Strong Inverse Correlation Between MicroRNA-125b and Human Papillomavirus DNA in Productive Infection

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Abstract

Infection by the human papillomavirus (HPV) is a cause of cervical intraepithelial neoplasia (CIN) and cancer. microRNA (miRNA) in situ analysis of the transformation zone epithelia, the site of initial cervical HPV infection, showed that miRNAs let-7c, — 99a, 26a, and 125b were the most abundantly expressed. In situ testing of CIN 1 showed a dramatic reduction in miR-125b expression in the koilocytes, the cytologic marker of productive HPV infection. A marked reduction in miR-125b was likewise observed in the HPV-infected cells of the condyloma acuminatum, verruca vulgaris, and epidermodysplasia verruciformis. Reverse transcriptase in situ polymerase chain reaction (PCR) showed that the pre-miRNA 125b was present in the koilocyte, suggesting direct inactivation of the mature miRNA. HEK cells transfected with only the antimiR-125b showed perinuclear halos equivalent to HPV-infected koilocytes. NIH 3T3 cells transfected with the HPV 16 full-length genome and mimetic miR-125b showed a marked reduction in viral DNA and protein synthesis by quantitative PCR and in situ-based analyses, respectively (*P*=0.002). Alternatively, cotransfection with anti-miR-125b and HPV 16 markedly increased HPV DNA (*P*=0.002). Sequence analyses showed strong homology between L2 of different HPV genotypes and miR-125b. Transfection with HPV 16 L2 resulted in a marked reduction in miR-125b levels in the NIH 3T3 cells. HPV L2-induced inactivation of miR-125b is associated with the classic cytologic changes of the koilocyte, and the exogenous application of mimetic miR-125b markedly inhibits HPV DNA synthesis.

Keywords

human papillomavirus; microRNA; in situ hybridization; L2

Human papillomavirus (HPV) infection is one of the most common sexually transmitted diseases. Early, productive HPV infection of the cervix (cervical intraepithelial neoplasia grade 1, CIN 1) is marked by disorganized cell growth, nuclear atypia, and the classic perinuclear halo creating the diagnostic koilocyte.[1–4] The koilocytic cell contains hundreds to thousand of copies of the HPV genome in the nucleus, and has been associated with over 25 different HPV types.[2,3] The analogous histologic/cytologic changes at other sites owing to productive HPV infection include condyloma acuminatum (vulva, vagina, penis, perianal region) that is associated with HPVs 6 and 11, verruca vulgaris (nonsexually transmitted with nongenital tract, often fingers and face), owing primarily to HPV 2, and epidermodysplasia verruciformis (sun exposed skin), a disease affecting immunocompromised patients as a result of HPVs 5 and 8.[4] HPV ORFs E6 and E7 are classic viral oncoproteins that function, in part, by inactivating p53 and retinoblastoma (Rb) antioncogenes.[5,6] At present, there is no antiviral agent that can reduce HPV DNA proliferation in vitro. The purpose of this paper was to determine whether there was a strong association between HPV DNA proliferation and a specific microRNA(s), with a focus on the koilocyte as the marker of high HPV copy number, to explore possible anti-HPV effects of microRNAs.

miRNAs are small, noncoding sequences of approximately 20 to 23 nucleotides that regulate cell processes by inactivating specific mRNAs by annealing to their 3′ untranslated region. miRNAs are capable of serving as "master switches" of many cell processes, including oncogenesis, by being able to modulate the expression of a series of functionally integrated mRNAs/proteins.[7,8]

It has been documented that certain miRNAs play a central role in the host's susceptibility to viral infection. One example is miRNA-122 and hepatitis C infection.[9] To our knowledge, no study has examined miRNA expression in productive HPV infection. This study shows that miR-125b has a central role in productive HPV infection.

Materials and Methods

Cell and Tissue Samples

The target cells of HPV infection in the cervix are the metaplastic and squamous cells in the transformation zone.[2,3] Hence, we obtained a series of TMAs of formalin-fixed, paraffin-embedded cervical tissues that showed histologically unremarkable transformation zones. All tissues were obtained through an approved protocol from the Ohio State University Internal Review Board. The resultant miRNA in situ data were compared with TMAs of CIN 1 lesions. The mean number of normal tissues studied for microRNA expression in the TMAs was 69, whereas the median number for the CIN 1 lesion analyses was 39. To determine whether the dysregulation of the miRNA expression patterns in CIN 1 was a

phenomenon generalized to early HPV infection, we also examined 10 condyloma acuminata, 5 verruca vulgaris, and 4 epidermodysplasia verruciformis lesions.

We studied the cell line HEK (ScienCell Research Laboratories, Carlsbad, CA), which is derived from the normal squamous cells of the lower genital tract, and NIH 3T3 cells (ATCC), a mouse fibroblast cell line that is susceptible to infection by the related virus bovine papillomavirus.[10] The latter cell line grew much more rapidly as it was more amenable to study HPV DNA synthesis. The HEK and NIH 3T3 cells were cultured in keratinocyte medium and Dulbecco's modified Eagle's medium plus 10% fetal bovine serum, respectively. After growth/transfection, the cells were fixed for 8 to 15 hours in 10% buffered formalin for in situ analysis, Trizol reagent (Invitrogen, Carlsbad, CA) for miRNA analysis, or Qiagen Plasmid Midi Kit (Valencia, CA) for DNA quantitative PCR analysis of HPV 16 copy number.

Transfection of Mimetic and Anti-miRNA-125b and of HPV 16 L2 ORF

Lipofectamine transfection of miRNA-125b was carried out using a protocol published earlier.[11] The protocol was modified using a 5′ digoxigenin tagged LNA-modified microRNA mimetic (TCCCTGAGACCCTAAC TTGTGA) and antimiR-125b (TCACAAGTTAGGGT CTCAGGGA) at concentrations of 100 nM and 50nM, respectively, to document the efficiency of the transfection; all probes were from Exiqon (Woburn, MA).

The full length of the HPV-16 L2 viral gene ($\sim$ 1.4 Kb) was inserted into the pcDNA3.1/ Zeo(+) (Invitrogen) vector. The primers used to clone the full-length ORF were forward: TGGCAAGCTTATGCGACACA AACGT and reverse: CTGCGAATTCCTAGGCAGC CAAAG. NIH 3T3 cells (2×10^5) were seeded in 6-well tissue culture plates supplemented with Dulbecco's modified Eagle's medium media containing 10% fetal bovine serum. One milligram and 3 μg plasmids were used to transfect the cells by lipofectamine 2000 (Invitrogen) according to the standard protocol.[11] After 48 hours transient transfection, total RNA were isolated by Trizol and used to detect miRNA expression.

In Situ Hybridization/RT In Situ PCR Analyses

Our in situ hybridization protocol for HPV 16 DNA detection has been published earlier.[3] We used two different HPV 16 in situ systems. One was the manual system (Pathogene HPV tissue typing) from Enzo Clinical Laboratory (Farmingdale, NY) and the other the automated system (HPV HR III system) from Ventana Medical Laboratories (Tuscon, AZ); they yielded equivalent results. Our in situ protocol for the in situ detection of microRNAs has also been published.[12,13] We used the 5′ digoxigenin tagged LNA modified microRNA probes from Exiqon (Woburn, MA). For each in situ method, the chromogen was nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP) that gives a blue signal in comparison to the pink of the counterstain nuclear fast red. Similarly, the reverse transcriptase RT in situ polymerase chain reaction (PCR) protocol we used to detect the pre-miRNA-125b and pri-miRNA-125b has been published earlier;[12] the following primers were used: GTCCCTGAGACCCTAACTTG, AG CCTAACCCGTGGATTT, and CAGAAATTGCCTGTC ATTCTT.

We initially focused on the normal transformation zone of the cervix, as this is the site of initiation of over 95% of CIN 1 lesions.[1–4] We carried out in situ hybridization for the following miRNAs because they were reported to be produced in high abundance in the normal cervix: miRNAs 125b, 26a, let7c, and 99a.[14] We also examined miRNAs reported to be produced either in intermediate/low abundance or not at all in the cervix (miRNAs-200a, 302a,b,c,d, 96, 802, 21, 155, 29b, 205; 206, 221, 222, 130a,b, 133a, 1, 16, 19b, 126, 128a, 143, 145, and 34b).[14]

Immunohistochemical/Colabeling Analyses

Our immunohistochemical protocol has been published earlier.[12] We used the Ultrasensitive Universal Fast Red or DAB system (Benchmark LT) from Ventana Medical Systems (Tuscon) according to the manufacturer's specifications. Colabeling of the miRNA and protein (p53 and p16) was carried out according to our recently published protocol[12] using the Nuance system from CRI (Boston, MA) according to their specifications. Similarly, the antibodies [p53 (Ventana) and the p16 from MTM (Heidelberg, Germany)] were pre-made and used according to the manufacturer's recommendations.

qPCR Analysis for HPV 16 DNA and qRT-PCR Analysis for miR-125b

Both HPV 16 DNA and miR-125b were detected by Taqman real-time PCR. The primers and probe sequence for the quantification of HPV 16 DNA were HPV 16 forward primer: TTGCAGATCATCAAGAACACGTAGA; the HPV 16 reverse primer: CAGTAGAGATCAGTTGT CTCTGGTTGC; and the HPV 16 probe: FAM-AATCATGCATGGAGATACACCTACATTGCATGA-TAMRA[15] and mouse β-actin as internal control P (Applied Biosystems, Foster City, CA). All experiments were conducted in triplicate for qPCR testing, with an additional well for HPV DNA and protein expression by in situ analyses. The real-time stem-loop RT-PCR analysis for miR-125b expression was designed according to the protocol [11] with the 5s RNA as the internal control. The miR-125b forward primer was: GCCCTCCCTGAGACCCTAAC, the miR-125b reverse primer: GTGCAGGGTCCGAGGT and the probe for the qRT-PCR for miR-125b: FAM-TGGATACGACTCACA AGT-MGB.

Results

Cases were scored as positive by in situ hybridization for a given microRNA if at least 10% of the epithelial cells (both mature squamous and metaplastic squamous cells) in the transformation zone yielded a signal. Thus, many cases were deemed negative even if the miRNA was produced in abundance in non-HPV target cells, such as inflammatory cells. One notable example was miR-155, produced in abundance in the inflammatory cells, which is consistent with its documented role in immune response[16] (Fig. 1D). In comparison, panels 1E and 1F show the abundant expression of miR-125b and miR-let-7-c in the epithelia cells of the normal transformation zone, respectively. Note that in each case the signal is cytoplasmic in the basal cells and nuclear-based in the cells in the middle and superficial aspect of the epithelia. A summary of our data for the epithelia of the transformation zone is presented in Figure 1A to C, blue bar. The n values were miR-1 (74), miR-16 (136), miR-19b (41), miR-125b (84), miR-26a (98), miR-let-7-c (68), miR-99a (60),

miR-126 (26), miR-34b (31), miR-205 (56), miR-206 (103), miR-451 (33), miR-221 (101), miR-222 (45), miR-130a (71), miR-133a,b (66), miR-200a (76), miR-302a,b,d (68), miR-802 (68), miR-21 (82), miR-155 (79), miR-145 (16), miR-29b (103), scrambled probe (72), and RNA consensus probe (68).

We theorized that microRNAs important for the onset of HPV infection in the cervix would be markedly upregulated or downregulated in the koilocytes relative to the data in the epithelia of the transformation zone. Thus, we then examined the same 24 miRNAs noted in Figure 1 by in situ hybridization in CIN 1 lesions with the data presented in Figure 1A-C (red bar); a positive result required that at least 10% of dysplastic (HPV-infected) cells have a signal. Note that miR-206, 200a, and 155 were increased in the dysplastic cells of the CIN 1 lesions. Further, the expression of miR-451, miR-1, and miR-19b were significantly decreased in the CIN 1 lesions (P values from 0.010 to 0.005). However, the most dramatic difference was evident with miR-125b that was nearly completely eliminated in the CIN 1 lesions ($P<0.001$). This marked reduction was also noted in CIN 2 lesions (0/10); however, miR-125b expression was much increased in invasive cervical cancers (27/33=81%) (data not shown). The n values were miR-1 (30), miR-16 (31), miR-19b (16), miR-125b (54), miR-26a (39), miR-let-7-c (78), miR-99a (68), miR-126 (20), miR-34b (23), miR-205 (44), miR-206 (33), miR-451 (49), miR-221 (57), miR-222 (39), miR-130a (32), miR-133a,b (28), miR-200a (30), miR-302a,b,d (53), miR-802 (21), miR-21 (43), miR-155 (37), miR-145 (29), miR-29b (40), scrambled (41), and RNA consensus probe (39).

CIN 1 is associated with over 25 distinct HPV genotypes.[1–4] To determine whether the marked reduction in miR-125b expression in CIN 1 lesions was a more generalized phenomenon of productive HPV infection, we analyzed a series of condyloma acuminatum, verruca vulgaris, and epidermodysplasia verrucaformis. In each case the corresponding in situ hybridization test for HPV DNA showed a very high copy number (Fig. 2F). Of the total of 19 cases of verruca vulgaris, condyloma acuminatum, or epidermodysplasia verrucaformis, miR-125b was not present in 15 cases (Fig. 2A) and very weakly expressed in rare cells in the other 4 cases. In comparison, miR-125b was highly expressed, as expected, in normal vulvar epithelium (10 cases) (data not shown). The HPV-infected cells did express high copy numbers of other miRNAs, such as miR-let-7-c, miR-99a, and miR-26a (Fig. 2D).

We addressed whether the marked reduction in miR-125b was a result of reduced synthesis of the pri-miR-125b or pre-miR-125b by carrying out RT in situ PCR using primers that can detect either molecule. RT in situ PCR did document that the precursors to miR-125b were present in the nucleus of the HPV-infected cells of the CIN 1 and condyloma lesions, indicating that the reduced miR-125b was a result of a direct effect on the mature miRNA per se (data not shown). To determine whether the marked miR-125b reduction was specific for HPV-infected cells, or a response to viral infections in general, we examined 13 tissues from either the genital tract or the oral cavity (also susceptible to HPV infection [2,3]) infected with herpes simplex virus (n=4), molloscum contagiosum (n=6), or EBV (n=3). In each case, the cells that contained high copy numbers of the respective virus were highly positive for miR-125b (Fig. 2 B, C).

The marked reduction in miR-125b in the koilocyte next led us to examine p16 expression, as overexpression of this protein is an indicator of cell transformation and, in the cervix, suggests a high-risk HPV infection as its overexpression is expected with pRb inactivation secondary to ample ORF E7 production.[2,3,16,17] This is underscored in Figures 2E and F, where it can be observed that the histologic pattern of HPV DNA was equivalent to that of p16. In addition, p53 is an important component of HPV infection,[5] and has been shown to be down-regulated by miR-125b.[18] Thus, we carried out colocalization of miR-125b and p16, as well as p53, to determine whether the presence of the miRNA and either of these 2 tumor suppressor genes was mutually exclusive. Ten CIN 1 lesions and 10 normal transformation zones were analyzed for each protein, and in each of the analyses the p16-positive or p53-positive cells did not colabel with miR-125b. In comparison, it was evident that other miRNAs, such as miR-let-7-c, did colocalize with p16 or p53 (Figs. 2G, H). Although this raises the possibility that miR-125b may be regulating p16, as it regulates p53,[18] the association may not be direct, but rather through other intermediaries. As the focus of this paper was miRNA expression as it relates to early HPV infection, the basis of this association was not further explored.

Given that the classic cytologic feature of early HPV infection is the koilocyte, we next addressed the issue of whether the inactivation of miR-125b in normal squamous cells would induce the koilocytotic change without the virus. We cultured HEK cells and determined by in situ hybridization that they were HPV-negative (data not shown). We then transfected these cells with either the digoxigenin-tagged antimiR-125b or a tagged scrambled miRNA. Two days after transfection, qRT-PCR showed that there was a 6.7-fold reduction in the amount of miR-125b in the HEK cells transfected with anti-miR-125b versus the scrambled miRNA (Fig. 3A). Scattered miR-125b-transfected HEK cells (and none transfected with the scrambled miRNA) showed a large, well-defined perinuclear halo characteristic of an HPV-infected cell (Fig. 3B). The tagged anti-miR-125b localized specifically to the perinuclear halo (Fig. 3C), as did p16 (Fig. 3C), suggesting that the formation of the halo per se was related to the loss of activity of miR-125b.

Given the absence of miR-125b in HPV-infected cells, we next addressed the following question: Can transfection with mimetic miR-125b reduce HPV proliferation in a cell culture model? We transfected 0.8μg/mL of genomic HPV 16 genome into the 3T3 cells and compared the detection rate of HPV 16 alone with HPV 16 with the mimetic miR-125b at days 3 and 6. The analyses were first carried out by qPCR. As evident in Figure 3D, there was a highly significant reduction at both time points in HPV 16 DNA synthesis as a result of cotransfection with mimetic miR-125b.

To corroborate the qPCR data, and to visualize HPV DNA and protein expression in the cells per se, we next carried out in situ analyses. Specifically, we compared the in situ detection rate of HPV transfection alone with the empty vector (puC19), and with HPV 16 with either the mimetic miR-125b or antimiR-125b (each digoxigenin tagged) at days 3 and 6 days. Analysis for the digoxigenin tag after transfection showed that 43% and 45% of the 3-day transfected cells contained the mimetic-125b or anti-miR-125b, respectively. The HPV in situ hybridization data are presented in Table 1. Note that at days 3 and 6 there was a significant decrease in the percentage of cells that contained detectable HPV 16 DNA if

cotransfected with the mimetic miR-125b. As just indicated, 43% of the 3T3 cells at day 3 contained detectable mimetic digoxigenin miR-125b and 36% of cells transfected only with HPV 16 contained detectable viral DNA. Thus, the percentage of cells that would theoretically contain both HPV 16 and the mimetic miR-125b is 16%. Hence, the efficiency of the mimetic transfection in reducing HPV 16 proliferation would equal the measured reduction in HPV 16 proliferation/ theoretical maximum efficiency=15/16=94%. Also note that the anti-miR-125b when cotransfected with HPV 16 markedly increased the percentage of cells with detectable HPV 16 DNA (Table 1) at 3 days. The cells were too degenerated at day 6 for evaluation, which could reflect cell death from the much-increased viral DNA synthesis.

To determine whether a specific HPV transcript could bind to miR-125b and thus, potentially inactivate it, we conducted a search using RNA hybrid version 2.2.[19] This search did indeed show that the L2 region of the HPV genotypes examined in this study (HPV 2, 5, 6, 8, and 16) shared high homology with miRNA-125b, and thus was capable of forming a strong hybridized complex under physiologic conditions (Fig. 3E). To further investigate the putative relationship between miR-125b and HPV L2 ORF, we transfected the NIH 3T3 cells with HPV ORF L2 versus the empty vector and then measured the relative expression of miR-125b by qRT-PCR. The primers used to clone the L2 fragment were (forward): tggcAAGCTTatgcgacacaaacgtt and (reverse): ctgcGAAT TCctaggcagccaaag. The data are presented in Figure 3 F and shows that L2 transfection much reduced miR-125b expression relative to the negative control (empty vector).

Discussion

miR-125b is capable of markedly reducing HPV DNA synthesis in NIH 3T3 cells that are susceptible to papillomavirus infection.[10] This miRNA is highly conserved among mammals, fish, and fowl (http://microrna.sanger.ac.uk/sequences/). It has been shown to be downregulated in a variety of cancers and to have possible targets that include several oncogenes, such as the growth factor receptor FGFR2, and several members of the mitogen-activated signal transduction pathway.[20] To our knowledge, there has been no documented interaction between miR-125b and any antiviral host defense. Nonetheless, we were able to provide evidence that miR-125b has an important role in the marked viral proliferation that is obligatory in productive (high-copy) HPV infection. Further, a near 7-fold decrease in miR-125b through transfection of the antimiRNA in normal genital tract squamous cells led to koilocytes, even though no viral DNA was present, suggesting that the viral-induced inactivation of this microRNA is responsible for the perinuclear halo in the Pap smear that signals the presence of the virus.

The papillomavirus ORF L2 is a logical target for a microRNA. L2 is responsible for wide-spectrum immunity against HPV and other papillomaviruses, such as bovine papillomavirus.[21] Further, L2 protein plays a critical role by binding to cellular elements T(κ, α and β) and HPV DNA to the import of viral DNA to the nucleus. The nuclear import is dependent on the interaction of L2 with the microtubule motor protein dynein.[22–24] Importantly, mutations in L2 lead to a 10 fold decrease in viral DNA copy number per virion and a 100-fold decrease in viral infectivity. [25] Thus, it is likely that miR-125b inactivation of

L2 through RNA-RNA complexing would reduce the infectivity of the virus, and this is consistent with our finding that exogenous mimetic miR-125b greatly reduced HPV DNA synthesis, presumably, in part, by not allowing synthesis of the L2 protein in the nucleus with the concomitant loss of viral infectivity. This process is highly specific for miRNA-125b, as miR-99a and let-7-c had no relationship with HPV DNA proliferation, on the basis of the in situ data, yet are on the same chromosome[22] as miR-125b and only approximately 2000 base pairs apart (Elton T, personal communication). Further in situ-based evidence of an interaction between miR-125b and HPV L2 RNA is that the latter localizes to the nucleus of the mid- and superficial zone of CIN 1 as shown by Stoler et al,[26] which is the same distribution we noted for miR-125b in the transitional zone epithelia. However, we did note that miR-125b was also not evident in the basal cells of CIN 1 lesions where HPV L2 RNA is usually not present. Whether this reflects relatively less miR-125b synthesis in the basal cells relative to the mid/superficial cells, as evident in Figure 1F, will require more study. On the basis of this information, we present a schematic theoretical model for the role of HPV L2 and miR-125b in early HPV infection in Figure 3 G and H.

miR-125b can inactivate p53.[18] This is consistent with our in situ colocalization data showing that p53 and miR-125b were expressed in mutually exclusive cervical epithelial cells. Hence, inactivation of miR-125b by HPV may result in a concomitant increased expression of this tumor suppressor gene, which, presumably, would be disadvantageous to continued viral proliferation. This may explain the fact that HPV 16 E6 directly targets p53. Interestingly, the benign HPV types 6 and 11 E6 are not effective in targeting p53,[5] and it is possible that the increased p53 expression found in these tissues, in part resulting from miR-125b inactivation, may relate to the very low risk of progression to cancer with these genotypes.[1–4]

The majority of HPV types, including HPVs 1, 2, 5, 8, 13, 30, 31, 33, 35, 42, 43, 44, 45, 51, 52, 56, 68, and 70, associated with human disease are not those included in the HPV vaccine.[2] The current strategies for treating the productive manifestation of HPV infection (CIN 1, genital tract condyloma acuminatum, and nongenital tract verruca vulgaris) include cryotherapy, laser therapy, and topical keratinolytic agents. The recurrence rate of condyloma acuminatum and verruca vulgaris treated by any of these modalities is approximately 30% to 50%.[2,3] The usual treatment for CIN 1 (laser therapy) is expensive and has a recurrence rate of approximately 15%.[2,3] Our data suggest that direct treatment at the mucosal or cutaneous sites of HPV infection with mimetic miR-125b may be able to reduce viral infectivity, as such infections are marked by continuous infection of squamous cells contiguous with the lesion.[2,3] Clearly, this will require additional testing with clinical trials.

It is well documented that CIN and thus cervical cancer are sexually transmitted diseases.[1–4] Thus, the histologic diagnosis of CIN will have strong emotional ramifications for the patient. Many studies have shown that the false-negative diagnosis of CIN on biopsy is approximately 10%, whereas the false-positive diagnosis of CIN on biopsy may be as high as 20%, depending on the study, and the expertise of the laboratory in making this diagnosis.[2,3] Immunohistochemistry for p16 in cases equivocal for CIN 1 is commonly carried out, but has a high false-positive and negative rate.[2,17] Our data suggest a possible

role for the in situ detection of miR-125b in pathologically equivocal lesions as a way to differentiate early cytologically "borderline" HPV infection from its mimics.

Several recent studies have shed some important light on the possible interactions between HPV and microRNAs. Wang et al[27] showed that HPV infection in cancer cells is able to negatively influence miR-34a levels through E6 inhibition of p53. Martinez et al[28] recently showed that E6/E7 can downregulate the tumor suppressor miR-218. However, these studies have focused on cervical cancers, whereas we focused on the initiation of HPV infection. The virology of HPV 16 is markedly different in cervical cancers, where viral DNA is integrated, typically low copy, and L1/L2 transcription very low.[2–4] Indeed, we noted that miR-125b is not decreased in cervical cancer, which may relate, in part, to the paucity of L2 expression in these tumors.[2]

In sum, we have shown that miR-125b can directly and markedly reduce HPV DNA proliferation in an in vitro model. This is consistent with the near total eradication in vivo of miR-125b in the koilocyte from a wide variety of sites of clinically obtained tissues infected by HPV. A corollary of this observation was that inactivation of miR-125b without the virus induces the cytologic changes of the koilocyte. These observations should induce clinical studies to determine whether mimetic miR-125b therapy is as effective an inhibitor of HPV proliferation in vivo as is it in vitro.

Acknowledgments

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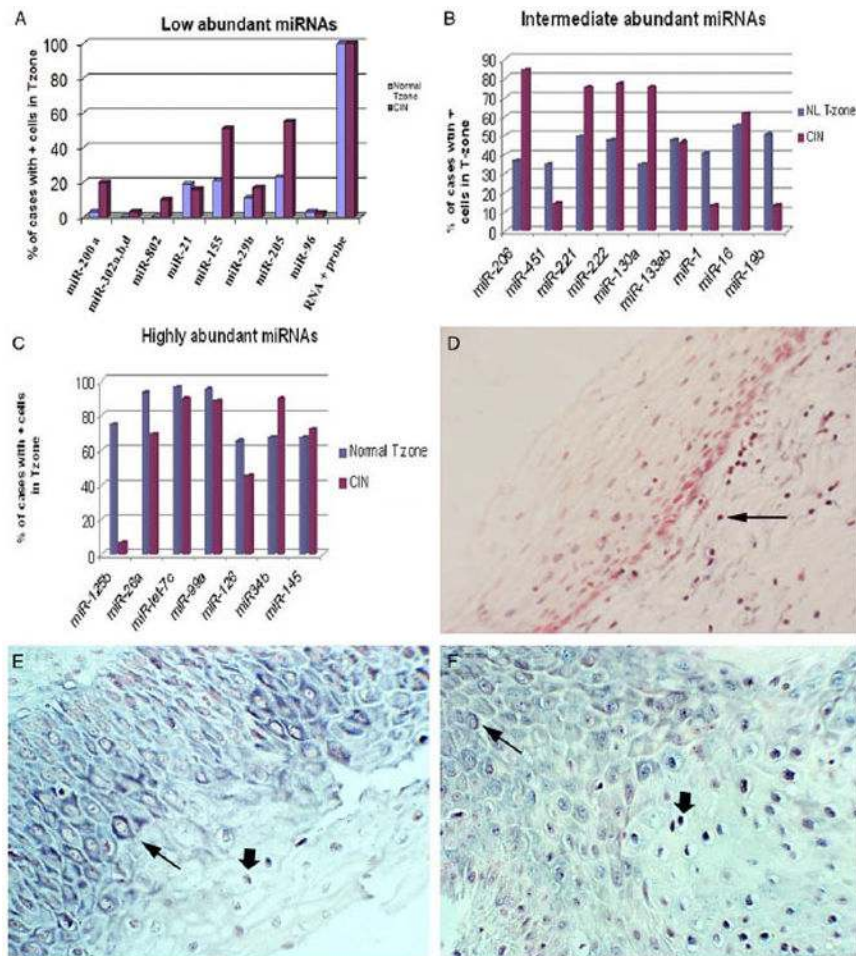
Figure 1.
The in situ detection of microRNAs in the transformation zone of the cervix. Panels A to C show the percentage of cases of normal transformation zone (blue bar) or cervical intraepithelial neoplasia 1 (CIN 1) in the zone (red bar) for a variety of miRNAs as determined by in situ hybridization. The results are stratified according to low, intermediate, and highly abundant miRNAs in the transformation zone, respectively. Panel D shows the detection of miR-155 in the stromal inflammatory cells (arrow), but not the epithelia of the zone. Panels E and F show serial sections of the normal squamous epithelia of the transformation zone analyzed for miR-let-7-c and miR-125b, respectively. Note that the signal toward the basal zone (large arrow) is cytoplasmic and stronger for miR-let-7c, whereas the signal in the mid-to-superficial zone (arrowhead) is nuclear and more intense for miR-125b.
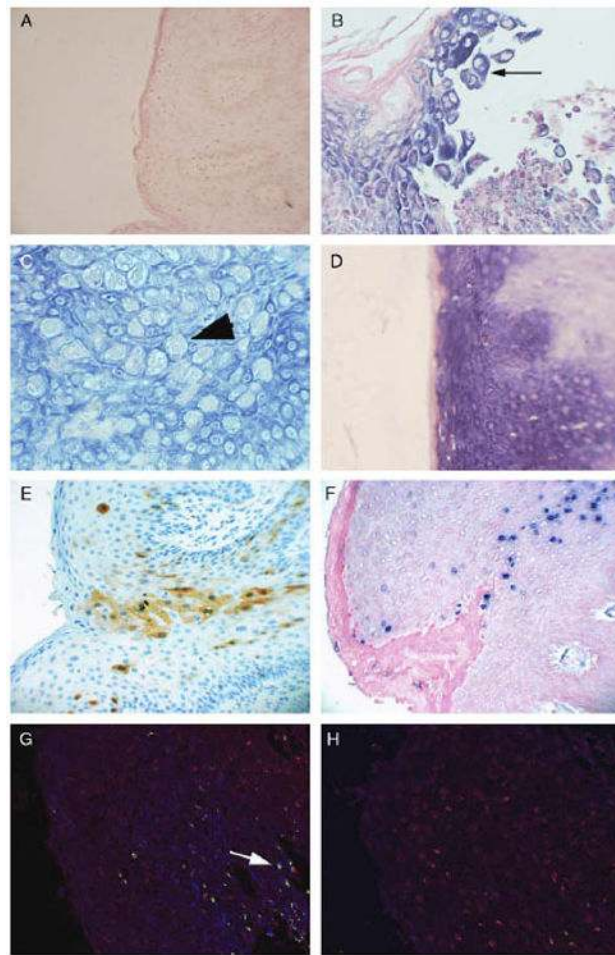
Figure 2.
The in situ detection of miR-125b in viral infections of the genital tract and correlation to p16 and p53 detection. Panel A shows an area of a vulvar condyloma that was negative for miR-125b. In comparison, note the strong signal in the same lesion for miR-26a (panel D). The loss of miR-125b expression in the genital tract was unique to productive HPV infection, as an intense signal for this miRNA was evident in the exfoliating cells with large, glassy nuclei diagnostic of herpes simplex virus infection (panel B, arrow) and in the vulvar epithelia with the large cytoplasmic inclusions classic for molloscum contagiosum (panel C). Whereas miR-125b was much reduced in condyloma, p16 is upregulated (panel E), and this protein shows the same distribution pattern as HPV DNA (serial section, panel F). Colocalization of p53 with 2 different miRNAs in cervical intraepithelial neoplasia 1 (CIN 1) lesions showed that miR-let-7c did colocalize with p53 (panel G, arrow shows green signal indicative of colocalization of blue (miR-let-7-c) and red (p53) signals). In comparison, no colocalization was evident between miR-125b (blue) and p53 (red) (panel H).
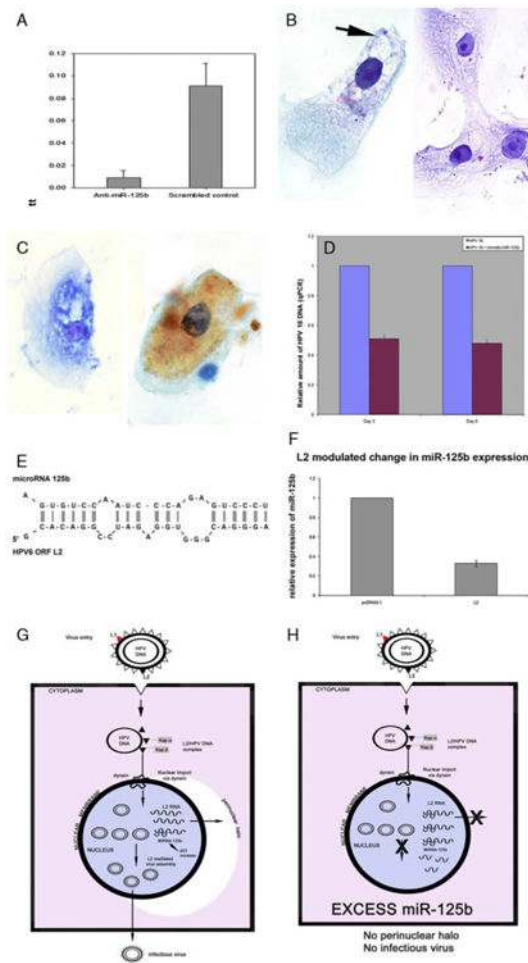
Figure 3.
Correlation among miR-125b and koilocytotic change, p16 expression, HPV DNA proliferation, and HPV RNA L2. Panel A shows the marked reduction in miR-125b expression in HEK cells after transfection with a digoxigenin-tagged anti-miR-125b compared with the scrambled probe. The marked reduction in mature miR-125b was associated with a perinuclear halo typical of a koilocyte (panel B, left image; the right image is the corresponding scrambled probe). Detection of the digoxigenin-tagged anti-miR-125b and p16 showed both localized to the halo (panel C, left and right image, respectively). If the NIH 3T3 cells were cotransfected with miR-125b and HPV 16, there was a significant reduction in HPV DNA copy number at both days 3 and 6 after cotransfection compared with HPV transfection alone. Analysis of possible miR-125b and HPV ORF interactions showed strong homology in the 3′ untranslated region (UTR) regions of a wide variety of HPV genotypes; HPV 6 L2 ORF and miR-125b annealing is shown in panel E. If the NIH 3T3 cells were transfected with HPV 16 ORF L2, there was a significant reduction in the relative expression of mature miR-125b compared with transfection with the empty vector pCDNA3.1 (panel F). Panel G shows the importance of HPV L2 to successful HPV infection on the basis of this work and the references 21 to 24. The L2 protein binds to the circular naked viral DNA and, with cellular factors, transports it to the nucleus and HPV

DNA and RNA (including L2) synthesis occurs. The latter inactivates miR-125b, which, in turn, upregulates p53, which is degraded by high-risk HPVs but not low risk HPVs. We theorize that if the cell contains excess miR-125b (panel H), this can block the ability of L2 RNA to produce L2 protein and, thus, block the formation of infectious virions.

Table 1

Effect of miR-125b (Mimetic and Anti-miRNA) on HPV 16 Transcription in NIH 3T3 Cells: In Situ Hybridization Analyses

Treatment	% Cells+HPV 16 (SEM)	Statistical Analysis[*]
Day 3		
No transfection	0	
puC (empty vector)	0	
HPV 16	36.3 (4.0)	
HPV 16+antimiR-125b	54.6 (2.9)	Increased, $P = 0.041$[†]
HPV 16+mimetic miR-125b	20.6 (3.1)	Decreased, $P = 0.072$[‡]
Day 6		
No transfection	0	
puC (empty vector)	0	
HPV 16	59.0 (2.0)	
HPV 16+antimiR-125b	ND	
HPV 16+mimetic miR-125b	33.5 (3.2)	Decreased, $P = 0.0005$[†]

HPV indicates human papillomavirus.

[*] Statistical analyses based on the Bonferroni corrected P value with values <0.05 highly significant.

[†] Highly significant difference.

[‡] Value 0.072 marginally significant.