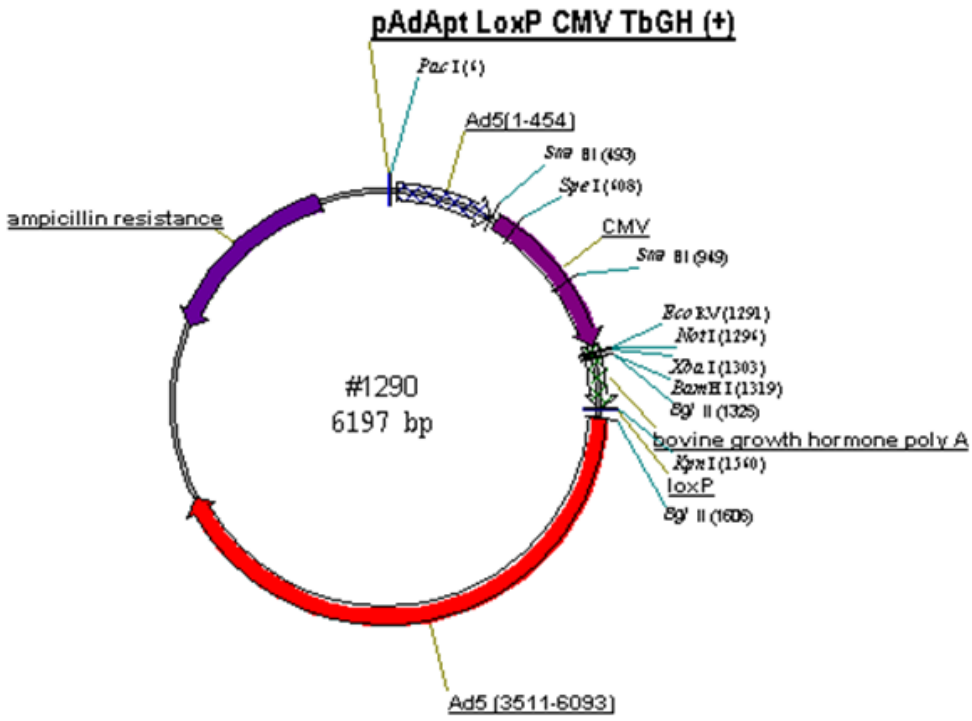


Figure 36: pAdCMVLoxP vector

3.4.1. Subcloning *Krt8* cDNA to pAdCMVLoxP vector

pBS-*Krt8*.5 clone containing *Krt8* cDNA was grown with midi-culture and plasmid DNA was isolated with midi-prep. Then, pBS-*Krt8*.5 was digested with EcoRV and XbaI. EcoRV and XbaI are zero cutters (online program : NEB cutter) of *Krt8* cDNA while they are single cutters of pBluescript vector inside its MCS (Figure 20). After digestion with EcoRV and XbaI , *Krt8* cDNA is released from the vector and has a 5' blunt-end (EcoRV) along with a 3' sticky-end (XbaI). (Figure 37).

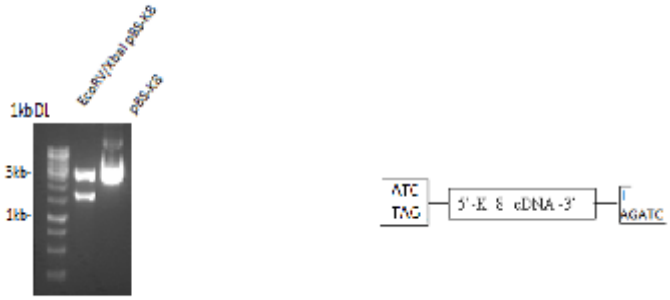


Figure 37: **pBS-*Krt8* digested with EcoRV and XbaI.**
After EcoRV/XbaI digestion of pBS-*K8* (4,6kb) there are 2 DNA bands , a 3kb (vector) and 1,6kb (released *krt8* cDNA fragment).

Released *krt8* cDNA fragment was then, extracted and purified from the gel. In the continuity , pAdCMVLoxP vector (6,2kb) was digested also with EcoRV/XbaI (Figure 38).

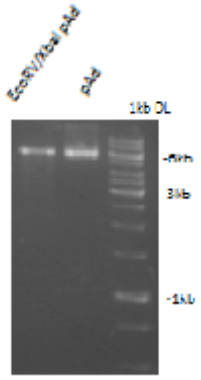


Figure 38: Digestion of pAdCMVloxP (pAd) with EcoRV/XbaI. Digested pAd DNA band is slightly higher (6,2kb) than undigested supercoiled pAd.

EcoRV/XbaI pAdCMVLoxP vector was also extracted and purified from the gel. Then, it was dephosphorylated with antarctic phosphatase to inhibit self-ligation and purified with mini-column (PCR product purification protocol). After DNA concentration was measured for EcoRV/XbaI *Krt8* cDNA and EcoRV/XbaI dephoshorylated pAd vector, ligation with T4 DNA ligase was performed. In this case blunt-sticky ligation was performed and exactly as previously described positive colonies with the correct orientation (5' → 3') of *Krt8* cDNA were identified with restriction digestions and they were selected.Finally, pAdCMVLoxP vector (6,2kb) contains the cDNA of *Krt8* (1,6kb) with 5' → 3' orientation inside the MCS downstream of the CMV promoter and is named pAd-*Krt8* plasmid (7,8kb).The subcloning process of *Krt8* cDNA from pBS-*Krt8*.5 to pAdCMVLoxP vector is figured below (Figure 39).

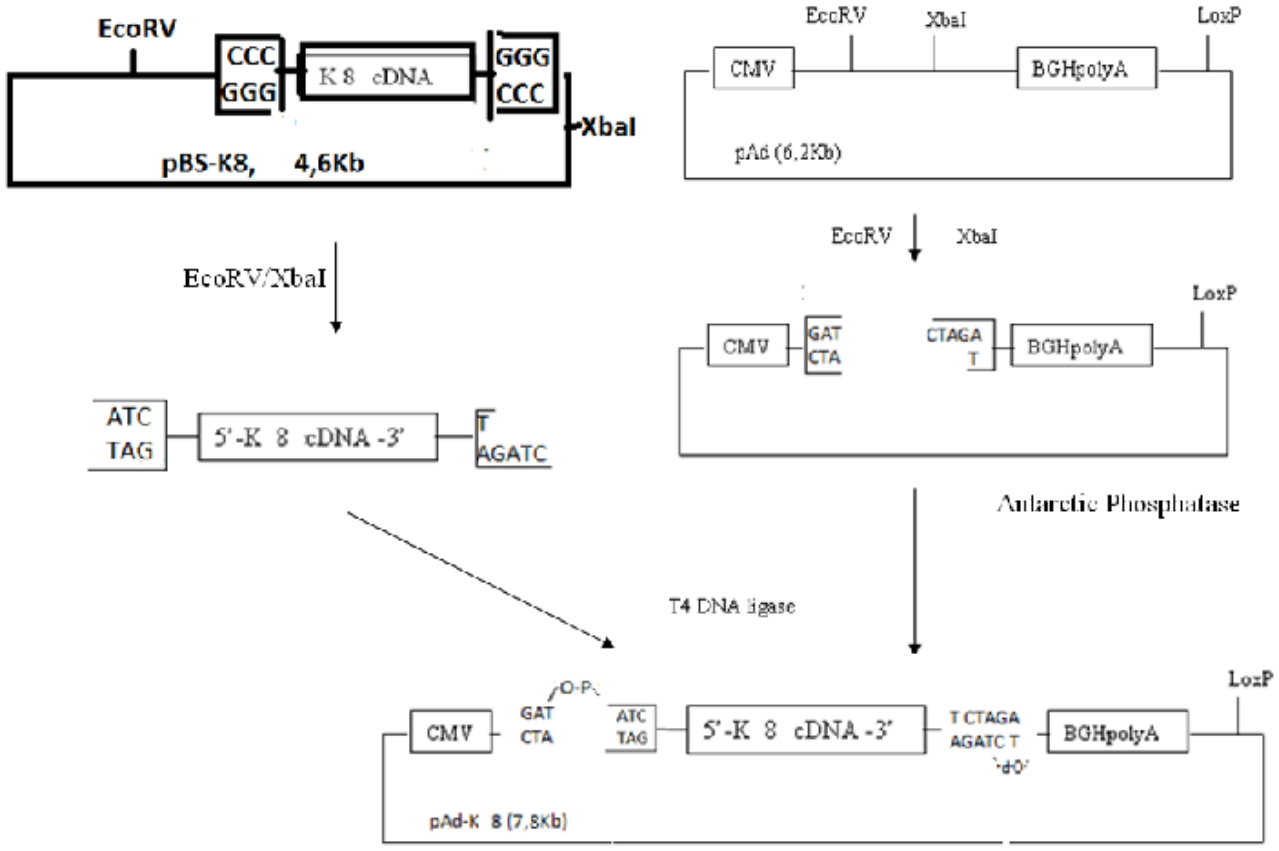


Figure 39 : Subcloning *Krt8* cDNA to pAdCMVLoxP vector.
Adenoviral shuttle vector with *Krt8* cDNA is formed (pAd-*Krt8*).

3.4.2 Subcloning *Krt18* cDNA to pAdCMVLoxP vector

pTP-*Krt18*.1 clone was grown and DNA was purified. Then, pTP-*Krt18*.1 (5,4kb) was digested and linearized with SpeI restriction enzyme (Figure 40). SpeI is a zero cutter of *Krt18* cDNA (1,4kb) and a single cutter of pTOPO vector as well as pTP-*Krt18*.1.

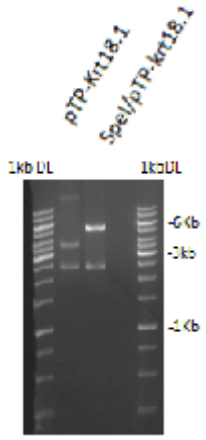


Figure 40: pTP-*Krt18*.1 digested with SpeI.
After SpeI digestion there are 2 DNA bands, a 5,4kb band (linearized pTP-*Krt18*.1 and a 2,5kb band (undigested supercoiled pTP-*Krt18*.1)

Afterwards , linearized pTP-*Krt18*.1 was gel extracted and purified. Then, it was treated with DNA Polymerase I, Large (Klenow) Fragment and sticky-ends of SpeI digestion transformed into blunt-ends. Afterwards, linearized blunt-ended pTP-*Krt18*.1 was purified and then digested with XbaI. XbaI is a zero cutter of *Krt18* cDNA while it is a single cutter of pTOPO vector as well as pTP-*Krt18*.1 . XbaI digestion resulted in the release of *Krt18* cDNA with a 5' blunt-end (Klenow reaction) and a 3' sticky (XbaI) end (Figure 41).

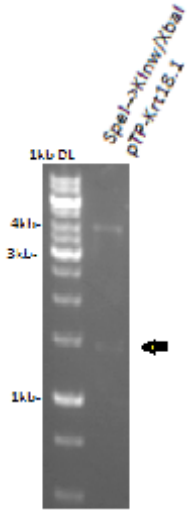


Figure 41: SpeI→Klnw/pTP-*Krt18*.1 digested with XbaI.
After XbaI digestion of SpeI → Klenow treated pTP-*Krt18*.1 , there are 2 DNA bands , a 4kb (TOPO vector) and a 1,4kb which corresponds to *krt18* cDNA.

Then ,released *Krt18* cDNA (1,4kb) was extracted from the gel and purified. Then, it was ligated with EcoRV/XbaI pAdCMVLoxP (6,2kb) (isolated previously during pAd-*krt8* subcloning) through a blunt-sticky ligation mediated by T4 DNA ligase. After the ligation, pAdCMVLoxP vector would contain *Krt18* cDNA in the MCS (pAd-*krt18*). Colonies with *Krt18* cDNA and a 5' → 3' orientation of this insert downstream of CMV promoter were isolated after identifying digestions. The subcloning process of *Krt18* cDNA from pTP-*Krt18*.1 to pAdCMVLoxP vector is figured below (Figure 42).

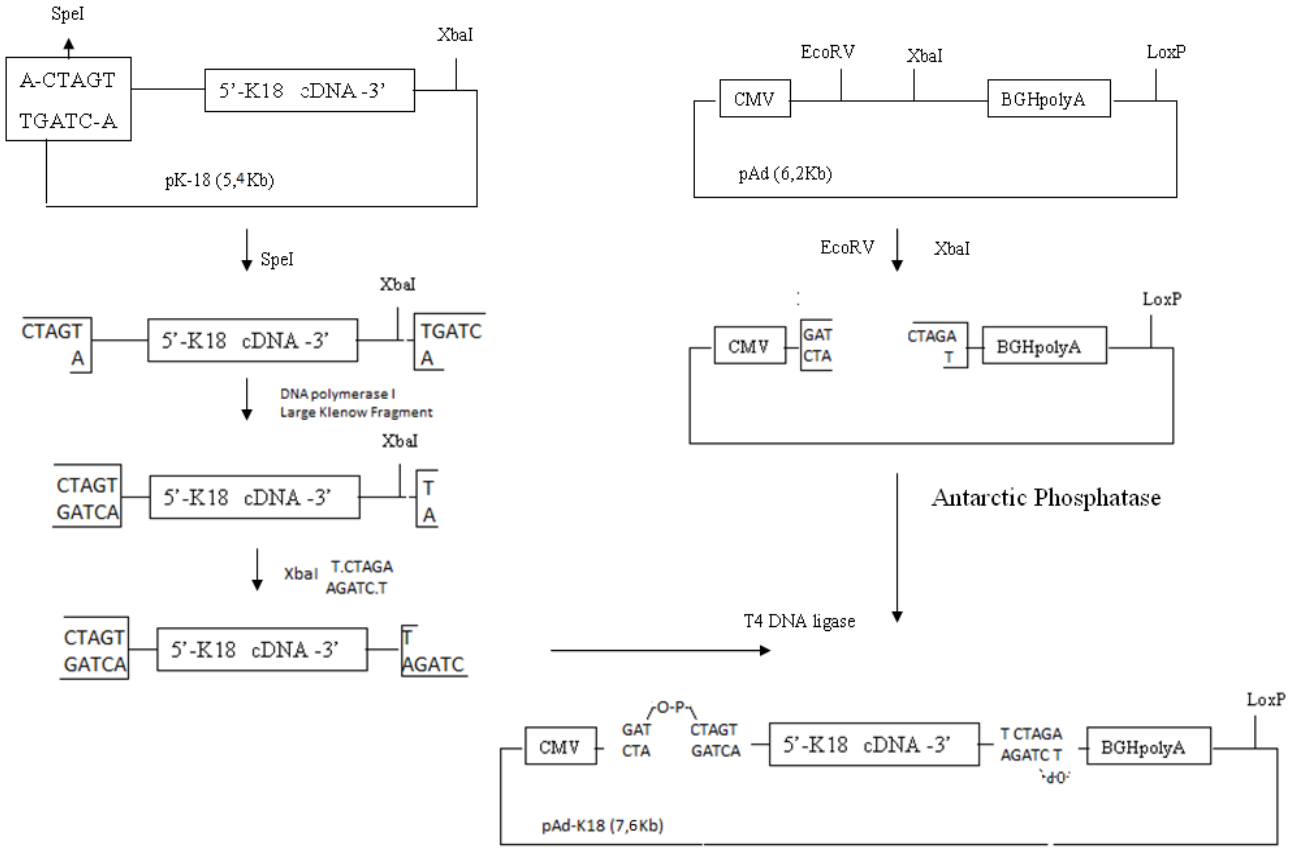


Figure 42: Subcloning *Krt18* cDNA to pAdCMVLoxP vector.
Adenoviral shuttle vector with *Krt18* cDNA is formed (pAd-*Krt18*).

Finally, pAd-*Krt8* and pAd-*Krt18* with the desired 5' → 3' orientation of the insert are indicated below (Figure 43). PacI is a single cutter of pAd and a zero cutter of *Krt8* cDNA. SpeI is a zero cutter of *Krt8* cDNA. However, at the left arm of *Krt8* cDNA a SpeI restriction site was added because a part of pTOPO MCS was incorporated. As far as pAd-*Krt18* is concerned, HindIII is a zero cutter of pAd but a single cutter of *Krt18* (-1,2kb position).

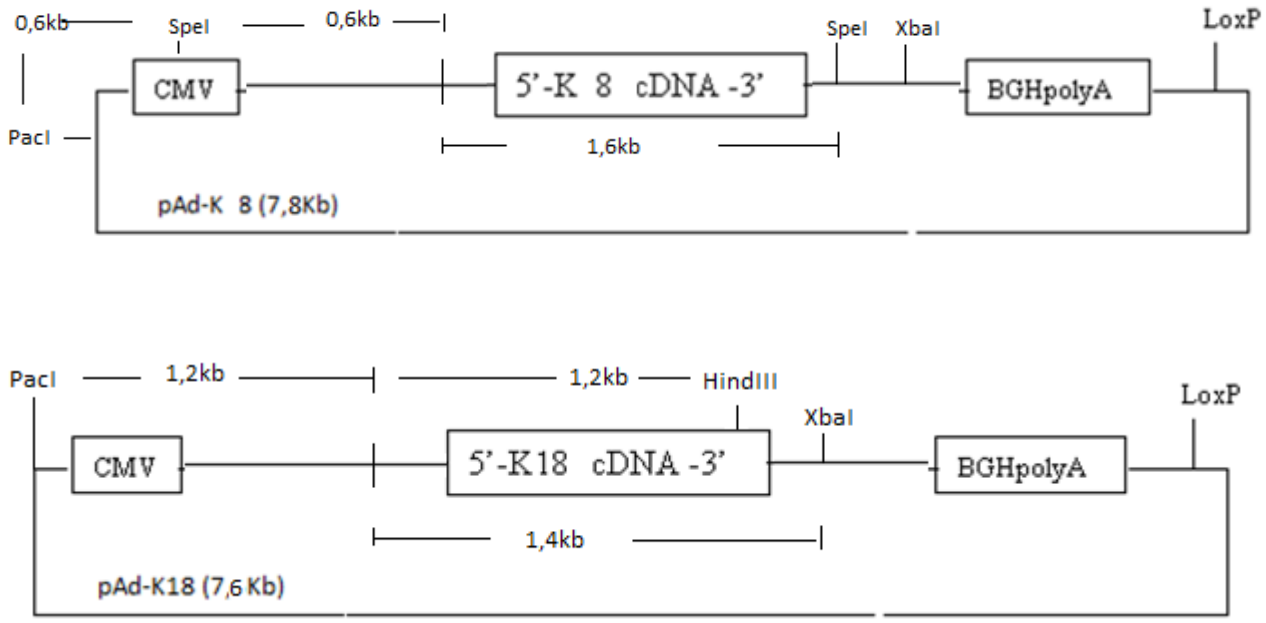


Figure 43: pAd-*Krt8* and pAd-*Krt18* along with restriction sites used for identification

Restriction Enzymes	Plasmid DNA (Kb)	PCR product orientation	Digestion DNA Fragments (kb)
PacI	pAd-*Krt8* (7,8Kb)	5' → 3' or 3' → 5'	7,8Kb
	Self ligated pAd (6,2kb)	No insert	6.2Kb
SpeI	pAd-*Krt8* (7,8Kb)	5' → 3'	5,6 + 2,2
	pAd-*Krt8* (7,8Kb)	3' → 5'	7,2 + 0,6
	Self ligated pAd vector (6,2Kb)	No insert	6,2kb
PacI	pAd-*Krt18* (7,6kb)	5' → 3' or 3' → 5'	7,6kb
	PAd(6,2kb)	No insert	6,2kb
PacI/ HindIII	pAd-*Krt18* (7,6kb)	5' → 3'	5,2kb+2,4kb
	pAd-*Krt18* (7,6kb)	3' → 5'	6,2kb+1,4kb
	pAd	No insert	6,2kb

Figure 44: Restriction digestions either with PacI , SpeI or PacI / HindIII of pAd-*krt8* or pAd-*Krt18*. According to the presence of insert or its orientation in pAdCMVLoxP , distinct digestion DNA fragments are produced.

Although, subcloning of either *Krt8* or *Krt18* cDNA was performed with blunt-sticky ligation , orientation identifying digestions as SpeI/pAd-*Krt8* and PacI/HIndIII pAd-*Krt18* confirmed the expected 5' → 3' orientation (Figure 44-45) . Below it is indicated that the 6th pAd-*Krt8*.5 (pAd-*Krt8*.5/6) and the 3rd pAd-*Krt18*.1 (pAd-*Krt18*.1/3) clones do have *Krt8* and *Krt18* cDNAs respectively with 5' → 3' orientation downstream the CMV promoter.

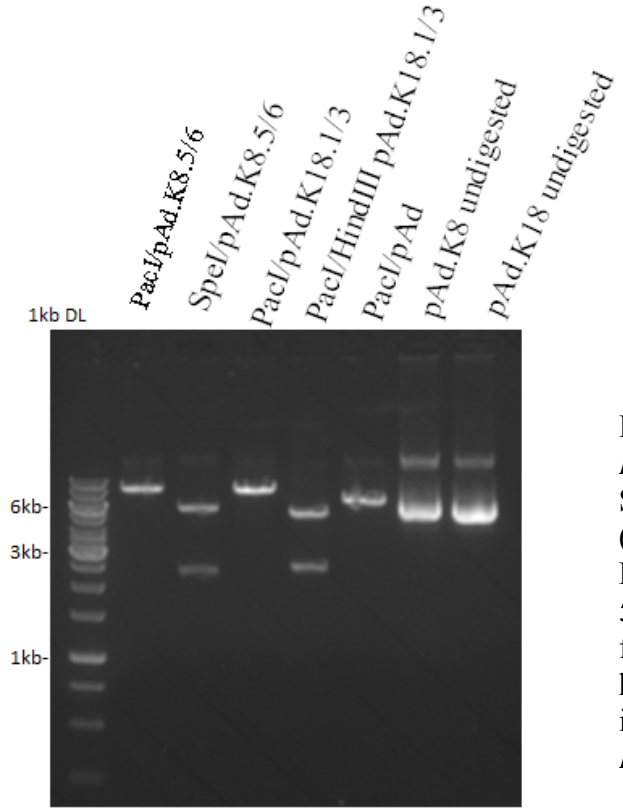


Figure 45: Identifying digestions of pAd-*Krt8*.5/6 and pAd-*Krt18*.1/3 .
SpeI/pAd-*Krt8*.5/6 produced 2 bands, a 5,6kb (pAd fragment) and a 2,2kb (*Krt8* fragment) .
PacI/HindIIIpAd-*Krt18*.1/3 produced 2 bands, a 5,2kb (pAd fragment) and a 2,4kb (*krt18* fragment). PacI/pAd-*K8* or PacI/pAd-*K18* gave a higher band than PacI/pAd since there is an insert. PacI/pAd and undigested pAd-*K8* or pAd-*K18* were used as controls.

3.5. Transfection of pAd-*Krt8* and pAd-*Krt18* to NIH3t3 cell line

The process of introducing nucleic acids into eukaryotic cells by nonviral methods is defined as transfection. Using various chemical, lipid or physical methods, this gene transfer technology is a powerful tool to study gene function and protein expression in the context of a cell.Transfection is a method that neutralizes or obviates the issue of introducing negatively charged molecules (e.g., phosphate backbones of DNA and RNA) into cells with a negatively charged membrane. Plasmid DNA is most commonly transfected into cells, but other macromolecules can be transferred as well.

Plasmid DNAs were previously constructed and each one contain a keratin cDNA either *K8* (pAd-*Krt8*) or *K18* (pAd-*Krt18*).Each adenoviral shuttle plasmid contains a CMV promoter as well as SV40 poly(A) sequence. SV40 PolyA (Simian virus 40 PolyA, also called PolyA) sequence is a 240bp DNA sequence that possesses the activity of transcription termination and can add PolyA tail to mRNA.Polyadenylation (polyA) confers mRNA stability and promotes an mRNA's translational efficiency.Additionaly, cytomegalovirus (CMV) immediate early enhancer/promoter is a powerful promoter frequently used for driving the expression of transgenes in mammalian cells. These characteristics of recombinat adenoviral shuttle plasmid vectors provide the capability to express keratin transgene in mammalian cells.

3.5.1 Experiment Design

In order to study keratin 8 and 18 expression and their characteristics , expression analysis study was performed in NIH 3T3 cell line. NIH 3T3 cell line contains modified mouse embryonic fibroblasts and do not express keratins [69] . Instead the major intermediate filament of fibroblasts is vimentin. NIH 3T3 cell line was thawed , cultured and splitted to well plates in order to perform transfection.Polyethylenimine (PEI) was used as transfection agent.Transfection either with single keratin or both of them was performed. Additionally, pAdCMVLoxP vector-transfected as well as untrasfected NIH 3T3 cells (transfection media with no plasmid component) were used as negative controls. Moreover, pAdCMVLoxP with GFP cDNA (pAd-GFP) was used as a positive control for trasfection assay with PEI. Finally, human embryonic kidneys 293 cells (HEK293) are simple epithelium cells and their major intermediate filaments are keratins 8 and 18. For this reason, HEK293 cells were the positive control for keratin 8/18 expression. Below , the design of the experiment is indicated (Figure 46) .

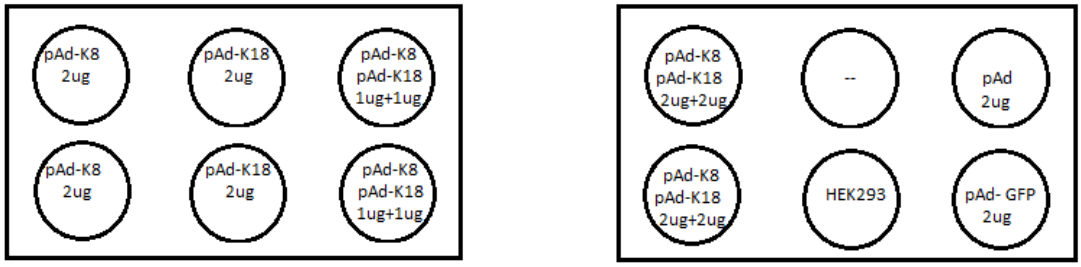


Figure 46: Expression analysis experiment for pAd-*K8* and pAd-*K18*.

3.5.2. NIH 3T3 transfected with pAd-GFP

Although transfection with PEI was performed , the efficiency of this method was low. NIH 3T3 cells were transfected with pAd-GFP and the percentage of green fluorescence protein (GFP) - expressing cells was low, approximately 5% per high optical field (HOF) (Figure 47).

Since, soluble GFP has been shown to be extracted by methanol fixation [70] , images for GFP expression were taken prior to that step.

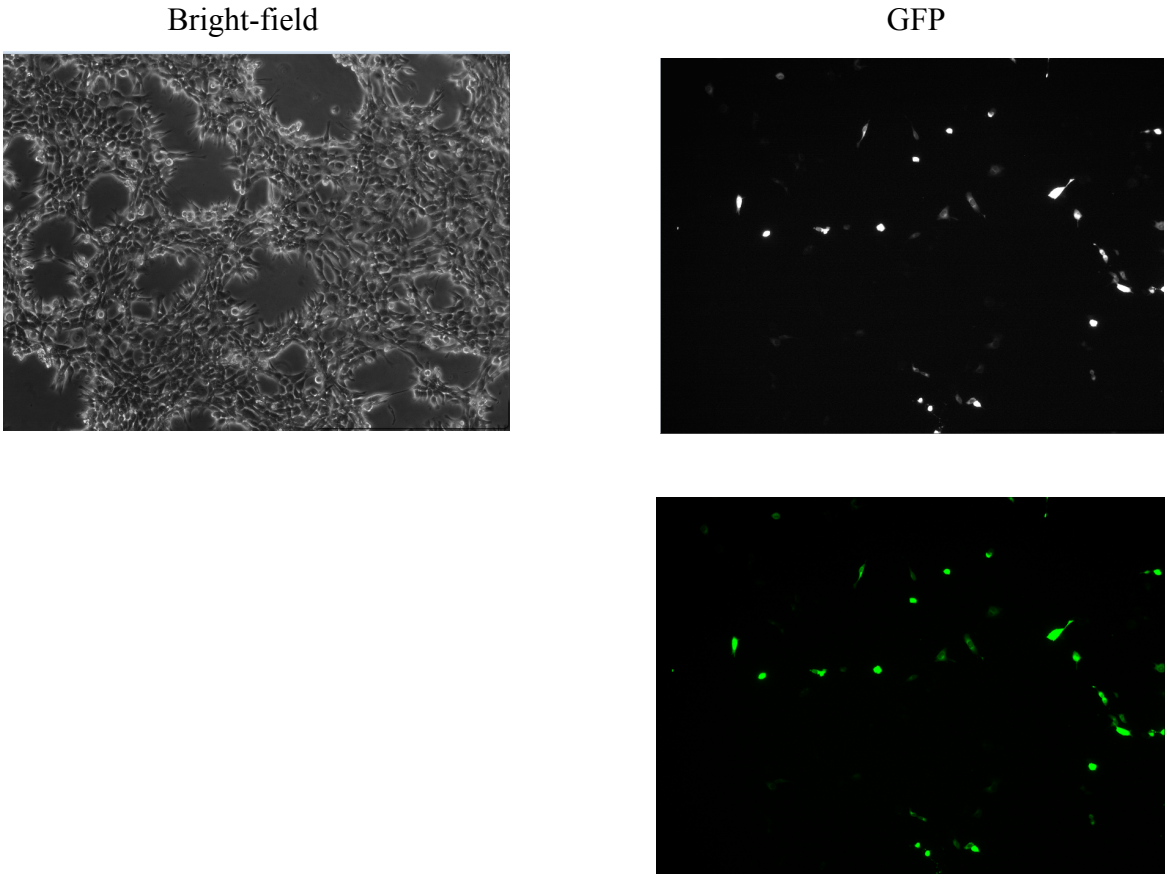


Figure 47: **NIH 3T3 transfected with pAd-GFP.** Low transfection efficiency with polyethylimine agent.

3.5.3. K8-K18 expression profile in HEK vs NIH cells

Keratin 8 and 18 (K8/18) are the major components of the intermediate filaments of simple or single layered epithelia found in the gastrointestinal tract, liver, exocrine pancreas, mammary gland and kidney[37] . HEK 293 cell line is composed by adeno-transformed human embryonic kidney (HEK) cells and HEK cells express keratin 8 and 18 IF proteins as it is shown below (Figure 48) . Keratin 8 along with keratin 18 form a filamentous network that surrounds the nucleus , spans the cytoplasm reaching the cellular periphery. Keratin 8 may form filaments with other keratin partners and it spans more distal than keratin 18 (Figure 48-49). Generally, it is known that keratins form heteropolymeric filamentous networks that are attached to the cytoplasmic plaques of the typical epithelial cell-cell junctions , the desmosomes [29] .

In contrast to simple epithelial cells , NIH 3T3 fibrobast-like cells do not express keratin 8 and 18. The major intermediate filament protein in NIH 3T3 cells is vimentin [71, 69].

The prototypic type III gene, vimentin, is expressed in a plethora of non-epithelial cell types, and its product can homopolymerize to form 10-nm filaments *in vitro* as well as *in vivo*. Few other proteins in assembly group B are capable of this feat; most require the involvement of another 'group B' sequence, often vimentin, for proper assembly [72] .

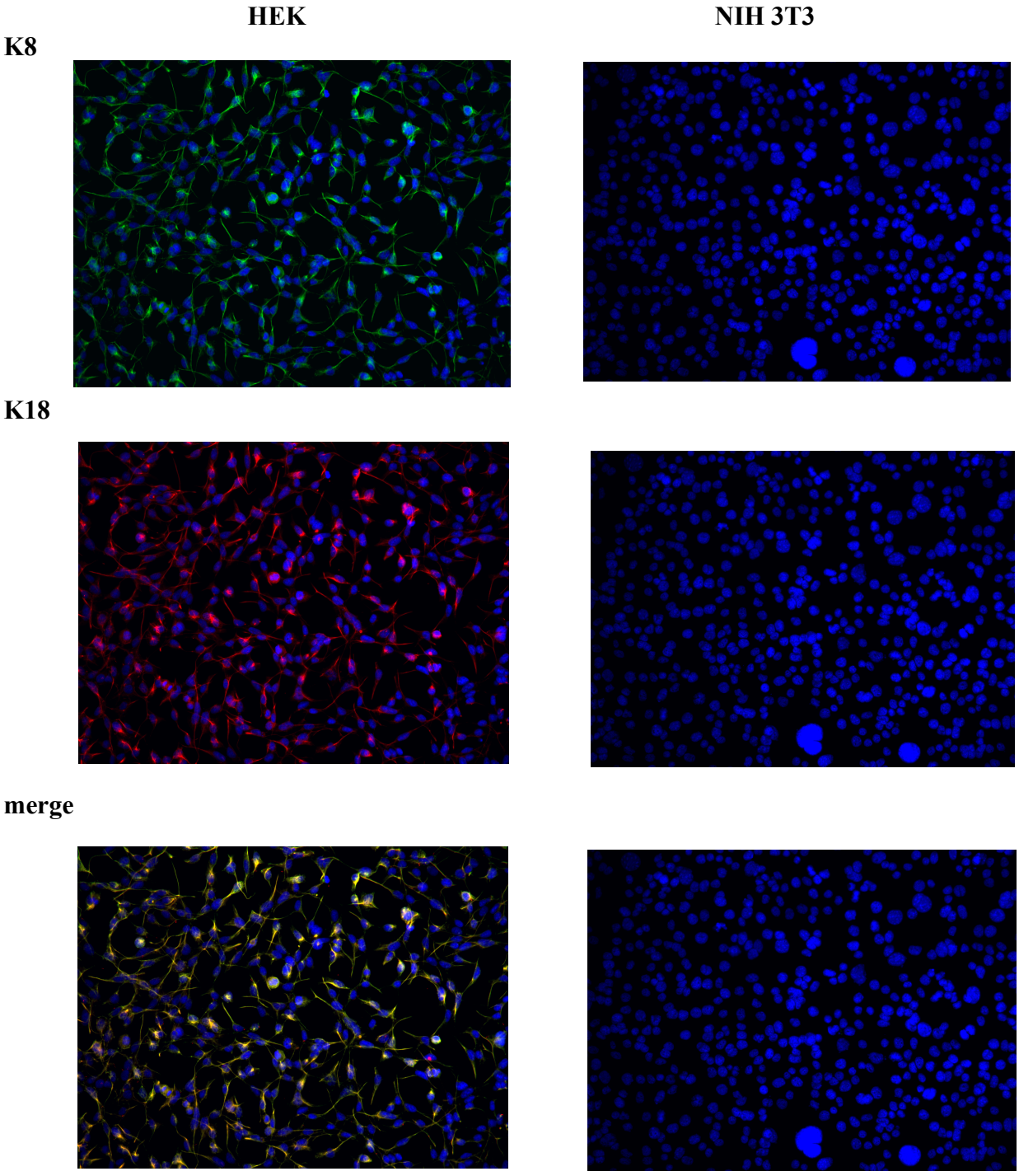


Figure 48: Keratin 8 and 18 expression in HEK 293 cells. NIH 3T3 cells do not express keratins 8 and 18.

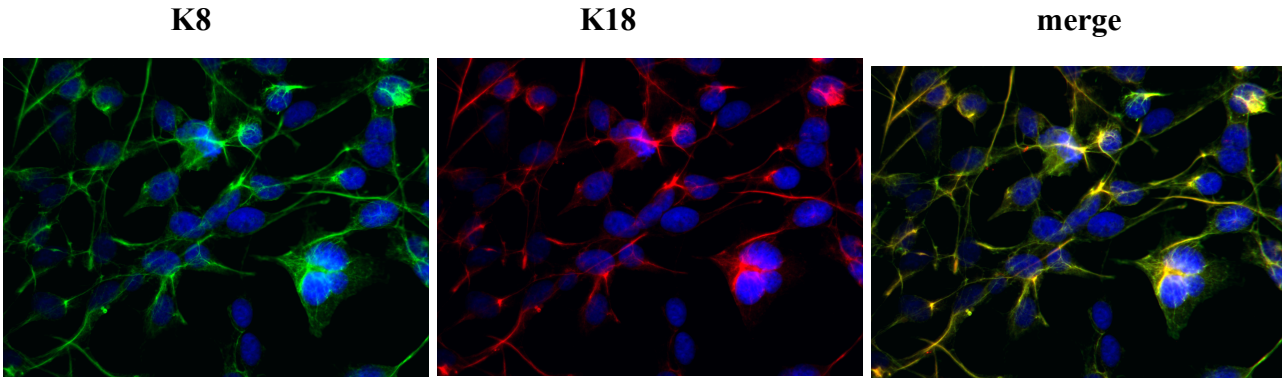


Figure 49: Keratin 8 (K8) along with K18 form a filamentous network in HEK 293 cells.

3.5.4. Vimentin Network in NIH 3T3 fibroblasts

Below, NIH 3T3 cells were transfected with pAdCMVLoxP and expression of keratin 8 and vimentin was studied with immunofluorescence. pAdCMVLoxP is a blank vector and it does not contain any transgene (neither *Krt8* nor *Krt18*). In NIH 3T3 cells, vimentin surrounds the nucleus , spans through the cytoplasm and forms filaments (figure 50).

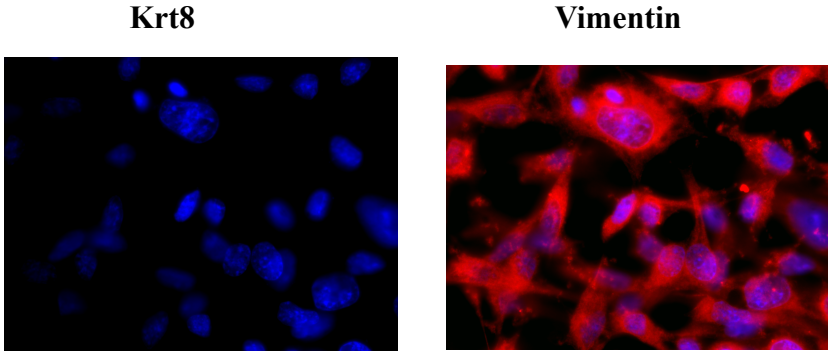


Figure 50: Vimentin Network in NIH 3T3 fibroblasts . NIH 3T3 fibrobast-like cells do not express keratin 8. The major intermediate filament protein of NIH 3T3 cells is vimentin.

3.5.5. *K8*-transfected NIH 3T3 fibroblasts

In contrast to vimentin protein which usually homopolymerize, keratins have to form specific heteropolymeric pairs between type I and type II keratins in order to form filaments. Expression of keratin 8 was studied in NIH 3T3 cells after transfection with pAd-*Krt8* vector.

Merged images after Krt8 and Krt18 IF

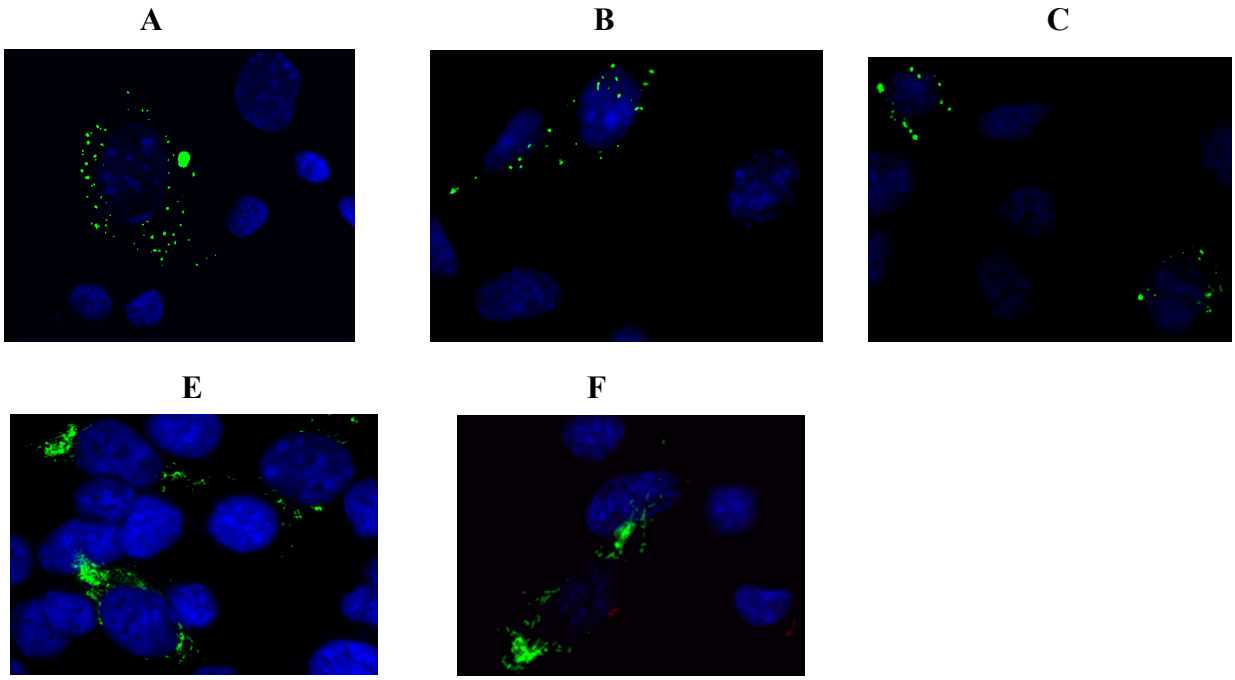


Figure 51: *K8*-transfected NIH 3T3 fibroblasts. (A-F:merged images) Single transfection with pAd.*Krt8* induces keratin 8 expression but no keratin18 expression. Keratin 8 single expression forms mainly perinuclear aggregates but no filaments. (green → Krt8, red → krt18, blue → nucleus)

Keratin 8 is expressed in fibroblast-like NIH 3T3 cells after single transfection with *Krt8* vector. However, keratin 8 does not form cytoplasmic filaments but aggregates that are located mostly perinuclearly. Furthermore, it seems that keratin 8 does not induce the expression of its heteropolymeric partner keratin 18. Since expression of a heteropolymeric keratin partner is necessary for filament protein formation, it seems that there is not expression or induction of any keratin protein partner in NIH 3T3 cells (Figure 51).

3.5.6 *K18*-transfected NIH fibroblasts

Then, keratin 18 expression was studied after single transfection with pAd.*Krt18* vector in NIH 3T3 cell line. It seems that there is either no expression of keratin 18 or rapid degradation of Krt18. Furthermore, there is not expression of keratin 8 after Krt18 transfection. Additionally, the rapid degradation of keratin 18 could be explained by the absence of any heteropolymeric keratin partner as Krt8 that could stabilize its expression and therefore filament protein formation (Figure 52) .

Merged Images after K8 and K18 IF

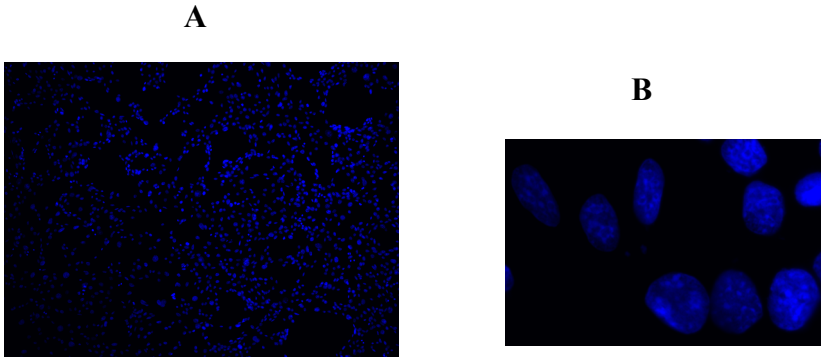
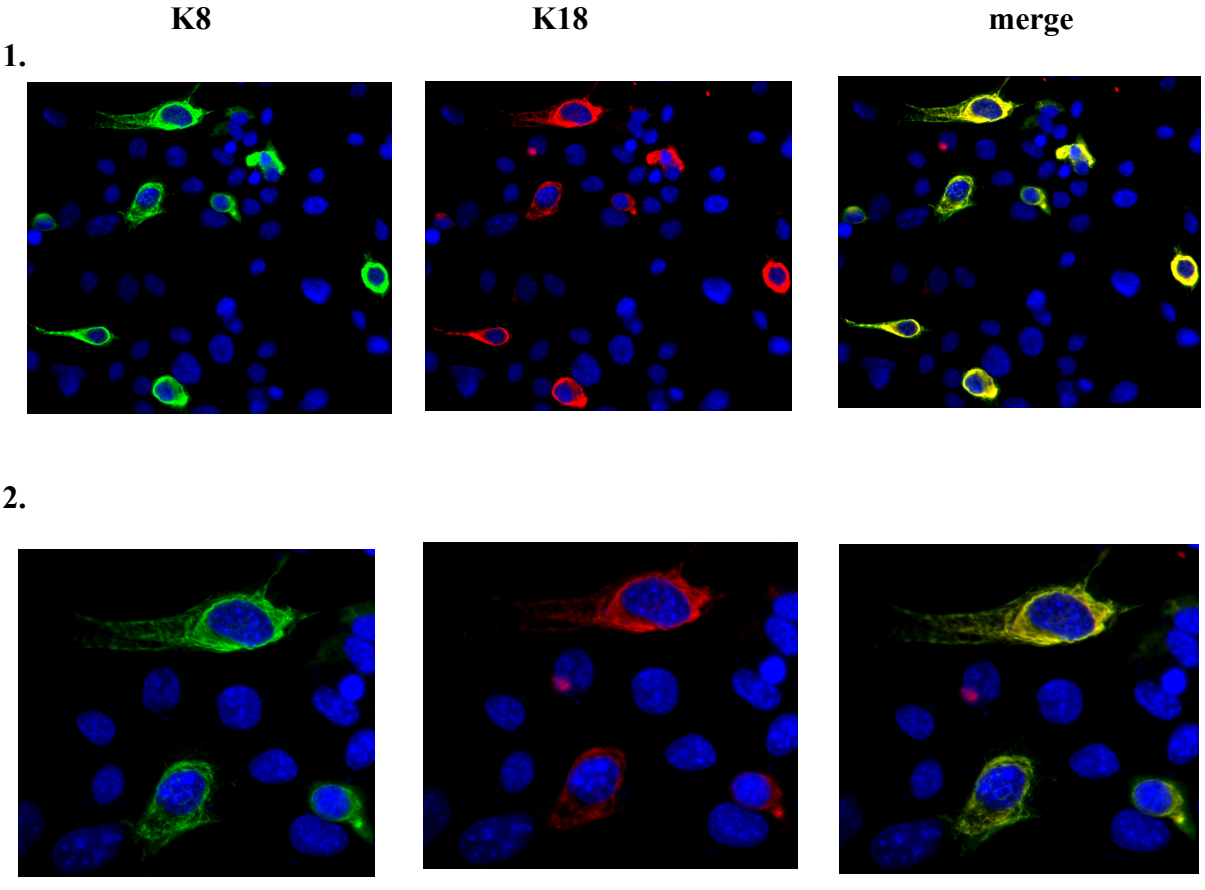


Figure 52: ***K18*-transfected NIH fibroblasts (A-B).** Single transfection with pAd.*Krt18* does not induce keratin 8 expression. Keratin 18 is not found intracellularly when keratin 8 is not present and it seems that is degraded rapidly in Krt8 absence (green → Krt8, red → krt18, blue → nucleus). (B image is a magnification).

3.5.7. *K8* and *K18*-transfected NIH 3T3 fibroblasts

In the continuity, NIH 3T3 cells were transfected with pAd.*Krt8* and pAd.*Krt18* simultaneously. Then, keratin 8 and 18 expression was studied.



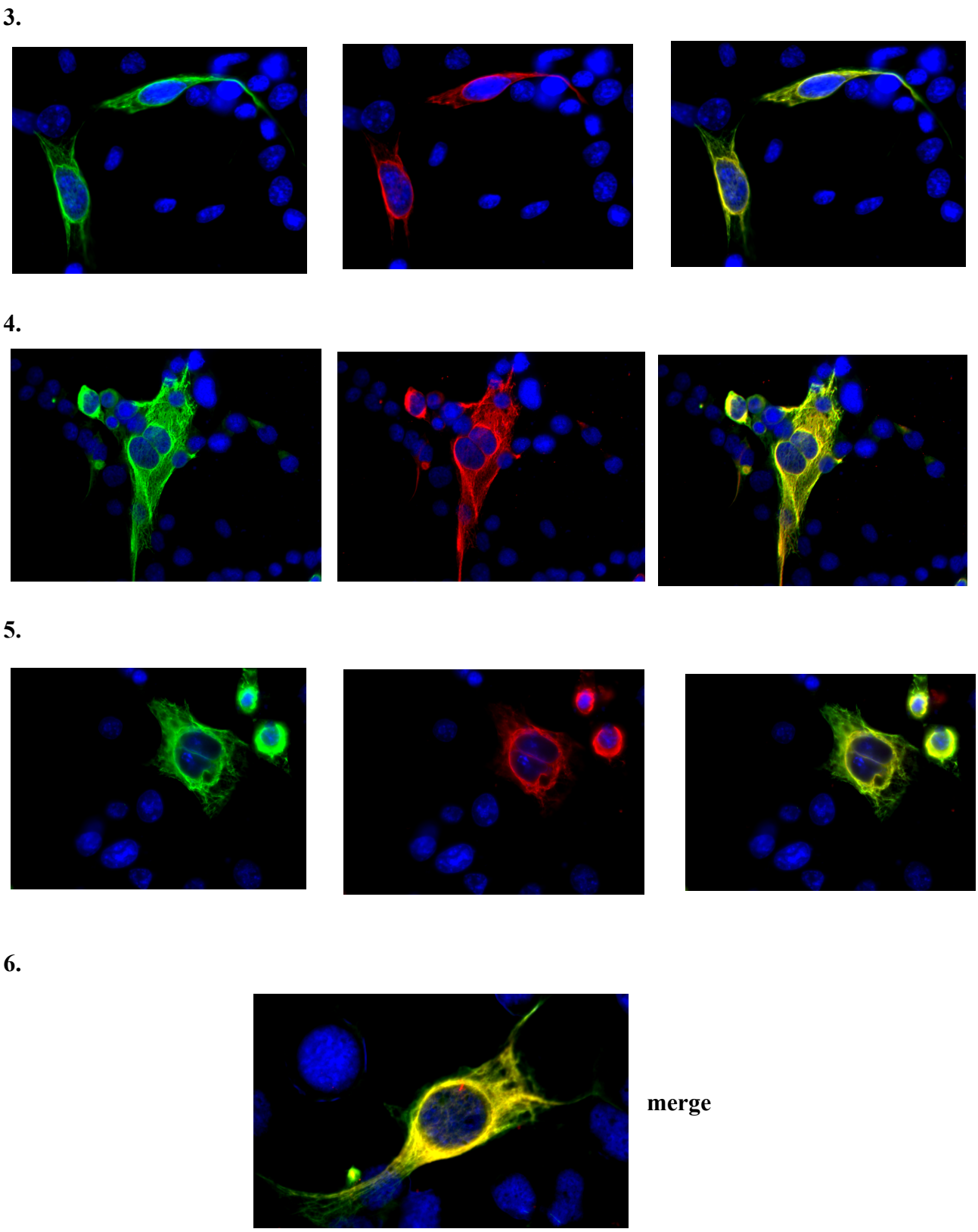


Figure 53: ***K8* and *K18*-transfected NIH 3T3 fibroblasts (1-6).** Double transfection of NIH 3t3 with pAd.*Krt8* and pAd.*Krt18* constructs. Keratin 8 and 18 are expressed in the cytoplasm and form a filamentous network (green → Krt8, red → krt18, blue → nucleus), (6: merge).

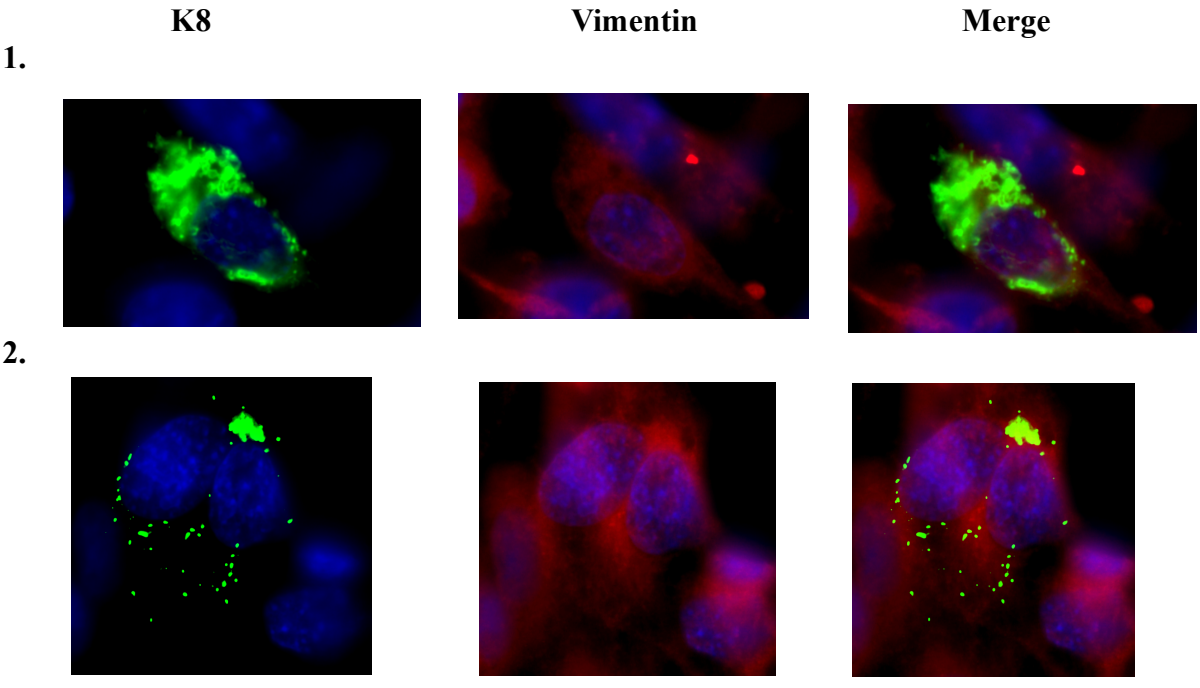
Keratin 8 and 18 are expressed after double transfection with pAd.*Krt8* and pAd.*Krt18*. Keratin 8 along with its heteropolymeric protein partner keratin 18 are co-localized inside the cytoplasm and form a filamentous network that surrounds nucleus and spans the cytoplasm reaching the cellular periphery (Figure 53). In contrast to single expression with Krt8 and formation of aggregates, keratin 18 expression attenuates aggregate formation. Moreover, expression of keratin 8 stabilizes keratin 18 expression and attenuates its rapid degradation when single expression of Krt18 is present. Furthermore, Krt8 reaches more the cellular periphery than krt18 possibly interacting with cytoplasmic membrane proteins like desmosomes in case of epithelial cells. Additionally, among the transfected cells there is differential DNA transfection (DNA amount or ratio) that may lead to increased signal or abnormal network formation. It is noteworthy to mention that fibroblast-like NIH 3T3 cells have the essential protein machinery to construct keratin network. Therefore, pAd.*Krt8* and pAd.*Krt18* vectors contain the necessary coding genes for keratin expression and their expression characteristics are similar according to previous studies.

3.5.8. Vimentin and keratin network

As it was mentioned previously, the major intermediate filament protein in NIH 3T3 cells is vimentin. Although keratin 8 and 18 intermediate filaments are mainly found in simple epithelial cells , fibroblast-like NIH 3T3 cells have the capability to express and form a keratin 8/18 filamentous network after transfection assay. According to that, it would be interesting to study if keratin expression interacts with the native vimentin intermediate filament network.

3.5.8.1. *K8*-transfected fibroblasts and vimentin

In the absence of keratin 18, keratin 8 is not able to form keratin filaments but it forms cytoplasmic aggregates that do not interfere with the native vimentin filamentous network (Figure 54).



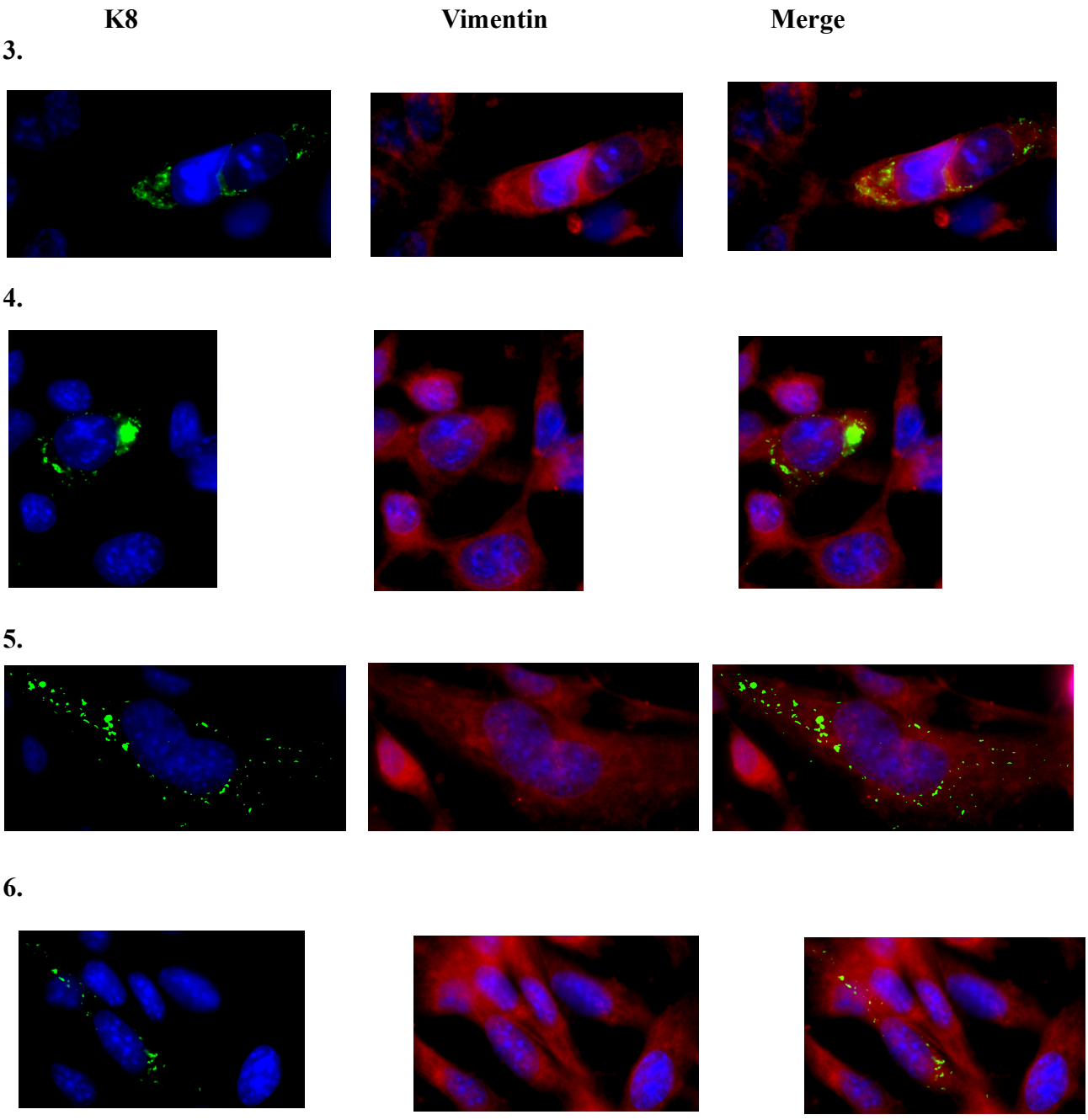


Figure 54: *K8*-transfected fibroblasts and vimentin. Keratin 8 and vimentin in single transfected NIH 3T3 cells with pAd.*Krt8*. Keratin 8 forms perinuclear aggregates that do not interfere with vimentin filamentous network in the absence of keratin 18.

According to previous results, keratin 18 is rapidly degradated after single transfection with pAd.Krt18 and it does not induce keratin 8 expression.

3.5.8.2. *K18*-transfected fibroblasts and vimentin

Below, it seems that native vimentin network is not influenced after pAd-*Krt18* transfection in NIH 3T3 cells (Figure 55).

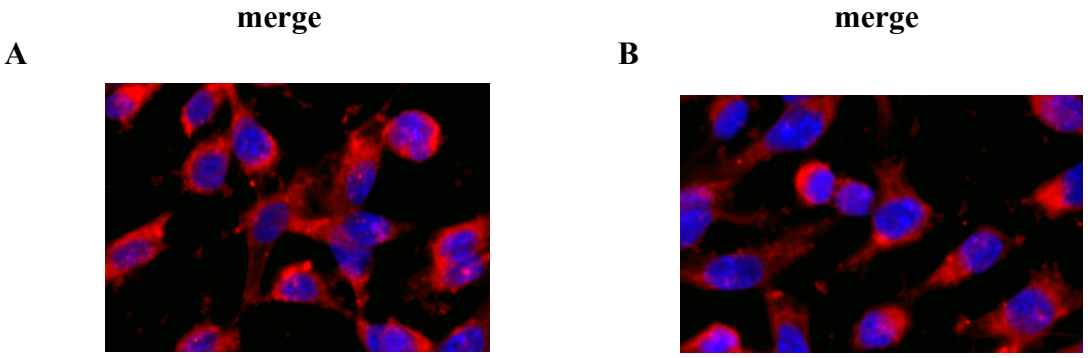


Figure 55: ***K18*-transfected fibroblasts and vimentin (A-B).** Immunofluoresence for Krt8 and Vimentin after pAd.*Krt18* transfection to NIH 3T3. Keratin 18 is rapidly degraded in the absence of keratin 8 while native the vimentin filamentous network of NIH 3T3 remains unchanged. (Krt8 → green, vimentin → red, nucleus → blue).

3.5.8.3 *K8* and *K18*-transfected fibroblasts and vimentin

Finally, keratin 8/18 network was compared with native vimentin filament network in NIH 3T3 cells. In the presence of keratin 18 , keratin 8 along with keratin 18 form a filamentous cytoplasmic network besided the native vimentin filamentous network. It seems that vimentin presence does not inhibit keratin network expression and formation. Keratin 8/18 as vimentin filamentous network spans surround nucleus and spans through cytoplasm to cellular periphery but it reaches the outer edges of cytoplasm more than vimentin (Figure 56).

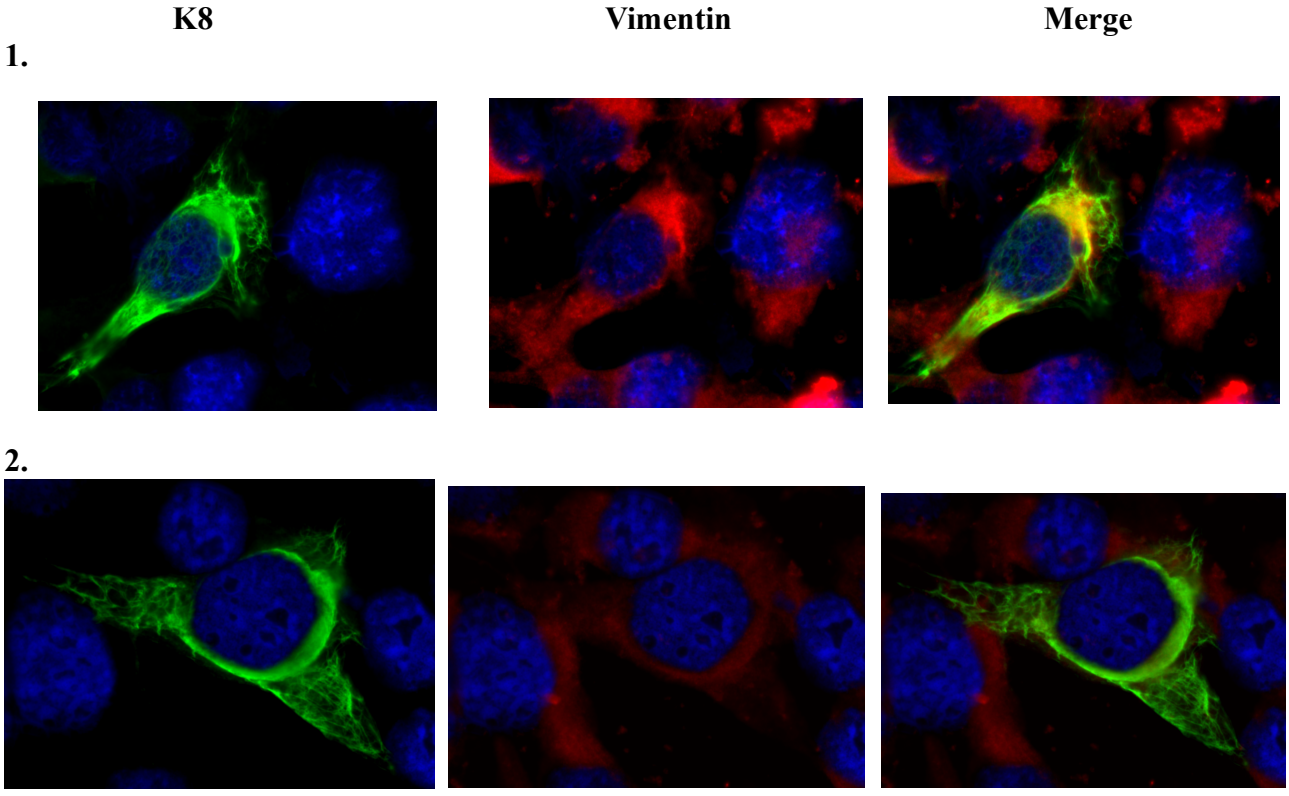


Figure 56: *K8* and *K18*-transfected fibroblasts and vimentin (1-2). Immunofluoresence for Krt8 and Vimentin double transfection with pAd-*Krt8* & pAd.*Krt18* to NIH 3T3. Keratin 8 along with

keratin 18 form a filamentous cytoplasmic network beside fibroblast's vimentin network (Krt8 → green, vimentin → red, nucleus → blue).

4. Discussion

4.1. Keratin 8 and 18 expression

Many factors influence the expression of foreign genes in animal cells, and an understanding of these factors allows transgene expression to be controlled. In this study, it seems that the expression of keratin 8 and 18 IF proteins appears both necessary and sufficient for the formation of filament structures which is also supported by Kulesh et al. [69] . Eukaryotic mRNAs comprise a coding region (which actually encodes the gene product) bracketed by untranslated regions (UTRs) of variable lengths. Both the 5′ and 3′ UTRs can influence gene expression in a number of ways [73] . For example, the 5′ UTR may contain one or more AUG codons upstream of the authentic translational start site, and these are often detrimental to translational initiation. The 3′ UTR may contain regulatory elements that control mRNA stability (e.g. AU-rich sequences that reduce stability have been identified [74] . Furthermore, both the 5′ and 3′ UTRs may be rich in secondary structure, which prevents efficient translation.In animal systems , UTR sequences are generally removed from transgene constructs to maximize expression . Although we constructed keratin 8 and 18 transgenes with parts of UTRs , their expression was not inhibited. According to the principles of regular cellular expression, construction of full cDNA transgenes seems to be related more relevant to overexpression assays. Moreover, expression of a transgene is also dependent on its promoter. Strong promoters and enhancers that function in a wide range of cell types were found in certain viruses, and several of these have been subverted for use in plasmid vectors. The elements most commonly used in mammalian cells are the SV40 early promoter and enhancer [75] , the Rous sarcoma virus long-terminal-repeat promoter and enhancer [76] and the human cytomegalovirus immediate early promoter [77] . Although these function widely, they are not necessarily active in all mammalian cells, e.g. the SV40 promoter functions poorly in the human embryonic kidney line 293 [78] . Particularly, in NIH 3T3 fibroblasts, SV40 promoter is fuctionable [69] as well as CMV promoter. Furthermore, polyadenylation signals (terminators) are required in eukaryotic genes to generate a defined 3′ end to the mRNA. This poly(A) tail is required for the export of mRNA into the cytoplasm, and also increases its stability. In the absence of such a site, the level of recombinant protein produced in transformed cells can fall by as much as 90% [79] . Therefore, the presence of CMV promoter and the Poly(A) site from the SV40 early transcription unit in the adenoviral K8 and K18 vectors along with NIH 3T3 fibroblast transcriptional machinery was necessary for their expression.

4.2. Keratin 18 stabilizes K8 expression

Keratin 8 along with K18 form obligatory heterodimers like the other Type I and II keratin family members[23]. This requirement underlies the pairwise transcriptional regulation of keratin genes. Moreover, it was identified that, single keratin proteins deviating from equimolar type I/type II amounts are rapidly degraded [28.] Since, equimolar ratio is necessary , equal amount of pAd.*K8* and pAd.*K18* were combined. Keratin 8 and 18 expression was dose-dependent on the amount of vector DNA transfected to NIH 3T3 fibroblasts (not shown). Particularly, 1ug *K8* : 1ug K18 transfection was resulted in 1% while 2ug-2ug in 4% K8-K18 expression respectively. K8 and K18 expression in *K8* and *K18* transfected NIH 3T3 cells resulted in filamentous network formation that

surrounds the nucleus and spans through the cytoplasm to the periphery. As we exprected, keratin 8 and 18 protein are co-localized. Furthermore, Krt8 reaches more the cellular periphery than krt18 possibly interacting with cytoplasmic membrane proteins like desmosomes in case of epithelial cells. Among *K8* and *K18* transfected fibroblasts, there were also some cells staining weakly and other staining more strongly that could be explained by differential intracellular amount of *K8* and *K18* DNA vector. Additionally, although the possibility of equimolar ration per cell in culture is not known , we observed that the case of perinuclear aggregates inside *K8* and *K18* transfected NIH cells was minimal.

4.3. Single K18 is degraded rapidly

Although keratin 18 is normally restricted in expression in adult animals to simple epithelium, Kulesh et al. identified that transfection of the isolated K18 gene into fibroblasts resulted in the expression of both K18 mRNA and K18 protein without induction of the endogenous homologous keratin genes [69] . Since immunofluorescent staining of *K18* transfected NIH 3T3 cells didn't detect keratin 18 filaments , it seems that keratin 18 is rapidly degraded in mouse fibroblasts. These results combined with those for K18 and Endo B (K18 mouse homolog) expression in somatic cell hybrids and chromatin and methylation studies of Endo B , suggest that expression of K18 is not restricted in fibroblasts because of the absence of transcriptional regulators but rather that K18 expression may be modulated either by an additional post-translational modification or an epigenetic inactivation mechanism. Furthermore , as it is generally known , gene expression may be regulated by several protein factors but in this study we observed that keratin 18 expression didn't induce the expression of its heteropolymeric partner , keratin 8 [69]. Futhermore, K18 is rapidly degraded in fibroblasts when K8 is absent and it seems that fibroblasts have the necessary machinery to degrade rapidly K18 without the formation of K18 aggreagates. Therefore, K18 protein stability and filament formation depends on the presence of K8.

4.4. Single K8 is degraded slowly

On the other hand, immunofluorescent staining of *K8* transfected NIH 3T3 fibroblasts revealed that K8 didn't induced the expression of endogenous *K18* gene. In contrast to *K18* transfected fibroblasts, expression of *K8* cDNA in fibroblasts resulted in formation of cytoplasmic punctuations which resemble as aggregates that were mainly located perinuclearly. Kulesh et al. identified that K8 appeared relatively stable in NIH 3T3 fibroblasts when expressed alone because it was not degraded rapidly [69] . This event was not related with an expression of keratin filament partner since K8 expression neither induced expression of K18 nor another keratin filament [69] . K8 aggregation may reflect a dysfuction of proteolytic machinery in *K8* transfected fibroblasts. Both K8 and K18 have candidate PEST signal sequences, which are postulated to be responsible for the degradation of various intracellular proteins [80] . However, these sequences are located in the head and tail domains of K8 and near the center of K18. Some PEST proteins may be shielded from proteolytic degradation by complexing with a second protein [81] . The interaction of K8 and K18 or the interaction of higher-order intermediates of IF formation may similarly safeguard these keratins from degradation. Moreover, in several *K8*-transfected fibroblasts it was detected K8 in the form of nonfilamentous deposits and therefore K8 aggregates may reflect precipitated K8 form [69] .

4.5. Keratin 8/18 IF network

According to these results and the work of Kulesh et al. , acidic and basic keratins are stabilized when expressed together.Moreover, the induction of a type I keratin by the expression of a type II keratin in fibroblasts as reported by other investigators [82] appears not to be a general mechanism for all keratins. Additionally, although Kulesh et al. used human *K8* and *K18* cDNA in transfection assays , murine homologes seem to obey the same principles in murine fibroblasts. K18 expression is stabilized when K8 is present and keratin intermediate network is created.NIH 3T3 fibroblasts do have the necessary protein machinery to construct another IFs such as keratins. No synthesis of a complementary , endogenous keratin is detected in NIH 3T3 fibroblasts expressing only K8 or K18. Generally, according to the related studies, there is the suggestion that tight coordinate regulation of the mRNA levels of the two keratin proteins may not be necessary to ensure the equal amounts of the proteins commonly found in the cytoskeletons of epithelial cells. However, construction of a single vector expressing simultaneously *Krt8* and *Krt18* transgenes may be useful. Simultaneous transfection with different transgenes may result in contamination by single-transfected cells. The use of a multicistronic expression cassette such as internal ribosomal entry site (IRES) or foot-and-mouth disease virus (FMDV) 2A segment would enable the co-expression of multiple genes. Although equivalent expression of multiple genes is achieved with 2A segment, addition of amino acids at the end of the 1st and at the beginning of the 2nd transgene may alter the regulation and protein interaction in case of keratins[83]. In contrast to 2A segment, IRES does not alter protein sequence of the transgenes but its primary disadvantage is poor downstream gene translatio relative to the upstream gene[84]. Since keratin 18 is degraded rapidly and its overexpression attenuates Mallory Body formation which are rich in keratin 8 aggregates[54], construction of the following K18-IRES-K8 construct would be interesting to study. Moreover, keratin 8/18 IF network on fibroblasts seem to play a mechanical role in cellular integrity but whether it performs another possible functions according to its properties in different tissues should be under investigation.

4.6. Keratin network parallel to vimentin

Cell architecture is largely based on the interaction of cytoskeletal proteins, which include intermediate filaments (IF), microfilaments, microtubules, as well as their type-specific membrane-attachment structures and associated proteins.Recent studies support that an ectopic IF network may be expressed without interfering with the native cytoskeleton and may rescue a defective native IF network. Ectopic expression of desmin led to the formation of an additional, keratin-independent IF cytoskeleton and did not interfere with the keratin-desmosome interaction as well as with the normal epidermal architecture and the program of terminal differentiation [85] . Another study supported functional improvement of mutant keratin cells on addition of desmin [86] . In our study, immunofluorescent staining revealed that either keratin 8 or keratin 18 expression or both didn't affect vimentin expression and its network. There are studies that support coexpression of simple epithelial keratins and vimentin as for example in human mesothelium or in in the human eye corneal endothelium under pathological conditions, [87,88] . Furthermore, Krt8 reaches more the cellular periphery than vimentin possibly interacting with cytoplasmic membrane proteins like desmosomes in case of epithelial cells. When both IF systems are present in the same cell, keratins have been reported to preferentially interact with desmosomes [89] . The globular end domains of keratin and vimentin IF are significantly different [90] ; these differences may be responsible for differential interactions with desmoplakin [91] .Therefore, since keratin 8 and 18 IF network didn't interact with the native IF network of fibroblasts and it seems that the keratin network retains its natural characteristics. Expression of an ectopic IF network may attenuate cell damage by

mechanical or regulatory functions upon stress.

4.7. Keratin 8/18 IF network and potential cardioprotection

As described in the introduction, keratin 8/18 network attenuates TNFa mediated apoptosis, regulates mitochondrial function and retains cellular integrity that may provide protection to cardiomyocytes upon stress and especially, when native desmin IF network is impaired. Therefore, keratin 8/18 IF network expression may be used as cardioprotective agent. Recently, Panagopoulou et al. identified that desmin is a substrate since TNFa-dependent caspase activation induces desmin cleavage in aMHCsTNFa mice.This cleavage provokes intercalated disk re-organization and formation of desmin aggregates in cardiomyocytes [20] . Among K8 and K18 , K18 has a conserved caspase cleavage motif as desmin (VEVD) [33] . However, keratin 8-18 filament network is not impaired in TNFa desmin-/- mice and this event may reflect that post-translational K18 modifications may inhibit cleavage. Keratin hyperphosphorylation occurs early-on upon an apoptotic signal and provides significant protection (but not prevention) from caspase-mediated degradation, as demonstrated *in vitro* for K18 [92] . Post-translational modifications in a stressed myocardium may regulate the function of keratin IF network. Recently, K18 glycosylation was associated with protection in epithelial injury by promoting the phosphorylation and activation of cell-survival kinases [93] .

K8/18 ectopic expression may regulate TNFR2 signaling pathway in cardiomyocytes .Recently, Yoshihiro Higuchi et al. identified that TNFR2 role is crucial in TNFa-mediated heart failure in transgenic mice since ablation of the TNFR2 gene exacerbates heart failure and reduces survival. Consequently, signaling via TNFR2 may play a cardioprotective role in the pathogenesis of cytokine-mediated heart failure [94]. A scientific group identified that K8/18 deficiency is associated with increased targeting (distribution) of Fas ligand to the plasma membrane from Golgi apparatus as well as related with enhanced Fas-mediated susceptibility to apoptosis [93] . Furthermore, K8 and K18 both bind to the cytoplasmic domain of TNF receptor type 2 (TNFR2) and moderate the effects of TNF. K8/18 may regulate similarly TNFR2 distribution on plasma membrane either stabilizing it or reducing its recycling. Otherwise, K8/18 ectopic expression may regulate TRAF2 (degradation) in TNFR2 signaling pathway. Particularly, K8/18 may protect from apoptosis and against stress possibly by functioning as a phosphate “sponge” for stress-activated kinases [44] .As a consequence, K8/18 ectopic expression may modulate TRAF degradation, regulating either phosphorylation pattern of TNFR2 or degradation by the proteasome and lysosome.

K8/18 ectopic expression in cardiomyocytes may attenuate TNFa-induced apoptosis modulating TNFR1 signaling pathway. Inada et al. identified that K8/18 attenuate the TNFa-induced cytotoxity through association with TRADD [51] . K18 could affect TNF-induced death signaling in a step upstream of caspase- 8 activation, which strongly supports that K18 sequesters TRADD and inhibits the recruitment of TRADD to activated TNFR1, leading to the inhibition of DISC formation (TRADD–FADD–caspase-8) and caspase- 8 activation.

Therefore, studing the keratin 8/18 intermediate filament network in the failing heart may reveal their potential cardioprotective role. Cardiac cytoskeleton is involved in the pathogenesis of heart failure. Intermediate filaments , a member of cytoskeleton maintains the mechanical cellular integrity and the normal homeostasis of regulatory pathways inside the cell. Cardiac muscle specific IF, desmin , acts particularly, as a mechanochemical coordinator in cardiomyocyte. In failing heart , desmin cytoskeleton is impaired. Expression of compensatory IF proteins such as keratins 8/18 acting as stress proteins may attenuate native desmin network dysfunction. Therefore, construction of adenoviral particles that express K8 and K18, infection of desmin-/- cardiomyocytes and investigation of their potential anti-apoptotic effect are promising events. Adenovirus-mediated *K8* and *K18* gene delivery may act as a gene therapy in heart failure.

References

1. Dickstein, K. *et al.* ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the diagnosis and treatment of acute and chronic heart failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). *Eur. J. Heart Fail.* **10,** 933–989 (2008).
2. Mosterd, A. & Hoes, A. W. Clinical epidemiology of heart failure. *Heart Br. Card. Soc.* **93,** 1137–1146 (2007).
3. Ho, K. K., Anderson, K. M., Kannel, W. B., Grossman, W. & Levy, D. Survival after the onset of congestive heart failure in Framingham Heart Study subjects. *Circulation* **88,** 107–115 (1993).
4. Mann, D. L. & Bristow, M. R. Mechanisms and models in heart failure: the biomechanical model and beyond. *Circulation* **111,** 2837–2849 (2005).
5. Chen, W. & Frangogiannis, N. G. The role of inflammatory and fibrogenic pathways in heart failure associated with aging. *Heart Fail. Rev.* **15,** 415–422 (2010).
6. McKinsey, T. A. & Kass, D. A. Small-molecule therapies for cardiac hypertrophy: moving beneath the cell surface. *Nat. Rev. Drug Discov.* **6,** 617–635 (2007).
7. Lazarides E & Capetanaki Y. The striated muscle cytoskeleton: Expression and assembly in development. *Molecular Biology of Muscle Development* (1986).
8. Capetanaki, Y. & Milner, D. J. Desmin cytoskeleton in muscle integrity and function. *Subcell. Biochem.* **31,** 463–495 (1998).
9. Capetanaki, Y. Desmin cytoskeleton in healthy and failing heart. *Heart Fail. Rev.* **5,** 203–220 (2000).
10. Eriksson, J. E. *et al.* Introducing intermediate filaments: from discovery to disease. *J. Clin. Invest.* **119,** 1763–1771 (2009).
11. Fuchs, E. & Weber, K. Intermediate filaments: structure, dynamics, function, and disease. *Annu. Rev. Biochem.* **63,** 345–382 (1994).
12. Lazarides, E. & Hubbard, B. D. Immunological characterization of the subunit of the 100 A filaments from muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* **73,** 4344–4348 (1976).
13. Granger, B. L. & Lazarides, E. The existence of an insoluble Z disc scaffold in chicken skeletal muscle. *Cell* **15,** 1253–1268 (1978).
14. Milner, D. J., Weitzer, G., Tran, D., Bradley, A. & Capetanaki, Y. Disruption of muscle architecture and myocardial degeneration in mice lacking desmin. *J. Cell Biol.* **134,** 1255–1270 (1996).
15. Milner, D. J. *et al.* The absence of desmin leads to cardiomyocyte hypertrophy and cardiac dilation with compromised systolic function. *J. Mol. Cell. Cardiol.* **31,** 2063–2076 (1999).
16. Levine, B., Kalman, J., Mayer, L., Fillit, H. M. & Packer, M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N. Engl. J. Med.* **323,** 236–241 (1990).
17. Sivasubramanian, N. *et al.* Left ventricular remodeling in transgenic mice with cardiac restricted overexpression of tumor necrosis factor. *Circulation* **104,** 826–831 (2001).
18. Diwan, A. *et al.* Targeted overexpression of noncleavable and secreted forms of tumor necrosis factor provokes disparate cardiac phenotypes. *Circulation* **109,** 262–268 (2004).
19. Haudek, S. B., Taffet, G. E., Schneider, M. D. & Mann, D. L. TNF provokes cardiomyocyte apoptosis and cardiac remodeling through activation of multiple cell death pathways. *J. Clin. Invest.* **117,** 2692–2701 (2007).
20. Panagopoulou, P. *et al.* Desmin mediates TNF-alpha-induced aggregate formation and intercalated disk reorganization in heart failure. *J. Cell Biol.* **181,** 761–775 (2008).
21. Weisleder, N., Taffet, G. E. & Capetanaki, Y. Bcl-2 overexpression corrects mitochondrial defects and ameliorates inherited desmin null cardiomyopathy. *Proc. Natl. Acad. Sci. U. S. A.*

101, 769–774 (2004).
22. Schweizer, J. *et al.* New consensus nomenclature for mammalian keratins. *J. Cell Biol.* **174,** 169–174 (2006).
23. Bernot, K. M., Coulombe, P. A. & Wong, P. Skin: an ideal model system to study keratin genes and proteins. *Methods Cell Biol.* **78,** 453–487 (2004).
24. Hesse, M., Zimek, A., Weber, K. & Magin, T. M. Comprehensive analysis of keratin gene clusters in humans and rodents. *Eur. J. Cell Biol.* **83,** (2004).
25. Moll, R., Franke, W. W., Schiller, D. L., Geiger, B. & Krepler, R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **31,** (1982).
26. Letai, A. & Fuchs, E. The importance of intramolecular ion pairing in intermediate filaments. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 92–96 (1995).
27. Steinert, P. M. & Gullino, M. I. Bovine epidermal keratin filament assembly in vitro. *Biochem. Biophys. Res. Commun.* **70,** 221–227 (1976).
28. Lu, X. & Lane, E. B. Retrovirus-mediated transgenic keratin expression in cultured fibroblasts: specific domain functions in keratin stabilization and filament formation. *Cell* **62,** 681–696 (1990).
29. Waschke, J. The desmosome and pemphigus. *Histochem. Cell Biol.* **130,** (2008).
30. Langbein, L., Spring, H., Rogers, M. A., Praetzel, S. & Schweizer, J. Hair keratins and hair follicle-specific epithelial keratins. *Methods Cell Biol.* **78,** 413–451 (2004).
31. Langbein, L. & Schweizer, J. Keratins of the human hair follicle. *Int. Rev. Cytol.* **243,** (2005).
32. Smith, E. A. & Fuchs, E. Defining the interactions between intermediate filaments and desmosomes. *J. Cell Biol.* **141,** 1229–1241 (1998).
33. Coulombe, P. A. & Omary, M. B. 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr. Opin. Cell Biol.* **14,** 110–122 (2002).
34. Magin, T. M., Vijayaraj, P. & Leube, R. E. Structural and regulatory functions of keratins. *Exp. Cell Res.* **313,** 2021–2032 (2007).
35. DePianto, D. & Coulombe, P. A. Intermediate filaments and tissue repair. *Exp. Cell Res.* **301,** (2004).
36. Toivola, D. M., Strnad, P., Habtezion, A. & Omary, M. B. Intermediate filaments take the heat as stress proteins. *Trends Cell Biol.* **20,** (2010).
37. Oshima, R. G., Baribault, H. & Caulin, C. Oncogenic regulation and function of keratins 8 and 18. *Cancer Metastasis Rev.* **15,** 445–471 (1996).
38. Savtchenko, E. S., Schiff, T. A., Jiang, C. K., Freedberg, I. M. & Blumenberg, M. Embryonic expression of the human 40-kD keratin: evidence from a processed pseudogene sequence. *Am. J. Hum. Genet.* **43,** 630–637 (1988).
39. Huitfeldt, H. S. & Brandtzaeg, P. Various keratin antibodies produce immunohistochemical staining of human myocardium and myometrium. *Histochemistry* **83,** 381–389 (1985).
40. Moll, R. *et al.* Immunocytochemical analysis of Ewing's tumors. Patterns of expression of intermediate filaments and desmosomal proteins indicate cell type heterogeneity and pluripotential differentiation. *Am. J. Pathol.* **127,** (1987).
41. Jaquemar, D. *et al.* Keratin 8 protection of placental barrier function. *J. Cell Biol.* **161,** 749–756 (2003).
42. Caulin, C., Ware, C. F., Magin, T. M. & Oshima, R. G. Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. *J. Cell Biol.* **149,** (2000).
43. Zatloukal, K. *et al.* Cytokeratin 8 protects from hepatotoxicity, and its ratio to cytokeratin 18 determines the ability of hepatocytes to form Mallory bodies. *Am. J. Pathol.* **156,** 1263–1274 (2000).
44. Ku, N.-O. & Omary, M. B. A disease- and phosphorylation-related nonmechanical function

for keratin 8. *J. Cell Biol.* **174,** 115–125 (2006).
45. Toivola, D. M. *et al.* Disturbances in hepatic cell-cycle regulation in mice with assembly-deficient keratins 8/18. *Hepatol. Baltim. Md* **34,** 1174–1183 (2001).
46. Ku, N.-O., Michie, S., Resurreccion, E. Z., Broome, R. L. & Omary, M. B. Keratin binding to 14-3-3 proteins modulates keratin filaments and hepatocyte mitotic progression. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 4373–4378 (2002).
47. Galarneau, L., Loranger, A., Gilbert, S. & Marceau, N. Keratins modulate hepatic cell adhesion, size and G1/S transition. *Exp. Cell Res.* **313,** 179–194 (2007).
48. Zatloukal, K. *et al.* The keratin cytoskeleton in liver diseases. *J. Pathol.* **204,** 367–376 (2004).
49. Owens, D. W. & Lane, E. B. Keratin mutations and intestinal pathology. *J. Pathol.* **204,** 377–385 (2004).
50. Zatloukal, K. *et al.* From Mallory to Mallory-Denk bodies: what, how and why? *Exp. Cell Res.* **313,** 2033–2049 (2007).
51. Inada, H. *et al.* Keratin attenuates tumor necrosis factor-induced cytotoxicity through association with TRADD. *J. Cell Biol.* **155,** 415–426 (2001).
52. Gilbert, S., Loranger, A. & Marceau, N. Keratins modulate c-Flip/extracellular signal-regulated kinase 1 and 2 antiapoptotic signaling in simple epithelial cells. *Mol. Cell. Biol.* **24,** 7072–7081 (2004).
53. Tao, G.-Z. *et al.* Keratins modulate the shape and function of hepatocyte mitochondria: a mechanism for protection from apoptosis. *J. Cell Sci.* **122,** 3851–3855 (2009).
54. Harada, M., Strnad, P., Resurreccion, E. Z., Ku, N.-O. & Omary, M. B. Keratin 18 overexpression but not phosphorylation or filament organization blocks mouse Mallory body formation. *Hepatol. Baltim. Md* **45,** (2007).
55. LEDERBERG, S. & MESELSON, M. DEGRADATION OF NON-REPLICATING BACTERIOPHAGE DNA IN NON-ACCEPTING CELLS. *J. Mol. Biol.* **8,** 623–628 (1964).
56. Gumport, R. I. & Lehman, I. R. Structure of the DNA ligase-adenylate intermediate: lysine (epsilon-amino)-linked adenosine monophosphoramidate. *Proc. Natl. Acad. Sci. U. S. A.* **68,** 2559–2563 (1971).
57. Sambrook, J. & Russell, D. W. Cloning PCR products by addition of restriction sites to the termini of amplified DNA. *CSH Protoc.* **2006,** (2006).
58. Mead, D. A., Pey, N. K., Herrnstadt, C., Marcil, R. A. & Smith, L. M. A universal method for the direct cloning of PCR amplified nucleic acid. *Biotechnol. Nat. Publ. Co.* **9,** 657–663 (1991).
59. Sambrook, J. & Russell, D. W. Cloning PCR products into T vectors. *CSH Protoc.* **2006,** (2006).
60. Langley, K. E. & Zabin, I. beta-Galactosidase alpha complementation: properties of the complemented enzyme and mechanism of the complementation reaction. *Biochemistry (Mosc.)* **15,** 4866–4875 (1976).
61. Boussif, O. *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7297–7301 (1995).
62. Godbey, W. T., Wu, K. K. & Mikos, A. G. Poly(ethylenimine)-mediated gene delivery affects endothelial cell function and viability. *Biomaterials* **22,** 471–480 (2001).
63. Lungwitz, U., Breunig, M., Blunk, T. & Gopferich, A. Polyethylenimine-based non-viral gene delivery systems. *Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Pharm. Verfahrenstechnik EV* **60,** 247–266 (2005).
64. Hitt, M. M., Addison, C. L. & Graham, F. L. Human adenovirus vectors for gene transfer into mammalian cells. *Adv. Pharmacol. San Diego Calif* **40,** (1997).
65. Hillgenberg, M., Hofmann, C., Stadler, H. & Loser, P. High-efficiency system for the construction of adenovirus vectors and its application to the generation of representative

adenovirus-based cDNA expression libraries. *J. Virol.* **80,** 5435–5450 (2006).
66. Ali, M., Lemoine, N. R. & Ring, C. J. The use of DNA viruses as vectors for gene therapy. *Gene Ther.* **1,** 367–384 (1994).
67. Hatanaka, K. *et al.* A simple and efficient method for constructing an adenoviral cDNA expression library. *Mol. Ther. J. Am. Soc. Gene Ther.* **8,** 158–166 (2003).
68. Shuman, S. Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase. *J. Biol. Chem.* **269,** 32678–32684 (1994).
69. Kulesh, D. A., Cecena, G., Darmon, Y. M., Vasseur, M. & Oshima, R. G. Posttranslational regulation of keratins: degradation of mouse and human keratins 18 and 8. *Mol. Cell. Biol.* **9,** 1553–1565 (1989).
70. Wang, D. S., Miller, R., Shaw, R. & Shaw, G. The pleckstrin homology domain of human beta I sigma II spectrin is targeted to the plasma membrane in vivo. *Biochem. Biophys. Res. Commun.* **225,** 420–426 (1996).
71. Franke, W. W., Schmid, E., Osborn, M. & Weber, K. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. U. S. A.* **75,** 5034–5038 (1978).
72. Coulombe, P. A., Ma, L., Yamada, S. & Wawersik, M. Intermediate filaments at a glance. *J. Cell Sci.* **114,** 4345–4347 (2001).
73. Kozak, M. Initiation of translation in prokaryotes and eukaryotes. *Gene* **234,** (1999).
74. Shaw, G. & Kamen, R. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46,** 659–667 (1986).
75. Mulligan, R. C. & Berg, P. Factors governing the expression of a bacterial gene in mammalian cells. *Mol. Cell. Biol.* **1,** 449–459 (1981).
76. Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I. & Howard, B. H. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. U. S. A.* **79,** 6777–6781 (1982).
77. Boshart, M. *et al.* A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41,** 521–530 (1985).
78. Gorman, C. M., Gies, D., McCray, G. & Huang, M. The human cytomegalovirus major immediate early promoter can be trans-activated by adenovirus early proteins. *Virology* **171,** 377–385 (1989).
79. Kaufman, R. J. Use of recombinant DNA technology for engineering mammalian cells to produce proteins. *Bioprocess Technol.* **10,** (1990).
80. Rogers, S., Wells, R. & Rechsteiner, M. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234,** 364–368 (1986).
81. Gronostajski, R. M., Goldberg, A. L. & Pardee, A. B. Energy requirement for degradation of tumor-associated protein p53. *Mol. Cell. Biol.* **4,** 442–448 (1984).
82. Giudice, G. J. & Fuchs, E. The transfection of epidermal keratin genes into fibroblasts and simple epithelial cells: evidence for inducing a type I keratin by a type II gene. *Cell* **48,** 453–463 (1987).
83. De Felipe, P. Skipping the co-expression problem: the new 2A 'CHYSEL' technology. *Genet. Vaccines Ther.* **2,** (2004).
84. Vagner, S., Galy, B. & Pyronnet, S. Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. *EMBO Rep.* **2,** 893–898 (2001).
85. Kirfel, J., Peters, B., Grund, C., Reifenberg, K. & Magin, T. M. Ectopic expression of desmin in the epidermis of transgenic mice permits development of a normal epidermis. *Differ. Res. Biol. Divers.* **70,** (2002).
86. D'Alessandro, M. *et al.* Functional improvement of mutant keratin cells on addition of desmin: an alternative approach to gene therapy for dominant diseases. *Gene Ther.* **11,** 1290–

1295 (2004).
87. LaRocca, P. J. & Rheinwald, J. G. Coexpression of simple epithelial keratins and vimentin by human mesothelium and mesothelioma in vivo and in culture. *Cancer Res.* **44,** 2991–2999 (1984).
88. Stock, E. L., Roth, S. I. & Morimoto, D. Desquamating endotheliopathy. An incipient iridocorneal endothelial syndrome? *Arch. Ophthalmol.* **105,** 1378–1381 (1987).
89. Kartenbeck, J., Schwechheimer, K., Moll, R. & Franke, W. W. Attachment of vimentin filaments to desmosomal plaques in human meningiomal cells and arachnoidal tissue. *J. Cell Biol.* **98,** 1072–1081 (1984).
90. Steinert, P. M. & Roop, D. R. Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem.* **57,** (1988).
91. Stappenbeck, T. S. *et al.* Functional analysis of desmoplakin domains: specification of the interaction with keratin versus vimentin intermediate filament networks. *J. Cell Biol.* **123,** (1993).
92. Ku, N. O. & Omary, M. B. Effect of mutation and phosphorylation of type I keratins on their caspase-mediated degradation. *J. Biol. Chem.* **276,** 26792–26798 (2001).
93. Ku, N.-O., Toivola, D. M., Strnad, P. & Omary, M. B. Cytoskeletal keratin glycosylation protects epithelial tissue from injury. *Nat. Cell Biol.* **12,** 876–885 (2010).
94. Higuchi, Y. *et al.* Tumor necrosis factor receptors 1 and 2 differentially regulate survival, cardiac dysfunction, and remodeling in transgenic mice with tumor necrosis factor-alpha-induced cardiomyopathy. *Circulation* **109,** 1892–1897 (2004).
95. Gilbert, S., Loranger, A., Daigle, N. & Marceau, N. Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis. The protection occurs through a receptor-targeting modulation. *J. Cell Biol.* **154,** 763–773 (2001).