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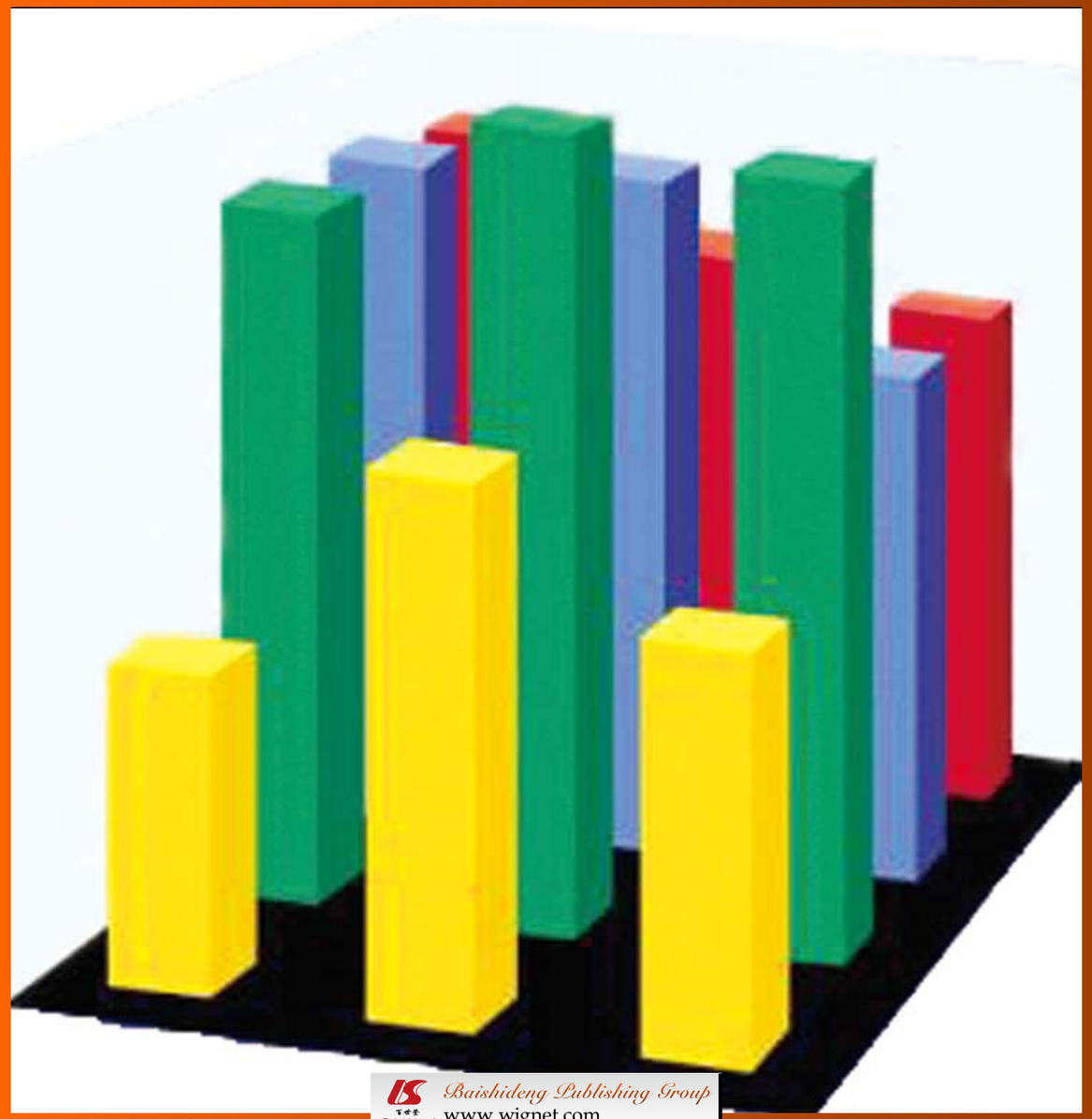

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EDITORIAL

Interplay among cellular polarization, lipoprotein metabolism and hepatitis C virus entry

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Abstract

Hepatitis C virus (HCV) infects more than three million new individuals worldwide each year. In a high percentage of patients, acute infections become chronic, eventually progressing to fibrosis, cirrhosis, and hepatocellular carcinoma. Given the lack of effective prophylactic or therapeutic vaccines, and the limited sustained virological response rates to current therapies, new approaches are needed to prevent, control, and clear HCV infection. Entry into the host cell, being the first step of the viral cycle, is a potential target for the design of new antiviral compounds. Despite the recent discovery of the tight junction-associated proteins claudin-1 and occludin as HCV co-receptors, which is an important step towards the understanding of HCV entry, the precise mechanisms are still largely unknown. In addition, increasing evidence indicates that tools that are broadly employed to study HCV infection do not accurately reflect the real process in terms of viral particle composition and host cell phenotype. Thus, systems that more closely mimic natural infection are urgently required to elucidate the mechanisms of HCV entry, which will in turn help to design antiviral strategies against this part of the infection process.

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Key words: Cellular polarization; Tight junctions; Lipoprotein metabolism; Hepatitis C virus

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-polarity RNA virus that belongs to the *Flaviviridae* family and infects mainly hepatocytes[1]. The HCV genome encodes a polyprotein that is processed by host and viral proteases to yield

ten mature products, which include three structural proteins [the capsid protein (core) and two envelope glycoproteins (E1 and E2)], the p7 protein, and the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The HCV particle consists of a nucleocapsid surrounded by a lipid bilayer harboring the two envelope glycoproteins, which heterodimerize and play a major role in HCV entry[1]. Cellular infection begins with the attachment of the viral particle to the host cell and, after interacting with cell surface molecules, the virus is subjected to clathrin-mediated endocytosis and its envelope is fused with the endosomal membrane, releasing the viral genome into the cytosol[1]. These steps involve a set of attachment factors and cellular co-receptors, including highly sulfated heparan sulfate, the low-density lipoprotein receptor (LDL-R), the scavenger receptor class B type Ⅰ (SR-BI), the tetraspanin CD81, and the tight junction (TJ) proteins, claudin-1 and occludin[2-4]. These molecules are not exclusively present in hepatocytes; therefore, HCV hepatotropism may be determined by other factors, such as the absence of inhibitory proteins[5].

In early reports, the soluble E2 envelope protein was employed as an approach to search for host cell HCV binding factors[6,7]. However, the conformation and function of the soluble glycoprotein may differ considerably from HCV envelope-anchored E2. In addition, envelope-anchored E2 is associated with E1[8], which may have additional implications in terms of receptor recognition and binding. The study of HCV entry was boosted by the use of HCV pseudotyped particles (HCVpp)[9], providing an infection system exclusively relying on the envelope glycoproteins. Since the establishment of a cell culture-derived HCV (HCVcc)[10-12], it has become the most powerful tool for studying HCV because it reflects the complete viral cycle. The use of both HCVpp and HCVcc has been of crucial importance in the discovery of new receptors, and has enabled high-throughput assays to test molecules for their ability to inhibit HCV entry.

INHIBITION OF HCV ENTRY AS A THERAPEUTIC ALTERNATIVE

HCV infection is the most frequent cause of liver failure worldwide[13,14]. Current therapies based on PEGylated interferon-alpha and ribavirin often fail to clear the infection and present a wide spectrum of systemic side effects[15]; therefore, alternative therapeutic options need to be developed. Among the different steps of HCV cycle, viral entry could be considered as a clinical target, especially in the context of orthotopic liver transplantation, where allograft reinfection occurs within hours after reperfusion and is followed by an accelerated chronic disease progression[16]. To date, several molecules have been found to inhibit HCV entry. Matsumura *et al*[17] showed that phosphorothioate oligonucleotides, previously described as HIV entry inhibitors[18], blocked HCV entry *in vivo*. Other inhibitors of HIV entry, such as cyanovirin-N, have also proved effective[19]. Furthermore, serum amyloid A, an acute phase protein mainly produced by the liver in response to different stimuli, including infections, has been demonstrated to inhibit HCV entry[20,21]. Moreover, the milk thistle (*Silybum marianum*)-derived silymarin and its purified flavonolignans have been recently shown to inhibit HCV infection both *in vitro* and in non-responder patients[22,23], blocking viral entry and transmission[24]. Therefore, increasing evidence suggests that blocking the entry step of HCV infection may be a good therapeutic alternative.

The genetic diversity of HCV contributes to its evasion from the host immune response[25], challenging the development of effective vaccines and virus-targeted inhibitors[26-28]. Nonetheless, this problem could be overcome by developing antiviral strategies aimed at blocking essential host factors for viral infection. To this end, multiple strategies have been pursued to inhibit HCV entry at different levels, including viral attachment, post-binding events, and fusion with the endosomal membrane[4,29]. One of these approaches consists of interfering with the interaction between the viral particle and cell surface co-receptors by the use of glycosaminoglycans, natural ligands, recombinant proteins, or blocking antibodies (Table 1). Notably, it has been demonstrated that antibodies against CD81 can prevent HCV infection of human liver-uPA-SCID mice[16], probably by inhibiting the E2-CD81 binding process[6,16,30]. As a more realistic and economical alternative, small molecules with similar properties could be used instead of blocking antibodies. In search of these compounds, several high-throughput screenings have been performed recently to identify molecules with the ability to inhibit HCV infection at the entry step[31-33]. Importantly, the possible cytopathic effects of these inhibitors should be assayed prior to starting clinical trials and considering them as potential therapeutic options. Chockalingam *et al*[32] developed a cell protection screen where cytotoxicity and inhibition of infection were evaluated simultaneously. As a more practical approach, Gastaminza *et al*[31] performed the screening with a set of drugs that had already been clinically approved.

The study of HCV infection and the search for inhibitory molecules are usually carried out with the use of HCVpp or HCVcc, and a highly permissive cell line, such as Huh7 and its derivatives. However, several conflicting results have arisen when attempting to validate the data in a more pathophysiologically relevant context (Table 1). A soluble form of CD81 was shown to prevent infection of Huh7.5 cells by HCVcc; however, it was not effective when primary human hepatocytes (PHH) were challenged with serum-derived virus[34]. Moreover, infection of hepatoma-derived cell lines with HCVpp and HCVcc does not seem to depend on LDL-R[35-37], whereas it has been demonstrated to participate in the infection of PHH with HCV from human plasma[38]. These facts stress the importance of being cautious with results obtained from HCV surrogates and cell lines, which should be validated *in vivo* whenever possible or at least in systems that more closely mimic real infection.

CELL POLARIZATION, TJ-ASSOCIATED PROTEINS, AND HCV ENTRY

In contrast to "simple" polarized cells, which present the

Table 1 Inhibition of infectivity by the blockade of hepatitis C virus co-receptors in different systems

		Host			Viral particle			Ref.
Co-receptor	Blocking agent	Cell lines	PHH	*In vivo*	HCVpp	HCVcc	Serum	
Heparan sulfate	Heparin	x			Y			[87]
		x				Y		[30,88,89]
			x[1]			Y		[90]
LDL-R	Anti-LDL-R	x			N			[36]
			x				Y	[38]
	Soluble LDL-R		x				Y	[38]
	LDLs/VLDLs	x			N			[9,37,91]
		x				N		[37]
			x				Y	[38]
CD81	Anti-CD81	x			Y			[9,36,92]
		x				Y		[30,88,89]
			x		Y			[9,92]
			x			Y		[34]
			x				Y	[34]
				x		Y		[16]
	CD81-LEL	x			Y			[9,36]
			x			Y		[34]
			x				N	[34]
	Knockdown	x			Y			[37]
		x				Y		[89]
			x				Y	[34]
SR-BI	Anti-SR-BI	x			N			[36]
		x			Y			[35,93]
		x				Y		[30,89]
	BLT-4	x			N/Y[2]			[91,93]
	ITX 5061/7650	x				Y		[94]
			x		Y			[94]
	oxLDLs	x			Y			[37]
		x				Y		[37]
	Knockdown	x			N/Y[2]			[37]
		x				Y		[89]
Claudin-1	Anti-claudin-1	x			Y			[30,56]
		x				Y		[30,56]
			x		Y			[56]
			x				Y	[56]
	Knockdown	x			Y			[42,43,95]
		x				Y		[42,43,95]
Occludin	Knockdown	x			Y			[43-45]
		x				Y		[43-45]

x: Experimental system employed; Y: Inhibition of infection; N: No inhibition of infection; [1]HCV-core immortalized PHH; [2]Only in the presence of high density lipoproteins. HCVpp: Hepatitis C virus pseudotyped particles; HCVcc: Cell culture-derived hepatitis C virus.

typical epithelial columnar phenotype with individual basolateral and apical domains, hepatocytic polarity is very peculiar and complex[39]. The plasma membrane of polarized hepatocytes is divided into several basolateral and apical poles, the latter forming a continuous network of bile canaliculi (BC) into which bile is secreted[40]. BC are delimited by TJs, which maintain cell polarity by separating apical from basolateral domains and form the intercellular barrier between bile and blood[41]. Claudin-1 was the first TJ-associated protein to be described as a HCV co-receptor[42]. Soon after, occludin was also shown to participate in HCV entry[43-45]. Despite these discoveries clearly pointing to a role of TJs in HCV cell entry, recent works have reported conflicting data about how cell junctions and polarity influence HCV infection. Perturbation of cellular junctions by calcium depletion promotes opposing effects depending on the system employed, e.g. it decreases viral entry in Huh7 cells[46] but increases it in "simply" polarized Caco-2 cells[47]. Furthermore, junctional accumulation of claudin-1 has been shown to either improve[48] or hinder[49] infection of Huh7.5 and polarized HepG2 cells, respectively. Collectively, these data suggest that the HCV entry process may vary considerably depending on the polarization state of the target cells.

Several studies have questioned the importance of TJ integrity in the function of claudin-1 and occludin as HCV co-receptors. For example, HCV infection is not affected after knocking down other TJ-associated proteins, such as ZO-1 and JAM-A[45], and claudin-1's association with CD81 at the basolateral membrane of HepG2 cells, but not at the TJ, defines HCV entry[50,51]. Furthermore, fluorescent HCV particle internalization generally occurs outside of cell-cell junctions[52], and VEGF induces a reduction of junctional occludin concomitant with an increase of HCV

infectivity[53]. Moreover, claudin-1 and occludin mutants lacking domains that are important for their correct junctional localization and function are still capable of rendering cells susceptible to HCVpp entry[42,54,55]. Finally, HCV infection of HepG2 cells is negatively regulated by cell polarity[49,53], but is not affected by TNF-α- and IFN-γ-mediated TJ disruption[49], and claudin-1 blocking antibodies inhibit HCV infection without perturbing TJs[30,56]. Taken together, these data strongly suggest that the role of claudin-1 and occludin in viral entry is relevant, but not necessarily when these proteins are part of functional TJs, which may indeed be a barrier for HCV infection. Interestingly, it has recently been demonstrated that hepatitis A virus infects HepG2-derived cells from the basolateral domain and that TJ-dependent polarization restricts infection[57].

The mechanisms by which claudin-1 and occludin participate in HCV entry have not been clearly established. In both cases, an extracellular loop of the protein has been shown to be indispensable for infection[42,44,55,58]. Several reports have shown that occludin precipitates with HCV E2 in infected, transfected, or replicon-containing cells[43,55,59], but its direct interaction with viral particles or envelope glycoproteins has not been demonstrated. Additionally, it has been shown that occludin interacts with dynamin Ⅱ, a well known regulator of endocytosis[55]. This observation, along with data obtained from cell-cell fusion experiments[45], suggests that occludin might participate in late steps of the HCV entry process. Interestingly, occludin endocytosis has been implicated in group B coxsackievirus infection, although not by directly interacting with the virus[60]. On the other hand, kinetic studies with blocking antibodies have shown that claudin-1 mediates an HCV entry step closely linked to CD81[30]. Indeed, it has been described that basolateral pools of claudin-1 are associated with CD81 in polarized HepG2 cells[50,51], and that disrupting this interaction, either by site directed mutagenesis or claudin-1 blocking antibodies, neutralizes HCV infection by reducing E2 association with the cell surface[30,51]. However, Cukierman *et al*[54] generated a mutant version of claudin-1 which, in spite of maintaining its interaction with CD81, no longer localized to cell-cell contacts and lost HCV receptor properties. This result suggests that, besides favoring E2 binding to the host cell, additional mechanisms involving claudin-1 participation in HCV entry may exist. Nevertheless, as these experiments were carried out in the non-hepatic, non-polarized HEK cell line, data should be carefully interpreted. This is a good example of how cell polarity may influence the results obtained, especially when studying features of TJ-associated proteins in a hepatocellular context.

LIPID METABOLISM AND HCV ENTRY

Cell polarization is crucial for the correct localization and function of TJ-associated proteins with HCV receptor activity, which could in turn be important for viral entry[41,61]. In addition, polarization may affect other steps of the HCV cycle, such as assembly and egress. Indeed, it has been shown that assembly of RNA enveloped viruses in MDCK cells is closely related to cell polarization[62]. It is also noteworthy that polarization is tightly linked to lipoprotein secretion[63,64], especially because some low density natural HCV particles have been found to be complexed with ApoB and/or ApoE-positive triglyceride-rich lipoproteins[65-67]. This association is believed to take place during viral egress[68] because HCVcc virions were found to be secreted in a manner that parallels the formation of VLDLs[69-71]. Thus, cell polarization may influence lipoprotein secretion, which is important for the generation of correctly assembled HCV progeny. Indeed, non-polarized Huh7-derived cells have been shown to be unable to secrete authentic, ApoB-containing VLDLs[72]. In addition, when HCVcc generated in these cells was subjected to isopycnic gradient ultracentrifugation, it was found to have higher average buoyant density than viral particles obtained from PHH[72]. Interestingly, the density profile of serum-derived viral particles obtained from HCVcc-infected animals was significantly lower than that of the initial HCVcc inoculum[73]. In both reports, specific infectivity of PHH and serum-derived HCV particles was shown to be greatly increased compared to standard HCVcc, and these properties were lost after passaging the virus again in Huh7.5 cells. These findings strongly suggest that HCVcc generated in Huh7-derived cells probably presents a defective lipoprotein association, which may in turn affect viral infection.

The lipoprotein composition and distribution within the viral particle may thus be important for the mechanisms underlying HCV entry[74]. Indeed, several reports have shown that lipoprotein lipase and hepatic triglyceride lipase alter both the physiological characteristics and the infectivity of HCV[75-77]. This notion is supported by the fact that LDL-R participates in the first steps of the entry process[38], and that viral particle density is inversely correlated to infectivity *in vivo*[66]. Additionally, the virus-host interaction could be affected in a context of a lipoprotein-defective viral particle because of changes in the exposure and accessibility of E2 to cellular co-receptors. In fact, this could explain the differences observed between HCVcc and serum-derived HCV infection in terms of inhibition by the soluble CD81 large extracellular loop[34]. Moreover, in contrast to serum-derived HCV[38], infection with HCVcc and HCVpp seems to be LDL-R independent[35-37], probably because of differences in lipoprotein composition.

Therefore, it is necessary to study HCV entry in systems that more accurately mimic the viral cycle in a pathophysiological context, from viral particle generation to target cell infection. The interplay between HCV and hepatocytes seems to be closely related to their particular phenotype (Figure 1); therefore, data obtained from models that do not reflect hepatocellular polarization and lipoprotein secretion should be cautiously interpreted.

TOWARDS A MORE PHYSIOLOGICALLY RELEVANT MODEL

The use of HCVpp and HCVcc has meant a great advance in HCV research. Their adaptability to high-throughput

analysis has enabled genome-wide screening for host proteins involved in the HCV cycle at different stages. In addition, they provide a valuable tool for testing the possible antiviral activity of large chemical libraries of compounds. However, it is important to bear in mind the limitations of these *in vitro* models, as both viral particles and target cells differ notably from serum-derived HCV and polarized hepatocytes, respectively. Thus, results obtained with HCVpp and HCVcc should be validated in systems that more closely mimic real HCV infection before establishing firm conclusions. To this end, significant work is being done by Molina and colleagues, who have already confirmed a role for LDL-R and CD81 in serum-derived HCV infection of PHH[34,38]. This model is considered an accurate *in vitro* system, as cells can be maintained in a differentiated phenotype to retain their polarity and drug metabolizing capacities. In addition, they can be infected with serum-derived HCV of any genotype and, in contrast to what is observed in hepatoma cell lines (e.g. Huh7 cells and derivatives), the innate immune response is fully preserved[78]. However, production of measurable titers of progeny virus in this system has not been achieved[79], indicating that the model fails to reflect the entire HCV cycle. Recently, it has been shown that HCVcc infection of PHH results in the robust production of infectious viral particles, which were in turn able to efficiently infect naïve PHH[72]. This primary cultured-derived HCV (termed HCVpc), compared to HCVcc, exhibited lower average buoyant density and higher specific infectivity, reminiscent of what is seen for virus recovered from the blood of animals infected with HCVcc[72,73]. Therefore, HCVpc infection of PHH emerges as a valuable tool for studying the complete HCV cycle in a more relevant context. Nevertheless, this system presents the drawbacks of working with PHH, such as restricted availability, the difficulty of studying long-term infections, and the heterogeneity of samples and results. Interestingly, Matrigel-embedded 3D cultures of Huh7 cells display a hepatocyte-like polarization and are susceptible to HCVcc infection. Progeny viruses generated by these cultures, similarly to HCVpc, also present a shift towards lower densities (our unpublished observations). This result suggests that if hepatocellular polarity is achieved, it is possible to generate viral particles that more closely mimic real HCV, even when cell lines are used as the source of virus.

Animal models are essential to validate *in vitro* data, because not only hepatocytes, but also the liver as a whole, may determine the mechanisms of HCV cell entry. Indeed, the liver sinusoidal endothelial cell-expressed protein, L-SIGN, has been shown to bind serum-derived HCV[80] and mediate trans-infection of Huh7 cells by HCVpp[81,82]. Additionally, co-culture of PHH with liver sinusoidal endothelial cells significantly increases the expression of the HCV co-receptor LDL-R[83]. Regarding *in vivo* obtained data, CD81 is the only co-receptor that has been demonstrated to participate in HCV infection using the human liver-uPA-SCID mouse model[16]. This system, albeit constituting a useful tool, is limited by the fact that the animals lack a functional immune system. This may be important,

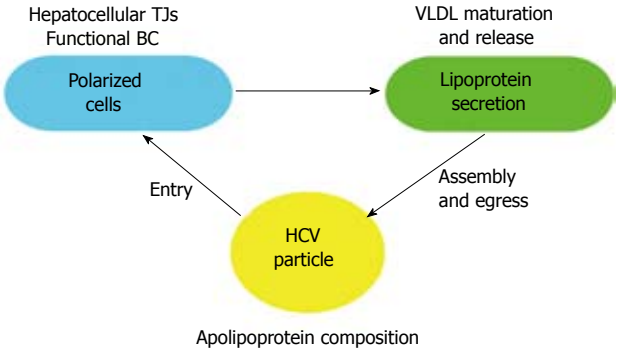

Figure 1 Interplay among cell polarization, lipoprotein secretion, and hepatitis C virus particle assembly, release, and entry into host cells. The correct polarization of hepatocytes, implying the presence of functional bile canaliculi delimited by TJs, may be important for the proper maturation and secretion of lipoproteins. This process is tightly associated with the composition and assembly of hepatitis C virus (HCV) lipoviroparticles and their exit from infected cells. Finally, HCV entry may be affected by the lipoprotein composition of the viral particle, the hepatocellular polarization of target cells, and the localization of the TJ-associated proteins claudin-1 and occludin.

not only for the outcome of the infection, but also for the entry process itself, because DC-SIGN, expressed on dendritic cells, has been shown to capture and transmit HCVpp to Huh7 cells[81,82]. More recently, peripheral blood B cells have been shown to exert a similar function[84]. To date, chimpanzees are the only immunocompetent *in vivo* system for studying HCV infection, but their use is limited by ethical concerns, restricted availability, requirement of special facilities, and very high costs[85]. In search of a small, HCV susceptible and immunocompetent animal model, it has been proposed to combine human liver chimeric models with mice harboring a human hematolymphoid system[85], although this approach depends on the availability of human primary cells. Thus, the ideal model would be an immunocompetent mouse susceptible to HCV infection without the need of harboring human cells. Given that HCV species tropism is restricted to human and primates[58,85], an alternative strategy consists of using an HCV variant able to infect murine cells. Bitzegeio et al. adapted HCVcc to mouse CD81 and identified three envelope glycoprotein mutations which together enhanced infection of cells with mouse or other rodent receptors by approximately 100-fold[86], thus overcoming the species-specific restriction of HCV cell entry. Another possible approach would be to employ genetically engineered mice bearing the human entry factors that confer species specificity[44]. However, mouse hepatocytes fail to initiate viral replication[85]; therefore, these models would not be able to mimic the entire HCV cycle.

CONCLUSION

During HCV infection, hepatocytes almost exclusively constitute both the target and the virus-producer cells. Thus, it is mandatory to perform HCV studies in systems that closely mimic the complex nature of hepatocyte pheno-

type. These models should enable the generation of viral particles that resemble the ones found *in vivo*, and reproduce the hepatocyte physiology as accurately as possible. Only the combination of these two factors will provide the necessary information to establish firm conclusions. Nevertheless, the choice of employing HCVpp, HCVcc, HCVpc, or serum-derived HCV to infect cell lines, PHH, or animals should depend on the stage of the research process: the *in vitro* systems are more adequate for high throughput screenings and the *in vivo* models are essential for validating the data. Once the mechanisms of HCV entry are deciphered in detail, this step of the viral cycle could be an effective target for the development of antiviral compounds. These inhibitors should ideally be effective for a broad range of HCV genotypes and subtypes, and even for other viruses, such as HIV, that might share some entry mechanisms and co-infect some patients. Thus, blocking cellular factors might be a good therapeutic alternative in the fight against viral genetic variability. However, targeting host molecules could alter their physiological functions and result in harmful side effects. In addition, this approach does not rule out the possible emergence of viral variants that would be able to circumvent the specific effect of the entry inhibitor. Moreover, HCV cell-to-cell transmission may bypass the inhibition of cell-free virus entry and allow viral spread. Therefore, clinical strategies based on broad-spectrum compounds or the combination of different therapeutic molecules should be developed to simultaneously interfere with several steps of the viral cycle to efficiently control infection with the minimal side effects.

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TOPIC HIGHLIGHT

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Inflammatory bowel disease in adolescents: What problems does it pose?

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Abstract

Adolescents with inflammatory bowel disease face daily and long-term challenges that may be difficult for teenagers to manage. The developmental and psychosocial changes unique to this age group include becoming more autonomous and being more vulnerable to peer influence. These changes may lead to problems in medical management such as poor medication adherence and risky behavior. Being aware of these issues will help the medical team provide anticipatory guidance to address these concerns.

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Key words: Inflammatory bowel disease; Adolescent; Patient compliance; Growth and development; Nutrition; Peer group; Risk behavior

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INTRODUCTION

Inflammatory bowel disease (IBD) is one of the more common chronic illnesses afflicting adolescents, with an estimated prevalence in those younger than 20 years old of 71 per 100 000[1]. Both Crohn's disease (CD) and ulcerative colitis (UC) present day-to-day as well as long-term challenges that can be particularly difficult for the adolescent patient. The developmental and psychosocial changes unique to adolescence, including establishing autonomy, risk-taking behavior, and undue susceptibility to peer influence make managing IBD in this age group more difficult.

MEDICATION ADHERENCE

Adhering to a prescribed medication regimen is difficult for teenagers. Adolescents need to remember to take medications amidst a busy lifestyle with school and extracurricular activities, and to accurately recall the types and number of medications to be taken at various times of the day. They can be particularly resistant to taking medications that cause cosmetic changes, choosing a better appearance over persistent IBD symptoms. The concept of maintenance therapy can also be challenging for teens who, once acute symptoms subside, often decide to stop taking medications "because I don't need them anymore".

A study by Hommel *et al*[2] examined the problem of medication nonadherence in 42 adolescents with IBD. In addition to recording an objective measure of adherence

using pill count, they further evaluated the problem by determining the adolescents' subjective assessments of their adherence through standardized interviews. Subjects reported that they missed 6% of their 6-mercaptopurine/azathioprine (6-MP/AZA) doses and only 3% of the 5-aminosalicylate (5-ASA) medications. However, pill counts revealed that 38% of 6-MP/AZA and 49% of 5-ASA doses had been missed. Only 14% of patients had therapeutic levels of thiopurine metabolites, further confirming their poor adherence to the prescribed 6-MP/AZA regimen. The subjects who were nonadherent to 6-MP/AZA were also likely to miss 5-ASA doses. The frequent dosing and large quantity of pills per dose were factors associated with poor adherence to 5-ASA treatment.

A survey study by Greenley *et al*[3] of 64 adolescents and their parents found that 48% of teenage patients took one IBD medication or supplement, 36% took two, and 11% took three. Only 65% of adolescents reported perfect adherence. Common barriers to medication adherence included lack of time, medication side effects, feeling well, or belief that the medication was ineffective. Patients on polytherapy had more barriers to successful medication-taking than those on monotherapy. A similar study by Ingerski *et al*[4] found that the most common barriers to medication nonadherence included forgetting, being away from home, and interference with an activity.

Based upon these studies, physicians may help increase medication adherence by minimizing the frequency of doses and number of pills. When prescribing a new treatment, physicians need to carefully explain to adolescents why they need to take their medications as prescribed, especially when patients have low expectations that the therapeutic regimen will be effective, or are likely to experience side effects[3]. Physicians must also clearly communicate the concept of maintenance therapy, both when medications are first prescribed, and more importantly, during routine follow-up visits so that teenagers understand why they need to continue to take medications even when they are feeling well. Suggestions to help teens remember to take their medications include using a pill box, carrying an extra supply of pills, and setting an alarm as a reminder on a watch, cell phone, or portable electronic device.

GROWTH AND DEVELOPMENT

IBD may lead to growth failure and delayed puberty during adolescence. Pfefferkorn *et al*[5] examined growth outcomes in children with CD at diagnosis, 1 year, and 2 years using data from the Pediatric IBD Registry, a prospective, multicenter observational database established in 2002. The study found that the mean height z-scores were approximately -0.50 standard deviations (SD) at each of the three time points despite improvement in disease activity over the course of the observation period. Although the mean height velocity z-score increased between the first and second years, the proportion of patients with a height velocity z-score less than -1 SD was similar and substantial (45% at 1 year and 38% at 2 years). Furthermore, all subjects in the study were Tanner Ⅰ to Ⅲ at diagnosis and at 1 year. At 2 years, 84% (92/110) of patients did not progress to Tanner Ⅳ. These data are disturbingly similar to those derived from patients diagnosed in the 1970s and 1980s, in whom periods of significant growth failure were demonstrated in 37% of young adults whose CD had been diagnosed before their adolescent growth spurt[6]. Despite advances in nutritional and anti-inflammatory therapy, promoting normal growth continues to be a significant challenge for the physician caring for adolescents with IBD.

Not only is height adversely affected in children with IBD, but bone mineral density (BMD) is as well. Sylvester *et al*[7] prospectively followed Caucasian children with IBD over a 2-year period from diagnosis. The mean total body BMD z-score at diagnosis was significantly lower for CD patients compared to healthy controls of similar age (-0.78 for CD, -0.46 for UC, and -0.17 for controls). CD patients with a BMD z-score lower than -1 tended to have a lower body mass index (BMI) and higher serum interleukin-6. Despite clinical improvement in the CD and UC patients over the follow-up period, the mean BMD z-score was unchanged for CD. Similarly, the mean BMD z-score for UC patients was unchanged from diagnosis to 1 year, but increased from the first to second year.

Sylvester's study[7] also found that both CD and UC patients had decreased serum concentrations of biochemical markers of bone formation at diagnosis compared to controls. In addition, CD patients had a lower concentration of N-telopeptide of collagen, a marker of bone resorption, compared to controls. Over the course of the study, improvement in clinical status and nutrition was associated with increased concentrations of markers of bone formation but not of bone resorption. However, mineralization rates did not significantly improve, particularly in CD patients.

Dubner *et al*[8] evaluated the bone and muscle of the left tibia using peripheral quantitative computed tomography (pQCT) in pediatric CD patients. pQCT provides a 3-dimensional assessment of trabecular and cortical volumetric BMD (vBMD) and geometry, and a cross-sectional area of muscle and fat. Patients with CD had musculoskeletal deficits (in trabecular vBMD, cortical bone geometry, and muscle mass) at diagnosis compared to healthy controls of similar age. Six months after diagnosis, there was improvement in trabecular vBMD and muscle mass, but worsening of cortical bone geometry. One year after diagnosis, all 3 were still low compared to controls.

In addition to physical appearance, growth failure and delayed puberty may also affect psychosocial aspects of adolescent life. Looking different from peers may render teens to feel self-conscious or "abnormal". Teens may have difficulty "fitting in" with peers or dating because they appear less mature. Having short stature may also affect their ability to participate in or excel at sports, or at least give adolescents this perception. All these

factors may lead teens to become repeatedly frustrated, have low self-esteem, or seclude themselves.

Poor nutrition may contribute to poor growth. Patients may not meet their nutritional requirements because of inadequate caloric intake, malabsorption, or increased metabolic needs from chronic inflammation. Patients with CD are more likely to be malnourished than patients with UC. It is important to monitor nutritional status by anthropometrics, and checking blood for albumin and micronutrient levels (e.g. iron, folate, and B12). Patients who are undernourished or have micronutrient deficiencies should receive supplementation with oral or nasogastric tube feeds, or with specific vitamins or minerals. In addition, it may be beneficial for patients to take a daily multivitamin. Patients receiving sulfasalazine and/or methotrexate should be given folic acid supplements[9]. Patients may also be referred to a nutritionist for evaluation of dietary intake and guidance on how to eat a healthy and appropriate diet.

PEER INFLUENCE

Peer influence is strong during adolescent years. This period is filled with the desire to be accepted, identity formation, intense introspection, and internal conflict. Teenagers may feel embarrassed or self-conscious about their body as a result of IBD, its complications, or side effects from medications. Patients may feel uncomfortable about their short stature, delayed puberty, body habitus (thin from malnutrition or weight gain from corticosteroids), surgical scars, or ostomy. Patients missing school or limiting extracurricular activities due to IBD flares or medical appointments may feel isolated from peers. Teenagers may not want to appear different from friends or raise suspicion about having a medical condition, especially one that involves potentially embarrassing topics such as diarrhea.

When adolescents are ready to share their medical condition with friends or someone they are dating, they must decide when is the appropriate time, and how to do so. Patients may want to let peers realize that disclosing this information demonstrates that patients trust their peers and that their relationship has reached a certain level. Patients may also explain that the disease does not define them as a person. In addition, adolescents should understand that they have the option of sharing only information which they are comfortable disclosing.

RISKY BEHAVIOR

Adolescents may participate in risky behaviors such as practicing unprotected sex, and abuse of alcohol and illicit drugs[10,11]. It is common to hear adolescents explain their risky behavior because they think that bad consequences "won't happen to me." Adolescents with IBD are no exception, but their risky behavior can also take the form of discontinuing their medications without consulting their physician. Therefore, it is important for the physician caring for adolescents with IBD to address risky behavior and provide anticipatory guidance. For example, adolescent IBD patients should be counseled about the importance of using protection if they decide to have sexual intercourse. If the female adolescent patient decides to use oral contraceptive pills, she should be aware of the possible increased risk of thrombosis[12]. It is also important to explain that, although sulfasalazine can lower sperm counts, it should not be considered a method of contraception[13].

If the teenage patient is pregnant, she may want to seek family planning to decide whether to continue the pregnancy. If the decision is to continue the pregnancy, it may be beneficial to transfer her to an adult gastroenterologist because internists have more experience with pregnancy and high-risk obstetrics than pediatricians. Patients should be encouraged to tell their physicians that they are pregnant because some IBD medications such as methotrexate are known teratogens and others may need to be monitored carefully during pregnancy. Conversely, some medications are safe during pregnancy but there may be a popular misconception that they are not (such as infliximab until 28 wk gestation)[14].

Toxic ingestion (including alcohol consumption) and illicit drug use are other risky behaviors that are important to address. Teens may abuse alcohol and illicit drugs for various reasons, such as peer pressure, recreational use, emotional escape, pain control, or appetite stimulation. Regardless of their reason, alcohol and illicit drug use may have multiple adverse health effects including damage to the liver, an organ vital in metabolizing some medications used to treat IBD. In addition, intravenous drug use, unprotected sex, and having tattoos or body piercing are risk factors for contracting hepatitis B and C[15-18]. Adolescents should also be informed that metronidazole must not be taken with alcohol, as it can cause a disulfiram-like reaction[19].

TRANSITION TO AN ADULT GASTROENTEROLOGIST

Transition to an adult gastroenterologist provides older teenagers and young adults with a hospital or clinic environment that is appropriate for their age. In addition, adult gastroenterologists are more experienced than their pediatric counterparts regarding issues frequently encountered in adults such as pregnancy, fertility, and cancer surveillance. In addition, the process of transition promotes independence and better adherence with therapy because patients must learn to take care of themselves, be proactive in their medical care, form decisions, communicate with the medical team, and be a self-advocate[20,21].

The transition may be stressful for everyone involved in the process: patients, parents, as well as pediatric and adult gastroenterologists. Patients and families must relinquish the familiarity of the pediatric medical team and institution to face a new one that may have a different practicing style and expect patients to be independent. The

adult gastroenterologist may subsequently be perceived as less concerned about the needs of the patients and their families. Pediatric gastroenterologists may be reluctant to transfer patients to an adult provider whose medical management may be different or who may be less familiar with the issues of adolescent patients. Adult gastroenterologists may see young adult patients as less mature or self-reliant, and their families as overly involved[20].

Hait *et al*[21] proposed a timeline consisting of competencies patients should achieve and tasks the medical team should implement based on patients' age, to help promote a successful transition. Pre-adolescents (ages 11-13 years) should learn to identify their disease and medications (names, dosages, and major side effects). The medical team should present the idea of independent visits in the future and discuss the effects of exercise, sexual intercourse, and substance abuse on IBD. Young adolescents (ages 14-16 years) should learn to identify their medical team and be knowledgeable about their medical history (including prior procedures and tests), relevant family history, and the risk of medication nonadherence. The medical team should start to help patients to become more independent by addressing patients first and having the family step out of the room for part of the visit. However, it is important to explain that the medical team has a legal obligation to share certain aspects with parents. Patients should be taught how to contact the medical team, schedule appointments, and fill prescriptions. In addition, physicians should inquire about their patients' plans after high school and introduce the idea of transition. Older adolescents (ages 17-19 years) should be able to manage their medical needs, which include scheduling appointments and having a plan to attend them, asking and answering questions during their private conversation with the gastroenterologist, filling prescriptions and picking them up at the pharmacy, and being knowledgeable about their insurance. Physicians should discuss possible difficulties encountered in the transition process and provide names of prospective adult gastroenterologists.

When patients are ready to transfer care, transition is easier when there is stability in their disease and life. Transition is more successful when patients in college are transferred to an adult gastroenterologist after graduation and have acquired a job or started graduate school. Patients who do not plan to attend college may be transferred after securing housing and a job. A medical summary should be provided at the time of transfer[21].

A survey by Hait *et al*[22] illustrated adult gastroenterologists' perspectives of the transition process. The survey of 363 adult gastroenterologists revealed that factors they believed were most important for a smooth transition were often lacking amongst their young adult patients. These factors included patients' knowledge about their medications (name, dose, and major side effects), medical history, and health effects of smoking, drugs, and alcohol. Adult gastroenterologists also considered it helpful that pediatric gastroenterologists should provide a medical summary of their patients. The vast majority of adult gastroenterologists thought it was important that adult providers be familiar with adolescent medical and developmental issues, but a significantly low proportion felt proficient in this area.

CONCLUSION

In summary, adolescents have unique issues such as poor medication adherence, growth failure, peer influence, and risky behavior, which make managing teenage IBD patients more challenging. It is important for physicians to recognize these issues so that they are prepared to address patients and parents early to help improve adherence to medications, nutritional status, complications of IBD, social well-being, and the transition process.

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TOPIC HIGHLIGHT

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Inflammatory bowel disease in pregnancy

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Abstract

Crohn's disease and ulcerative colitis affect women in their child-bearing years. Family planning has come to be a common discussion between the gastroenterologist and the inflammatory bowel disease (IBD) patient. Disease control prior to desired conception and throughout pregnancy is the most important thing to keep in mind when caring for the IBD patient. Continued medical management during pregnancy is crucial in optimizing outcomes. Studies indicate that quiescent disease prior to conception infer the best pregnancy outcomes, similar to those in the general population. Active disease prior to and during pregnancy, can lead to complications such as pre-term labor, low birth weight, and small for gestational age infants. Although there are no definitive long term effects of pregnancy on IBD, there are some limited studies that suggest that it may alter the disease course. Understanding the literature and its limitations is important in the modern era of IBD care. Educating the patient and taking a team approach with the obstetrician will help achieve successful outcomes for mother and baby.

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Key words: Inflammatory bowel disease; Pregnancy; Crohn's disease; Ulcerative colitis; Breastfeeding

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INTRODUCTION

Approximately fifty percent of patients are less than 35 years of age at the time of diagnosis and twenty five percent conceive for the first time after their diagnosis of inflammatory bowel disease (IBD)[1-3]. Advances in the field of IBD have made successful pregnancy outcomes a reality for many women. It is very important that as a gastroenterologist you have input into the conversation regarding management during pregnancy. It is important to understand the effect of pregnancy on IBD and the effect of IBD on a pregnancy. This review discusses the evidence for the important questions that female patients have in regards to this topic, and recommendations based on clinical experience of the authors has also been included.

HOW DOES IBD AFFECT THE OUTCOMES OF PREGNANCY?

The general consensus agrees that the impact of IBD

on pregnancy depends on disease activity at conception. Studies suggest that quiescent disease throughout a pregnancy leads to similar risks to those of the general population in regards to spontaneous abortion, pregnancy related complications, and adverse perinatal outcomes[4-6]. Disease activity at conception has been associated with preterm births, low birth weight (LBW), and fetal loss[4,7-10]. In addition, active disease during pregnancy results in the greatest risk of adverse perinatal outcomes[3,4,11]. This risk appears to be higher in women with Crohn's disease (CD) than in those with ulcerative colitis (UC). However, severe disease relapses during pregnancy in UC are associated with shorter gestation periods and lower birth weights[12]. In the study by Reddy *et al*[12] there was a higher risk of preterm births among women hospitalized for severe UC, with the mean gestational age being 35 wk *vs* 38.7 wk in the control group (without disease relapse).

In 1997, Kornfeld *et al*[13] studied 756 women with IBD and found that IBD was an independent risk factor for LBW and premature infants. However, they did not differentiate between CD and UC. Studies in the US and Denmark have demonstrated an increased risk of preterm delivery, small for gestational age (SGA), and LBW in infants to CD mothers[8,14,15]. In a study of Danish UC women, 1531 infants were included and there was no difference in rates of pre-term delivery, LBW, or SGA compared to controls[16]. The rate of healthy offspring to UC women was similar to the general population[3,8]. Despite a consistent trend of preterm delivery, most of the deliveries occurred after 35 wk with favorable outcomes[14].

Khosla *et al*[17] examined a cohort of 54 pregnant CD patients, and found that those with active disease at conception had rates of miscarriage up to 35% higher than those of patients who were in remission. In 2007, Nørgård *et al*[18] examined the impact of disease activity on birth outcomes in CD, and reported that activity during pregnancy only increased the risk of preterm birth. Furthermore, Moser *et al*[19] concluded that the presence of ileal disease in CD women was a strong predictor for LBW.

In a study by Dominitz *et al*[14] a greater risk of congenital abnormalities was seen in UC women compared to controls (7.9% *vs* 1.7%, $P < 0.001$). This study, however, failed to take into consideration disease activity or medication use, and this finding has not been replicated by other investigators. Indeed, most studies have found no greater risk of malformations in UC or CD[8,10,20]. For example, Lamah *et al*[21] found that there was no increased risk of spontaneous abortions, perinatal mortality, or congenital malformations in their UC cohort.

Using the 2005 Nationwide Inpatient Sample, Nguyen *et al*[22] examined 2372 CD deliveries and 1368 UC deliveries. In this population-based study, the adjusted odds of a cesarean section were higher in women with CD (aOR 1.72) and UC (aOR 1.29) compared to non-IBD controls. The risk of a venous thromboembolism was also substantially higher in IBD pregnancies. Interestingly, protein calorie malnutrition occurred more frequently in IBD women, as did blood transfusions in CD deliveries (aOR 2.82). To date, there are no associated complications of hypertension or proteinuria with IBD pregnancy[5].

Beniada *et al*[11] reported a series of 76 cases of women with their first IBD flare during pregnancy, and observed that they were at increased risk for pre-term delivery and/or LBW. The largest study to date on this topic is the meta-analysis by Cornish *et al*[23] that evaluated 12 studies in regard to the impact of IBD on pregnancy. This study comprised a total of 3907 patients with IBD and 320 531 controls. Based on this analysis, women with IBD were more likely to experience adverse pregnancy outcomes, such as premature birth and LBW. In fact, premature delivery was almost twice as likely compared to the general population. Women with IBD were also 1.5 times more likely to undergo cesarean section. Unfortunately, neither medication use nor disease activity was analyzed as a cofounder, which makes it difficult to put the results into proper perspective. The meta-analysis reported a 2.37 fold greater risk of congenital abnormalities (95% CI: 1.47-3.82, $P < 0.001$), but most of the studies included did not differentiate between minor and major malformations[23].

In 2006, a Spanish study of 124 pregnant CD women looked at pregnancies before and after diagnosis. They concluded that the course of IBD did not adversely affect pregnancy or the postpartum time period. The study determined that diagnosis prior to pregnancy did not influence the number of cesarean sections performed or increase the presence of LBW infants[24].

In 2007, Mahadevan *et al*[25] compared pregnancy outcomes between women affected with IBD and those unaffected. The study comprised of 461 pregnant IBD patients and a randomly selected cohort of age matched controls, and represents the largest US study to date. Women with IBD were more likely to have an adverse pregnancy complication compared to those women without IBD, but there was no difference in adverse newborn outcomes or congenital abnormalities. This difference was seen irrespective of the disease activity. The use of IBD medications was not found to be predictive of adverse outcome in this large, non-referral population. There was no statistically significant difference in newborn outcomes between the IBD and control pregnancies.

In the general population, smoking is a known risk factor for LBW infants and for disease activity in CD women[2]. Pregnant CD patients who smoke are at a substantially increase risk for LBW and preterm delivery[2,21]. Conversely, smoking in UC women does not increase their risk of preterm delivery[26]. However, given the known risk of smoking on the individual and the baby, smoking cessation should be encouraged in all scenarios.

Numerous studies continue to associate preterm birth and IBD; however the majority of these "preterm" deliveries occurred after 35 wk of gestation[14,26]. In UC, if resection is needed during pregnancy, Nielsen *et al*[8] found an increased risk of preterm delivery. Many theories have been put forth for this observation, but the etiology is unclear. One hypothesis is that an increase in circulating

prostaglandin levels during a flare could initiate pre-term labor with the induction of smooth muscle contraction[12,27]. Another theory is that the role of increased gut permeability during increased inflammation could alter nutritional and immunological factors affecting labor[12]. In addition, safety concerns for the mother and baby might prompt induction of early delivery, which would bias the end result of an LBW infant. Although each theory is plausible, more data are needed to clarify IBD and preterm infants.

While the data are conflicting at times, it appears that the disease activity is the main impetus of adverse pregnancy outcomes[28], as miscarriages are seen more frequently with active disease[13-15]. In our practice, we encourage women to be in remission before considering conception, and for those who become pregnant, we monitor closely and treat disease activity aggressively.

HOW DOES PREGNANCY AFFECT THE DISEASE COURSE?

A consistent finding in more recent literature is that the rate of disease flare during pregnancy (26%-34%) is similar to non-pregnant flare rates[7,10,29,30]. An exacerbation rate of 34% per year during pregnancy in UC women and 32% per year when not pregnant was observed by Nielsen *et al*[8]. These rates of relapse were similar in the CD population[10]. The Kaiser cohort, previously discussed, included women with inactive disease throughout their pregnancy, with no sudden increase in activity post-partum[25,29].

UC

When conception occurs during a quiescent state, 70%-80% of UC patients will remain in remission[2,8]. The rate of relapse is thus similar to a non-pregnant UC patient. Unfortunately, when flares do occur, the data is unreliable in relation to the stage of pregnancy. It was initially believed that an increase of disease flare occurred in both the first trimester and post-partum, but timing of a flare appears to more related to disease activity at conception and at term. Moreover, disease flare is often related to discontinuation of medical therapy (first trimester) or resuming smoking after delivery (post-partum)[2,11,31]. Active disease at conception can be associated with a worse prognosis. In a cohort of UC patients, Willoughby *et al*[32] noted that active disease during these times was more resistant to treatment.

The patient who has undergone an ileoanal anastomosis procedure presents a special situation. Ravid *et al*[33] examined 67 pregnancies in 38 UC women with ileal pouch anal anastomosis surgery. It was determined that pregnancy was safe with some alteration of pouch function, almost exclusively during the third trimester. For most of the women, the pouch function returned to its pre-pregnancy state. However, there was a small proportion of women that suffered long term disturbance of pouch function. This long- term effect was not related to the method of delivery. Although the mode of delivery in a pouch patient remains disputed, the method of delivery should not be determined by the presence of a pouch, but by patient and obstetric decisions. There are no long term data on pouch function after vaginal delivery but short-term data showed that pouch function, continence and quality of life are not affected by uncomplicated vaginal delivery[2,34].

CD

CD during pregnancy is similar to patients with UC. As with UC, the key to a good outcome is the disease state at conception and delivery. If the CD is quiescent at conception, 70% of pregnant CD patients will remain quiescent compared to the non-pregnant CD patient[2,17,35]. There has been some suggestion that CD symptoms might improve during gestation and that relapse is more common in the first trimester[2,17,26]. When disease is active at the time of conception, we follow "the rule of thirds". One third of women will get better, one third will stay the same, and one third will worsen. The biological mechanism of this finding has yet to be fully explained, but several studies have suggested that the immune disparity between mother and fetus might play a role in immune regulation, thereby altering immune function and pathology[36].

The recommended mode of delivery in CD patients is still controversial. In comparison to the general population, CD patients undergo cesarean sections more frequently, with the rate of cesareans increasing after diagnosis[37,38]. There are conflicting data on vaginal deliveries and perianal disease. The current recommendation, based on small observational studies, is to avoid vaginal deliveries in the setting of active perianal disease. CD patients with uncomplicated disease should be treated like the general population when deciding on delivery, but episiotomy should be avoided. The presence of a colostomy or ileostomy should not designate delivery choice. Despite the limited data, choice of delivery should be a collaborative decision between the patient, gastroenterologist, and the high-risk obstetrician.

Nursing

Any detriment to maternal health secondary to nursing after delivery is controversial. There have been some reported associations between nursing and increased disease activity, but this is unclear whether this is related to disease course or cessation of medication. Kane *et al*[31] found the odds ratio (OR) of disease flare for women who breastfeed was 2.2 (95% CI: 1.2-2.7) compared to those who did not breastfeed. However, once medication discontinuation was factored in, the OR became non-significant. Moffatt *et al*[39] published a population based study of breastfeeding and found no increased risk of flare in the postpartum period, and a possible protective effect once the discontinuation of medications was taken into account.

Long term effects of pregnancy on IBD

There have not been any data to suggest a long term detrimental effect of pregnancy on IBD and there is

never a role for elective termination. In the specific cases where methotrexate or thalidomide are involved, the decision for a therapeutic abortion may need to be addressed, given that it is a category X drug with known association with fetal abnormalities. Pregnancy has not been shown to definitively alter disease phenotype[38]. Riis *et al*[38] demonstrated that parous IBD patients experienced a reduction in relapse rate in the three years following pregnancy when compared to the three preceding years[2,40]. The rate of relapse decreased in the years following pregnancy in both UC and CD. Riis *et al*[38] looked at 580 IBD pregnancies in a European cohort. The pregnancy itself did not influence disease phenotype or surgery rates, but it was associated with a reduced number of flares in the following years (UC 0.34 flares/year *vs* 0.18 flares/year, $P = 0.008$ and CD 0.76 flares/year *vs* 0.12 flares/year, $P = 0.004$). Nwokolo *et al*[41] demonstrated a negative correlation between increasing parity and number of resections[2]. They found that in parous women with CD, the need for surgical resection was inversely correlated with increasing parity. Castiglione *et al*[42] studied parous IBD women and found that the incidence of relapses in the first three years after pregnancy was lower than that prior to pregnancy. Hormonal changes during and after pregnancy might account for a change in fibrosis and stricture formation[5]. Some studies suggest a down regulation of the immune system with maternal fetal HLA disparity[36]. Maternal immune response to paternal HLA antigens might result in immunosuppression that can in turn affect the maternal immune-mediated response. Kane *et al*[36] looked at 50 pregnancies in 38 women and found 42 disparate (84%) at the DRB1 locus, 34 (68%) at the DQ locus, and 31 (62%) at both loci. A significant difference was found in IBD activity between women mismatched at both loci *vs* only 1 or neither locus (OR 8.4, $P = 0.01$) Improvement of IBD symptoms during pregnancy was associated with disparity in HLA class Ⅱ antigens between mother and fetus. When logistic regression was performed, pre-partum disease activity and disparity at both DRB1 and DQ were significant predictors of overall disease activity during pregnancy. Pregnancy should never be discouraged or terminated in a patient with IBD, but instead, the goal of care should be early counseling and appropriate medical management.

IBD medications and pregnancy outcomes (Table 1)

It is believed that the greatest risk to IBD pregnancy is active disease and not active therapy. In addition to the effect on disease activity during pregnancy, the fear of a medication's effect on the fetus often prompts physician and patient to discontinue all medications. Pregnancy data on outcomes and disease course are complicated by cessation of drugs. One of the earliest available drugs for the treatment of colitis, sulfasalazine, readily crosses the placenta, but has not been linked to any fetal abnormalities in several large studies. However, patients taking sulfasalazine should be supplemented with folic acid to decrease the risk of neural tube defects. A dose of one milligram twice daily is appropriate.

Table 1 Use of medications during pregnancy in inflammatory bowel disease

Benefit clearly outweighs risk	Limited data	Contraindicated
5-ASA, oral and topical	Olsalazine	Methotrexate
Corticosteroids	Natalizumab	Thalidomide
Metronidazole, amoxicillin		
Azathioprine/6-MP		
Anti-TNF agents		

TNF: Tumor necrosis factor.

Aminosalicylates: The safety of 5-ASA compounds during pregnancy has been demonstrated in a number of trials, despite the fact that mesalamine and its metabolite, acetyl-5-aminosalicylic acid are found in cord plasma[43,44]. In two separate studies, women taking 2-3 g/d for either UC or CD had no increased incidence of fetal abnormalities compared to normal healthy women.

Immunomodulators: In the retrospective chart review by Francella *et al*[45], there were 79 women with 325 pregnancies. The compared patients on 6-MP during conception, those that stopped prior to conception, and patients never exposed to 6-MP were compared. Although they did not look at prematurity or LBW, there were no statistical differences in spontaneous abortions, major congenital abnormalities, neoplasia, or increased infection [RR 0.85 (0.47-1.55), $P = 0.59$]. Moskovitz *et al*[46] looked at IBD medications (including 6-MP and azathioprine) taken during pregnancy. In this age-controlled multivariate analysis of 113 patients with 207 conceptions, there was no evidence that medications affected pregnancy outcomes (abortions, premature birth, healthy full-term birth, ectopic pregnancy, congenital abnormalities, birth weight, or type of delivery). Nørgård *et al*[47] combined two large national data registries with a national prescription database to look at therapeutic drug use in women with CD and birth outcomes. Among the women that were exposed to 6-MP/azathioprine throughout their pregnancies, the risk of preterm birth and congenital abnormalities was 4.2 (95% CI: 1.4-12.5) and 2.9 (95% CI: 0.9-8.9), respectively. Preterm births were more prevalent among steroid and 6-MP/azathioprine exposed women compared to the reference group. However, they were unable to stratify disease activity to adverse birth outcomes due to the low number of hospital admissions. Due to model fitting issues, the authors could not adjust for the "disease activity" when looking at the LBW infants at term[48].

In has been our practice to continue immunomodulator therapy through pregnancy. While classified as an FDA category D medication, it received this designation in the 1950s when originally approved to treat leukemia. The doses used to treat IBD are much smaller and the above cited literature suggests this to be a low risk therapy at these doses. Stopping therapy once pregnancy is diagnosed only puts the mother's disease at risk of flaring, as organogenesis has already occurred at this point and the fetus has been exposed at its most vulnerable-

cessation for safety to the fetus has to be 6-8 wk before conception, not after.

Biologics: Biologics are now more commonly used for more aggressive disease, and sometimes are used as first line in the "top-down" therapeutic approach. The first series of intentional infliximab use throughout pregnancy by Mahadevan *et al*[49] examined outcomes in ten women with active CD during pregnancy. All ten pregnancies resulted in live births, with no congenital malformations. There were three pre-term births and one LBW infant, but these were not unexpected in a population of women with CD significant enough to require biologic therapy. Infliximab has been detected in the infants born to mothers receiving infliximab during the third trimester of pregnancy; however, to date, the long term effect of this placental transfer is unknown[50].

A published report for the successful use of adalimumab in pregnancy describes a patient with severely active disease at conception[51]. She was placed on adalimumab one month prior to conception and delivered of a normal growth infant without visible congenital anomalies. A case series recently presented suggested its safety in fetal outcomes in women treated for CD[52]. More recent data presented in abstract form showed that patients exposed to natalizumab during pregnancy had a spontaneous abortion rate comparable to what is expected in the general population[53]. However, the number of exposed patients was too low to draw any definitive conclusions. Therefore, it is important for the physician to discuss with each patient the risk to benefit ratio of biologic therapy to control disease in pregnancy.

It has been our practice to continue biologics at least through the second trimester of pregnancy. Placental transport of IgG begins around week 20 and increases thereafter. Theoretically therefore, no therapy is reaching the fetus until administrations after week 20. If the last infusion of infliximab is timed to be around week 32, then the next infusion can be after delivery. Adalimumab injections are held after week 36.

Other agents: Corticosteroids have not been associated with teratogenicity in humans and can be used as required to control disease activity. Prednisolone crosses the placenta less efficiently than other steroid formulations, such as betamethasone or dexamethasone. Only limited data are available regarding the safety of antibiotics as treatment for CD. Currently, ampicillin, cephalosporins, and erythromycin are believed low risk, as is ciprofloxacin. Metronidazole has been used to treat vaginitis in women during the first trimester of pregnancy but no controlled trials have definitively demonstrated its safety[54].

CONCLUSION

Over twenty years ago it was recognized that IBD patients flaring at the time of conception had a higher chance of spontaneous abortion, still birth, and premature delivery. The classic Miller[6] paper from 1986 is often referenced. For patients with active disease, one third will improve, one third will stay the same, and one third will worsen. This leaves two thirds of patients having to live with active disease during pregnancy[48]. Despite conflicting and sometimes confusing data, it is clear that there are potential risks involved with IBD pregnancies. It is crucial to understand the literature and also recognize its limitations. Ideally, conversations with the patient should occur before conception. Furthermore, decisions should be made with an informed approach with a team effort between the patient, the obstetrician, and her gastroenterologist. The key is continued monitoring and aggressive control of the disease prior to conception and throughout, to achieve optimal outcome for mother and baby.

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TOPIC HIGHLIGHT

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Extraintestinal manifestations of inflammatory bowel disease: Do they influence treatment and outcome?

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Abstract

Crohn's disease and ulcerative colitis are chronic inflammatory bowel diseases that often involve organs other than those of the gastrointestinal tract. Immune-related extraintestinal manifestations (EIMs) are usually related to disease activity, but sometimes may take an independent course. Globally, about one third of patients develop these systemic manifestations. Phenotypic classification shows that certain subsets of patients are more susceptible to developing EIMs, which frequently occur simultaneously in the same patient overlapping joints, skin, mouth, and eyes. The clinical spectrum of these manifestations varies from mild transitory to very severe lesions, sometimes more incapacitating than the intestinal disease itself. The great majority of these EIMs accompany the activity of intestinal disease and patients run a higher risk of a severe clinical course. For most of the inflammatory EIMs, the primary therapeutic target remains the bowel. Early aggressive therapy can minimize severe complications and maintenance treatment has the potential to prevent some devastating consequences.

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Key words: Inflammatory bowel disease; Immune-related extraintestinal manifestations; Treatment

INTRODUCTION

Inflammatory bowel diseases (IBDs) are clinically heterogeneous disorders with the potential for systemic involvement. The clinical spectrum of these extraintestinal manifestations (EIMs) varies from mild transitory to very severe lesions, sometimes more incapacitating than the intestinal disease itself.

Most of the EIMs, such as those that occur in the joints, skin, mouth and eyes, are related to the activity of the bowel disease and, consequently, have been referred to as "inflammatory". Others are associated with autoimmunity, while further manifestations result from nutritional or metabolic dysfunction.

Patients with ulcerative colitis (UC) are considered a more homogeneous group than patients with Crohn's disease (CD). The most important significant variables in UC are the extent of colonic involvement and the activity of disease.

The current clinical practice for management of CD patients should take into account a major number of events, such as age at diagnosis, location, behavior, intestinal complications, EIM symptoms and previous medical or surgical treatment.

The aim of the present manuscript is to review the common immune-related EIMs and to evaluate whether they are important targets of treatment or just an expression of disease activity.

PREVALENCE OF EIMS IN IBD

Globally, about one third of patients with IBD develop inflammatory systemic manifestations. The frequency of these manifestations in previous major studies ranged from 21% to 41%, depending on the patient population and criteria used[1-5]. In our series they were observed in 26% of the patients and were more common in CD than in UC patients. However, during the follow-up period the cumulative probability of developing EIMs increased from 12% at diagnosis up to 30%[2].

We have studied the course of CD according to the Vienna classification and clinical activity[6]. More recently, in this cohort of 480 patients followed from diagnosis, we investigated the cumulative probability of inflammatory EIMs, which varied from 22% at diagnosis to 40% 10 years after diagnosis. Here we showed that a single manifestation occurred in 80 patients, whereas multiple manifestations overlapping joints, skin, mouth, and eyes occurred in 89 patients (Figure 1).

The specific disease entities considered were peripheral and axial arthritis (joints), erythema nodosum and pyoderma gangrenosum (skin), aphthous stomatitis (mouth), and episcleritis and uveitis (eye). Thus, it was confirmed that two or more manifestations in the same patient occurred more frequently than would be expected by chance alone ($P < 0.001$)[2,4].

Patients with one of these EIMs not only run a high risk of a repetition of the same manifestation, but also of having any of the other associated manifestations. In our patients the cumulative probability of a second manifestation was about 70% at 10 years of follow-up.

Moreover, we have also studied the appearance of various EIMs throughout the clinical course and it was observed that some types of manifestations are more likely to appear during the early stages. This was particularly evident for skin manifestations, which tend to occur in most of the patients for the first time during the first 2 years of follow-up (Figure 2).

We have also studied the probability of EIMs in different subsets of CD patients[7]. The location of disease was significantly different between patients who had and who did not have EIMs and was higher in those with colonic involvement ($P < 0.005$) (Figure 3).

To calculate the clinical course, we constructed a Markov model to study the probability of maintaining a similar state during the following year. Patients were divided into two exclusive groups, those who had and those who did not have manifestations. Patients with EIMs ran a higher risk of a severe clinical course ($P < 0.005$)[7].

In UC it is still controversial whether EIMs are correlated with the extent of colonic involvement, although they are usually related to the activity of the disease.

DESCRIPTION OF EIMS

Joint manifestations

Peripheral involvement of the joints is the most common of the EIMs and it affects between 5% and 20% of IBD patients[8-11]. In our series, arthritis occurred frequently, twice as often in CD as in UC (20% *vs* 11%)[2]. The joint involvement is mostly monoarticular, asymmetrical, transitory and migrating. Generally, it is of short duration and is unaccompanied by residual deformity or radiological change.

The diagnosis is based on clinical signs of synovitis, eventually associated with enthesitis. However, using articular scintigraphy, a sensitive indicator of active joint disease, asymptomatic and mild forms of synovitis could be detectable. We have evaluated articular involvement, clinically and by scintigraphy, in forty-two IBD patients. More affected joints have been diagnosed scintigraphically than

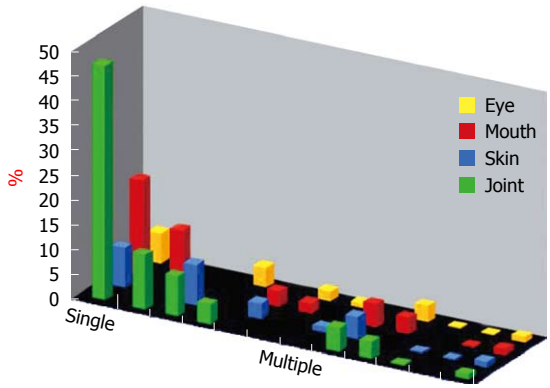

Figure 1 Associations of the four major inflammatory extraintestinal manifestations in 169 of 480 Crohn's disease patients.

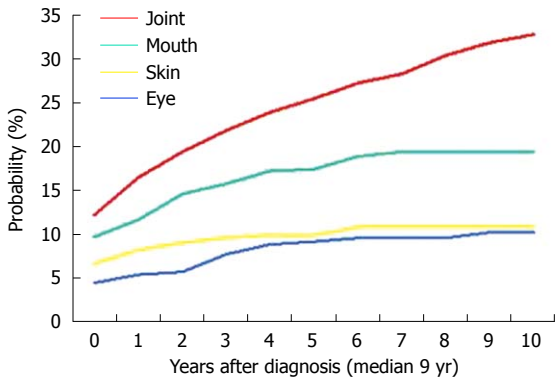

Figure 2 Cumulative probability of having a repetition of the same inflammatory extraintestinal manifestations.

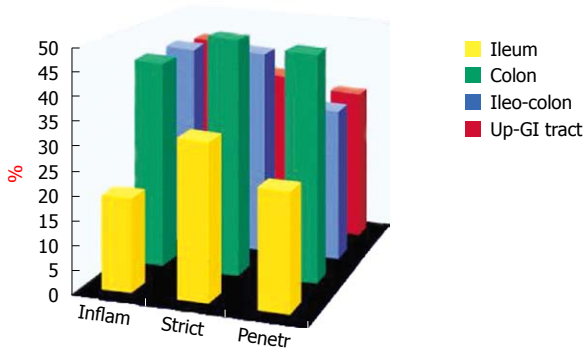

Figure 3 Distribution of inflammatory extraintestinal manifestations according to the Vienna classification in 169 Crohn's disease patients.

clinically, indicating that the prevalence of this complication could be much higher than generally believed[11].

The clinical activity of synovitis tends to parallel that of the intestinal disease. In most instances, the synovitis symptoms follow rather than precede IBD and recurrence is common, frequently coinciding with a flare-up of intestinal disease. Also, they are more common in patients with extensive colitis.

Regarding treatment, the main issue should be to control the clinical activity of the underlying bowel disease. Thereafter, there is agreement that this manifestation generally responds well to medical or surgical treatment targeted to the bowel disease. Conventional treatments for mild to moderate inflamed joints consist of acetaminophen, sulfasalazine, corticosteroids, and if necessary nonsteroidal anti-inflammatory drugs (NSAIDs). Furthermore, the majority of IBD patients with active intestinal disease and peripheral arthritis will notice an improvement of joint symptoms after receiving infliximab[12].

Ankylosing spondylitis, as defined by the Rome criteria, is associated with IBD in 5% of cases and it runs an independent course to the IBD[8-10]. In fact, the onset of ankylosing spondylitis is not related to the onset of IBD and usually precedes it. Also, there is no association between the severity of IBD and axial arthritis.

The proven efficacy of infliximab therapy in CD and rheumatoid arthritis has led to the suggestion that anti-tumor necrosis factor-α treatment might also be a useful agent in the management of spondyloarthropathies associated with CD.

In several open studies, infliximab has been used in patients with ankylosing spondylitis, leading to clinical, biologic and imaging improvement[12-15].

In our clinical practice the use of infliximab in patients with spondyloarthropaties associated with CD has resulted in an impressive improvement in clinical and laboratory abnormalities. The same has been reported in other uncontrolled studies[12,16]. In a multicentric, randomized, double-blind international trial to evaluate the efficacy of infliximab maintenance therapy in patients with moderate to severe active CD (ACCENT I), it has been reported that maintenance therapy with infliximab is superior to a single dose in resolving arthritis/arthralgia[17].

Clinical data suggest that intensive therapeutic strategies using biologic drugs should be tailored to the individual with severe or refractory articular disease. However, long-term studies with larger numbers of patients will be necessary to determine the patients who are most likely to benefit from these specific treatments. Furthermore, careful attention should be paid to the potential adverse events; mainly infections and malignancy.

Skin manifestations

There is a wide range of dermatological manifestations reported in patients with IBD, of which erythema nodosum is the most common form of reactive eruption. The prevalence of erythema nodosum has been reported to occur in up to 15% of patients[1], although other, more recent studies have reported that the prevalence may be considerably lower[10,18]. In fact, in a previous report from our group it was seen in 8% and 3% of CD and UC patients, respectively[2]. Clinically, erythema nodosum appears in conjunction with symptoms of active bowel disease and is most common in females, patients with large intestine involvement and peripheral arthritis[2].

Figure 4 Severe lesions of erythema nodosum treated with infliximab.

Erythema nodosum lesions usually respond to treatment with corticosteroids used to control the bowel flare-up. In refractory cases, potassium iodide, colchicine, hydroxychloroquine, and thalidomide have been used[19]. More recently a successful response to infliximab in severe erythema nodosum has also been reported[20]. We have used this treatment in a patient with colonic CD complicated with severe lesions of erythema nodosum and arthritis, refractory to high doses of steroids, which resulted in a rapid and complete resolution of skin and articular manifestations (Figure 4).

Pyoderma gangrenosum is a severe and debilitating skin disorder, characterized by the occurrence of nodules and pustules that rapidly enlarge and ulcerate. Lesions typically occur on the extensor surfaces of the lower extremities, but may also be found elsewhere. Since there is no specific test, the diagnosis can be difficult, only being established when the clinical and histopathological pictures are consistent with pyoderma gangrenosum, and other pustular or ulcerative dermatoses have been excluded.

This chronic ulcerating skin disorder can be found in 0.4%-2% of patients with IBD[10,19]. Patients with severe disease and colonic involvement are most likely to develop this complication. Pyoderma gangrenosum may run a course independent of the IBD, but sometimes coincides with an exacerbation of the underlying intestinal disease.

Management of pyoderma gangrenosum continues to be a therapeutic challenge and usually requires aggressive local and systemic therapy. High doses of oral prednisone and/or intralesional injections of corticosteroids are generally effective in the management[21]. However, the best form of treatment, particularly in patients with inactive intestinal disease, has not been established yet. Several clinical cases have been successfully treated with intravenous cyclosporine, oral and topical tacrolimus, or mycophenolate mofetil and granulocytapheresis[22-27].

In several reports patients treated with infliximab have shown rapid healing of their lesions[28]. We have successfully treated one patient in this way (Figure 5). In a recent controlled study, 6 of 13 patients with pyoderma

Figure 5 Pyoderma gangrenosum before, during and after treatment with infliximab.

Figure 6 Aphthous ulcers in the buccal mucosa and on the tongue.

gangrenosum were successfully treated with infliximab[29]. Thus, infliximab treatment is highly effective in healing refractory lesions and has become the first choice for the treatment of pyoderma gangrenosum.

Oral ulceration

Aphthous ulcers are the most common oral lesions in IBD; they occur in about 10% of patients. Aphthae are shallow, round ulcers with a central fibrinous membrane and erythematous halo (Figure 6). Their onset is usually sudden, coinciding with the flare-up of intestinal disease and occurring simultaneously with other EIMs[2,30].

Many similarities exist between CD and intestinal Behçet's disease. Both diseases have similar EIMs, particularly aphthous ulcers, which are an initial symptom in the majority of patients with Behçet' disease[31].

Oral ulcers usually respond to the treatment of the underlying bowel disease, but sometimes they may be resistant to conventional therapy. In these situations topical applications of steroids or tacrolimus may be of use[32]. Oral thalidomide has been reported to be beneficial in the treatment of aphthous stomatitis related to HIV infection and could be tried for lesions in patients with IBD. Infiximab has also been used in the treatment of patients with CD complicated with gigantic oral ulcers[7].

Eye manifestations

Ophthalmologic complications have been reported to occur in up to 12% of patients according to the published literature and these complications seem to occur more frequently in CD patients[33]. The most common complications are episcleritis, scleritis, and uveitis. Uveitis is the most serious complication and may be the cause of significant morbidity. Often it occurs independently of the bowel disease and is particularly associated with the musculoskeletal EIMs[34] (Figure 7). Specific treatment may include topical and systemic steroids. In the management of ophthalmologic manifestations, immunosuppression is often required. Infliximab has been reported to have a beneficial effect in a few patients suffering from CD with uveitis and spondyloarthropathy, and is increasingly being used in both acute and chronic ocular inflammatory manifestations of IBD[12,35-37].

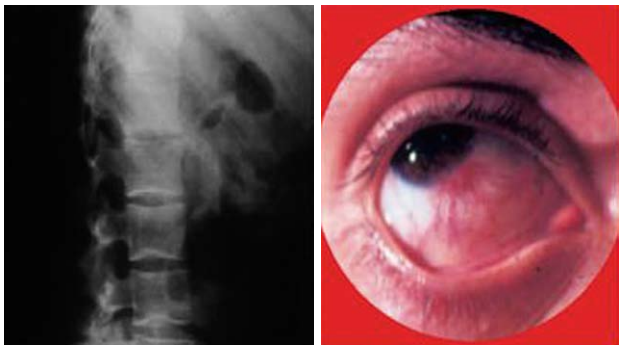

Figure 7 Ankylosing spondylitis and uveitis in the same patient.

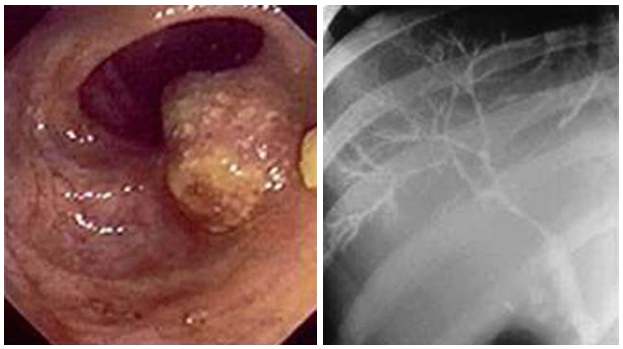

Figure 8 ERCP showing sclerosing cholangitis and endoscopic view of carcinoma of the colon observed in the same patient.

Hepatobiliary manifestations

Primary sclerosing cholangitis (PSC) is the most common immune-mediated hepatobiliary disease. It is a chronic, progressive, cholestatic disorder characterized by inflammation and fibrosis of the intrahepatic and extrahepatic bile ducts. The prevalence of IBD (mostly UC) is about 75% and PSC complicates approximately 2%-5% of cases of UC and 0.7%-3.4% of cases of CD[10,38].

Most patients with PSC are diagnosed when asymptomatic with abnormal liver function tests performed in routine follow-up. Symptoms of PSC usually consist of fatigue, pruritus, right upper quadrant pain, fever, jaundice, and weight loss. Magnetic resonance (MR) cholangiography is often used for diagnosis of PSC and avoids the complication of endoscopic retrograde cholangiopancreatography. Liver biopsy is not always necessary where typical features are present on MR cholangiography[39].

IBD-PSC patients have a specific phenotype, characterized by extensive, mild disease with a particularly high risk of colonic dysplasia and carcinoma (Figure 8), as well as an increased risk of bile duct cancer. Yearly surveillance

colonoscopy is recommended for those patients with colonic involvement[40,41].

The medical management of PSC remains controversial. Ursodeoxycholic acid is widely used for this indication, and the best results have been obtained with high dose (up to 22-25 mg/kg). Moreover, the evidence that this medication prevents progression of PSC and that it may reduce the high risk of colonic cancer in these patients may become an indication for its use[38]. Liver transplant is the only treatment for patients with advanced PSC, with a 5-year survival rate of approximately 80%. However, the optimum timing for transplantation remains a difficult decision.

CONCLUSION

During the course of IBD, EIMs related to the activity of intestinal inflammation are quite common. Phenotypic classification shows that certain subsets of patients are more susceptible to developing EIMs. They also have a higher probability of maintaining active disease during the clinical course. For most of the inflammatory EIMs, the primary therapeutic target remains the bowel. Early aggressive therapy with biologic drugs may be required for several EIMs and maintenance treatment has the potential to prevent some devastating consequences. Long-term studies with larger numbers of patients will be necessary to determine the patients who are most likely to benefit from specific treatments.

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TOPIC HIGHLIGHT

Belén Beltrán, MD, PhD, *Series Editor*

Inflammatory bowel disease in travelers: Choosing the right vaccines and check-ups

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Abstract

The majority of patients with inflammatory bowel disease (IBD) achieve good control of the inflammatory activity using available therapies. When remission is achieved and quality of life recovered, a considerable proportion of IBD patients express their desire to travel abroad, be it for business, academic or leisure purposes. Their physicians should help and encourage them whenever possible. However, preventive measures are warranted to minimize the risk, since IBD patients are exposed to the same infections affecting the general population, plus opportunistic infections (OI) related to the immunosuppression. There are a large number of potential OI that might affect patients with IBD. The true prevalence of these infections is unknown, and can vary from country to country. Therefore, reactivation or *de novo* acquisition of infections such as tuberculosis, malaria, and viral hepatitis will be much more frequent in endemic areas. Therefore, physicians should be aware of these aspects when planning specific preventive measures for patients traveling to a particular country. This includes good control of environmental exposure, chemoprophylaxis when indicated, and the use of a specific vaccination program to prevent endemic infections. In addition, it should be noted that, though the risk of acquiring an infectious disease is probably greater for IBD patients traveling from a developed to a developing country, the inverse situation can also occur; it depends on the previous acquired immunity of the host against infections in any particular environment.

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Key words: Inflammatory bowel disease; Vaccination; Opportunistic infections

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PREVENTIVE MEASURES AND CHECK-UPS BEFORE THE TRIP

Patients traveling abroad should adhere to a specific vaccination program depending on the country to which they are traveling. Specific advice by specialists in tropical medicine might be required at least 4-6 wk before the trip, to allow time for vaccines to take effect and to start taking chemoprophylaxis to prevent malaria when needed. Requirements for visiting/staying in any particu-

lar country can be found on the websites of the World Health Organization (WHO) (http://apps.who.int/tools/geoserver/www/ith/index.html) and of the Centers for Disease Control and Prevention (CDC) (http://wwwnc.cdc.gov/travel/destinations/list.aspx). An interactive map provides information on health risks for travelers to specific countries by clicking on any part of the country or a nearby country.

The vaccination status according to current recommended vaccination program for adult inflammatory bowel disease (IBD) patients[1-3] (Table 1) should be periodically monitored, particularly before the start of a trip. Concerns about the safety of live-attenuated vaccines in immunosuppressed patients have arisen due to the possibility of fatal reactivation of the infectious agent contained in the vaccine. However, there are no data regarding the required time free of immunomodulators for administering these vaccines with confidence. The risk of reactivation, which also occurs with opportunistic infections (OI), probably depends on the type, dose, and duration of immunosuppressants added to a particular host response, for example the existence or not of leucopenia and lymphopenia[4]. Thus, an immunosuppressant washing-out period of 2 mo before and after live attenuated vaccine administration seems to be prudent. Live attenuated vaccines include, among others, varicella and yellow fever vaccines (Tables 1 and 2). Additionally, there is limited information as to whether IBD patients, mainly those under immunosuppressants, acquire adequate levels of protection using the present recommended vaccination schedule. For example, in one study including adult patients with rheumatological diseases and Crohn's disease vaccinated against two strains of influenza (A/H3N3 and B), the majority achieved protective titers ⩾ 40, irrespective of whether or not they were taking anti-tumor necrosis factor (TNF) therapy[5]. However, impaired response against a single dose of 23-valent pneumococcal polysaccharide vaccine[6] and against standard three doses of hepatitis B vaccine[7] was reported, mainly in those patients treated with combined immunosuppressants and biologics. Therefore, it remains to be established whether higher doses of vaccine and booster administration might afford better immunization against specific infections.

For IBD travelers coming from countries with well-developed immunization programs, vaccination against tetanus, diphtheria, and inactivated poliomyelitis should be administered every 10 years and a booster dose is recommended before traveling. A booster dose against pertussis is also advisable in combination with the former, as a resurgence of pertussis has been documented in many industrialized countries, such as the United States, Australia, and Canada. Estimating rates of pertussis in developing countries is difficult, because of a lack of access to diagnostic methods and under-reporting. However, epidemiologic estimates by the WHO from Asia, Africa, and South America demonstrate that these areas have the highest disease burden, and there is a public health problem in all age groups[8].

Antibodies against hepatitis B should be checked when planning a trip to high prevalence areas (China, Southeast Asia, and tropical Africa) or intermediate prevalence areas (Eastern Europe, the Mediterranean, Russia, and Central and South America). For immunocompromised patients, regular testing and booster administrations, when anti-HBs antibody levels fall below 10 mIU/mL, are recommended[9]. This is important because not only can the response to standard dose of vaccination be impaired, but antibody titters can decrease with time in immunosuppressed patients who have achieved an adequate previous immunization. There is a combined Hepatitis A inactivated and hepatitis B recombinant vaccine for the immunization of adults. The effect of hepatitis A immunization in IBD patients is not known, but it seems to be severely impaired in immunosuppressed patients for other disease conditions[4].

Influenza viruses change rapidly from season to season, consequently Influenza vaccine must be manufactured and administered every year. This vaccine includes the three strains that cause the most illness in the upcoming season based on virus samples and patterns collected from around the world. Influenza viruses circulate worldwide and IBD travelers should be aware of the outbreaks of specific influenza viruses when they plan a trip to a particular country. They should be vaccinated for the specific strain affecting this particular area at a given moment. In this sense, the pandemic caused by the H1N1 swine-derived influenza strain, or the previously emerged avian influenza strains still circulating, constitute good examples of the need for specific protection[10].

One of the biggest problems for IBD patients traveling to tropical areas of South America and Sub-Saharan Africa is how to prevent yellow fever. It is caused by an RNA hepatotropic virus that causes a pansystemic disease, with fever, hepatic, renal and myocardial injury, hemorrhage, shock, and mortality as high as 50%. Vaccination against yellow fever is mandatory when visiting 16 countries and strongly recommended for all endemic countries. Although limited studies indicate that vaccine immunity lasts for at least 45 years, the WHO requires booster immunizations every 10 years to maintain adequate protection. The yellow fever vaccine 17D contains live attenuated virus and is contraindicated for IBD patients who cannot stop immunosuppressants for at least 4 mo. Hence, traveling to endemic areas should be discouraged for patients requiring continuous immunosuppression to keep their IBD under control. However, the long-lasting immunization effect of yellow fever vaccine allows its administration at any time that is convenient for immunosuppressant discontinuation when a trip to endemic areas is expected in the future. Risk of serious adverse events following yellow fever vaccination is very low, but increases with age (4 per 100 000 doses for people aged 60-69 and 7.5 per 100 000 doses for people 70 and older)[11].

Neisseria meningitidis causes endemic meningococcal disease worldwide, with a specific serotype distribution

Table 1 Current recommended vaccination program for adult inflammatory bowel disease patients[1-3]

Illness	Vaccine	Recommendation	Schedule
Tetanus Diphteria	Purified anatoxin	Recommended	Every 10 yr
Poliomyelitis	Injectable: inactivated	Recommended	Every 10 yr
Pertussis	Acellular antigen	Authorized	Every 10 yr
Hepatitis B	Recombinant peptide	Recommended	Single/double doses? Booster?
Pneumococcal disease	23-valent purified antigen	Recommended	Every 5 yr Single/double doses? Booster?
Influenza	Inactivated virus	Recommended	Annually
Human papillomavirus infection	Recombinant L1 protein	Authorized	??
Measles,mumps and rubella	Live attenuated	Contraindicated during immunosuppression	??
Varicella	Live attenuated	Contraindicated during immunosuppression	Double dose (4 wk interval)
Haemophilus influenzae B disease	Conjugated capsular polyosidique antigen	Authorized	Single dose

Table 2 World distribution of travel-related preventable illnesses and current recommended vaccination program for adult inflammatory bowel disease patients

Illness	Regions with high and intermediate endemicity	Vaccine/schedule	Recommendation
Hepatitis A	High: Africa South America, Middle East Southeast Asia, China Intermediate: Southern and Eastern Europe	Inactivated virus (every 10 yr)	Authorized
Yellow fever	Africa: Sub-Saharan Africa America: Central and South America	Live attenuated (every 10 yr) 17D strain (17D-204 /17DD)	Contraindicated during immunosuppression
Meningococcal disease	Europe: Serogroups B, C Americas: Serogroups B, C, Y Africa and Asia: serogroups A, C, W135	Conjugate polysaccharide C Polysaccharide combined A+C Polysaccharide combined A+C+W+Y (single dose among persons aged 11-55)	Authorized Authorized Authorized
Typhoid	High: Southern Africa Western, Eastern South central and Southeastern Asia Intermediate: Eastern, Middle and Northern Africa, Western Asia, Latin America/Caribbean, Oceania	Vi Capsular polysaccharide (single dose IM. Booster dose every 2-3 yr for those at risk)	Authorized
Cholera	Africa: Congo, Kenya, Mozambique, Uganda, Tanzania and West Africa	Oral Killed (2 doses at 1-6 wk interval with a buffer to protect the B-subunit against stomach acidity)	Authorized
	South and Central America: Peru, Ecuador, Guatemala, Nicaragua Asia: Afghanistan, India, Cambodia, Malaysia, Nepal, Sri Lanka	Oral live	Contraindicated during immunosuppression
Rabies	High: Africa, Asia, parts of Central and South America Intermediate: Eastern Europe, parts of central and South America (Chile, Argentina)	Cell culture-derived vaccine (travellers, not handling animals: 2 doses, at days 0-28. If risk continues booster dose at 6-12 mo)	Authorized
Japanese encephalitis	Southeast Asia Far East	Cell culture-derived vaccine (2 doses, at days 0-28 booster dose?)	Authorized
Tick-borne encephalitis	Europe: Central and Eastern Europe, Russia Asia: China, Siberia, Russian Fareast	Inactivated virus (3 doses at 0,1 and 12 mo)	Use with caution

per continent (Table 2). Polysaccharide vaccines against meningococcal serogroups A, C, Y, and W135 have been available for several decades, but have been little used due to poor immunogenicity and minimal effects on nasopharyngeal carriage. The recent advent of a quadrivalent conjugate vaccine including A, C, Y, and W135 ensures a broad coverage for travelers and should always be considered before the polysaccharide vaccine[12]. However, effective global prevention of meningococcal disease will not be achievable without the availability of a vaccine against Group B meningitis (predominant in Europe and America), for which outer membrane protein vaccines are under development[13].

Travel to the Indian subcontinent is associated with the highest risk of contracting enteric fever. There are two available vaccines against *S. typhi*: the live attenuated oral vaccine containing the *S. typhi* strain Ty21a (Ty21a vaccine) and the parenteral capsular polysaccharide vaccine based on the *S. typhi* Vi antigen (Vi vaccine). Thus, the Vi vaccine is recommended for IBD patients. It is

available for children ⩾ 2 years old, conferring protection 7 d after injection with a maximum neutralizing antibody concentration demonstrated 28 d after vaccination[14]. Percentages of efficacy in immunocompetent individuals range from 55% to 72%, but the figure is unknown for IBD patients taking immunosuppressants. The gastroenterologist should discuss with the patient the efficacy of the vaccine and reinforce the necessity of strict food and water precautions. The same control measures are required to prevent cholera and all diarrheal illnesses. However, when access to clean water and sanitation are not guaranteed, cholera vaccine should be administered, conferring 85% short-term protection, and 60% protection up to 3 years following vaccination. IBD patients taking immunosuppressants should receive the oral-killed vaccine licensed in more than 20 countries, including the European Union (Dukoral®). Another available oral-killed vaccine (Vabiotech, ORC-Vax®) was initially licensed only in Vietnam[15].

Rabies is a viral zoonosis, almost invariably fatal in humans. Rabies is widely distributed throughout the world and present in all continents. However, the probability of rabies exposure is directly related to the incidence of rabies in the area and the probability of contact with infected animals. In areas of high-risk exposure, such as most parts of Africa, Asia, and Latin America, human rabies occurs from the bite of domestic and stray dogs and cats without owners. By contrast, in low-risk areas (North America, southern Africa, parts of the Caribbean, and Europe), the principal mammalian reservoir species are wild carnivores[16]. Currently, cell-culture derived vaccines are used, and these are authorized for use by IBD patients traveling to high risk areas, particularly where there is limited access to medical care[1,3].

Japanese encephalitis (JE) is a leading cause of viral meningoencephalitis transmitted by *Aedes* mosquitoes in large parts of Asia. It is mainly a problem in rural rice growing and pig farming regions, but can also be found at the outskirts of cities. It occurs more commonly in the rainy season (roughly May-September), when the mosquitoes are most active[17]. Individuals under chronic conditions and under anti-TNF therapy are particularly considered candidates for JE vaccine[18]. A novel inactivated cell culture-derived vaccine (IXIARO®) has recently been licensed in the United States and Europe and can be safely administered to IBD patients.

Tick-borne encephalitis (TBE) is a disease of the central nervous system caused by a tick-borne viral infection. A recent systematic review has demonstrated that the three currently licensed TBE vaccines (Encepur children®, Encepur Adults®, and FSME-IMMUN"new"®) gave seroconversion rates of over 87%[19]. However, the relationship between seroconversion and clinical protection has not been established. As all the vaccines may produce commonly but generally mild adverse effects, their use in IBD patients is authorized with caution. In addition, the TBE vaccine has been suspected of causing an exacerbation of autoimmune diseases, but a cause-and-effect relationship has not been confirmed. Taking all these factors together, risk-benefit should be weighed and vaccine administered to those patients traveling to high-risk rural or forested areas, especially in the spring or summer.

PREVENTIVE MEASURES DURING THE TRIP

Though vaccination or chemoprophylaxis remains the most effective means of traveler infection prevention, some additional measures upon exposure to environmental factors during the trip should be taken. These include the avoidance of insect bites and the ingestion of safe foods and beverages. Insects are vectors of infections such as malaria, dengue, filariasis, Chagas, leishmaniasis, onchocerciasis, and trypanosomiasis, among others. At the WHO and CDC websites there is updated information about diseases that should be prevented per country, the type of insect vector, and the best means of prevention. Some of these diseases may be partly prevented by the application of insect repellents. However, the ideal mosquito repellent remains to be identified. It should repel multiple species of arthropods, have long-lasting effect, cause no local or systemic toxicity, be resistant to abrasion and rub-off, and be greaseless and odorless. DEET (N,N-diethyl-m-toluamide) remains the gold standard of currently available insect repellents[19]. Used at variable concentrations ranging from 10% to 75%, it is considered that a concentration higher than 50% offers no additional benefit. Other measures, such as avoidance of outdoor exposure during the period of maximum insect activity (for example crepuscular periods for malaria mosquitoes), and wearing long-sleeved shirts, long pants, and a hat outdoors, are also advisable.

It is important to prevent animal bites and scratches to avoid rabies. If suspected exposure to rabies occurs, prompt and thorough cleansing of the wound, together with administration of immunoglobulin added to immunization with the above-mentioned vaccine starting immediately after exposure, virtually guarantees complete protection[16].

IBD patients should pay particular attention to preventing traveler's diarrhea (TD), as there is evidence showing that intestinal infections can trigger the disease[20] or induce relapses[21]. However, infection by enteropathogenic bacteria does not appear to be associated with a poorer clinical outcome of the IBD flare[21]. There is no evidence supporting the need for chemoprophylaxis for IBD travelers to prevent diarrhea, and the majority of specialists prefer not to recommend it to travelers[22]. However, a recent expert review included IBD patients as potential candidates for chemoprophylaxis[22]. An intermediate and very reasonable position might be early self-treatment when a gastrointestinal infection is suspected[4,23]. Fluoroquinolones are the drugs of choice in IBD, followed by azithromycin in patients who take fluoroquinolone as a part of their treatment[4]. The efficacy of Rifaximin against enteroinvasive bacteria (*Campilobacter*,

Table 3 Preventive measures for inflammatory bowel disease patients coming from developing countries (mainly while taking immunosuppressants or before starting)

	South America	Maghreb and Western Orient	Sub-Saharan Africa	Southeast Asia and India	Other
Thick drop	Consider	No	Always	Consider	No
Stool parasite	Always	Consider	Always	Always	No
Urine parasite	No	No	Always		No
Strongyloides (culture, serology)	Always	Consider	Always	Always	No[1]
Trypanosoma (serology)	Always	No	No	No	No
Histoplasma (serology)	Always	No	Always	No	No
HBV and HCV (serology)	Always	Always	Always	Always	Always
Tuberculin skin test or IGRA	Always	Always	Always	Always	Always

[1]Consider in case of Chinese individuals with eosinophilia. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Shigella, and *Salmonella*) has never been proved and consequently it is not an appropriate drug for IBD patients with bloody diarrhea and fever[4,22].

The evidence for the use of vaccines against enterotoxigenic *Escherichia coli* (ETEC) is scarce and it cannot be recommended at the present time for TD prevention. In addition, studies should be performed to demonstrate which foods and beverages have the lowest and the highest risk for TD. Though firm evidence is lacking demonstrating the value of dietary and beverage selection in the prevention of TD, uncooked fruits and vegetables and untreated drinking water are considered to hold the highest risk[22].

PREVENTIVE MEASURES AND CHECK-UPS TO DETECT INFECTIONS HARBORED DURING THE TRIP

Patients with IBD are likely to be treated with steroids, immunomodulators, or biologicals. It has been reported that these therapies can increase the risk of severe infections[24] or OI[25], especially when administered in combination, and even more so in the elderly or when narcotics are also prescribed. Thus, those patients on maintenance therapy with these drugs who travel to developing countries are, at least hypothetically, at increased risk for OI. Of course, the time spent in those countries may be one of the most decisive factors in increasing the risk of such infections, and it should be always taken into account when evaluating these patients. Most OI will present as acute manifestations soon after (or even while) the patient returns home. However, some parasitic or protozoal infections can remain latent for years (e.g. strongyloidosis, Chagas' disease, or histoplasmosis); we must remember this especially in patients who stay for a long period of time in developing countries or, even more importantly, for individuals coming from developing countries who are diagnosed with IBD once in a developed country.

Another important factor is familiarizing oneself with the endemic infections affecting countries from which the patients are returning (Table 3). Depending on the geographic area, our investigations should be directed towards some infective agents or others. However, no specific recommendations are yet available for returning travelers; of course, there is no definition for "long-term" travel or stay in developing countries. Therefore, in this section the authors will try to draw a modest picture of which infections should be investigated, and how.

The most common clinical setting should be that of a patient returning from a short stay in a developing country who presents with diarrhea[26]. In this case, stool samples for microbiological evaluation using conventional cultures (mainly to detect enteric pathogens) should be taken and examination for fecal parasite detection should be carried out. It is important to collect at least three samples from different bowel movements (preferably from different days). If infestation by *Giardia lamblia* or *Criptosporidium parvum* is suspected, immunofluorescence is necessary. The presence of fecal *Entamoeba* cysts must be corroborated by serology; if positive, combined therapy with metronidazole and paromomycin should be started. However, in seronegative patients, paromomycin alone might be sufficient[27].

Among chronic infections, strongyloidosis might reactivate in case of immunosuppression[28]. Infection by *Strongyloides stercolaris* is endemic in tropical and subtropical regions, and it can be also seen anecdotally in Europe, the United States, Japan, and Australia. Evaluation of stool samples remains of paramount importance, although with limited diagnostic yield because of the intermittent presence of ova and larva in feces. Collection of repeated stool samples is strongly warranted, as is the use of concentration techniques or specific cultures that can achieve 80% sensitivity. Although serologic tests are highly sensitive, they do not differentiate past from current infection. Strongyloidosis might present not only by diarrhea, but also by abdominal pain or rectal bleeding. It should be especially suspected in patients coming from endemic areas, with a compatible clinical picture and peripheral eosinophilia[29]. Similarly, schistosomiasis is a prevalent chronic infestation in tropical and subtropical regions. Diagnosis is based in the visualization of ova in urine samples. Chagas' disease, or American trypanosomiasis, is a zoonosis found all over South America. The infection can remain latent for 10 to 30 years, and 30%-40% of individuals will then develop a chronic disease[30]. Interestingly, clinical symptoms will be those

related to the development of visceral involvement; in the case of the GI tract, dysphagia due to megaesophagus and constipation, abdominal pain and bloating due to megacolon make up the most common clinical picture[31]. Parasitological studies are of low diagnostic yield, and serological tests (indirect hemaglutination, indirect immunofluorescence, and ELISA) are preferred; in fact, two positive serological tests are necessary to establish the diagnosis[32]. Although digestive involvement is not usually life threatening, cardiac involvement can result in a fatal outcome.

Malaria and tuberculosis are the most common infectious diseases. *Plasmodium falciparum* can persist as a latent infection for 1 to 5 years, *P. ovale* and *P. vivax* for 3 to 5 years, and *P. malariae* for up to 40 years. Thick drop test in peripheral blood remains the diagnostic test of choice, although PCR tests can be useful in cases of low parasitemia[33,34].

Tuberculosis is nowadays systematically ruled out in patients who might be treated with anti-TNF agents. However, this diagnosis should always be taken into account in IBD patients returning from or coming from developing countries. Tuberculin skin tests and interferon gamma assays can be used for the diagnosis of latent or active TB, as recently stated in the European Crohn's and Colitis consensus[4,35]. Finally, histoplasmosis is widely distributed, although predominantly in America and Africa. Primoinfection is often subclinical, but reactivation can be life threatening. Diagnosis is based on direct observation on microscopy, and it is corroborated by a positive culture of biological samples (sputum, skin lesions, liver biopsy, or bone marrow aspiration). Antigenuria is useful to monitor therapeutic response[36,37].

CONCLUSION

In summary, patients with IBD on immunosuppressanttherapy (including steroids) must be carefully evaluated in case of fever, diarrhea, abdominal pain, or rectal bleeding when returning from or coming from developing countries. Learning about the geographical area and time spent in the area are of paramount importance in identifying one or another OI and appropriately targeting the diagnostic tests. In addition, an accurate medical history, as well as a complete physical examination, will be of great value.

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TOPIC HIGHLIGHT

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Familial aggregation in inflammatory bowel disease: Is it genes or environment?

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Abstract

Inflammatory bowel disease (IBD) develops in genetically susceptible individuals due to the influence of environmental factors, leading to an abnormal recognition of microbiota antigens by the innate immune system which triggers an exaggerated immune response and subsequent bowel tissue damage. IBD has been more frequently found in families, an observation that could be due to either genetic, environmental or both types of factors present in these families. In addition to expanding our knowledge on IBD pathogenesis, defining the specific contribution to familial IBD of each one of these factors might have also clinical usefulness. We review the available evidence on familial IBD pathogenesis.

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Key words: Inflammatory bowel disease; Familial aggregation; Familial clustering; Environmental factors; Genetics; Genome wide association studies

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INTRODUCTION

Our present understanding of inflammatory bowel disease (IBD) pathogenesis considers ulcerative colitis (UC) and Crohn's disease (CD) as complex conditions that develop in genetically susceptible individuals due to the influence of various environmental factors[1-6]. An abnormal recognition of certain antigens of the bowel microbiota by elements of the innate immunity is thought to play a key role, leading to an exaggerated immune response, release of pro-inflammatory molecules and, ultimately, bowel tissue damage.

In past decades a greater incidence of IBD cases among UC and CD relatives, referred to as family aggregation or familial IBD, has been clearly demonstrated[7-11]. The reason for such an increased risk is not straightforward, but genes, the environment or a combination of both could, in theory, account for family aggregation, considering their contribution to the development of IBD. A better understanding of the factors leading to familial IBD might result in clinical applications.

FAMILLIAL IBD

Familial aggregation among IBD patients or "familial IBD" is defined by the occurrence of a trait in more fam-

ily members than expected by chance. Multiple population studies have demonstrated that relatives of an IBD patient have a much higher risk of developing the same condition, compared to the general population[7-11]. The magnitude of familial aggregation depends on several factors, including: (1) type of IBD; (2) population studied; and (3) family relationship.

In respect to type of IBD, familial aggregation has been more frequently reported in CD than UC[7,9]. In first degree relatives, the age-adjusted relative risk of developing the same type of IBD ranges from 2-8 for UC and from 5-10 in the case of CD[7-9]. As elegantly demonstrated by Yang *et al*[9], affected relatives can develop both forms of IBD, although the greatest risk is associated with the appearance of the same disease type occurring in the index case. Additionally, CD patients tend to have a much higher frequency of relatives affected with UC when compared to UC patients having relatives affected with CD[7].

In respect to different populations, it has been shown that Jewish families present more than twice the number of multiple affected families when compared to the non-Jewish population[9]. However, we cannot rule out the possibility that differences amongst other geographical populations are due to their study design.

Finally, frequency of familial IBD also varies according to the degree and type of kinship. The prevalence of IBD in second-degree relatives appears to be lower than in first-degree relatives, especially in those with discordant disease[7]. In addition, although only a few studies have estimated the age-adjusted risk of IBD in relatives, it seems that the risk of IBD in offspring is higher than in parents and similar, or even slightly higher, than in siblings[8,9,11]. A potential source of bias could result from underreported cases in second-degree relatives and older generations, which might have influenced these differences.

In addition to an increased risk of developing the disease, first-degree relatives of IBD patients also have an increased likelihood of sharing the same phenotype[8,12]. This seems to be partly true in CD owing to the striking clinical concordance in families, in respect to disease location and behavior. On the contrary, literature is scarce and presents mixed results for CD severity and complications[8,9,13-16]. In UC families, the phenotype concordance data is less consistent, but a high concordance rate related to colonic extent and extra-intestinal manifestations has been reported[12,14].

The possibility that IBD develops at an earlier age in offspring than in their parents, a phenomenon known as anticipation, has been controversial[17-21]. Although different studies have reported such differences in age of onset, it seems that multiple biases could account for these findings. Whether familial IBD is a different clinical entity was the subject of debate. However, in the largest population-based study including 654 sporadic and familial IBD patients, a positive familial IBD history did not significantly influence clinical course or risk of developing IBD-related complications[22].

ROLE OF GENES IN FAMILIAL IBD

Evidences supporting the role of genetic factors in IBD pathogenesis

The fact that IBD is a genetically mediated disease was initially derived from the physician's perception of a higher prevalence of UC and CD cases among the relatives of IBD patients. This hypothesis was initially supported by case report studies showing clustering in IBD families and was subsequently confirmed by several population-based studies[3-7]. In one of these studies, Yang *et al*[9] described a risk of developing UC and CD among first degree relatives of IBD patients of 1.6% and 5.2%, respectively. The risk of developing IBD also varies according to the ethnic origin of individuals, a fact that is likely to be linked to their genetic background. In that regard, the prevalence of IBD among the Jewish population is 2 to 4 times higher than in any other ethnic group, being greater in Ashkenazi than in Sephardic or Oriental Jewish, with no influence from their geographical location[9,11].

Another source of evidence underlying the key role of genetic factors in IBD steams from twin studies. In these studies the greatest IBD concordance rate was found in monozygotic twins, ranging from 20% to 50% in CD and from 14% to 19% in UC twins, whereas in dizygotic twins concordance rates dropped to 0%-7% in both CD and UC twins[23-26]. The degree of monozygotic-dizygotic twin concordance found in CD point towards a genetically mediated condition with a non-Mendelian inheritance pattern. Of note, the concordance rates observed in CD are greater than these found in type 1 diabetes, asthma or schizophrenia, all of them diseases with a well-established genetic background.

Genes involved in CD pathogenesis

Several genome linkage studies identified a number of CD susceptibility regions in chromosomes 1, 3, 4, 5, 6, 7, 10, 12, 14, 16, 19 and X[27-34]. After subsequent confirmation, regions on chromosomes 16, 12, 6, 14, 5, 19 were named IBD1 to IBD7, respectively[35]. Of these 7 original loci, only IBD1 (chromosome 16q12) was replicated in all studies, whereas another three loci, IBD2 (chromosome 12), IBD3 (chromosome 6) and IBD4 (chromosome 14) were replicated in some of the studies[36]. Later on, several CD susceptibility genes, such as NOD2, NOD1, toll-like receptors (TRLs) genes, and novel organic cation transporter (OCTN) genes were identified using either a candidate gene approach or positional cloning techniques[37].

The first and most relevant CD susceptibility gene described to date is NOD2. The carriage of one or more of the three main NOD2 variants (Arg702Trp, Gly908Arg and Leu1007incC) is found in 25%-45% of CD Caucasian patients and in only 15%-20% of healthy subjects[38]. A clear gene-dose effect has been described for NOD2 in CD patients. While the risk of developing CD is increased by 2-3-fold in subjects carrying one NOD2 variant allele, it reaches a 20-40-fold increase in subjects with two or more NOD2 variant alleles. In addition to increasing CD

susceptibility, the *NOD2* gene variants can also influence CD behaviour, phenotypes and need of surgery[39]. Similarly, the carriage of NOD2 variants has also been linked to a slight increase in familial CD risk[40]. In spite of being the most powerful CD susceptibility gene found to date, it must be underlined that the *NOD2* gene only accounts for a small proportion of the genetic inheritance of CD in Caucasians. Moreover, *NOD2* gene variants are infrequent in some geographic areas, such as Scotland, Ireland and Scandinavia, or even completely absent in subjects with an Asian and African-American genetic background[37]. In these cases other genes must account for the genetic predisposition to develop CD. This is in keeping with the highly polygenic nature of this disease.

In the last 3 years, the field of IBD genetics has experienced a dramatic transformation. Completion of the human genome project and development of tools capable of simultaneously studying a great number of genes has resulted in a much higher number of genes influencing CD and UC susceptibility than expected. Several genome-wide association studies (GWAS) have been undertaken in CD patients and healthy controls[41-45] and a meta-analysis has been recently published[46]. In this meta-analysis more than 30 independent loci are found to be convincingly associated with CD, providing an extraordinary insight into CD pathogenesis. Interestingly, most CD susceptibility genes are involved in either recognition of bowel microbiota antigens by the innate immunity, the IL-17/IL-23 pathway or autophagy, suggesting that these molecular mechanisms play a key role in CD pathogenesis.

Several studies have evaluated the frequency of CD-related mutations in affected families[47-49]. Jess *et al*[47] studied NOD2 mutation frequency in a population of Danish twins with IBD. In this study, a high prevalence of NOD2 mutation was observed in both CD twins and their healthy siblings. A Swedish study on monozygotic twin pairs reported a NOD2 frequency in both concordant CD siblings of only 22%, although the prevalence of NOD2 was indeed higher in concordant than in discordant twin pairs[48]. Joossens *et al*[49] investigated the prevalence of genetic markers (NOD2, NOD1, TLR4, CARD8) in multiplex and single-case families, healthy relatives and controls. The authors found a significant correlation between the number of genetic mutations per family and an increasing number of first-degree relatives with CD. However, these results could not discriminate between single-case and multiplex families.

Genes involved in UC pathogenesis

Two studies aimed at describing the influence in UC of the well-established CD susceptibility genes reported very interesting findings[50,51]. It became clear there is a genetic overlap between the two forms of IBD, with some genes involved in the development of both CD and UC (3p21.31, NKX2-3, CCNY). On the contrary, other genes have been only associated with UC, but not CD (ECM1 HERC2, STAT3 and PTPN2). In that regard, a very recently published UC GWAS meta-analysis has demonstrated that roughly half of the known CD susceptibility gene loci are shared by UC[52]. In addition, more than 20 exclusive UC loci have been recognized to date including, among others, IL10, ARPC2 and ECM1[52-55]. To summarize our present understanding, we believe that some genetic factors influence the global predisposition to develop IBD, whereas other genes are related to the risk of developing either UC or CD, specifically. Such genetic overlap between UC and CD probably contributes to the existence of a 5% of non-classifiable or indeterminate colitis among the IBD population. Similarly, it also contributes to the fact that first degree relatives of CD or UC patients not only have an increased risk of developing the same type of IBD, but also the other form of IBD, although with a lower frequency in the latter case.

EVIDENCE SUPPORTING AN ENVIRONMENTAL AETIOLOGY IN FAMILIAL IBD

Evidences supporting the role of environmental factors in IBD pathogenesis

The remarkable increase in the IBD incidence in the last few decades cannot be explained by changes in the genetic background of a certain population. Instead, it clearly points towards the existence of potent environmental factors playing a key role in IBD pathogenesis. Several studies performed in Europe that have evaluated IBD prevalence in immigrant populations from low IBD risk areas found that immigrants present a similar or higher risk of developing IBD when compared to the indigenous population[56-58]. These results suggest that differences in prevalence are probably associated with lifestyle and environmental factors, and not with a specific genetic background. It is remarkable though that only very few studies have addressed environmental etiologic factors in respect to familial IBD.

Eating habits, pets and previous infections

One of the largest controlled studies addressing the impact of environmental factors on familial IBD was conducted in Belgium by Van Kruiningen *et al*[59] who investigated 21 families with 3 or more first-degree relatives affected with CD. Subjects were interviewed using an extensive questionnaire on potential environmental factors. In this study affected families and controls presented some remarkable differences in eating habits, domestic factors and medical history. IBD patients ate fewer oats, rye and bran, consumed more unpasteurized cheese and drank more well water, compared to controls. Additionally, an increased frequency of smoking habit, appendectomy and fecal-oral transmitted infections was found in a subject who later developed CD. In respect to domestic habits, affected families also presented a lower daily contact with pets during childhood. Taken together, these results point towards a role of certain gastro-intestinal infections as triggering events contributing to the development of IBD, whereas

the contact with pets during childhood seem to have a protective role favoring the immune system modulation.

Another familial aggregation study by the same Belgian group reported on a large Moroccan family with multiple CD cases[60]. Potential environmental, genetic and serological markers were studied in all family members. No differences in CD susceptibility genes or serological antibodies described in Caucasian populations were found between CD patients and healthy subjects. The study of environmental factors revealed the consumption of a large amount of unpasteurized milk in all family members, which was an environmental factor previously associated with occurrence of familial CD[59].

Another questionnaire-based study on environmental factors in a large monozygotic and dizygotic twin population included more than 300 twin pairs who were discordant for IBD diagnosis[61]. Twins with UC and CD reported recurrent gastrointestinal infections more frequently than their healthy siblings. These findings are in keeping with an increased frequency of fecal-oral transmitted infections reported in multiplex CD families[59] and suggest that past gastrointestinal infections might influence the risk of IBD.

Appendectomy

Appendectomy is associated with a lower risk of developing UC, although the exact mechanisms of this protective role are still not elucidated[62]. While controversial, it seems that the effect of appendectomy requires a certain degree of inflammation (appendicitis or lymphadenitis) and also applies to subjects undergoing appendectomy before the age of 20 years[63]. However, in a recently published study evaluating the usefulness of appendectomy as a therapeutic strategy for distal UC, 40% of patients experienced a complete symptoms resolution after elective appendectomy[64]. Conversely to UC, appendectomy seems to be associated with an increased risk of developing CD, although the studies addressing this issue yield conflicting results[65-70]. These discrepancies might be due to the inclusion of appendectomies performed at CD diagnosis or to methodological differences. More recently, data from large Swedish and Danish cohorts and a meta-analysis have demonstrated that the risk of developing CD is markedly increased only during a short period following appendectomy, disappearing after 5 years[71,72]. This behavior suggests that the association of appendectomy with CD might be a diagnostic bias, instead of a true risk factor.

Tobacco

Smoking habit, particularly cigarette smoking, is the most indisputable example of the influence of the environment on IBD[73,74]. Smoking has striking opposite effects on CD and UC, supporting the notion that distinct mechanisms underlie the pathogenesis of each form of IBD[74]. Subjects who have never smoked and former smokers are at a higher risk of developing UC, whereas present smokers have an increased risk of CD. In addition to the impact on disease susceptibility, smoking habit also modifies the clinical course of disease, increasing the risk of experiencing a relapse and the need for surgery[75-78]. Moreover, it has been demonstrated that tobacco discontinuation improves CD course[79]. Tuvlin *et al*[80] conducted a survey on tobacco use in UC and CD patients in familial IBD. In this study, smokers had double the risk of CD and former smokers had double the risk of UC, in younger age groups. A Danish case-report study on 2 monozygotic female twins with ileo-colonic CD and their non-affected brother and parents showed that though the healthy father. brother and twins all presented a NOD2 variant related to CD, only the affected twins were smokers, had undergone appendectomy and were on oral contraceptive use[81]. To further evaluate the influence of smoking habit in familial IBD, Bridger *et al*[82] analyzed 658 IBD patients, including 339 affected sibling pairs of whom 89 were discordant for smoking when diagnosed. Siblings who were discordant for smoking and IBD type almost always show CD in the smoker and UC in the non-smoker patient. The authors also suggest that the protective effect of tobacco on UC is due to a shift towards development of CD, in subjects prone to undergo bowel inflammation, rather than true protection of from the development of UC.

Oral contraceptive and non-steroidal anti-inflammatory drugs

Several studies have addressed the potential contribution of contraceptive pills to the development of IBD. It has been demonstrated that the risk of IBD in women taking oral contraceptives is greater that in controls, although there is no direct evidence for a causal relationship[83-85]. Data from two meta-analysis suggest a modest association between the use of oral contraceptives and development of IBD, with a pooled relative risk adjusted for smoking habit of 1.46 for CD and 1.26 for UC[86,87]. The most recent meta-analysis also suggests that the risk disappears once the medication is discontinued[87]. Other frequently used types of drug that have also been associated with IBD are non-steroidal anti-inflammatory drugs (NSAIDs)[88-95]. Due to their inhibitory action on protective prostaglandins, these drugs could enhance intestinal permeability facilitating disease activity. A growing body of evidence suggests a true association between NSAIDs and IBD activity, although the existence of multiple confounding factors makes it difficult to establish a formal relationship[96]. These confounding factors include selection of inadequate control groups, publication bias and intestinal tissue damage due not only to IBD activity but also to NSAIDs. Unfortunately, there are no studies evaluating the role of oral contraceptives and NSAIDs in familial IBD.

IBD as an infectious disease

Van Kruiningen *et al*[97] analyzed the pedigrees and time course of IBD development in a group of patients with familial IBD. They found that first-borns and subsequently born siblings were more frequently affected[87,97]. In addition, they described a statistically significant CD clustering that would indicate an infectious etiology supported by

which family members were affected and time to develop symptomatic disease. Another study by Van Kruiningen *et al*[98] assessed 2 French families with multiple CD cases, in an attempt to identify the suspected infectious cause. However, *Campylobacter*, *Yersinia*, *mycobacteria*, *mycoplasma*, *torovirus*, *coronavirus, Brucella*, *Influenza* and animal enteropathogenic infections were all ruled out and no pathogen could be identified. Even though an abnormal recognition of antigens of the intestinal microbiota by innate immunity is thought to play a key role in IBD pathogenesis, there are no studies addressing specifically the interaction between bowel microbiota and familial IBD.

Intestinal permeability

An abnormal gut barrier function, with an increased intestinal permeability, could contribute to CD pathogenesis[99]. It that regard, it has been demonstrated that small intestinal permeability is increased not only in patients with CD but also in their healthy relatives[100,101]. Peeters *et al*[100] reported that 25% of healthy first-degree relatives of CD patients had an increased small intestinal permeability. The mechanisms responsible for these disturbances in bowel permeability are not fully elucidated and its pathogenesis is a much debated topic. In their study in familial and sporadic CD, Peeters *et al*[100] reported no specific genetic pattern accounting for the abnormal permeability found in patients and relatives. In addition, there were no significant differences between families with multiple members affected and families with only one individual affected. Interestingly, almost half of the spouses presented increased intestinal permeability, which clearly suggests that this abnormality is due to environmental and not to genetic mechanisms. In keeping with this finding, Fries *et al*[102] studied the prevalence of intestinal permeability and some CD genetic markers (including NOD2 main variants) in 23 families of CD patients. Authors found no association between increased intestinal permeability and genetic markers in their population. In contrast, other studies have found an association between an abnormal bowel permeability and the presence of NOD2 variations[101,103].

CONCLUSION

IBD is a complex polygenic disorder modulated by a series of environmental factors, some of which are likely yet to be determined. In spite of the significant progress done in the study of both the genetic and environmental factors associated, the exact contribution of each one of the many factors involved is still largely unknown. From a practical point of view, at present no specific recommendations to IBD families, in respect to genetic or environmental counselling, are available. Large, prospective, population-based studies following IBD patients and their healthy relatives will be necessary to untie the knots in this tangled web of genetic and environmental factors.

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TOPIC HIGHLIGHT

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Comorbidity in inflammatory bowel disease

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Abstract

Patients with inflammatory bowel disease (IBD) can be affected by other unrelated diseases. These are called comorbid conditions, and can include any secondary health problem that affects a person suffering from a primary or main disease, and which is neither linked physiopathologically to the primary condition, nor is it due to the treatments used for the primary condition or to its long-term anatomical or physiological consequences. Different comorbid conditions, as well as their influence on IBD, are discussed.

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Key words: Comorbidity; Comorbid conditions; Crohn's disease; Inflammatory bowel disease; Ulcerative colitis

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COMORBODITY, THE CONTEXT, DEFINITION

It has always been difficult for physicians to attain a balance between specialization and the possession of general medical knowledge that makes it possible to optimize and expand the quality of care delivered to patients. The amount of information that a physician is supposed to manage is huge, and always growing. This is why, even when information tools are better than ever, we have perhaps reached a point in which Medicine achieves such a deep level of knowledge in specific areas, that sometimes the global perspective is lost. Or at least, is lost by physicians.

Health care professionals entrusted to assist patients with inflammatory bowel disease (IBD) must remember that our patients are also unfortunately exposed to other health problems (Table 1). We are not dealing here with the extraintestinal manifestations of IBD, whose importance we recognize and that we have probably learned to manage in a satisfactory way. Neither are we concerned with the adverse effects caused by the diverse therapies applied to our patients, such as osteoporosis or infections in the immunocompromised patients, or with the long-term consequences of the anatomical and physiological alterations induced by the disease. The problem that now demands our attention relates to a series of health problems that do not have a direct relationship to IBD as such, but that could alter the diagnosis, presentation and management of the intestinal disease. These conditions are generically known as "comorbidities". A working definition of comorbid conditions could include in this concept any secondary health problem that affects a person suffering from a primary or main disease, and which is neither linked physiopathologically to the primary condition, nor is it due to the treatments used for the primary condition or to its long-term anatomical or physiological consequences. The delimitation of what is and is not a comorbid condition is not easy, and we are

sure that many readers will think that this review should have perhaps addressed a different scope of diseases. In fact, agreement between the authors has required some debate. Our selection may be incomplete, but we are sure that all the conditions that we have included deserve important consideration when caring for a patient with IBD.

It is of paramount importance to differentiate comorbidity from multimorbidity. The first concept deals with the association of a group of diseases with a given condition, whereas multimorbidity refers to the association of multiple diseases in a single person[1]. Another term bordering the concept of comorbidity, is that of patient complexity. In this concept, other extramedical factors are taken into account, such as personal, cultural or social situations that might significantly influence the way in which the health system has to take care of a given person.

Why is comorbidity important?

Firstly, it is obvious that comorbid conditions cannot be overlooked in a patient with IBD. The existence of comorbidity can significantly change several scenarios of medical practice[2]. (1) Clinical manifestations of IBD and its activity can be altered or confused by associated diseases; (2) Prognosis of IBD will also be influenced; (3) Whenever a patient with significant comorbidity is seen by us, we step outside the realms of medical evidence. As a rule, randomized controlled trials exclude patients with comorbidity, and so their conclusions are not necessarily applicable to such situations; (4) The use of drugs for the treatment of IBD is limited by the increased importance their pharmacologic or collateral effects can have on a person with comorbid conditions; and (5) Frequently, it is more important than ever to set up multidisciplinary teams to empower patient care, or, at least, to assure that channels of collaboration and mutual consultation are as fluid and reliable as possible.

Is it possible to quantify comorbidity? What are its generic consequences?

Different sets of clinical indices have been developed and proposed for the specific study of comorbidity. Not all of them have been developed in the same way, nor have they been applied to similar populations. This is why they are not always comparable, and to pick one or another must be done very carefully. Charlson's index is widely used[3], probably due to its unique combination of simplicity and performance. However, it has been validated in multiple populations, but not in IBD patients.

In the general population, multimorbidity is associated with a significantly worse quality of life[4-6]. The specific case of IBD has not been addressed yet, but it is known that in other chronic conditions there is an increased cost of care and a higher complexity of medical activities[7], which are accompanied by poorer outcomes and unfavorably influence indices such as emotional impact, ability to cope, mortality, days of admission or postoperative complications[8-11].

What is the quantitative importance of comorbidity in IBD?

Data on the importance of comorbidity in IBD patients are scarce. Its prevalence has been poorly studied, and refers to other related diseases[12] such as pulmonary thromboembolism[13], arthritis[14] or immune-based conditions[15]. These immune-mediated inflammatory disorders, which include IBD itself, associate with each other and determine a higher risk for other chronic diseases, with a corresponding increase in resource costs.

Although the main determinant of quality of life in patients with IBD is activity of the disease[16], other comorbidities not directly related also have an impact on the physical scores, especially cardiovascular diseases[17-20].

It is not known whether the presence of several chronic diseases can determine poorer results following the medical treatment of this disease; however, it has been well described that they strongly influence surgical outcomes, because preoperative comorbidities are, alongside with age, the main predictors of the occurrence of postoperative complications[18-20].

Finally, among the identified causes of mortality in IBD are several processes that reflect the patient's comorbidity, such as postsurgical cardiovascular complications, age and infections[19]. It is true that many factors favor infections in IBD patients, such as immunosuppressants and malnutrition, but both age and comorbidity probably have their corresponding effects[20].

HEPATIC COMORBIDITY

The potential association between primary sclerosing cholangitis (PSC) and IBD is well known. However, we shall not deal with this condition here, and instead will focus on other liver diseases which can be observed in our patients, and may well alter the course of their disease.

During the long-term follow-up of a patient with IBD, a transient elevation in liver function tests is frequently observed. The cross-sectional prevalence of high aminotransferase levels varies between 5% and 50%, but more adjusted figures show, that around 20%-40% of patients have elevated aminotransferases at some time during the course of their disease, whereas a chronic alteration in such values can be detected in approximately 10% of these patients[21-23]. These elevations in liver function tests are usually discrete, in a range below twice the upper normal level[21,24]. There is no clear correlation between the degree of alteration of liver function tests and the presence of active IBD. An investigation of the underlying cause is frequently frustrating and unyielding, with a small percentage of definitive diagnoses[22].

The causes of altered hepatic biochemistry are manyfold, but the most frequent causes are steatosis and drug toxicity[21]. The evaluation of such patients has to be sensible and reassuring. The first step is to categorize the type and degree of altered hepatic biochemistry. Four different situations could perhaps be defined: (1) Slight (< 2

Table 1 Putting comorbidity into context: secondary health problems in patients with inflammatory bowel disease

Extraintestinal manifestations	Adverse effects of treatments	Comorbid conditions	Direct consequences of the disease
Peripheral and axial arthritis	Steroids: cataracts, glaucoma, mood changes, osteoporosis…	Cardiovascular	Abdominal and retroperitoneal scarring: hydronephrosis, intestinal obstruction, female infecundity…
Erythema nodosum, pyoderma gangrenosum, oral aphtae	Immunosuppressors: infections, neoplasia, liver toxicity, myelosuppression…	Hepatic, biliary, pancreatic, digestive	Consequences of intestinal resection: malabsorption, short bowel syndrome, oxalate nephrolithiasis
Uveitis, episcleritis, blepharitis	Biologics: infections, neoplasia, demyelinizing disease, infusion	Metabolic: obesity	Persistent inflammation: osteoporosis, amyloidosis
Primary sclerosing cholangitis	Reactions, drug-induced lupus	Neuropsychiatric	

× upper normal level) and transient elevation of aminotransferases (aspartate aminotransferase/alanine aminotransferase), γ-glutamyl transferase, alkaline phosphatase or bilirubin: it is probably appropriate not to alarm the patient and check the altered values after a short period, proceeding to investigate the cause if the alteration persists; (2) Sustained elevation has to be approached as it would in the general population, with special attention to the usual data in the anamnesis (epidemiological sources of exposure, potential liver toxics…) and ordering a battery of tests that could pinpoint the cause of such elevation. No precise indications have been published to determine which tests should be explored, and in what order. A possible selection could include the following: hepatitis B and C serology, and anti-neutrophil-cytoplasma, antitransglutaminase, antinuclear, anti-smooth-muscle and antimitochondrial antibodies; exploring copper and iron metabolism and investigating other less frequent or characteristic causes of altered liver function tests, could be left for a second step. A liver ultrasound should be obtained at an early stage, to explore the presence of a bright pattern, indicative of steatosis[25], detect cholelithiasis and its complications, explore the bile ducts and rule out signs of chronic liver disease or portal hypertension; (3) Some alterations in the liver function tests tend to be more specific, and deserve a different approach, such as predominant cholestasis, suggesting PSC, or elevation of aminotransferases more than 10 times the upper normal level, indicative of acute hepatocellular damage, which should bring to mind the possibilities of acute viral or toxic hepatitis; and (4) The alteration of liver tests in a patient with previously normal values, after starting a new therapy (notably thiopurine immunusuppressors) has to be approached and managed as possible liver toxicity[26].

Some causes of altered liver tests deserve a special comment, due to their prevalence or their importance.

The range of lesions collectively known as steatosis (including both uncomplicated fatty liver and steatohepatitis) are more and more frequent in the general population, but also in IBD patients, in which it accounts for the majority of diagnoses when investigating altered liver tests[21]. The usual causes of liver steatosis in the general population will also be present in IBD patients, such as metabolic syndrome, overweight/obesity and alcohol abuse. However, other possible reasons should be considered in our patients, notably glucocorticosteroid exposure, malnutrition and parenteral nutrition[27]. It is an exclusion diagnosis, and only the presence of a bright liver pattern in the ultrasound examination has been signaled as relatively specific[25]. More specific causes of liver disease have to be ruled out, notoriously those that can be appropriately managed, such as the viral hepatitides, autoimmune liver disease and drug-induced liver injury. A liver biopsy is rarely needed to confirm steatosis, but ultrasound follow-up will detect the potential development of portal hypertension or hepatocarcinoma.

Viral hepatitides B and C are, in older series, somehow more frequent in IBD patients than in the general population[28-31]. However, the current situation is different. In a Spanish national prospective study[32], the presence of markers of hepatitis B virus (HBV) or hepatitis C virus (HCV) infection was prospectively studied in a series of more than 2000 patients. Evidence of current or past infection was present in 9.7% of cases, which is similar to figures observed in the general population: in ulcerative colitis (UC), hepatitis B surface antigen (HBsAg) was present in 0.8%, anti-HBc in 8% and anti-HCV in 1.3%, and in Crohn's disease (CD) HBsAg was present in 0.6%, anti-HBc in 7.1% and anti-HCV in 2.3%. 12% of patients had serological evidence of effective anti-hepatitis B vaccination (anti-HBs without anti-HBc). Multivariate analysis determined that age, family history of hepatitis and moderate to severe IBD were associated with HBV infection or contact, whereas HCV was mainly associated with previous transfusion of blood or blood products, but only if this was performed before 1991, when anti-HCV screening became widespread in local blood banks. Similar data have been communicated by other authors[33]. It is important to remember, that any prevalence of HCV and, especially, HBV markers has to be kept in mind, because the infection can reactivate under immunosuppressive therapy, necessitating prophylactic measures in some cases (antiviral therapy in HbsAg+ patients), and close follow-up in the remaining patients[34].

GASTROINTESTINAL COMORBIDITY

Helicobacter pylori disease

The prevalence of infection by *Helicobacter pylori* (*H. pylori*), as determined by serology, ranges between 15% and 50%. It is always lower than the corresponding prevalence in the general population, and is inversely correlated with sulfasalazine exposure[35-38]. The diminishing use of this drug makes it possible that this situation has

changed lately. The positivity of the urea breath test is also lower then in the general population[39]. However, the prevalence of peptic ulcer in transversal studies can reach 5%[38]. It is important to remember, that about 15% of CD patients will show histologic or even endoscopic lesions in the upper gastrointestinal endoscopy, and that the absence of *H. pylori* in these lesions is an important criterion when identifying them as truly related to IBD.

The indication for gastroprotection in patients exposed to glucocorticoids deserves a mention. The decision to co-prescribing proton pump inhibitors with glucocorticoids is quite usual, at least in our environment, but it is not sustained by medical evidence[40]. Gastroprotection should only be indicated on rare occasions when NSAIDs and glucocorticoids are prescribed jointly. To automatically prescribe proton pump inhibitors in the general patient, will lead to unnecessary higher costs, exposure to yet another set of adverse events and, most importantly, an increased number of tablets taken daily, which generally adversely influences adherence to therapy[41].

Celiac disease /gluten-sensitive enteropathy

Celiac disease and CD share some physiopathological, epidemiological and clinical features[42]. They frequently form part of the differential diagnosis. In older times, it was difficult to rule out celiac disease in a population with IBD, mainly due to the clinical superposition, but also to the possibility of finding very similar histologic changes (duodenal mucosal infiltrate, villous atrophy in Crohn's) and some parallel serologic alterations (positivity of antigliadin antibodies). The higher specificity of both antiendomysium and antitransglutaminase antibodies in the diagnosis of celiac disease, has finally allowed the performance of studies on the prevalence of this condition in patients with an unequivocal diagnosis of IBD.

Following the communication of isolated cases of associated Crohn's and celiac disease[43], an intriguing study was published[44], in which a very high prevalence (15%-20%) of antigliadin or anti endomysium antibodies was detected in a short series of CD patients. The high specificity of these determinations could make it reasonable to deduce that this association is extraordinarily frequent. However, later investigations of better quality have been unable to confirm these results[45-47]. The systematic search for celiac disease in CD patients cannot be recommended, according to the available evidence.

On the other hand, and although data are scarce, it seems that both UC and CD are more frequent in persons with celiac disease than in the general population. This has been described in two clinical series, with an IBD prevalence about 10 times higher than expected[45,48].

Biliary and pancreatic diseases

Cholelithiasis is a very prevalent condition in the general population[49]. Some circumstances explain its increased incidence in IBD patients, mainly the distortion of bile metabolism induced by the functional or anatomical alterations of the gut. Thus, it cannot strictly be considered a comorbid condition. The interest in cholelithiasis as a comorbid condition is mainly due to the possible overlap between its manifestations and those of the IBD or of extraintestinal manifestations, such as PSC. Management does not differ from the approach in the general population.

Pancreatic disease can ensue following the use of medications, the extraintestinal activation of inflammatory phenomena or can appear as an associated autoimmune disease[50]. In any case, it cannot be listed as a comorbid condition.

OBESITY

Presently, obesity is considered an emerging epidemic in Western societies, where it is more prevalent in disadvantaged social classes[51]. It also affects affluent classes in countries with emerging economies. The prevalence of this condition is growing, and some of its causes are sedentary behaviour, changing socioeconomic status and variations in traditional diets[52].

An increasing prevalence of obesity in patients with IBD has been described recently. In the past, this was considered an exception, but today, figures range from 15% to 20% in some series, with a further 40% of patients being overweight[53]. Although there is no evidence that obesity alters the course of the disease, at least in CD[54], obesity is related to unfavorable outcomes such as colonic adenomas, surgical morbimortality, cardiovascular risk and thrombotic disease.

CARDIOVASCULAR COMORBIDITY

Cardiovascular disease (CVD) is the main cause of death in developed countries[55] and its prevalence increases with age. Therefore, IBD patients are very likely to experience these entities throughout their life or, at least, be affected by some of the associated risk factors. The impact of CVD on IBD is the same as for the general population, increasing complications and remaining a common cause of mortality[56], especially when the disease is more severe or surgery is needed[18]. Anecdotally, in the 1950s when Truelove *et al*[57] published the first clinical trial with corticosteroids in UC, which demonstrated a decrease in mortality, the cause of death in 2 of the 5 patients who died was CVD, namely pulmonary thromboembolism. We will discuss IBD as a predisposing factor for CVD in more detail and the possible effects of IBD treatments on cardiovascular morbidity together with the undesired consequences of drugs used in the management of CVD for EII.

Venous thrombosis

Venous thromboembolism (VTE) has been considered a manifestation which is directly related to intestinal inflammatory activity, but in other chronic inflammatory diseases such as rheumatoid arthritis the incidence of VTE is not greater than in the general population[58].

Table 2 Acquired thrombotic risk factors in inflammatory bowel disease

Inflammation
Immobility (surgery or hospitalization)
Surgery
Fluid depletion
Central venous catheters
Drug therapy: Corticosteroids, anti-TNF drugs?
Smoking
Hyperhomocysteinemia (vitamin deficiencies)
Oral contraceptives
Increased levels of lipoprotein A

TNF: Tumor necrosis factor.

Furthermore, IBD patients in remission also have an increased risk of VTE[59], therefore additional factors other than inflammation are involved. This is why we have considered it a manifestation not directly linked with IBD.

In a recent population study[59], the incidence of VTE in IBD was 26 cases/10000 person-years (PY) with a Hazard ratio of 3.4 (95% CI: 2.7-4.3). These data are similar to those observed in a previous Canadian population study, where the incidence of deep vein thrombosis (DVT) was 31.4/10000 PY and 10.3/10000 PY for pulmonary embolism (PE) for CD patients and 30/10000 PY and 19.8/10000 PY in patients with UC, respectively[60]. Again, the risk was 3 times higher in IBD compared with controls. There was also a time trend which increased with an annual average of 17% in odds of VTE[61]. There are no major differences between CD and UC, although the incidence in hospitalized patients may be somewhat higher in UC (OR = 1.32), probably because its frequency increases when the colon is involved[61]. IBD activity increases the likelihood of VTE with rates up to 8 times higher than controls[60]. Although VTE episodes are less frequent in outpatients, in this subgroup the differences with controls were even more marked (HR = 15.4) than during hospitalization periods (HR = 3.2)[59]. Similarly, although the majority of thromboembolic events occur in patients over 60 years, differences with controls are greatest in young patients (< 40)[60]. Other aspects that may increase the risk are involvement of the colon in the case of UC (pancolitis in 76% of episodes of VTE *vs* 2% of proctitis) and fistulizing pattern in CD[61].

Causes of thrombosis in IBD are many-fold; in most patients, recognized acquired prothrombotic factors can be identified, such as inflammation, immobilization, surgery, central catheters, corticosteroids, and smoking (Table 2)[62,63]. Nevertheless, in a significant percentage of patients (20%-50%) no obvious cause can be identified[64-66]. This supports the notion, that IBD itself acts as a predisposing factor for thrombosis. Inherited thrombophilias have no role in VTE associated with IBD because VTE is significantly less frequent than in thrombotic non-IBD subjects[67].

The appearance of VTE in a patient with IBD carries a poor prognosis reaching a mortality of 22%-25%[65,66], significantly higher than the control group (OR = 2.1). Hospital stay also increased by 48% and associated health costs doubled[61].

The most common location of VTE is lower extremity DVT with or without PE[58], which would include three-quarters of all episodes, but many locations have been described such as cerebral sinus[68-71], retinal vein[72-75], portal venous system[76-81] and hepatic or cava veins[82-87]. Portal system thrombosis is particularly important both for its potential complications and for its non-negligible frequency, being reported in up to 6% of patients after restorative proctocolectomy and ileal pouch-anal anastomosis[78,88]. A probable long-term complication is portal hypertension, which can be avoided with early anticoagulant therapy, for which clinical suspicion is fundamental, ordering an abdominal Doppler ultrasound and/or abdominal CT in the case of sudden onset of abdominal pain, fever or prolonged ileus after abdominal surgery[89].

Treatment of thromboembolic episodes of IBD is the same as in the general population and is based on the use of anticoagulation, first with heparin (usually low-molecular-weight heparins, LMWH) and then oral anticoagulants which should be sufficient for most patients[90]. Occasionally other measures may be required, such as thrombolysis or placement of an inferior cava filter that can be used as an effective means of preventing pulmonary embolus when anticoagulant therapy is contraindicated or thromboembolism recurs in spite of anticoagulant therapy. These drugs have proven safe in clinical trials where their effectiveness was evaluated as primary treatment. The optimal duration of anticoagulant therapy is unknown, but in general will vary depending on the severity of thrombosis and bleeding risk. In the first episode, 6 mo provided adequate coverage, but will be extended if the risk factor has not disappeared (surgery, immobilization) opting for lifelong anticoagulation in the setting of an inherited hypercoagulable state[89,91]. Despite treatment, up to 13%-26% of patients have a recurrent thromboembolic event[65,92]. Similar rates were found in patients with prior colectomy, thus surgery does not seem to prevent recurrence[65].

Treatment of thromboembolic episodes is important, but so is prevention. Possible measures include the control of disease activity, correcting vitamin and nutritional deficiencies, cessation of smoking, early mobilization after surgery or the use of intermittent pneumatic compression for patients at high risk of bleeding. Drug prophylaxis with LMWH is recommended in patients hospitalized with severe UC[93] or when planning surgery[94]. Outpatients with moderate flares and restricted mobility (old age, motor deficiencies…) are probably also candidates for pharmacologic prevention.

Atherosclerosis and arterial thromboembolic disease

Early atherosclerosis: Early atherosclerosis is a common phenomenon in several immune-based inflammatory diseases, particularly rheumatoid arthritis and systemic

lupus erythematosus[95], and is one of the most important causes of morbidity and mortality in these diseases, justifying the publication of specific recommendations[96]. The inflammatory process is behind the emergence of this phenomenon with a prominent role for the cytokines IL-6 and TNF that are significantly associated with the severity of subclinical atherosclerosis, independent of Framingham risk score[97]. Both cytokines are implicated in the pathogenesis of IBD, making possible the development of early atherosclerosis in these patients. Few data exist on this entity in IBD, although case reports of arterial occlusions in young patients with CD support this possibility[98]. Furthermore, to assess the presence of subclinical atherosclerosis at an early stage, several methods have been proposed, such as assessment of the intima-media thickness of the common carotid artery wall or the measurement of carotid artery stiffness; these have been shown to predict the occurrence of cardiovascular events[99,100]. Both parameters have been found to be altered in IBD patients compared to controls[101,102], although not uniformly in all studies[103].

Cardiovascular risk factors: There are few data on the prevalence of cardiovascular risk factors in IBD, apart from the known relationship with tobacco use[104]. Only in the cohort study by Ha *et al*[105], where the controls were randomly selected, IBD patients had a higher frequency of hypertension and hyperlipidemia, with similar rates of diabetes mellitus. Plasma lipid levels were inversely correlated with inflammatory activity[106], thus do not appear to have a particularly decisive role in predisposition to atherosclerosis in patients with IBD. As mentioned earlier, inflammatory activity *per se* could promote the development of atherothrombotic complications and in relation to this, C-reactive protein has been identified as a risk factor both in chronic inflammatory diseases[107] and in the general population[108,109]; unfortunately there are no specific data for IBD.

Arterial thrombotic events: Only two studies have evaluated the incidence of arterial events in IBD independently of venous thrombosis. The aforementioned study by Ha *et al*[105] found an overall increased incidence compared to the control group, mainly due to a marked increased in the risk of acute mesenteric ischemia (HR = 11.2, $P < 0.001$). The risk of myocardial infarction, conversely, was only discretely increased (HR = 1.6) in women over 40 years; in contrast, stroke was elevated in women below that age. However, Bernstein *et al*[110], found an overall increase in the prevalence of ischemic heart disease (IRR = 1.26) and cerebrovascular disease (IRR = 1.32) regardless of age group, although the latter only applied to patients with CD. In any case, except for acute mesenteric ischemia, in which local factors are probably involved, the rates found were quite similar to those of the control population, and not necessarily clinically relevant.

IBD therapy and cardiovascular diseases

The deleterious effects of glucocorticoids on the cardiovascular system are well known[111,112]: among their adverse effects are hypertension, hyperglycemia, hyperinsulinemia and hyperlipidemia, determining in some contexts increased cardiovascular morbidity, as in renal transplantation[113]. In other diseases, such as rheumatoid arthritis[114], inflammatory activity control can reduce cardiovascular complications. In spite of this, there is no direct evidence that IBD treatments alter the thrombotic tendency of the disease; however, some drugs show a favorable profile on some of the factors involved. For example, aminosalicylates reduce platelet activation and azathioprine inhibits formation of platelet-leukocyte aggregates[62]. There are no specific data for IBD, but methotrexate therapy in rheumatoid arthritis has been shown to decrease the prevalence of metabolic syndrome[115] in this disease and, more particularly, cardiovascular morbidity and mortality[114]. Regarding anti-TNF drugs, the results are somewhat contradictory: initially, endothelial function and insulin resistance transiently improve. On the other hand, infliximab in particular, shows a potential adverse effect on the lipid profile[116]. Likewise, thrombotic events associated with these drugs in patients without other risk factors have been described, although this needs to be confirmed[117].

Finally, it has to be noted, that some of the drugs used in the management of cardiovascular risk factors may be beneficial for IBD. Thus, the anti-diabetes drug rosiglitazone has been proven effective in the treatment of UC in two clinical trials[118,119], atorvastatin could have an antiinflammatory effect in CD[120] and ACE inhibitors have an antifibrogenic effect[121,122], not yet explored in CD.

PSYCHIATRIC COMORBIDITY

Anxiety and mood disorders have been extensively studied in IBD patients, whereas data on other conditions, such as psychoses, are scarce. However, there may be some connection, because some of the drugs used in the management of IBD, such as steroids[123], can precipitate psychotic manifestations and, on the other hand, the treatment of IBD may be followed by improvement of psychiatric illness[124].

As in other chronic diseases, the prevalence of anxiety and depression is higher in patients with IBD than in controls, both in hospital[125-128] and population cohorts[129-132]; their frequency is variable but approaches 24%-27%[129,133], which is two to three times higher than in a control population[129,131]. There are no great differences between UC and CD[130], or with other chronic diseases such as rheumatoid arthritis or diabetes[134].

Psychiatric comorbidity in IBD has been considered a risk factor for the onset of IBD itself, because it can precede its diagnosis[129,132]. However, the relative risks are low, and are limited to the year prior to diagnosis, which could represent the presence of symptoms of an undiagnosed somatic illness[135]. Moreover, the frequency of anxiety and depression increases after diagnosis of IBD,

suggesting that it is a consequence rather than a cause of the disease[132].

The role of daily stress and major life events in the precipitation of flares has also investigated, mainly because patients with higher perceived stress have a greater chance of disease reactivation[136,137]. However, there are several potential factors which may have contributed to this finding. In many studies, the sample size is not enough, and on the other hand, these results may be influenced by the fact that the influence of stress on disease activity is what patients, and probably many physicians, expect to be true[138]. Furthermore, anxiety and depressive symptoms predicted an increased likelihood of functional symptoms[139], and have a decisive influence on variables such as abdominal pain and overall well-being, used in the calculation of indices for most common activity indices. In addition, episodes of recurrence are limited to a relatively restricted period following the initial outbreak[137], which could be attributed to an incomplete improvement of IBD. Finally, several studies did not find such an association[130,140,141] and the results of trials of psychological interventions in IBD have been negative[140]. Thus, although anxiety and depression are more frequent around the periods of IBD activity, they did not seem to be risk factors for flares; also, more importantly, to wrongly assume that stress is responsible for disease exacerbation, may contribute in some way to induce unjustified and harmful feelings of guilt in some patients.

Psychiatric comorbidity has a marked impact on the management of IBD because it is, together with disease activity, the main determinant of quality of life in these patients[142]. Additionally, it is one of the factors associated with poorer adherence to treatment[41] and determines a greater use of health resources[139]. Thus, given the particular importance of these observations, and the frequent association of psychiatric symptoms with IBD, it is essential to identify and properly manage these conditions. In this sense, the use of screening tests with a good balance between sensitivity and specificity, such as the Anxiety and Depression Detector[143,144] or the Goldberg Anxiety and Depression Scale, could be recommended as an initial screening tool[134]. Treatment of psychiatric comorbidity in IBD does not differ from the general approach to these disorders, and the options include different types of psychotherapy[145] or second-generation antidepressants such as selective serotonin reuptake inhibitors or serotonin norepinephrine reuptake inhibitor, with fewer side effects than traditional drugs (tricyclics and MAO inhibitors) and that may be initiated by the gastroenterologist pending evaluation by the appropriate specialist.

CONCLUSION

Throughout the evolution of a patient with IBD, these diseases are frequently associated with other non-related diseases which may change management and prognosis. On the one hand, decision making is complicated because the available evidence does not always apply, as in most clinical trials such patients are excluded. On the other hand, their existence entails taking into account the possible consequences of treatment on other comorbidities, both by the possibility of interactions and by the facilitation of potential adverse effects. Prognosis also changes, especially in the presence of cardiovascular comorbidity, which is associated with a greater overall morbidity and mortality, especially in the surgical context. Finally, the concomitant presence of several diseases requires collaboration and coordination among professionals for joint decision making, and implementation of the proper clinical circuits to facilitate medical attention.

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TOPIC HIGHLIGHT

Belén Beltrán, MD, PhD, *Series Editor*

Old-age inflammatory bowel disease onset: A different problem?

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Abstract

Inflammatory bowel disease (IBD) in patients aged > 60 accounts for 10%-15% of cases of the disease. Diganostic methods are the same as for other age groups. Care has to be taken to distinguish an IBD colitis from other forms of colitis that can mimick clinically, endoscopically and even histologically the IBD entity. The clinical pattern in ulcerative colitis (UC) is proctitis and left-sided UC, while granulomatous colitis with an inflammatory pattern is more common in Crohn's disease (CD). The treatment options are those used in younger patients, but a series of considerations related to potential pharmacological interactions and side effects of the drugs must be taken into account. The safety profile of conventional immunomodulators and biological therapy is acceptable but more data are required on the safety of use of these drugs in the elderly population. Biological therapy has risen question on the possibility of increased side effects, however this needs to be confirmed. Adherence to performing all the test prior to biologic treatment administration is very important. The overall response to treatment is similar in the different patient age groups but elderly patients have fewer recurrences. The number of hospitalizations in patients > 65 years is greater than in younger group, accounting for 25% of all admissions for IBD. Mortality is similar in UC and slightly higher in CD, but significantly increased in hospitalized patients. Failure of medical treatment continues to be the most common indication for surgery in patients aged > 60 years. Age is not considered a contraindication for performing restorative proctocolectomy with an ileal pouch-anal anastomosis. However, incontinence evaluation should be taken into account an individualized options should be considered

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Key words: Inflammatory bowel diseases; Ulcerative colitis; Crohn's disease; Eldery population

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INTRODUCTION

Old-age colitis refers to patients over 60 years of age who are affected by a group of diseases of the colon, including infections, vasculitis, microscopic colitis, colorectal cancer, ischemic colitis, drug-associated colitis [particularly non-steroid anti-inflammatory drugs (NSAIDs)] and inflammatory bowel disease (IBD)[1]. Diagnosis of IBD in this age group may be difficult because it can be easily confused with other forms of colitis. Of particular relevance is so-called segmental colitis associated with diverticular disease (SCAD), which may simulate IBD both clinically, endoscopically, and histologically[1-3]. SCAD is characterized by the presence of diverticula, most often in the sigmoid colon, and affects middle and old

aged patients. A recent study in a German IBD elderly population showed that 8% of older patients diagnosed as IBD patients were in fact affected by SCAD[4]. Special precautions and attention must be taken in the diagnosis of these patients to avoid errors. New diagnostic tools, such as serological markers or advanced radiological examinations, may be useful in for a correct diagnosis.

FREQUENCY

Approximately 10% of patients at first flare of IBD are > 60 years old, with a similar distribution between ulcerative colitis (UC) and Crohn's disease (CD); 50% of them are diagnosed between 60-70 years of age[4,5]. However, it is difficult to establish the true incidence of IBD in older patients due to problems of case definition, population, and particularly because it may be confused with other clinical conditions, such as NSAID-associated colitis or ischemic colitis[1]. A bimodal age curve for IBD incidence has been suggested in epidemiological studies, with a second peak occurring at 50-70 years[5-7], but this point is currently the subject of debate, and it was not observed in the results of four population studies using strict radiological and endoscopic diagnostic criteria. A clear decrease in incidence occurs from age 40 in patients with CD and then stabilizes from age 60. Although there is a similar trend in patients with UC, the decrease is smaller in this group[8].

Thus, the incidence in patients over 60 years old represents approximately 10% of the cases of IBD; the bimodal distribution is questionable, and care should be taken to avoid including inadequately diagnosed patients.

CLINICAL MANIFESTATIONS

The clinical manifestations of the first flare of disease, both in UC and CD, are similar in the group aged > 60 years and younger age groups. It has been noted that the severity of the symptoms of UC, particularly rectal bleeding and diarrhea, may be lower in patients in this group, but it may also have atypical forms of presentation, such as constipation[9,10]. With regard to disease location, according to the Montreal classification, proctitis and left-sided UC are more common in patients > 60 years old than in younger patients. Severity of recurrence is usually higher in elderly patients (S2-S3 by the Montreal classification), with a longer duration of symptoms, which has influence on the need for steroid treatment[11-14].

In the case of Crohn's disease, the Montreal classification considers three age groups, establishing age > 40 years as the more advanced age group[11]. It has been observed that the form of disease presentation is similar between the different age groups, but that the L2 (colonic) disease location (clinical pattern) is the most common form in the older group (48% colon involvement in > 40 years group versus 28% and 20% in the groups diagnosed between 20-40 years and < 20 years, respectively)[1,15-19]. This frequency rises to 66% in patients aged > 60 years. With regard to the disease behavior, the most common is the inflammatory disease behavior (B1), the stricturing and penetrating pattern being less common than in the 18-60 years age group[1,5,19].

There are few references on the frequency of presentation of extraintestinal manifestations in this subgroup of patients. In the study by Hadithi[5], 17% of patients over 60 years of age had extraintestinal manifestations, which, in order of frequency, were: peripheral arthritis, uveitis, spondylitis, and erythema nodosum.

DIFFERENTIAL DIAGNOSIS

There are various clinical situations that may hinder diagnosis of IBD in elderly patients. IBD may be complicated by infections or by taking certain medications, such as NSAIDs and antibiotics[1,20-22]. Similarly, certain neoplasms, and specifically small bowel lymphoma, may have similar characteristics to those of IBD or be a complication of the disease itself[23-25]. The most common causes of chronic diarrhea and bowel inflammation in patients aged > 60 years are indicated in Table 1[25-31].

Segmental colitis associated with diverticular disease (SCAD) warrants special consideration[1,25-27]. Diverticulosis affects more than half of the people over 60 years of age. SCAD is clinically manifested by abdominal pain, rectal bleeding, and altered bowel habits, but only 3%-8% of patients have mucosal inflammation around the diverticula. It involves only the sigmoid colon, with sparing of the rectum and proximal colon. Endoscopic examination shows a circumferential distribution of erythema, granularity, and friability, and sparing of the diverticular ostium and cavity with a diffuse periosteal distribution of inflammation confined to the area of the diverticula[24,25]. Histopathologically, inflammation analogous to that seen in the UC and nonspecific chronic inflammation that may contain granulomas has even been reported, leading to misdiagnosis with CD. In general, SCAD may be suspected when colitis is confined to the diverticular segment of the left colon (excluding the rectum), the rectum and proximal colon are endoscopically and histologically normal, and if surgical resection of the affected segment is required, when no recurrence of segmental colitis occurs.

There are no randomized studies specifically assessing treatment of SCAD[1,5]. Overall response to oral (2.4-3 g/d) or topical (enemas 1-2 g/d) mesalazine was good, with a clinical response of 80% at six weeks and a maintenance of response in 90% of patients at six months[5,27]. The addition of fiber and antibiotics (rifaximin) during the acute phase may improve the response. If there is no improvement with this combination treatment, steroids applied topically as enemas may improve the clinical response. Exceptionally, oral prednisone has been evaluated in some cases of non-responders. These are patients who will rarely require surgery with segmental resection for SCAD.

MEDICAL TREATMENT

The principles governing medical management of IBD in patients aged > 60 years are the same as in other age groups. The location, extent, severity, and disease behavior,

Table 1 Differential diagnostic of colitis in patients older than 60 years

Disease	Clinical characteristics	Endoscopic findings
Colitis associated to diverticulosis	Rectal bleeding, abdominal pain, diarrhea	Segmentary distribution, peridiverticular, sigma affected, rectum and proximal colon are normal
Ischemic colitis	Abdominal pain rectorragia acute onset	Segmentary colitis (sigma/left colitis), majority are non-obstructive
Microscopic colitis	Watery diarrhea, no rectal bleeding, no fever, frequent cause of diarrhea in the eldery	Normal endoscopy, multiple biopsies from colon, celiac disease association
Infectious colitis	Disenteriforme diarrhea, different agents, *C.difficile* to be ruled out	Difuse effects on the colon Increased morbidity and mortality in older-aged population
NSAIDs colitis	Recurrent abdominal pain; obstruction, perforation, hemorrhage; chronic anemia	Any part of the intestine, isolated lesions, aggravate previous UC and CD

NSAIDs: Non-steroid anti-inflammatory drugs; UC: Ulcerative colitis; CD: Crohn's disease.

both in UC and CD, will determine the medical treatment options to be used[32-37]. Special precautions and the most generally used treatments in IBD old-aged patients are summarized below. In brief, depending on the kind and severity of IBD suffered by patients, we should choose the best treatment, taking special care of side effects caused by advanced age or by drugs associated with another diseases.

Mesalazine

Oral and/or topical mesalazine is the main option for medical treatment in patients with a mild to moderate flare of UC. The availability of new distal release mesalazine preparations with the MMX formulation may facilitate compliance and, therefore, adherence to treatment. It is similar in terms of efficacy to other oral preparations of mesalazine, regardless of its form of release[38]. However, in Crohn's disease, the efficacy of mesalazine is questionable and it has a limited role in the treatment of mild flares of ileal or colonic location[36,37].

Oral or topic steroids

Beclomethasone combined with mesalazine has been shown to be effective for treating moderate flares of left UC when administered orally as a single 5 mg dose for 2-4 wk, obtaining remissions in about 60% of patients[39]. Although there is no specific study by age subgroups, it remains an option to consider that may avoid the need for systemic steroids[40,41]. Nevertheless, we have to bear in mind that local or systemic steroids administered intrarectally are absorbed and therefore not free from their side effects.

Antibiotics

A detailed case history should be made in the group of patients > 60 years old, looking for symptoms suggestive of sensory neuropathy in the use of metronidazole[42].

Immunomodulators

The prevalence of corticosteroid resistance or dependence in this group of patients has not been specifically evaluated, but it is estimated at about 30%[43]. Although there is little objective data on which to base this practice, treatment was started with conventional immunomodulatory agents (azathioprine, mercaptopurine, and methotrexate) in this group of patients without evidence of differences in terms of efficacy, metabolism, and toxicity between the patients aged > 60 years and younger patients[35-37].

Biological agents

Their indications remain similar to those for patients aged < 60 years[44-46]. However, one of the most important aspects regarding the use of these agents is the safety profile, particularly that related to the risk of infections. The trend observed in the subgroup of elderly patients with rheumatoid arthritis > 65 years old to develop infections has not been confirmed in any controlled study. It is not uncommon for patients to be treated with combination therapies (corticosteroids, conventional immunomodulators, and biological agents) because of the disease's complexity. In this regard, the higher rate of infection found in the TREAT registry in patients treated with anti-TNF agents (infliximab (IFX) in this case) may have been due to the greater severity of these patients and the greater use of steroids[47]. In fact, the only independent risk factor for infections was the concomitant use of steroids (RR = 2.33, 95% CI = 1.50-3.62). However, the results of series describing the clinical experience in treatment with these drugs will best reflect what we would encounter in our clinical practice, particularly when they report observations collected over years. In this regard, the results of the Leuven group comparing adverse effects in 734 patients treated with IFX versus 664 control patients, with a follow-up of 58 to 144 mo, showed no significant differences in the risk of severe infections, mortality, and malignant conditions. No specific analysis by age subgroups was done[48].

In the Stockholm cohort study[44], it was observed that the use of infliximab in the subgroup of patients aged > 60 years was associated with an increased risk of serious side effects and higher mortality, which was also suggested in the multivariate analysis of the Lichtenstein registry[41]. However, more safety data are required on the use of biological therapies in patients aged > 60 years. In the meantime, it seems logical to use them according to recommended usage guidelines and with strict adherence to performing all tests prior to administration. It should be kept in mind that in this age group, and particularly in countries where the prevalence of tuberculosis is higher, it is more likely that an increased frequency of cases of latent TB will be observed.

Surgical indications

In older patients surgical indications are the same as in younger patients, with failure of medical treatment being the most common cause of surgery[49]. In general, the need for surgery is lower in CD patients with an age at diagnosis > 40 years and colonic disease location, and more common in the elderly. However, it is similar in ileocecal disease[49-51].

In UC, restorative proctocolectomy with ileal pouch-anal anastomosis continues to be the surgical technique of choice[52,53]. In general, results are not as good in older patients compared to younger patients, but they are both reasonably good in terms of efficacy and morbidity. Only an increased frequency of diurnal incontinence and nocturnal leakage has been reported among patients aged > 65 years. Generally, overall quality of life was good, to the extent that in the study by Delaney[52] 89% of patients over 65 years of age were satisfied with surgery and 96% would recommend it. Therefore, age is currently not considered a contraindication for performing an ileal-anal pouch.

MORTALITY

Mortality from UC is similar to that of the general population, with more controversial results in the case of CD[1,5,41,54,55]. In UC, age at diagnosis is not associated with increased mortality, whereas in CD, there appears to be a slight increase in the risk of mortality in older aged patients (> 55 years) with long-standing disease[56-58]. However, we should be cautious, because some of the results may be biased by the type of study, study population, follow-up period, *etc*.

There are a number of aspects to consider regarding possible factors that may be associated with an increased relative risk of mortality. In this regard, there were more hospitalizations in patients > 65 years than in the younger group, representing 25% of all admissions for IBD occurring in the USA (33% of hospitalizations for UC and 20% for CD)[41]. In general, the disease course of UC is more severe in elderly patients and mortality in hospitalized patients, both with UC and CD, is estimated to be 3-5 times higher than in age subgroups < 65 years. In the study by Ananthakrishnan *et al*[41], hospital mortality was higher (OR: 3.91; 95% CI: 2.50-6.11) and age was an independent risk factor in the group of IBD patients aged > 65 years; other variables significantly associated with hospital mortality were fistulizing disease, malnutrition, and need for intestinal resection for stenosing disease. Interestingly, in this study, a greater difference was observed in mortality between unoperated and operated patients, possibly because medical salvage therapies, including immunomodulatory and/or biological therapies, may increase morbidity and mortality, suggesting that "early" surgery is associated with lower mortality in older patients. However, hospital stays were longer, particularly in the group of operated patients. Although postoperative complications were similar between this age group and those younger than 60 years, the risk of cardiovascular or pulmonary complications was greater, these being aspects to be taken into account in the postoperative period of patients.

Table 2 Summary of inflammatory bowel disease onset in the elderly

Home messages
10%-15% patients with inflammatory bowel disease > 60 years
12% UC patients
16% CD patients
Differential diagnosis with colitis associated to diverticulosis, ischemic colitis, infectious colitis (*C.difficile*)
Ulcerative colitis: proctitis/left colitis is more frequent
CD: Granulomatous colitis > frequent than ileocecal
Medical treatment
Same treatment options as younger patients
Increased rate of complications with steroids
Similar response, but recurrence less frequent
Surgery: Equal indications, ileoanal reservoir is secure
More hospitalizations
Similar mortality, increased in hospitalized patients, comorbidity

UC: Ulcerative colitis; CD: Crohn's disease.

Related to the use of biological agents, there is a study[59] that points out that 3 out of 5 deaths attributable to infliximab treatment were older than 70 years. However, these patients had a longer evolution of disease (15-26 years) and they suffered from severe diseases and comorbidities.

The risk of suffering dysplasia and cancer is higher in extended Crohn's colitis patients > 45[60]. However there is an increased incidence of these complications related to disease duration (increased risk among patients with disease duration longer than 20 years). Therefore, older and long-evolution patients should be under colonoscopic surveillance, however, only being of advanced age when an IBD outcome occurs is not enough to maintain different colonoscopic surveillance compared to naïve younger patients.

Little is known about effects of age on colonoscopy risks. In a large retrospective study of safety, feasibility, and tolerability of ileonoscopy in IBD patients[61], few severe complications where detected (0.3%-0.8%) and none of them were related to the advanced age of patients or disease activity.

CONCLUSION

The main messages regarding IBD in the elderly are summarized in Table 2. The prevalence of IBD in patients aged > 60 years is difficult to determine, but accounts for 10%-15% of cases of the disease. Diagnosis may be difficult because there are a number of clinical conditions that may mimic IBD, particularly ischemic colitis or colitis associated with diverticular disease. Diagnostic methods are the same as for other age groups. The clinical pattern in UC is proctitis and left-sided UC, while granulomatous colitis is more common in CD. Its disease course is similar to that of the younger groups. The treatment options are those used in younger patients, but a series of considerations related to potential pharmacological interactions and side effects of the drugs must be taken into account.

The safety profile of conventional immunomodulators and biological therapy is acceptable, but more data are required on the safety of use of these drugs in the elderly population. The overall response to treatment is similar in the different patient age groups, but elderly patients have fewer recurrences. The number of hospitalizations in patients > 65 years is greater than in younger groups, accounting for 25% of all admissions for IBD. Mortality is similar in UC and slightly higher in CD, but significantly increased in hospitalized patients. Failure of medical treatment continues to be the most common indication for surgery in patients aged > 60 years. Age is not considered a contraindication for performing restorative proctocolectomy with an ileal pouch-anal anastomosis.

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TOPIC HIGHLIGHT

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Ulcerative colitis in smokers, non-smokers and ex-smokers

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Abstract

Smoking is a major environmental factor that interferes in the establishment and clinical course of ulcerative colitis (UC). Firstly, the risk of smoking status impact in the development of UC is reviewed, showing that current smoking has a protective association with UC. Similarly, being a former smoker is associated with an increased risk of UC. The concept that smoking could have a role in determining the inflammatory bowel disease phenotype is also discussed. Gender may also be considered, as current smoking delays disease onset in men but not in women. No clear conclusions can be driven from the studies trying to clarify whether childhood passive smoking or prenatal smoke exposure have an influence on the development of UC, mainly due to methodology flaws. The influence of smoking on disease course is the second aspect analysed. Some studies show a disease course more benign in smokers that in non-smokers, with lower hospitalizations rates, less flare-ups, lower use of oral steroids and even less risk of proximal extension. This is not verified by some other studies. Similarly, the rate of colectomy does not seem to be determined by the smoking status of the patient. The third issue reviewed is the use of nicotine as a therapeutic agent. The place of nicotine in the treatment of UC is unclear, although it could be useful in selected cases, particularly in recent ex-smokers with moderate but refractory attacks of UC. Finally, the effect of smoking cessation in UC patients is summarised. Given that smoking represents a major worldwide cause of death, for inpatients with UC the risks of smoking far outweigh any possible benefit. Thus, physicians should advise, encourage and assist UC patients who smoke to quit.

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Key words: Smoking; Ulcerative colitis; Nicotine; Inflammatory bowel disease; Colectomy; Pouchitis

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INTRODUCTION

The development of inflammatory bowel disease (IBD) is the result of an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host. Some environmental factors, such as cigarette smoking and appendectomy, have been shown to play a significant role in the pathogenesis of IBD. Cigarette smoking may cause lung cancer, atherosclerotic vascular disease, other kinds of cancers and chronic obstructive pulmonary disease.

Nowadays, smoking represents a major cause of preventable morbidity and is probably the most important and preventable cause of premature mortality in developed and developing countries.

As smoking is a major environmental factor that interferes in the clinical course of ulcerative colitis (UC), in this article we will review its influence on patients with UC.

RISK OF DEVELOPING UC

It is now fully accepted that UC predominantly affects non-smokers and former smokers, and that smoking exerts a universal protective effect against developing UC[1].

This relationship between smoking and UC has been described for more than 30 years. The first to report this association was Samuelsson[1], who noted a lack of smokers among UC patients compared with matched control subjects. The author attributed this observation to interaction with medication rather than to pathogenesis. In 1982, Harries *et al*[2] confirmed this observation; in a study on 23 UC patients, they found only 8% were current smokers compared with 44% of matched control subjects [patients with Crohn's disease (CD) or a cohort evaluated for fractures]. After the first reports, numerous other studies also confirmed this association. The first meta-analysis, including nine case-control studies, revealed a pooled odds ratio (OR) for non-smoking as the risk factor for acquiring UC of 2.9 (95% CI: 2.6-3.2)[3]. The authors also demonstrated a higher risk for former smokers compared to non-smokers with a pooled OR of 1.64 (95% CI: 1.36-1.98). Additionally, they found a dose-response association with an overall pattern of decreased risk of disease with increased level of smoking. A subsequent meta-analysis[4] that included new information from 11 741 patients with UC in 13 studies confirmed the previous data. When they compared current smoking with never smoking, all studies, with the exception of one, found an OR of less than 1.0, indicating a protective association of current smoking and UC; current smoking decreased the risk for UC (OR: 0.58, 95% CI: 0.45-0.75), suggesting that current smoking is associated with an approximately 42% reduced risk of an UC diagnosis. In contrast to current smoking, when former smokers were compared with never smokers among patients with UC *vs* controls, being a former smoker was associated with an increased risk of UC, with an overall OR of 1.79 (95% CI: 1.37-2.34).

The influence of smoking in genetically predisposed IBD patients has been analyzed in family studies. It is well known that there is high concordance within a family between smoking habits and the IBD phenotype, with UC developing in non-smokers and CD in smokers[5]. Thus, some of the apparent protection that smoking exerts on sporadic UC may be due not only to a therapeutic effect of tobacco usage, but rather that in some instances, it is CD rather than UC which develops as a result of the influence of smoking on the pathogenic processes. Bridger *et al*[6] examined 89 sibling pairs with CD or UC discordant for both smoking and IBD phenotype to investigate whether smoking determines the type of IBD that develops in individuals with very similar genetic susceptibility. Of 89 sibling pairs discordant for smoking at diagnosis, 23 were also discordant for disease type. In 21 of these, CD occurred in the smoker and UC in the non-smoker, suggesting that tobacco consumption may act on the IBD genetic predisposition to shift the phenotype from UC towards CD. This role of smoking habits on the IBD phenotype has been confirmed in twins[7]. Among 103 pairs (at least one twin who suffered from IBD), the frequency of smokers was lower among twins with UC. Furthermore, smoking habits were found to be of significance for discordance of disease.

Another issue that should be noted is whether gender may influence the effect of tobacco smoking on UC. Motley *et al*[8] noticed that current smoking delayed disease onset in men but not in women. The effect of gender was also described in the aforementioned article by Bridger *et al*[6], who noted different effects of smoking on the incidence of CD or UC in females and males. This paper showed more pronounced protection from UC (OR 0.18, 95% CI: 0.11-0.3) in females smoking at diagnosis compared with non-smokers (OR 0.47, 95% CI: 0.28-0.79). Later, Cosnes *et al*[9] studied a cohort of 1784 consecutive adult patients (978 with UC). In this study, the beneficial effect of smoking in UC was modulated importantly by gender: they described a more marked increase in disease presentation in men during the few years after smoking cessation.

One important question that should be resolved is whether childhood passive smoking or prenatal smoke exposure has the same influence on the development of UC. This issue was analyzed in a meta-analysis of 13 studies[10]; the results revealed that, in contrast to the inverse association between active smoking and UC development, there was no significant association between childhood passive smoke exposure or prenatal smoke exposure and the development of UC. A dose-response relationship is a possible explanation for the failure of this meta-analysis to show a protective effect of childhood passive smoke exposure on UC; thus it is possible that the level of exposure does not reach a threshold level which is required for the protective effect that has been well documented in active smokers. Moreover, the size of the studies regarding prenatal smoke exposure and the development of IBD were too small to reach a definitive conclusion. After the publication of the meta-analysis, van der Heide *et al*[11] published a new study that addressed this issue; the authors did not find that passive smoking had a beneficial effect. Furthermore, passive smoking UC patients had a higher prevalence of ileal disease (pouchitis and backwash ileitis) than non-passive smoking UC patients.

All the presented data support the view that current smoking is associated with a low risk of UC, but before drawing conclusions we should be aware of the possible

bias that may be present in the studies analyzed, such as differences in study methods, measurement error, or a lack of verification of self-reported smoking status. The lack of uniformity in smoking definitions is probably a serious weakness in many of the published studies. Another important issue that should be noted is that most of the studies are based strictly on the effects of smoking on the non-Jewish white population. Certain races have not demonstrated associations between smoking behaviors and IBD[12,13], suggesting that different races may have varying degrees of susceptibility to IBD, although these differences are probably more important in CD than in UC. In fact, in Israeli Jewish patients, there was no association between smoking and CD patients, although the opposite association exists in UC[14].

INFLUENCE OF SMOKING ON DISEASE COURSE

Active tobacco smoking has a protective effect on the severity of UC; the disease course is more benign in smokers than in non-smokers[15,16]. Flare-up, hospitalization rates, the need for oral steroids and, more importantly, colectomy rate are reported to be lower in smokers compared with non-smokers, though this has not been observed in all studies[17-19].

The link between smoking and colectomy in UC patients is controversial. In a retrospective analysis of a large series of UC patients, current smoking was found to decrease the 10-year cumulative colectomy risk from 0.42 to 0.32[18]. A subsequent meta-analysis with a total of 1489 UC patients found the risk for colectomy to be lower (OR: 0.57, 95% CI: 0.38-0.85) in current smokers compared with non-smokers[20].

In agreement with these results, a population-based cohort study performed in Europe[16] with 771 UC patients prospectively included and followed for 10 years revealed a lower relapse rate (Hazard ratio: 0.8, 95% CI: 0.6-0.9) in smokers compared with non-smokers. Another similar study carried out in the Netherlands by van der Heide *et al*[11] with 295 UC patients identified smoking after diagnosis as a protective factor for colectomy (OR 0.27, 95% CI: 0.11-0.67), whereas pancolitis at diagnosis (OR 3.18, 95% CI: 1.85-5.48) was a risk factor.

On the other hand, a study by Beaugerie *et al*[21], designed to determine the impact of cessation of smoking on the course of UC, analyzed the disease severity in 32 patients with UC who stopped smoking after the diagnosis compared with 32 non-smokers and 32 continuing smokers matched for sex, age, and age at onset. There was no significant difference in colectomy rate among quitters during the 5-year period after smoking cessation and either non-smokers or continuing smokers during the matched periods. Nevertheless, smokers who quit experienced an increase in disease activity, hospital admissions, and the need for major medical therapy (oral steroids, immunosuppressants) within the first years following the cessation of smoking.

As well as the study by Beaugerie *et al*[21], Boyko *et al*[17] reported a lower hospitalization rate in patients who were smoking at the onset of UC, but could not identify a difference in the colectomy rate between smokers and non-smokers.

In agreement with the presented data, improvements in symptoms and a milder course of disease have been reported in ex-smokers who returned to smoking[22,23]. In fact, many patients noted symptom exacerbation when they stopped smoking, followed by symptom relief when they smoked again[8].

Interestingly, some studies reported a gender association; when compared to non-smokers, male UC who smoked ran a more benign disease course as assessed by the decreased need for immunomodulators, whereas this difference was not observed in females[3,9,20]. Additionally, smoking delayed the onset of the disease, although only in males.

Smoking seems to be a protective factor against proximal extension. In patients with distal UC at diagnosis, retrograde extension of the disease process was less frequent in smokers than in non-smokers[24]. A retrospective analysis in France showed that in a subgroup of patients with limited disease at onset of symptoms, the percentage of smokers developing pancolitis was lower than among non-smokers[18]. More recently, Meucci *et al*[25], in a cohort of 273 patients, described proximal extension of the disease in 27.1%. The cumulative rate of proximal extension was higher in non-smokers, in patients with more than three relapses per year and in patients requiring systemic steroid or immunosuppressive treatment.

Not all the studies could identify a protective effect of smoking on the retrograde extension of UC. Pica *et al*[26] retrospectively reviewed 138 patients with ulcerative proctitis; in this series, proximal extension of the disease was seen in 30% of patients. The prevalence of smoking habit was not higher in patients with extended disease.

Finally, it is important to note that many studies failed to identify a beneficial effect of smoking on the course of UC[27-30]. For instance, Roth *et al*[31] included 102 consecutive patients in a survey to assess the natural history of the disease and to determine predictors of future disease severity at the time of diagnosis. Delay from symptoms to diagnosis of UC, gender, family history of IBD, smoking status and disease severity at the time of diagnosis did not significantly predict the disease severity. Similar results were described by Romberg-Camps *et al*[32] in a population-based survey designed to predict the disease course in 630 UC patients. In this study, disease severity, cumulative medication use, and "surgical" and "nonsurgical" recurrence rates were calculated as outcome parameters. A protective effect of smoking on disease recurrence in UC could not be confirmed in this study.

Tobacco smoking also influences other clinical scenarios in patients with UC. Smoking may also prevent the development of primary sclerosing cholangitis (PSC), or pouchitis after colectomy and ileal pouch anal anastomo-

Table 1 Studies of the use of nicotine in ulcerative colitis

Ref.	Formulation	Dose (mg)	*n*	Type of disease	Study	Comparator	Results
[44]	Nicotine gum	4	1	Active UC	Uncontrolled	--	100%
Perera *et al*[45]	Nicotine gum	4	11	Active UC	Uncontrolled	--	27%
Watson *et al*[46]	Nicotine gum	--	1	Active UC	Uncontrolled	--	100%
Srivastava *et al*[47]	Transdermal nicotine	22	18	Active UC	Uncontrolled	--	78%
Guslandi *et al*[48]	Transdermal nicotine	15	3	Active UC	Uncontrolled	--	66%
Guslandi *et al*[49]	Transdermal nicotine	15	10	Active UC	Uncontrolled	--	70%
Lashner *et al*[50]	Nicotine gum	20	7	Active UC	Cross-over	Placebo	NI 43% *vs* PL 0%
Pullan *et al*[51]	Transdermal nicotine	15-25	72	Active UC	Controlled	Placebo	NI 49% *vs* PL 24%
Sandborn *et al*[52]	Transdermal nicotine	11-24	64	Active UC	Controlled	Placebo	NI 39% *vs* PL 9%
Thomas *et al*[53]	Transdermal nicotine	15-25	61	Active UC	Controlled	Prednisone	NI 20% *vs* PR 45%
Guslandi *et al*[54]	Transdermal nicotine	15	38	UC in remission	Controlled	Prednisone	NI 71% *vs* PR 88%
Thomas *et al*[55]	Transdermal nicotine	15	80	UC in remission	Controlled	Placebo	NI 45% *vs* PL 50%
Green *et al*[57]	Nicotine enemas	4	22	Active UC	Uncontrolled	--	73%
Sandborn *et al*[58]	Nicotine enemas	6	7	Active UC	Uncontrolled	--	71%

NI: Nicotine; PL: Placebo; UC: Ulcerative colitis.

sis[33-36]. PSC is a chronic cholestatic liver disease of unknown etiology that is associated with UC. Loftus *et al*[33] published a case-control study to determine whether the relationship between smoking and PSC is similar to that found between smoking and UC. Like UC, PSC was found to be a disease of non-smokers, as the odds of having PSC in current smokers compared with never-smokers was 0.13. The protective effect was independent of whether or not the PSC patient had underlying IBD; the odds of having disease among former and current users of any tobacco relative to never-users were 0.41 regardless of the presence or absence of IBD. Another study published recently by van der Heide *et al*[11] found that never smoking was a risk factor for the development of PSC in UC patients (OR 4.32, 95% CI: 1.52-12.25). In this article, proctitis (OR 0.09, 95% CI: 0.02-0.39) and left-sided colitis at diagnosis (OR 0.35, 95% CI: 0.13-0.93) were associated with a lower risk for PSC.

NICOTINE AS A THERAPEUTIC AGENT IN UC

Four different formulations of nicotine have been used as a therapeutic agent in patients with UC: nicotine gum, transdermal nicotine, delayed release oral capsule and enema (Table 1).

Chewing polacrilex gum containing up to 4 mg of nicotine results in peak blood nicotine concentrations that are usually observed with cigarette smoking. However, the extraction of nicotine from gum is incomplete and variable (53%-72%) for a variety of reasons, including: variable nicotine extraction because of differences in chewing intensity and duration, variable saliva production (the basic pH of saliva enhances absorption), variability in the amount of nicotine retained in the mouth for buccal absorption *vs* the amount swallowed (which undergoes first pass hepatic metabolism) and inconsistent administration schedules[37,38].

Transdermal nicotine administration provides a steady plasma nicotine concentration that is directly proportional to the nicotine dose and leads to peak nicotine concentrations of approximately two thirds of those measured during smoking[39]. Nevertheless, compared with smoking cigarettes, no sharp increases in blood nicotine concentrations have been observed using transdermal application.

Topical administration of nicotine directly to the colon *via* enema or delayed release oral capsule formulations decrease the systemic absorption and side effects, and may be clinically beneficial. Nicotine is rapidly and extensively metabolized, primarily in the liver *via* the cytochrome P450 enzyme pathway[40,41]. When nicotine is ingested orally, bioavailability is low (20%-44%) due to the first pass hepatic metabolism[42,43].

A pharmacokinetic study in healthy volunteers demonstrated that nicotine tartrate administered as a liquid enema had low systemic absorption (mean systemic bioavailability for various formulations ranged from 15% to 25%) and was well tolerated after a single dose[42].

Uncontrolled studies reported that nicotine gum 4 mg, 5-7 sticks/d was beneficial in some ex-smokers with active UC[44-46] but, in contrast, nicotine gum had no benefit and was poorly tolerated in those with active UC who had never smoked[45]. Similarly, uncontrolled studies reported that transdermal nicotine 15 mg/24 h and 30 mg/24 h (22 mg delivered) was beneficial in patients with active and steroid-dependent UC[47-49]. As happened with the nicotine gum, patients who had never smoked were reported to tolerate nicotine poorly[47]. Following the uncontrolled studies, six controlled trials have assessed the utility of nicotine therapy in UC patients[50-56].

A study in seven patients with active UC confirmed that nicotine gum 2 mg administered 5-7 times/d was beneficial compared with placebo in three of four ex-smokers and in none of three who had never smoked[50].

Two randomized, placebo-controlled trials of transdermal nicotine for active UC showed that nicotine was useful when compared with placebo[51,52]. Both studies began with lower nicotine doses (15 mg and 11 mg) to improve patient tolerance, followed by dose escalation after 1-2 wk. The first study, conducted by Pullan *et al*[51]

treated 72 patients with active left-sided UC with either transdermal nicotine patches (up to 25 mg/24 h) or placebo over a period of 6 wk. The standard medication, which included mesalamine (all patients) and glucocorticoids (12 patients), continued to be administered during the study. Most patients tolerated the nicotine doses. Patients in both the nicotine and placebo groups improved, although the improvement in clinical and histological grades was greater in the group treated with nicotine. Seventeen of 35 (48.6%) patients in the nicotine group had complete remission, compared with only nine of 37 (24.3%) patients in the placebo group.

Similar results were found by Sandborn *et al*[52], who compared transdermal nicotine (22 mg/24 h) for 4 wk with placebo. Nicotine treatment led to a clinical benefit in 39% of patients compared with 9% receiving placebo. No improvement in histology was observed.

A fourth randomized controlled trial compared transdermal nicotine at the highest tolerated dose (up to 25 mg/16 h) with prednisolone 15 mg/d in active UC[53]. No significant differences were found between the two groups, although the corticosteroid therapy tended to be more efficacious.

A fifth randomized controlled trial compared transdermal nicotine 15 mg/24 h for 5 wk to prednisone 30 mg/d tapered over 5 wk and demonstrated equivalence, although again there was a tendency towards prednisone being more efficacious[54]. After following up 30 patients with remission of distal UC after therapy with either mesalazine plus transdermal nicotine or mesalazine plus oral prednisolone for 12 mo, Guslandi *et al*[56] suggested that nicotine-induced remission of UC lasts longer than that obtained by therapy with oral corticosteroids. With respect to the maintenance of remission in patients with UC, no beneficial effect was noted with a lower dose of transdermal nicotine (up to 15 mg/16 h) compared with placebo over a period of 6 mo in the sixth randomized, placebo-controlled trial[55].

In two studies in which liquid nicotine enemas were used to treat patients with active distal UC, the results suggested a clinical benefit in the absence of detectable serum nicotine concentrations[57,58]. Adverse events were of minimal consequence. Nevertheless, the liquid enemas were difficult to retain. Further controlled trials are needed to determine the therapeutic value of topical nicotine administration.

Finally, in patients with PSC, a pilot study of oral nicotine tartrate capsules delivered to the gastroduodenum did not report a beneficial effect[59].

Adverse events when nicotine is therapeutically administered are common (> 50%), but generally mild. They include contact dermatitis, nausea, lightheadedness, headache, sleep disturbance, central nervous system stimulation, sweating, tremor, and tachycardia. The side effects are more frequent in non-smokers than in ex-smokers. Non-smokers have fewer nicotine-associated adverse events if they are initially administered a low dose patch, subsequently escalating the nicotine dose, presumably because they have not yet developed a tolerance to nicotine side effects[60].

Table 2 Possible mechanisms in the pathogenic interactions of smoking and inflammatory bowel disease[64]

Modulation of cellular and humoral immunity
Changes in cytokine levels
Modification of eicosanoid-mediated inflammation
Reduction of antioxidant capacity, production of oxygen free radicals
Release of endogenous glucocorticoids
Colonic mucus effects
Alteration of mucosal blood flow
Pro-thrombotic effects and promotion of microvascular thrombosis
Alteration of gut permeability
Modification of gut motility

PATHOGENIC MECHANISMS OF SMOKING ON UC PATIENTS

The exact mechanisms of action of nicotine and smoking in UC patients is not well known (Table 2)[61]. Tobacco smoke contains hundreds of different substances including nicotine, free radicals and carbon monoxide. It is suspected that the main metabolite responsible for the impact on the course of UC is nicotine, however there is no absolute proof of nicotine being the sole active moiety. In consequence, probably the mechanisms are diverse and considering that the pathogenesis of UC is only partially understood, any dissertation on their possible mechanisms can only be hypothetical.

Nicotine increases mucin synthesis in UC patients[62]. Patients with UC are shown to have a significantly thinner mucus layer in the left colon and rectum compared with healthy (smoking and nonsmoking) control subjects[63]. Nevertheless, no effect of transdermal nicotine on mucin gene expression in UC patients could be demonstrated by Louvet *et al*[64].

Nicotine also affects the immune system. Heavy smokers may have reduced levels of immunoglobulin A in both saliva and intestinal secretions[65,66]. Heavy smoking also influences cellular immune-defense mechanisms, leading to a state resembling immune suppression with an increased level of suppressor CD8+ T cells and a diminished ratio of CD4/CD8 T cells, which is reversible after the cessation of smoking[67]. Nicotine has been shown to decrease the synthesis of proinflammatory molecules, for instance, interleukin (IL)-1β and tumor necrosis factor (TNF)-α by mouse colonic mucosa as well as the production of mucosal eicosanoids[68] and some proinflammatory cytokines by human mononuclear cells (e.g. IL-2[69], IL-8, and TNF-α[70]).

Smokers with UC have a significant reduction in mucosal cytokine levels, specifically, IL-1b and IL-8[71]. Beneficial effects of nicotine in active UC may be associated with a decrease in IL-8 expression. In rats, DNBS-induced colonic damage was improved in passive-smoking rats involving changes in colonic cytokine levels[72]. The increase

of leukotriene B4, TNF-α, IL-1β, and IL-6 levels was alleviated. In contrast, the deprivation of IL-10 during UC was preserved. The exact meaning of these changes in the cytokine balance still remains poorly understood.

Smoking has a deleterious effect on phagocyte function, decreasing their bactericidal and bacteriostatic activity[73]. Chronic exposure of rats to nicotine inhibits the antibody-forming cell response, impairs the antigen mediated signaling in T-cells and induces T-cell anergy[74].

Smokers have a greater capacity for the generation of free oxygen radicals, with reduced antioxidant capacity[75]. This fact correlates with the harmful effect of smoking on patients with CD but does not offer an explanation for the beneficial effects on UC patients.

Other effects of nicotine or smoking on the intestine include hypoperfusion of the rectum and of acutely damaged colonic tissue[76], the alteration of gut motility, the reduction of smooth muscle tone and contractility (modulated by nitric oxide)[77], altered permeability[78], and alterations in the microcirculation[79]. Patients with IBD have an increased intestinal permeability[80]. Smoking was found to decrease the intestinal permeability in healthy control subjects[81]. However, no such observation could be made in smokers with UC compared with nonsmokers with UC[82], refuting the notion that the protective effect of smoking on UC is due to the moderation of an increased intestinal permeability.

SMOKING CESSATION

Based on all the available data, it is clear that smoking cessation in a patient with UC could exacerbate its symptoms as well the disease activity. For this reason, in order to decide whether we should recommend that UC patients quit smoking, we need to balance the decision with the patient.

First and foremost, the patient has to be aware that active cigarette smoking causes a broad spectrum of diseases; it is a major cause of vascular disease, cancer and chronic obstructive pulmonary disease. In addition to these, cigarette smoking also causes other respiratory symptoms, adversely affects reproductive outcomes and is a cause of diminished health status. Furthermore, exposure to second-hand smoke is an established cause of coronary heart disease and lung cancer, as well as a host of other adverse health effects.

Secondly, patients should be given truthful information; it is important to explain to them that there is the possibility of disease exacerbation (flares, hospitalization or the need for oral steroids) following cessation of smoking. We should also explain that the risk of colectomy in the short term appears not to be increased in the case of quitting smoking.

Additionally, patients should be given other important information about the effects of smoking on the course of UC. Patients with UC had a significantly decreased risk of pulmonary cancer, which may primarily be explained by the smoking habits of the patients with UC[83]. Patients should also be informed about the influence of smoking on the risk of colon cancer (CRC). It is well accepted that UC is associated with an increased risk of colon cancer. A recent meta-analysis of 36 studies showed that current smokers had a significantly increased risk of CRC incidence[84].

Finally, the patient should be aware that nowadays there is an increasing number of treatments that can be used to control inflammation in the case of disease exacerbation after smoking cessation, such as immunosuppressants, leukocyte apheresis or anti-TNF-α products[85].

Based on all the deleterious health effects combined with the substantial prevalence, cigarette smoking represents a major worldwide cause of death. In patients with UC, the risks of smoking far outweigh any possible benefit, so physicians should advise, encourage and assist UC patients who smoke to quit.

CONCLUSION

(1) Smoking is protective in UC, with a lower incidence of disease in smokers; (2) Current smoking protects against UC and, after onset of the disease, improves its course, reducing the need for colectomy; (3) Ex-smokers have an increased risk of developing an unfavorable clinical course; (4) PSC is observed almost exclusively in nonsmokers; (5) Nicotine is efficacious for active UC, but in most studies, side-effects (nausea, headache, dermatitis) were frequent and tended to overcome the clinical benefit; (6) The place of nicotine in the algorithm of treatment of UC patients is unclear, although it may be useful in selected cases, particularly in recent ex-smokers with moderate but refractory attacks of UC; and (7) Since smoking is associated with several additional deleterious effects (e.g. cardiovascular, lung cancer risk), gastroenterologists should encourage both UC and CD patients to quit smoking. Before stopping, UC patients should be informed about the potential risk of an increase in disease activity, without a higher risk for surgery.

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ORIGINAL ARTICLE

miRNA studies in *in vitro* and *in vivo* activated hepatic stellate cells

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Abstract

AIM: To understand which and how different miRNAs are implicated in the process of hepatic stellate cell (HSC) activation.

METHODS: We used microarrays to examine the differential expression of miRNAs during *in vitro* activation of primary HSCs (pHSCs). The transcriptome changes upon stable transfection of rno-miR-146a into an HSC cell line were studied using cDNA microarrays. Selected differentially regulated miRNAs were investigated by quantitative real-time polymerase chain reaction during *in vivo* HSC activation. The effect of miRNA mimics and inhibitor on the *in vitro* activation of pHSCs was also evaluated.

RESULTS: We found that 16 miRNAs were upregulated and 26 were downregulated significantly in 10-d *in vitro* activated pHSCs in comparison to quiescent pHSCs. Overexpression of rno-miR-146a was characterized by marked upregulation of tissue inhibitor of metalloproteinase-3, which is implicated in the regulation of tumor necrosis factor-α activity. Differences in the regulation of selected miRNAs were observed comparing *in vitro* and *in vivo* HSC activation. Treatment with miR-26a and 29a mimics, and miR-214 inhibitor during *in vitro* activation of pHSCs induced significant downregulation of collagen type Ⅰ transcription.

CONCLUSION: Our results emphasize the different regulation of miRNAs in *in vitro* and *in vivo* activated pHSCs. We also showed that miR-26a, 29a and 214 are involved in the regulation of collagen type I mRNA.

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Key words: Hepatic stellate cells; miRNA; miR-146a; Nuclear factor-κB

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INTRODUCTION

Liver fibrosis, characterized by an overproduction of extracellular matrix (ECM), is a common outcome of

different chronic liver diseases[1]. Hepatic stellate cells (HSCs) are one of the major cell types responsible for the production of ECM molecules like collagens, laminin, proteoglycans and fibronectin[2]. The production of different ECM molecules is increased upon transdifferentiation (activation) of HSCs from a quiescent to an activated myofibroblast-like state[3,4]. Consequently, the regulation of the complex process of HSC activation is of great interest to the research community. Understanding this process should lead to the discovery of therapeutic strategies for liver fibrosis. Due to the complexity of the activation of HSCs, the number of regulatory steps is expected to be overwhelming[5], and requires addressing many different targets at the same time, either with different compounds or with one compound that is able to work on many different targets.

miRNAs are small approximately 23-nt non-coding RNAs, which are able to regulate hundreds of different proteins. The versatility of miRNAs is attributed to the imperfect binding (seed region) to the 3'-UTR of mRNAs, which results in, contrary to siRNA, many binding partners. The regulation by miRNAs is also different to siRNAs because it leads to a translational repression and/or mRNA destabilization[6,7]. That miRNAs fulfill regulatory functions has been established by their involvement in many different processes and diseases[8,9]. Therefore, it is tempting to use these molecules in order to treat liver fibrosis; a condition that is caused by a deregulation of biological processes. To succeed in this attempt, we need to identify the miRNAs, which are differentially regulated in the normal and diseased liver, and more specifically in the HSCs; one cell type that is responsible for the fibrotic process.

The purpose of this study was to identify differentially regulated miRNAs in *in vitro* activated HSCs, in order to study them in an *in vivo* animal model, and finally, to determine their role in the activation process.

MATERIALS AND METHODS

Isolation of rat primary HSCs and cell culture conditions

Wistar rats were used to isolate primary HSCs (pHSCs) according to a published pronase/collagenase *in situ* perfusion protocol[10]. The isolation protocol was approved by the Institutional Animal Care and Use Committee under #080389. For *in vitro* activation, the cells were seeded into 75-cm^2 culture flasks and harvested after 3, 5, 7 or 10 d. Primary cells and the HSC-2 cell line were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ in a 5% CO_2 humidified incubator.

HSC-2 is a spontaneous immortalized cell line derived from the pHSCs of a male Wistar rat. The primary cells were passaged several times before clonal selection by limiting dilution[11].

The purity of pHSCs from rats on normal and choline-deficient ethionine supplemented (CDE) diet was assessed using vitamin A autofluorescence or real-time polymerase chain reaction (PCR), respectively (Figure 1A). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA).

In vivo activation of rat HSCs

Six- to eight-week-old male Wistar rats were fed the CDE diet (CDE model) (MP Biomedicals, Solon, OH, USA, #0296021410) for 4 wk (Figure 2). Livers were isolated, perfused with PBS and fixed in neutralized formalin (paraffin embedding) or *in vivo* activated pHSCs were isolated.

Isolation of miRNA for microarray and analysis

miRNA was extracted from quiescent (freshly isolated) and 10-d *in vitro*-activated pHSCs using the PureLink purification kit (K1570-01; Invitrogen). The miRNA microarray (NCode Multi-Species miRNA microarray V2) was performed according to the manufacturer's manual (MIRLS-20; Invitrogen). For each experiment, a dye swap was performed. The arrays were scanned using a GenePix 4200AL array scanner. The raw datasets were deposited under #GSE19463 at the Gene Expression Omnibus (GEO) repository[12]. For two-color miRNA arrays, averaging of dye-swapped arrays was performed to minimize the dye effects prior to normalization using the Cross-Correlation method[13]. The targets of differentially regulated miRNAs (Table 1) were predicted by three different methods, TargetScan 5.1[14], mirBASE target[15], and miRNA Viewer[16] using default parameters. Targets predicted by at least two tools were selected and grouped into upregulated and downregulated miRNAs, respectively. These two groups of targets were subjected to pathway analysis using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA). A ratio was calculated whereby the number of predicted targets in a given pathway was divided by the total number of molecules in that pathway. The Fisher's exact test was used by the software to calculate a *P* value. This *P* value represented the probability that the association between the predicted targets and the pathway could not be explained by chance alone. The *P* value cutoff was set at $P \leqslant 0.001$. The x axis was the negative logarithm of *P* value with a base of 10 ($-\log_{10} P$ value).

Real-time PCR

The verification of the microarray data and subsequent miRNA assessments were performed for let-7b, let-7c, miR-16, 26a, 29a, 31, 125b, 143, 146a, 150 and 214 by using the respective Taqman MicroRNA assays (P/N 4427975, Applied Biosystems, Foster City, CA, USA). The U6 snRNA assay (ID 001973) served as a normalization control. Total RNA was isolated using the NucleoSpin RNAII kit (Macherey-Nagel, Germany). Total RNA and miRNA were isolated using the same kit but with a small modification. Briefly, the cell lysate was adjusted to contain 35% ethanol and passed through the RNAII column to bind the total RNA. The ethanol concentration of the flow through was then adjusted to > 70% and passed through the same column in order to bind the miRNA.

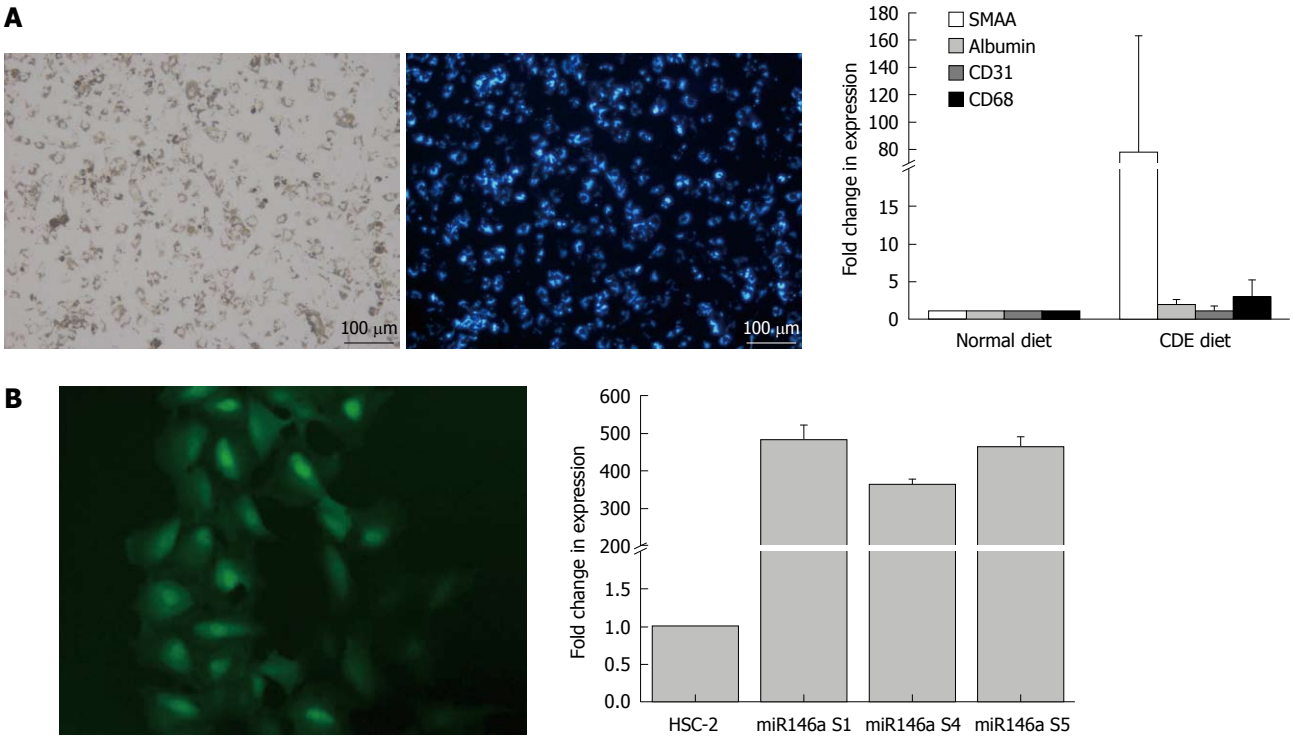

Figure 1 Primary hepatic stellate cells and over-expression of miR-146a in hepatic stellate cell-2 cell line. A: Bright-field image of 1 d cultivated primary hepatic stellate cells and the corresponding vitamin A autofluorescence image are shown. Scale bar represents 100 μm. Real-time polymerase chain reaction (PCR) for *in vivo* activated hepatic stellate cells from rats on normal (n = 2) and choline-deficient ethionine supplemented (CDE) diet (n = 4). The mean ± SE for each diet model is shown; B: A representative image for the over-expression of miR-146a as visualized by the reporter GFP is shown. Real-time PCR for three independent clones confirmed the expression of miR-146a. The data represent the mean ± SE of triplicate reactions. SMAA: Smooth muscle α-actin.

The Cells-to-Ct kit (Invitrogen, P/N 4391848) was used for some experiments to quantify the miRNA expression with the respective miRNA assays. The reverse transcription and real-time PCR were performed according to the assays protocol using the ABI 7500 Fast Real Time PCR System (Applied Biosystems). Taqman assays used were smooth muscle α-actin (SMAA) (Rn01759928_g1), Col1a1 (Rn01463849_g1), interleukin (IL)-6 (Rn00561420_m1), cyclooxygenase-2 (Cox-2) (Rn00568225_m1), RelA (Rn01502266_m1), CD31 (Rn01467259_m1), Albumin (Rn01413833_m1), CD68 (Rn01495643_g1) and tissue inhibitor of metalloproteinase (TIMP)-3 (Rn00441826_m1).

Nuclear factor-κB siRNA transfection

HSC-2 cells were seeded at a density of 10^6 per 100 mm cell culture dish and incubated at 37℃. The siRNA was mixed at a final concentration of 10 nmol/L with 1 mL DMEM without serum and 120 μL HiPerfect transfection reagent (Qiagen, Germany) and incubated for 10 min. The mixture was added drop-wise to the cells and incubated for 48 h. For the mock control, only the HiPerfect reagent was used. The ON-Targetplus nuclear factor (NF)-κB siRNAs used were J-080033-11 and J-080033-12 (Dharmacon, Lafayette, CO, USA). These conditions were tested for transfection efficiency using FITC-labeled siRNA and FACS analysis.

Overexpression of miR-146a in an HSC cell line

The vector was constructed by amplification of a 487-bp fragment containing the rno-miR-146a from rat genomic DNA using the following primer pair: sense 5'-*AAGCTT*GCCACCAGTCCCATCCTTCACC-3' (*Hin*dIII), anti-sense 5'-*GGATCC*TTCCTCTGTGCTGGGATTACAGGGTG-3' (*Bam*HI). After sub-cloning, the rno-miR-146a was excised using *Bam*HI/*Eco*RV and cloned into pcDNA6.2/GW EmGFP-miR (Invitrogen). The HSC-2 cells were stably transfected with the construct using Lipofectamine 2000 (Invitrogen) and selected in cell culture medium supplemented with 10 μg/mL Blasticidin. The clonal selection was achieved using FACS.

Gene expression array and analysis

Total RNA from HSC-2 cells overexpressing miR-146a and control cells (two different passages) were used to study the transcriptome changes using the GeneChip Rat Genome 230 2.0 (Affymetrix, USA). The preparation of the samples was performed according to the technical manual P/N 702232 Rev. 3 (Affymetrix) using one-cycle cDNA and target labeling. The chips were scanned using a Genechip Scanner 3000 (Affymetrix). The raw datasets were deposited under #GSE19463 at the GEO repository[12].

The microarray probe set data was summarized using the Robust Multi-Array Average expression measure method, and pre-processed to correct unreliable (small) intensities for each array. The pre-processed data were then normalized using the Cross-Correlation method[13]. For

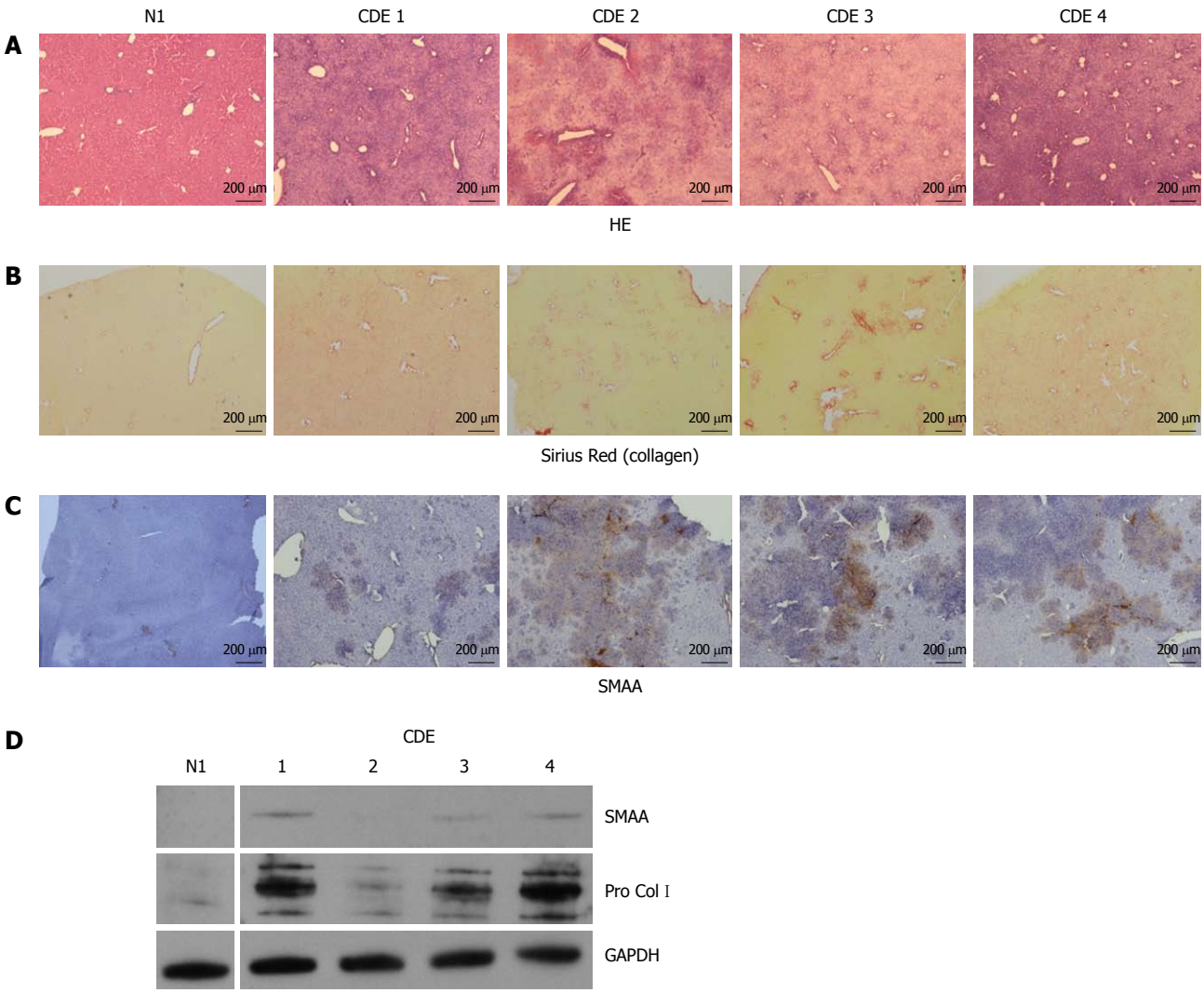

Figure 2 Histological and immunohistochemical analysis of livers from rats receiving choline-deficient ethionine supplemented diet for 4 wk. A: HE staining shows the structural changes between control and choline-deficient ethionine supplemented (CDE) diet livers. No severe steatosis is observed; B: The Sirius Red staining depicts the deposition of collagen around the portal area and the whole liver; C: The increase in smooth muscle α-actin (SMAA) staining reflects the increasing number of myofibroblasts seen in patches throughout the liver. Scale bar represents 200 μm; D: The Western blotting data confirm the increase in SMAA and Col Ⅰ.

each gene, a fold change value was calculated for samples *vs* control. Differentially expressed genes (DEGs) were selected based on the criterion of fold change > 2. The *P* values of DEGs were obtained using one-tailed Student's *t* test. Pathway analysis was carried out on the DEGs using Ingenuity Pathway Analysis (Ingenuity Systems).

Transfection of miRNA mimics and hairpin-inhibitor

Cells were seeded at 20 000 per well in 48-well plates 24 h prior to transfection. The miRNA mimics or hairpin-inhibitor were added at the required final concentration (miR-26a, 146a, controls and quadruple transfection: 50 nmol/L each; miR-29a and 214: 200 nmol/L each) to 750 μL DMEM without serum, followed by 10 μL Hi-Perfect transfection reagent. The mixture was incubated for 10 min. The medium from each well was aspirated and replaced by 250 μL of the mixture. The transfection was performed in triplicate. Controls were either Hi-Perfect reagent only (mock) or control miRNAs for the mimic and/or inhibitor.

SDS-PAGE and Western blotting

Cells were lysed in ProteoJet lysis buffer (#K0301; Fermentas, Glen Burnie, MD, USA) and the protein concentration was estimated using the BCA method (Thermo Scientific, USA). The samples were separated in 4%-12% Bis-Tris NuPage gels (Invitrogen) and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h at room temperature using 5% non-fat milk in TBS-Tween (TBS-T). The primary antibodies were applied in the following dilutions: interleukin receptor associated kinase 1 (IRAK1) (sc-7883; Santa Cruz Biotechnology, Santa Cruz, CA, USA,) 1:400; tumor necrosis factor receptor associated factor 6 (TRAF6) (sc-7221; Santa Cruz Biotechnology) 1:400; IκBα (#4814; Cell Signaling, Danvers, MA, USA) 1:1000; pIκBα (#2859; Cell Signaling) 1:750; Cox-2 (sc-1747; Santa Cruz Biotechnology) 1:5000; and β-actin (ab-8227; Abcam, Cambridge,

Table 1 Differentially regulated miRNAs as identified by miRNA microarray

miRNA name	Fold change	*P* value
Upregulated compared to day 0		
rno-let-7b	4.70	0.0242
rno-let-7c	3.75	0.0236
rno-let-7e	2.77	0.0340
rno-miR-125b	11.98	0.0113
rno-miR-132	1.97	0.0184
rno-miR-143	17.05	0.0014
rno-miR-145	2.29	0.0483
rno-miR-152	3.01	0.0255
rno-miR-199a	3.46	0.0415
rno-miR-21	5.73	0.0142
rno-miR-210	2.34	0.0186
rno-miR-214	18.44	0.0011
rno-miR-22	3.11	0.0392
rno-miR-221	10.09	0.0007
rno-miR-222	2.67	0.0317
rno-miR-31	8.91	0.0013
Downregulated compared to day 0		
rno-let-7f	-2.17	0.0327
rno-miR-10a	-3.53	0.0417
rno-miR-122a	-349.63	0.00002
rno-miR-125a	-2.36	0.0474
rno-miR-126	-170.32	0.0003
rno-miR-146a	-16.83	0.0352
rno-miR-150	-9.81	0.0325
rno-miR-151*	-3.72	0.0345
rno-miR-16[1]	-4.38	0.0366
rno-miR-181a[1]	-4.50	0.0346
rno-miR-192	-6.08	0.0206
rno-miR-194	-6.08	0.0168
rno-miR-195	-14.50	0.0130
rno-miR-207	-1.93	0.0449
rno-miR-26a	-5.17	0.0163
rno-miR-26b	-4.81	0.0300
rno-miR-296	-1.93	0.0292
rno-miR-29a[1]	-2.38	0.0644
rno-miR-30a-5p	-5.35	0.0327
rno-miR-30b	-10.51	0.0075
rno-miR-30c	-9.69	0.0138
rno-miR-30d	-8.68	0.0093
rno-miR-335	-3.74	0.0500
rno-miR-422b[1]	-8.49	0.0455
rno-miR-483	-2.49	0.0451
rno-miR-99a	-2.97	0.0383

[1]Data from two experiments.

UK) 1:5000. After three washes in TBS-T, the appropriate HRP-conjugated secondary antibody was given at 1:2000 dilution in blocking solution. After three washes in TBS-T, the membrane was developed using the chemiluminiscence substrate (Millipore, Billerica, MA, USA). Primary and secondary antibodies were incubated at 4℃ overnight and 1 h at room temperature, respectively.

Electrophoretic mobility shift assay

Nuclear protein extract from rno-miR-146a-overexpressing clones was obtained using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). The electrophoretic mobility shift assay (EMSA) was performed using the NF-κB(I) EMSA kit according to its protocol (AY1030; Panomics, USA), as described previously[17]. The samples were separated in a 6% non-denaturing polyacrylamide gel (Invitrogen) and transferred to a nylon membrane.

Immunohistochemistry and staining of liver sections

Slides were de-paraffinized and the antigen retrieved by heat exposure in the Target Retrieval Solution pH 9 (S2367; Dako, Glostrup, DK) using a 2100-Retriever retrieval steamer for 45 min. The endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 15 min. Protein was blocked in 10% normal goat serum in PBS for 20 min. The slides were incubated with mouse anti-human SMAA (M0851; Dako) at 1:100 dilution for 1 h, washed and incubated with an anti-mouse HRP-conjugated antibody (K4001; Dako) for 30 min, and developed with DAB (K3468; Dako). All incubations were carried out at room temperature. Nuclei were counter stained with hematoxylin. Hematoxylin and eosin and Sirius Red staining was performed according to standard protocols on paraffin sections. Bright-field images were taken with the LEICA RMB-DM epifluorescence microscope (LEICA, Germany).

Statistics

All quantitative data were presented as mean ± SE. Experimental data were analyzed using the two-tailed Student's *t* test assuming equal variances. $P \leqslant 0.05$ was considered significant. The time-dependent changes during *in vitro* HSC activation were tested for significance at the 0.05 level using one-way ANOVA and Bonferroni's *post-hoc* test. The array data were normalized and analyzed as described in the respective sections above.

RESULTS

Identification of differentially regulated miRNAs in in vitro activated pHSCs and comparison to in vivo activated pHSCs

In 10-d *in vitro* activated pHSCs, 16 miRNAs were upregulated and 26 were downregulated significantly in comparison to quiescent pHSCs (Table 1). We included miR-29a, although the *P* value was above the threshold of 0.05, for further studies because of its predicted targets, which consisted of a number of collagens. The microarray data were confirmed for a number of chosen miRNAs (let-7b, 7c, miR-16, 26a, 29a, 31, 125b, 143, 146a, 150 and 214) using real-time PCR in three additional experiments (Figure 3A). Using isolated *in vivo* activated pHSCs from rats on CDE diet, we found that only miRNAs let-7b, 7c, miR-31, 143 and 214 showed the same regulation as observed for the *in vitro* activated pHSCs (Figure 3B).

Pathway analysis for differentially regulated miRNAs in in vitro activated pHSCs

We performed a pathway analysis using the predicted targets of the differentially regulated miRNAs. The enrich-

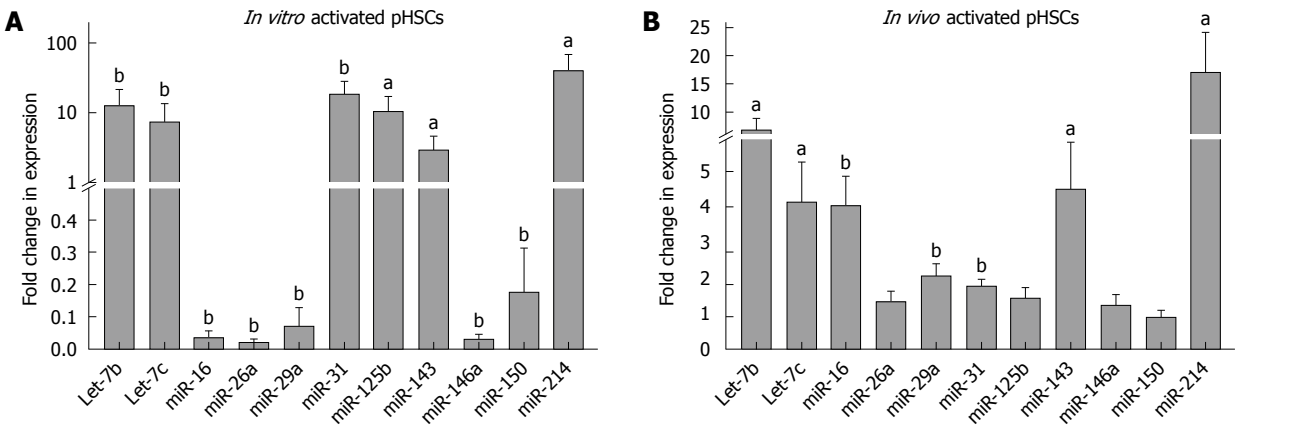

Figure 3 Verification of microarray data by real-time polymerase chain reaction of 11 differentially regulated miRNAs and their regulation upon *in vivo* activation of hepatic stellate cells. A: The graph depicts the changes in the miRNA expression of 11 miRNAs detected by real-time polymerase chain reaction, comparing quiescent with 10-d culture activated primary hepatic stellate cells (pHSCs). The data represent the mean ± SE of three independent experiments ($^aP \leqslant 0.05$, $^bP \leqslant 0.005$); B: The graph illustrates the relative expression levels of miRNAs in isolated *in vivo* activated pHSCs ($n = 4$, choline-deficient ethionine supplemented diet) compared to normal diet ($n = 2$) ($^aP \leqslant 0.05$, $^bP \leqslant 0.005$).

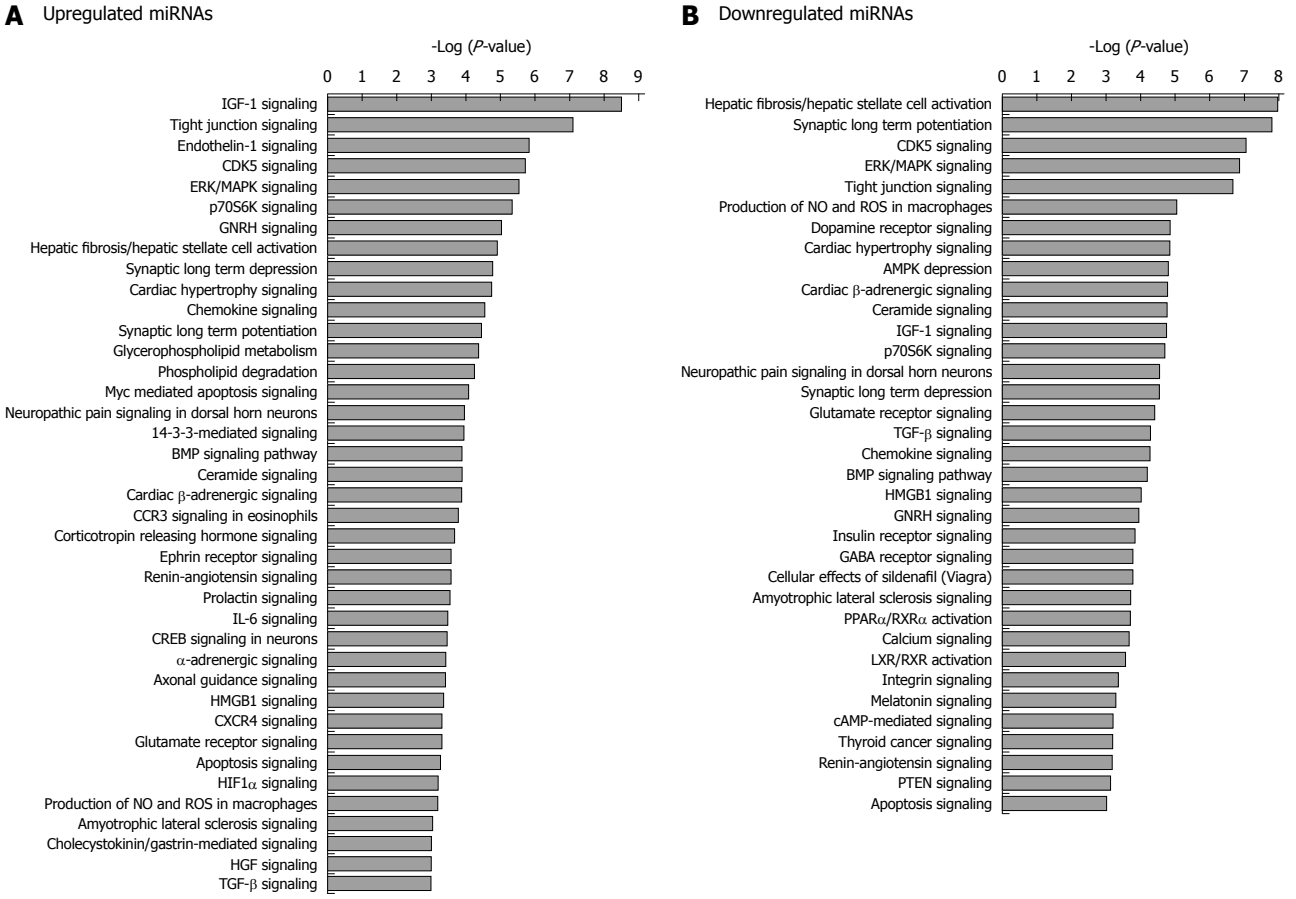

Figure 4 Predicted targets of all differentially regulated miRNAs during *in vitro* activation of primary hepatic stellate cells (Table 1) were analyzed. The two charts represent the enrichment of molecules in affected pathways for the upregulated (A) and downregulated (B) miRNAs. Only pathways with $P \leqslant 0.001$ are shown. IGF-1: Insulin-like growth factor-1; ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; BMP: Bone morphogenetic protein; IL: Interleukin; ROS: Reactive oxygen species; AMPK: AMP activated protein kinase; TGF: Transforming growth factor; PTEN: Phosphatase and tensin homolog; LXR: Liver X receptor; RXR: Retinoid X receptor; PPAR: Peroxisome proliferator-activated receptor; HGF: Hepatocyte growth factor.

ment of genes in single pathways is shown as the -log of the P value ($P \leqslant 0.001$). Signaling pathways which were affected include endothelin-1, cyclin-dependent kinase 5, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), $p70^{S6K}$, chemokine, bone morphogenetic protein (BMP) and IL-6 for the upregulated miRNAs, as well as ERK/MAPK, production of NO and reactive oxygen species (ROS), AMP

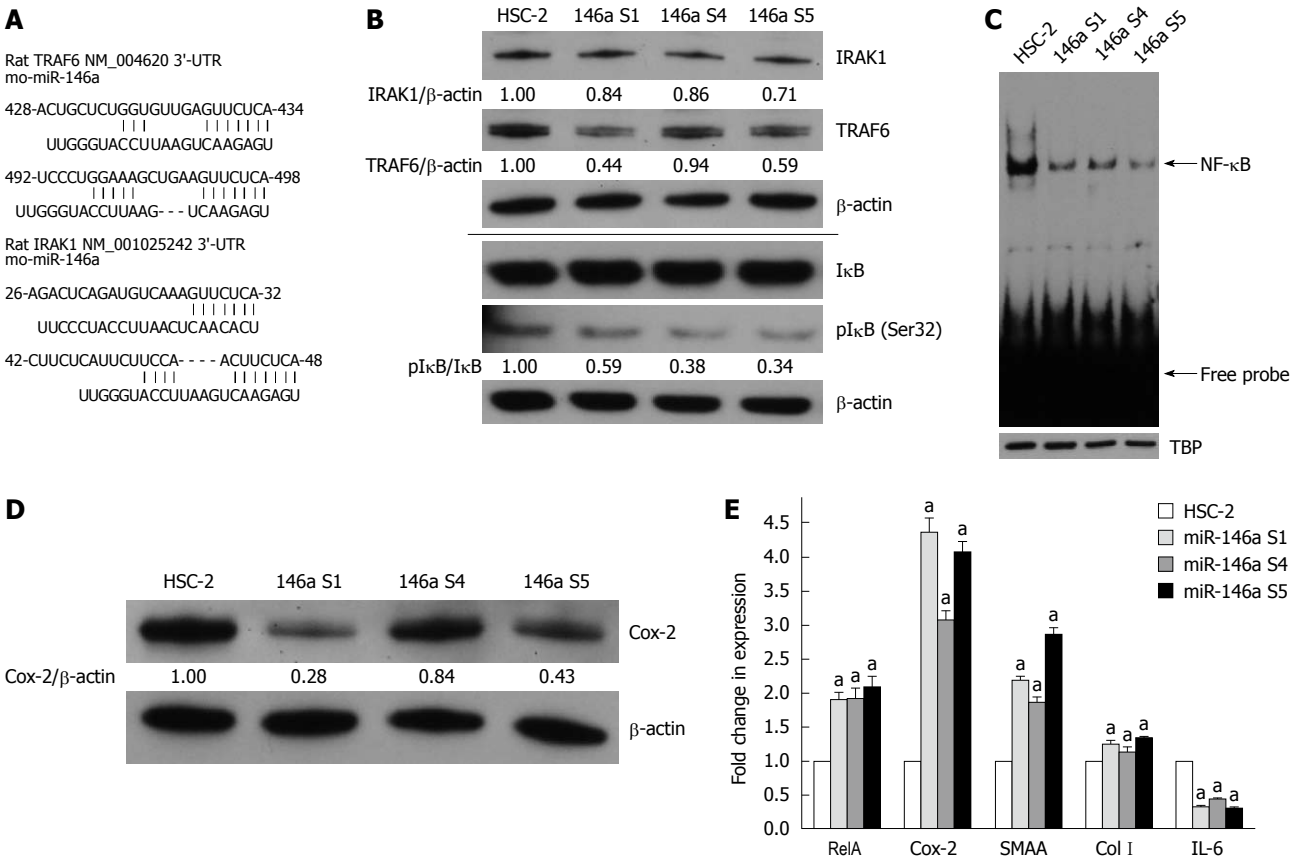

Figure 5 Changes during overexpression of rno-miR-146a in the hepatic stellate cell-2 cell line. A: Depicted are two putative binding sites of miR-146a to the 3'-UTR of rat tumor necrosis factor receptor associated factor 6 (TRAF6) and rat interleukin receptor associated kinase 1 (IRAK1), respectively; B: The Western blotting data show the suppression of TRAF6 and IRAK1, resulting in the decreased phosphorylation of IκB, although the expression of IκB remained unchanged. A representative Western blotting for two independent experiments is shown; C: Electrophoretic mobility shift assay (EMSA) results demonstrated a decrease in nuclear factor (NF)-κB DNA binding activity due to the overexpression of miR-146a. TATA binding protein (TBP) showed equal loading of samples. A representative EMSA experiment is shown out of three independent samples for each clone; D: miR-146a-overexpressing clones showed a reduced level of cyclooxygenase-2 (Cox-2) protein. The Western blotting shown is representative of two independent experiments; E: The relative fold change in mRNA expression between hepatic stellate cell (HSC)-2 and miR-146a-overexpressing HSC-2 cells for five different targets [NF-κB (RelA), Cox-2, smooth muscle α-actin, Col Ⅰ, interleukin-6] is shown. The data represent the mean ± SE of two independent experiments ($^aP \leqslant 0.005$).

activated protein kinase (AMPK), transforming growth factor (TGF)-β, integrin, cAMP-mediated signaling and phosphatase and tensin homolog (PTEN) for the downregulated miRNAs (Figure 4A and B).

Overexpression of miR-146a in HSC-2 and transcriptome analysis

Studies have shown that miR-146a is linked to inflammation and the NF-κB pathway through the two known targets IRAK1 and TRAF6[18,19]. In order to study the function of miR-146a in activated HSCs *in vitro*, we overexpressed this miRNA in a HSC cell line HSC-2[11]. The level of miR-146a in this cell line is very low, making it suitable for the overexpression. The expression of the reporter green fluorescent protein and the real-time PCR validation of the miR-146a expression (Figure 1B) provided evidence for the successful overexpression of miR-146a in three different clones (S1, S4 and S5).

IRAK1 and TRAF6 are direct targets of miR-146a with two target sites for each mRNA (Figure 5A). We were able to show downregulation of these proteins in all three clones (Figure 5B). The functional consequence of this downregulation can be seen by suppression of the phosphorylation of IκB at Ser32 (Figure 5B). The reduced phosphorylation of IκB in turn should lead to the retention of NF-κB in the cytoplasm. Indeed, our EMSA illustrated that there was reduced nuclear binding activity of NF-κB to an NF-κB probe in all clones (Figure 5C). One of the genes regulated by NF-κB is Cox-2, which is functionally related to HSCs due to its pro-apoptotic effect on HSCs[20,21]. Therefore, we investigated the protein level of Cox-2 in the miR-146a-overexpressing clones, and found the expected downregulation (Figure 5D). Surprisingly, further investigation revealed that the mRNAs of NF-κB and Cox-2 were upregulated (Figure 5E). In contrast, we observed a significant downregulation of IL-6 mRNA, another target of NF-κB, in the clones S1, S4 and S5 (Figure 5E). We also found a significant upregulation of SMAA and collagen Ⅰ (Col Ⅰ) mRNAs, a HSC activation and a fibrotic marker, respectively (Figure 5E).

In order to establish a link between the regulation of

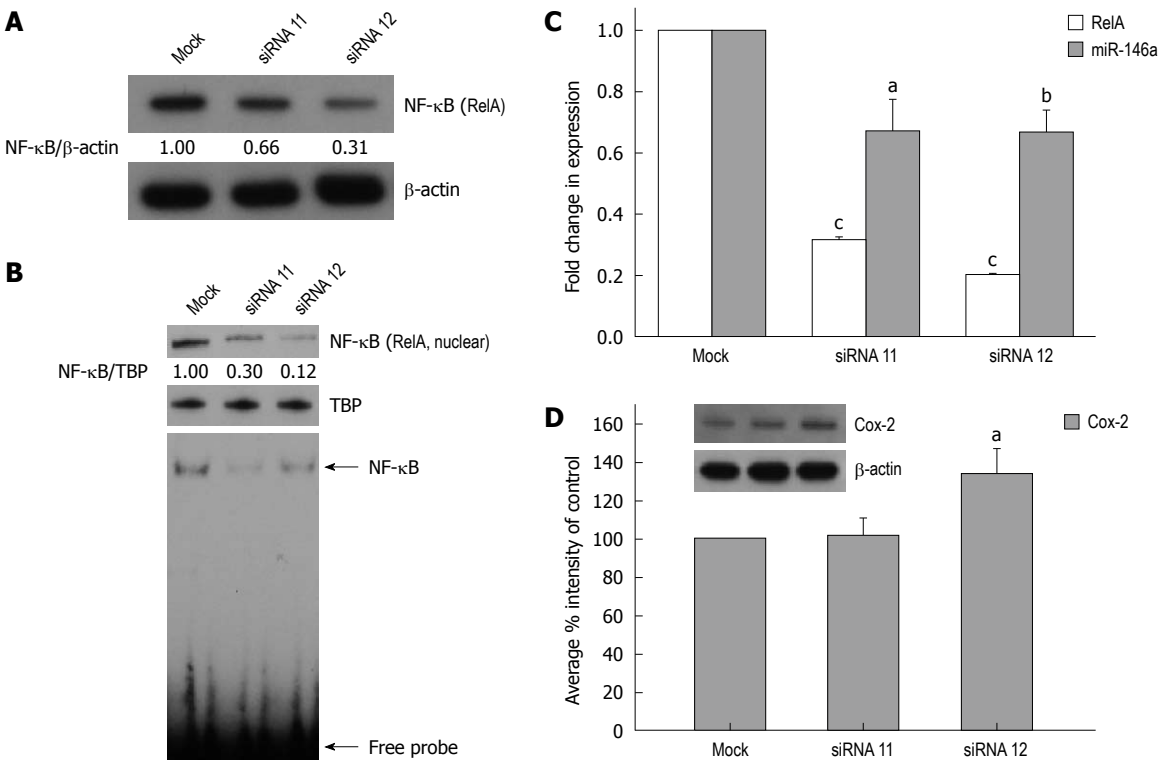

Figure 6 Regulation of miR-146a by nuclear factor-κB. A: Knock-down experiments using nuclear factor (NF)-κB siRNAs showed a reduced level of cellular NF-κB (RelA) protein; B: The nuclear level of NF-κB (RelA) was decreased and showed a diminished DNA binding activity. Depicted is a representative Western blotting and electrophoretic mobility shift assay from three independent experiments; C: Downregulation of NF-κB (RelA) mRNA due to NF-κB siRNA transfection was accompanied by a decrease in miR-146a after 24 h. The data represent the mean ± SE of two independent experiments ($^aP \leqslant 0.05$, $^bP \leqslant 0.01$, $^cP \leqslant 0.001$); D: Cyclooxygenase-2 (Cox-2) protein was upregulated after NF-κB siRNA transfection. Shown are a representative Western blotting and the densitometric analysis of six independent experiments ($^aP \leqslant 0.05$).

miR-146a and NF-κB activity, as proposed by Taganov *et al*[18], we transfected NF-κB siRNAs into HSC-2 cells. The efficiency of the transfection was shown by the downregulation of NF-κB in total cell lysates and nuclear extracts, which resulted in a decrease in NF-κB DNA binding activity (Figure 6A and B, respectively). We also found downregulation of miR-146a in NF-κB siRNA-transfected cells, thereby confirming a regulation of miR-146a by NF-κB in HSCs (Figure 6C). Surprisingly, we noticed an increase in the Cox-2 protein expression (Figure 6D), which implied a yet unclear involvement of miR-146a in the regulation of this enzyme.

The differences in the NF-κB-dependent regulation of Cox-2 and IL-6 have already hinted at the intricacy of the influence of the miR-146a overexpression has on the gene expression in activated HSCs. In order to get an overview of the transcriptome changes, we performed a gene expression analysis of the three miR-146a-overexpressing clones, and compared them with control cells using a cDNA microarray. The analysis yielded 485 up- and 309 downregulated transcripts (Supplementary Tables 1 and 2), which satisfied a P value $\leqslant 0.05$ and at least twofold change. Among the upregulated genes were Lmcd1, CD81, FGF13, Col4a1, Cadherin 11 and BMP-4. The highly downregulated genes included Col15a1, MMP-2, Thy-1, IL-1RL1 and Cadherin 13.

We further analyzed the pathways which were significantly enriched, using a P value $\leqslant 0.05$ as a threshold. Here, we observed enrichment for signaling pathways like integrin-linked kinase, hepatic fibrosis/HSC activation and caveolar-mediated endocytosis, calcium, cAMP-mediated signaling, integrin, endothelin-1 for the upregulated genes (Figure 7 A), and hepatic fibrosis/HSC activation, lipopolysaccharide (LPS)/IL-1-mediated inhibition of retinoid X receptor (RXR) function and nitrogen metabolism, and liver X receptor/RXR activation for the downregulated genes (Figure 7 B).

The most interesting finding was the robust upregulation of TIMP-3 mRNA (Supplementary Tables 1), verified by real-time PCR (Figure 8 A), which is an inhibitor of the tumor necrosis factor-α converting enzyme[22], and has been proposed as a tumor suppressor. Similarly, pHSCs treated with miR-146a mimic also showed induction of TIMP-3 mRNA (Figure 8 B).

Time-dependent expression of different miRNAs during in vitro activation of pHSCs

The fact that miR-146a was not downregulated in *in vivo* activated pHSCs (CDE diet) prompted us to study the time-dependent expression of this miRNA during the *in vitro* activation of pHSCs, together with miR-26a, 29a and 214. The expression of miR-146a was indeed downregulated at day 3 already, and recovered subsequently until day 10. Although the miR-146a level at day 10 was

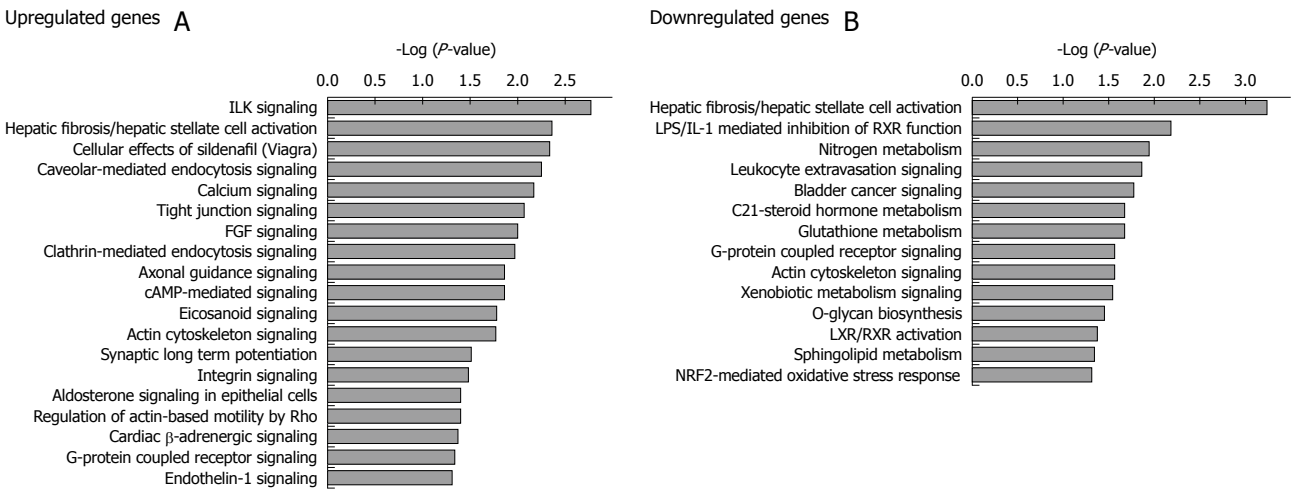

Figure 7 Pathway analysis for the differentially expressed genes of miR-146a over-expressing clones. The charts depict the pathways affected by the (A) or (B) of genes upon stable transfection of miR-146a into hepatic stellate cell-2 cells. Only pathways with $P \leqslant 0.05$ are shown. ILK: Integrin-linked kinase; FGF: Fibroblast growth factor; IL: Interleukin; LPS: Lipopolysaccharide; LXR: Liver X receptor; RXR: Retinoid X receptor.

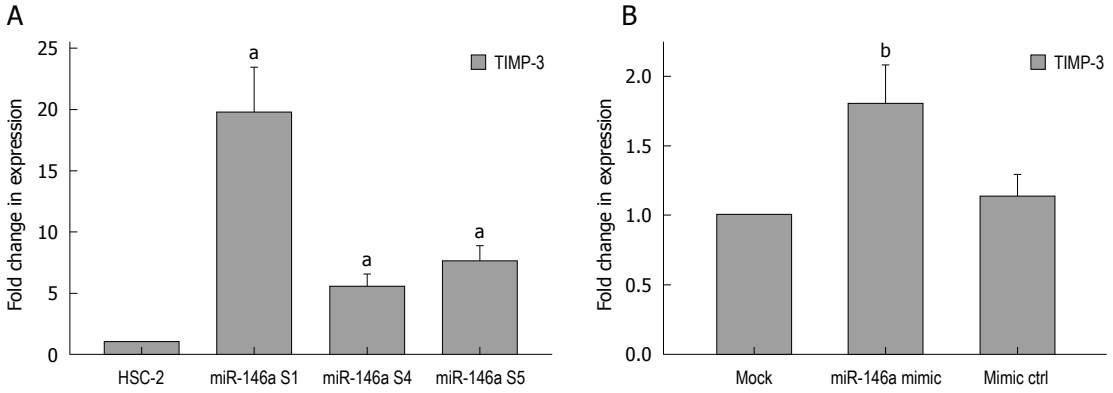

Figure 8 Relative expression of tissue inhibitor of metalloproteinase-3 mRNA in rno-miR-146a-overexpressing hepatic stellate cell-2 cells. A: The graph depicts the relative changes in tissue inhibitor of metalloproteinase (TIMP)-3 mRNA of three rno-miR-146a-overexpressing clones detected by real-time polymerase chain reaction (PCR). The data represent the mean ± SE of two different passages for each clone ($^aP \leqslant 0.005$); B: Primary hepatic stellate cells were treated with 50 nmol/L miR-146a mimic, and the expression of TIMP-3 mRNA was analyzed by real-time PCR and expressed as fold change relative to mock controls. The data represent the mean ± SE of three independent experiments ($^bP \leqslant 0.01$). HSC: Hepatic stellate cell.

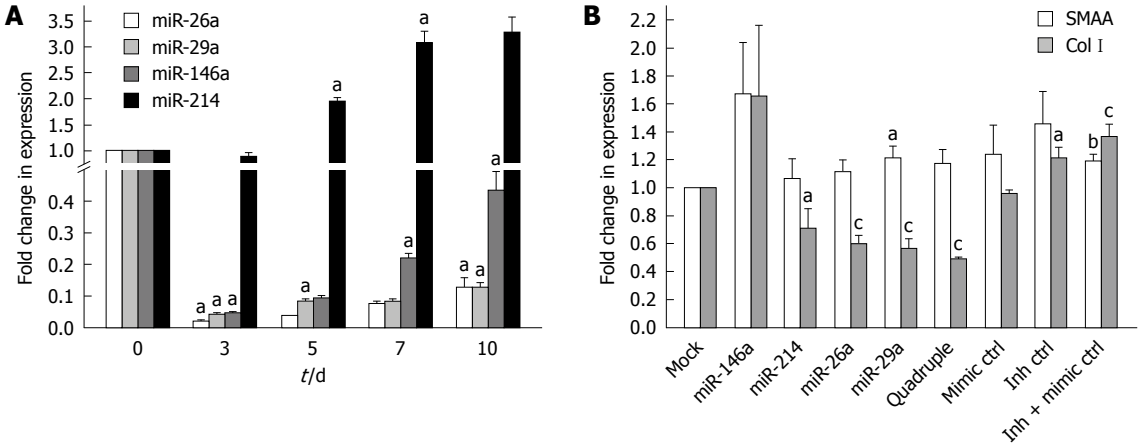

Figure 9 Time-dependent changes in the expression of different miRNAs during *in vitro* activation and miRNA mimic and inhibitor transfection of primary hepatic stellate cells. A: The relative changes in the expression level after 3, 5, 7 and 10 d of *in vitro* primary hepatic stellate cells (pHSCs) activation is shown for miR-146a, 26a, 29a and 214 ($^aP \leqslant 0.05$, one-way ANOVA). The data represent one of two independent experiments performed in triplicate; B: pHSCs were transfected with miR-146a, 26a, 29a mimics or miR-214 inhibitor or in combination. The control transfection consisted of control miRNAs for the mimic and/or inhibitor. The smooth muscle α-actin and Col I mRNA expression was analyzed as fold change relative to mock controls. The data represent the mean ± SE of two independent experiments, each performed in triplicate ($^aP \leqslant 0.05$, $^bP \leqslant 0.01$, $^cP \leqslant 0.001$). SMAA: Smooth muscle α-actin.

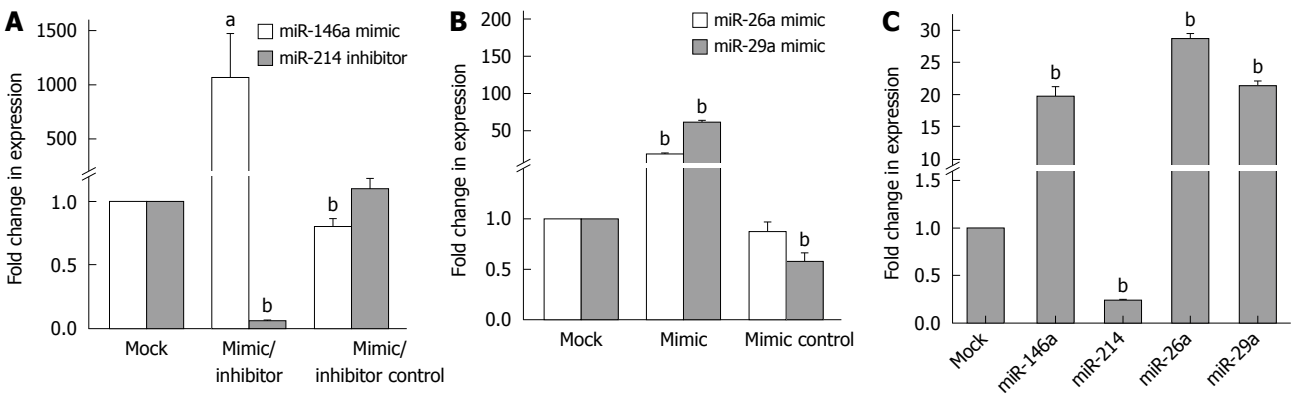

Figure 10 Transfection of primary hepatic stellate cells with different miRNA mimics and inhibitor. The miRNA expression was analyzed as fold change relative to mock transfected primary hepatic stellate cells (pHSCs). Shown are data for the transfection of miR-146a mimic and miR-214 inhibitor with respective control (A), for the transfection of miR-26a and miR-29a mimic with respective control (B) and for the quadruple transfection of miR-146a, miR-26a, miR-29a mimic and miR-214 inhibitor (C) ($^aP \leqslant 0.05$, $^bP \leqslant 0.005$).

still lower than that in quiescent pHSCs at day 0, there was still a 10-fold increase between day 3 and 10 (Figure 9A). In contrast, the expression of miR-26a and 29a did not change as dramatically from day 3 to day 10. We also noticed that miR-214 started to increase only from day 5 onwards (Figure 9A).

Regulation of SMAA and Col I transcripts in pHSCs by different miRNA mimics and inhibitor

In order to study the effect of different miRNA mimics or inhibitor on the *in vitro* activation process, we transfected 3-d *in vitro* activated pHSCs for 3 d with miR-146a, 26a, 29a mimics, miR-214 hairpin inhibitor or all combined. The impact on the HSC activation was followed using real-time PCR to study the changes on the mRNA levels of SMAA and Col I . The high efficiency of transfection was demonstrated by real-time PCR (Figure 10). We found moderate upregulation of the activation marker SMAA by miR-146a (Figure 9B); an observation seen also for the miR-146a-overexpressing clones (Figure 5E and cDNA microarray data). In fact, all cells transfected with the mimics, the inhibitor or combined showed an upwards trend for SMAA mRNA compared to the mock control, although the level did not always change significantly (Figure 9B). For the Col I expression, we noted again an increase caused by miR-146a mimic (not significant) and a decrease by miR-26a, 29a mimic and miR-214 inhibitor. The quadruple transfection led to a suppression of Col I mRNA (Figure 9B).

DISCUSSION

The aim of this study was to gain a deeper insight into the regulation of miRNAs during the activation process of pHSCs, as well as the influence of up- or downregulation of miRNAs on the gene expression and activation of HSCs.

The expression analysis of miRNAs between quiescent and *in vitro* activated pHSCs yielded a number of induced and suppressed miRNAs (Table 1), some of which (miR-143, 16, 122, 146a, 92b, 126) confirmed the findings of Guo *et al*[23]. On the other hand, there were some differences in the regulation of certain miRNAs (miR-328, 207), which could be attributed to the dynamic nature of miRNA regulation and the different use of quiescent pHSCs (day 0 *vs* day 2).

When evaluating the miRNAs expression profile of the *in vitro* and *in vivo* activated pHSCs, a clear distinction was seen in the expression of miR-16, 26a, 29a, 125b, 146a and 150 (compare Figure 3A and B); a phenomenon which could be explained by the distinct HSC activation process. This has been shown at the gene expression level by De Minicis *et al*[24]. The *in vivo* activation was performed over a period of 4 wk, whereas the *in vitro* activation was monitored over 10 d, which could also account for some differences in the miRNA expression, assuming a dynamic regulation.

On the other hand, we found that certain miRNAs (let-7b, 7c and miR-214) were regulated in the same way during *in vitro* and *in vivo* activation of pHSCs. It also became clear to us that miR-214 could be a potential candidate for a diagnostic approach, because this miRNA always shows robust upregulation.

Pathway analysis of the miRNA microarray data was performed to obtain information on signaling cascades involving predicted targets of the differentially regulated miRNAs in *in vitro* activated pHSCs (Figure 4A and B). NO and ROS are known to play a role in the activation process and apoptosis of HSCs[25,26]. The pathways for AMPK, ERK/MAPK, PTEN and TGF-β are also implicated in HSC activation[27-30]. We noticed that a number of pathways were present in the charts for both up- and downregulated miRNAs, which could denote the complexity of regulated targets by each single miRNA, and possibly a cooperative effect between up- and downregulated miRNAs.

A number of publications have shown that miR-146a is involved in inflammatory diseases, regulation of the immune response and NF-κB[19,31-33]. In the early events of liver fibrosis, the activation of HSCs is in part driven by

the hepatic inflammatory process, during which different cytokines are secreted by various liver cells, like Kupffer cells, endothelial cells and hepatocytes[34,35]. Involvement of NF-κB in HSC activation has also been shown in several research papers[36,37]. Therefore, we overexpressed miR-146a in an HSC cell line and observed changes consistent with the findings from Bhaumik *et al*[19]. The detected increase in the NF-κB transcript (Figure 5E) could be explained by a feedback mechanism to the reduced nuclear activity, which leads to the upregulation of the mRNA.

Cox-2 is inducible in activated HSCs by various stimuli and is thought to regulate proliferation[21]. Others have shown that the inhibition of this enzyme has a beneficial antifibrotic effect[20,38,39]. The seemingly discrepant findings of the protein (lower) and transcript (elevated) level for Cox-2 in the miR-146a-overexpressing HSCs (Figure 5D and E) hint at independent pathways for the regulation of Cox-2. These pathways have been shown for intestinal myofibroblasts[40] and during ischemic injury of ileal mucosa[41]. Lasa *et al*[42] and others have shown that the p38 MAPK signaling cascade is able to stabilize the Cox-2 mRNA[43], which could also explain an elevated transcript level. We also cannot exclude that other mechanisms could be involved in stabilizing the Cox-2 mRNA and/or a regulation of Cox-2 by other miRNAs like miR-26a or 143, which are also present in the cell line HSC-2 and for which Cox-2 is a predicted target.

In contrast, the IL-6 mRNA, another molecule regulated by NF-κB, was downregulated (Figure 5E). This observation implies that IL-6 regulation in HSCs is more tightly associated with NF-κB than that of Cox-2.

We were also interested to know whether downregulation of the NF-κB DNA binding activity triggered by miR-146a overexpression could facilitate a feedback loop in HSCs; a notion supported by the fact that the promoter region of miR-146a contains a number of NF-κB binding sites[18]. As expected, a reduction in the NF-κB DNA binding activity (Figure 6B) leads to a decrease in miR-146a (Figure 6C). The observed upregulation of Cox-2 protein (Figure 6D) was somewhat surprising and again substantiated the speculation that other pathways such as p38 MAPK, C-Jun N-terminal kinase and ERK could participate in the regulation of Cox-2 in HSCs[40,44,45].

The microarray analysis revealed that the transcriptome changes caused by miR-146a overexpression are complex and numerous pathways are affected (Figure 7, Supplementary Tables 1 and 2). We found that several DEGs coincided with data from earlier publications on HSC activation[24,46], suggesting that a number of genes affected by miR-146a overexpression are also involved in the activation process. Pathway analysis of the DEGs (Figure 7) confirms a link between miR-146a and inflammation (LPS/IL-1 mediated inhibition of RXR function, eicosanoid signaling, nitrogen metabolism and NRF2-mediated oxidative stress response pathways). That the miR-146a overexpression in HSC-2 cells leads to changes in the pathway called hepatic fibrosis/HSC activation emphasizes that these changes are specific for the HSCs. The upregulation of TIMP-3 (Supplementary Table 1 and Figure 8) again emphasizes the involvement of miR-146a in inflammatory processes and immunity, by linking it to the TNFα activity[47].

We noticed a robust downregulation of miR-146a during *in vitro*, but a missing regulation of miR-146a during *in vivo* activation of pHSCs (CDE diet). We hypothesized that there is a dynamic component in the regulation of miR-146a. We effectively found that there is a time-dependent regulation of miR-146a over 10 d of *in vitro* activation of pHSCs. From an *in vivo* perspective, it could be a possibility that miR-146a is decreased following the first insult to the liver, but reaches almost a normal level during the developing fibrosis, as seen for the *in vivo* activated pHSCs (CDE diet). The mechanism behind this miR-146a regulation is not clear, but the involvement of different transcription factors [NF-IL6, interferon regulatory factor (IRF 3/7)] binding to its promoter region is conceivable[18].

The dynamic nature of miRNA regulation during the *in vitro* activation of pHSCs could also partially explain the differences in the expression pattern of the miRNAs *in vitro* and *in vivo*. The dynamic nature of miRNA expression has been shown for the T-cell development[48], and it makes sense if we consider the multitude of effects a single miRNA can have due to the imperfect complementarity to its target sequence.

The *in vivo* targets of a miRNA treatment are pHSCs, therefore, we assessed the effects of several miRNAs mimics (miR-26a, 29a, 146a) and inhibitor (miR-214) on the activation state of pHSCs. The transfection with a combination of all mimics and inhibitor was performed so as to examine possible cooperative effects between different miRNAs, as a first step to understand the cooperativity of miRNA expression changes during HSC activation. The miR-26a, 29a mimics and miR-214 inhibitor showed a significant suppression of the Col I mRNA (Figure 9B). This is somewhat surprising because even though a number of collagens are predicted targets for miR-26a and 29a, none has a perfect binding site, which would explain regulation by mRNA degradation. Therefore, we conclude that the mechanism by which miR-26a, 29a and 214 downregulate the Col I mRNA is indirect, as also suggested by van Rooij *et al*[49] for miR-29a. The downregulation of Col I by the quadruple transfection shows some synergistic effect between the miRNAs.

Our findings showed the differential regulation of miRNAs in *in vitro* and *in vivo* activation of pHSCs, and particularly, the involvement of miR-26a, 29a and 214 in the regulation of Col I mRNA. Moreover, miR-146a overexpression or treatment with miR-146a mimic upregulates TIMP-3 mRNA, which suggests an association between miR-146a, TNFα activity and inflammation. In conclusion, our observations help build a global picture of the miRNA regulation during HSC activation *in vitro* and *in vivo*, and may have important implications when considering a therapeutic approach for treating liver fibrosis using miRNAs.

Supplementary Table 1 Upregulated genes in miR-146a-transfected hepatic stellate cell-2

Probe ID	Representative public ID	Gene symbol	Gene title	Log2	Fold change	*P*-value
1383164_at	AW524366			5.065854	33.494550	3.49E-05
1379902_at	BE108170			4.568453	23.726920	0.000154
1382211_at	AI602542			4.401705	21.137100	0.008197
1394456_at	AW525722			3.905818	14.988850	0.000414
1373740_at	AA851385			3.777603	13.714240	0.002694
1393437_at	AW142608			3.417765	10.686850	3.36E-05
1389579_at	BI284372			3.412511	10.648010	0.010102
1374065_at	BG378920			3.327195	10.036580	0.000137
1393018_at	AI071984			3.323743	10.012590	0.000201
1392105_at	AW527533			3.229460	9.379166	0.005625
1373062_at	BM388650			3.174909	9.031143	0.000330
1373776_at	AI406341			3.127483	8.739090	3.23E-06
1378457_at	AI179450			2.993031	7.961450	0.001547
1391428_at	AI639162			2.959079	7.776274	0.003728
1393314_at	BI289840			2.826721	7.094600	8.14E-05
1379382_at	AI144865			2.810339	7.014492	0.017550
1391481_at	BE104424			2.742723	6.693324	0.000235
1376435_at	BI303340			2.688229	6.445217	0.000280
1395327_at	AW522341			2.651476	6.283098	0.017329
1371506_at	AA891207			2.611623	6.111911	0.013546
1394833_at	BE120930			2.604706	6.082676	3.53E-06
1375230_at	AA800192			2.512936	5.707806	0.000375
1374811_at	AA858705			2.505232	5.677406	0.035302
1377934_at	BF387289			2.461691	5.508619	0.005853
1383240_at	BE110753			2.374072	5.184023	0.004602
1372921_at	AI073219			2.355729	5.118529	0.036703
1376800_at	AA892496			2.321059	4.996990	0.000124
1384137_at	AI030318			2.265411	4.807915	0.000711
1398597_at	AI044699			2.200111	4.595148	0.000220
1393782_at	BF396790			2.190070	4.563275	0.003901
1382330_at	BE116838			2.188725	4.559023	0.000179
1398657_at	AI045896			2.164482	4.483053	5.91E-05
1376617_at	BE107482			2.109346	4.314957	0.001578
1378111_at	AI576002			2.104523	4.300555	0.023000
1379936_at	AA875132			2.096645	4.277136	0.005892
1377946_at	BF420043			2.071766	4.204010	3.19E-05
1377675_at	AI177743			2.063136	4.178936	0.005112
1380940_at	BF402603			2.046820	4.131942	0.007683
1381335_at	BE349658			2.046725	4.131669	7.30E-05
1374971_at	AA818954			2.036171	4.101556	0.000603
1397781_at	BF414751			1.968385	3.913299	0.004705
1391841_at	BE103537			1.948096	3.858649	0.003749
1388546_at	AI013328			1.918544	3.780415	0.005524
1379444_at	BF283694			1.836166	3.570599	0.000940
1392627_x_at	BI282114			1.828818	3.552459	9.63E-05
1374432_at	BE118251			1.820423	3.531847	0.023594
1389239_at	BM384377			1.820056	3.530949	3.85E-06
1394578_at	BI299761			1.783117	3.441689	0.019487
1376734_at	BI279030			1.776063	3.424903	0.021384
1392820_at	BI285064			1.763119	3.394312	0.000366
1382212_at	AI385201			1.761883	3.391405	0.008303
1374273_at	BG665433			1.740316	3.341083	0.001848
1390471_at	BM383411			1.732112	3.322138	0.003308
1396009_at	BE108258			1.726446	3.309117	2.68E-05
1382294_at	AI576111			1.698452	3.245526	0.004164
1390459_at	BG670247			1.683832	3.212802	0.000503
1381577_at	AI170131			1.674706	3.192544	9.98E-05
1388720_at	BM390713			1.669188	3.180356	0.000841
1371394_x_at	BG664827			1.660846	3.162018	0.000385
1379719_at	AI408386			1.629839	3.094784	8.76E-06
1392876_at	BG375098			1.597297	3.025758	0.000425
1377881_at	AA997027			1.583295	2.996534	0.000249
1392924_at	BG371591			1.573984	2.977258	2.15E-05
1378152_at	AI170349			1.572157	2.973490	0.000818
1390987_at	AI406858			1.539611	2.907161	0.005124
1390300_at	BM383635			1.539317	2.906568	0.001988

1381996_at	BG666712	1.524793	2.877454	0.007289
1392893_a_at	AA926239	1.492644	2.814042	1.82E-05
1378462_at	BE107396	1.484439	2.798083	0.027693
1391028_at	AI511126	1.480597	2.790641	0.002716
1385978_at	AI072788	1.480070	2.789622	0.015570
1392813_at	AI548994	1.472777	2.775556	0.028907
1379903_at	AI059853	1.456459	2.744339	0.005294
1382291_at	AI454332	1.447251	2.726879	0.021889
1396539_at	BE119221	1.444627	2.721924	0.003250
1395381_at	BF542239	1.428428	2.691532	0.018331
1394709_at	AI406967	1.426754	2.688412	0.013879
1378780_at	BF410325	1.420940	2.677598	0.009832
1374172_at	AI010883	1.407256	2.652321	0.005074
1377551_at	BE118580	1.406921	2.651706	0.000613
1378172_at	AI008119	1.390207	2.621164	9.29E-05
1389744_at	AW527194	1.371756	2.587854	0.029149
1393728_at	AA964541	1.361978	2.570373	0.006640
1389284_at	BI275747	1.352525	2.553586	0.009308
1377309_at	AA963085	1.344539	2.539490	0.000217
1394012_at	BI303933	1.325072	2.505453	9.03E-06
1396886_at	BF387869	1.319644	2.496045	0.000671
1389172_at	AI179391	1.318725	2.494456	5.03E-06
1376011_at	AI411359	1.315430	2.488765	0.013276
1374171_at	AI170507	1.313956	2.486224	0.003317
1382802_x_at	AW920828	1.313169	2.484867	0.003200
1395211_s_at	BE118557	1.294366	2.452692	0.014857
1391727_at	BG662710	1.294339	2.452646	0.001351
1373079_at	BI296427	1.291969	2.448621	0.040034
1375005_at	BF403824	1.291727	2.448210	0.001959
1382174_at	AI227996	1.290673	2.446421	0.006458
1380088_at	AW533021	1.284168	2.435415	5.37E-05
1383910_at	BF398220	1.280890	2.429889	0.003117
1381498_at	AA956116	1.226183	2.339473	0.001176
1391936_a_at	BI289110	1.212511	2.317406	0.041362
1379089_at	BM382838	1.208370	2.310764	0.000681
1372993_at	BI299621	1.204492	2.304561	0.000924
1390671_at	AI044666	1.203424	2.302856	0.001722
1394916_at	AW526714	1.202724	2.301739	0.001978
1389397_at	AI234012	1.202426	2.301263	0.000195
1376637_at	AI102401	1.198002	2.294217	0.027098
1386552_at	BF284027	1.197145	2.292856	0.003348
1377792_at	AW524891	1.195075	2.289568	0.013872
1384952_at	AI028968	1.184453	2.272772	0.001744
1376768_at	BM386807	1.182682	2.269984	0.008644
1389127_at	BF552908	1.178770	2.263837	0.001451
1392140_at	BF419584	1.174126	2.256561	0.010974
1375707_at	AA817993	1.171668	2.252720	0.017511
1377994_at	AI501237	1.165986	2.243866	2.56E-05
1372027_at	AI009713	1.165036	2.242388	0.004861
1374290_at	AI408191	1.161834	2.237416	0.000514
1372820_at	BE109102	1.157167	2.230190	6.76E-05
1395629_at	BE105336	1.152754	2.223379	0.001122
1373628_at	AA818342	1.148702	2.217143	0.003268
1383697_at	AW530905	1.147530	2.215342	0.000199
1384269_at	BF386887	1.144996	2.211455	0.039498
1379733_at	BF396474	1.144326	2.210428	0.000479
1379682_at	BI281668	1.138058	2.200846	0.001290
1393334_at	AW528448	1.134957	2.196120	0.000111
1372583_at	AI009094	1.134834	2.195932	0.000776
1390193_at	BF389884	1.132371	2.192188	0.007448
1373114_at	AI408442	1.132006	2.191632	0.026188
1395586_at	BF545930	1.130756	2.189735	0.009671
1389085_at	BI296359	1.122011	2.176502	0.033565
1378898_at	BE109293	1.117104	2.169111	0.018502
1384433_at	AI072153	1.116005	2.167459	0.008374
1392778_at	AA891634	1.112387	2.162030	0.000734
1372515_at	BI281177	1.109794	2.158148	0.000373
1394727_at	AI407942	1.104267	2.149896	0.000884
1391617_at	AI171103	1.103278	2.148422	0.001730
1384680_at	AA924336	1.103174	2.148268	1.20E-05
1374246_at	BF402392	1.100807	2.144746	0.001207

1395350_at	AW919190			1.100062	2.143640	0.026720
1397343_at	BE113258			1.098815	2.141787	0.000702
1384051_at	BF390066			1.096884	2.138923	0.000370
1381654_at	BF398637			1.086982	2.124292	0.008757
1392619_at	BE118107			1.081379	2.116058	0.010153
1376747_at	BE107075			1.079554	2.113382	0.014697
1380763_at	AI101194			1.078476	2.111804	0.008553
1393653_at	BM384831			1.077241	2.109997	0.029779
1379844_at	AW531072			1.073984	2.105239	0.004020
1394948_at	BI303527			1.073384	2.104364	0.028955
1393911_at	AI502300			1.069942	2.099349	0.001054
1378006_at	AI233832			1.069145	2.098190	0.000230
1379451_at	AI549081			1.069122	2.098155	0.025226
1391643_at	BI290758			1.065858	2.093415	0.009922
1390136_at	BE109274			1.062274	2.088220	0.003245
1389256_at	BG381256			1.061409	2.086969	0.027360
1376465_at	BI295240			1.055842	2.078931	0.025628
1390515_at	AA998383			1.052460	2.074063	0.003395
1390205_at	BE108876			1.052183	2.073665	0.000145
1374728_at	BG671786			1.050605	2.071399	0.043671
1394883_at	AI179616			1.050405	2.071111	0.038398
1372104_at	BF289002			1.043745	2.061573	0.000585
1379252_at	AW522833			1.041529	2.058408	0.000451
1377151_at	AI102833			1.040228	2.056553	0.003640
1377880_at	AI170633			1.038506	2.054099	0.000230
1389908_at	BE107167			1.035223	2.049431	0.015670
1373082_at	AA893743			1.033904	2.047558	0.017691
1373537_at	BE113175			1.028307	2.039629	0.005797
1392578_at	AI070875			1.021081	2.029439	0.010470
1377705_at	BF549971			1.019922	2.027809	0.001107
1371854_at	BG374451			1.019759	2.027580	0.012677
1375647_at	BG671943			1.006159	2.008556	0.017266
1385903_at	AA859627			1.004338	2.006023	0.000426
1398265_at	NM_013040	Abcc9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	1.437830	2.709130	0.001785
1387287_a_at	D83598	Abcc9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	1.223092	2.334465	0.004574
1397375_at	BM384537	Acsl5	Acyl-CoA synthetase long-chain family member 5	1.153318	2.224249	0.015680
1386926_at	NM_053607	Acsl5	Acyl-CoA synthetase long-chain family member 5	1.053592	2.075692	0.015907
1370857_at	BI282702	Acta2	Smooth muscle α-actin	2.229514	4.689760	0.000129
1398294_at	NM_031005	Actn1	Actinin, α 1	1.131925	2.191509	0.001329
1368223_at	NM_024400	Adamts1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.048565	4.136942	0.028144
1376481_at	BF416285	Adamts9	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9	2.392634	5.251154	0.000223
1374535_at	BI283881	Afap1l2	Actin filament associated protein 1-like 2	2.203032	4.604460	0.000634
1368869_at	BG663107	Akap12	A kinase (PRKA) anchor protein 12	1.529713	2.887284	0.019479
1368868_at	NM_057103	Akap12	A kinase (PRKA) anchor protein 12	1.010475	2.014575	0.013205
1387493_at	NM_133515	Akap5	A kinase (PRKA) anchor protein 5	1.569515	2.968049	0.003304
1370043_at	NM_031753	Alcam	Activated leukocyte cell adhesion molecule	1.603863	3.039561	0.010096
1383469_at	BG377269	Aldh1a3	Aldehyde dehydrogenase 1 family, member A3	1.116847	2.168725	0.002910
1370638_at	AF069525	Ank3	Ankyrin 3, epithelial	1.818441	3.526998	0.008330
1367664_at	NM_013220	Ankrd1	Ankyrin repeat domain 1 (cardiac muscle)	1.668973	3.179881	0.020907
1367665_at	L81174	Ankrd1	Ankyrin repeat domain 1 (cardiac muscle)	1.503501	2.835300	0.035713
1372069_at	BF284716	Ankrd15	Ankyrin repeat domain 15	1.333618	2.520339	0.003331
1367974_at	NM_012823	Anxa3	Annexin A3	3.372176	10.35443	7.22E-05
1367975_at	BF283732	Anxa3	Annexin A3	1.914777	3.770554	0.000113
1395313_s_at	AI179982	Anxa3	Annexin A3	1.717191	3.287957	0.000365
1373654_at	BM389254	Anxa8	Annexin A8	1.142590	2.207770	0.000179
1392815_at	BE114489	Arap2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	1.501088	2.830562	0.000231
1387018_at	NM_053770	Argbp2	Arg/Abl-interacting protein ArgBP2	1.513378	2.854778	0.039786
1373315_at	AI176425	Arnt2	Aryl hydrocarbon receptor nuclear translocator 2	2.041125	4.115662	0.023949
1378134_at	BI291629	Atp8b1	ATPase, Class Ⅰ, type 8B, member 1	1.061725	2.087426	7.68E-05
1368485_at	NM_024401	Avil	Advillin	2.121568	4.351667	0.012237
1370823_at	AF387513	Bambi	BMP and activin membrane-bound inhibitor, homolog (Xenopus laevis)	1.526089	2.880041	0.003285
1372613_at	AI232784	Bdh2	3-hydroxybutyrate dehydrogenase, type 2	1.177434	2.261742	0.013257
1387232_at	NM_012827	Bmp4	Bone morphogenetic protein 4	3.074538	8.424189	0.000456
1380459_at	AI555023	Btbd14a	BTB (POZ) domain containing 14A	1.357670	2.562709	3.53E-06
1386995_at	BI288701	Btg2	B-cell translocation gene 2, anti-proliferative	1.228055	2.342509	0.004008
1377086_at	AI233530	C1qtnf3	C1q and tumor necrosis factor related protein 3	2.513168	5.708723	0.002654
1376657_at	BE117767	Cadm1	Cell adhesion molecule 1	1.040910	2.057525	0.001137
1393452_at	BM391835	Car9	Carbonic anhydrase 9	2.223630	4.670671	0.000110

1390101_at	AI170609	Ccdc107	Coiled-coil domain containing 107	1.152965	2.223704	0.000166
1398827_at	NM_013087	Cd81	Cd81 molecule	3.901095	14.93986	0.000330
1388936_at	BI296340	Cdh11	Cadherin 11	3.493679	11.26425	0.016743
1370371_a_at	U23056	Ceacam1 /// Ceacam10	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) /// carcinoembryonic antigen-related cell adhesion molecule 10	2.350728	5.100814	0.001261
1393142_at	BF562621	Cep70	Centrosomal protein 70 kDa	1.414308	2.665318	5.96E-05
1368675_at	NM_032084	Chn2	Chimerin (chimaerin) 2	1.111248	2.160324	0.00013
1389368_at	AW253242	Cnksr3	Cnksr family member 3	1.115693	2.166991	0.002136
1376868_at	BM389293	Cobll1	Cobl-like 1	2.807857	7.002435	0.002758
1372439_at	AI176393	Col4a1	Collagen, type Ⅳ, α 1	3.587058	12.01745	4.28E-05
1373245_at	BE111752	Col4a1	Collagen, type Ⅳ, α 1	3.148631	8.868134	9.98E-05
1388494_at	BI281705	Col4a2	Collagen, type Ⅳ, α 2	2.800595	6.967276	0.000478
1393891_at	BE128699	Col8a1	Collagen, type Ⅷ, α 1	1.151470	2.221401	0.003468
1367782_at	NM_012812	Cox6a2	Cytochrome c oxidase, subunit Ⅵa, polypeptide 2	2.245734	4.742785	0.001312
1386921_at	NM_013128	Cpe	Carboxypeptidase E	2.564823	5.916823	0.021482
1382037_at	AI600057	Crim1	Cysteine rich transmembrane BMP regulator 1 (chordin like)	2.041047	4.115440	0.002850
1391448_at	BI289620	Crim1	Cysteine rich transmembrane BMP regulator 1 (chordin like)	1.915600	3.772706	0.000387
1398622_at	AI703807	Crim1	Cysteine rich transmembrane BMP regulator 1 (chordin like)	1.808390	3.502512	0.000527
1376457_at	AI175861	Crispld2	Cysteine-rich secretory protein LCCL domain containing 2	1.377770	2.598664	0.018183
1387922_at	AF109674	Crispld2	Cysteine-rich secretory protein LCCL domain containing 2	1.195518	2.290270	0.003604
1368059_at	NM_053955	Crym	Crystallin, mu	1.288402	2.442574	0.000395
1383590_at	AA963863	Csgalnact1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	3.304007	9.876547	0.022018
1370057_at	NM_017148	Csrp1	Cysteine and glycine-rich protein 1	1.030407	2.042601	0.001628
1388583_at	BF283398	Cxcl12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	2.092111	4.263716	0.042845
1387655_at	AF189724	Cxcl12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	1.643136	3.123441	0.028532
1369633_at	AI171777	Cxcl12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	1.567476	2.963858	0.045339
1368290_at	NM_031327	Cyr61	Cysteine-rich, angiogenic inducer, 61	1.261651	2.397699	0.022554
1371436_at	AI176924	Ddah2	Dimethylarginine dimethylaminohydrolase 2	1.710173	3.272001	0.006371
1368013_at	NM_080399	Ddit4l	DNA-damage-inducible transcript 4-like	1.056235	2.079498	0.002531
1389894_at	BF399476	Dlc1	Deleted in liver cancer 1	1.068336	2.097013	0.002194
1377835_at	BM390876	Dock8	Dedicator of cytokinesis 8	2.230319	4.692377	0.005526
1388506_at	AW144509	Dsp	Desmoplakin	1.830721	3.557148	9.48E-05
1368146_at	U02553	Dusp1	Dual specificity phosphatase 1	1.560299	2.949149	0.003438
1368949_at	NM_053820	Ebf1	Early B-cell factor 1	1.316466	2.490553	0.000315
1369519_at	NM_012548	Edn1	Endothelin 1	1.366023	2.577591	0.009786
1368541_at	NM_053719	Emb	Embigin	1.064032	2.090767	0.031817
1377752_at	BE112998	Emp2	Epithelial membrane protein 2	1.392599	2.625512	0.000189
1373617_at	AA818807	Emp2	Epithelial membrane protein 2	1.347238	2.544246	0.000131
1377311_at	AI045616	Emx2	Empty spiracles homeobox 2	1.032095	2.044992	0.001019
1369096_at	NM_134331	Epha7	Eph receptor A7	1.427798	2.690358	0.001357
1385788_at	AW534949	Ephb3	Eph receptor B3	1.321665	2.499544	0.000783
1369182_at	NM_013057	F3	Coagulation factor Ⅲ (thromboplastin, tissue factor)	2.945169	7.701657	8.99E-05
1377940_at	BF398271	Fam101b	Family with sequence similarity 101, member B	1.069187	2.098250	0.047471
1384507_at	AA817708	Fam105a	Family with sequence similarity 105, member A	1.169405	2.249190	0.005950
1389146_at	BF283267	Fam107b	Family with sequence similarity 107, member B	1.016680	2.023257	0.000409
1393910_at	BF563961	Fam13a1	Family with sequence similarity 13, member A1	4.490284	22.475530	0.003831
1379625_at	BG664461	Fam164a	Family with sequence similarity 164, member A	1.550421	2.929026	0.001732
1384648_at	AA963844	Fam164a	Family with sequence similarity 164, member A	1.219281	2.328307	0.000456
1391944_at	BI296237	Fam184a /// RGD1560557	Family with sequence similarity 184, member A /// similar to minichromosome maintenance protein 8 isoform 1	1.268560	2.409209	0.001168
1373286_at	AA875261	Fblim1	Filamin binding LIM protein 1	1.055977	2.079126	0.000141
1376500_at	AI639044	Fbxo23	F-box only protein 23	1.064218	2.091036	0.001619
1386614_at	BG671466	Fbxo23	F-box only protein 23	1.016912	2.023583	0.010871
1368114_at	NM_053428	Fgf13	Fibroblast growth factor 13	3.654623	12.593640	0.004204
1370106_at	NM_019199	Fgf18	Fibroblast growth factor 18	2.489420	5.615522	0.016971
1369313_at	NM_031677	Fhl2	Four and a half LIM domains 2	1.491713	2.812227	0.011052
1371951_at	AA800031	Fhl2	Four and a half LIM domains 2	1.319164	2.495215	0.010936
1372825_at	BI290551	Fnbp1	Formin binding protein 1	1.481808	2.792985	0.003539
1376784_at	BI274481	Fnbp1	Formin binding protein 1	1.407572	2.652903	0.007946
1369471_at	NM_138914	Fnbp1	Formin binding protein 1	1.142482	2.207604	0.012674
1377342_s_at	BE105446	Fnbp1	Formin binding protein 1	1.043905	2.061801	0.023433
1370829_at	M69056	Fntb	Farnesyltransferase, CAAX box, β	2.001250	4.003468	0.001272
1368711_at	NM_012743	Foxa2	Forkhead box A2	3.125513	8.727165	0.004980
1380387_at	BE105492	Foxp2	Forkhead box P2	1.808179	3.501999	0.000372
1383721_at	AI556075	Fzd8	Frizzled homolog 8 (Drosophila)	1.891668	3.710641	8.54E-05
1372016_at	BI287978	Gadd45b	Growth arrest and DNA-damage-inducible, β	1.287102	2.440374	0.007019
1369735_at	NM_057100	Gas6	Growth arrest specific 6	2.030906	4.086614	0.000694

1367627_at	NM_031031	Gatm	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	1.704989	3.260264	0.001931
1390557_at	BF394809	Gca	Grancalcin	1.294802	2.453433	0.015190
1379031_at	BM390697	Gca	Grancalcin	1.132824	2.192876	0.016810
1374903_at	AI234819	Gcnt2	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	1.780391	3.435192	0.000760
1370375_at	J05499	Gls2	Glutaminase 2 (liver, mitochondrial)	1.381886	2.606088	0.000222
1392888_at	AI071251	Gpc4	Glypican 4	1.149833	2.218883	0.017197
1373773_at	BF394166	Gpm6a	Glycoprotein m6a	1.492990	2.814717	0.004487
1370389_at	AB036421	Gpm6b	Glycoprotein m6b	1.729687	3.316558	0.041126
1382955_at	BI284296	Gpr126	G protein-coupled receptor 126	1.691914	3.230851	0.002139
1373693_at	BF414143	Gprc5c	G protein-coupled receptor, family C, group 5, member C	1.172596	2.254169	0.000245
1368618_at	NM_031623	Grb14	Growth factor receptor bound protein 14	2.263062	4.800093	0.001165
1368401_at	M85035	Gria2	Glutamate receptor, ionotropic, AMPA 2	1.185577	2.274543	0.038866
1383897_at	BE117477	H2afy2	H2A histone family, member Y2	1.467448	2.765322	0.003118
1384541_at	BM391441	Hapln1	Hyaluronan and proteoglycan link protein 1	2.720224	6.589753	0.021358
1370125_at	NM_019189	Hapln1	Hyaluronan and proteoglycan link protein 1	2.532368	5.785206	0.010848
1368983_at	NM_012945	Hbegf	Heparin-binding EGF-like growth factor	1.057962	2.081988	0.018982
1376867_at	BE095833	Hspc159	Galectin-related protein	2.821242	7.067707	0.000688
1373515_at	BI275737	Hspc159	Galectin-related protein	2.640040	6.233487	0.000522
1387028_a_at	M86708	Id1	Inhibitor of DNA binding 1	1.097822	2.140313	0.005605
1390507_at	BI296097	Isg20	Interferon stimulated exonuclease 20	2.272905	4.832953	0.040035
1394824_at	BF398348	Itga11	Integrin, α 11	1.885728	3.695395	0.000279
1393558_at	AI137931	Itga6	Integrin, α 6	1.341112	2.533464	0.001146
1382439_at	AI070686	Itgb6	Integrin, β 6	1.316970	2.491424	0.005869
1387907_at	J05510	Itpr1	Inositol 1,4,5-triphosphate receptor, type 1	1.709204	3.269803	0.001386
1368725_at	NM_019147	Jag1	Jagged 1	1.045878	2.064622	0.010981
1398124_at	AI071356	Jazf1	JAZF zinc finger 1	1.325135	2.505564	1.36E-05
1396701_at	BE110052	Kalrn	Kalirin, RhoGEF kinase	1.050512	2.071265	0.000530
1369144_a_at	NM_031739	Kcnd3	Potassium voltage gated channel, Shal-related family, member 3	1.978834	3.941744	0.019750
1394039_at	BM382886	Klf5	Kruppel-like factor 5	1.168271	2.247422	0.001471
1368363_at	NM_053394	Klf5	Kruppel-like factor 5	1.107290	2.154406	0.000169
1388932_at	BI274917	Lama5	Laminin, α 5	1.499220	2.826898	0.008496
1367880_at	NM_012974	Lamb2	Laminin, β 2	1.055487	2.078420	0.000777
1370993_at	AA997129	Lamc1	Laminin, γ 1	1.144416	2.210566	0.001299
1388422_at	BI275904	Lims2	LIM and senescent cell antigen like domains 2	3.226389	9.359224	0.000228
1376632_at	AI602501	Lmcd1	LIM and cysteine-rich domains 1	3.919424	15.130880	0.000893
1381798_at	BE114958	Lmo7	LIM domain 7	1.648918	3.135983	0.000383
1375726_at	BI284480	Lmo7	LIM domain 7	1.223048	2.334393	0.000945
1381190_at	AI598833	Lmo7	LIM domain 7	1.051248	2.072321	0.001218
1375523_at	BE108178	LOC294446	Similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (ACAMP-81)	1.286331	2.439070	0.000713
1370948_a_at	M59859	LOC294446 /// LOC681252 /// Marcks	Similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (ACAMP-81) /// similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (Protein kinase C substrate 80 kDa protein) /// myristoylated alanine rich protein kinase C substrate	1.113014	2.162971	3.48E-05
1370949_at	M59859	LOC294446 /// LOC681252 /// Marcks	Similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (ACAMP-81) /// similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (Protein kinase C substrate 80 kDa protein) /// myristoylated alanine rich protein kinase C substrate	1.078213	2.111419	5.02E-05
1381434_s_at	AW253721	LOC302022	Similar to nidogen 2 protein	1.462223	2.755326	0.002301
1373232_at	AI008975	LOC302022	Similar to nidogen 2 protein	1.153904	2.225152	0.007223
1390158_at	BI290752	LOC304903	Similar to Pappalysin-2 precursor (Pregnancy-associated plasma protein-A2) (PAPP-A2) (Pregnancy-associated plasma protein-E1) (PAPP-E)	1.089462	2.127947	0.013140
1384907_at	AI411835	LOC306096	Similar to Dachshund homolog 1 (Dach1)	2.365997	5.155086	0.015526
1383888_at	AA998264	LOC307495	Similar to biliverdin reductase B (flavin reductase (NADPH))	1.345301	2.540832	6.23E-05
1379465_at	AW527596	LOC311134	Hypothetical LOC311134	1.691930	3.230885	0.004884
1392074_at	AA926082	LOC500046	Similar to hypothetical protein FLJ21986	1.886585	3.697589	0.042067
1392592_at	AI137045	LOC679869	Similar to transcription factor 7-like 2, T-cell specific, HMG-box	1.098548	2.141391	0.000254
1394497_at	AI535239	LOC679869 /// LOC683733	Similar to transcription factor 7-like 2, T-cell specific, HMG-box /// similar to Transcription factor 7-like 2 (HMG box transcription factor 4) (T-cell-specific transcription factor 4) (TCF-4) (hTCF-4)	1.055505	2.078445	0.000768
1373088_at	BI295811	LOC682888	Hypothetical protein LOC682888	1.243831	2.368265	1.11E-06
1388447_at	AA800701	LOC683626	Similar to limb-bud and heart	1.193694	2.287377	0.007020
1379815_at	AI713959	LOC683733	Similar to Transcription factor 7-like 2 (HMG box transcription factor 4) (T-cell-specific transcription factor 4) (TCF-4) (hTCF-4)	1.406898	2.651664	1.15E-05

1377156_at	BI273936	LOC683733	Similar to Transcription factor 7-like 2 (HMG box transcription factor 4) (T-cell-specific transcription factor 4) (TCF-4) (hTCF-4)	1.332040	2.517583	0.001391
1383488_at	AA817785	LOC687536	Similar to Forkhead box protein F1 (Forkhead-related protein FKHL5) (Forkhead-related transcription factor 1) (FREAC-1) (Hepatocyte nuclear factor 3 forkhead homolog 8) (HFH-8)	1.534125	2.896127	0.003143
1386120_at	BF393607	LOC689147	Hypothetical protein LOC689147	1.753785	3.372422	0.003982
1393414_at	AW142650	LOC689176	Similar to transmembrane protein 64	1.201660	2.300042	0.047653
1376691_at	AI103213	LOC689176	Similar to transmembrane protein 64	1.150453	2.219836	0.044249
1374016_at	AI502597	Lpar1	Lysophosphatidic acid receptor 1	1.299750	2.461863	0.003803
1370048_at	NM_053936	Lpar1	Lysophosphatidic acid receptor 1	1.268034	2.408331	0.001243
1389913_at	BI276990	Lrrfip1	Leucine rich repeat (in FLII) interacting protein 1	1.260408	2.395635	0.000794
1368448_at	NM_021586	Ltbp2	Latent transforming growth factor β binding protein 2	2.071905	4.204415	0.002220
1367768_at	NM_031655	Lxn	Latexin	1.381643	2.605649	0.000525
1374933_at	BI277043	Mcam	Melanoma cell adhesion molecule	1.008050	2.011191	0.034411
1369218_at	NM_031517	Met	Met proto-oncogene	1.253867	2.384798	0.000472
1384617_at	AI385260	MGC72614	Hypothetical LOC310540	1.318517	2.494096	0.001888
1398387_at	AI009530	MGC72614	Hypothetical LOC310540	1.263746	2.401184	0.001149
1367568_a_at	NM_012862	Mgp	Matrix Gla protein	3.353436	10.220800	0.010514
1384150_at	AA901038	Mid1	Midline 1	1.062584	2.088669	0.000603
1370072_at	NM_012608	Mme	Membrane metallo endopeptidase	3.646308	12.521260	0.003799
1372457_at	BF284182	Mtus1	Mitochondrial tumor suppressor 1	1.629140	3.093285	5.84E-06
1380321_at	BI287786	Mtus1	Mitochondrial tumor suppressor 1	1.402213	2.643066	0.000159
1378970_at	AW252385	Mybphl	Myosin binding protein H-like	1.080283	2.114451	0.043933
1370158_at	AA946388	Myh10	Myosin, heavy chain 10, non-muscle	1.192392	2.285314	0.005978
1388298_at	BI279044	Myl9	Myosin, light chain 9, regulatory	1.368369	2.581786	0.025217
1389507_at	AI072446	Nedd4l	Neural precursor cell expressed, developmentally down-regulated 4-like	1.010244	2.014252	0.002318
1369679_a_at	AB060652	Nfia	Nuclear factor I/A	1.018188	2.025374	0.008943
1388618_at	BM389302	Nid2	Nidogen 2	1.740644	3.341843	0.002285
1368883_at	NM_030868	Nov	Nephroblastoma overexpressed gene	1.914944	3.770993	0.010007
1371412_a_at	BE107450	Nrep	Neuronal regeneration related protein	1.740346	3.341152	0.003311
1369783_a_at	U02319	Nrg1	Neuregulin 1	1.341273	2.533748	0.000850
1370607_a_at	U02323	Nrg1	Neuregulin 1	1.322000	2.500124	0.000298
1371211_a_at	U02315	Nrg1	Neuregulin 1	1.304653	2.470242	0.000467
1382814_at	AW521702	Odz3	Odz, odd Oz/ten-m homolog 3 (Drosophila)	2.654920	6.298115	0.004174
1377702_at	BG380173	P2ry5	Purinergic receptor P2Y, G-protein coupled, 5	1.073159	2.104036	0.000211
1367687_a_at	M25719	Pam	Peptidylglycine α-amidating monooxygenase	1.192716	2.285826	0.021615
1398487_at	BF419639	Pbx1	Pre-B-cell leukemia homeobox 1	1.092533	2.132481	0.006868
1393966_at	AW530825	Pbx1	Pre-B-cell leukemia homeobox 1	1.018972	2.026474	0.000957
1370490_at	L43592	Pcdhb12	Protocadherin β 12	1.403580	2.645572	0.026763
1377042_at	BI288196	Pcgf5	Polycomb group ring finger 5	1.189904	2.281376	0.002754
1392773_at	AA859578	Pcsk5	Proprotein convertase subtilisin/kexin type 5	1.589348	3.009134	0.002877
1393467_at	BF549923	Pcsk5	Proprotein convertase subtilisin/kexin type 5	1.332597	2.518556	0.000461
1387812_at	NM_012999	Pcsk6	Proprotein convertase subtilisin/kexin type 6	3.748654	13.441790	0.001431
1382345_at	AA955299	Pctk2	PCTAIRE protein kinase 2	1.020874	2.029147	5.29E-05
1374157_at	AA858930	Pde4b	Phosphodiesterase 4B, cAMP specific	2.492088	5.625918	0.004862
1369044_a_at	AF202733	Pde4b	Phosphodiesterase 4B, cAMP specific	1.307088	2.474415	0.025797
1374616_at	BM384311	Pdgfrl	Platelet-derived growth factor receptor-like	1.872122	3.660707	1.96E-06
1368703_at	NM_053326	Pdlim5	PDZ and LIM domain 5	1.360465	2.567680	0.000723
1386913_at	NM_019358	Pdpn	Podoplanin	2.706385	6.526842	0.015510
1374969_at	AA799832	Pgm5	Phosphoglucomutase 5	1.912300	3.764087	4.83E-05
1368860_at	NM_017180	Phlda1	Pleckstrin homology-like domain, family A, member 1	1.236279	2.355901	0.031298
1378106_at	AI029402	Phlda2	Pleckstrin homology-like domain, family A, member 2	1.063574	2.090104	0.007195
1388539_at	BE113268	Pkp2	Plakophilin 2	1.216333	2.323553	0.004529
1382659_at	BF289229	Pla2r1	Phospholipase A2 receptor 1	1.819950	3.530690	5.56E-05
1387122_at	NM_012760	Plagl1	Pleiomorphic adenoma gene-like 1	6.377022	83.114140	0.001611
1386962_at	NM_024353	Plcb4	Phospholipase C, β 4	1.727990	3.312660	0.008873
1370489_a_at	U57836	Plcb4	Phospholipase C, β 4	1.479277	2.788090	0.007828
1369029_at	NM_057194	Plscr1	Phospholipid scramblase 1	1.321345	2.498990	0.002555
1370247_a_at	AA943163	Pmp22	Peripheral myelin protein 22	1.312653	2.483979	0.000418
1372531_at	BE106488	Ppfibp2	PTPRF interacting protein, binding protein 2 (liprin β 2)	1.798887	3.479517	0.023491
1393082_at	AI044747	Ppp1r14c	Protein phosphatase 1, regulatory (inhibitor) subunit 14c	1.263639	2.401006	0.009828
1368716_at	NM_133425	Ppp1r14c	Protein phosphatase 1, regulatory (inhibitor) subunit 14c	1.085918	2.122725	0.010686
1370012_at	NM_031557	Ptgis	Prostaglandin I2 (prostacyclin) synthase	1.878328	3.676486	0.007631
1368527_at	U03389	Ptgs2	Prostaglandin-endoperoxide synthase 2	1.723796	3.303044	0.037120
1377427_at	BE104739	Ptpn14	Protein tyrosine phosphatase, non-receptor type 14	1.140142	2.204028	1.10E-05
1374774_at	BF552241	Ptpn14	Protein tyrosine phosphatase, non-receptor type 14	1.065112	2.092332	3.73E-05
1368035_a_at	X83505	Ptprf	Protein tyrosine phosphatase, receptor type, F	2.196463	4.583543	0.005199

1384227_at	AI044031	Ptprk	Protein tyrosine phosphatase, receptor type, K, extracellular region	2.172519	4.508097	4.14E-05
1390034_at	BF393945	Ralgps2	Ral GEF with PH domain and SH3 binding motif 2	1.192882	2.286089	0.025134
1367791_at	NM_031645	Ramp1	Receptor (G protein-coupled) activity modifying protein 1	1.907320	3.751116	0.017043
1368660_at	NM_021690	Rapgef3	Rap guanine nucleotide exchange factor (GEF) 3	1.018953	2.026448	0.003623
1390159_at	AA819332	Rasgrp3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	1.433832	2.701633	0.016442
1383322_at	BG375198	Rasl11b	RAS-like family 11 member B	1.757780	3.381775	0.008884
1387581_at	NM_022959	Rassf9	Ras association (RalGDS/AF-6) domain family (N-terminal) member 9	2.580119	5.979890	2.33E-07
1383247_a_at	BI291029	rCG_35099	Spinster homolog 2	1.270831	2.413004	0.006005
1388791_at	BI275911	RGD1309930	Similar to 2810022L02Rik protein	1.327569	2.509794	0.016780
1395336_at	BE098691	RGD1309930	Similar to 2810022L02Rik protein	1.309761	2.479005	0.004198
1374898_at	AW527473	RGD1311422	Similar to CG8841-PA	1.039847	2.056009	0.002294
1373584_at	BE113205	RGD1559643	Similar to hypothetical protein A430031N04	1.019520	2.027245	0.000710
1372380_at	AI231308	RGD1561067	Similar to RNA binding protein gene with multiple splicing	3.350875	10.202670	0.002486
1375898_at	AW252379	RGD1561067	Similar to RNA binding protein gene with multiple splicing	3.091972	8.526606	0.004328
1376619_at	AI412803	RGD1561090	Similar to protein tyrosine phosphatase, receptor type, D	2.033850	4.094962	1.02E-06
1374591_at	AI409042	RGD1561090	Similar to protein tyrosine phosphatase, receptor type, D	1.973152	3.926251	2.32E-05
1376919_at	BG665267	RGD1562317	Similar to expressed sequence AW212394	1.376378	2.596157	0.000204
1388879_at	BG669292	RGD1562717	Similar to ABI gene family, member 3 (NESH) binding protein	1.796298	3.473277	0.026049
1388906_at	BM389311	RGD1564174	Similar to novel protein similar to Tensin Tns	1.157511	2.230722	4.32E-05
1383398_at	AI059150	RGD1564327	Similar to integrin α 8	1.649388	3.137005	0.000121
1385354_at	BE120766	RGD1564327	Similar to integrin α 8	1.472111	2.774276	9.05E-07
1376546_at	BE120498	RGD1565432	Similar to hypothetical protein	1.921295	3.787629	0.009661
1396347_at	BF395640	RGD1565926	RGD1565926	1.020952	2.029258	0.010695
1371731_at	AI408151	RGD1566215	Similar to Coatomer γ-2 subunit (γ-2 coat protein) (γ-2 COP)	1.928401	3.806332	5.12E-05
1380425_at	AI012859	Rnasel	Ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)	1.671401	3.185237	0.002887
1377116_at	BI301478	Rnasel	Ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)	1.626917	3.088523	0.001458
1381533_at	AI144754	Rnd1	Rho family GTPase 1	1.196579	2.291955	0.000495
1379693_at	AI409154	Robo2	Roundabout, axon guidance receptor, homolog 2 (Drosophila)	1.245877	2.371626	0.001543
1390632_at	BE107414	Rspo3	R-spondin 3 homolog (Xenopus laevis)	1.049128	2.069279	0.005955
1388356_at	AI406499	S100a16	S100 calcium binding protein A16	1.404636	2.647510	0.004150
1368379_at	NM_054001	Scarb2	Scavenger receptor class B, member 2	2.124624	4.360893	0.001346
1393338_at	AW528719	Scx	Scleraxis	1.644999	3.127476	0.007845
1368394_at	AF140346	Sfrp4	Secreted frizzled-related protein 4	1.610004	3.052527	0.003747
1367802_at	NM_019232	Sgk1	Serum/glucocorticoid regulated kinase 1	1.265947	2.404849	0.007546
1389779_at	AA800626	Sh2d4a	SH2 domain containing 4A	1.417920	2.672000	0.032404
1392301_at	AI237897	Sh3tc1	SH3 domain and tetratricopeptide repeats 1	1.214738	2.320987	0.006644
1392556_at	BF410961	Shroom3	Shroom family member 3	1.454496	2.740607	9.57E-05
1376040_at	BI290044	Sipa1l2	Signal-induced proliferation-associated 1 like 2	1.185619	2.274609	0.012637
1368565_at	NM_019225	Slc1a3	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	1.926317	3.800836	0.003174
1376165_at	BE098153	Slc24a3	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	1.145511	2.212244	0.000291
1398295_at	NM_031684	Slc29a1	Solute carrier family 29 (nucleoside transporters), member 1	1.222426	2.333387	0.003869
1369074_at	NM_130748	Slc38a4	Solute carrier family 38, member 4	2.257810	4.782650	0.000540
1392349_at	BE116021	Slc5a3	Solute carrier family 5 (inositol transporters), member 3	1.031672	2.044392	0.000967
1387968_at	L22022	Slc6a15	Solute carrier family 6 (neutral amino acid transporter), member 15	2.581534	5.985759	0.018733
1368920_at	NM_031321	Slit3	Slit homolog 3 (Drosophila)	1.652522	3.143828	0.030840
1384437_at	AI576309	Smarca1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	1.832940	3.562623	4.14E-05
1377695_at	BF281135	Smtnl2	Smoothelin-like 2	2.018633	4.051996	0.047065
1375349_at	BI295776	Sorbs1	Sorbin and SH3 domain containing 1	2.508532	5.690409	0.000169
1372728_at	BE103745	Sort1	Sortilin 1	1.319517	2.495825	0.006034
1371004_at	AI070124	Sort1	Sortilin 1	1.117923	2.170343	0.001420
1379611_at	BF416979	Spsb1	splA/ryanodine receptor domain and SOCS box containing 1	1.025012	2.034976	0.009283
1373554_at	BE349698	Spsb1	splA/ryanodine receptor domain and SOCS box containing 1	1.010972	2.015269	0.008275
1389142_at	AI013361	Sqrdl	sulfide quinone reductase-like (yeast)	2.278473	4.851642	0.001121
1368109_at	NM_031337	St3gal5	ST3 β-galactoside α-2,3-sialyltransferase 5	1.683094	3.211158	5.13E-05
1370907_at	M83143	St6gal1	ST6 β-galactosamide α-2,6-sialyltranferase 1	1.294325	2.452622	0.000575
1389420_at	BI279446	Stap2	Signal transducing adaptor family member 2	1.208781	2.311423	0.019358
1370680_at	AF483620	Stau2	Staufen, RNA binding protein, homolog 2 (Drosophila)	1.015615	2.021764	0.000276
1372602_at	BI295979	Stbd1	Starch binding domain 1	2.761499	6.781006	2.53E-05
1373590_at	BI295949	Stom	Stomatin	1.267382	2.407243	0.003323
1379732_at	AW920037	Stx11	Syntaxin 11	1.976724	3.935983	9.77E-05
1368771_at	NM_134378	Sulf1	Sulfatase 1	2.238326	4.718492	0.000659

1376572_a_at	AI045848	Svil	Supervillin	1.320708	2.497887	0.019303
1367570_at	NM_031549	Tagln	Transgelin	1.696733	3.241660	0.002280
1367859_at	NM_013174	Tgfb3	Transforming growth factor, β 3	1.509832	2.847768	0.003273
1375951_at	AA818521	Thbd	Thrombomodulin	1.311404	2.481829	0.020049
1370474_at	J03819	Thrb	Thyroid hormone receptor β	2.219205	4.656368	0.002376
1387983_at	J03933	Thrb	Thyroid hormone receptor β	1.184729	2.273206	0.003624
1383623_at	BM383909	Thyn1	Thymocyte nuclear protein 1	1.043826	2.061688	0.002044
1375138_at	AA893169	Timp3	TIMP metallopeptidase inhibitor 3	6.264691	76.888260	0.015514
1389836_a_at	AI599265	Timp3	TIMP metallopeptidase inhibitor 3	5.105264	34.422120	0.013029
1372926_at	AI009159	Timp3	TIMP metallopeptidase inhibitor 3	4.004944	16.054920	0.025103
1368989_at	NM_012886	Timp3	TIMP metallopeptidase inhibitor 3	2.253301	4.767725	0.015049
1373847_at	AW435343	Tm4sf1	Transmembrane 4 L six family member 1	2.114633	4.330798	0.000242
1378305_at	AI578087	Tm4sf1	Transmembrane 4 L six family member 1	1.938512	3.833100	0.000317
1390832_at	BI294696	Tmcc3	Transmembrane and coiled-coil domain family 3	2.119391	4.345106	0.005139
1376623_at	AI409186	Tmem204	Transmembrane protein 204	1.085094	2.121514	7.30E-05
1383314_at	BE110098	Tmem51	Transmembrane protein 51	1.344562	2.539531	3.99E-05
1371361_at	BI278826	Tns1	Tensin 1	1.158595	2.232399	0.000276
1370288_a_at	AF372216	Tpm1	Tropomyosin 1, α	2.037181	4.104428	0.000937
1395794_at	BF395218	Tpm1	Tropomyosin 1, α	1.997138	3.992073	0.008961
1371241_x_at	AF370889	Tpm1	Tropomyosin 1, α	1.663566	3.167985	0.013541
1370287_a_at	M23764	Tpm1	Tropomyosin 1, α	1.590740	3.012039	5.95E-05
1368724_a_at	NM_019131	Tpm1	Tropomyosin 1, α	1.054891	2.077561	0.018900
1372219_at	AA012755	Tpm2	Tropomyosin 2	1.064563	2.091537	2.16E-05
1398759_at	NM_013043	Tsc22d1	TSC22 domain family, member 1	1.051368	2.072494	0.000844
1377630_at	AI408602	Tspan13	Tetraspanin 13	1.481687	2.792752	0.019678
1375057_at	BG377313	Tspan18	Tetraspanin 18	1.454071	2.739802	0.030870
1398476_at	AW527349	Vcl	Vinculin	1.244474	2.369321	0.000134
1372905_at	AW433888	Vcl	Vinculin	1.136321	2.198197	3.11E-05
1369098_at	NM_013155	Vldlr	Very low density lipoprotein receptor	1.505396	2.839027	0.001107
1387455_a_at	NM_013155	Vldlr	Very low density lipoprotein receptor	1.471028	2.772194	0.001621
1389611_at	AA849857	Vldlr	Very low density lipoprotein receptor	1.435945	2.705593	0.000947
1368854_at	AI227991	Vsnl1	Visinin-like 1	2.472695	5.550796	0.007104
1368853_at	NM_012686	Vsnl1	Visinin-like 1	2.077806	4.221647	0.004903
1387873_at	BI279661	Wfdc1	WAP four-disulfide core domain 1	1.449292	2.730739	0.003744
1370221_at	BF419320	Wisp1	WNT1 inducible signaling pathway protein 1	1.058567	2.082861	0.000154
1393613_at	BE117871	Zfp462	Zinc finger protein 462	1.088387	2.126361	0.004351
1383462_at	BF566263	Znf294	Zinc finger protein 294	1.010246	2.014255	0.000352

Supplementary Table 2 Downregulated genes in miR-146a-transfected hepatic stellate cell-2

Probe ID	Representative public ID	Gene symbol	Gene title	Log2	Fold change	*P*-value
1374345_at	AI111707			-3.40046	-10.55940	1.80E-07
1397317_at	BI296984			-3.25476	-9.54513	3.34E-07
1397400_at	BM391846			-3.11991	-8.69335	5.36E-05
1382027_at	BI296880			-3.05526	-8.31237	3.85E-07
1380245_at	AI411847			-2.90249	-7.47715	8.46E-07
1381129_at	BF392367			-2.52142	-5.74147	0.018162
1383211_at	BE109736			-2.46677	-5.52805	0.006284
1380057_at	BE097091			-2.33498	-5.04543	0.000685
1381048_at	BF398435			-2.31590	-4.97916	0.000443
1381064_at	AI137604			-2.26573	-4.80896	1.32E-07
1373583_at	BF396317			-2.26180	-4.79589	1.23E-07
1377240_at	AW526305			-2.19645	-4.58351	0.001977
1394468_at	BF287020			-2.12335	-4.35705	0.000157
1377678_at	BI283757			-2.12273	-4.35519	0.000117
1375101_at	BI292651			-2.05823	-4.16476	0.000442
1380712_at	AI406475			-2.04987	-4.14068	0.006175
1372110_at	BE113148			-1.95331	-3.87261	0.000463
1394517_at	AW522148			-1.88952	-3.70513	0.006671
1390530_at	AI169239			-1.87482	-3.66755	0.001510
1376463_at	AA955579			-1.87022	-3.65587	0.001028
1384743_at	BF418132			-1.86231	-3.63589	2.42E-14
1377114_at	AI410861			-1.83910	-3.57788	8.88E-06
1377161_at	BG378317			-1.83484	-3.56731	0.039081
1383220_at	BE114231			-1.73514	-3.32911	0.000179
1393143_at	AI045866			-1.69968	-3.24828	0.005065

1376324_at	BF406329			-1.68113	-3.20679	0.004765
1390429_at	BF398114			-1.62771	-3.09021	6.83E-05
1382431_at	AI103530			-1.61690	-3.06716	1.15E-06
1391251_at	BI290666			-1.60415	-3.04016	1.73E-05
1383580_at	AA859643			-1.58751	-3.00530	0.000305
1372011_at	BI292028			-1.57490	-2.97915	0.000487
1377232_at	BF406608			-1.54704	-2.92217	0.003334
1398457_at	AI146156			-1.50417	-2.83662	0.005925
1381161_a_at	BI301117			-1.47549	-2.78078	0.008417
1382142_at	AI029975			-1.45644	-2.74431	3.98E-05
1382472_at	AI502459			-1.44328	-2.71939	8.76E-05
1375266_at	BG380633			-1.43613	-2.70595	0.010349
1381862_at	AW524296			-1.39645	-2.63253	0.004828
1393235_at	AI059968			-1.39173	-2.62392	0.000172
1385181_at	AI029337			-1.39139	-2.62332	1.30E-07
1377686_at	AA859337			-1.37593	-2.59535	3.53E-07
1372449_at	AW253616			-1.37158	-2.58753	0.009120
1377556_at	AW535380			-1.36924	-2.58334	0.004323
1372637_at	AI169241			-1.36639	-2.57825	0.000419
1375957_at	AW434654			-1.30351	-2.46828	4.46E-06
1383936_at	BM386842			-1.29935	-2.46118	1.09E-05
1384724_at	AA850766			-1.28208	-2.43190	3.20E-05
1379510_at	BF546306			-1.20859	-2.31112	9.04E-05
1382296_at	BF291041			-1.20836	-2.31075	1.53E-05
1382544_at	AI058746			-1.20774	-2.30975	3.79E-06
1376816_at	BF284903			-1.19854	-2.29507	0.000155
1383019_at	BF558478			-1.18776	-2.27798	0.000454
1374558_at	AI010316			-1.17751	-2.26186	8.04E-06
1389250_at	AW915115			-1.16975	-2.24972	9.96E-05
1377328_at	BI290012			-1.16378	-2.24044	0.009615
1380602_at	AI764190			-1.16264	-2.23867	0.001006
1384812_at	AI229409			-1.15815	-2.23172	0.006986
1396217_at	BF542447			-1.13720	-2.19954	0.000345
1397668_at	H34328			-1.13718	-2.19950	3.26E-07
1374932_at	BI282731			-1.13055	-2.18943	0.001048
1385381_at	AA996491			-1.12644	-2.18320	0.000161
1394047_at	BE107848			-1.10947	-2.15767	0.035398
1397452_at	AI112776			-1.10848	-2.15618	0.000172
1393730_at	BI277836			-1.10819	-2.15575	0.033820
1381975_at	BG371767			-1.10049	-2.14427	0.001469
1380699_at	BE110761			-1.09332	-2.13364	0.000497
1374920_at	AI228955			-1.07203	-2.10239	2.65E-05
1375473_at	BI296644			-1.05345	-2.07548	0.001418
1373914_at	BM389075			-1.05092	-2.07186	0.001887
1383436_at	BG376768			-1.04917	-2.06933	0.000656
1390405_at	AA942765			-1.04540	-2.06394	0.003275
1376911_at	BM386385			-1.04165	-2.05858	0.000165
1384183_at	AA996869			-1.03133	-2.04391	0.004095
1377469_at	AI103161			-1.02160	-2.03016	0.006072
1393030_at	BE115641			-1.01825	-2.02547	0.000652
1377113_at	BF415786			-1.01680	-2.02343	0.002724
1382960_at	BE108047			-1.01496	-2.02084	9.24E-05
1377955_at	AI137602			-1.01226	-2.01707	4.55E-07
1391853_at	AA998997			-1.00175	-2.00243	0.000388
1384086_at	BG671196			-1.00024	-2.00033	0.031992
1385235_at	AA818804	A2bp1	Ataxin 2 binding protein 1	-1.24533	-2.37073	4.71E-05
1383130_at	BF555795	A2bp1	Ataxin 2 binding protein 1	-1.07712	-2.10983	0.000947
1394490_at	AI502114	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-1.80354	-3.49076	3.72E-07
1384381_at	BF284523	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-1.27132	-2.41382	0.000215
1383355_at	AW918387	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-1.21129	-2.31545	5.71E-07
1369928_at	NM_019212	Acta1	Actin, α 1, skeletal muscle	-3.10676	-8.61449	4.42E-05
1370856_at	AA800705	Actc1	Actin, α, cardiac muscle 1	-1.26863	-2.40933	2.02E-05
1394483_at	AW535310	Adamts5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	-1.53094	-2.88975	1.96E-05
1390383_at	BI285616	Adfp	Adipose differentiation related protein	-2.49834	-5.65037	3.30E-08
1382680_at	BG673602	Adfp	Adipose differentiation related protein	-2.22186	-4.66496	1.20E-05
1387395_at	NM_017161	Adora2b	Adenosine A2B receptor	-2.08666	-4.24763	0.000258
1395695_at	BE126420	Aebp1	AE binding protein 1	-1.59720	-3.02555	0.005916
1372301_at	BI278482	Aebp1	AE binding protein 1	-1.55735	-2.94313	0.011670
1368342_at	NM_031544	Ampd3	Adenosine monophosphate deaminase 3	-1.65434	-3.14779	0.004546
1377783_at	BI294141	Angpt4	Angiopoietin 4	-1.64644	-3.13060	0.001964
1397579_x_at	BI294552	Apc2	Adenomatosis polyposis coli 2	-1.91631	-3.77455	0.001510

1395461_at	BI294552	Apc2	Adenomatosis polyposis coli 2	-1.30834	-2.47656	0.002146
1391083_at	BM384457	Arhgap22	Rho GTPase activating protein 22	-1.09826	-2.14096	0.007774
1377385_at	BE100015	Arhgap27	Rho GTPase activating protein 27	-1.30775	-2.47556	6.57E-07
1387959_at	AB009372	Aspg	Asparaginase homolog (S. cerevisiae)	-1.29918	-2.46089	0.000534
1380726_at	BI290633	Aspn	Asporin	-2.13124	-4.38092	0.032454
1381504_at	AI639412	Aspn	Asporin	-1.90669	-3.74947	0.029180
1368477_at	NM_012914	Atp2a3	ATPase, Ca++ transporting, ubiquitous	-1.60464	-3.04119	0.000593
1369664_at	NM_053019	Avpr1a	Arginine vasopressin receptor 1A	-2.07931	-4.22605	1.62E-05
1375941_at	BI292120	Baiap2l1	BAI1-associated protein 2-like 1	-1.64069	-3.11816	1.70E-06
1369807_at	NM_030851	Bdkrb1	Bradykinin receptor B1	-1.11926	-2.17236	0.000155
1391345_at	BI293047	Bmper	BMP-binding endothelial regulator	-1.85131	-3.60828	3.59E-06
1387540_at	NM_012514	Brca1	Breast cancer 1	-1.02475	-2.03461	0.000898
1381995_at	AW530502	Brunol4	Bruno-like 4, RNA binding protein (Drosophila)	-2.20211	-4.60152	7.21E-08
1387893_at	D88250	C1s	Complement component 1, s subcomponent	-1.26395	-2.40152	0.048179
1375569_at	BM386267	Ccdc92	Coiled-coil domain containing 92	-1.03224	-2.04520	0.000197
1367973_at	NM_031530	Ccl2	Chemokine (C-C motif) ligand 2	-2.02826	-4.07912	0.002595
1379935_at	BF419899	Ccl7	Chemokine (C-C motif) ligand 7	-1.07909	-2.11271	0.006482
1370810_at	L09752	Ccnd2	Cyclin D2	-1.31667	-2.49091	0.005098
1389490_at	BI274335	Cd248	CD248 molecule, endosialin	-3.64068	-12.47250	1.26E-06
1389755_at	BM391858	Cdca7l	Cell division cycle associated 7 like	-1.44180	-2.71661	0.000141
1369425_at	NM_138889	Cdh13	Cadherin 13	-3.44410	-10.88370	4.56E-08
1375719_s_at	BG381748	Cdh13	Cadherin 13	-3.20680	-9.23301	2.00E-08
1373102_at	BI282750	Cdh13	Cadherin 13	-3.03097	-8.17360	6.92E-06
1373054_at	AA801076	Cdw92	CDW92 antigen	-1.00321	-2.00445	0.000362
1396034_at	BF402373	Ces7	Carboxylesterase 7	-2.24147	-4.72879	7.58E-05
1389179_at	BF284899	Cidea	Cell death-inducing DNA fragmentation factor, α subunit-like effector A	-1.13966	-2.20329	1.12E-05
1367740_at	M14400	Ckb	Creatine kinase, brain	-1.40456	-2.64738	0.000293
1392672_at	AI576758	Clec11a	C-type lectin domain family 11, member a	-1.45028	-2.73262	0.004619
1368571_at	NM_021997	Clip2	CAP-GLY domain containing linker protein 2	-1.09569	-2.13715	4.77E-07
1372584_at	BG672517	Cnrip1	Cannabinoid receptor interacting protein 1	-1.11448	-2.16517	1.59E-08
1379345_at	BM386752	Col15a1	Collagen, type XV, α 1	-6.09695	-68.44850	3.18E-06
1388939_at	AA800298	Col15a1	Collagen, type XV, α 1	-4.48588	-22.4071	2.71E-05
1384969_at	BE109107	Col24a1	Collagen, type XXIV, α 1	-1.82710	-3.54824	0.002725
1371349_at	AI598402	Col6a1	Collagen, type Ⅵ, α 1	-1.66097	-3.16228	0.001251
1371369_at	BI287851	Col6a2	Collagen, type Ⅵ, α 2	-1.80919	-3.50445	9.31E-05
1372818_at	BI284441	Colec12	Collectin sub-family member 12	-2.38156	-5.21100	3.25E-07
1372774_at	AI170570	Coq6	Coenzyme Q6 homolog (yeast)	-1.57329	-2.97583	1.94E-06
1369964_at	NM_130411	Coro1a	Coronin, actin binding protein 1A	-1.66410	-3.16916	7.57E-05
1392996_at	BG668435	Cpeb1	Cytoplasmic polyadenylation element binding protein 1	-1.42707	-2.68901	3.55E-05
1368293_at	NM_031766	Cpz	Carboxypeptidase Z	-1.17127	-2.25210	0.020505
1376051_at	BI293393	Cryl1	Crystallin, lambda 1	-2.8568	-7.24406	4.30E-05
1383575_at	BG376561	Ctnnd2	Catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)	-2.38434	-5.22105	9.56E-05
1369947_at	NM_031560	Ctsk	Cathepsin K	-1.00677	-2.00941	0.001608
1387969_at	U22520	Cxcl10	Chemokine (C-X-C motif) ligand 10	-2.22859	-4.68674	4.90E-05
1368738_at	D11354	Cyp11b1	Cytochrome P450, subfamily 11B, polypeptide 1	-1.73100	-3.31958	0.003738
1387305_s_at	NM_012539	Cyp11b1 /// Cyp11b2	Cytochrome P450, subfamily 11B, polypeptide 1 /// cytochrome P450, subfamily 11B, polypeptide 2	-1.73027	-3.31790	4.54E-05
1387276_at	NM_021584	Dclk1	Doublecortin-like kinase 1	-1.05016	-2.07076	0.000907
1384971_at	BI289108	Depdc7	DEP domain containing 7	-1.10481	-2.15070	0.000468
1371732_at	BI285485	Dpt	Dermatopontin	-1.89757	-3.72585	0.009977
1383853_at	BE103067	Dyrk3	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	-1.76949	-3.40934	1.66E-07
1383641_at	BF414702	Ednra	Endothelin receptor type A	-2.19922	-4.5923	0.014670
1378342_at	BF284819	Ednra	Endothelin receptor type A	-1.78129	-3.43733	0.004867
1393415_at	BF548891	Ednra	Endothelin receptor type A	-1.51332	-2.85467	0.007430
1391442_at	AA957585	Ehd3	EH-domain containing 3	-1.71662	-3.28665	3.90E-05
1367905_at	NM_019370	Enpp3	Ectonucleotide pyrophosphatase/phosphodiesterase 3	-1.33397	-2.52096	9.07E-05
1382434_at	AI059015	Entpd5	Ectonucleoside triphosphate diphosphohydrolase 5	-1.63931	-3.11518	2.50E-06
1370503_s_at	AB032828	Epb4.1l3	Erythrocyte protein band 4.1-like 3	-1.94250	-3.84372	0.005443
1368515_at	NM_053927	Epb4.1l3	Erythrocyte protein band 4.1-like 3	-1.52656	-2.88098	0.000173
1369422_at	NM_138850	Fap	Fibroblast activation protein, α	-1.69721	-3.24274	0.001039
1376561_at	AW523739	Fbxo16	F-box protein 16	-1.11142	-2.16058	6.95E-06
1367850_at	NM_053843	Fcgr2a /// LOC498276 /// LOC498277	Fc fragment of IgG, low affinity IIa, receptor (CD32) /// Fc γ receptor Ⅱ β /// similar to Low affinity immunoglobulin γ Fc region receptor Ⅲ precursor (IgG Fc receptor Ⅲ) (Fc-γ RⅢ) (FcRⅢ)	-1.23098	-2.34726	0.007866
1392865_at	BG371594	Fgf9	Fibroblast growth factor 9	-2.37371	-5.18274	0.002748
1373882_at	AI170324	Figf	C-fos induced growth factor	-2.48251	-5.58869	0.000198
1387709_at	AY032728	Figf	C-fos induced growth factor	-2.21994	-4.65873	5.44E-07

1374726_at	AI411941	Fndc1	Fibronectin type Ⅲ domain containing 1	-2.18189	-4.53749	0.005581
1370248_at	AA851939	Fxyd6	FXYD domain-containing ion transport regulator 6	-1.91583	-3.77331	0.004165
1385636_at	AI029226	Fzd3	Frizzled homolog 3 (Drosophila)	-1.30152	-2.46488	6.39E-06
1388395_at	AI406939	G0s2	G0/G1switch 2	-1.69782	-3.24410	6.68E-05
1382314_at	BE096523	G1p2	Interferon, α-inducible protein (clone IFI-15K)	-1.43064	-2.69565	0.012403
1370963_at	AJ131902	Gas7	Growth arrest specific 7	-2.12746	-4.36946	0.001866
1387221_at	NM_024356	Gch1	GTP cyclohydrolase 1	-1.03450	-2.04840	0.001025
1368085_at	NM_133595	Gchfr	GTP cyclohydrolase I feedback regulator	-1.09278	-2.13284	1.23E-05
1368770_at	NM_022276	Gcnt1	Glucosaminyl (N-acetyl) transferase 1, core 2	-1.35748	-2.56237	0.000674
1387659_at	AF245172	Gda	Guanine deaminase	-1.54413	-2.91629	0.000209
1377761_at	BI296057	Gfpt2	Glutamine-fructose-6-phosphate transaminase 2	-2.38870	-5.23685	0.003422
1387007_at	NM_012959	Gfra1	GDNF family receptor α 1	-1.49726	-2.82306	0.000202
1367954_at	U59486	Gfra1	GDNF family receptor α 1	-1.10817	-2.15573	0.000600
1397461_at	BF416400	Glt8d2	Glycosyltransferase 8 domain containing 2	-1.11036	-2.15899	8.28E-06
1386870_at	BI275294	Glul	Glutamate-ammonia ligase (glutamine synthetase)	-1.18385	-2.27182	0.000113
1367632_at	NM_017073	Glul	Glutamate-ammonia ligase (glutamine synthetase)	-1.11961	-2.17288	0.003082
1369302_at	NM_133573	Gper	G protein-coupled estrogen receptor 1	-1.10018	-2.14381	0.001057
1387241_at	NM_031696	Gpr88	G-protein coupled receptor 88	-1.11226	-2.16184	0.002865
1369926_at	NM_022525	Gpx3	Glutathione peroxidase 3	-2.05946	-4.16829	1.35E-06
1374488_at	AI175700	Gramd1b	GRAM domain containing 1B	-1.12881	-2.18678	0.001788
1368180_s_at	NM_017013	Gsta2 /// LOC494499	Glutathione S-transferase A2 /// LOC494499 protein	-2.31111	-4.96266	6.68E-05
1371298_at	BF284168	H19	H19 fetal liver mRNA	-1.87119	-3.65833	0.000303
1391575_at	BG380566	Hapln4	Hyaluronan and proteoglycan link protein 4	-1.12321	-2.17831	0.002326
1368255_at	NM_017354	Hnt	Neurotrimin	-2.33813	-5.05648	0.004300
1367816_at	NM_133621	Hopx	HOP homeobox	-1.32323	-2.50226	0.003008
1393592_at	AA998087	Hs3st5	Heparan sulfate (glucosamine) 3-O-sulfotransferase 5	-1.19206	-2.28479	0.004675
1368578_at	NM_017265	Hsd3b1	Hydroxy-delta-5-steroid dehydrogenase, 3 β- and steroid delta-isomerase 1	-2.13200	-4.38324	0.000250
1387282_at	NM_053612	Hspb8	Heat shock protein 8	-1.47457	-2.77901	2.14E-07
1388721_at	BG380282	Hspb8	Heat shock protein 8	-1.28154	-2.43098	1.12E-06
1376908_at	AW531805	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	-1.35357	-2.55544	0.019235
1382220_at	AI180454	Igf2bp2	Insulin-like growth factor 2 mRNA binding protein 2	-1.40203	-2.64272	0.007370
1387180_at	NM_053953	Il1r2	Interleukin 1 receptor, type II	-2.39783	-5.27012	0.000126
1387273_at	NM_013037	Il1rl1	Interleukin 1 receptor-like 1	-3.65216	-12.57210	0.013461
1370692_at	U04317	Il1rl1	Interleukin 1 receptor-like 1	-1.22822	-2.34278	0.004082
1387504_at	NM_133575	Il1rl2	Interleukin 1 receptor-like 2	-1.52580	-2.87946	7.08E-05
1377163_at	BM385741	Inhbb	Inhibin β-B	-1.23015	-2.34591	0.014339
1369043_at	NM_012971	Kcna4	Potassium voltage-gated channel, shaker-related subfamily, member 4	-4.17720	-18.09100	8.01E-08
1390404_at	BF556962	Lama2	Laminin, α 2	-2.54141	-5.82159	9.68E-05
1370138_at	NM_130429	Lef1	Lymphoid enhancer binding factor 1	-1.10908	-2.15708	0.000657
1378179_a_at	AW524864	Lhfpl2	Lipoma HMGIC fusion partner-like 2	-1.03801	-2.05339	0.002170
1371094_at	L06804	Lhx2	LIM homeobox 2	-1.06797	-2.09648	0.000653
1389885_at	BI294855	Limd2	LIM domain containing 2	-1.05098	-2.07194	6.73E-05
1376871_at	AA891475	LOC680910 /// LOC681069 /// LOC681182 /// LOC681196 /// LOC685030 /// LOC685048 /// LOC685111 /// LOC685262 /// LOC685305 /// LOC686848 /// LOC686899 /// RGD1559588 /// RGD1561143 /// RGD1561730 /// RGD1562525 /// RGD1563400 /// RGD1566006	Similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to cell surface receptor FDFACT /// similar to cell surface receptor FDFACT /// similar to cell surface receptor FDFACT /// similar to cell surface receptor FDFACT /// similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β	-1.06939	-2.09855	2.14E-06
1385047_x_at	AI012782	LOC685048 /// LOC685111 /// RGD1559588 /// Vom2r61	Similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to cell surface receptor FDFACT /// vomeronasal 2 receptor, 61	-2.82049	-7.06400	5.90E-06
1393688_at	AI012782	LOC685048 /// LOC685111 /// RGD1559588 /// Vom2r61	Similar to paired immunoglobin-like type 2 receptor β /// similar to paired immunoglobin-like type 2 receptor β /// similar to cell surface receptor FDFACT /// vomeronasal 2 receptor, 61	-2.75933	-6.77080	2.26E-07

1371293_at	AI103218	LOC688228	Similar to Myosin light polypeptide 4 (Myosin light chain 1, atrial isoform)	-1.30459	-2.47013	6.68E-07
1398732_at	BF553297	LOC688273	Hypothetical protein LOC688273	-3.37961	-10.40790	0.000276
1384540_at	BE101066	Lrfn3	Leucine rich repeat and fibronectin type III domain containing 3	-1.05498	-2.07769	0.000430
1388347_at	AI233210	Ly6e	Lymphocyte antigen 6 complex, locus E	-2.76880	-6.81539	1.82E-05
1376184_at	BG381127	Lynx1	Ly6/neurotoxin 1	-1.98858	-3.96846	8.46E-08
1393645_at	BI288003	Mageb16	Melanoma antigen family B, 16	-1.53459	-2.89706	0.000131
1388152_at	BG374290	Map2	Microtubule-associated protein 2	-1.77330	-3.41836	0.008563
1382046_at	AA963495	Map3k3	Mitogen activated protein kinase kinase kinase 3	-1.24191	-2.36511	1.42E-06
1392547_at	AI716211	MGC105649	Hypothetical LOC302884	-2.31654	-4.98136	0.004157
1388300_at	AA892234	Mgst3	Microsomal glutathione S-transferase 3	-1.00700	-2.00973	0.017898
1393836_at	BE097933	Mitf	Microphthalmia-associated transcription factor	-1.09560	-2.13702	0.010323
1368590_at	NM_080776	Mmp16	Matrix metallopeptidase 16	-1.01729	-2.02411	0.000829
1370301_at	U65656	Mmp2	Matrix metallopeptidase 2	-3.98031	-15.78310	0.000568
1382190_at	BF405725	Mrgprf	MAS-related GPR, member F	-3.60572	-12.17390	3.94E-07
1368441_at	NM_031658	Msln	Mesothelin	-1.36403	-2.57403	0.001477
1376648_at	BI275570	Mycn	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	-1.62357	-3.08136	0.002089
1368415_at	NM_012604	Myh3	Myosin, heavy chain 3, skeletal muscle, embryonic	-1.24039	-2.36262	0.003414
1387787_at	NM_012605	Mylpf	Myosin light chain, phosphorylatable, fast skeletal muscle	-2.12252	-4.35453	0.000567
1398655_at	AA955902	Myod1	Myogenic differentiation 1	-2.39143	-5.24677	9.65E-05
1373839_at	BG372386	Nope	Neighbor of Punc E11	-1.24338	-2.36752	0.009522
1371036_at	BG671431	Nrcam	Neuronal cell adhesion molecule	-1.60969	-3.05187	2.40E-06
1384112_at	BI289470	Nt5e	5' nucleotidase, ecto	-1.12050	-2.17423	0.004069
1392780_at	BF283270	Nxf7	nuclear RNA export factor 7	-3.39721	-10.53570	0.005424
1377497_at	BF419319	Oasl	2'-5'-oligoadenylate synthetase-like	-1.36533	-2.57636	2.34E-05
1369008_a_at	NM_053573	Olfm1	Olfactomedin 1	-3.58878	-12.0318	0.000169
1368940_at	NM_017255	P2ry2	Purinergic receptor P2Y, G-protein coupled 2	-1.21116	-2.31523	0.009814
1383273_a_at	AA956005	Pcbp3	Poly(rC) binding protein 3	-2.10678	-4.30729	4.21E-06
1383274_at	AA956005	Pcbp3	Poly(rC) binding protein 3	-1.86620	-3.64572	0.000552
1385116_at	BF386807	Pcdhb21	Protocadherin β 21	-1.13304	-2.19321	0.007625
1373368_at	BI279680	PCOLCE2	Procollagen C-endopeptidase enhancer 2	-1.80500	-3.49430	7.61E-07
1368145_at	NM_013002	Pcp4	Purkinje cell protein 4	-4.28374	-19.47750	0.000401
1370941_at	AI232379	Pdgfra	Platelet derived growth factor receptor, α polypeptide	-1.62538	-3.08523	0.011837
1377100_at	AI172172	Pds5b	PDS5, regulator of cohesion maintenance, homolog B (S. cerevisiae)	-1.14971	-2.21869	1.40E-05
1388634_at	BI277505	Pgm1	Phosphoglucomutase 1	-1.66935	-3.18071	9.06E-09
1369473_at	NM_017033	Pgm1	Phosphoglucomutase 1	-1.50694	-2.84207	3.02E-06
1383749_at	AI112954	Phospho1	Phosphatase, orphan 1	-1.16307	-2.23934	0.003657
1370445_at	D88666	Pla1a	Phospholipase A1 member A	-1.35014	-2.54937	0.001836
1390190_at	BI293691	Plac1	Placenta-specific 1	-2.19087	-4.56582	0.000174
1384558_at	BI276313	Plac9	Placenta-specific 9	-1.07069	-2.10043	0.007797
1367800_at	NM_013151	Plat	Plasminogen activator, tissue	-1.15024	-2.21951	0.004677
1391187_at	BI303019	Ppl	Periplakin	-1.11393	-2.16434	0.000262
1368259_at	NM_017043	Ptgs1	Prostaglandin-endoperoxide synthase 1	-2.42323	-5.36370	3.32E-05
1381806_at	BF418208	Ptgs1	Prostaglandin-endoperoxide synthase 1	-1.06169	-2.08737	5.08E-05
1372084_at	AI104546	Ptp4a3	Protein tyrosine phosphatase 4a3	-1.02120	-2.02961	2.54E-05
1368350_at	NM_013080	Ptprz1	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1	-1.04165	-2.05858	0.022268
1373646_at	BM384841	Rab15	RAB15, member RAS oncogene family	-1.17275	-2.25440	7.21E-05
1374035_at	BI296482	Rem2	RAS (RAD and GEM) like GTP binding 2	-1.00521	-2.00723	0.017185
1368080_at	NM_054008	Rgc32	Response gene to complement 32	-1.00087	-2.00121	0.032818
1392883_at	AI013730	RGD1305269	Similar to hypothetical protein	-1.04588	-2.06463	1.81E-05
1373226_at	BF400995	RGD1308019	Similar to hypothetical protein FLJ20245	-1.10844	-2.15613	0.015649
1381757_at	AA965058	RGD1309501	Hypothetical LOC305552	-1.28517	-2.43712	2.17E-05
1373596_at	AI230766	RGD1310423	Similar to hypothetical protein FLJ31737	-2.01177	-4.03277	6.86E-08
1398577_at	BI297744	RGD1310507	Similar to RIKEN cDNA 1300017J02	-1.72535	-3.30661	0.000821
1390397_at	BF413152	RGD1310753	Similar to chromosome 20 open reading frame 39	-2.10856	-4.31261	5.11E-05
1393191_at	BF554733	RGD1561205	Similar to RIKEN cDNA 2610200G18	-1.39361	-2.62736	0.000183
1376693_at	AA998964	RGD1563091	Similar to OEF2	-1.00045	-2.00063	0.047169
1395145_at	BF544481	Rgl1	Ral guanine nucleotide dissociation stimulator,-like 1	-1.54730	-2.92270	0.000329
1394472_at	BF282814	Rgl1	Ral guanine nucleotide dissociation stimulator,-like 1	-1.20268	-2.30166	0.005425
1391075_at	AI179271	Rgs17	Regulator of G-protein signaling 17	-1.50436	-2.83699	0.004883
1368373_at	NM_019343	Rgs7	Regulator of G-protein signaling 7	-3.29254	-9.79836	1.82E-05
1370142_at	NM_022175	Rhox5	Reproductive homeobox 5	-3.12793	-8.74178	2.44E-09
1383554_at	AW142796	Rnf128	Ring finger protein 128	-1.47300	-2.77598	1.47E-05
1389735_at	BE107296	Rps6ka6	Ribosomal protein S6 kinase polypeptide 6	-1.18452	-2.27288	0.013943
1384707_at	AI600020	Scara5	Scavenger receptor class A, member 5 (putative)	-1.46379	-2.75833	9.19E-06
1392856_at	AI549470	Serf1	Small EDRK-rich factor 1	-1.52508	-2.87802	0.000710
1375084_at	BF419780	Serinc2	Serine incorporator 2	-1.64207	-3.12113	0.000591

1377034_at	BF411331	Serpinb1a	Serine (or cysteine) proteinase inhibitor, clade B, member 1a	-1.17504	-2.25799	8.45E-05
1369547_at	NM_130404	Serpinb7	Serine (or cysteine) peptidase inhibitor, clade B, member 7	-1.40940	-2.65626	0.001746
1393620_at	AI113325	Sesn3	Sestrin 3	-1.17758	-2.26198	0.001718
1390119_at	BF396602	Sfrp2	Secreted frizzled-related protein 2	-1.64679	-3.13135	0.011642
1367881_at	NM_013016	Sirpa	Signal-regulatory protein α	-1.88267	-3.68756	1.79E-06
1392789_at	BI296353	Slc25a36	Solute carrier family 25, member 36	-2.25714	-4.78042	4.14E-05
1372341_at	AI233213	Slc25a36	Solute carrier family 25, member 36	-2.07958	-4.22685	0.000633
1369237_at	NM_053996	Slc6a7	Solute carrier family 6 (neurotransmitter transporter, L-proline), member 7	-1.12269	-2.17753	0.001140
1368322_at	NM_012880	Sod3	Superoxide dismutase 3, extracellular	-2.20485	-4.61028	1.15E-05
1368254_a_at	AB049572	Sphk1	Sphingosine kinase 1	-1.09627	-2.13802	0.004730
1368655_at	NM_020074	Srgn	Serglycin	-2.51283	-5.70737	0.006656
1373146_at	AI716240	Ssx2ip	Synovial sarcoma, X breakpoint 2 interacting protein	-1.23239	-2.34956	0.001168
1387174_a_at	AB006007	Star	Steroidogenic acute regulatory protein	-3.29954	-9.84599	0.000194
1368406_at	NM_031558	Star	Steroidogenic acute regulatory protein	-2.92085	-7.57291	7.29E-07
1377672_at	BI300997	Sult1c2	Sulfotransferase family, cytosolic, 1C, member 2	-3.45192	-10.94290	3.19E-06
1369531_at	NM_133547	Sult1c2	Sulfotransferase family, cytosolic, 1C, member 2	-2.77927	-6.86504	2.07E-05
1369627_at	L10362	Sv2b	Synaptic vesicle glycoprotein 2b	-2.39744	-5.26866	0.000178
1385637_at	AI029494	Svep1	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	-1.08524	-2.12172	0.000482
1383686_at	BE111537	Syngr1	Synaptogyrin 1	-1.73204	-3.32197	0.005240
1384202_at	BI287326	Tesc	Tescalcin	-1.69209	-3.23124	0.004681
1371913_at	BG379319	Tgfbi	Transforming growth factor, β induced	-1.05453	-2.07704	0.023537
1369652_at	AI145313	Thy1	Thy-1 cell surface antigen	-3.93458	-15.29070	2.03E-07
1369651_at	NM_012673	Thy1	Thy-1 cell surface antigen	-3.67268	-12.75220	7.68E-08
1392980_at	AI716456	Tiam1	T-cell lymphoma invasion and metastasis 1	-1.56449	-2.95774	7.40E-06
1382222_at	BI293607	Tmem163	Transmembrane protein 163	-2.11820	-4.34153	8.73E-06
1376106_at	AI010157	Tmem178	Transmembrane protein 178	-3.28823	-9.76912	0.007054
1377554_at	BF394106	Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	-1.95931	-3.88877	1.88E-05
1370332_at	AF159356	Unc13d	Unc-13 homolog D (C. elegans)	-2.36452	-5.14980	0.000313
1368474_at	NM_012889	Vcam1	Vascular cell adhesion molecule 1	-3.24587	-9.48643	1.90E-05
1388142_at	AA850991	Vcan	Versican	-1.54149	-2.91096	0.000288
1388054_a_at	AF072892	Vcan	Versican	-1.53261	-2.89308	0.000536
1371232_a_at	AF084544	Vcan	Versican	-1.47614	-2.78204	0.000764
1388265_x_at	AF084544	Vcan	Versican	-1.45114	-2.73423	0.001864
1389253_at	BI289085	Vnn1	Vanin 1	-1.15392	-2.22518	0.000172
1382283_at	BF283711	Wipf1	WAS/WASL interacting protein family, member 1	-1.01562	-2.02176	3.13E-05
1387227_at	NM_057192	Wipf1	WAS/WASL interacting protein family, member 1	-1.00508	-2.00706	8.16E-05
1389119_at	AI105018	Xirp1	Xin actin-binding repeat containing 1	-1.17137	-2.25226	9.90E-05
1372989_at	BI296586	Zdhhc14	Zinc finger, DHHC-type containing 14	-3.38007	-10.4112	1.23E-07

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COMMENTS

Background

miRNAs are a relatively new and exciting tool to control the expression of multiple genes. During liver injury and subsequent wound healing involving hepatic stellate cells (HSCs), complex regulatory processes occur and have to be tightly regulated in this cell type. miRNAs could be one tool to control these processes, and therefore, it is of interest to the research community to gain information about the expression of miRNAs during liver fibrosis in HSCs.

Research frontiers

Liver fibrosis and subsequently cirrhosis are common outcomes of chronic injuries to the liver. HSCs are involved in liver fibrosis and repair. The tools for the treatment of liver fibrosis are limited and are still under development. In this study, the authors aimed to gain information for the possible role of miRNAs in liver fibrosis and whether they could become a future tool to develop a treatment for liver fibrosis by addressing the changes in HSCs.

Innovations and breakthroughs

Different publications have analyzed the miRNA expression in HSCs *in vitro* and studied the effect of various differentially regulated miRNAs in HSCs. The authors analyzed the miRNA expression in an *in vivo* model of hepatic fibrosis, namely choline-deficient ethionine supplemented diet. Furthermore, they studied the transcriptome changes upon overexpression of miR-146a and found that, in particular, tissue inhibitor of metalloproteinase-3 showed robust up-regulation, a hitherto unreported effect, which emphasizes its involvement in inflammation. Another important finding was the dynamics of miRNA regulation during the *in vitro* activation of HSCs.

Applications

miRNAs are becoming a promising tool for the regulation of gene expression. In order to use this tool, it is necessary to understand the role and regulation of the targeted miRNA. In this study, the authors describe the dynamic regulation of specific miRNAs. The results of this study show clearly that the use of miRNAs as target molecules will have to take this dynamic component into consideration. The same is valid for the use of miRNAs as therapeutic agents.

Terminology

miRNAs are small non-coding RNAs that are about 23 nucleotides long. The versatility of miRNAs depends on the imperfect binding (seed region) to the 3'-UTR of mRNAs. This imperfect binding results in many different binding partners. The regulation by miRNAs leads to a translational repression and/or mRNA destabilization.

Peer review

The field of miRNA research as well as HSC activation mechanisms are very up to date and important areas of research, in order to find new strategies against liver fibrosis. The methods used are comprehensive and convincing. In all, the study was fairly well conducted and interesting.

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ORIGINAL ARTICLE

Influence of chitosan nanofiber scaffold on porcine endogenous retroviral expression and infectivity in pig hepatocytes

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Abstract

AIM: To investigate the influence of chitosan nanofiber scaffold on the production and infectivity of porcine endogenous retrovirus (PERV) expressed by porcine hepatocytes.

METHODS: Freshly isolated porcine hepatocytes were cultured with or without chitosan nanofiber scaffold (defined as Nano group and Hep group) for 7 d. The daily collection of culture medium was used to detect reverse transcriptase (RT) activity with RT activity assay kits and PERV RNA by reverse transcription-polymerase chain reaction (PCR) and real time PCR with the PERV specific primers. And Western blotting was performed with the lysates of daily retrieved cells to determine the PERV protein gag p30. Besides, the *in-vitro* infectivity of the supernatant was tested by incubating the human embryo kidney 293 (HEK293) cells.

RESULTS: The similar changing trends between two groups were observed in real time PCR, RT activity assay and Western blotting. Two peaks of PERV expression at 10H and Day 2 were found and followed by a regular decline. No significant difference was found between two groups except the significantly high level of PERV RNA at Day 6 and PERV protein at Day 5 in Nano group than that in Hep group. And in the *in-vitro* infection experiment, no HEK293 cell was infected by the supernatant.

CONCLUSION: Chitosan nanofiber scaffold might prolong the PERV secreting time in pig hepatocytes but would not obviously influence its productive amount and infectivity, so it could be applied in the bioartificial liver without the increased risk of the virus transmission.

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Key words: Chitosan nanofiber scaffold; Porcine hepatocyte; Porcine endogenous retrovirus; Bioartificial liver

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INTRODUCTION

Although liver transplantation is currently recognized as the most effective treatment for acute liver failure and end-stage liver diseases, its application has been seriously limited because of the lack of donor organs[1,2]. Therefore, bioartificial liver (BAL) has been proposed as a temporary liver support for patients awaiting liver transplantation[3-5].

At present, porcine hepatocytes were still a major cell source because of their adequate resources, accessibility and characters similar to human hepatocytes[6-8]. However, the clinical application of BAL based on porcine hepatocytes was not very optimistic[4]. So how to increase the *in-vitro* function of hepatocytes with a suitable scaffold was always an attractive issue and some previous reports showed the enhanced function of procine hepatocytes with their scaffold, including the chitosan nanofiber scaffold developed in our institute[9-11].

Nevertheless, as xenogeneic cells, there were some problems with porcine hepatocytes. The security of the transmission of porcine endogenous retrovirus (PERV) has been one of the most essential concerns that can not be ignored, since PERV infection of human cells *in vitro* was widely reported[12]. Therefore, it raised new questions that whether the production and infectivity of PERV would be influenced and whether the risk of PERV infection in BAL would be increased when the function of porcine hepatocytes was enhanced with certain scaffolds.

Previously, we had proved the chitosan nanofiber scaffold could enhanced hepatocytes adhesion, viability and function *in vitro*[13]. This study was focused on the influence of chitosan nanofiber scaffold on PERV expression and infectivity in pig hepatocytes.

MATERIALS AND METHODS

Animals and reagents

Outbred white pigs with an average weight of 15-20 kg received humane care and all animal procedures were performed according to institutional and national guidelines and approved by the Animal Care Ethics Committee of Nanjing University and Nanjing Drum Tower Hospital. All cell culture-related reagents were purchased from GIBCO (USA). Lactobionic acid (LA) and chitosan (low molecular weight, brookfield viscosity 20 000 cps, 85% deacetylation) were purchased from Sigma-Aldrich (Saint Louis, USA). N-Hydroxysuccinimide was purchased from Thermo-Pierce (Rockford, USA). 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide and N,N,N0N0-tetramethylethylenediamine were obtained from TCI (Tokyo, Japan). Poly(ethylenoxide) (PEO, MWz1_106) was supplied by Guoren Chemical Co. (Beijing, China). All other reagents were of analytical reagent grade.

Preparation of chitosan nanofiber scaffold via electrospinning

The chitosan nanofiber scaffold was prepared according to the previous reference[13]. In brief, Chitosan (Sigma) and PEO powders (9:1 w/w) were dissolved in formic acid/ethanol (7:3 v/v) to give 2.6% (w/v) at room temperature. The stock solution was then filled into a 5 mL glass syringe fitted with a 20 G needle and then expressed at 5 mL/h using a syringe pump. The nanofibers were collected on 24 mm diam. coverslips located at a fixed distance of 10-20 mm from the needle tip. A non-coated cover slip was also prepared and tested in the same manner as control.

Hepatocytes isolation, characterization and culture

Primary pig hepatocytes were harvested by a two-step *in situ* collagenase perfusion technique[14,15]. First, the prewarmed (37℃) Ca^2+ and Mg^2+ free Hanks balanced salt solution was perfused into the livers of the anesthetized pigs *in vivo via* the portal vein at a flow rate of 80-100 mL/min for 30-40 min, followed by 0.05% Type IV collagenase (37℃) at a rate of 10 mL/min for 40-50 min. The released cells were filtered through nylon mesh with 100-mm openings and washed *via* three centrifugations (50 g). The viability of the isolated primary hepatocytes determined by trypan blue exclusion was more than 95%. Nonparenchymal cells, as judged by their size (< 10 mm in diameter) and morphology (nonpolygonal or stellate), were less than 1%, which was also verified by immunocytochemical analysis of albumin and cytokeratin 18 (data not shown). Freshly isolated hepatocytes were seeded at a density of 10^6 cells/mL in the substratum of 2 mL RPMI-1640 without serum and incubated in 6-well microtiter plates with or without chitosan nanofiber scaffold (defined as Nano group and Hep group) at 37℃ and 5% CO_2. Culture medium was replenished daily without the growth medium containing low-glucose Dulbecco's modified Eagle's medium (DMEM-LG) supplemented with 10% fetal bovine serum (FBS) for 7 consecutive days. The daily collection of culture medium and cells were deposited at -80℃ for later use.

Reverse transcription-polymerase chain reaction

Total RNA was extracted respectively from the centrifuged supernatant with Trizol (Invitrogen, US) and then treated with DNase I (Invitrogen, US) according to the manufacturer's instructions. For each sample, 60 ng extracted RNA with the value of OD 260/280 among 1.60 and 2.00 was reversed transcribed to cDNA using the reverse transcriptase (RT) kits (Biouniquer, China) in accordance with the instructions. Then polymerase chain reaction (PCR) was completed with the protease-specific primers (forward, 5'-GCTACAACCATTAGGAAAACTAAAAG-3'; and reverse, 5'-AACCAGGACTGTATATCTTGATCAG-3'), polymerase-specific primers (forward, 5'-CTACAACCA TTAGGAAAACTAAAAG-3'; and reverse, 5'-AACCAGGACTGTATATCTTGATCAG-3'), and porcine glyceraldehyde 3-phosphate dehydrogenase-specific primers (forward, 5'-CATCACCATCTTCCAGGAG-3'; and reverse, 5'-TGCCCACAGCCTTGGCAGC-3') and its conditions were as follows: 50℃ for 30 min and then 95℃ for 5 min followed

by 35 cycles of 94℃ for 30 s, 55℃ for 45 s, 72℃ for 30 s, and a final extension step of 72℃ for 5 min[16,17]. Amplified production was detected by 2% agarose gel electrophoresis and ethidium bromide (EB) staining.

PERV-specific real time PCR

Quantitative real time PCR was performed with the obtained cDNA, the Roche SuperScript Ⅲ platinium system, the MX3000P thermocycler (Stratagene), and the primers specific for PERV protease gene (GenBank accession number U77599) (forward, 5'-AGTGCTGCTACAACCATTAGGAAA-3'; and reverse, 5'-AGGGATGACCAGAAACGAGTG-3') and for porcine β-actin gene (GenBank accession number DQ845171) (forward, 5'-GGACTTCGAGCAGGAGATGG-3', and reverse, 5'-AGGAAGGAGGGCTGGAAGAG-3').The conditions were as follows: 95℃ for 10min followed by 40 cycles of 95℃ for 15 s, 60℃ for 1 min and a final cycle of 95℃ for 1 min, 65℃ for 30 s, 95℃ for 30 s. PERV expression was normalized to the amount of the expression of the house-keeping genes β-actin and denoted with the relative value of protease/β-actin.

Western blotting analysis

The lysates of the cultured cells harvested every day were analysed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE) and Western blotting with polyvinylidene difluoride membranes (Millipore, Eschborn, Germany). The membranes were then incubated with a 1:1500 dilution of mouse anti-FeLV p27 IgG antibody (ABcam, San Francisco, US) which had been proven cross-reactivity with protein gag p30 of PERV[18] overnight at 4℃, followed by a 1:10000 dilution of goat protein anti-mouse IgG conjugated horseradish peroxidase (KeyGEN, Nanjing, China) for 1 h at 37℃. Immunoreactive proteins on membranes were detected with the enhanced chemiluminescence system and exposured for 15 to 20 s on hyperfilm ECL (Kodak, US) Meanwhile, The protein β-actin was detected as controls. Software ImageJ was used to measure the lightness of each band.

In vitro infection experiments

The *in vitro* infection experiments were performed according to the reference with some modification[19,20]. Human embryo kidney 293 (HEK293) cells (as gifts from Professor Hua, Nanjing University) were passed overnight in 24-well plates and then incubated in the supernatant of the cell culture (0.5 mL/well) for 6 h in the presence of 8 mg/mL of polybrene after the supernatant was centrifuged at a speed of 5000 r/min for 5 min to remove the cells and cell debris. Meanwhile, the supernatant of porcine kidney 15 (PK15) cells and 0.8 g/mL polybrene was inoculated into the culture of HEK293 cells as positive control. After 4 h of exposure at 37℃, the incubating medium was removed and the cell monolayer was washed with phosphate buffered solution for two times. Then the cells were cultured with the DMEM-HG supplemented with 10% FBS and passed upon confluence for 1 mo before collection.

DNA extraction and PCR

Total DNA was extracted from the treated HEK293 cells with the DNA extracting kits (Axygen, California, US) according to their instructions. PCR was completed with the human β-actin primers (forward, 5'-GCTCGTCGTCGACAACGGCTC-3'; and reverse, 5'-CAAACATGATCTGGGTCATCTTCTC-3'), Sus scrofa cytochrome B (SsCytB) primers (forward, 5'-CATTGGAGTAGTCCTACTATTTACCG-3'; and reverse, 5'-GTAGGATTAGTATTATAAATAAGGCTCCT-3'), above mentioned protease-specific primers and polymerase-specific primers and its conditions consisted with the proceeding of PCR in reverse transcription-PCR (RT-PCR). Amplified production was detected by 2% agarose gel electrophoresis and EB staining.

RT activity assay

The RT activity of the supernatant from the cultured hepatocytes in both group and the treated HEK293, was detected by the C-type RT activity kits (Cavidi-Tech, Uppsala, Sweden) according to the quantitative and qualitative protocols of the manufacturer's instructions, respectively. The RT activity was examined twice for each collected supernatant sample.

Statistics

Both culture conditions of this study were completed in quintuplicate. All values were expressed as mean ± SD. The two-tailed unpaired Student's *t*-test was used to evaluate the statistical significance of differences which was set with a *P*-value less than 0.05.

RESULTS

PERV RNA in the supernatants

From the results of electrophoresis (shown in Figure 1A and B, representatively), it could be found that the changing trend of the protease sequence and the polymerase sequence was accordant. In real time PCR, the relative value of protease/β-actin was defined as the normalized amount of PERV RNA. Two PERV secreting peaks at 10H and Day 2 was observed, and then the amount of PERV RNA declined gradually after Day 2. No PERV expression was found after Day 6 in Hep group and Day 7 in Nano group, respectively (Figure 1C). There were significant differences at Day 6 between two groups (0.071 ± 0.0348 *vs* 0.014 ± 0.0193, $P < 0.05$).

Western blotting

Figure 2A demonstrated a representative result in Western blotting. In Hep group, positive gag p30 proteins were found at 10H and from Day 2 to Day 4, while in Nano group positive bands were observed at 10H and from Day 2 to Day 5. The lightness of each band was measured by the software ImageJ and the amount of the expressed

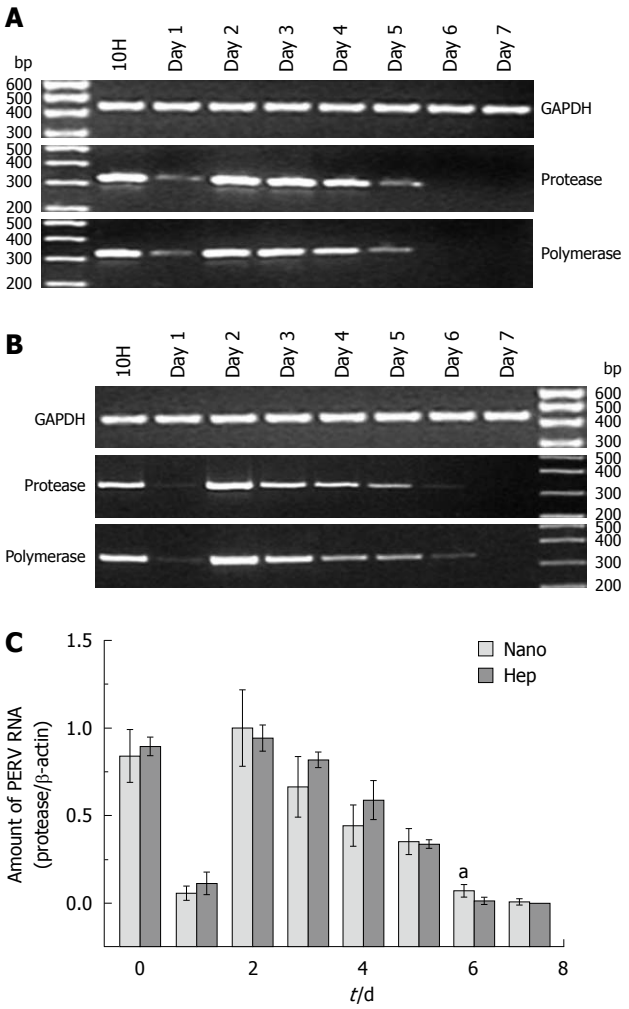

Figure 1 Porcine endogenous retrovirus RNA in the supernatants. A and B: The representative results of reverse transcription-polymerase chain reaction (PCR) electrophoresis with the RNA extracted from the supernatants in Hep group and Nano group, respectively; C: The results of real time PCR. $^aP < 0.05$. PERV: Porcine endogenous retrovirus; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

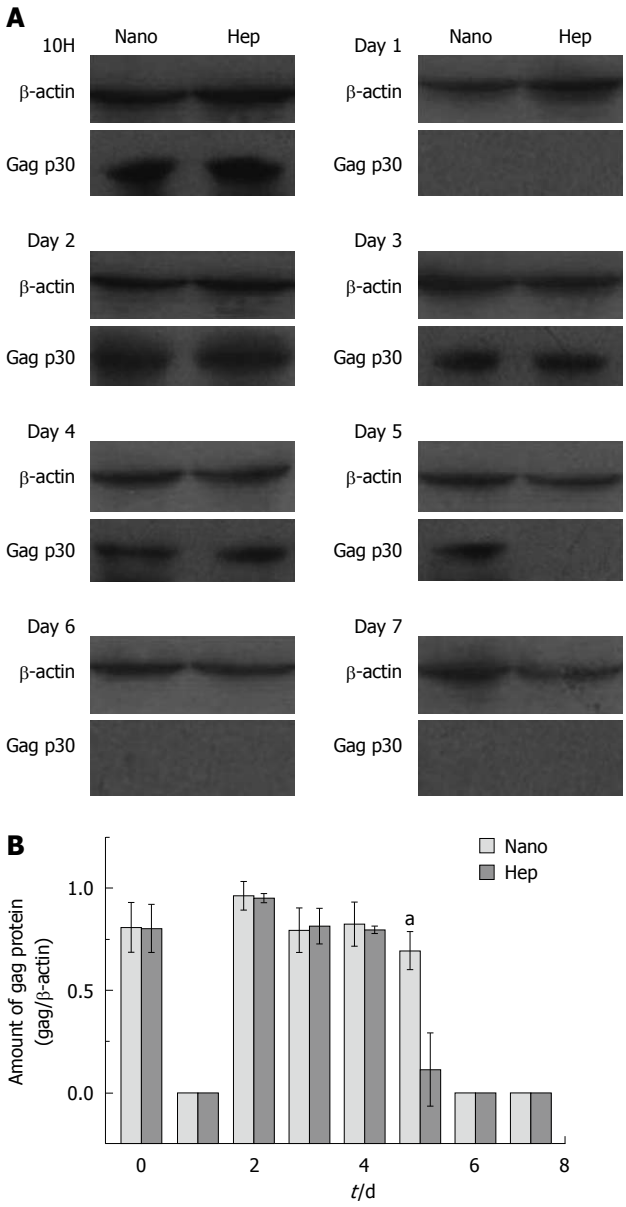

Figure 2 Western blotting of the porcine endogenous retrovirus gag protein in the cell lysates. A: Representative results of Western blotting with the cell lysates in both groups; B: The normalized protein amount in different days. $^aP < 0.05$.

gag p30 protein was normalized with the relative value of gag/β-actin, which was depicted in Figure 2B. Except for the significant differences found at Day 5 (0.70 ± 0.0929 *vs* 0.11 ± 0.180, $P < 0.01$), there were no remarkable differences between two groups in other days.

Infection of HEK293 cells in vitro

No PERV DNA sequence including protease and polymerase genes and SsCytB sequence were found in the HEK293 DNA (Figure 3). The DNA extracted from PK15 infected HEK293 cells and the pure water was used as positive control and negative control, respectively. And the RT activity in the culture supernatant of the treated HEK293 cells was all negative.

RT activity assay

The RT activity in the supernatants from the cultured hepatocytes was demonstrated in Figure 4. No significant difference was found between two groups in each day. And there was no positive RT activity in the supernatant of the HEK293 cells incubated with the supernatant from Hep group or from Nano group.

DISCUSSION

Porcine hepatocytes were still a major cell source for BAL at present[6-8]. How to enhance their function was always an attractive issue and some progress has been made[9-11]. The chitosan nanofiber scaffold fabricated in our previous study had been proved its superior abilities on cell adhesion and biocompatibility, thereby greatly improved the functions of hepatocytes[13]. However, there had been a new and un-ignorable issue that whether the porcine hepatocytes with improved function would secrete more

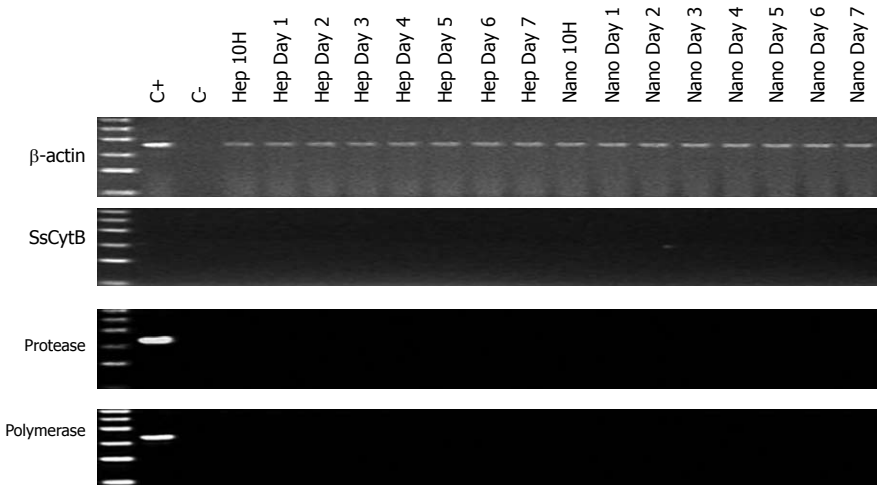

Figure 3 Representative results of polymerase chain reaction electrophoresis with the DNA extracted from human embryo kidney 293 cells of *in vitro* infection experiments. C+: Porcine endogenous retrovirus-infected human embryo kidney 293 cells; C-: Pure water. The ladder ranged from 100 to 600 bp. "Hep" and "Nano" meant the incubating supernatant from the simple culture of hepatocytes and the hepatocyte culture on chitosan nanofiber scaffolds. SsCytB: Sus scrofa cytochrome B.

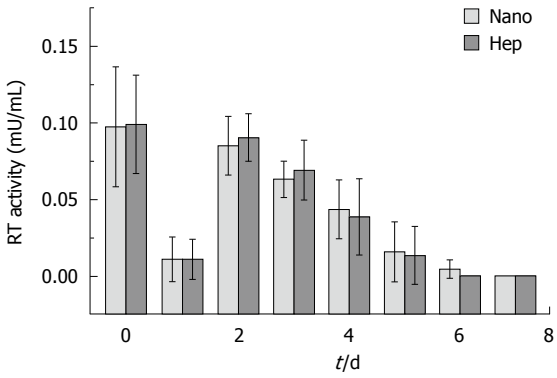

Figure 4 Reverse transcriptase activity of the cultured porcine hepatocytes on different substrata. RT: Reverse transcriptase.

PERV with enhanced infectivity, which might limit its application in BAL.

PERV was first discovered in 1971 in porcine kidney (PK15) cells[21]. In 1997, Patience *et al*[22] found for the first time that PERV released from PK15 could infect HEK293 cells *in vitro*. And then PERV is known to exist regularly in the porcine genome and various porcine cells can excrete PERV particulates[12]. Meanwhile, it was found that PERV successfully infected a variety of human cells *in vitro*, such as endothelial cells, fibroblasts and bone marrow stromal cells and so on, and virus replication was observed in some of these cells as well[23-28]. Nyberg *et al*[19] reported that freshly isolated porcine hepatocytes secreted the PERV which didn't infect HEK293 cells *in vitro*, at the same time, the production of PERV by cultured pig hepatocytes was unaffected by exposure to growth factors and cytokines present in human FHF sera. Fortunately, there was no evidence of PERV transmission into the patients treated with BAL so far[20,29-32]. However, a recent article[33] claimed PERV released from a BAL infected primary human cells by short-term contact of primary porcine liver cell supernatants with primary human cells, which increased the anxiety about the problem of PERV infection in the application of BAL.

In this study, we sought to find the influence of chitosan nanofiber scaffold on the production and infectivity of PERV expressed by porcine hepatocytes. PERV RNA, RT activity and PERV gag protein was detected for analysis of PERV production by porcine hepatocytes. At present, RT-PCR was the most specific and sensitive method for PERV detection[12]. And it had been identified that the RT activity was related with the retrovirus particles[34], so the result of low RT activity implied a small number of secreted PERV particles. In addition, in previous reports[18,35], sucrose gradient-purified PERV from the culture supernatant was used to qualitatively detect the protein gag p30 by Western blotting, but the condensed PERV was not suitable for hemi-quantitative analysis of the protein. Then we considered the supernatant as the samples directly and attempted to do all the test again. Much to our regret, no positive bands were observed. The possible reason may lay on the microamount of the virus. Therefore, the cell lysate was chosen as samples in Western blotting and the expressing level of the protein in cell lysate was detected to reflect the level of virus replication indirectly.

The results of real time PCR, RT activity assay and Western blotting presented a similar changing trend with some minor differences. Positive PERV RNA but no gag protein at Day 6 might result from less sensitivity of Western blotting. From these results, it could be seen that two peaks of PERV expression at 10H and Day 2 were followed by a regular decline in both groups, and no obviously increased expression level with chitosan nanofiber scaffold was found in first 5 d. The first secreting peak might be regarded as the expression of fresh

isolated hepatocytes with some contents of the ruptured non-adherent cells. In order to reduce the influence of the ruptured non-adherent cells, 10H was chosen as the first time point for detecting. But the second peak should be the real secreting peak and the low PERV level in Day 1 might be attributed to the short supernatant collecting interval between 10H and Day 1. On the other hand, the level of PERV RNA at Day 6 and the amount of PERV protein at Day 5 in Nano group was significantly more than that in Hep group, implying a prolonged expression time, which might be due to the superior activity maintaining of hepatocytes cultured on chitosan nanofiber scaffold[13].

The most important concern was no doubt the effect of chitosan nanofiber scaffold on the infectivity of PERV secreted by hepatocytes. Nyberg *et al*[19] had reported PERV released from hepatocytes didn't infect the HEK293 cells *in vitro* and its infectivity would not influenced by human fulminant hepatic failure sera. Likewise, our *in vitro* infection experiments demonstrated no PERV gene sequence and even no porcine specific SsCytB gene existed in the DNA of HEK293 cells, and no positive RT activity could be detected in the supernatant of treated HEK293 cells either, which implied no PERV transmission into the HEK293 cells and no microchimerism. So it could be concluded that there was no obvious infectivity of the PERV secreted by porcine hepatocytes.

In conclusion, porcine hepatocytes could express PERV for 5 d with normal culture condition, but the secreting time might be prolonged to 6 d when cells were cultured with chitosan nanofiber scaffold. Nevertheless, the productive amount and infectivity of the PERV expressed by porcine hepatocytes would not be obviously influenced by chitosan nanofiber scaffold. Therefore, it could be applied in BAL without enhanced risk of the virus infection.

COMMENTS

Background

Bioartificial liver (BAL) carrying porcine hepatocytes which were still a major cell source at present has been proposed as a temporary liver support for patients waiting for liver transplantation or even as a treatment for liver failure. However, the clinical application of BAL was not very optimistic. As a result of that, much progress had been made on cellular function improvement.

Research frontiers

Some scaffold materials were reported to be capable enhancing the *in vitro* function of hepatocytes. And chitosan nanofiber scaffold fabricated in our previous study had demonstrated superior abilities on cell adhesion and biocompatibility, thereby greatly enhanced the functions of hepatocytes. So it became a potential material which could be applied in BAL and a new bioreactor based on chitosan nanofiber scaffold was developed in our institute.

Innovations and breakthroughs

Although much progress had been made cellular function improvement, there were few researches on the influence of the materials on the expression and infectivity of porcine endogenous retrovirus (PERV) in porcine hepatocytes. So this study was focus on the safety of chitosan nanofiber scaffold, finding probable prolonging of PERV expression within chitosan nanofiber scaffold but no increased productive amount and infectivity.

Applications

The study identified no obvious influence of chitosan nanofiber scaffold on PERV expression and infectivity in porcine hepatocytes, and concluded that it could be applied in BAL without enhanced risk of the virus infection.

Terminology

PERV was a porcine specific C-type retrovirus which was capable infecting human cells *in vitro*. Chitosan nanofiber scaffold was a scaffold material made with chitosan by nanotechnology, and it was proved to enhance the hepatocyte function *in vitro*.

Peer review

Generally speaking, the paper presents a laboratory-based study in which a number of highly sophisticated procedures took place, especially in terms of the methods utilized to demonstrate the presence and infectivity of retroviral agent. In this sense, efforts made by the authors deserve particular attention, making this manuscript very interesting.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

March 21-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

June 14-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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Instructions to authors

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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