A ROLE FOR HIGH-RISK HPV TYPE 16 E6 AND E7 ONCOPROTEINS IN COLORECTERAL CARCINOGENESIS

Riccardo Pietro Ricciardi

Department of Experimental Medicine
McGill University
Montreal, Quebec, Canada
October 2007

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies of McGill University in partial fulfillment of the requirements of the degree of Master of Science.

Your file Votre référence
ISBN: 978-0-494-51329-3
Our file Notre référence
ISBN: 978-0-494-51329-3

Acknowledgements

These days were counted, but cherished.

First and foremost, I must thank my parents who have supported me, in every sense of the word, my entire life. At times I wonder if I will ever have their patience. They have shown me infinite amounts of unconditional love that I hope to reciprocate. With this degree I feel slightly independent but not the least bit detached. I must truly thank them for making everything possible. Believe it or not, my brothers as well: Nicola and Pietro. I love you all very much. (Please do not bring this acknowledgment up in any discussion, ever. I thank you for that too).

I would like to thank Dr. Ala Eddin Al Moustafa and Dr. Tarek Bismar for giving me the opportunity to study with them. They offered me a wonderful learning experience and provided a large stepping stone for my future. Thank you very much and good luck with future endeavors.

I will never forget anyone from the 4th floor, Pavillion-E. Beginning with those from E-424: Mike and Tayma. I could always count on one for coffee and the other for a different beverage. The Pollakians: Carolyne and Mattei, who were cleverly conned into a PhD. The Gallipeans: Patrick and Moutih, who taught us that people from worlds apart can unite as one - so long as they ignore each other. The Barranquillans: Orlando and Julio, who taught us how to equate professionalism with a good time. For all those with Dr. Miller, fearing for my life I'll avoid saying too much. A special thank you to Anna, Susan, Jess and, of course, Andy a.k.a Luca Petrucelli. You've been my partner in crime for the last 5 years and now that we're apart I graduate ahead of time... Nonetheless, I couldn't, and wouldn't, have done it without you. From the movies before aced exams (1 that is) to the hi-fi stores after them, neither of us we'll forget those days. I'll see you soon and under more profitable circumstances, one way or another! Speaking of profit, I could never forget Emy and Khalil who, along with Andy, helped me produce a multi-million dollar idea for which we are yet to receive a penny. Alex, who sabotaged the competition, thank you.

A special thanks goes to everyone from room E-421: Dr. Basik, Min, Adriana, Maggie, Saima, Amelie, Sophie, Aline and Cristiano – whose name is in a smaller font for no apparent reason. I can't possibly forget my historical/political and religious discussions with a double steak and cheese – Thanks Abdel. Thank you all for putting up with me every single day. You made my Master's possible with helpful advice and great company. There wasn't a day that went by without at least one good laugh! I hope every work environment I become a part of is similar to what we made; but I doubt it.

And now for the brothers and sisters. A special thank you to Amal Kassab. Always helpful, always cheerful and always a little reserved!

I can never forget my dear friend Reyhan "Le Citron" El-Kares; much better known as, Le Rey. Je vous remercie pour toute ce que tu m'as offris, come collègue, amis et plus important, grand frère.

Finally, Amber Yasmeen Baluch. I don't know where to begin. If I could re-write my Master's I would discuss the blessings of a great sister – in the presence and absence of high-risk HPV E6 and E7 oncoprotein transduction. I must thank you *forever* for all you have done for me. You are a mentor, a sister and a very best friend. You are most certainly my Baji. I am happy to be part of your family forever; Wassim's as well! Thank you indefinitely.

~ Riccardo Pietro Ricciardi

Table of contents

Results and Discussion:

Summary and Conclusion:

Bibliography

LIST OF FIGURES AND TABLES

Abstract:

Human papillomavirus (HPV) infections play a crucial role in human carcinogenesis. Greater than 96% of all cervical carcinomas are positive for high-risk HPV infections; especially types 16 and 18. High-risk HPV onco-proteins, E6 and E7, are consistently expressed in such cancers and function by inactivating p53 and pRb tumor suppressors, respectively. The presence of high-risk HPVs is also correlated with anogenital cancers. In this study, we examined the effect of high-risk HPV type 16 E6 and E7 oncoproteins in two normal human colorectal epithelial cell lines, NCE1 and NCE5. We report that the expression of E6/E7 proteins, alone, induced cellular transformation of both cell lines; consequently, NCE1-E6/E7 and NCE5-E6/E7 form colonies in soft agar with respect to their wild type cells. This is accompanied by cell cycle deregulation, as is demonstrated by the over-expression of cyclin dependant kinases (cdks) and their respective cyclins. Furthermore, we demonstrate that E6/E7 oncoprotein transduction induces migration of colorectal epithelial cells. More still, well analyzed Id gene expression, a family member of the helix-loop-helix (HLH) transcription factors involved in the regulation of cell invasion and metastasis of human cancer cells. In parallel, using tissue microarray analysis we found that the four members of the Id protein family are correlated with the presence of HPV type 16 and 18 in human colon cancer tissues. Our data suggests that high-risk HPV infections are sufficient to induce cellular transformation of normal human colorectal cells, *in vitro*. Furthermore, the correlation with the Id family of proteins may present a novel set of markers associated with HPV induced colorectal carcinogenesis. Our results may suggest a new approach to detect and prevent colorectal cancer.

Résumé :

Les infections au virus du papillome humain (VPH) jouent un rôle clé dans la carcinogenèse chez l'humain. Plus de 96% des cancers du col de l'utérus sont associés à une infection par un des types de VPH à risque élevé, en particulier les types 16 et 18. Les oncoprotéines E6 et E7 des VPH à risque élevé sont exprimées de façon constitutive dans ces cancers et agissent en inactivant respectivement les supresseurs de tumeurs p53 et pRb. La présence des VPH à risque élevé corrèle aussi avec les autres cancers anogénitaux. Dans cette étude, nous évaluons l'effet des oncoprotéines E6 et E7 dans deux lignées colorectales humaines normales, NCE1 et NCE5. Nous démontrons que l'expression des protéines E6/E7 à elles seules induisent une transformation cellulaire dans les deux lignées. Conséquemment, NCE1-E6/E7 et NCE5-E6/E7 forment des colonies dans la gélose d'agar molle lorsque comparées aux cellules sauvages. Cet effet est accompagné d'une dérégulation du cycle cellulaire, tel que démontré par la surexpression des kinases cycline-dépendantes et leurs cyclines correspondantes. De plus, nous démontrons que la transduction de signaux par les oncoprotéines E6/E7 induit la migration des cellules colorectales épithéliales. Également, nous avons analysé l'expression du gène Id, un membre de la famille des facteurs de transcription de type hélice-boucle-hélice impliqué dans la régulation de l'invasion cellulaire et les métastases des cellules cancéreuses humaines. En parallèle, en utilisant un microarray de tissu, nous avons observé que les quatre membres de la famille des protéines Id corrèlent avec la présence des VPH de type 16 et 18 dans les tissus humains de cancers du côlon. Nos résultats suggèrent que les infections au VPH à risque élevé sont suffisantes pour l'induction de la transformation des cellules colorectales humaines *in vitro*. Également, la corrélation avec les protéines de la famille Id pourrait représenter un nouvel ensemble de marquers associés avec la carcinogenèse colorectale. Nos résultats suggèrent une nouvelle approche pour la détection et la prévention du cancer colorectal.

Introduction

What is the Human Papillomavirus?

The human papillomavirus (HPV) is one of the most common family of viruses in the world today; over 120 different types of HPV have been characterized. Papillomaviruses are a genus of encapsulated, double-stranded DNA-based viruses from the Papovavirus (Papillomavirus and Polyoamavirus) family. With virions of approximately 55nm (cancerweb.ncl.ac.uk), Papillomaviruses are known to infect a variety of mammals including dogs, cattle, chimpanzees and humans. They infect cutaneous and mucosal epithelial tissues. These include surfaces of the skin, lining of the mouth, tongue, throat, tonsils, vagina, penis, cervix, and anus. HPV is also the world's most common sexually transmitted infection. A group of about 30-40 HPV's infect the anogenital region. Such HPVs fall into two categories: low-risk and high-risk (arhp.org, Tommasino et al., 1995).

There exist a far greater number of low-risk HPVs then high-risk HPVs. The most common low-risk papillomavirus infection outcomes are asymptomatic clearance or cutaneous papillomas (for which the virus family is named after). Less common are the genital warts caused by HPV types 6 and 11. These do not harm the patient; however, they are distressing and highly contagious. The treatment includes several topical agents to eradicate the lesions, cryosurgery, laser therapy or surgical removal. Safe sex practice helps decrease the risk of infection.

High-risk HPV infection may also produce papillomas; however, they are fundamentally different from low-risk HPV's via their association with cancer. High-risk HPV's are important etiological factors in the development of cervical cancer as they are prevalent in approximately 99% of cervical carcinomas - higher than the association

between smoking and lung cancer (Burd et al., 2003). Furthermore, recent studies have demonstrated that high-risk HPVs may be important risk factors in other human cancers. More specifically, studies have demonstrated an 80% correlation with colorectal cancer (Damin et al., 2007), a 35% association with head and neck cancers (Venuti et al., 2004) (oropharyngeal and esophageal) and a similar correlation with breast cancers (Damin et al., 2004). Moreover, the presence of high-risk HPV serves as a prognostic factor in early-stage cervical, head/neck, and colorectal cancers; in addition, they have been associated with vascular invasion, lymph node metastases and tumor size (Begum et al., 2003, Umudum et al., 2007, Varnai et al., 2006).

From Transmission to Cancer

A unifying theme of the entire papovavirus group is that infection can be cleared or contained by a healthy immune response. Thus, HPVs must go to extraordinary lengths to hide from immune surveillance. In doing so, the host may be vulnerable to repeat infections – a fundamental requirement in the development of HPV associated cancers.

To begin, the viral genome exists in a circular episomal configuration with three core regions: the Long Control Region (LCR, a.k.a Upstream Regulatory Region, a.k.a Non-coding Region), the early region (E), and the late region (L) (arhp.org). The long control region contains transcriptional and replication regulatory elements. It controls viral replication and gene expression of some of the "early" genes. The late genes are the genes of greatest genetic conservation. There exist 2 late genes: L1 and L2. The L1 gene produces 72 pentamers which assemble into the major capsid protein. L2 produces the

minor capsid protein that is required for the encapsidation of the viral genome. The early region contains 6 different genes that give the virus the utilities it needs to setup and survive. They include: *E1, E2, E4, E5, E6 and E7*. *E1* is important for viral DNA replication. *E2* is a cofactor for viral DNA replication and also plays a role in transcriptional regulation. *E4* is involved in the maturation process of the virus, ultimately releasing it from the host cell. *E5* mimics mitogenic signalling from the epidermal growth factor receptor. *E6* binds to and targets p53 for degredation whereas *E7* binds and inhibits the function of Retinoblastoma, pRb. It should be noted that E6 and E7 are the prevalent onco-proteins associated with high-risk HPV infections. They are intricately associated with cervical cancers, as well as other forms of human cancer (Gillison et al., 2000, Burd et al., 2003). Their mechanism of action is well characterized and will be discussed briefly.

Initial HPV infection can result from sexual or cutaneous contact (Burd et al., 2003). HPVs enter their hosts through sites of microtrauma in the skin, such as scars on hands and feet. With regard to the anogenital region, the vaginal introitus and perineum are highly susceptible (Shroyer et al., 1992). Other areas with increased vulnerability for HPV infection are the epithelial layers of immature metaplasia found in the transformation zone of the cervix and anal verge (arhp.org). Once the virus has entered the host it must reach basal squamous cells in the basal layer of the epithelium (Burd et al., 2003). To enter the host cell, the virus must first shed its protein capsid and inject naked, viral DNA into the cytoplasm. Once the viral DNA is exposed, it travels to the host cell's nucleus. The infected cell remains a basal cell - competent to replicate and not

yet differentiated. At this point, the virus begins a pattern of viral gene expression that intimately correlates its life cycle with the differentiation of the host cell.

The episomal viral DNA begins its life cycle by transcribing the three essential early genes, *E5, E6* and *E7* which work in tandem (Stanley et al., 2006). Firstly, E5 lodges in the host cell membrane and mimics mitogenic signaling from the epidermal growth factor receptor. This allows the virus to use the host cell's DNA replication machinery. E6 then sequesters p53. In a normal cell, p53 is a "checkpoint" molecule — a gatekeeper that halts the cell cycle if the cell's genome is damaged. In cells with damaged DNA, p53 accumulates to high levels and can induce a pause during either of the resting phases of the replication cycle (G1 and G2) (Voet et al., 2005). A checkpoint arrest gives the cell's repair machinery an opportunity to correct the problem before the cell embarks upon DNA replication (S phase) or division (M phase). If the problem cannot be repaired, p53 sets off a chain of biochemical events that ends in apoptosis. In the case of HPV infection, the invading virus must inactivate p53, since the gatekeeper would block the cell division on which the virus depends (Vermeulen et al., 2002). The E6 proteins of high-risk strains handle p53 with particular efficiency. High-risk E6 not only sequesters p53, but actually labels it for destruction by the proteasome (Tommasino et al., 1995). The host is thus tricked into destroying its own tumor-suppressing machinery so that the virus can have free reign over the cell cycle. Meanwhile, protein E7 sequesters pRb, the second major tumor suppressor protein in mammalian cells. pRb is also a gatekeeper protein. It binds the replication-initiating transcription factor E2F-1 and DP-1, holding them imprisoned until the cell provides specific signals indicating that the time has come to divide (Tommasino et al., 1995, arhp.org). Once released, E2F-1

and DP-1 enter the nucleus and activate the expression of many of the DNA replication genes. Thus, the combined effect of E5, E6 and E7 allows both the virus and the host cell to replicate uncontrollably after which E1, E2 and E4 will begin their roles followed by the late gene expression during the final phases of the viral life cycle.

Interestingly, however, in the vast majority of HPV infections the simultaneous inactivation of p53 and pRb tumor suppressor proteins does not have dangerous consequences for the host (arhp.org). In fact, one may remain asymptomatic throughout infection with either low or high-risk HPV and completely rid themselves of the virus within two years (arhp.org). This is due in part to nature's skin renewal system as well as a healthy immune response.

A deficient immune system raises the concern for high-risk HPV infections. To reiterate, the replication of HPV is tied to the differentiation of the host cell. As basal cells differentiate and move outward to become mature squamous epithelium, any infected cells will also mature and move outward concurrently. HPV directs them not only to divide at an accelerated rate, eventually piling up into a recognizable wart, but also to express an increasing number of HPV genes (Syrjanen et al., 2002). Those cells that reach the endpoint of differentiation (squamous epithelium) are exposed on the skin surface and produce mature HPV virions that can finally stimulate an immunogenic response. The new viruses flake off with dying skin cells and go on to infect new hosts or new sites on the same host. For the majority of patients, a phase of host containment begins following immune recognition of HPV. A successful immune response results in viral control (latency) or clearance. Because the immunogenic HPV virions are not produced until the infected cells are on the bloodless outside surface of the skin, immune

surveillance may miss an HPV infection for years. In such a case where an individual's immune system is not sensitive enough to recognize the viral antigen, one may be susceptible to the consequences of re-infection. Re-infection for an extended period of time allows the rare event of viral integration into the host cell's genome (Greenblat et al., 2005). In doing so, the virus may lose all its functional gene sequences and die. However, if E6 and E7 manage to integrate into the host cell genome then the cell has all the necessary components for uncontrolled proliferation. This is why E6 and E7 are considered the gold-standard oncoproteins involved with HPV induced cancers. Their complementary functions allow them to fully transform an otherwise normal cell. This is the case with Cervical Intraepithelial Neoplasia (CIN), a common malignancy strongly associated with high-risk HPV types 16 and 18.

Much research has been done to address the role of E6/E7 genes in high-risk HPV-associated carcinogenesis. For example, several transgenic mice have been developed expressing E6/E7 of HPV type 16. For example, Herber et al. (1996), Song et al. (1999) and Riley et al. (2003) generated transgenic mice carrying E6 or E7 individually, as well as E6/E7 together (Herber et al., 1996, Song et al., 1999, Riley et al., 2003). The transgenic mice developed skin tumors as well as cervical cancer with chronic estrogen administration. (It should be noted that estrogen is a required co-factor for the development of HPV-related cervical cancer). The E6 and E7 mice developed low-grade cervical dysplasia and high-grade cervical carcinomas, respectively. However, the cervical cancers were larger, more extensive and invasive in the E6/E7 double transgenic mice.

HPV, Cervical Cancer and Colorectal Cancer

In many women, the resulting effect of CIN is cervical cancer. Invasive cervical cancer resulting from HPV integration is a major women's health concern with approximately 500,000 cases diagnosed each year worldwide. HPV infection is a necessary factor in the development of nearly all cases of cervical cancer. Persistent infection with a subset of about 13 high-risk sexually transmitted HPVs, including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 can lead to the development of cervical cancer, or penile cancer in men. Type 16 and 18 account for approximately 70% of cervical cancers as well as the majority of HPV-induced vaginal/vulvar cancers, penile cancers and head and neck cancers (Greenblat et al., 2005, Gillison et al., 2000, Shah et al., 1992, Shroyer et al., 1992). However, the vast majority of high risk HPV infections are transient. It is estimated that roughly 90% of high risk HPV infections are eliminated by healthy systems, as mentioned above. However, 10% of persistent infections develop into low and high grade neoplasias (Burd et al., 2003). Considering that 80% of North American individuals are estimated to contact HPV in their lifetime, it is crucial that proper testing is performed. Unfortunately, no HPV test exists for men. However, a clinical procedure known as a pap-smear test (a.k.a the papanicolaou test) should be performed annually for sexually active women. A pap-smear is a clinical examination that tests for cellular abnormalities induced by high risk HPVs. More specifically, it involves microscopically examining cells of the cervix for pre-malignant and malignant lesions. It is 70% to 80% effective in detecting HPV-caused cellular abnormalities (Wikipedia.org). As such, it is highly effective in reducing the number of cervical

cancers and is said to be one of the most successful screening tests in the history of medicine.

Recently, an even greater achievement in the fight against cervical cancer has been accomplished in the form of a vaccine. In June, 2006, the FDA approved Gardasil – a prophylactic HPV vaccine. In addition, in early 2007 Australia approved Cervarix – another prophylactic HPV vaccine. Each confers resistance to infection from HPV types 16 and 18 greatly reducing the risk of cervical carcinogenesis. Furthermore, Gardasil provides resistance against HPV types 6 and 11 which are responsible for the majority of genital warts. Cervarix, however, offers resistance to high-risk HPV types 31 and 45 which are more prevalent in Australian women. The underlying mechanism behind each vaccine's anti-HPV type 16/18 effect lies in the aforementioned HPV genome. More specifically, the vaccines express recombinant L1 in eukaryotic cells (Gardasil.com). As such, the vaccines allow the formation of virus-like particles with no DNA. This leads to a safe and effective immunogenic response. However, because the vaccines do not protect against all high-risk HPV strains, women are still urged to perform annual pap-smear examinations. To fully eliminate the transmission of high-risk HPV infections studies are being conducted to administer such vaccines to young boys. At present, it is being proposed to all girls aged 9 and up.

Colon cancer is another major threat, especially in North America. In 2007, an estimated 20,800 Canadians will be diagnosed with colorectal cancer and 8,700 will die of it (cancer.ca). On average, 400 Canadians will be diagnosed with colorectal cancer every week and 167 will die of it (cancer.ca). One in 14 men is expected to develop colorectal cancer during their lifetime and one in 28 will die of it (cancer.ca). For

women, one in 16 is expected to develop colon cancer during their lifetime and one in 31 will die of it (cancer.ca). Overall, colorectal cancer is the second leading cause of death from cancer in Canada and is estimated to account for 10% of all cancer-related deaths, worldwide (cancer.ca). Despite remarkable progress in the battle against major cancers, such as breast and cervical, little has been accomplished in the fight against colon cancer in the last 10 years. To add insult to injury, the number of people affected continues to increase. Although many risk factors for the development of colorectal cancer have been identified the molecular mechanisms related to colorectal carcinogenesis remain under investigation.

Sexually transmitted HPVs are associated with a major fraction of anal cancers. Engaging in anal sex with an HPV-infected partner may increase the risk of developing such cancers. As such, many clinical and laboratory studies have investigated the potential correlation between HPV and colorectal cancer. Some authors were able to detect the presence of HPV DNA in colorectal adenocarcinomas by different laboratory techniques; such as PCR and in situ hybridization (Kirgan et al., 1990, McGregor et al., 1993, Cheng et al., 1995, Lee et al., 2001, Perez et al., 2005). Of particular interest was a recent publication by the laboratory of Damin et al. They conducted PCR and nested PCR experiments on 72 primary colorectal cancer patients (32 male, 32 female) with no history of cervical cancer or dysplasia or clinical evidence of previous HPV infection. Furthermore, patients with any sort of familial adenomatous polyposis, hereditary non-polyposis colon cancer, inflammatory bowel disease or immunosuppressive conditions were excluded from the study. The results demonstrated that HPV DNA was found in a staggering 60 out of 72 patients (83.3%) with cancer but in none of the patients without

cancer (Damin et al., 2007). Furthermore, high-risk HPV types 16 and 18 were present in 41/60 (70%) of HPV positive tissues; strikingly similar to cervical cancer data.

Considering our understanding of HPV induced cervical cancer it does not require much imagination to extrapolate the potential role for HPV in colorectal carcinogenesis. Despite the potential implication of high risk HPVs in anal cancers, little, if any, research has been conducted to verify its transformation potential in colorectal cancers. If such a hypothesis is proven then great strides can be made in detection, prevention and prognosis of colorectal cancer.

A brief review of the Cell cycle:

The cell cycle is regulated by a plethora of enzymes in which kinases are of particular importance. Fundamental to the integrity and proper progression of the cell cycle are a family of kinases known as cdks; cyclin-dependant kinases. Cdk's, of which nine exist, are small (30-40kDa) serine/threonine protein kinases that consist of little more than their kinase core (Voet et al., 1995). Of the nine, however, only four are fundamental in human cell cycle regulation: cdk1, cdk2, cdk4 and cdk6 (Vermeulen et al., 2002). Cdk3, 7, 8 and 9 are involved in controlling basal gene transcription by RNA polymerase II, whereas cdk5 has been implicated in controlling nerve cell differentiation. Due to slight conformational differences with regular kinases, cdks require the binding of a regulatory cyclin subunit for their activation (Voet et al., 2005). That is, specific time points throughout the cell cycle require activation of a particular cdk by its respective cyclin for proper progression. In some cases, full activation requires phosphorylation of a threonine residue near the kinase active site. This is the role of cdk7, a.k.a cyclin

dependant kinase activating kinase - CAK. Sixteen cyclins have been identified so far but not all of them are cell cycle related (Vermeulen et al., 2002). Those relevant to cell cycle regulation include cyclins D1, D2, D3, A, B, E and H. Cyclin levels vary depending on the stage of the cell cycle (except for D-type cyclins which are stimulated by growth factors). There are three such stages: Interphase, Mitosis (karyokinesis) and Cytokinesis; together they allow the body to replicate one daughter cell into a second, identical cell (Vermeulen et al., 2002).

Interphase can be subdivided into three distinct phases: G1-phase, S-phase and G2-phase (Voet et al., 1995). Cells in G1 carry out basic cell functions while making the crucial decision to either re-enter or exit the cell cycle. A cell that exits G1 enters G0 and is said to be dormant. That is, it will perform its assigned functions, it will no longer replicate and it will eventually be subject to apoptosis. Some cells, however, receive the appropriate signals to enter G1. More specifically, the three D-type cyclins (D1, D2 and D3) bind to cdk4 and cdk6; the cyclin-D-cdk4/6 complexes are necessary for entry into G1 (Vermeulen et al., 2002). During early G1, the cyclin-D-cdk4/6 complexes phosphorylate pRb which release its contact inhibition on the transcription factors E2F-1 and DP-1 (ncbi.gov). Once E2F-1 and DP-1 are released they positively regulate transcription of genes whose products are necessary for progression into the S-phase: they include cyclin A, cyclin E and Cdc25 (Voet et al., 2005). pRb must remain phosphorylated throughout the remainder of the cell cycle. Cdk2-cyclin-E helps maintain hyperphosphorylation of pRb while inducing proteasome-dependant degradation of its own regulator, p27 (Vermeulen et al., 2002). Cdk2-cyclin-E is also required at the G1/S phase transition. Through its association with NPAT (nuclear protein mapped to the

ATM locus) the cdk2-cyclin-E complex is linked to the regulation of replication dependant histone gene transcription (nbci.gov). Cdk2-cyclin-E phosphorylates histone H1 which is important for chromosome condensation required during DNA replication. At this point, cyclin-A associates with cdk2 to regulate the initiation of DNA replication, i.e, entry into S-phase. This is accomplished via phosphorylation of DNA polymerase alpha primase. When the DNA replication is complete the cells enter G2. Cyclin-A associates with cdk-1 during G2 to allow cells to transition from G2 into mitosis. The cyclin-A-cdk1 complex phosphorylates histone H1 to keep chromosomes condensation active throughout mitosis (Voet et al., 2005). Furthermore, cyclin-A-cdk1 phosphorylates cytoskeletal proteins, such as nuclear lamins and microtubules, which are also essential for mitosis. Cyclin-B associates with cdk-1 during mitosis and performs the same functions as the cyclin-A-cdk-1 complex (Voet et al., 2005). Once the cell has completed mitosis the duplicated cells undergo cytokinesis to divide into two identical cells. At this point, the cell either re-enters G1 or becomes dormant.

The cell cycle is a complex process and its regulation is no less complicated. Despite the aforementioned cdk/cyclin interactions there are also cyclin dependant kinase inhibitors (CKI). Two distinct families of cdk inhibitors have been discovered: the INK4 family and the Cip/Kip family. The INK4 family includes p15 (INK4b), p16(INK4a), P18 (INK4C) and p19 (INK4d) (Prabhu et al., 1997). The INK4 family is characterized by forming stable complexes with unbound G1-cdks (cdk4 and cdk6) preventing cyclin D binding and, ultimately, avoiding entrance into G1. The second family of inhibitors, the Cip/Kip family, includes p21 (Waf1, Cip1), p27 (Cip2) and p57 (Kip2). These inhibit cdk/cyclin complexes both at the G1 stage as well as cdk1/cyclin B complexes. CKI's

are regulated by internal and external signals. For example, the p53 tumor suppressor gene directly promotes transcription of p21 (Prabhu et al., 1997). p15 and p27, however, are regulated by transforming growth factor β (TGF-β) (Vermeulen et al., 2002).

In a nutshell, cell cycle arrest can occur at essentially any stage of the cell cycle and for any reason. For example, at the G1/S transition there is a "checkpoint" controlled by p53 that is inducible upon DNA damage. In doing so, the cell can repair damaged DNA or call for apoptosis in a situation where repair is impossible. A similar checkpoint exists at the G2-M transition. Different regulatory signals can elucidate different regulatory pathways to inhibit different stages of the cell cycle and, if necessary, completely aborting cell cycle replication via apotosis. However, such quality control is lost in a cancerous cell.

HPV and the Cell Cycle:

In cancer, there are fundamental alterations to cell cycle control that result in unrestrained cellular proliferation. Mutations mainly occur in proto-oncogenes or tumor suppressor genes (such as pRb and p53) ultimately promoting tumor growth. During high-risk HPV infection E6 and E7 function to simultaneously disable p53 and pRb, respectively, leading to uncontrolled cellular proliferation and accumulated DNA damage (Burd et al., 2003). E6 promotes degradation of p53 while E7 sequesters pRb by efficient binding to an LxCxE sequence on pRb (Sdek et al., 2006). The two work together and are expressed from bicistronic mRNA initiated at the viral early promoter (p97) (Al Moustafa et al., 2004).

E7 can also associate with other proteins involved in cell proliferation including histone de-acetylases (Longworth et al., 2004), components of the AP-1 transcription complex (Antinore et al., 1996) and the CKI's p21 and p27 (Funk et al., 1997). Despite E7 stimulating cellular proliferation, during productive infection only a small subset of parabasal layers are mitotically active. This is because of high concentrations of p21 and p27. It appears that during natural infection, only those cells with low levels of p21 / p27 or with high enough levels of E7 to overcome the block imposed by such CKI's are able to stimulate S-phase progression (Longworth et al., 2004).

E6 functions to complement the role of E7. Although the association with p53 is well characterized, E6 can also associate with other pro-apoptotic proteins such as Bak (Thomas et al., 1998) and Bax (Li et al., 2000). E6 has also been shown to stimulate cell proliferation independently of E7 through its C-terminal PDZ-ligand domain (Thomas et al., 2002). E6-PDZ binding is sufficient to mediate suprabasal cell proliferation and may contribute to the development of metastatic tumours by disrupting normal cell adhesion.

Recent studies have demonstrated a deregulation in cyclin D1 as well as cdk4/6 in oral epithelial cells (Sdek et al., 2006) resulting from high-risk HPV infection. Yasmeen et al. have reported that D-type cyclins are the downstream target of E6/E7 in normal oral epithelial (NOE) cells (Yasmeen et al., 2007). Furthermore, Al Moustafa et al. have confirmed that cyclin D1 is essential for neoplastic transformation induced by E6 and E7 cooperation in normal cells (Al Moustafa et al., 2004). More specifically, normal embryonic fibroblast cells (NEF-wt) cells transduced with E6/E7 retrovectors form tumors in nude mice and colonies in soft agar. However, in $D1^{-/-}$ cells, neither tumor nor

colony formation is observed. Furthermore, using anti-sense cyclin D1 they completely inhibited tumor and colony formation in NEF-wt-E6/E7 cells. These data suggest that D-type cyclins are important downstream mediators of the cellular transformation induced by high-risk HPV oncoproteins E6 and E7.

ID proteins:

Id (Inhibitors of DNA binding) proteins are members of the basic Helix-loop-helix (b-HLH) family of transcription factors. The b-HLH transcription factors are characterized by a helix-loop-helix motif required for homo/hetero-dimerization and a basic domain required for DNA binding (Minn et al., 2005). b-HLH family members are involved in differentiation of many processes including myogenesis, neurogenesis and heart development (Barone et al., 1994). Id proteins lack the basic region but share the HLH motif allowing them to bind and inhibit other b-HLH family members. The best characterized Id protein interaction is with the ubiquitously expressed E-protein family of transcription factors, as well as with Ets proteins. To date, four members of the Id-family have been discovered: Id1, Id2, Id3, and Id4.

The involvement of Id-proteins in cancer progression has been investigated, yet no mechanism of action has been defined (Norton et al., 1998). They have been implicated in tumorigenesis, angiogenesis, invasiveness and metastases in many different types of cancer including breast, prostate, ovary, rectum and colon. Furthermore, Id gene expression has been linked to both the MYC and RAS pathways (Norton et al., 1998). Studies have suggested their implication in promoting the progression through the cell cycle. It was demonstrated by Hara and colleagues that quiescent fibroblasts are

stimulated to produce high levels of Id1 and Id2 mRNA with mitogenic signaling. Furthermore, if this response is inhibited by anti-sense oligonucleotides directed against Id1 and Id2 then cells are prevented from entering the S-phase (Hara et al., 1994, Barone et al., 1994).

In addition, Id1 has been shown to delay replicative senescence despite being insufficient to independently immortalize cells (Norton et al., 1998, Alani et al., 1999, Tang et al., 2000, Tang et al., 2002). Id1 has also been shown to inhibit ETS-1, a non b-HLH protein predicted to inhibit INK4a, consequently allowing the cyclin D-cdk4 complex to phosphorylate and inhibit pRb (Ohtani et al., 2001). Furthermore, Id1 also inhibits E2A-dependant expression of p21, ultimately alleviating inhibition on cyclin-E, as well as cyclin-A complexes with cdk2. This leads to pRb phosphorylation allowing an increased progression through the cell cycle. It has also been shown that overexpression of Id1 in cultured SCp2 mammary, epithelial cells makes them negligent to differentiation signals and also induces rapid proliferation. Furthermore, reducing Id1 using antisenses slows cell proliferation (Desperez et al., 1995), facilitates differentiation (Desperez et al., 1995) and inhibits invasiveness (Fong et al., 2003). SCp2 cells with increased Id1 expression are thought to invade the basement membrane by expressing a 120kDa gelatinase (Desperez et al., 1998). In addition, Munger et al. demonstrated that Id1 localizes to centrosomes and rapidly induces abnormal centrosome numbers (Haaskarl et al., 2004). This may promote malignant growth through genomic instability. Cell cycle progression has also been correlated to Id levels *in vivo*. The association between loss of Id and the up-regulation of INK4a was first demonstrated in mouse embryos that lacked both Id1 and Id3. A recent publication demonstrated that Id1 is a

component of metastasis in human breast cancer. Its overexpression can drive metastasis in breast cancer cell lines injected into animals (Minn et al., 2005).

Extensive work has also been done to analyze the role of Id2 in driving the cell cycle. Iavarone et al. demonstrated that Id2 is a direct target of pRb (Lasorella et al., 2000). pRb is essential in activating targets that are naturally repressed by Id2, namely the b-HLH and Ets-family members involved in cell fate determination. They also report that Id2 is able to bind pRb and abolish its growth-suppressing activity. *In-vitro* analysis showed that growth arrest by pRb was not antagonized in transfection studies using Id2 lacking the HLH domain required for hetero-dimerization. In addition, Id2 has been shown to associate with p107 and p130 (other members of the pRb family) both *in-vitro* and *in-vivo*. Finally, Id2 was shown cause tumor initiation, proliferation and angiogenesis in pRb null mice (Lasorella et al., 2005).

The roles of Id3 and Id4 in cancer progression have been far less characterized. Nonetheless, some correlations have been made. In *Xenopus laevis*, a reduction of Id3 leads to the up-regulation of p27 (cell-type specific), the inhibition of cell growth and increased apoptosis (Kee et al., 2005a). Furthermore, epigenetic *inactivation* of Id4 has been correlated with poor differentiation and poor prognosis in colorectal carcinomas. These results suggest a potentially different role for Id4 (at least in colorectal carcinomas) considering their down-regulation was associated with unfavorable circumstances.

Despite the aforementioned data, much remains unresolved regarding the mechanism of Id operation. Further analysis is required to decipher their proper function

and potentially direct anti-cancer agents against Ids and related pathways. Regardless, their implication in cancer is heavily supported.

Interestingly, Yasmeen et al. have correlated Id expression with HPV. Moreover, Yasmeen et al. have shown that Id's are a target of E6/E7 transformation in normal oral epithelial cell lines. More specifically, Id1 was up regulated in E6/E7 transduced cell lines. Furthermore, $Id1^{-/-}$ cell lines, as well as Id1 anti-sense retroviruses, completely inhibited invasion in normal embryonic fibroblast and normal oral epithelial cell lines. In parallel, Id-1 over-expression was correlated with the presence of HPV type 16 in human invasive and metastatic breast cancer (Yasmeen et al., 2007b).

The Hypothesis:

If HPV *is* involved in colorectal carcinogenesis then great success can be achieved in detection, prevention and prognosis. Despite evidence of a correlation, little work has been done to explain the potential role of HPV infection in colorectal carcinogenesis.

The aim of this project is to provide a qualitative characterization of high-risk HPV type16 E6 and E7 oncoproteins in colorectal carcinogenesis. Thus, two primary colorectal cell lines were established by extracting non-cancerous tissue from patients that underwent colorectal surgery to generate normal, wild-type (WT) colorectal epithelial cell lines. They were transduced with high-risk HPV type 16 E6 and E7 retro-vectors. We performed *in-vitro* analysis and compared the proliferation, transformation, migration and cell cycle regulation in transduced colorectal cell lines with respect to their WT counterparts. Furthermore, because the expression of Id proteins is correlated with

the expression of p53 in colorectal adenocarcinomas and regulated by E6/E7 in NOE cells, we investigated their correlation with HPV expression in ten primary, colorectal cancer patients.

We concluded that high-risk HPV type 16 E6/E7 transduction was sufficient to induce transformation, migration, increased proliferation and a deregulation of many common cell cycle regulators in colorectal epithelial cell lines. Furthermore, we illustrate a strong correlation between the presence of high-risk HPVs and Id proteins 1, 2, 3 and, to a lesser extent, 4 in primary colon cancer patients.

These data suggest a cause and effect relationship between high-risk HPV infection and colorectal carcinogenesis and suggest a potentially new approach in detection, prevention and treatment of colorectal cancer.

Materials and Methods

1. Cell lines:

A) Tissue samples and primary colorectal epithelial cell culture

Normal colorectal tissues were obtained with informed consent from patients undergoing surgery. The biopsies were macroscopically normal. The specimens were washed immediately in cold, sterile phosphate-buffered saline (PBS). After removing excess and damaged epithelium and stromal tissue, the healthy specimen was cut into small pieces and incubated for 10 min at 37°C in 0.05% trypsin in 0.53mM EDTA (Gibco/BRL). Surface epithelium was mechanically separated to dissociate the cells into a single cell suspension. The normal colorectal epithelial (NCE) cells were collected after centrifugation and resuspended in mammary epithelial basal medium (MEBM, Clonetics). Cells were seeded on plastic dishes (Falcon) and fed every 48 h.

Two normal colorectal epithelial cell lines were generated and named as follows: NCE1-WT: normal colorectal epithelial cell line 1, NCE5-WT: normal colorectal epithelial cell line 5.

B) Transduction of colorectal epithelial cells with E6/E7 genes of HPV type 16

NCE cells, at 60% confluency, were transfected with E6/E7 ORFs using a recombinant retroviral system as previously described (Al Moustafa, 2004). HPV E6/E7 ORFs were cloned into the murine-based retroviral vector PLXSN. The constructs were transfected into a packaging cell line PA317 and recombinant retrovirus collected in the supernatant. The resulting PLXSN virus was used to infect the early passage NCE cells. Cells were selected with G418 at 1000 mg/ml and passaged in culture. Normal control cells and normal cells transduced with PLXSN vector were also treated with G418.

Over 95% of the E6/E7-immortalized cells were healthy after the G418 treatment. NCE-E6/E7 cells were trypsinized and passaged twice, while maintained on G418. The NCE normal cells died after approximately four days of treatment with G418; whereas PLXSN-transduced cells underwent senescence after approximately 10 passages.

Two transduced cell lines were generated as such and named as follows: NCE1-E6/E7: normal colorectal epithelial 1 – E6/E7 transduced, and NCE5-E6/E7: normal colorectal epithelial 5 – E6/E7 transduced.

C) Maintenance

Cell lines were maintained in long-term culture with MEBM with 5% Fetal Bovine Serum (FBS) and 1% antibiotics in a 75cm (Burd, 2003) Corning flask. Cell lines were maintained in an atmosphere of 5% CO_2. They were passed 25 times at 70 % confluency and stored at -80°C in freezing buffer medium. All experiments were performed between the 5^{th} and the 20^{th} passages.

2. Soft-Agar colony formation assay

Approximately $2x10^3$ cells from each cell line were placed in MEBM medium containing 0.4% agar and plated over a layer of MEBM medium containing 0.7% agar. The cells were examined under light microscopy every 1-2 days for 21 days.

3. Cell wound-healing assay

Each cell line was grown until complete confluency and serum-starved for 24hrs. The cells were finally wounded with a sterile 2mL pipette. Cells were examined under light microscopy after 24hrs and 48 hrs for cell migration.

4. RNA extraction

RNA extractions were performed when flasks were 70% confluent. Cells were washed twice with PBS buffer and trypsinized for 5 mins. Cells were then collected and pelleted in RPMI medium at 1,200 RPM for 4 mins. RNA extraction was finally performed using the Qiagen RNeasy Mini Kit (Qiagen, Mississauga, Ontario). RNA was collected in 30-50μL RNase-free water.

The purity of RNA was measured using the nano-drop spectrophotometer (nano drop technologies). 1μL of RNA solution was placed on the nano-drop spectrophotometer and the absorbance ratio between 260nm/280nm was measured. Only RNA samples with ratios in the range of 1.8-2.2 were selected for experimentation.

5. Reverse Transcription (RT) Polymerase Chain Reaction (PCR)

RT-PCR amplification was performed using the following primer sets: HPV type 16 E6 right primer: 5'-ATGCACCAAAAGAGAACTGCA-3' and left primer: 5'-TTACAGCTGGGTTTCTCTACG-3'; E7 right primer: 5'-TTACAGCTGGGTTTCTCTACG-3' and left primer: 5'-GTTTCTGAGAACAGATGGGGCACAC-3'. Primers for the GAPDH gene were as follows: GAPDH right: 5'-GAAGGCCATGCCAGTGAGCT-3' and left primer: 5'-

CCGGGAAACTGTGGCGTGAT-3'. GAPDH was used to control the amounts of cDNA generated from each sample. Synthesis of the first-strand of cDNA using reverse script II Reverse transcriptase was carried out using a cDNA kit for RT-PCR (Invitrogen life Sciences, ON. Canada). One fifth of the RT product was amplified for 30 cycles (1min at 95 °C, 1min at 58 °C, and 1 min at 72°C) followed by an extension of 7 min at 72 °C. RT-PCR amplification products were analysed on a 1% agarose gel (Sigma-Aldrich) stained with ethidium bromide (Sigma-Aldrich).

6. Protein extraction and Quantification

Protein extractions were performed when flasks were 70% confluent. Cell lines were washed twice with phosphate buffered saline (PBS) buffer and trypsinized. Detached cells were collected with RPMI solution and pelleted by centrifugation at 1,200 RPM for 4 minutes. Pelleted cells were re-suspended in 150-400 µL of RIPA (according to the size of the initial pellet) containing 100µL of V-Sodium (1mM stock), 10µL PMSF (1.25mM stock), 5µL Aprotinin (10mg/mL), 5µL Leupeptin (10mg/mL). Re-suspended cells were rotated on ice for 30 mins and re-centrifuged at 12,500 RPM for 15mins. The supernatant was collected into another tube.

Protein samples were quantified using Bradford BioRAD protein quantification assay (Bio-Rad Laboratories). A standard curve was produced using 0.1mg/mL BSA standard solution (Sigma-Aldrich). 800µL of distilled water and 200µL of Bio-RAD protein assay dye reagent along with 1.0µL of protein sample were incubated for 5mins at room temperature and the absorbance was measured 595nm. Protein concentration was extrapolated from the standard curve.

7. Western Blot

Equal amounts of protein (100 μg) were subjected to electrophoresis (Bio-Rad) through a 7.5% SDS-PAGE gel (BioShop) and transferred to a nitrocellulose membrane (Xymotech) overnight at 4°C. The membrane was then blocked for 2hrs in T-BST (24.2g Tris-base, 80g NaCl, 1% Tween, pH 7.6) containing 5% dry milk. The membrane was then probed overnight at 4°C with anti-cdk 2 and 4 (Chemicon int.) anti-cdk6 (Santa-Cruz), anti-cyclin D1, D2, D3, E, B (Santa-Cruz) and anti-cyclin A (Calbiochem), anti-Id1, 2, 3, 4, anti-β-catenin (Santa-Cruz) and anti-actin (Bio-Rad) (all dilutions were 1:1000 in 10mL of T-BST). The membrane was then washed three times with T-BST and incubated for 1 hr with anti-rabbit or mouse IgG (Bio-Rad) (all dilutions were 1:3000 in 10mL of T-BST) coupled to alkaline phosphatase (Canadian Life Technologies, Inc.). The membrane was finally washed 3 more times followed by alkaline phosphatase substrate detection (Vector Laboratories, Inc.).

8. MTT (Thiazol Blue Tetrazolium) proliferation assay

Approximately $2x10^3$ cells were seeded in 96 well corning plates. Three hours prior to the absorbance reading, 5mg/mL MTT (Sigma-Aldrich) dissolved in PBS was added to each well and incubated at 37°C in a CO2 incubator. The solution was aspirated and 200μL of dimethyl sulfoxide (DMSO, Fisher-Scientific) was added to each well and incubated for 5 mins. The absorbance was read at 550nm. The procedure was repeated every 2 days for 7 days and the results were standardized to the first (24hr) reading and graphed in excel.

9. Hematoxylin stain

Cells were stained according to Mayer's hematoxylin staining kit. Cells were hydrated with water and exposed to Mayer's hematoxylin dye and counterstained with eosin. Cells were dehydrated in 95% and absolute alcohol, cleared in xylene (Fisher Scientific) and mounted with permount (Fisher Scientific).

10. Tissue Micro-Array (TMA) and Immunohistochemistry

For histological analysis, tumors were excised and fixed in neutral-buffered formalin 10% (EM Science, NJ, USA) overnight at room temperature prior to being embedded in paraffin. Duplicate samples of each tissue was taken, at a radius of 5μm, and embedded in a separate paraffin block. An array of ten tissues was generated. The TMA (Leica RM2255) was sectioned at 5μm. Paraffin-embedded TMA sections were labelled using the avidin–biotin method (Vector Laboratories, Inc.). Endogenous peroxidase activity was quenched by incubation in 1.5% hydrogen peroxide for 30 min at room temperature. Sections were immersed in 10mM sodium citrate buffer (pH 6.0) and subjected to heat-induced antigen retrieval. To block binding of endogenous biotin, sections were incubated with an endogenous avidin–biotin-blocking kit (Zymed Laboratories, Inc.) according to the manufacturer's instructions. The sections were then incubated with anti-HPV type 16, anti-Id1, 2, 3 and 4 (Santa cruz) at a dilution of 1:50 in blocking buffer (5% goat serum in P-BST) overnight at 4°C. After being rinsed extensively with PBS, the sections were incubated with the appropriate biotinylated secondary antibodies at a dilution of 1:200 for 1hr at room temperature (Vector Laboratories, Inc.), followed by a 30-min incubation with the avidin–biotin–horseradish

peroxidase complex at a dilution of 4:10,000 (Vector Laboratories, Inc.) in PBS. Final color development was achieved with diaminobenzidine (DAB) substrate kit peroxidase (Vector Laboratories, Inc.). Sections were then lightly counterstained with Hematoxylin and analysed by conventional light microscopy and photographed with the use of a Leica DMLB2 microscope.

Results and Discussion

1. Verification of High-risk HPV E6/E7 transduction of colorectal epithelial cells

We confirmed the presence of HPV type 16 E6 and E7 oncoproteins in the transduced cell lines as well as their absence in the wild type (WT) cell lines. RT-PCR analysis was used to detect HPV type 16 E6 (475bp) and E7 (290bp) in the transduced cell lines in contrast to their WT counterparts (Fig.1). We used human normal mammary epithelial cells transduced with E6/E7 (HNME-E6/E7) as a positive control and distilled water (dH_20) as a negative control. Upon verification we were able to use the cell lines for further characterization.

2. Characterization of primary colorectal epithelial cell lines

We established primary normal colorectal epithelial (NCE) cell lines from explants of two fresh human normal colorectal patients that underwent surgery. More than 95% of the primary NCE cells showed an epithelial-like morphology (Fig. 2a and 2c) and doubled in approximately 4-5 days. We transduced NCE1-WT and NCE5-WT cells with E6/E7 retro-vectors as described earlier. We characterized the transduced cell lines as myo-epithelial-like (Fig. 2b and 2d).

We further characterized our cells with markers via western blot analysis. As mentioned in table 1, we found that our WT cells expressed greater amounts of epithelial markers, E-cadherin and α-catenin. Furthermore, the transduced cells expressed similar amounts of Vimentin but greater amounts of Fibronectin and N-cadherin. These results suggest an epithelial to mesenchymal (fibroblastic) transition in the presence of high-risk HPV type 16 E6/E7 oncoproteins.

3. Hematoxylin stain

Hematoxylin analysis gives insight as to whether cells are cancerous or not based on their nuclear staining. Hematoxylin is positively charged and, therefore, binds to basophilic structures (structures that attract positive charges). Because DNA is negatively charged it is considered basophilic and stains purple in the presence of hematoxylin. The intensity of the stain is directly proportional to the amount of DNA present in the cell. Cancerous cells undergo increased DNA replication and, as such, they stain more intensely than normal cells.

Fig. 3 shows that an increase in the intensity of purple staining was present in both of the transduced cell lines (Fig. 3b and 3d) as compared to the WT cells (Fig. 3a and 3c). In general, the transduced cell lines also contained larger cells with larger nuclei. The arrows in Fig. 3b illustrate such cells in the NCE1-E6/E7 transduced cell line. The tendency for both darker and larger nuclei suggests an increased amount of DNA replication associated with E6/E7 transduction. In addition, the transduced cell lines seemed to exhibit a complete loss of contact inhibition – another common characteristic of cancerous cells. The circled area in Fig. 3d clearly illustrates loss of contact inhibition in the NCE5-E6/E7 transduced cell line. Furthermore, the transduced cell lines exhibit a far greater number of cells than the WT cell lines; as observed in Fig. 3b and 3d.

In summary, these data suggest that high risk HPV type 16 E6 and E7 oncoproteins were sufficient to induce uncontrolled proliferation associated with cellular transformation of normal colorectal epithelial cells, *in vitro*.

4. High-risk HPV E6/E7 transduction and colorectal epithelial cell proliferation

To confirm the affect of E6/E7 on cell proliferation, we used the MTT proliferation assay. Approximately $3x10^3$ cells were plated in triplicate and assayed for 7 days. The absorbance was measured at 550nm and standardized at 24hrs. The resulting relative absorbance was directly proportional to the number of cells present. The rate of proliferation was thus quantified as the relative absorbance after 3, 5 and 7 days.

There is a marked increase in the proliferation of NCE1-E6/E7 and NCE5-E6/E7 cell lines when compared to their WT cells, as seen in Fig. 4a and 4b, respectively. After 7 days, the NCE1-E6/E7 cell lines demonstrated a two-fold increase while the NCE5-E6/E7 demonstrated a three-fold increase. This was also observed in cell culture. Another observation made during cell culture was the doubling periods. That is, the transduced cell lines had doubling periods of approximately 3-4 days whereas the WT cell lines took approximately 6-7 days.

The NCE5-E6/E7 cell line proliferated slightly more aggressively than did the NCE1-E6/E7 cell lines (2-fold vs. 3-fold increase). This could be reasoned by the genetic background differences between the patients. However, a significant increase in proliferation is seen in both cases.

An increase in proliferation as a result of E6/E7 transduction is well documented. Numerous reports have explained E7's inhibitory effect on Rb, as well as E6's degradation effect on p53. This mechanism is well recognized to induce benign papillomas, in the case of low-risk HPV infection, and malignant transformation due to high-risk HPV infection. Our data suggests that the same mechanism may be taking place in colorectal epithelial cells, *in vitro*.

5. High-risk HPV E6/E7 transduction and colorectal epithelial cell transformation

The purpose of the soft-agar assay is to illustrate anchorage-independent cell growth. This is a common characteristic of many cancerous cells and, as such, suggests if a cell has been transformed *in*-vitro; transformed cells form colonies in soft agar whereas normal cells do not. We assayed the colony formation abilities of each of the high risk HPV type 16 E6/E7 transduced cell lines in comparison to their WT cells.

We found that E6/E7 transduced cell lines form colonies in comparison with the WT cell lines. The ability to transform can be attributed to the presence of high risk HPV type 16 E6 and E7 oncoproteins in the transduced cell lines.

To reiterate, it is well documented that high-risk HPV E6 and E7 oncoproteins complement each other in inducing transformation *in vitro* and *in vivo.* In a normal cell, pRb inhibits entry into G1 interphase by holding essential cdk's and their respective cyclins hostage until the appropriate growth signals are present. In the case of high-risk HPV infection, E7 efficiently binds to and inhibits pRb providing a scenario that mimics mitogenic growth stimulation. Of vital importance to high-risk HPV induced carcinogenesis is the simultaneous binding and degradation of p53 by E6. The infected cell then loses its ability to regulate its progression through the cell cycle and uncontrolled proliferation ensues. Such a transformation is demonstrated by the formation of colonies in soft-agar assays; Fig. 5b and 5d.

Similar high risk HPV soft agar results have been reported in cervical epithelial cells (Brake et al., 2005) as well as normal oral epithelial cells (Al Moustafa et al., 2004) and BT20 breast cancer cell lines (Yasmeen et al., 2007b) each transduced with high-risk

HPV retro-vectors. Interestingly, these results also display a loss of contact inhibition which complements our previous results.

Our data has uniquely demonstrated that colorectal epithelial cells may be transformed by high-risk HPV type 16 E6 and E7 oncoproteins, *in vitro.*

6. High-risk HPV E6/E7 transduction and colorectal epithelial cell migration

To further characterize the *in vitro* effect of high risk HPV type 16 E6/E7 oncoproteins on colorectal cells we used the wound healing assay. This assay provides a qualitative assessment of cells that have migratory potential. By causing a space ('wound') between cells we can monitor whether cells tend to migrate toward each other (across the 'wound-space') or not. Both NCE1-E6/E7 and NCE5-E6/E7 transduced cells migrate across the wound as compared to the WT cell lines; Fig. 6a. The results were quantified in Fig. 6b. This may be explained by the myo-epithelial transformation induced by the E6/E7 transduced cell lines.

Interestingly, these results are consistent with work performed in parallel to this research that reported the migratory potential induced by high risk HPV type 16 E6/E7 transduced BT20 breast cell lines, *in vitro* (Yasmeen et al., 2007b). Similar studies have also shown that E6/E7 cooperation induces cell invasion and metastases in human breast cancer cells (Yasmeen et al., 2007c). Furthermore, earlier reports show that HPVs are associated with vascular invasion and lymph node metastases (Begum et al. 2003, Umudum et al. 2007, Varnai et al. 2006). A matrigel invasion would prove useful to further characterize the effect in colorectal epithelial cells.

Nonetheless, these data suggest that high risk HPV type 16 E6/E7 oncoproteins affect the *in vitro* migration of colorectal epithelial cells.

7. High-risk HPV E6/E7 transduction and cell cycle deregulation in colorectal epithelial cells

Uncontrolled cellular proliferation is the hallmark of cancer. This may result from the over-expression of oncoproteins or by the inhibition of tumor-suppressor proteins. Often, there is a combination of both; as is the case with carcinogenesis induced by high-risk HPV's. Regardless of how a cell is transformed, cell cycle deregulation is inevitable. The expression of common cell cycle regulators, including cdks and cyclins, is affected accordingly. As previously discussed, high-risk HPV infection impinges the function of common cell cycle regulators; pRb and p53. In addition, earlier studies have reported the link between high-risk HPV E6 and E7 with cell cycle regulators and their implication in carcinogenesis of several cancers including oropharyngeal, esophageal and cervical cancer (Burd et al., 2003, Sdek et al. 2006, Tommasino et al. 1995). To assess the affect of high-risk HPV infection on colorectal epithelial cell cycle regulation, western blots were used to detect changes in protein levels of different cell cycle regulators.

We reveal that several cdks and their respective cyclins show increased protein expression in normal colorectal epithelial cell lines transduced with high-risk HPV type 16 E6 and E7 oncoproteins, *in vitro* (Fig. 7a and 7b).

Interestingly, E6/E7 cooperation has been shown to affect D-type cyclin expression in normal oral epithelial (NOE) cells and normal embryonic fibroblast (NEF)

cell lines, *in vitro* (Yasmeen et al., 2007a). More specifically, earlier data has demonstrated that D-type cyclins are the downstream targets of high-risk HPV type 16 induced transformations. E6/E7 transduced colorectal epithelial cell lines exhibit a similar up-regulation in the D-type cyclins. In parallel, there is also an up-regulation of cdk4 and cdk6 – targets of the D-type cyclins. More still, literature has corroborated that human epithelial cells expressing HPV type 16 E6 and E7 oncoproteins did not decrease in pRb protein levels, but rather, were hyper-phosphorylated (Vermeulen et al. 2002, Demers et al. 1994). This indicates increased activity for activated cdk4/cdk6/D-type cyclin kinase complexes as seen in Fig. 7a and 7b. In addition, elevated levels of G1/S-phase transition cdks and associated cyclins has already been correlated with enhanced colorectal dysplasia (Bartkova et al., 2001). Our results suggest that high-risk HPV infection may induce a similar pattern in colorectal epithelial cells.

Furthermore, Kanaoa et al. reported that cervical cancer tissues positive for high-risk HPV type 16 and 18 infections revealed an over-expression of cyclin-dependant kinase inhibitor (CKI) mRNA (Kanao et al., 2004). However, their inhibitory functions were over-ruled; in some cases the over-ruling of CKI inhibition was attributed to an increased expression of E7 (Noya et al., 2001). This can be hypothetically explained by an increase in cdk/cyclin-complex kinase activity in the presence of E7; as seen in the transduced cell lines. However, to fully characterize HPV induced carcinogenesis in colorectal epithelial cells, one must assess the regulation of tumor suppressor proteins, such as TGF-β and p53, and their respective CKIs. Theoretically, p53 should be severely reduced in E6/E7 transduced cell lines, and associated CKI's should be down-regulated and hyper-phosphorylated (Vermeulen et al. 2002, Demers et al. 1994). Furthermore,

TGF-β regulated CKI's should also be hyper-phosphorylated. Subsequently, E6 and E7 are responsible for the up-regulation in cdk/cyclin-complex kinase activity and the associated increase in cellular proliferation.

In addition to cell cycle regulators, β- catenin expression was also investigated because it is associated with cyclin in colorectal cancer cells. More specifically, β-catenin activates transcription of the cyclin D1 promoter in colon cancers (Tetsu et al., 1999). Therefore, increased β- catenin expression may contribute to an increase in cell cycle progression via the regulation of G1-phase regulators. The E6/E7 transduced cell lines exhibited increased levels of β- catenin in contrast to the WT cell lines (Fig. 7b).

β- catenin has also been associated with the regulation of Id proteins (Rockman et al., 2001). As mentioned earlier, Id proteins are a family of helix-loop-helix (HLH) proteins that are intricately connected to the carcinogenesis of different cancers. Our results display an up-regulation of Id proteins 1, 2 and 3 (to a lesser extent) but decreased expression of Id4 in high risk HPV type 16 E6/E7 transduced cell lines (Fig. 7b).

Interestingly, high risk HPV type 16 E6/E7 transduction has been correlated with up-regulation of Id1 in NOE and NEF cell lines (Yasmeen et al., 2007c). In addition, Id1$^{-/-}$ and anti-sense retroviruses targeted against Id1 inhibit cell invasion in these cell lines. The results indicate that Id1 expression correlates with invasion. This may help explain the migration results seen in Fig.6. Id2, another member of the helix-loop-helix family, has been linked to tumour initiation, proliferation, and angiogenesis in pRb mutant mice (Lasorella et al., 2005). Id2 is also documented as being up-regulated by the β-catenin/T cell factor pathway in colon cancer (Rockman et al., 2001). Considering the deregulation of pRb by E7, as well as the up-regulation of β- catenin, it is imaginable that the up-

regulation of Id2 in our transduced cell lines results in similar cancer promoting functions. Though less characterized, Id3 has also been associated with cancer promotion via the deregulation of P16 (INK4a) – a well known CKI. This may also contribute to the aforementioned over-ruling of CKI induced cell cycle inhibition in the presence of E7. Finally, Id4 has been ambiguously described as both tumor promoting and tumor suppressing. Hoon et al. have correlated the inactivation of Id4 with poor differentiation and unfavourable prognosis in colorectal carcinomas (Umetani et al., 1994). Reduced levels of Id4 have been attributed to DNA methylation of the Id4 promoter (Al Moustafa et al., 2004). The results show that Id4 is markedly decreased in our transduced cell lines. It may be hypothesized that Id4 is acting as a tumor suppresser in E6/E7 transduced cell lines. As such, the characterization of Id4 methylation patterns in primary colorectal epithelial cell lines would be intricate in further dissecting the mechanism of HPV induced colorectal carcinogenesis.

Our results suggest interesting information regarding cell cycle regulation in HPV induced colorectal cancers. We begin to dissect the pathway not only with common cell cycle deregulation, but further still, with the Id family of proteins. Such information may prove useful in prevention and prognosis of colorectal cancer.

8. Tissue Micro-Array analysis of High-Risk HPV infection and Id protein expression in colorectal cancer tissues

In addition, we detected a correlation between the expression of HPV type 16/18 and the Id proteins in colorectal cancer patients. We immunostained ten tissues from

colon cancer patients with an HPV type 16/18 probe, as well as Id1, 2, 3 and 4. We compared the intensities between HPV and Id staining.

The upper part of Fig. 8 shows that, in the absence of HPV neither Id1, Id2 nor Id3 are expressed; however, Id4 is up-regulated. The results were present in two out of ten tissues. In addition, the bottom of Fig. 8 shows a positive correlation between the intensity of HPV staining and Id1, Id2 and, to a lesser extent, Id3. Id4, however, seems down-regulated. The results were consistent in four out of ten tissues. These data suggest that there is a direct correlation between the expression of high-risk HPVs and Id proteins in colorectal carcinomas. Interestingly, the correlation fully reflects our western results.

However, the over-expression of Id1, Id2 and Id3, as well as the down-regulation of Id4, has been documented in prior studies of colorectal cancers in the absence of HPV (Herber et al., 1996). Thus, HPV may not be *necessary* for Id protein expression in colorectal cancer but results suggest it may have a compounding effect.

Overall, the data suggests a correlation between HPV and Id regulation. However, certain tissues were undetectable due to technical error. Furthermore, patient history was unavailable. This would allow us to determine if the cancers are primary or metastasized. If the cancer was metastasized, then the expression of any proteins, including Id proteins, may not be conclusively attributed to the presence of local HPV. More still, tissue from a distant, non-cancerous region of each patient's colon was not available. This would provide a control to differentiate between the expressions of HPV, and the respective expression of Id proteins, in cancerous versus non-cancerous tissues. In essence, a larger array with an in-depth patient history and proper controls is necessary for full characterization and provide a statistically significant correlation.

Nonetheless, our results provide interesting preliminary data that inspires further investigation to characterize the relationship between high-risk HPV infections and Id proteins to dissect the mechanism of HPV induced colorectal carcinogenesis.

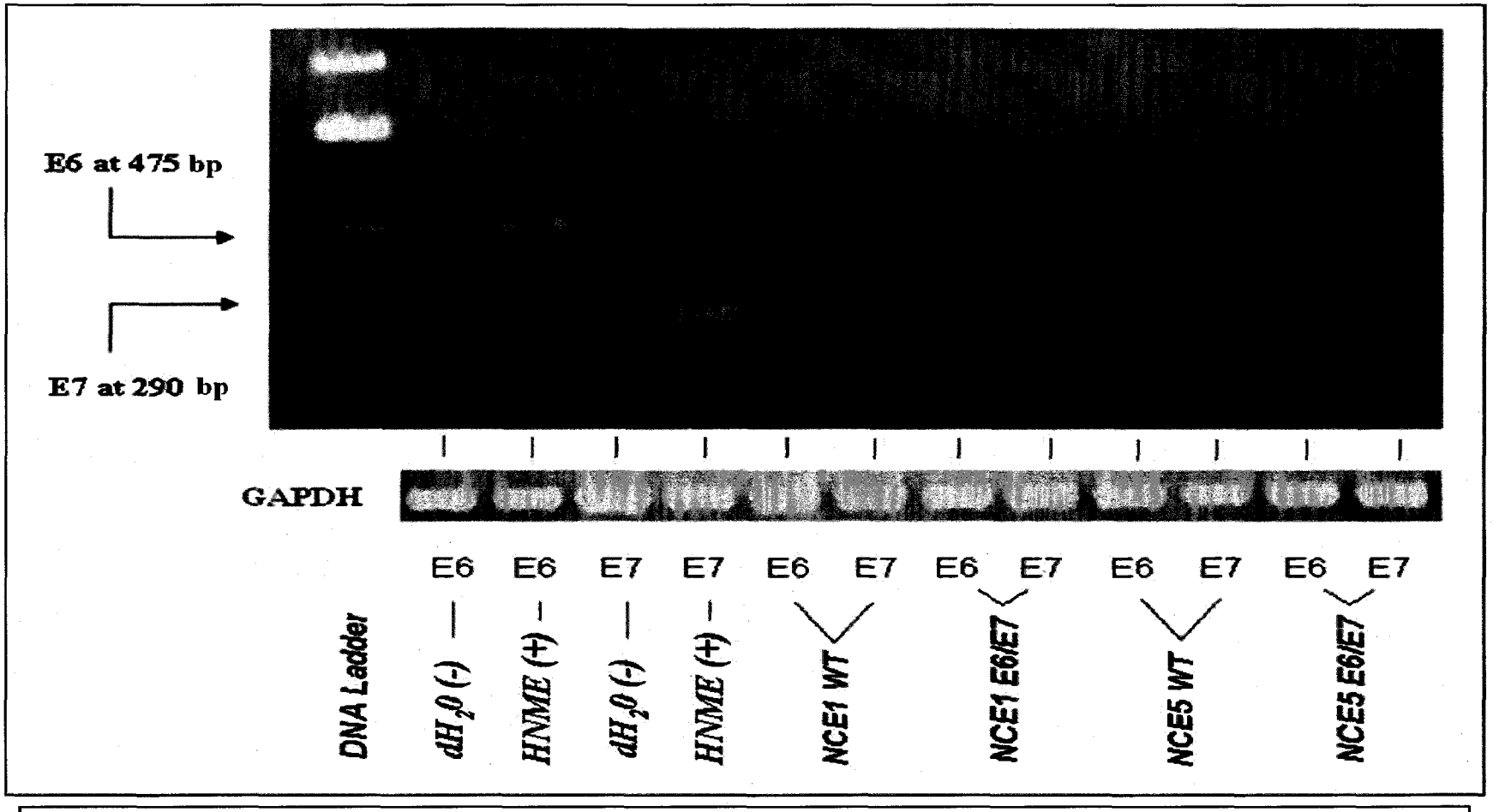

Figure 1. **High-Risk HPV type 16 E6/E7 transduction of Colorectal Epithelial Cells**

Verification of HPV type 16 E6 and E7 onco-protein transduction in colorectal epithelial cell lines. Bands are present in both transduced cell lines, NCE1-E6/E7 and NCE5-E6/E7, at 475 Kb (E6) and 290 Kb (E7). Bands are absent in both WT cell lines, NCE1 and NCE5.

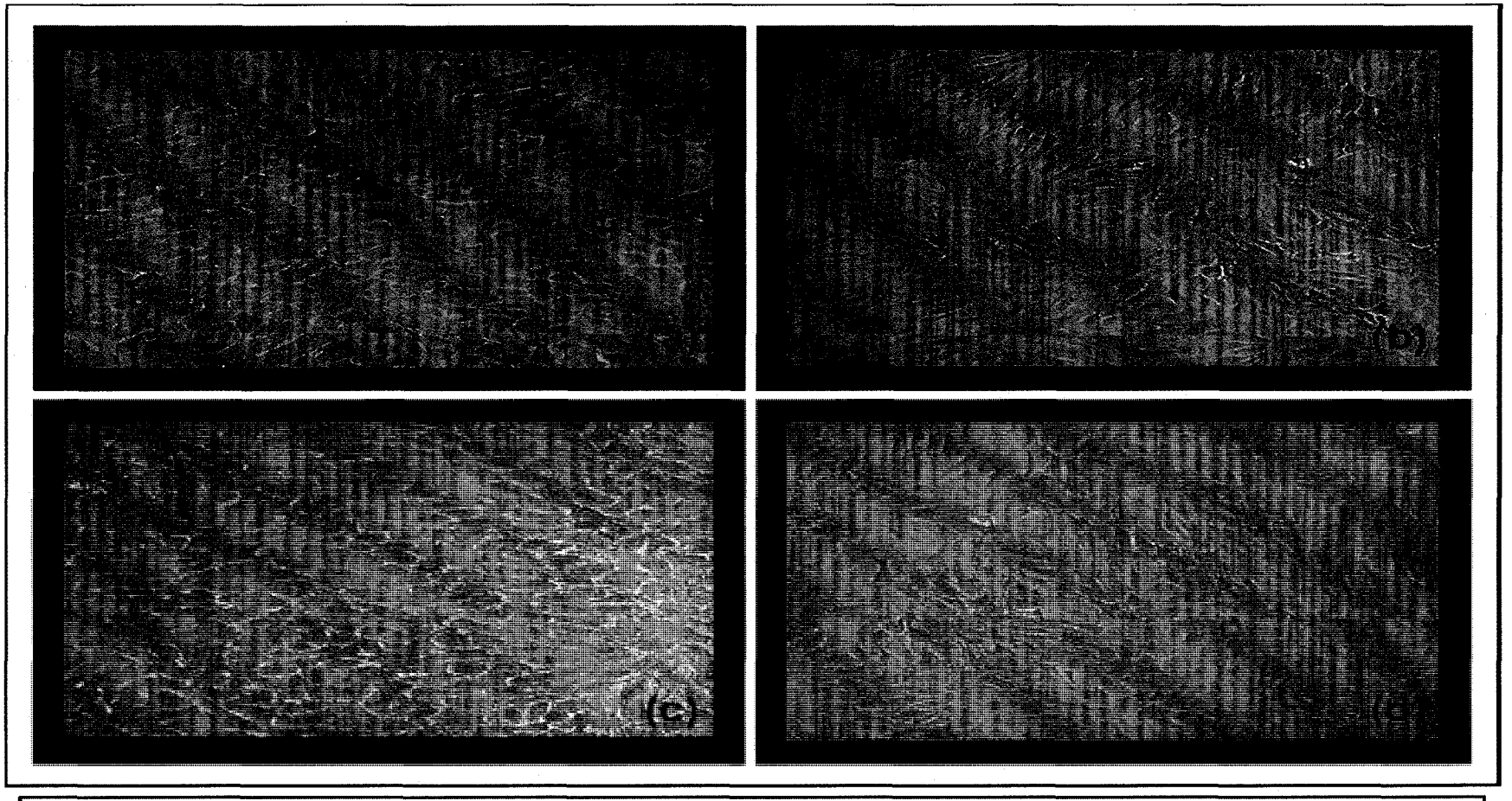

Figure 2. **Generation of Primary Colorectal Epithelial Cell lines**

(a) NCE1-WT epithelial-like cell line as compared to **(b)** NCE1-E6/E7 myo-epithelial-like cell line. **(c)** NCE5-WT epithelial cell line photo as compared to **(d)** NCE5-E6/E7 myo-epithelial cell line. All photos were taken at 10X zoom.

	Markers	NCE1	NCE1E6/E7	NCE5	NCE5E6/E7
Epithelial	E-cadherin	++	+	++	+
	α-catenin	++	+	++	+
Fibroblast	Vimentin	+	+	+	+
	Fibronectin	+	++	+	++
	N-cadherin	+	++	+	++

Table 1. **High-Risk HPV type 16 E6/E7 regulates Epithelial to Mesenchymal (Fibroblast) Transition**

Table 1 characterizes the differential expression of epithelial and fibroblastic markers.

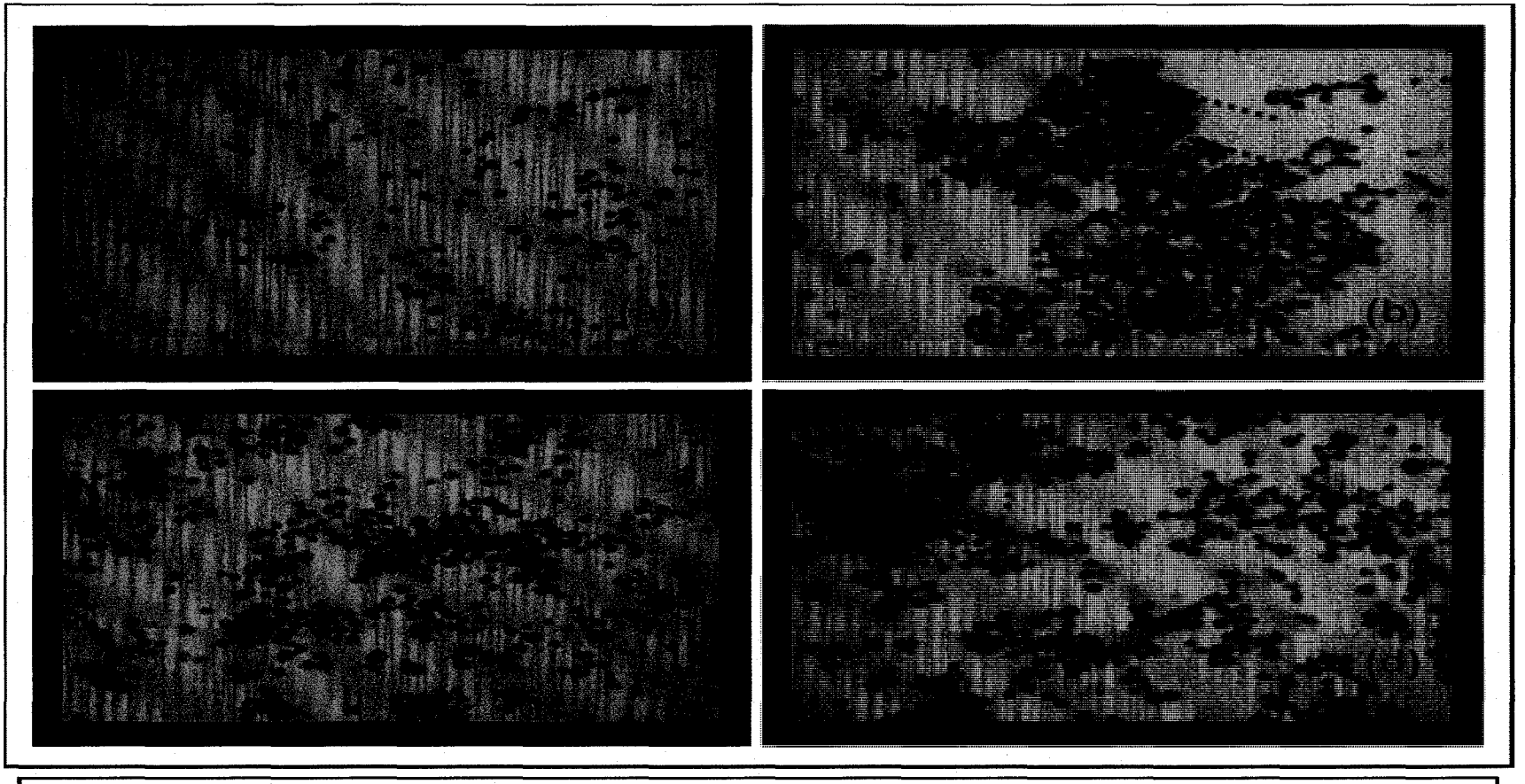

Figure 3. **Hematoxylin Stain**

(a) Hematoxylin staining for NCE1-Wt vs. **(b)** NCE1-E6/E7 cell lines. The latter illustrates three examples of large cells with larger nuclei which was associated with E6/E7 transduction (arrows). **(c)** NCE5-Wt vs. **(d)** NCE5-E6/E7 transduced cell line. The latter demonstrates loss of contact-inhibition prevalent in the E6/E7 transduced cell lines (circled area).

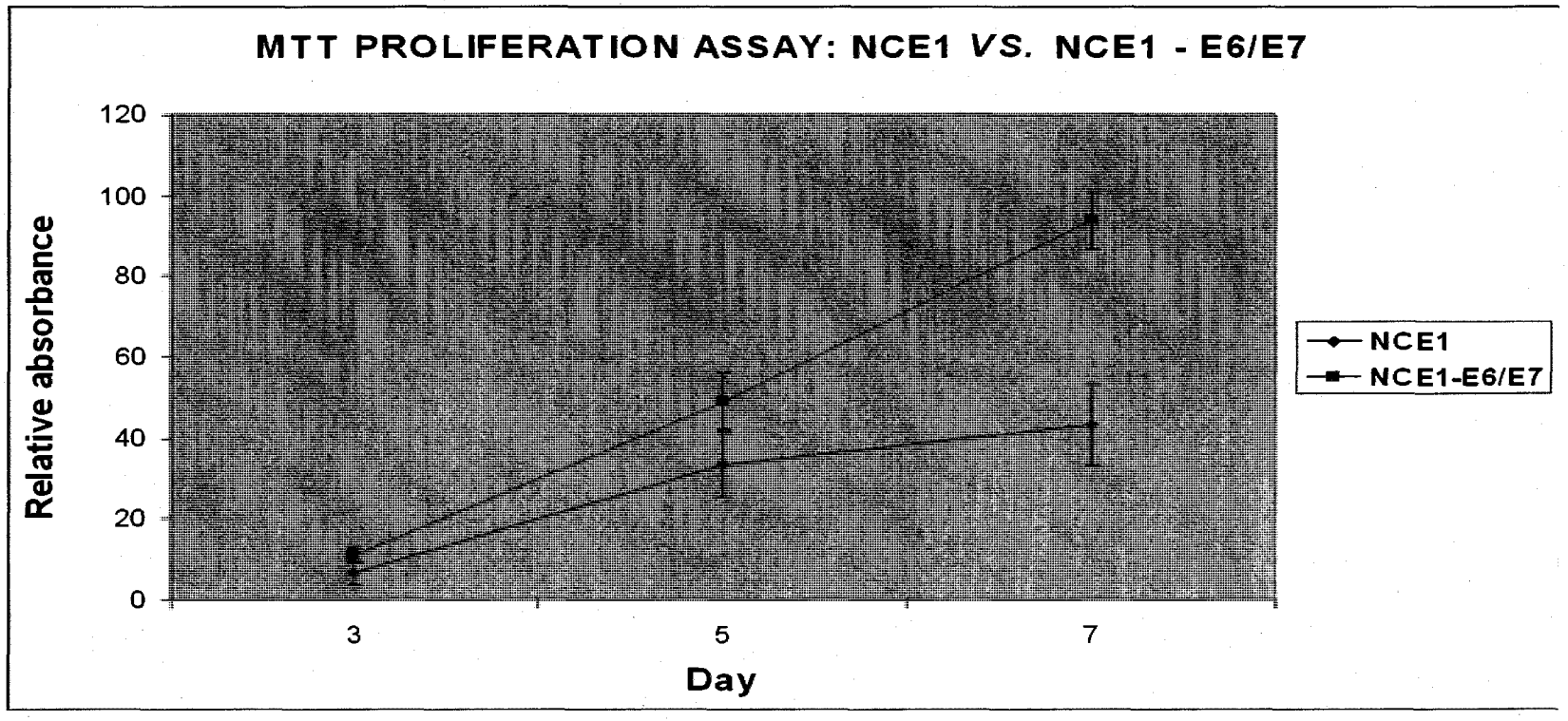

Figure 4a. **High-Risk HPV type 16 E6/E7 transduction and Colorectal Epithelial Cell Proliferation**

MTT proliferation assays measured the affect of high-risk HPV E6/E7 transduction on the rate of colorectal epithelial replication. NCE1-E6/E7 cells (top line) demonstrated a 2-fold increase in proliferation by day 7 with respect to NCE1-WT cells (bottom line). Transduced cells displayed in-culture doubling periods of 3-4 days; in contrast, WT cells displayed in-culture doubling periods of 6-7 days.

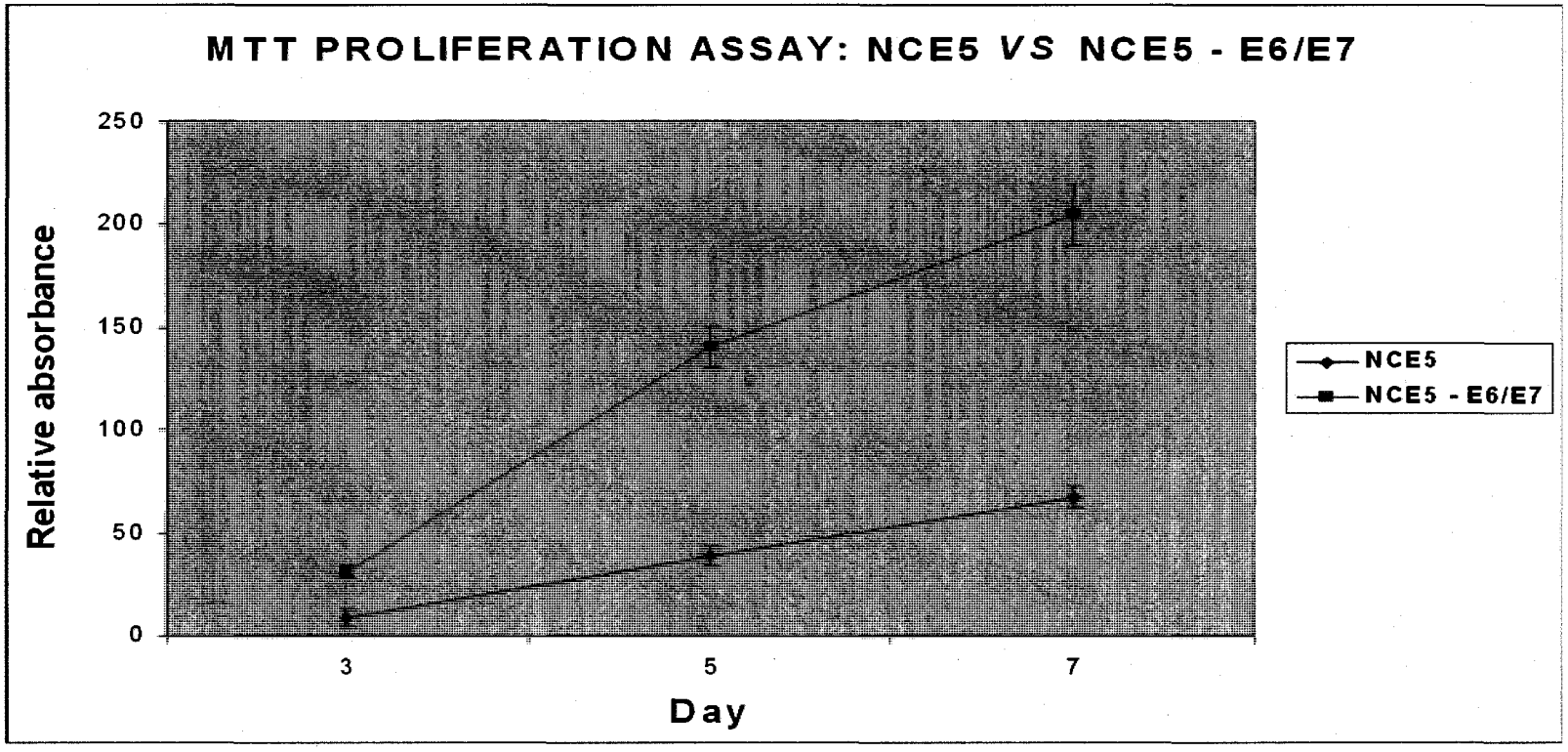

Figure 4b. **High-Risk HPV type 16 E6/E7 transduction and Colorectal Epithelial Cell Proliferation**

MTT proliferation assays measured the affect of high-risk HPV infection on the rate of colorectal epithelial replication. NCE5-E6/E7 cells (top line) demonstrated a 3-fold increase in proliferation by day 7 with respect to NCE5-WT cells (bottom line). Transduced cells displayed in-culture doubling periods of 3-4 days; in contrast, WT cells displayed in-culture doubling periods of 6-7 days.

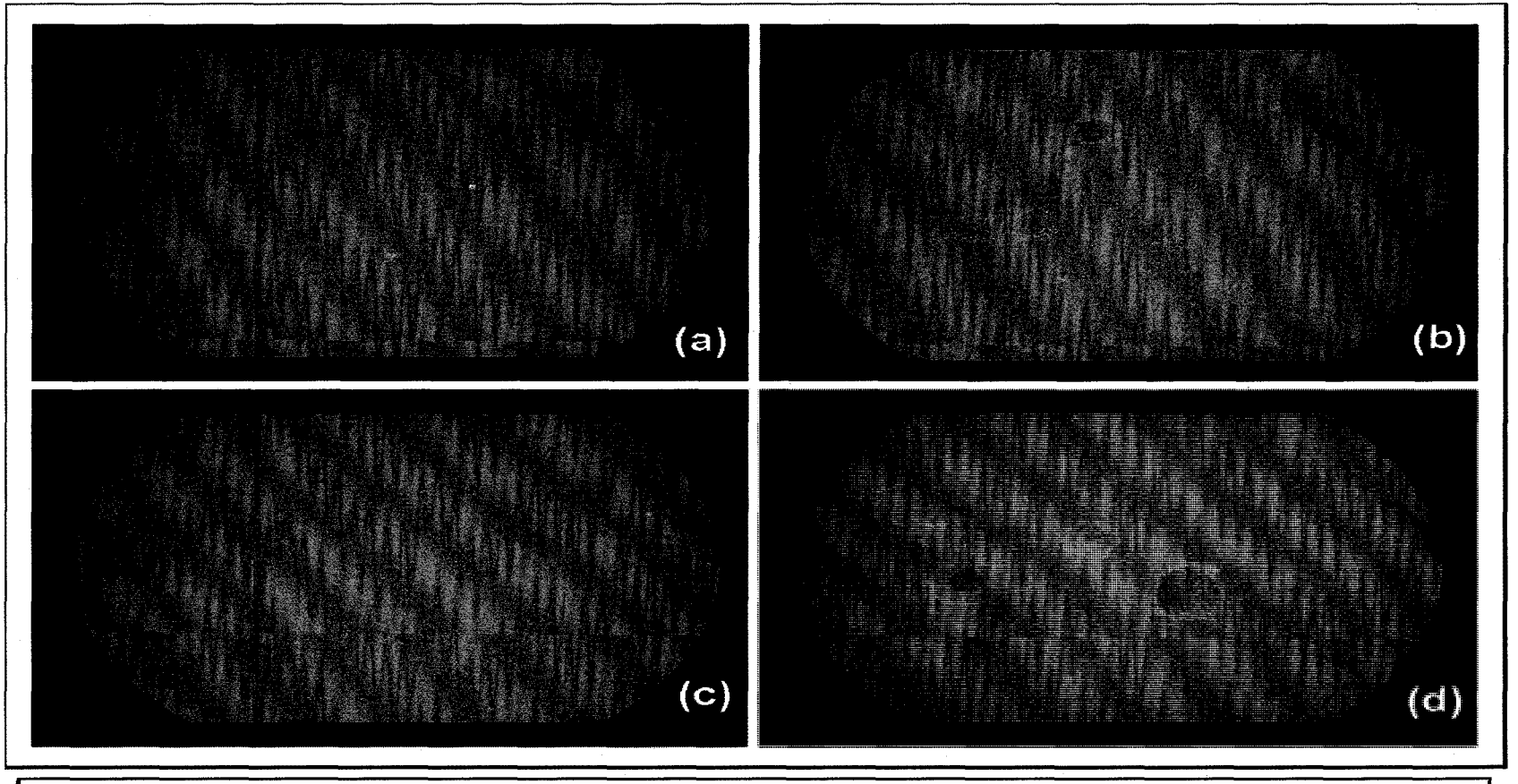

Figure 5. **High-Risk HPV type 16 E6/E7 transduction and Colorectal Epithelial Cell Transformation**

(a) and **(c)** NCE1-WT and NCE5-WT colony formation vs. **(b)** and **(d)** NCE1-E6/E7 and NCE5-E6/E7 transduced cell lines, respectively. Transduced cell lines display colony formation after 14 days whereas the WT cells do not.

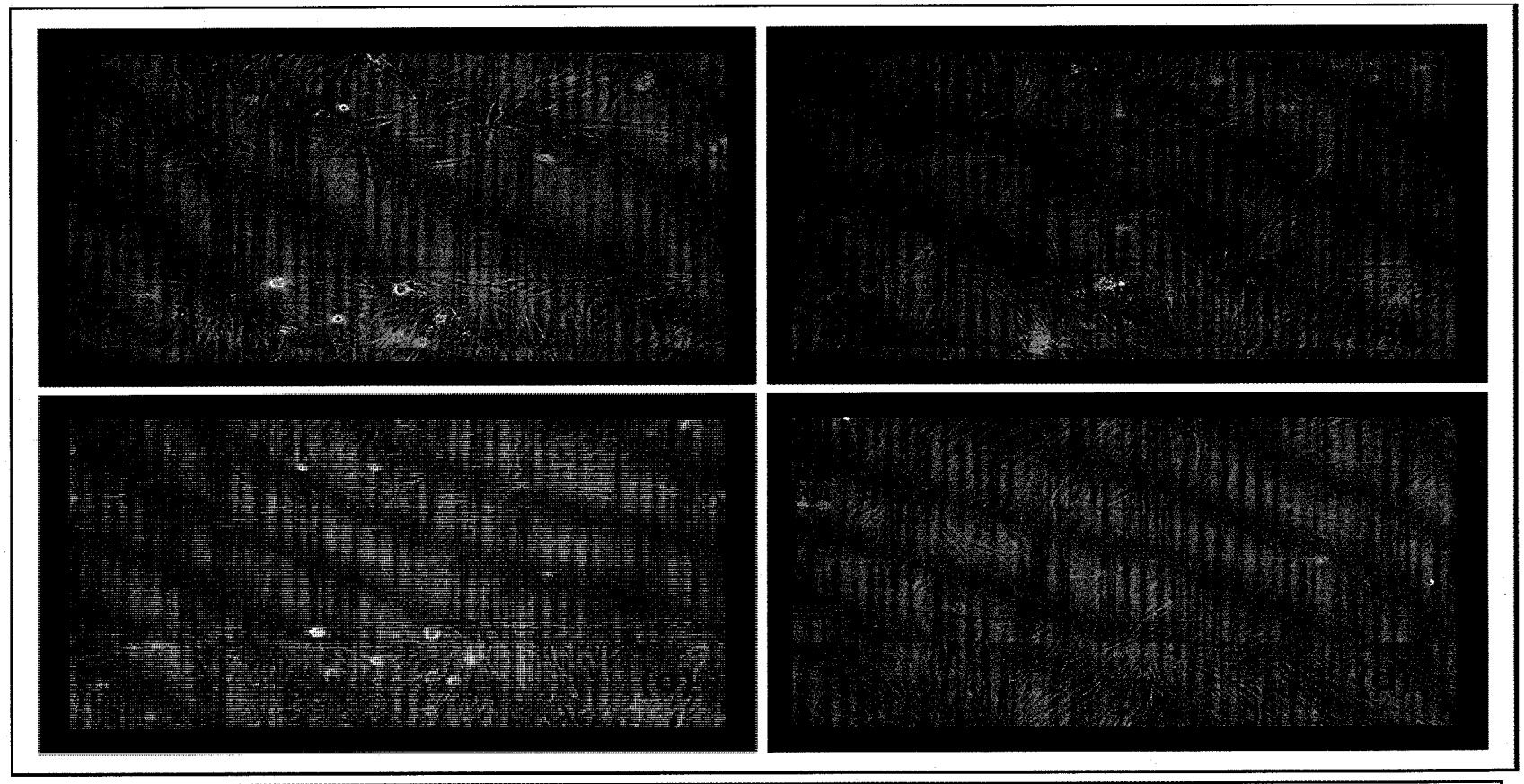

Figure 6a. **High-Risk HPV type16 E6/E7 transduction and Colorectal Epithelial Cell Migration**

(a) and **(c)** NCE1-WT and NCE5-WT cell migration vs. **(b)** and **(d)** NCE1-E6/E7 and NCE5-E6/E7 transduced cell lines, respectively. Transduced cells tend toward one another after 24hrs. of assay; in contrast, WT cells do not.

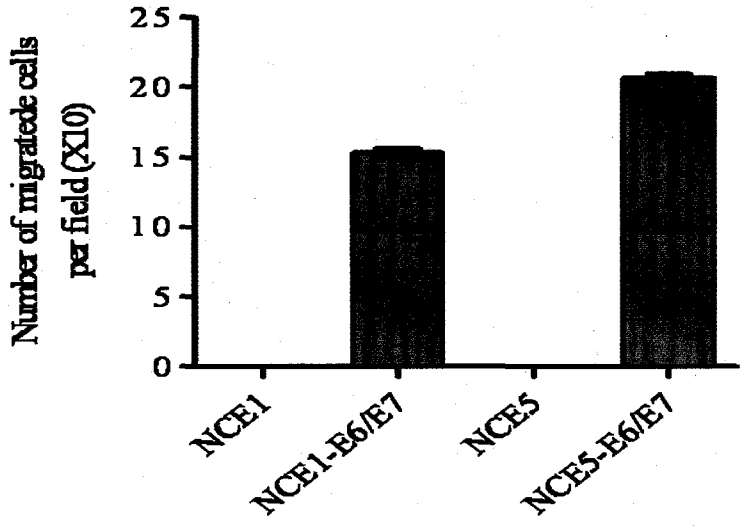

Figure 6b. **High-Risk HPV type16 E6/E7 transduction and Colorectal Epithelial Cell Migration**

All cells were seeded in triplicate for a 24 hour wounding healing assay. The cells were observed and all migrated cells were counted and graphed.

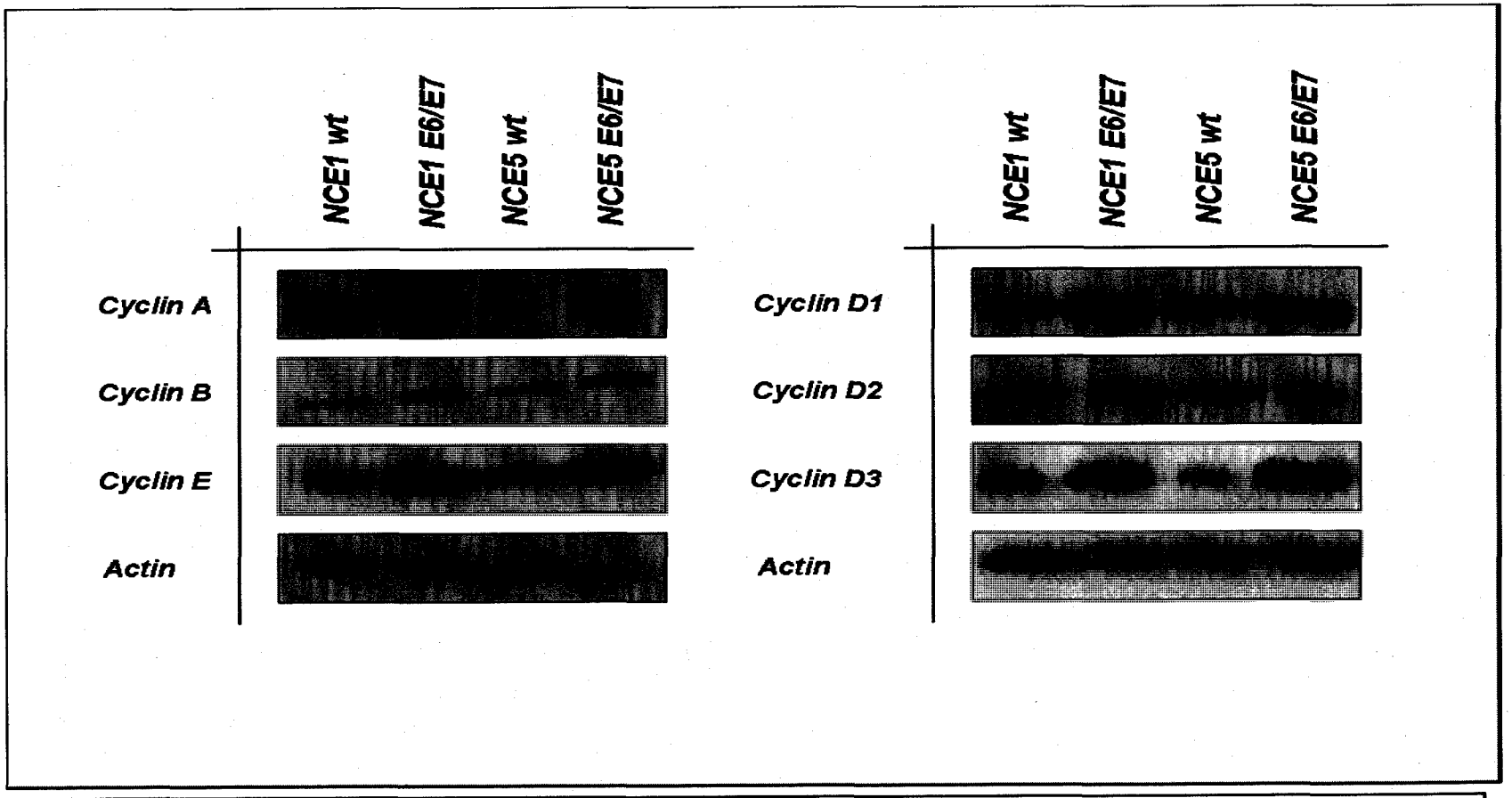

Figure 7a. **High-Risk HPV type 16 E6/E7 transduction and Cell Cycle deregulation in Colorectal Epithelial Cells**

All cyclins were up-regulated in the transduced cell lines with respect to the WT cells.

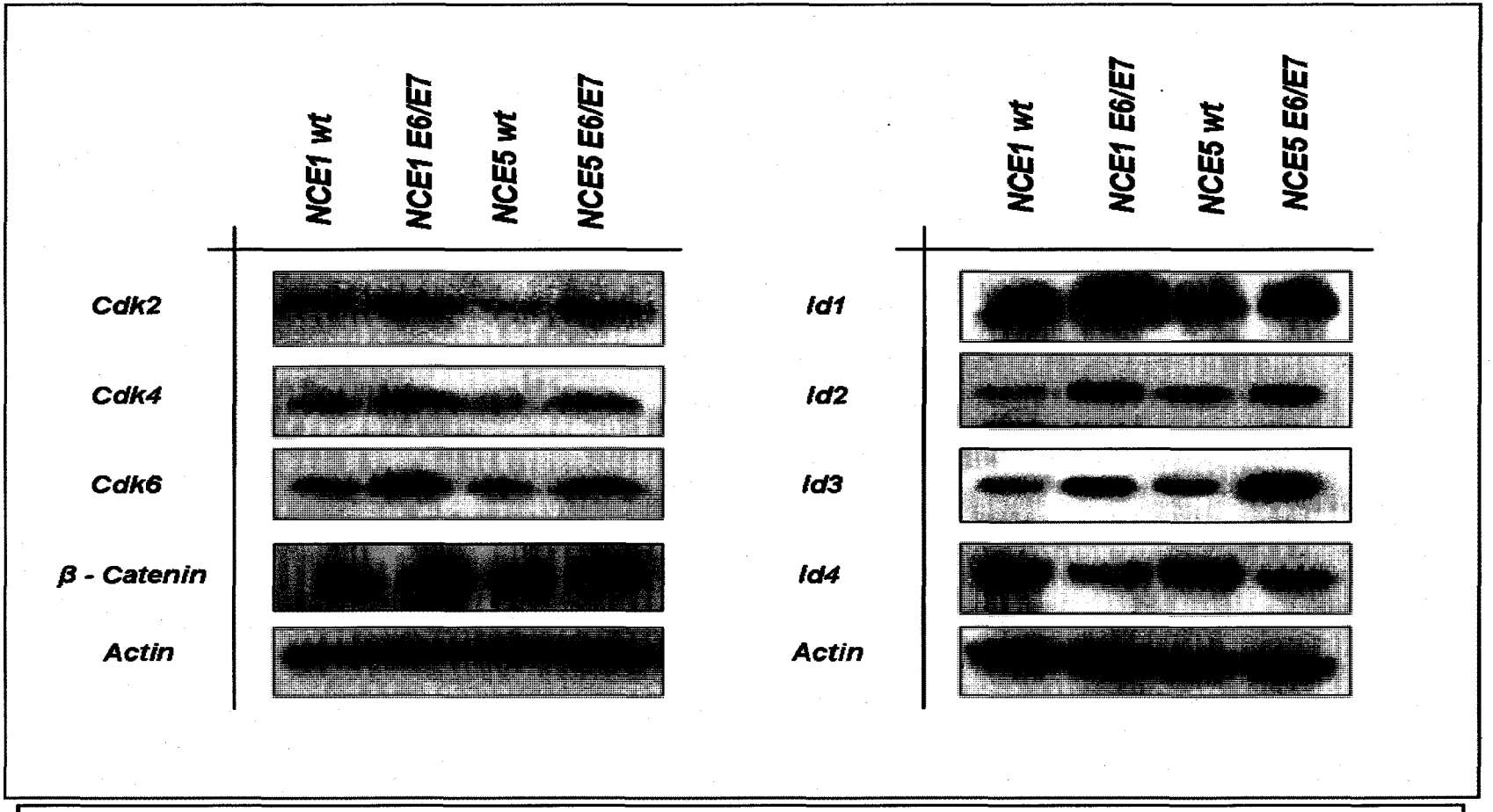

Figure 7b. **CdKs, β-catenin and Id protein expression in Colorectal Epithelial Cells transduced with high-risk HPV type 16 E6\E7.**

The blots were probed with antibodies for cdk2, cdk4, cdk6 , beta-catenin , Id proteins and subsequently for actin.

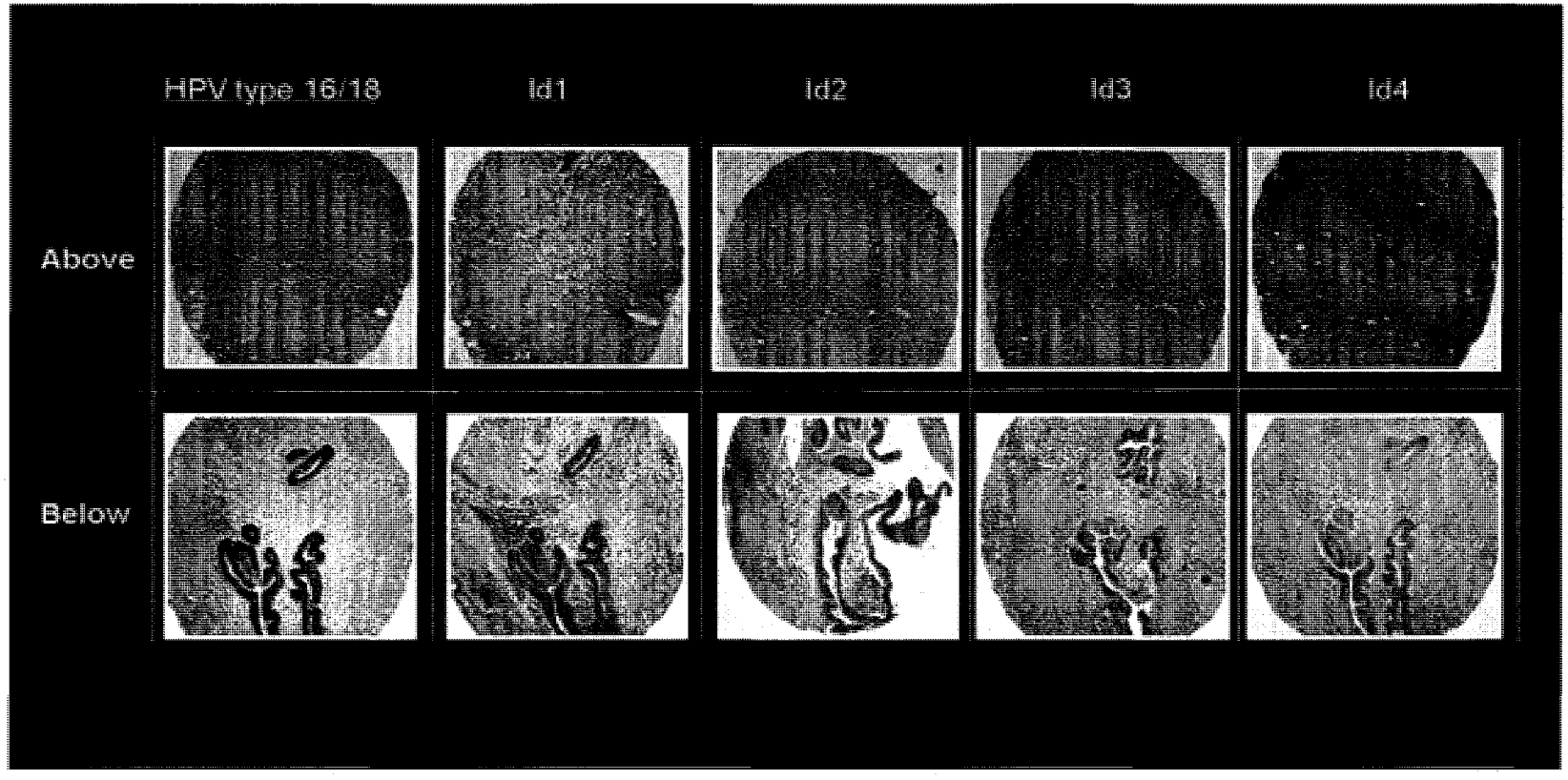

Figure 8. **TMA analysis of High-Risk HPV Infection and Id protein expression in colorectal cancer tissues**

Figure 8 shows a correlation between the expression of Id proteins and the presence of HPV. **Above:** The lack of high-risk HPV expression is associated with a lack of Id1,2 and 3 expression but an increase in Id4. **Below:** The presence of high-risk HPV is associated with an increase in Id1, 2 and 3 but a decreased Id4 expression.

Summary and Conclusion

In this study we report that high-risk HPV type 16 E6 and E7 oncoprotein transduction induced transformation of normal colorectal epithelial cells, *in vitro*. The transformation was accompanied by a deregulation of common cell cycle regulators, as well as a tendency towards migration. In addition, immunohistochemical staining provided a qualitative correlation between the presence of high-risk HPV infection and the Id family of helix-loop-helix proteins.

Many studies have reported strong correlations between the presence of high-risk HPVs and colorectal cancer. This study implies that such a correlation may be attributed to the process of carcinogenesis in colorectal cancers.

Interestingly, early detection of high-risk HPVs has been proven useful as a marker for the tumorgenesis of different cancers. Our results suggest that it may be useful in the early detection of colorectal carcinogenesis as well. In addition, the process of transformation in colorectal epithelial cells demonstrates similarities with transformation observed in cervical epithelial cells. Our results suggest that similar preventative strategies may prove useful against colon cancer. For example, the application of the pap-smear exam to colorectal epithelial cells may be possible if similar morphological characteristics are demonstrated between cervical and colorectal epithelial cells transformed with HPV. Furthermore, novel vaccines directed against high-risk HPV infections may applicable against colorectal cancer as well; both in males and females. One may also consider Id proteins as markers and therapeutic targets in colorectal cancer as well.

However, much needs to be determined before we can conclusively establish an indubitable role for high-risk HPV infections in colorectal carcinomas. First and

foremost, *in vivo* assessment of the transformation ability of E6 and E7 oncoproteins is essential. Once established, one may assess the affect of high-risk HPV vaccines on the formation of such tumors, *in vivo*. This may prove invaluable with respect to the potential for human treatment. Furthermore, a large tissue micro-array analyzing a statistically significant number of tissues should be tested for the presence of HPVs, Ids, and other potentially implicated proteins. In doing so, one may further decipher the mechanism and proteins involved in HPV induced colorectal carcinogenesis to provide valuable markers and therapeutic targets. More still, our migration assays implied the potential for high-risk HPV infection to promote a metastatic environment in colorectal epithelial cells. However, a matrigel invasion assay is necessary for an assertive connection between HPV and metastatic potential.

In essence, our results provide qualitative, preliminary information with regard to high-risk HPV infection and colorectal carcinogenesis, *in vitro*, and suggest the need for further research. Given the successes against cervical cancer, our results encourage similar approaches in the battle against colorectal cancer. With the potential aforementioned applications, we could improve diagnostic, prognostic and over-all survival in colorectal cancer patients.

Bibliography

A.S. Damin, K. Rachid, C.G. Zettler, M. Caleffi and C.O.P. Alexandre. **Evidence for an association of human papillomavirus and breast carcinomas.** *Breast Cancer Res Treat* (2004); **86**: 131–137.

Al Moustafa AE, Foulkes WD, Wong A, Jallal H, Batist G, Yu Q, Herlyn M, Sicinski P, Alaoui-Jamali MA. **Cyclin D1 is essential for neoplastic transformation induced by both E6/E7 and E6/E7/ErbB-2 cooperation in normal cells.** *Oncogene*. 2004, Jan 15; **23**(2): 350-8.

Alani RM, Hasskarl J, Grace M, Hernandez MC, Israel MA, Münger K. **Immortalization of primary human keratinocytes by the helix-loop-helix protein, Id-1.** *Proc.Natl Acad. Sci.* (1999) **96**: 9637 – 9641.

Anna Lasorella, Gerson Rothschild, Yoshifumi Yokota, Robert G. Russell, and Antonio Iavarone. **Id2 Mediates Tumor Initiation, Proliferation, and Angiogenesis in *Rb* Mutant Mice.** *Mol Cell Biol.* (2005) May; **25**(9): 3563-74.

Antinore MJ, Birrer MJ, Patel D, Nader L, McCance DJ. **The human papillomavirus type 16 E7 gene product interacts with and trans-activates the AP1 family of transcription factors.** *EMBO.* (1996); **15**: 1950-60.

Barone, M. V., Pepperkok, R., Peverali, F. A. & Philipson, L. **Id proteins control growth induction in mammalian cells.** *Proc. Natl Acad. Sci.* (1994) **91**: 4985 – 4988.

Bartkova J, Thullberg M, Slezak P, Jaramillo E, Rubio C, Thomassen LH, Bartek J. **Aberrant expression of G1-phase cell cycle regulators in flat and exophytic adenomas of the human colon.** 1: *Gastroenterology.* (2001) Jun; **120**(7): 1680-8.

Begum S, Gillison ML, Ansari-Lari MA, Shah K, Westra WH. **Detection of human papillomavirus in cervcial lymph nodes: a highly effective strategy for localizing site of tumor origin.** *Clin Cancer Res.* (2003) Dec 15; **9**(17): 6469-75.

Brake T, Lambert PF. **Estrogen contributes to the onset, persistence, and malignant progression of cervical cancer in a human papillomavirus-transgenic mouse model.** *Proc Natl Acad Sci.* (2005) Feb 15; **102**(7): 2490-5.

Burd EM. **Human papillomavirus and cervical cancer.** *Clin Microbiol Rev.* (2003); **16**(1): 1-17.

D. Kirgan, P. Manalo, M. Hall and B. McGregor. **Association of human papillomavirus and colon neoplasm.** *Arch Surg* (1990); **125:** 862–865.

Damin DC, Caetano MB, Rosito MA, Schwartsmann G, Damin AS, Frazzon AP, Ruppenthal RD, Alexandre CO. **Evidence for an association of human papillomavirus infection and colorectal cancer.** *Eur J Surg Oncol.* (2007) Jun; **33**(5): 569-74.

Demers GW, Foster SA, Halbert CL, Galloway DA. **Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7.** *Proc Natl Acad Sci* (1994) May 10; **91**(10): 4382-4386.

Desprez PY, Lin CQ, Thomasset N, Sympson CJ, Bissell MJ, Campisi J. **A novel pathway for mammary epithelial cell invasion induced by the helix-loop-helix protein Id-1.** *Mol. Cell Biol.* (1998) **18**: 4577–4588.

Desprez, P. Y., Hara, E., Bissell, M. J. & Campisi, J. **Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1.** *Mol. Cell Biol.* (1995); **15**: 3398 – 3404.

Fong S, Itahana Y, Sumida T, Singh J, Coppe JP, Liu Y, Richards PC, Bennington JL, Lee NM, Debs RJ, Desprez PY. **Id-1 as a molecular target in therapy for breast cancer cell invasion and metastasis.** *Proc. Natl Acad. Sci.* (2003), **100**: 13543–13548.

Funk JO, Waga S, Harry JB, Espling E, Stillman B, Galloway DA. **Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV16 E7 oncoprotein.** *Genes & Development.* (1997); **11**: 2090-3000

Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D. **Evidence for a causal association between human papillomavirus and a subset of head and neck cancers.** *J Natl Cancer Inst* (2000); **92**: 709–720.

Greenblat R. J. **Human Papillomavirus: Diseases, Diagnosis and a possible vaccine.** *Clin. Microbiology Newsletter.* (2005) Sept 15; **27**(18); 139-145.

Hara E, Yamaguchi T, Nojima H, Ide T, Campisi J, Okayama H, Oda K. **Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts.** *J. Biol. Chem.* (1994); **269**: 2139 – 2145.

Hasskarl J, Duensing S, Manuel E, Münger K. **The helix-loop-helix protein ID1 localizes to centrosomes and rapidly induces abnormal centrosome numbers.** *Oncogene.* (2004) Mar 11; **23**(10): 1930-8.

Herber R, Liem A, Pitot H, Lambert PF. **Squamous epithelial hyperplasia and carcinoma in mice transgenic for the human papillomavirus type 16 E7 oncogene.** *J Virol.* (1996) Mar; **70**(3): 1873-81.

J.Y. Cheng, L.F. Sheu, C.L. Meng, W.H. Lee and J.C. Lin, **Detection of human papillomavirus DNA in colorectal carcinomas by polymerase chain reaction.** *Gut* (1995); **37**: 87–90.

K.J. Syrjanen. **HPV infections and oesophageal cancer.** *J Clin Pathol* (2002); **55**: 721–728.

K.R. Shroyer, J.G. Kim, M.M. Manos, C.E. Greer, N.W. Pearlman and W.A. Franklin. **Papillomavirus found in anorectal squamous carcinoma, not in colon adenocarcinoma,** *Arch Surg* (1992); **127**: 741–744.

K.V. Shah, R.W. Daniel, J.W. Simon and B. Vogelstein. **Investigation of colon cancers for human papillomavirus genomic sequences by polymerase chain reaction.** *J Surg Oncol* (1992); **51**: 5–7.

Kanao H, Enomoto T, Ueda Y, Fujita M, Nakashima R, Ueno Y, Miyatake T, Yoshizaki T, Buzard GS, Kimura T, Yoshino K, Murata Y. **Correlation between p14ARF/p16INK4A expression and HPV infection in uterine cervical cancer.** *Cancer Lett.* (2004) Sep 15; **213**(1): 31-7.

Kee, Y. & Bronner-Fraser, M. **To proliferate or to die: role of Id3 in cell cycle progression and survival of neural crestprogenitors.** *Genes Dev.* (2005); **19**: 744–755.

L.O. Perez, M.C. Abba, R.M. Languens and C.D. Golijow. **Analysis of adenocarcinomas of the colon and rectum: detection of human papillomavirus (HPV) DNA by polymerase chain reaction.** *Coloretal Dis.* (2005); **5**: 492–495.

Lasorella, A., Noseda, M., Beyna, M., Yokota, Y. and Iavarone, A. **Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins.** *Nature.* (2000): **407**: 592-598.

Li B, Dou QP. **Bax degradation by the ubiquitin/proteasome-dependant pathway: involvement in tumor survival and progression.** *Proc Natl Acad Sci* (2000) Apr 11; **97** (8): 3850-3855.

Longworth MS, Laimins LA. **The binding of histone deacetylases and the integrity of zinc finger-like motifs of the E7 protein are essential for the life cycle of human papillomavirus type 31.** *J Virol.* (2004); **789**: 3533-3541.

Lopez-Beltran, Al. Escudero, J.C. Carrasco-Aznar and L. Vicioso-Recio. **Human papillomavirus infection and transitional cell carcinoma of the bladder.** *Pathol Res Pract* (1996); **192**: 154–159.

McGregor, P. Byrne, D. Kirgan, J. Albright, P. Manalo and M. Hall. **Confirmation of the association of human papillomavirus with human colon cancer.** *Am J Surg* (1993); **163**: 738–742.

Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J. **Genes that mediate breast cancer metastasis to lung.** *Nature.* (2005) Jul 28; **436** (7050): 518-524.

Norton, J. D. & Atherton, G. T. **Coupling of cell growth control and apoptosis functions of Id proteins.** *Mol. Cell Biol.* (1998); **18**: 2371–2381.

Noya F, Chien Wm, Broker TR, Chopw LT. **p21cip1 degradation in differentiated keratinocytes is abrogated by costabilization with cyclin E inuced by human papillomavirus E7.** *J Virol.* (2001); **75**: 6121-6134.

Ohtani N, Zebedee Z, Huot TJ, Stinson JA, Sugimoto M, Ohashi Y, Sharrocks AD, Peters G, Hara E. **Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence.** *Nature* (2001); **409**: 1067–1070.

Prabhu, S., Ignatova, A., Park, S. T. & Sun, X. H. **Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins.** *Mol. Cell Biol.* (1997); **17**: 5888–5896.

Riley RR, Duensing S, Brake T, Munger K, Lambet PF, Arbeit JM. **Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis.** *Cancer Res.* (2003) Aug 15; **63**(16): 4862 – 71.

Rockman SP, Currie SA, Ciavarella M, Vincan E, Dow C, Thomas RJ, Phillips WA. **Id2 is a target of the beta-catenin/T cell factor pathway in colon carcinoma.** *J Biol Chem.* (2001) Nov 30; **276**(48): 45113-45119.

Sdek P, Zhang ZY, Cao J, Pan HY, Chen WT, Zheng JW. **Alteration of cell-cycle regulatory proteins in human oral epithelial cells immortalized by HPV16 E6 and E7.** *Int J Oral Maxillofac Surg.* (2006) Jul; **35**(7): 653-657.

Song S, Pitot HC, Lambert PF. **The human papillomavirus type 16 E6 gene alone is sufficient to induce carcinomas in transgenic animals.** *J Virol.* (1999) Jul; **73**(7): 5887 – 5893.

Stanley M.A, **Human papillomavirus vaccines,** Reviews in medical virology, (2006); 16: 139-149.

Tang J, Gordon GM, Nickoloff BJ, Foreman KE. **Id-1 delays senescence but does not immortalize keratinocytes.** *J. Biol. Chem.* (2000); **275**: 27501– 27504.

Tang, J., Gordon, G. M., Nickoloff, B. J. & Foreman, K. E. **The helix-loop-helix protein id-1 delays onset of replicative senescence in human endothelial cells.** *Lab Invest.* (2002); **82**: 1073–1079.

Tetsu O, McCormick F. **Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells.** *Nature.* (1999) Apr 1; **398**(6726): 422-426.

Thomas M, Banks L. **Inhibition of bak-induced apoptosis by HPV-18 E6.** *Oncogene.* (1998); **17**: 2943-2954.

Thomas M, Laura R, Hepner K, Guccione E, Sawyers C, Lasky L, Banks L. **Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degredation.** *Oncogene.* (2002); **21**: 5088-5096.

Tommasino M, Crawford L. **Human papillomavirus E6 and E7: proteins which deregulate the cell cycle. Bioessays.** (1995) Jun; **17**(6): 509-518.

Umetani N, Takeuchi H, Fujimoto A, Shinozaki M, Bilchik AJ, Hoon DS. **Epigenetic inactivation of ID4 in colorectal carcinomas correlates with poor differentiation and unfavorable prognosis.** *Clinical cancer research.* (2004); **10**: 7475-7483.

Umudum H, Rezanko T, Dag F, Dogruluk T. **Human papillomavirus genome detection by in situ hybridization in fine-needle aspirates of metastatic lesions from head and neck squamous cell carcinomas.** *Cancer.* (2005) Jun 25; **105**(3): 171-178.

Varnai AD, Bollmann M, Griefingholt H, Speich N, Schmitt C, Bollmann R, Decker D. **HPV in anal squamous cell carcinoma and anal intraepithelial neoplasia (AIN). Impact of HPV analysis of anal lesions on diagnosis and prognosis.** *Int J Colorectal Dis.* (2006) Mar; **21**(2): 135-42.

Venuti A, Badaracco G, Rizzo C, Mafera B, Rahimi S, Vigili M. **Presence of HPV in head and neck tumours: high prevalence in tonsillar localization.** *J Exp Clin Cancer* Res. (2004) Dec; **23**(4): 561-566.

Vermeulen K, Van Bockstaele D. R, Berneman Z. N. **The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer.** *Cell Prolif.* (2003); **36**: 131 – 149.

Voet D, Voet J.G, **Biochemistry**, John Wiley & Sons, Inc. (2005). 4th Ed.

Y.M. Lee, S.Y. Leu, H. Chiang, C.P. Fung and W.T. Liu. **Human papillomavirus type 18 in colorectal cancer.** *J Microbiol Immunol Infect* (2001); **34**: 87–91.

Yasmeen A, Bismar T.A, Dekhil H, Ricciardi R, Kassab A, Gambacorti P.C. and Al Moustafa A. **ErbB-2 receptor cooperates with E6/E7 oncoproteins of HPV type 16 in breast tumorigenesis.** *Cell* Cycle. (2007b) Dec, In Press.

Yasmeen A, Bismar TA, Kandouz M, Foulkes WD, Desprez PY, Al Moustafa AE. **E6/E7 of HPV type 16 promotes cell invasion and metastasis of human breast cancer cells.** *Cell Cycle.* (2007c) Jun; **6**(16): 2038-2042.

Yasmeen A, Hosein AN, Yu Q, Al Moustafa AE. **Critical role for D-type cyclins in cellular transformation induced by E6/E7 of human papillomavirus type 16 and E6/E7/ErbB-2 cooperation.** *Cancer Sci.* (2007a) Jul; **98**(7): 973-977.

Online references:

www.arhp.org/healthcareproviders/cme/onlinecme/hpvcp/infection.cfm: *Association of Reproductive Health Professionals* (2003)

www.cancer.ca/ccs/internet/standard/0,3182,3543_14279__langId-en,00.html: *Canadian Cancer Society* (2007)

www.cancerweb.ncl.ac.uk/: *CancerWeb* (1995-1998)

www.Gardasil.com: *Merck & Co.* (2008)

www.ncbi.nlm.nih.gov/books/bv.fcgi?call=bv.View..ShowTOC&rid=mcb.TOC&depth=10 *W.H Freeman & Co.* (2000)

www.wikipedia.org/wiki/Cervical_cancer: *Wikimedia Foundation Inc.* (2008)