Published in final edited form as:
Mol Genet Metab. 2008 February ; 93(2): 95–103.

A Humanized Mouse Model for the Reduced Folate Carrier

David Patterson‡, **Christine Graham**‡, **Christina Cherian**#, and **Larry H. Matherly***,#

‡*Eleanor Roosevelt Institute, Department of Biological Sciences, University of Denver, Denver, CO*

**Department of Pharmacology, Barbara Ann Karmanos Cancer Institute and Wayne State University School of Medicine, Detroit, MI*

#*Department of Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute and Wayne State University School of Medicine, Detroit, MI*

Abstract

The ubiquitously expressed reduced folate carrier (RFC) or SLC19A1 is recognized to be an essential transport system for folates in mammalian cells and tissues. In addition to its generalized role as a folate transporter, RFC provides specialized tissue functions including absorption across intestinal/colonic epithelia, transport across the basolateral membrane of renal proximal tubules, transplacental transport of folates, and folate transport across the blood-brain barrier. The human RFC (hRFC) gene is regulated by 5 *major* upstream non-coding regions (designated A1/A2, A, B, C, and D), each transcribed from a unique promoter. Altogether, at least 14 distinct hRFC transcripts can be envisaged in which different 5' untranslated regions (UTRs) are fused to a common splice acceptor region (positions -1 to -49) within the first coding exon with a common 1776 bp coding sequence. The 5' non-coding regions are characterized by alternate transcription start sites, multiple splice forms, and selective tissue distributions. Alternate 5'UTRs impact mRNA stabilities and translation efficiencies, and result in synthesis of modified hRFC proteins translated from upstream AUGs. In this report, we describe production and characterization of transgenic mice (TghRFC1) containing a functional hRFC gene and of humanized mice in which the mRFC gene is inactivated and an active hRFC gene has been introduced. The mice appear to be healthy and to breed well. Analysis of tissue specificity of expression in both the TghRFC1 and humanized hRFC mice by real-time RT-PCR demonstrates that the hRFC gene is expressed with a specificity closely resembling that seen in human tissues. For the humanized hRFC mice, levels of B and A1/A2 5'UTRs predominated in all mice/tissues, thus resembling results in normal human tissues. Lower levels of A and C 5'UTRs were also detected. The availability of humanized mouse models for hRFC will permit investigators to address critical unanswered questions pertinent to human health and disease. These include the ability to analyze the hRFC gene *in vivo*, to control dietary and other environmental conditions that may impact levels of gene expression, and to control the genetics of the mice in order to assess the effects of hRFC gene alterations on tissue folate uptake and distribution, none of which can be easily achieved in human populations.

Keywords

reduced folate carrier; mouse models; folate homeostasis; promoter usage

Address Correspondence to: David Patterson, Ph.D., Eleanor Roosevelt Institute, 1899 Gaylord St., Denver, CO 80206, Tel: 303-871-5650, Fax:303-871-5699, Email: dpatter2@du.edu.

Introduction

Folic acid is the stable, synthetic parent form of the biologically important reduced folates. These cofactors differ in the oxidation state of the pteridine ring, the one-carbon substituent attached at the 5- and/or 10-positions, and the extent of conjugation with additional gamma-linked glutamates. The nutritional requirement for folates derives from their participation in one-carbon transfer reactions leading to biosynthesis of purine nucleotides, thymidylate, serine, and methionine (Stokstad, 1990). Mammalian cells cannot synthesize folates *de novo*, so they must accumulate these derivatives from the outside environment.

Since folates are hydrophilic molecules that are anions at physiologic pH, they cross biological membranes only poorly by diffusion. Although folate cofactor uptake into cells and tissues can be mediated by folate receptors (FRs), the family of organic anion transporters (OATs), and a proton-coupled folate transporter (PCFT), by far the best characterized folate transporter is the ubiquitously expressed reduced folate carrier (RFC; SLC19A1) (Matherly and Goldman, 2003).

Dietary folates are absorbed mostly in the duodenum and upper jejunum, primarily by PCFT (Qiu et al., 2006). However, the expression of RFC throughout the intestine (Chiao *et al.*, 1997; Wang *et al.*, 2001; Said, 2004) implies that it contributes to intestinal folate uptake as well, particularly in the lower intestine. Renal tubular secretion and reabsorption of folates in proximal tubules involves specific roles for folate transporters on the basolateral (e.g., OAT1, OAT3, RFC) and apical (e.g., OATP1, FR alpha) membranes (Russel *et al.*, 2002; Nozaki *et al.*, 2004). FR alpha is localized to the basal (blood) side of the choroid plexus and likely mediates uptake of folates from blood (Spector and Johanson, 2006). RFC is localized on the apical membrane of the bulbous microvilli of the choroid plexus epithelium adjacent to the ventricular membrane (Wang *et al.*, 2001), suggesting its role in transporting folates into the cerebral spinal fluid at the apical surface. The recent finding that at least some cases of hereditary folate malabsorption syndrome are accompanied by low levels of central nervous system (CNS) and plasma folates and a loss of functional PCFT (Qui *et al.*, 2006), implies a role for PCFT in CNS folate transport along with those for FR alpha and RFC. Finally, both FR alpha and RFC are involved in transplacental transport of folates (Sweiry and Yudlievich, 1985; Barber *et al.*, 1999). Thus, while the RFC is the major transport system for folates in mammalian cells and plays an integral role in *in vivo* folate homeostasis and tissue-specific folate transport, this is often in concert with other folate transport systems such as PCFT and FR alpha.

The structure of the human RFC (hRFC) gene was first described in 1998 (Zhang *et al.*, 1998; Tolner *et al.*, 1998). Five coding exons with conserved boundaries and high homology between humans and rodents were identified (Zhang *et al.*, 1998; Tolner *et al.*, 1998). hRFC is regulated by tissue-specific utilization of up to 5 *major* alternately spliced upstream non-coding regions (A1/A2, A, B, C, D) and multiple promoters spanning over 35 kb (Whetstine and Matherly, 2001; Whetstine *et al.*, 2002a,b; Liu *et al.*, 2004; Payton *et al.*, 2005a,b). Thus, hRFC expression is regulated by combinatorial effects of multiple diverse families of transcription factors (i.e., Sp, bZip, basic Helix-loop-helix leucine zipper, Ikaros, Ap2, and C/EBP) (Whetstine and Matherly, 2001; Whetstine *et al.*, 2002b; Liu *et al.*, 2004; Payton *et al.* 2005a,b), coupled with posttranscriptional effects on translational efficiency, transcript stability, and the synthesis of modified hRFC proteins initiated from upstream AUGs (Flatley *et al.*, 2004; Payton *et al.*, 2007). For mouse RFC (mRFC), 4 unique 5' non-coding regions were identified (mRFC-*a*, -*b*, -*c*, and -*d*), flanking unique promoters (Liu *et al.*, 2005). There appears to be at least some conservation of putative *cis* elements in the mRFC promoters compared to those identified in the hRFC-A1/A2, -B, and -C minimal promoters. The mRFC

gene was localized to chromosome 10 (Roy *et al.*, 1998) and the hRFC gene to 21q22.3 (Yang-Feng *et al.*, 1995; Moscow *et al.*, 1995) by fluorescence *in situ* hybridization (FISH).

Given its important role to tissue folate homeostasis, transport defects involving hRFC can easily be envisaged to contribute to pathophysiologic conditions associated with folate deficiency including cardiovascular disease, fetal abnormalities, neurologic disorders, and cancer (Matherly, 2004). These may be compounded by changes in catalytic activities of folate-dependent interconverting and biosynthetic enzymes (e.g., 5, 10-methylenetetrahydrofolate reductase) that impact cellular distributions of individual tetrahydrofolate forms [e.g., 5, 10-methylenetetrahydrofolate and 5-methyltetrahydrofolate] (Lucock, 2000; Matherly, 2004) and by functionally important (G/A80) polymorphisms in the hRFC locus (Chango *et al.*, 2000; Whetstine *et al.*, 2002b; O'Leary *et al.*, 2006). The cumulative effects of these alterations might be cellular or tissue folate deficiencies that result in impaired nucleotide biosynthesis and repair of DNA damage, and/or DNA hypomethylation due to decreased S-adenosyl methionine.

Given the challenges of studying these conditions in humans, we reasoned that mice in which the mRFC gene has been replaced with the hRFC gene would provide significant benefits in understanding the critical role played by RFC in folate homeostasis in health and disease. These include the ability to analyze the hRFC gene *in vivo* and to control dietary and other environmental conditions that may impact levels of gene expression, and to control the genetics of the mice, in order to assess the effects of hRFC gene alterations on tissue folate uptake and distribution, none of which can be easily achieved in human populations. We describe herein the production and initial characterization of such "humanized" hRFC mice.

Materials and Methods

Isolation and characterization of a P1 clone containing the hRFC gene

A human genomic P1 BAC library (Shepherd *et al.*, 1994) was screened as previously described (Brodsky *et al.*, 1997; Kraus *et al.*, 1998) using standard PCR conditions with a pair of oligonucleotide primers which amplify hRFC. The forward primer was 5'-CTCCTTCTCCACGCTCAAC and the reverse primer was 5'-GAAGCCGAGGTTTCGCACC. These primers produce an amplicon of 135 nt from positions 350-484 of the second coding exon of the hRFC gene. A P1 clone designated P33A12 (77.4 kb insert in 16 kb vector pAd10SacBII), containing 33.2 kb of the hRFC gene and including coding exons 1-5 and non-coding regions/promoters A1/A2, A, B, and C (Whetstine *et al.*, 2002a), was isolated. The locations of the insert ends were determined by end sequencing by the University of Colorado Cancer Center DNA Sequencing and Analysis Core and BLAST analysis of the human genome (Altschul *et al.*, 1990).

To test for hRFC gene expression, we used Lipofectamine 2000 (Invitrogen) to transfect P33A12 DNA into R5 HeLa cells (courtesy of I. David Goldman, Albert Einstein School of Medicine), which are null for the hRFC gene (Zhao *et al.*, 2004). R5 hRFC transfectants were selected for a functional hRFC gene by growing the cells in folate-free RPMI 1640 media with L-glutamine (Gibco) at pH 7.4 containing 10% charcoal-stripped fetal calf serum (Valley Biomedical), 20 μM 2-mercaptoethanol, antibiotics, 25 nM (6R,S)5-formyltetrahydrofolate (leucovorin) (5-CHO-THF) (Sigma), and 75 nM of the lipid-soluble antifolate, trimetrexate (Drug Development Branch, National Cancer Institute, Bethesda, MD) (Zhao *et al.*, 2004). Clones were isolated and tested for growth in medium containing 25 nM 5-CHO-THF and 200 nM trimetrexate.

Mouse husbandry

All animal experimentation was approved by the University of Denver Institutional Animal Care and Use Committee. Mice were fed LabDiet autoclavable diet 5K52 (PMI Nutrition International, LLC) *ad libitum* which was autoclaved prior to use. This diet has 2 mg/kg folic acid added but also probably contains significant amounts of methylfolates, which are the forms typically found in cereals and in other dietary components.

Isolation of transgenic mice carrying the hRFC gene

P1 P33A12 DNA was digested with Sal I and the 64,979 nt linear DNA fragment (48,968 nt human insert plus 16011 nt vector) containing hRFC was purified by electrophoresis. Transgenic mice carrying the hRFC gene were produced from FVB mice using this fragment as described previously (Butler *et al.*, 2006). Transgenic mice were genotyped by PCR. The forward primer was 5'AGGCAGCTGAATTCCTGAGC and the reverse primer was 5'TTCTGAACACCGTCGCTTGG. These primers generate an amplicon of 169 nt from positions 301 to 469 of hRFC and do not amplify mRFC. PCR conditions were 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, for 30 cycles. Amplicons were analyzed on a 1% agarose gel stained with ethidium bromide.

To determine the number of integration sites of the transgene and to verify that the hRFC transgene had not integrated into mouse chromosome 10 (the location of the mRFC gene), metaphase spreads were prepared from transgenic mouse spleens and analyzed by FISH as described previously (Butler *et al.*, 2006).

A Nick Translation Kit (Vysis) was used to directly label P33A12 (hRFC) DNA and DNA from a BAC (RP23-56K17) containing mRFC, with Spectrum Orange (Vysis) and Spectrum Green (Vysis), respectively, to use as FISH probes. Metaphase spreads were analyzed as described previously (Butler *et al.*, 2006). A minimum of 10 metaphase spreads per slide were analyzed, and representative metaphase spreads were captured with a Cytovision imaging system (Applied Imaging Corp.).

Mice from two matings of the transgenic line (one-copy transgenic x one-copy transgenic; therefore carrying the same transgene integration site) were analyzed by FISH to establish whether mice with two hRFC integration sites could be generated. This provides evidence that the integration of the hRFC transgene did not interrupt an essential mouse gene. Blood smears were prepared from each of the transgenic pups on pre-cleaned Superfrost/Plus microscope slides (Fisher). The smears were fixed for 2 minutes in methanol:glacial acetic acid (1:1 vol:vol) and 2 minutes in methanol:glacial acetic acid (3:1 vol:vol). Probe was prepared by incorporating Spectrum Orange (Vysis) direct-labeled dUTP into P33A12 DNA using a Nick Translation Kit (Vysis). Fixed cells were co-denatured with 1 μl probe in 4 μl LSI/WCP Hybridization Buffer (Vysis) for 5 minutes at 80°C. This was followed by a 16-hour hybridization period at 37°C in a humidified chamber. Slides were post-washed in 0.4X SSC at 72±1°C for 1 minute, then in 2X SSC/0.1% NP-40 at room temperature for 1 minute. Slides were allowed to dry, then counterstained with DAPI (4',6-diamidino-2-phenylindole)/Antifade (Vysis) and stored at 4°C. Hybridized nuclei were visualized using a Zeiss Axiovert 200M Microscope with DAPI and Cy3 filters (to detect DAPI counterstain and Spectrum Orange-labeled P33A12 probe, respectively). Representative nuclei were analyzed with a Zeiss Axiovert 200 inverted epifluorescence microscope with Zeiss Axiovision image analysis software.

Generation of mice containing hRFC but no functional mRFC (humanized mice)

To produce humanized hRFC mice, mice transgenic for hRFC (designated TghRFC1) were mated with C57BL/6 mice with one active and one inactive mRFC allele that were graciously

supplied by Dr. I. David Goldman (Zhao *et al.*, 2001). Detection of the inactivated mRFC allele was as previously described (Zhao *et al.*, 2001). Mice carrying the human transgene and one inactivated and one normal mRFC allele were then crossed. These crosses generated some mice with two inactivated mRFC alleles (double knockouts) and one or two copies of the hRFC transgene. The double knockout mRFC genotype is lethal in mice without the hRFC transgene (Zhao *et al.*, 2001), but mice with two inactivated mRFC alleles that are transgenic for hRFC appear healthy and breed well. Double knockouts with both one and two copies of the hRFC transgene were crossed. All offspring from these crosses were double knockouts. Those with two copies of the hRFC transgene (identified by FISH as described above,) were bred to generate a line of humanized mice.

Real-time RT-PCR analysis of mRFC and hRFC transcript levels

mRNAs from normal human tissues were purchased from Clontech (Mountain View, CA). RNAs from TghRFC1 mice and humanized hRFC mice were isolated from selected tissues using Trizol (Invitrogen). Total levels of hRFC and mRFC transcripts, as well as levels of hRFC transcripts with the individual 5' UTRs (A1/A2, A, B, C), were measured by real-time RT-PCR (*q*PCR). hRFC and mRFC transcript levels were normalized to levels of mouse 18S RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. Conditions and primers are identical to those previously described (Liu *et al.*, 2005, 2006). For mouse GAPDH, the primers were 5'TGCACCACCAACTGCTTAG and 5'GGATGCAGGGATGATGTT. All *q*PCR measurements were repeated 2-3 times.

Results

Isolation and characterization of a P1 clone containing a functional hRFC gene

The P1 clone P33A12 was isolated as described in Materials and Methods. The insert of P33A12 spans from nucleotide (nt) 45,714,910 to 45,792,261 of chromosome 21, and is 77,351 nt long. P33A12 contains 9960 nt of DNA 5' (telomeric) of the translation start codon of hRFC and 44,157 nt 3' of the hRFC 3' UTR, and does not contain any other functional genes or hypothetical genes.

To test whether the hRFC gene in P33A12 is functional, we transfected HeLa R5 cells, which are null for the hRFC gene (Zhao *et al.*, 2004) with P33A12 DNA and isolated clones that grew in 25 nM 5-CHO-THF and 75 nM trimetrexate. Several of these clones were screened by PCR for the presence of the hRFC gene and then tested against growth in 25 nM 5-CHO-THF and 200 nM trimetrexate. Figure 1 presents typical results for one of these clones and shows obvious and significant growth for wild type HeLa and P33A12-transfected cells, and no growth for untransfected R5 cells. This demonstrates that the hRFC gene in P33A12 is functional.

Production of transgenic mice containing hRFC using P33A12

P33A12 DNA was digested with Sal I to generate a 64,979 nt linear DNA fragment (48,968 nt human insert plus 16,011 nt vector) containing the hRFC gene. The 5' Sal I site resides in the P1 vector, while the 3' site lies at nt 45,743,298 of chromosome 21. Thus, the Sal I fragment containing the hRFC gene also contains 9960 nt 5' of the hRFC coding region, along with hRFC 5' non-coding regions/promoters A1/A2 through C and 15,259 nt 3' of the hRFC 3' UTR. This fragment was gel purified and used to produce transgenic hRFC mice. Three independent lines of hRFC-containing transgenic mice were generated and one, designated TghRFC1, was selected for further analysis.

To determine the number and chromosomal location(s) of P33A12 integrations sites, we performed dual color FISH. Figure 2 shows that there is a single integration site of P33A12

DNA in TghRFC1 mice (indicated in red in Figure 2) and that this is not located on mouse chromosome 10 (indicated by the green hybridization signal in Figure 2).

To assess whether integration of hRFC into the TghRFC1 mouse genome might have inactivated an important mouse gene, we mated TghRFC1 mice and examined offspring for mice containing two copies of the hRFC integration site by FISH. Since the parental mice are from the same transgenic line, they have the same chromosomal integration site. Thus, if the integration of the transgene does not inactivate an essential mouse gene, roughly one-fourth of the offspring would be expected to inherit both chromosome homologues containing the integrated transgene. Analysis of 12 mice from two independent TghRFC1 x TghRFC1 crosses revealed that 3 of the mice had 2 copies of the hRFC transgene integration site. These mice had no visible deleterious phenotype and appeared healthy. A representative interphase FISH result using fluorescently tagged P33A12 DNA for two mice, each with two integration sites, is shown in Figure 3.

We measured transcript levels for hRFC and mRFC in various tissues of TghRFC1 mice using *q*PCR methods (Liu *et al.*, 2005, 2006). Total RNAs were prepared from several tissues, and species-specific PCR primers were used to measure expression of hRFC and mRFC in the same tissue RNAs. In TghRFC1 mice, we found that hRFC exhibits a tissue-specific expression pattern that resembles that observed in humans (e.g. liver≫kidney) (Whetstine *et al.*, 2002a), while the mRFC expression pattern in these same samples is typical of mRFC patterns (e.g. kidney≫liver) in normal mice (Liu *et al.*, 2005) (Figure 4). For the TghRFC1 mice, the absolute expression levels for hRFC were approximately 10-20% of those for mRFC in the individual tissues. Most importantly, it appears that in the TghRFC1 mouse, the human *cis*-regulatory regions of hRFC function as if they were in a *human background*, while the *cis*-regulatory regions for the endogenous mouse gene function as expected in the mouse.

Production of a humanized mouse expressing hRFC but not mRFC

We accomplished our goal of producing a humanized hRFC mouse by breeding TghRFC1 mice with C57BL/6 mice heterozygous for an active and an inactive mRFC. Then, mice carrying the human transgene and one copy of the inactive mRFC gene and one normal mRFC gene were crossed. Of each 16 offspring, we would theoretically predict 2 mice carrying no active mRFC gene and one hRFC transgene, and one mouse carrying no active mRFC gene and two copies of the hRFC transgene. We obtained 3 mice with no active mRFC and one copy of the hRFC transgene, and one mouse with no active mRFC gene and two copies of the hRFC transgene from the first 26 offspring. Figure 5 (left panel) shows PCR amplification from genomic DNAs demonstrating disruption of the endogenous mRFC gene. Figure 5 (right panel) shows PCR analysis with hRFC-specific primers of the same samples in the left panel. Figure 6 shows an interphase FISH experiment of two of these mice using fluorescently labeled hRFC-containing P1 clone P33A12 DNA.

Figure 7 shows *q*PCR analysis of total hRFC transcripts in the humanized hRFC mice. All mice were 2.5 to 3 months old. Mice A2812, A2858, and A2834 were males and mice A2975 and A2835 were female. The results clearly show robust expression of hRFC with the highest level in liver, consistent with published results (Whetstine *et al.*, 2002a).

Since the hRFC gene in the P33A12 construct includes 4 of the major hRFC non-coding regions and promoters (A1/A2, A, B, and C) (Figure 8, upper panel), we used *q*PCR to also measure levels of the individual transcript forms and by inference, promoter usage for comparison with normal human tissues. Figure 8 (lower panel) shows *q*PCR analysis of the hRFC 5'untranslated regions (5'UTRs) from commercial mRNAs obtained from normal human tissues that establishes hRFC-B and −A1/A2 as the major transcript forms, although lower levels of other 5'UTRs were detectable and were preferentially expressed in certain tissues. In Figure 9,

*q*PCR results in the humanized mice are shown. Again, the A1/A2 and B 5'UTRs predominated. The finding that the hRFC A 5'UTR was detected in some samples is particularly interesting given that there is no apparent mouse homolog (Liu *et al.*, 2005). As there were only 2 female mice, it was not possible to broadly study 5'UTRs in placental hRFC transcripts. However, when tested, transcripts including the C 5'UTR were detected in placenta (not shown). Thus, with minor caveats, the results obtained with the humanized hRFC mouse closely resemble those obtained with human tissues.

Discussion

In this manuscript, we describe production and characterization of transgenic mice containing a functional hRFC gene and of humanized mice in which the mRFC gene is inactivated and an active hRFC gene has been introduced. The mice appear to be healthy and to breed well. Analysis of tissue specificity of expression in the TghRFC1 mice by *q*PCR demonstrates that the hRFC gene is expressed with a specificity closely resembling that seen in human tissues whereas, as expected, the tissue specificity of mRFC is that typical of mRFC in normal mice.

Although the hRFC gene construct used for generation of the mice includes 5' non-coding regions and promoters A1/A2 through C, such that exons D and E and promoters are missing, these latter transcript forms are comparatively rare. Indeed, no overt deleterious effects appeared to result from the loss of the D and E hRFC transcripts, and the tissue hRFC levels and patterns of hRFC usage closely resemble those seen in normal human tissues despite the absence of the upstream D and E regions. For instance, levels of B and A1/A2 5'UTRs predominated in all mice/tissues, thus resembling results in normal human tissues. Lower levels of A and C 5'UTRs were also detected in some mice/tissues. In our experiments with a small number of humanized hRFC mice, there was some variability in hRFC levels and transcript forms from mouse to mouse. While the basis for this is not entirely clear, a likely explanation is that the humanized hRFC mice have a mixed genetic background (FVB X C57BL/6). This will likely be resolved with additional crosses in future studies.

Understanding the genetic determinants that contribute to *in vivo* folate homeostasis is an increasingly pressing public health issue. Clearly, supplementation of the food supply with folate beginning in the U.S. in 1998 has resulted in a pronounced decrease in the incidence of neural tube defects in newborns. However, the potential long term adverse effects of chronic folate supplementation with respect to other health issues, including cancer, heart disease, and cognitive function are not yet known (Lucock and Yates, 2005; Kim, 2006). (i) Evidence exists that folate supplementation can, under some circumstances, accelerate development of pre-existing malignancies, including colorectal cancer and breast cancer (Ulrich and Potter, 2006; Kotsopoulos *et al.*, 2005), two of the most common cancers in humans. One study suggested that folate supplementation in pregnancy could increase the risk of breast cancer later in life (Charles *et al.*, 2004). A recent study demonstrated a temporal association between the introduction of folic acid supplementation in the U.S. and Canada and an increase in colorectal cancer rates of about 4 to 6 per 100,000 people (Mason *et al.*, 2007). These authors hypothesized that folate supplementation may have been responsible at least in part for the increase in colorectal cancer. (ii) Additional evidence suggests that high folate levels can have deleterious effects on cognitive ability in older people (Ulrich and Potter, 2006; Morris *et al.*, 2005). (iii) Even in the case of neural tube defects and other birth defects, there is some controversial evidence linking folate supplementation (or folate containing multivitamin supplementation) to increased risk of abortions, twinning, and chromosomal anomalies like Down syndrome (Canfield *et al.*, 2005; Czeizel *et al.*, 1994; Hook, 2001; Czeizel, 2001; Muggli and Halliday, 2006). (iv) Elevated homocysteine levels have been associated with increased risk of cardiovascular disease. It is also accepted that supplementation with B vitamins including folate is effective in reducing homocysteine levels and may reduce the risk of

cardiovascular disease. However, two major studies have now been published showing that, while these supplements do decrease homocysteine levels, they do not necessarily decrease risk of cardiovascular disease and may even increase the risk of certain types of cardiovascular events (Bonaa *et al.*, 2006; Lonn *et al.*, 2006). Manipulation of dietary folates in the mice described here presents an alternative, complementary approach to examining these issues in an easily controlled animal model.

Given the central role of hRFC in *in vivo* folate homeostasis, it is not surprising that this gene is under complex and exquisite control. A key feature of this regulation involves the use of at least five major 5' non-coding regions and associated promoters (Whetstine and Matherly, 2001; Whetstine *et al.*, 2002a,b; Liu *et al.*, 2004; Payton *et al.*, 2005a,b). hRFC promoter/5' non-coding region utilization is highly tissue-specific and is controlled by both ubiquitous and tissue-specific transcription factors. Moreover, hRFC protein levels are subject to additional posttranscriptional controls including alternate splicing, translation efficiencies, transcript stabilities, and synthesis of N-terminally modified hRFCs (Flatley *et al.*, 2004; Payton *et al.*, 2007). Collectively, these mechanisms can significantly impact tissue and plasma levels of folate cofactors. An important unexplored premise concerns the possible contributions of modifications in hRFC gene structure, expression, or function, involving promoter, 5' non-coding region, or coding sequence alterations (including naturally occurring polymorphisms) on normal folate homeostasis and pathophysiologies associated with folate deficiency. For instance, polymorphisms involving hRFC promoter A (Whetstine *et al.*, 2002b; O'Leary *et al.*, 2006) or the hRFC coding (G/A80) region (Chango *et al.*, 2000) have been implicated in an assortment of human conditions (Matherly *et al.*, 2004).

We posit that the availability of humanized mouse models for hRFC will permit investigators to address critical unanswered questions pertinent to human health and disease. For instance, dietary studies with our humanized mouse models could provide important insights into the extent and mechanism by which folate supplementation may under some circumstances be harmful, as noted above. It should be possible to prepare additional humanized hRFC mice using transgenes including polymorphisms or mutations in the hRFC coding and non-coding regions to identify their roles in folate homeostasis in response to dietary manipulations. Other questions relate to the role of ontogeny in determining patterns and levels of hRFC expression and promoter usage, and the possibility that loss of hRFC or defects in transcriptional/posttranscriptional controls may contribute to human pathophysiologies typically associated with folate deficiencies. If hRFC levels or function are compromised, these conditions could occur independent of the intake of dietary folates. Clearly, future studies with humanized mouse models for hRFC will complement those with cultured cells or with mRFC homo- and hemizygous knockout mouse models and, likewise, published correlative epidemiologic investigations in humans.

Acknowledgements

We would like to thank Dr. I. David Goldman (Albert Einstein School of Medicine) for his gifts of hRFC-null R5 HeLa cells and C57BL/6 mice with one active and one inactive mRFC allele, and Jean M. Smith and Lynne Meltesen (Colorado Genetics Laboratory, Department of Pathology, University of Colorado at Denver and Health Sciences Center) for FISH analysis of metaphase spreads of transgenic mice. DNA sequencing was performed at the University of Colorado Cancer Center DNA Sequencing & Analysis Core. This study was supported in part by grant CA53535 from the National Cancer Institute and grant HD17449 from the National Institute of Child Health and Human Development, National Institutes of Health, a grant from the Jerome Lejeune Foundation, and a grant from the Lowe Fund of the Denver Foundation.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1999;215:403–410. [PubMed: 2231712]

Barber RC, Bennett GD, Greer KA, Finnell RH. Expression of patterns of folate binding proteins one and two in the developing mouse embryo. Mol Genet Metab 1999;66:31–39. [PubMed: 9973545]

Bonaa KH, Njolstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, Wang H, Nordrehaug JE, Arnesen E, Rasmussen K. NORVIT Trial Investigators, Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med 2006;354:1578–1588. [PubMed: 16531614]

Brodsky G, Barnes T, Bleskan J, Becker L, Cox M, Patterson D. The human GARS-AIRS-GART gene encodes two proteins which are differentially expressed during human brain development and temporally overexpressed in cerebellum of individuals with Down syndrome. Hum Mol Genet 1997;6:2043–2050. [PubMed: 9328467]

Butler C, Knox AJ, Bowersox J, Forbes S, Patterson D. The production of transgenic mice expressing human cystathionine beta-synthase to study Down syndrome. Behav Genet 2006;36:429–438. [PubMed: 16541333]

Canfield MA, Collins JS, Botto LD, Williams LJ, Mai CT, Kirby RS, Pearson K, Devine O, Mulinare J. Changes in the birth prevalence of selected birth defects after grain fortification with folic acid in the United States: findings from a multi-state population-based study. Birth Defects Research (Part A) 2005;73:679–689.

Chango A, Emery-Fillon N, Potier de Courcy G, Lambert D, Pfister M, Rosenblatt DS, Nicolas J-P. A polymorphism (80G->A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. Mol Genet Metab 2000;70:310–315. [PubMed: 10993718]

Charles D, Ness AR, Campbell D, Davey Smith G, Hall MH. Taking folate in pregnancy and risk of maternal breast cancer. British Medical Journal 2004;329:1375–1376. [PubMed: 15591563]

Chiao JH, Roy K, Tolner B, Yang CH, Sirotnak FM. RFC-1 gene expression regulates folate absorption in mouse small intestine. J Biol Chem 1997;273:11165–11170. [PubMed: 9111015]

Czeizel AE. Miscarriage and use of multivitamins or folic acid. Am J Med Genet 2001;104:179–180. [PubMed: 11746052]

Czeizel AE, Metneki J, Dudas I. The higher rate of multiple births after periconceptual multivitamin supplementation; an analysis of causes. Acta Genet Med Gemellol (Roma) 1994;43:175–184. [PubMed: 8588492]

Flatley RM, Payton SG, Taub JW, Matherly LH. Primary acute lymphoblastic leukemia cells use a novel promoter and 5'non-coding exon for the human reduced folate carrier that encodes a modified carrier translated from an upstream translational start. Clin Cancer Res 2004;10:5111–5122. [PubMed: 15297414]

Hook EB. Statistical and logical considerations in evaluating the association of prenatal folic-acid supplementation with pregnancy loss. Am J Med Genet 2001;104:181–182. [PubMed: 11746053]

Kim YI. Folate: a magic bullet or a double edged sword for colorectal cancer prevention? Gut 2006;55:1387–1389. [PubMed: 16966698]

Kotsopoulos J, Medline A, Renlund R, Sohn K-J, Martin R, Hwang SW, Lu S, Archer MC, Kim Y-I. Effects of dietary folate on the development and progression of mammary tumors in rats. Carcinogenesis 2005;26:1603–1612. [PubMed: 15888495]

Kraus JP, Oliveriusova J, Sokolova J, Kraus E, Vlcek C, de Franchis R, MacLean KN, Bao L, Bukovska G, Patterson D, Paces V, Ansorge W, Kozich V. The human cystathionine-beta synthase (CBS) gene: complete sequence, alternative splicing, and polymorphisms. Genomics 1998;52:312–324. [PubMed: 9790750]

Liu M, Ge Y, Cabelof DC, Aboukameel A, Heydari AR, Mohammad R, Matherly LH. Structure and regulation of the murine reduced folate carrier gene: identification of 4 non-coding exons and promoters and regulation by dietary folates. J Biol Chem 2005;280:5588–5597. [PubMed: 15579899]

Liu M, Whetstine JR, Payton SG, Ge Y, Flatley RM, Matherly LH. Roles of USF, Ikaros, and Sp proteins in the transcriptional regulation of the human reduced folate carrier B promoter. Biochem J 2004;383:249–257. [PubMed: 15214842]

Liu M, Ge Y, Payton SG, Aboukameel A, Buck S, Flatley RM, Haska C, Mohammad R, Taub JW, Matherly LH. Transcriptional regulation of the human reduced folate carrier in childhood acute lymphoblastic leukemia cells. Clin Cancer Res 1996;12:608–616. [PubMed: 16428507]

Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, McQueen MJ, Probstfield J, Fodor G, Held C, Genest J. Heart Outcomes Prevention Evaluation (HOPE) 2 Investigators, Homocysteine lowering

with folic acid and B vitamins in vascular disease. N Engl J Med 2006;354:1567–1577. [PubMed: 16531613]

Lucock M, Yates Z. Folic acid - vitamin and panacea or genetic time bomb? Nat Rev Genet 2005;6:235–240. [PubMed: 15738964]

Lucock M. Folic acid: Nutritional biochemistry, molecular biology, and role in disease processes. Mol Genet Metabol 2000;71:121–138.

Mason JB, Dickstein A, Jacques PF, Haggarty P, Selhub J, Dallal G, Rosenberg IW. A temporal association between folic acid fortification and an increase in colorectal cancer rates my be illuminating important biological principles: a hypothesis. Cancer Epidemiol Biomarkers Prev 2007;16(7):1325–1329. [PubMed: 17626997]

Matherly LH, Goldman ID. Membrane transport of folates. Vitamins and Hormones 2003;66:403–456. [PubMed: 12852262]

Matherly LH. Human reduced folate carrier gene and transcript variants: functional, physiologic, and pharmacologic consequences. Current Pharmacogenetics 2004;2:287–298.

Morris MC, Evans DA, Bienas JL, Tangney CC, Herbert LE, Scherr PA, Schneider JA. Dietary folate and vitamin b12 intake and cognitive decline among community-dwelling older persons. Arch Neurol 2005;62:641–645. [PubMed: 15824266]

Moscow JA, Gong MK, He R, Sgagias MK, Dixon KH, Anzick SL, Meltzer PS, Cowan KH. Isolation of a gene encoding a human reduced folate carrier (RFC1) and analysis of its expression in transport-deficient, methotrexate-resistant human breast cancer cells. Cancer Res 1995;55:3790–3794. [PubMed: 7641195]

Muggli EE, Halliday JL. Folic acid and risk of twinning: a systematic review of the recent literature, July 1994 to July 2006. Med J Aust 2007;186:243–248. [PubMed: 17391087]

Nozaki Y, Kusuhara H, Endou H, Sugiyama Y. Quantitative evaluation of the drug–drug interactions between methotrexate and nonsteroidal anti-inflammatory drugs in the renal uptake process based on the contribution of organic anion transporters and reduced folate carrier. J Pharmacol Exp Ther 2004;309:226–234. [PubMed: 14722319]

O'Leary VB, Pangilinan F, Cox C, Parle-McDermott A, Conley M, Molloy AM, Kirke PN, Mills JL, Brody LC, Scott JM. Members of the Birth Defects Research Group, Reduced folate carrier polymorphisms and neural tube defect risk. Molecular Genetics and Metabolism 2006;87:364–369. [PubMed: 16343969]

Payton SG, Haska CL, Flatley RM, Ge Y, Matherly LH. Effects of 5' untranslated region diversity on the posttranscriptional regulation of the human reduced folate carrier. Biochim Biophys Acta 2007;1769:131–138. [PubMed: 17306382]

Payton SG, Whetstine JR, Ge Y, Matherly LH. Transcriptional regulation of the human reduced folate carrier promoter C: Synergistic transactivation by Sp1 and E/EBP beta and identification of a downstream repressor. Biochim Biophys Acta 2005b;1727:45–57. [PubMed: 15652157]

Payton SG, Liu M, Ge Y, Matherly LH. Transcriptional regulation of the human reduced folate carrier A1/A2 promoter: Identification of critical roles for the USF and GATA families of transcription factors. Biochim Biophys Acta 2005a;1731:115–124. [PubMed: 16225938]

Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, Tsai E, Sandoval C, Zhao R, Akabas MH, Goldman ID. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. Cell 2006;127:917–28. [PubMed: 17129779]

Roy K, Chiao JH, Spengler BA, Tolner B, Yang CH, Biedler JL, Sirontak FM. Chromosomal localization of the murine RFC-1 gene encoding a folate transporter and its amplification in an antifolate resistant variant overproducing the transporter. Cancer Genet Cytogenet 1998;105:29–38. [PubMed: 9689927]

Russel FG, Masereeux R, van Aubel RA. Molecular aspects of renal anionic drug transport. Annu Rev Physiol 2002;64:563–594. [PubMed: 11826280]

Said HM. Recent advances in carrier mediated intestinal absorption of water soluble vitamins. Annual Review of Physiol 2004;66:419–446.

Shepherd NS, Pfrogner BD, Coulby JN, Ackerman SL, Vaidyanathan G, Sauer RS, Balkenhol TC, Sternberg N. Preparation and screening of an arrayed human genomic library generated with the P1 cloning system. Proc Natl Acad Sci USA 1994:2629–2633. [PubMed: 8146166]

Spector C, Johanson C. Micronutrient and urate transport in choroid plexus and kidney: Implications for drug therapy. Pharmaceutical Res 2006;23:2515–2524.

Stokstad, ELR. Historical perspective on key advances in the biochemistry and physiology of folates. In: Picciano, MF.; Stokstad, ELR.; Gregory, JF., editors. Folic Acid Metabolism in Health and Disease. Wiley Liss; New York: 1990. p. 1-21.

Sweiry JH, Yudlievich DL. Transport of folates at maternal and fetal sides of the placenta. Lack of inhibition by methotrexate. Biochim Biophys Acta 1985;821:497–501. [PubMed: 4074742]

Tolner B, Roy K, Sirotnak FM. Structural analysis of the human RFC-1 gene encoding a folate transporter reveals multiple promoters and alternatively spliced transcripts with 5' end heterogeneity. Gene 1998;211:331–341. [PubMed: 9602167]

Ulrich CM, Potter JD. Folate supplementation: too much of a good thing? Cancer Epidemiol Biomarkers Prev 2006;15:189–193. [PubMed: 16492904]

Wang Y, Zhao R, Russell RG, Goldman ID. Localization of the murine reduced folate carrier as assessed by immunohistochemical analysis. Biochim Biophys Acta 2001;1513:49–54. [PubMed: 11427193]

Whetstine JR, Flatley RM, Matherly LH. The human reduced folate carrier gene is ubiquitously and differentially expressed in normal human tissues: Identification of seven non-coding exons and characterization of a novel promoter. Biochem J 2002a;367:629–640. [PubMed: 12144527]

Whetstine JR, Matherly LH. The basal promoters for the human reduced folate carrier gene are regulated by a GC-box and a CAMP-response element/AP-1-like element. Basis for tissue-specific gene expression. J Biol Chem 2001;276:6350–6358. [PubMed: 11078737]

Whetstine JR, Witt TL, Matherly LH. The human reduced folate carrier gene is regulated by the AP2 and Sp1 transcription factor families and a 61 base pair polymorphism. J Biol Chem 2002b;277:43873–43880. [PubMed: 12228234]

Yang-Feng TL, Ma YY, Liang R, Prasad PD, Leibach FH, Ganapathy V. Assignment of the human folate transporter gene to chromosome 21q22.3 by somatic cell hybrid analysis and in situ hybridization. Biochem Biophys Res Commun 1995;210:874–879. [PubMed: 7763259]

Zhang L, Wong SC, Matherly LH. Structure and organization of the human reduced folate carrier gene. Biochim Biophys Acta 1998;1442:389–393. [PubMed: 9804996]

Zhao R, Gao F, Hanscom M, Goldman ID. A prominent low-pH methotrexate transport activity in human solid tumors: contribution to the preservation of methotrexate pharmacologic activity in HeLa cells lacking the reduced folate carrier. Clin Cancer Res 2004;10:718–727. [PubMed: 14760095]

Zhao R, Russell RG, Wang Y, Liu L, Gao F, Kneitz B, Edelmann W, Goldman ID. Rescue of embryonic lethality in reduced folate carrier-deficient mice by maternal folic acid supplementation reveals early neonatal failure of hematopoietic organs. J Biol Chem 2001;276:10224–10228. [PubMed: 11266438]

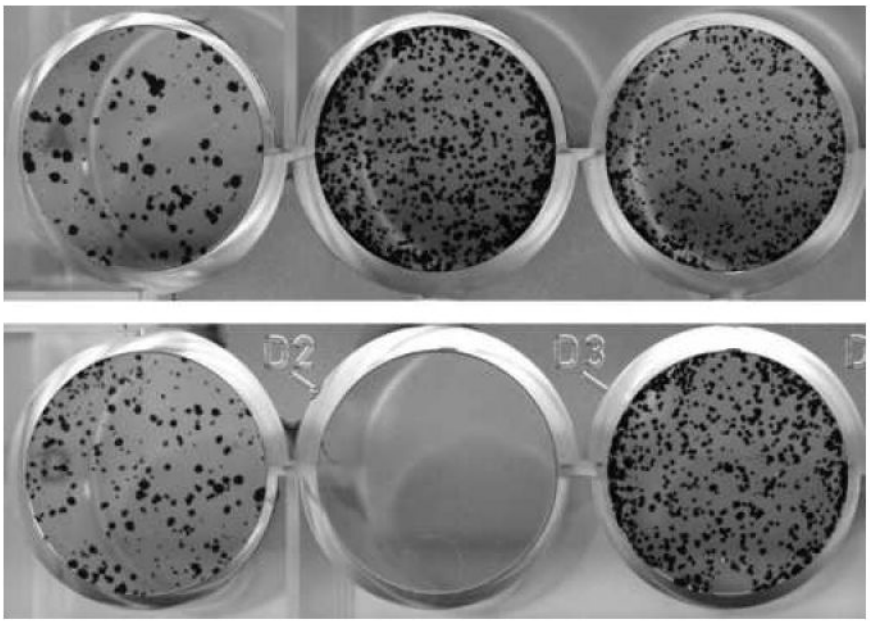

Figure 1. Stable expression of the hRFC gene contained in P1 clone P33A12

hRFC-null HeLa R5 cells were transfected with P33A12 DNA, stable clones picked as described in the text, and cells plated in folate-free media (pH 7.4) containing 25 nM leucovorin with (bottom row) and without (top row) 200nM trimetrexate (TMQ). Left panel, wild type HeLa cells; center panel, R5 cells; right panel, R5 cells stably transfected with P33A12 DNA.

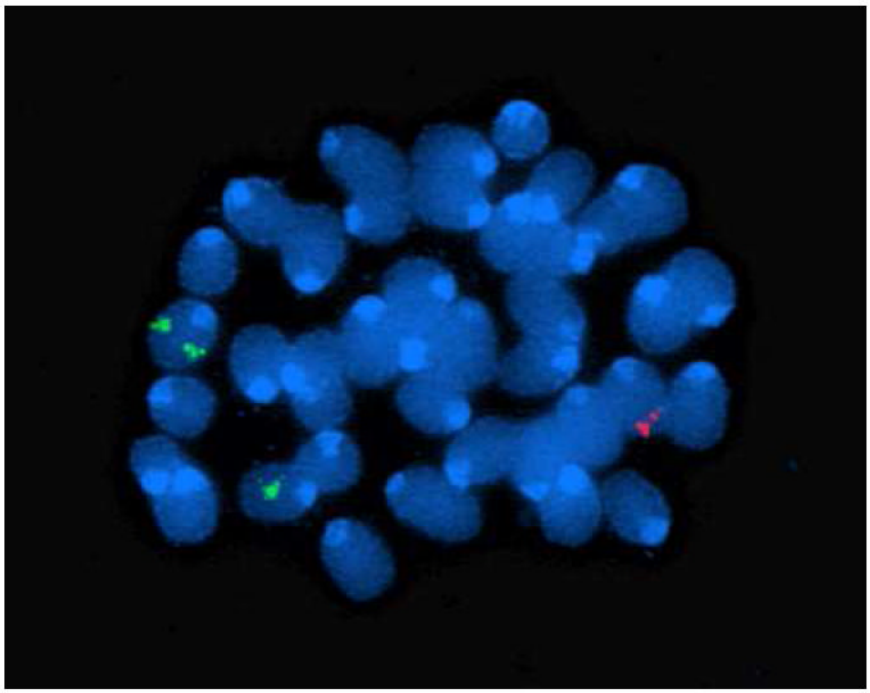

Figure 2. Dual label FISH of a metaphase spread of P33A12 hRFC transgenic mouse spleen cells

P33A12 DNA was labeled with Spectrum Orange and mouse BAC DNA containing the mRFC gene was labeled with Spectrum Green by direct labeling (Vysis). Chromosomes were stained with DAPI. Hybrid metaphase spreads were visualized using an Olympus epifluorescence microscope. Images were captured with a Cytovision imaging system (Applied Imaging Corp).

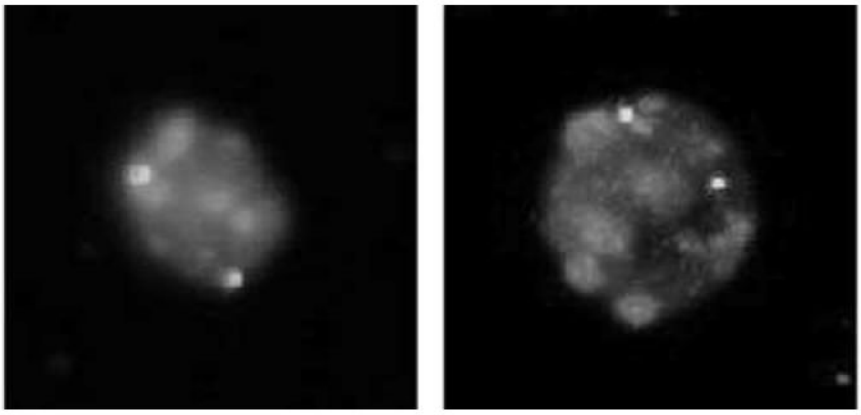

Figure 3. Interphase FISH demonstrating TghRFC1 mice with two hRFC integration sites
Interphase FISH was carried out as described in Materials and Methods.

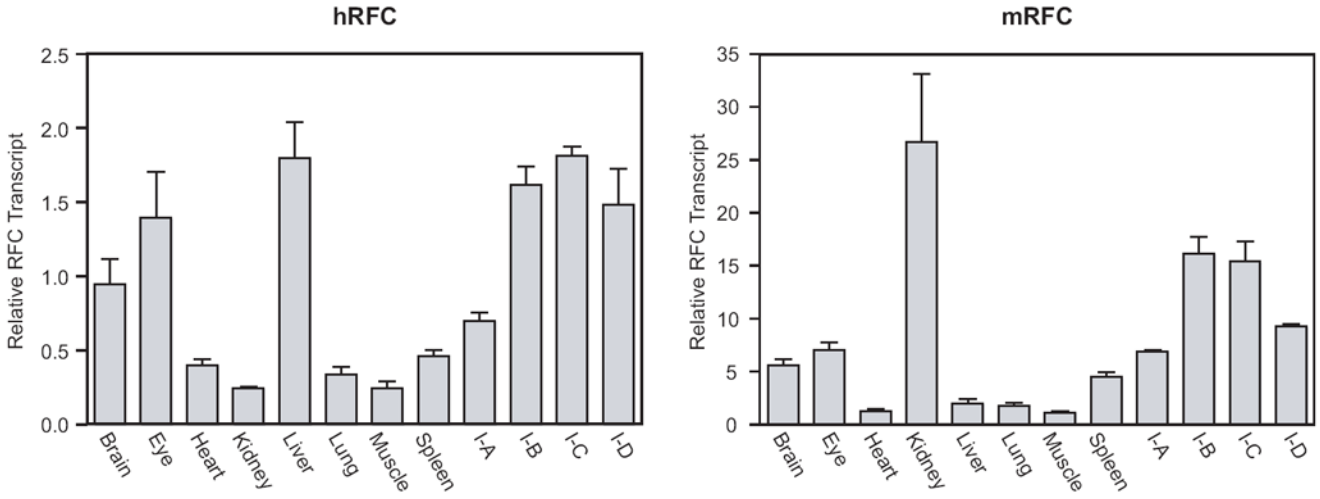

Figure 4. Comparison of hRFC and mRFC expression in Tg mouse tissues

Human-specific (left) and mouse-specific (right) PCR primers were used for *q*PCR to detect relative levels of hRFC and mRFC transcripts in RNAs prepared from tissues from the same hRFC Tg mouse. Normalization was with 18S rRNA. I A through D designate different regions of small intestine (proximal to distal). Only relative transcript levels are reported for hRFC and mRFC. Absolute transcript levels differed by approx. 5- to 10-fold. Results are presented as mean values plus/minus standard error form 3 measurements.

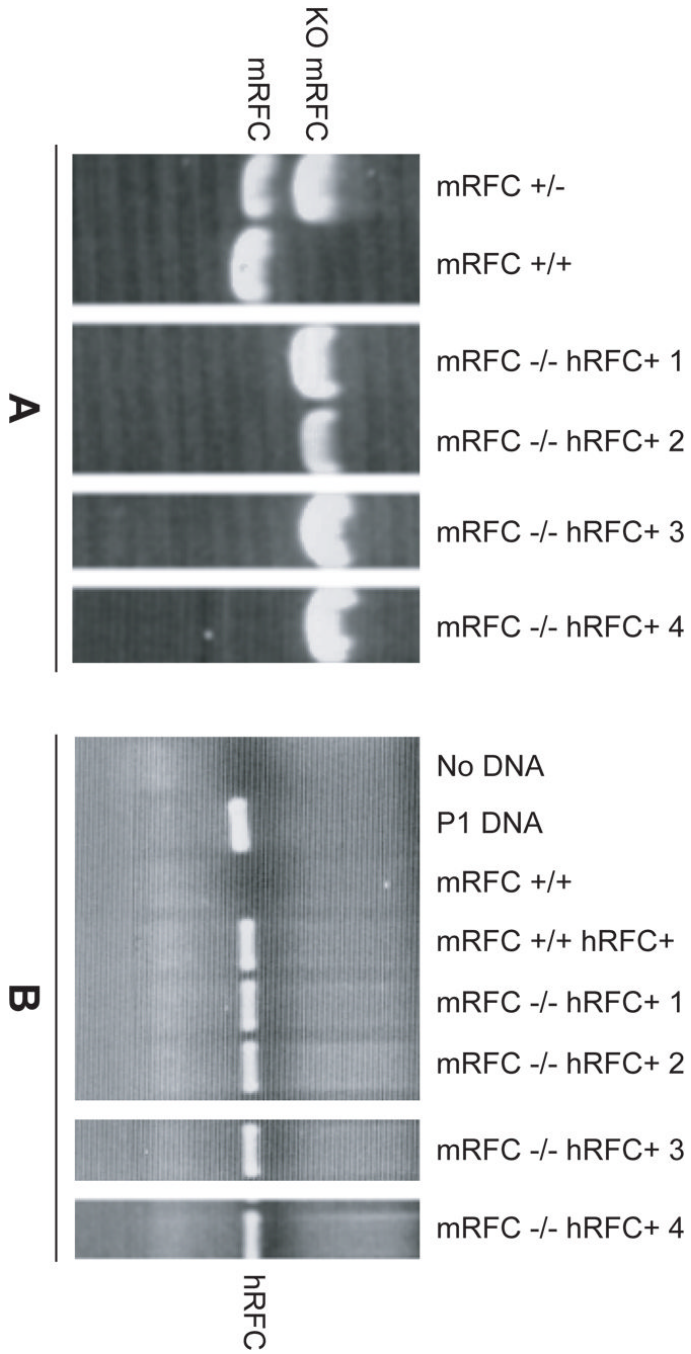

Figure 5. Isolation of mice containing hRFC but no mRFC

A: PCR amplification from genomic DNAs with primers specific for mRFC and for the knocked out (KO) mRFC. mRFC+/- = mouse with one normal mRFC allele and one mRFC KO allele; mRFC+/+ = mouse with two normal mRFC alleles; mRFC-/- hRFC+ 1-4 = different mice with two KO mRFC alleles and one or two hRFC alleles. B: PCR analysis of DNAs with primers specific for hRFC. No DNA = a no DNA control; P1 DNA = DNA isolated from P1 clone P33A12 used to make hRFC transgenic mice; the other lanes are the same DNA samples indicated by the labels in A. The 4 humanized (mRFC-/- hRFC+) mice are from three different litters. There are two male and two female humanized mice.

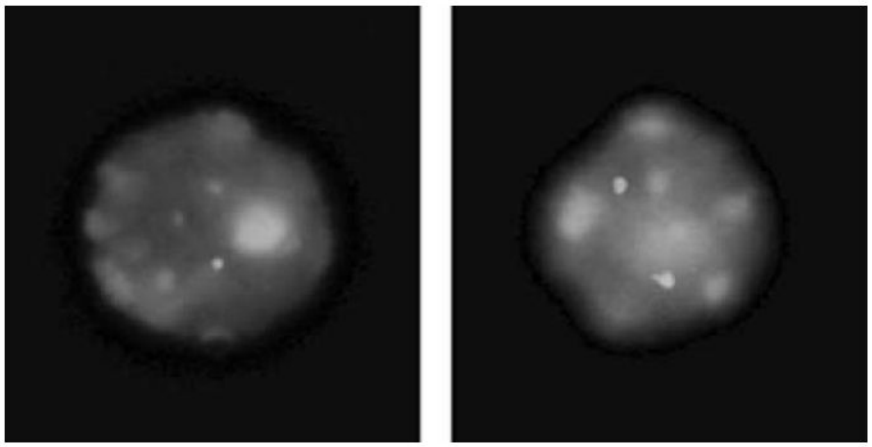

Figure 6. Interphase FISH demonstration of humanized hRFC mice containing one (left) or two (right) integration sites for the P33A12 hRFC containing P1 clone

Interphase FISH was carried out as described in Materials and Methods.

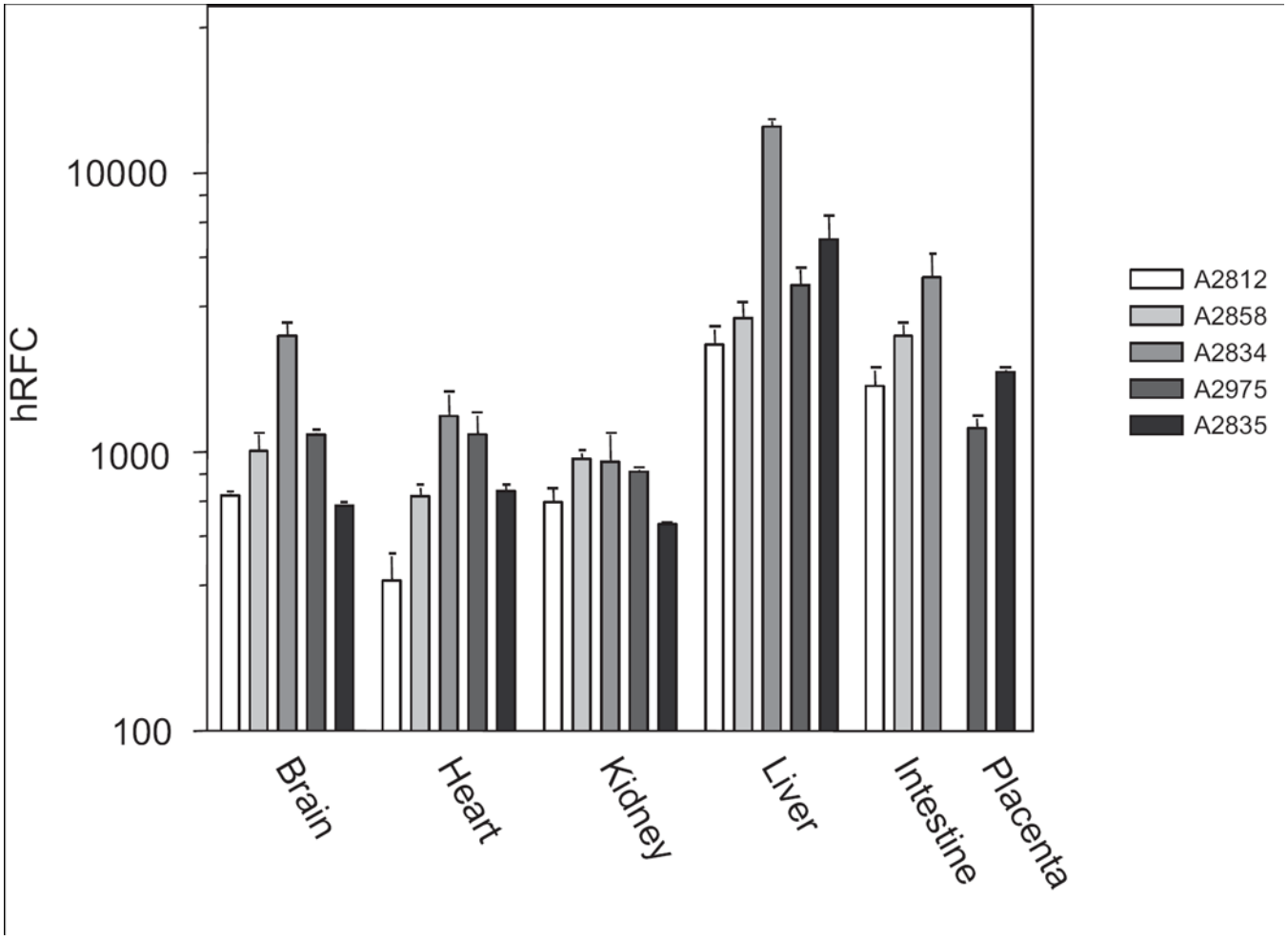

Figure 7. *q*PCR analysis of hRFC expression in humanized mice

Results for total hRFC transcripts for five different humanized mice are shown. RNAs were prepared from tissues isolated from the humanized mice including brain, heart, kidney, liver, intestine, and placenta. RNAs were reverse transcribed and analyzed for total hRFC transcripts by *q*PCR. hRFC transcript levels were normalized to mouse GAPDH transcripts. Intestinal RNAs were not analyzed for mice A2975 and A2835. Results are presented as average values plus/minus ranges from duplicate measurements.

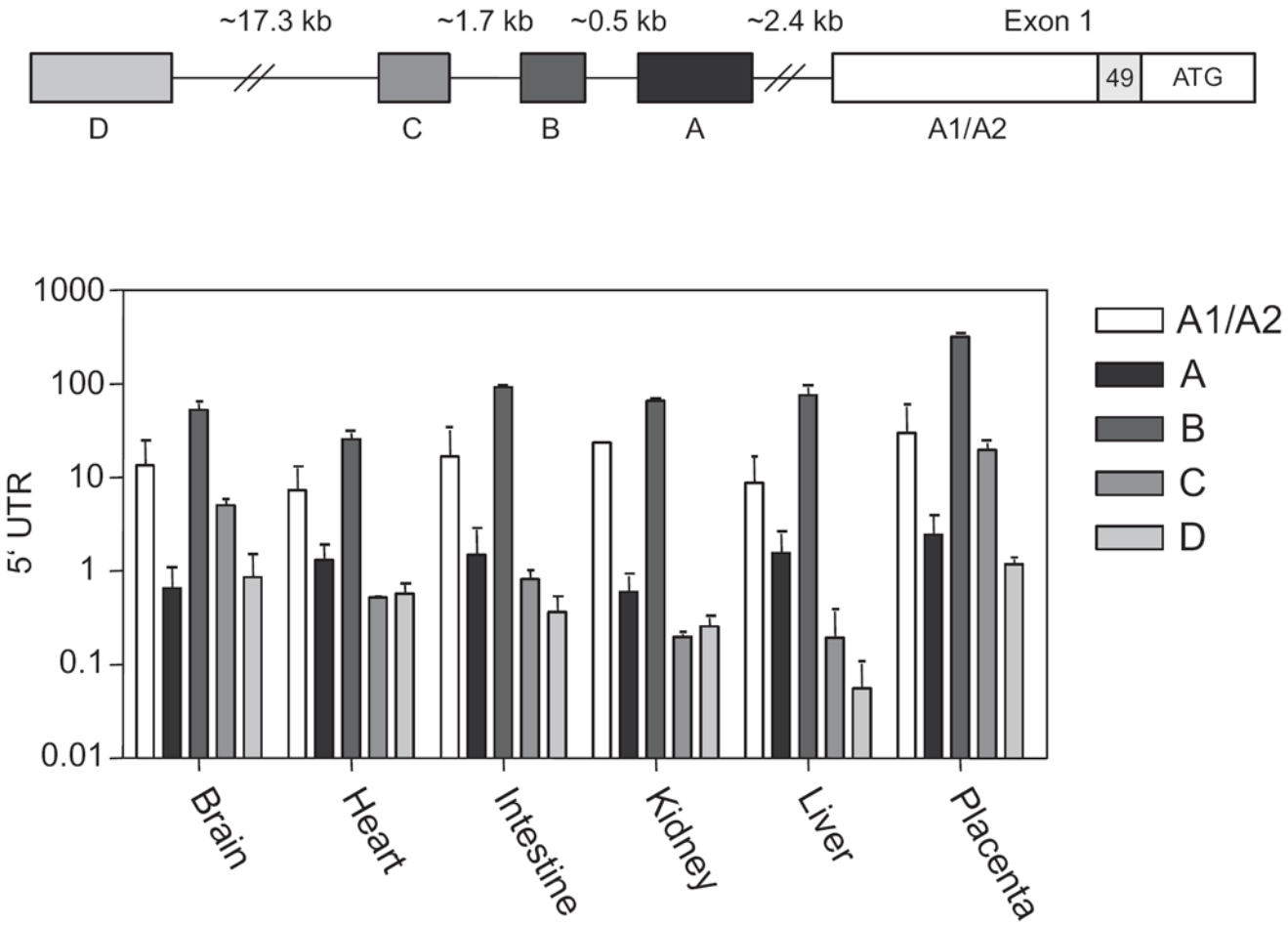

Figure 8. Schematic representation of the hRFC upstream region and *q*PCR analyses of assorted tissue hRFC 5'UTR usage

Upper panel: the upstream region of the hRFC gene is shown including five major non-coding regions (A1/A2 and A-D) and the first coding exon (Exon 1) including the ATG translation start site at position +1. The approximate distances between each of the exons were determined from those in chromosome 21 contig HS21C102 (accession no.AL163302). Lower panel: relative transcript levels of the various hRFC 5' UTRs (A1/A2 and A-D) for assorted human tissues were measured by *q*PCR analysis. Levels of hRFC transcripts were normalized to GAPDH transcript levels. Results are presented as the average values plus/minus ranges from duplicate measurements.

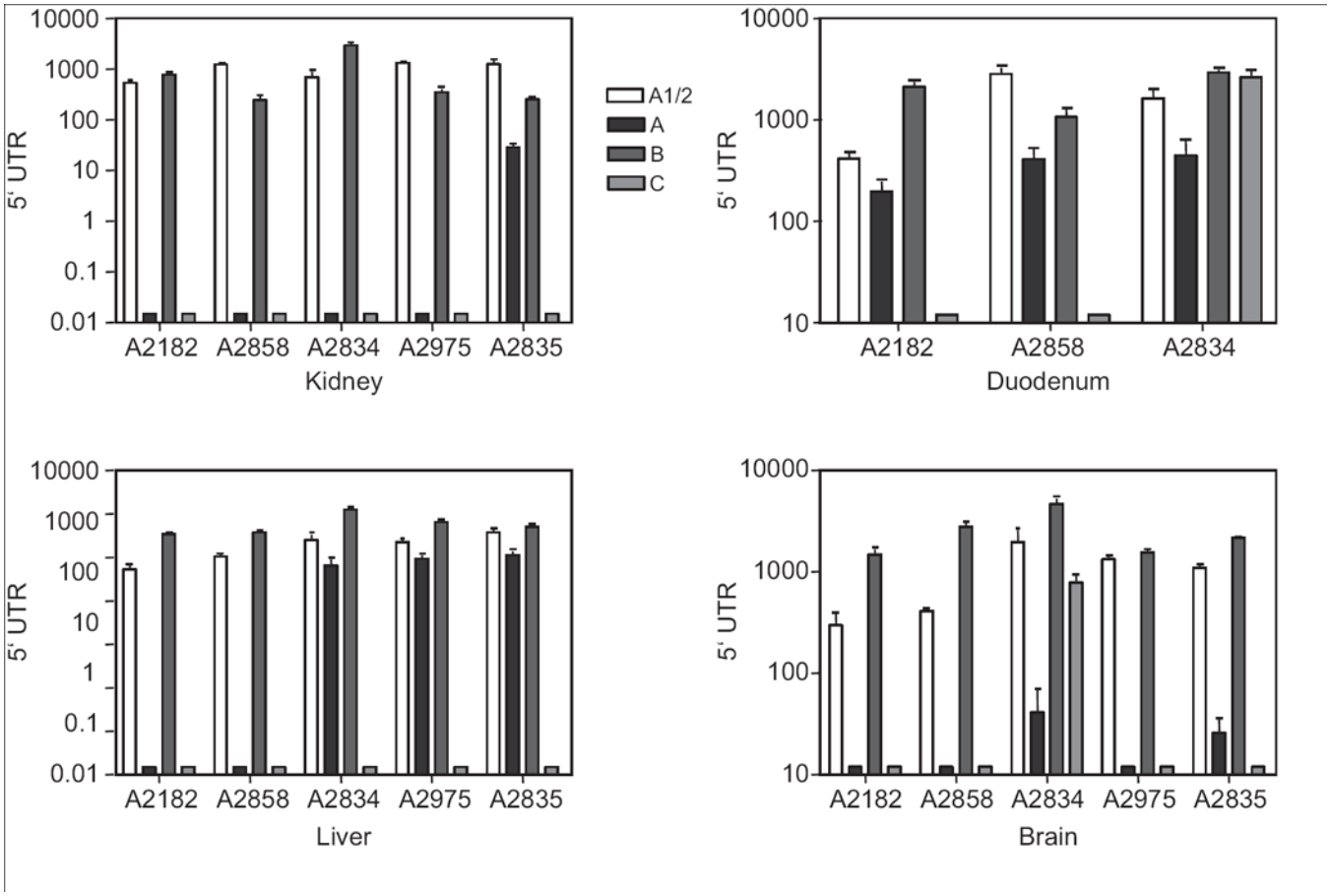

Figure 9. Tissue specific 5' UTR usage in humanized hRFC mice

RNAs were prepared from select tissues isolated from the humanized mice including brain, kidney, liver, and duodenum. RNAs were reverse transcribed and analyzed for hRFC 5'UTRs by *q*PCR. hRFC transcript levels were normalized to mGAPDH transcripts. The results show that the A1/A2 and B are consistently the major 5'UTRs used with lower levels of A and C. Thus, our results in the humanized mice resemble those in human tissues. In one female mouse (A2835) a significant level of transcripts including the C 5'UTR was detected (not shown). Results are presented as average values plus/minus ranges from duplicate measurements.