Universidade de São Paulo
Faculdade de Medicina de Ribeirão Preto
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O papel modulador do gene Aire
(autoimmune regulator) sobre redes de
expressão gênica em células tímicas
epiteliais medulares.

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Ribeirão Preto

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epiteliais medulares.

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RESUMO

A expressão de antígenos restritos a tecidos (TRAs do inglês *tissue restricted antigens*) no timo pelas células epiteliais medulares (mTECs de *medullary thymic epithelial cells*) é essencial para a tolerância central das células T.

Devido à sua heterogeneidade em termos de representação de autoantígenos, esse fenômeno foi denominado como expressão gênica promíscua (PGE de *promiscuous gene expression*), no qual o gene Aire (de *autoimmune regulator*) desempenha um papel como principal regulador transcricional positivo sobre um grande conjunto de TRAs dependentes de Aire.

A proteína Aire tem a capacidade de interagir com seqüências específicas de DNA desempenhando um papel como regulador direto.

Neste estudo utilizamos o método dos cDNA *microarrays* para acessar a PGE em células mTEC CD80+ murinas cultivadas *in vitro*. O agrupamento hierárquico dos dados permitiu a observação de que os genes de TRAs foram diferencialmente expressos. Para testar essa hipótese, inicialmente silenciámos o gene Aire pelo método de RNA interferente (RNAi) nas células mTEC.

O agrupamento hierárquico dos dados de cDNA *microarray* mostrou um conjunto de genes de TRAs dependentes de Aire, os quais foram reprimidos após o silenciamento deste último. Redes gênicas reconstruídas desses dados permitiram a identificação de um nó gênico (*Gucy2d*) estabelecendo regulação positiva sobre genes *downstream* nas células mTEC normais. Entretanto, sob efeito do silenciamento de Aire, *Gucy2d* passou a ser um repressor.

Esses resultados evidenciaram que genes da PGE estão conectados em rede, que um nó gênico pode atuar como intermediário no seu controle e que Aire na rede PGE desempenha seu controle como regulador *upstream*.

Promiscuous gene expression in medullary thymic epithelial cells is connected in network where the Aire gene is an upstream modulator

ABSTRACT

The expression of tissue restricted antigens (TRAs) in thymus by medullary thymic epithelial cells (mTECs) is essential for the central self-tolerance of T cells.

Due to heterogeneity of autoantigen representation this phenomenon has been termed promiscuous gene expression (PGE), in which the autoimmune regulator (Aire) gene plays a role as main positive transcriptional regulator on a large set of Aire-dependent TRAs.

Aire protein is able in binding to specific DNA sequence motifs and plays a role as a direct regulator.

Here we used the cDNA microarray method to access PGE in murine CD80⁺ mTECs cultured *in vitro*. Hierarchical clustering of the data allowed observation that TRA genes were differentially expressed. To further investigate the control of PGE, we hypothesize that TRA genes establish networks contributing it selves to modulate their transcriptional levels. Aire in this case plays a role as upstream positive modulator. To test this hypothesis, initially we silenced Aire by gene knockdown (RNA interference) in mTECs.

Hierarchical clustering of cDNA microarray data showed a set of Aire-dependent TRAs genes, which were down regulated after Aire silencing. Gene networks reconstructed from these data allowed the identification of a gene node (Gucy2d) establishing positive regulation upon downstream genes in normal mTECs. Nevertheless, under silencing of Aire, Gucy2d has become a repressor.

These finding evidentiates that, genes features in PGE are connected in network; a gene node may act as intermediate in their control and that Aire in PGE network plays a role as an upstream regulator.

Introdução

1. INTRODUÇÃO

1.1. A maturação e o papel das células tímicas epiteliais medulares (mTECs)

1.1.1. Ontogenia do timo

A origem do desenvolvimento do epitélio do timo tem sido debatida por décadas. Inicialmente, Cordier & Haumont (1980) sugeriram que o compartimento epitelial do timo seria derivado da ectoderme (córtex) e da endoderme (medula).

A hipótese de “origem dual” baseia-se na formação de um rudimento tímico de natureza reticuloepitelial e outro de origem endo-ectodérmica derivadas da terceira bolsa faríngeana e fenda branquial. O retículo do epitélio medular tímico é de origem endodérmica, enquanto que o retículo do epitélio cortical tímico é de origem ectodérmica. Nesta bolsa se formam tubos de células epiteliais que vão crescendo em forma de cordões para baixo até o tórax. Os cordões perdem a comunicação com sua origem e se transformam na medula do timo. Durante sua migração, decorrente de sua aderência ao pericárdio visceral, o rudimento tímico é invadido por vasos sanguíneos que serão os portadores dos precursores de timócitos, células dendríticas, macrófagos e mastócitos. As células reticulares epiteliais emitem prolongamentos e formam os septos, corpúsculos de Hassal e as áreas onde vão ser ocupadas pelos linfócitos T em maturação no córtex. Posteriormente, inicia-se a tração pelo pericárdio, que leva o timo até sua posição anatômica no mediastino anterior na maioria dos animais, sendo que o timo se mantém no

extramediastino cervical nas aves e branquial nos peixes (van Ewijk *et al.*, 2000; Gray *et al.*, 2005).

Embora num primeiro momento estudos digam que o timo tenha se derivado das células epiteliais de ambas as origens endodérmica e ectodérmica, experimentos em aves e camundongos, via transplante ectópico, tem demonstrado conclusivamente que as células epiteliais tímicas são exclusivamente de origem endodérmica, deste modo, refutando o modelo de “origem dual” de ontogenia epitelial tímica e aceitando um novo modelo de “origem única” (Anderson & Jenkinson, 2001; Blackburn & Manley, 2004; Barthlott *et al.*, 2006).

Recentemente, análises clonais utilizando embriões com 12 dias de desenvolvimento (Rossi *et al.*, 2006) e experimentos com mapeamentos indicando um progenitor comum para as células corticais e medulares (Bleul *et al.*, 2006), têm conclusivamente mostrado que somente um folheto germinativo contribui para o compartimento epitelial tímico e esse folheto seria a endoderme. Contudo, não se podem excluir sinais de indução e/ou sobrevivência de outro folheto germinativo (Manley *et al.*, 2003; Gordon *et al.*, 2004; Boehm & Bleul, 2006).

Em camundongos, o timo surge como estruturas bilaterais da terceira bolsa faríngeana no intestino primário embrionário (Manley, 2000; Gordon *et al.*, 2001) e são colonizados por progenitores hematopoéticos por volta do 11.5 dia do desenvolvimento embrionário (Itoi *et al.*, 2001). O primeiro marcador descrito para essa região foi o *Foxn1*, necessário para o desenvolvimento de células epiteliais tímicas funcionais, para a formação do timo e, portanto, para a formação da diversidade do repertório de células T auto-tolerantes (Nehls *et al.*, 1996; Boehm *et al.*, 2003; Bleul *et al.*, 2006).

Recentemente surgiram evidências da presença de um segundo timo em algumas linhagens de camundongos, chamado cervical, que se desenvolve após o nascimento e tendo o mesmo tamanho de um linfonodo. As células epiteliais no timo cervical também expressam Foxn1, indicando sua relação com o compartimento epitelial do timo torácico (Dooley *et al.*, 2006; Terszowski *et al.*, 2006). Essa descoberta pode mudar todo nosso conhecimento a cerca deste órgão, porém muitos estudos ainda deverão ser realizados a fim de se chegar a conclusões definitivas.

A involução alométrica do timo inicia-se pouco antes do nascimento e a involução absoluta ocorre na puberdade, entretanto o timo adulto continua a receber células precursoras, vindas da medula óssea e a lançar células colonizadoras das áreas T periféricas (Bodey *et al.*, 1997). Esse fenômeno é conhecido e frequentemente associado à idade, sendo caracterizado pela substituição do estroma tímico por tecido adiposo (Montecino-Rodriguez *et al.*, 2005).

1.1.2. Estrutura do timo

O timo é um órgão linfóide primário em que os precursores de células T derivados da medula óssea se submetem a um processo complexo de maturação no contexto do microambiente tímico, representado por células não linfóides e linfóides e por componentes da matriz extracelular (ECM) (Villa-Verde *et al.*, 1995). É formado por dois lobos (direito e esquerdo), sendo ambos divididos em múltiplos lóbulos por septos fibrosos. Anatomicamente, cada lóbulo é dividido em uma região subcapsular, um córtex, que contém uma densa coleção de linfócitos T em maturação e uma medula, com uma população mais

esparsa de linfócitos (tecido conjuntivo frouxo e células reticulares epiteliais), e onde ocorrem os processos finais de maturação dos linfócitos T (Abbas & Lichtman, 2005; Boehm & Bleul, 2006) (Figura 1).

Figura 1. Desenvolvimento do microambiente epitelial do timo. A) Capacidade de desenvolvimento das células TEC progenitoras. À esquerda estão indicadas as quatro bolsas faríngeas (i-iv); a origem comum da paratireóide (amarelo) e do timo (azul) está situada na terceira bolsa faríngeal. À direita, as células suprimidas com o gene funcional *Foxn1* apresentam timopoiese normal. Ao centro, um modelo para a diferenciação das células epiteliais do timo. Se existe ou não progenitoras epiteliais bipotentes para células medulares (MEP) e/ou corticais (CEP) não é sabido. B) Estrutura de um timo adulto encapsulado e dividido pelos septos mesenquimais em lóbulos individuais. CMJ = junção córtico-medular (cortico-medullary junction) (Extraído e modificado de Boehm & Bleul, 2006)

O córtex proporciona um microambiente necessário para a seleção positiva dos timócitos imaturos enquanto que a medula é responsável pela seleção negativa das células T auto-reativas. Os progenitores linfóides entram no timo adulto pela junção córtico-medular e então

migram para a região subcapsular antes, porém, retornando pelo córtex em direção a medula (Anderson & Jenkinson, 2001).

Cada um destes compartimentos forma um microambiente estromal especializado que é crucial para controlar a maturação das células T. Este estroma é composto essencialmente de células epiteliais corticais (cTECs) e medulares (mTECs), fibroblastos reticulares, células dendríticas e macrófagos derivados da medula óssea, além de estruturas conhecidas como corpúsculos de Hassal, localizados especificamente na medula tímica e composto de espirais compactas de células epiteliais remanescentes de células em degeneração (Figura 2). O timo tem um suprimento vascular abundante e vasos linfáticos eferentes que drenam para os linfonodos do mediastino (Abbas & Lichtman 2005).

Figura 2. Esquema representativo da estrutura do timo e de todos os seus componentes celulares. (Extraído e modificado de Human Embriology - <http://www.embryology.ch/anglais/qblood/lymphat03.html>).

A manutenção do microambiente tímico requer interações recíprocas entre timócitos e células estromais, denominadas de "cross

talk” tímico (van Ewijk *et al.*, 1994; van Ewijk *et al.*, 2000; Germeraard *et al.*, 2003; Gray *et al.*, 2006).

A interação dos precursores de timócitos com o microambiente estromal permite o comprometimento desses com as linhagens celulares T e essencialmente elimina ou permite a supressão de células auto-reativas (Boehm & Bleul, 2006; Anderson *et al.*, 2007).

As células epiteliais tímicas são os componentes celulares principais do microambiente tímico, e influenciam diferentes aspectos da diferenciação dos timócitos, vias de interações célula-célula e das secreções de fatores solúveis, tais como os hormônios tímicos timulina (*thymulin*), timopoetina (*thymopoietin*) e timosina- $\alpha 1$ (*thymosin-a1*) (Savino, 2006).

Diversas moléculas foram identificadas que são importantes no desenvolvimento inicial do timo, incluindo *forkhead box N1* (FOXN1), *T-box 1* (TBX1) e *pre-B-cell leukaemia transcription factor 1* (PBX1) (Boehm & Bleul, 2006)

Dados recentes mostram que o hormônio do crescimento (GH) modula pleiotropicamente funções tímicas como, por exemplo, aumenta a proliferação de timócitos e de células epiteliais tímicas (Savino, 2007).

Um complexo linfoepitelial localizado corticalmente no timo, as células “nurse” tímicas (TNC), são estruturas multicelulares linfoepiteliais formadas por uma célula epitelial tímica (TEC) que abriga 20-200 timócitos, carregando primeiramente o fenótipo duplo-positivo CD4/CD8, embora timócitos imaturos duplo negativos, bem como, células maduras simples positivas também possam ser encontradas. TNCs criam provavelmente um microambiente especial para a diferenciação e/ou proliferação dos timócitos, com timócitos sendo

expostos aos antígenos do complexo principal de histocompatibilidade (MHC) e aos hormônios tímicos. Tal diferenciação ocorre em paralelo com a migração da célula dentro e fora do complexo (Savino, 2006).

O mesênquima tímico, derivado da crista neural, também pode ser capaz de influenciar diretamente o desenvolvimento dos precursores de células T CD4⁺CD8⁻ por apresentação de fatores de crescimento solúveis sobre componentes da matriz extracelular (ECM). Além de influenciar o desenvolvimento das células epiteliais tímicas imaturas, e assim ter um papel direto no estabelecimento do microambiente tímico (Jenkinson *et al.*, 2003).

1.1.3. Migração das células precursoras linfóides para o timo

Os precursores de células T, ou células imaturas da linhagem de células T, derivados da medula óssea entram no timo na junção cortico-medular, e então migram à região subcapsular da glândula através dos vasos sanguíneos. O desenvolvimento começa no córtex e, conforme os timócitos vão evoluindo eles migram para a medula tímica, de forma que esta contém principalmente células T maduras. Desta por sua vez, deixam o timo e entram no sangue e nos tecidos linfóides periféricos. As quimiocinas são uma grande família de citocinas estruturalmente homólogas que regulam a migração de linfócitos e outros leucócitos através dos tecidos linfóides periféricos (Blackburn & Manley, 2004; Abbas & Lichtman 2005).

As células do microambiente tímico produzem dois grupos de moléculas, as quimiocinas, incluindo Cxcl12 (*Chemokine C-X-C motif ligand 12*), a qual preferencialmente atrai timócitos imaturos CD4⁺CD8⁻ e

CD4⁺CD8⁺, e Ccl21 (*Chemokine C-C ligand 21*), que exerce quimioatração para timócitos simples positivos maduros, e as proteínas da matriz extracelular, visto que os timócitos em desenvolvimento expressam os receptores correspondentes. Além disso, embora as quimiocinas e a matriz extracelular possam dirigir a migração dos timócitos por si mesmo, uma função combinada para estas moléculas parece contribuir para os padrões de migração resultantes dos timócitos em seus vários estágios de diferenciação (Savino *et al.*, 2003; Savino *et al.*, 2006).

1.1.4. Maturação dos timócitos em células T

O processo de maturação dos linfócitos consiste numa complexa seqüência de eventos biológicos, compreendendo a proliferação das linhagens celulares precursoras, a expressão diferencial de proteínas de membrana, rearranjos gênicos do receptor de antígeno, a seleção do repertório de linfócitos maduros, e conseqüente morte celular programada dos linfócitos não selecionados e finalmente a migração celular. Durante cada um desses estágios, as células sofrem significativas mudanças celulares e genéticas (Abbas & Lichtman, 2005).

A análise do desenvolvimento da célula T no timo conduziu à identificação de uma série de *checkpoints* durante o desenvolvimento inicial do timócito. Durante os estágios mais adiantados do desenvolvimento da célula T, a seleção positiva e negativa de timócitos CD4⁺CD8⁺, que conduz à produção de um repertório de células T tolerantes ao próprio, é de importância crítica em evitar respostas não desejadas da célula T aos antígenos próprios que podem se manifestar como auto-imunidade (Anderson *et al.*, 2007).

A maturação inicial caracteriza-se por um aumento da produção de todas as células sanguíneas na medula óssea e por uma alta atividade mitótica, estimulada principalmente pela interleucina 7 (IL-7) que é produzida pelas células do estroma da medula óssea e do timo e pelos ligantes Notch (Harman *et al.*, 2005; Zambich *et al.*, 2005). A atividade proliferativa cessa depois que os genes para os receptores de antígeno são expressos. Tais genes surgem por um processo de recombinação somática gerando diversidade do repertório dos linfócitos após junção de segmentos gênicos diferentes.

A interação entre células estromais e linfóides dentro do microambiente tímico é um fator crítico para formar a correta especificidade antigênica do repertório de células T, assim como para capacitá-lo a responder à apresentação de antígenos estranhos pelas moléculas de MHC e não responder a antígenos próprios (Barthlott *et al.*, 2006).

Os mecanismos que regulam o desenvolvimento e a seleção de células T expressando TCR- $\alpha\beta$ são governados por sinais de células estromais tímicas. Isto é ilustrado por estudos das linhagens de camundongo onde as mutações genéticas que afetam o desenvolvimento ou a função da célula estromal têm um efeito dramático na imunocompetência da célula T. Fenotipicamente as subpopulações distintas de timócitos ocupam áreas específicas dentro do timo, sugerindo que as células estromais dão forma aos microambientes especializados dentro do timo que compartimentaliza o desenvolvimento de timócitos e suportam estágios distintos do desenvolvimento da célula T (Anderson *et al.*, 2007). Por exemplo, a zona subcapsular fornece um ambiente para os precursores duplo-negativos da célula T CD4-CD8-CD44-CD25⁺ e a seleção mediada pelo pré-TCR (Lind *et al.*, 2001), o córtex tímico contém

os timócitos duplo-positivos CD4⁺CD8⁺ que são sujeitos aos eventos de seleção da célula T, enquanto que na medula tímica, células T simples-positivas CD4⁺ e CD8⁺ são selecionadas quanto à reatividade ao próprio antes de sua saída do timo (Takahama, 2006).

Os timócitos imaturos corticais recém-chegados ao timo contêm os genes de TRs na configuração germinativa e, portanto, ainda não expressam o TCR, CD3, as cadeias ζ e os co-receptores CD4 e CD8. Essas células são chamadas de timócitos duplo-negativos (DN) e se submetem à múltiplos ciclos de proliferação e de progresso ao estágio duplo-negativo CD4⁻CD8⁻ (DN), aproximadamente 5% do total de timócitos, com discretos passos de maturação distinguidos pela expressão de marcadores de superfície celular CD44^{high}CD25⁻ (DN1), CD44^{high}CD25⁺ (DN2), CD44^{low}CD25⁺ (DN3), e CD44^{low}CD25⁻ (DN4). As proteínas do complexo recombinase (Rag-1 e Rag-2) são expressas pela primeira vez nesse estágio, e os rearranjos V(D)J se iniciam. Durante a transição da fase DN para duplo-positiva CD4⁺CD8⁺ (DP), aproximadamente 85% dos timócitos se redistribuem à região cortical (Ramialison *et al.*, 2002). O rearranjo dos genes da cadeia α e a expressão do heterodímero TCR $\alpha\beta$ ocorrem na população CD4⁺CD8⁺, exatamente antes ou durante a migração dos timócitos do córtex para a medula (Figura 3).

Figura 3. Estrutura e função do timo. Dividido em duas regiões, córtex e medula, povoados por diferentes subtipos celulares epiteliais tímicos (TECs). Nos adultos, os precursores de células T entram no timo pela junção cortico-medular, e começam então um programa altamente complexo de diferenciação, o qual está ligado à migração através do estroma tímico. Os subtipos diferentes de timócitos são encontrados conseqüentemente em regiões espacialmente restritas do timo. O córtex tímico foi separado em quatro regiões: *Região 1*, junção cortico-medular, local de entrada dos precursores de células T; *Região 2*, células diferenciadas em DN2 as quais se submetem a expansão clonal proliferativa; *Região 3*, inicia-se os rearranjos V(D)J dos genes TRs em células no estágio DN3; *Região 4*, transição de DN para o estado de DP (CD4+CD8+). Células DP migram do córtex para a medula tímica, onde se diferenciam em simples positivas (SP CD4+ ou CD8+). A seleção positiva ocorre no córtex entre as TECs corticais (cTECs), e a seleção negativa ocorre principalmente na medula, entre as TECs medulares (mTECs) e células dendríticas (DCs). Células SP que completaram o programa de diferenciação saem da medula tímica em direção a periferia. [Extraído e modificado de Blackburn & Manley, 2004].

A recombinação V(D)J dos receptores de células T é um evento molecular essencial na maturação destas células. É um processo de recombinação sítio específico da molécula de DNA que só ocorre nos estágios iniciais de desenvolvimento dos linfócitos T e B. A geração de

grande diversidade nas regiões variáveis do receptor de antígeno de linfócitos T é crucial para que os organismos possam reconhecer e responder contra, virtualmente, todo e qualquer antígeno estranho.

Cada locus TCR consiste de segmentos gênicos de regiões variáveis (V), *joinig* (J) e constante (C), e para as cadeias β e δ incluem segmentos gênicos de diversidade (D). De modo geral, o rearranjo dos segmentos gênicos, ocorre através da ligação de um segmento J a um dos segmentos D, formando uma união D-J, posteriormente um dos segmentos gênicos V se liga ao segmento D-J unidos. Toda sequência de DNA interveniente aos segmentos gênicos que se ligaram passam por um processo de deleção (Paul, 1993). Os segmentos gênicos rearranjados são transcritos em um RNA primário, que se torna um RNA maduro após o seu processamento. O rearranjo D-J e V-DJ ocorre entre os segmentos gênicos da cadeia β e δ de TCR; entre os segmentos gênicos da cadeia α e γ onde não há segmentos D, ocorre apenas o rearranjo V-J (Abbas & Lichtman, 2005).

As primeiras células que expressam os TCRs estão no córtex tímico, e essa expressão é baixa em comparação com a célula T madura. Em virtude da expressão de seus complexos TCR completos, as células duplo-positivas podem responder ao antígeno e ficam sujeitas à seleção positiva e negativa. A maioria das células DP, as quais constituem cerca de 80% daquela população, morre por negligência porque não reconhecem moléculas disponíveis de MHC expressas por células estromais tímicas (Abbas & Lichtman, 2005; Savino, 2006).

As células que passam com sucesso pelos processos de seleção amadurecem em células T CD4⁺ ou CD8⁺ e são chamadas de timócitos simples positivos (SP), e compreendem aproximadamente 10% dos timócitos no timo (Ramialison *et al.*, 2002). Assim, os estágios de maturação das células T podem ser rapidamente distinguidos pela análise da expressão

das moléculas CD4 e CD8. Essa maturação fenotípica é acompanhada da maturação funcional (Abbas & Lichtman, 2005).

Finalmente, os timócitos maduros com positividade única (células T CD4 e CD8 restritas ao MHC) entram na medula do timo e deixam esse órgão pelos vasos sanguíneos para colonizar os tecidos linfóides periféricos (Haks *et al.*, 1998). Apenas 15% dos timócitos que vivem no timo nesta fase vão formar a maioria do repertório de células T periféricas (Savino, 2006).

1.1.5. As células medulares epiteliais tímicas (mTECs).

Os dois subconjuntos principais de células epiteliais tímicas (TECs) - TEC corticais (cTEC) e medulares (mTEC) - definem também os dois compartimentos principais do timo, do córtex e da medula. Ambas as linhagens originam de uma célula comum progenitora. As células T passam durante seu desenvolvimento através de ambos os compartimentos em um processo espacial e temporalmente ordenado. Durante a fase cortical, um repertório altamente diverso de células T é gerado e sujeito à seleção positiva com relação à restrição do MHC próprio. A fase medular subsequente impõe a tolerância das células T ao repertório nascente por meio da seleção negativa de células efetoras auto-reativas e da seleção positiva de células T regulatórias (T_{regs}). Mas cabe lembrar que as cTECs e mTECs são essenciais mas não suficientes para estes eventos da seleção (Glaser *et al.*, 2007).

Sinais derivados de cTEC podem regular a seleção positiva de timócitos que reconhecem complexos MHC-peptídeos próprios, no entanto mTECs expressando AIRE ajuda a assegurar a tolerância aos antígenos próprios (Anderson *et al.*, 2007).

As células mTECs são peculiares com relação à expressão de vários antígenos de superfície incluindo MHC de classe II, CD80, *Ulex europaeus* aglutinina (UEA), claudin 3/4 (Glaber *et al.*, 2007) e moléculas co-estimulatórias como consequência de sua progressiva maturidade e agem como células apresentadoras de antígenos (APCs) no timo (Hollander, 2007; Kont *et al.*, 2008).

Um subconjunto de mTECs expressa o gene *Aire* (*autoimmune regulator*) e em sua superfície as proteínas chamadas *claudins* (Cld) 3 ou 4, que são moléculas de adesão e são as principais proteínas que contribuem para a arquitetura molecular das junções celulares. Todas as células TECs Cld3⁺, Cld 4⁺ e Aire⁺ adultas apresentam alta densidade em sua superfície de moléculas MCH de classe II, com forte expressão de CD80 (Hollander, 2007).

As precursoras TECs surgem exclusivamente do forro endodérmico do terceiro folheto faríngeo; lá eles invaginam para dar forma a um epitélio separado inicial (figura 4). Depois da imigração de precursores linfóides para o timo, essas células epiteliais são reorganizadas de uma estrutura plana a uma arquitetura tridimensional, uma orientação única de TECs. Embora agora esteja estabelecido que todos os subconjuntos de TECs são derivados de um precursor comum, a origem específica de mTECs Aire⁺ e sua relação precursor-progênie com outros subconjuntos mTECs ainda não é clara (Hollander, 2007).

Figura 4. Diferenciação das células epiteliais tímicas (retirado de Hollander, 2007)

Ao contrário do que se assume, as mTECs não formam um esqueleto estático, mas de maneira similar ao epitélio estratificado da pele ou dos intestinos, têm um “turnover” com meia-vida de 3 a 4 semanas (Booth *et al.*, 2000; Alonso *et al.*, 2003; Boehm *et al.*, 2003).

Estudos recentes documentam uma dinâmica complexa da população de mTECs. Quando o *pool* maduro de mTEC apresenta um *turnover* dentro de 3 semanas, o *pool* imaturo contém ambos, uma fração ciclando rapidamente e uma fração em repouso ou passando por um ciclo maior (Glaser *et al.*, 2007).

Isto torna as mTECs um compartimento tímico altamente dinâmico. Apesar deste *cell turnover* altamente dinâmico, o espectro dos antígenos próprios expressos não se altera, segundo o que observa Kyewski &

Derbinski (2004). Os timócitos que entram na medula do timo sempre encontrarão um espectro completo de “genes promíscuos” apesar da geração contínua de mTECs.

As mTECs "imaturas" CD80-/MHC-II- expressam somente um conjunto limitado de TRAs, enquanto que mTECs "maduras" CD80+/MHC-II+ apresentam maior diversidade de TRAs incluindo aqueles genes cuja expressão é Aire-dependentes (Derbinski *et al.*, 2005). Estes achados conduziram alguns pesquisadores a propor "o modelo de diferenciação terminal", que diz que as mTECs se submetem a um processo contínuo de diferenciação similar à pele ou ao epitélio intestinal e que o complemento completo da expressão gênica promiscua (PGE) é contingente deste processo (Gabler *et al.*, 2007).

Nosso grupo de pesquisa, tem contribuído com observações sobre a expressão gênica promiscua no timo de camundongos isogênicos empregando a tecnologia dos cDNA microarrays. Num trabalho recente, usando culturas *in vitro* de timo fetal (FTOC), mostramos que o gene Aire começa sua expressão aos 16 dias de gestação e que a PGE também ocorre em FTOC, mas somente após a expressão do gene Aire, ou seja, aos 20 dias de gestação (Sousa Cardoso *et al.*, 2006). Comparando o tempo de gestação e a duração das culturas, foi possível determinarmos a época da emergência da indução de genes de TRAs os quais representaram 57 diferentes órgãos parenquimais e 7 órgãos linfóides. Mostramos pela primeira vez que FTOC também reproduz a expressão gênica promiscua (Sousa Cardoso *et al.*, 2006).

Foi possível observar que o conjunto inclui genes com diferentes ontologias (funções biológicas e moleculares) cujas respectivas localizações genômicas incluem praticamente todos os cromossomos, mostrando que o fenômeno é bastante abrangente. Tais observações

foram realizadas usando RNA extraído de timos intactos, quer dos mantidos em culturas FTOC ou dos recentemente removidos dos animais.

A observação da PGE foi possível, mas como sabemos que este fenômeno ocorre nas células mTEC, no presente trabalho procuramos refinar ainda mais nosso sistema modelo e passamos a estudar células mTEC da linhagem 3.10.

Tais células foram estabelecidas a partir do timo de camundongos da linhagem C57Bl/6 e o fenótipo de células medulares foi determinado por imunofenotipagem com anticorpo Th-4 (Mizuochi *et al.*, 1992). Elas também foram avaliadas usando um painel de anticorpos monoclonais anti-citoqueratina, confirmando o fenótipo medular distinto (Nihei *et al.*, 2003). Além disso, confirmou-se pela técnica de FACS que estas células são CD80⁺ e, portanto, aptas a PGE.

Por estes motivos, decidimos optar pelo sistema modelo de cultura de células mTECs 3.10 para estudarmos a expressão do gene Aire e sua relação com o fenômeno da PGE.

1.2. Expressão gênica promíscua no timo e a indução de tolerância central

Quando da fundação da teoria da seleção clonal há mais de 100 anos, Paul Ehrlich conceituou o problema da reatividade ao próprio como inerente ao sistema imune adaptativo e postulou a existência de mecanismos que podem prevenir uma reatividade deletéria ao próprio (Ehrlich & Morgenroth, 1901).

A discriminação entre o próprio e o não-próprio tem sido considerada como um pré-requisito essencial do sistema imune dos

invertebrados multicelulares e vertebrados. Com relação aos vertebrados, é sempre vista como a essência do sistema imune.

A formulação da teoria da seleção clonal da formação de anticorpos, o conceito de discriminação entre próprio e não-próprio, a demonstração experimental de tolerância adquirida ao próprio e a demonstração direta da deleção clonal de linfócitos auto-reativos foram importantes marcos no alcance do nosso conhecimento atual sobre tolerância ao próprio, cujo estabelecimento e a manutenção são uma exigência intrínseca do sistema imune adaptativo.

A discriminação do próprio/não-próprio é uma propriedade essencial do sistema imune que dirige uma variedade de mecanismos efetores contra agentes patogênicos enquanto ignora os constituintes próprios do organismo.

Somente quando a tolerância ao próprio está finamente balanceada é que a integridade do corpo é garantida. O timo é visto como o principal local de indução de tolerância a antígenos próprios que são expressos pelas células tímicas (tolerância central), enquanto que a tolerância a antígenos restritos a outros tecidos e órgãos parenquimais tem sido associada a mecanismos extra tímicos (tolerância periférica) (Medzhitov & Janeway, 1997; Kyewski & Derbinski, 2004).

Estudos prévios têm demonstrado que antígenos relacionados a tecidos próprios (TRAs) são expressos no timo e que sua expressão é necessária para a deleção de células T auto-reativas (Kyewski & Klein, 2006). Uma característica central neste processo é a *expressão gênica promíscua* de TRAs pelas células epiteliais na região medular tímica, onde os TRAs são apresentados e encontrados pelos timócitos, conduzindo à indução da tolerância por deleção clonal ou inativação funcional.

As doenças auto imunes, incluindo lúpus eritematoso sistêmico (LES), diabetes melitus do tipo 1 (DM-1) e artrite reumatóide (AR), representam desafios científicos e médicos dos mais intrigantes.

É difícil, até hoje, concluir se o problema gerado pelo desequilíbrio de reconhecimento entre o próprio e o não próprio é iniciado pelos tecidos-alvos da reação auto imune (constituintes do núcleo celular como DNA, RNA, histonas etc no LES; células β do pâncreas no DM-1 e tecido sinovial das juntas e articulações na AR) ou se o problema está nas células T auto-reativas ou ainda se a origem dessas doenças está no descontrole da indução da tolerância central.

Existem evidências para as duas primeiras possibilidades, porém um ponto em comum amplamente aceito, independente da origem da auto-imunidade, é a existência dos auto-antígenos, ou seja, proteínas celulares do próprio indivíduo que são reconhecidas pelo sistema imune.

Vários trabalhos mostraram que células tímicas epiteliais medulares (mTECs) expressam moléculas que normalmente caracterizam outros tecidos (~ 10% do genoma do camundongo ou humano) representando a quase totalidade de tecidos e órgãos parenquimais.

Suspeita-se que o descontrole desta expressão promíscua pode implicar na indução deficiente de tolerância com conseqüências no surgimento de doenças auto-imunes.

Por isto, esse fenômeno foi chamado de *expressão gênica promíscua* (Figura 5). O conjunto de genes 'promiscuamente' expressos no timo parece ser altamente diverso, incluindo genes tecido- e sexo-específicos e genes particularmente envolvidos com o desenvolvimento (Kyewski & Klein, 2006).

Figura 5. Representação de diversos tecidos e órgãos parenquimais no timo murino é garantida pela expressão dos antígenos relacionados a tecidos (TRAs) pelas células epiteliais (mTECs), fenômeno chamado expressão gênica promíscua (Retirado de Kyewski & Derbinski, 2004).

A partir do ano de 2005 nosso grupo de pesquisa vem contribuindo com esta área de estudos produzindo trabalhos sobre PGE no timo murino. Descrevemos sua ocorrência *in vitro* em cultura de timo fetal de BALB-c (*fetal thymus organ culture*, FTOC) mostrando a emergência deste tipo de expressão durante o desenvolvimento ontogenético (Sousa Cardoso *et al.*, 2006). Além disso, demonstramos que o gene Aire tem sua transcrição iniciada no timo fetal aos 16 dias de gestação e que a PGE emerge no vigésimo dia. Num outro trabalho, analisamos o efeito do *background* genético de linhagens isogênicas de camundongos sobre a diversidade da PGE e a distribuição genômica dos genes expressos (Magalhães *et al.*, 2006).

Atualmente focamos as análises de PGE do timo de linhagens de camundongos que reproduzem doenças auto-imunes tais como a NOD que reproduz diabetes melitus do tipo 1 (DM-1) (Silveira 2005; Fornari 2008) e DBA-1 que reproduz artrite reumatóide mediante imunização com colágeno (Donate 2008).

Sem reflexão poderíamos até pensar que estaríamos observando o óbvio. Mas ao invés disso, a imunologia molecular encontrou uma oportunidade ímpar de descrever e mensurar a expressão de cada um dos genes de TRAs quando passou a utilizar a tecnologia dos cDNA microarrays.

A tolerância do repertório de células T ao próprio é especificamente adquirida em cada indivíduo por caminhos diferentes no desenvolvimento das células T imaturas, dependentes da intensidade da interação entre o receptor de célula T e o peptídeo ligante de MHC próprio. Como resultado, a diversidade de antígenos próprios que são acessíveis ao repertório nascente no timo determinará a extensão e especificidade da tolerância central ao próprio (Kyewski & Derbinski, 2004).

Vários exemplos de expressão ‘aberrante’ de TRAs no timo com um possível papel na tolerância de células T foram reportados há vários anos atrás. Porém, as implicações da noção ainda herética de regulação gênica celular tipo-específica no timo ainda não foram exploradas a contento (Kyewski & Derbinski, 2004).

A maior implicação desta expressão heterogênea de genes no timo se associa à manutenção da homeostase imunológica do corpo, controlando as reações auto-imunes patológicas.

1.3. O papel do gene *Autoimmune regulator* (Aire)

Um importante regulador da expressão TRAs em mTECs é o gene Aire (*autoimmune regulator*) localizado no cromossomo 10C1/10 41.6 em camundongos e 21q22.3 no homem (Mathis & Benoist, 2007; Kont *et al.*, 2008).

O gene Aire é um regulador de transcrição (Pitkanen & Peterson, 2003) e controla a expressão de centenas, senão milhares de genes de TRAs, com uma tendência a genes cuja expressão é preferencial de células diferenciadas (Anderson *et al.*, 2002; Cheng *et al.*, 2007; Hollander, 2007). O produto do gene Aire (RNAm) encontra-se preferencialmente em órgãos linfóides, especialmente no timo sendo que dentro deste ele é expresso na maior parte em um conjunto de células medulares epiteliais (mTECs) mas também, em níveis muito menores, em células dendríticas (DCs) e, quanto a localização intra celular, sua proteína situa-se no núcleo (Mathis & Benoist, 2007).

A evidência direta que Aire tem um papel na seleção negativa de células T veio dos modelos de camundongos TCR-transgênicos (Liston *et al.*, 2003; Liston *et al.*, 2004; Anderson *et al.*, 2005).

Ainda utilizando modelos com camundongos TCR-transgênicos, Cheng *et al.* (2007) mostraram a correlação direta entre a perda da seleção negativa no timo e a susceptibilidade aumentada ao diabetes auto-imune, fazendo assim, uma ligação direta entre o processo de seleção aire-dependente no timo e a susceptibilidade auto-imune.

A purificação de mTECs de camundongos tipo selvagem e Aire-*knockout*, seguida da análise da expressão gênica, revelou que Aire certamente promove a transcrição de um grande número genes que

codificam antígenos de tecido periféricos (PTA) (Anderson *et al.*, 2002 e Anderson *et al.*, 2005) associados com a seleção negativa de células T auto-reativas (Nagafuchi *et al.*, 2006). Mas Aire também regula positiva ou negativamente a transcrição em mTECs de um conjunto de genes que não codificam PTAs (Mathis & Benoist, 2007).

Na ausência do gene Aire funcional, há falha das mTECs suprirem timócitos que expressam receptores TCR específicos para antígenos próprios. Como consequência, um grupo de células T auto-reativas ilude a seleção negativa no timo (e possivelmente na periferia) e aparentemente provoca as patologias auto-imunes órgão-específicas como a síndrome da poliendocrinopatia auto-imune tipo 1, diabetes insulino-dependente, doença de Addison, vitiligo e alopecia (Hollander, 2007).

Os genes que são controlados pela proteína AIRE são altamente diversos com relação à estrutura e função, o que dificulta conclusões sobre seu modo de ação detalhado. Dados recentes indicaram que a proteína AIRE contém domínios de ligação com a molécula de DNA (Kumar *et al.*, 2001), mas ainda nenhuma evidência foi gerada mostrando que tais domínios se ligam diretamente nos genes conhecidos controlados por AIRE (Yunxia *et al.*, 2006).

O papel de Aire em regular a expressão promíscua de TRAs envolvida na seleção tímica encontra-se bem estabelecido, mas os mecanismos moleculares precisos da ação não estão inteiramente elucidados. A sequência da proteína AIRE sugere que esta pode mediar a regulação transcricional. AIRE contém diversos domínios estruturais comuns às proteínas nucleares e aos fatores de transcrição, incluindo um sinal nuclear de localização, um domínio de dimerização, e domínios DNA-ligantes putativos. Os domínios DNA-ligantes de Aire são compostos de um domínio SAND (Sp100, Aire-1, NucP41/75 e DEAF-1),

definido pela atividade transcricional modulatória dos membros da família Sp100, e dois homeodomínios *plant* tipo (PHD)-*zinc fingers* (Cheng *et al.*, 2007).

Uma suposição de como a proteína AIRE pode modular a seleção negativa está na ativação da expressão promíscua de proteínas periféricas nas células tímicas apresentadoras de antígeno (APC). Estudos recentes sugerem fortemente que os antígenos tecido-específicos são comumente expressos no timo e que este mecanismo é crítico em dar forma à tolerância central para proteínas periféricas (Kyewski *et al.*, 2002). Mais especificamente, esta função foi atribuída a um subconjunto das mTECs (G8.8+ CDR-), que expressam uma gama diversa de antígenos incluindo auto-antígenos do diabetes tipo I tais como GAD65, GAD67 e insulina (Derbinski *et al.*, 2001).

Deve-se mencionar, contudo que além das mTECs, as células dendríticas e os macrófagos intra-tímicos também expressam o gene Aire e antígenos promíscuos (Pietropaolo *et al.*, 2002; Pugliese *et al.*, 2002).

Entretanto, a significância destes achados para o entendimento preciso da ação molecular do gene Aire *in vivo* ainda não está clara. São necessárias evidências diretas de co-regulação dos genes de TRAs (expressão promíscua) devida a seqüências consenso em suas regiões de promotores/enhancers, nas quais atuaria a proteína AIRE ou mesmo outros complexos reguladores da transcrição (Kyewski & Derbinski, 2004). Ou talvez imaginar outros mecanismos plausíveis para tentar explicar este modo de regulação gênica não convencional.

Nossa idéia é que o gene Aire regula a transcrição dos genes de TRAs por meio de interações em rede em duas etapas, ou seja, regulando primeiramente alguns genes mais importantes (nós gênicos) e estes por sua vez, regulariam um número maior de genes.

1.4. Testando a hipótese da ação indireta do gene Aire: Construção de redes gênicas a partir de dados de microarrays

Antes de discorrer sobre redes gênicas a partir de dados de *microarrays*, julgamos pertinente focarmos uma das questões atuais sobre o mecanismo de ação do gene Aire contextualizando assim a necessidade da construção das mesmas.

O fato de Aire ser um gene codificador de um fator de transcrição faz sentido com o controle da expressão gênica nas células mTECs, isto não representa um problema. O que tem intrigado os pesquisadores é a grande abrangência de sua ação. A proteína AIRE apresenta domínios de ligação com a molécula de DNA idênticos a fatores de transcrição clássicos.

Entretanto, enquanto os fatores de transcrição controlam a expressão de um grupo reduzido de genes, senão de um gene específico, tudo indica que a proteína AIRE controla a expressão da maioria dos TRAs, além de outras proteínas no timo. Pode ter uma ação indutora da expressão destes genes como de inibir a de outros. Foi estimado que pode controlar a expressão de até 10% do genoma do camundongo nas células mTEC salientando que isto não tem precedentes na literatura .

Precisamente como o gene Aire (ou a proteína AIRE) controla a expressão ectópica de uma gama tão diversa de genes codificadores de TRAs, cuja regulação transcricional é bem diferente em suas localizações teciduais ? (Mathis & Benoist, 2007).

Nossa idéia é que o gene Aire (ou a proteína AIRE) atuaria sobre o vasto número de genes de TRAs indiretamente e em rede (ou em cascatas gênicas). Como as medidas de PGE são dados de *microarrays* passou a ser de muito interesse a construção de redes gênicas a partir destes dados

para abordarmos nossa proposta. Neste trabalho, construímos um sistema modelo de inibição parcial do transcrito do gene Aire pela técnica de RNA interferente nas células mTEC, determinação da PGE por *microarrays* e análise dos dados com o algoritmo de redes.

Os experimentos sobre a determinação da expressão gênica com *microarrays* oferecem uma lista de genes que governam o comportamento celular. A análise dessa lista provê uma grande quantidade de informações relevantes às quais precisam ser organizadas de maneira coerente para dar um sentido biológico a tais resultados. No contexto do estudo do comportamento celular a descrição dos mecanismos regulatórios atingiu prioridade máxima.

A regulação transcricional (DNA $\rightarrow$ RNAm) é um dos primeiros pontos de controle da expressão gênica e, num sentido amplo, pressupõe-se que o controle da transcrição influencia diretamente os níveis das respectivas proteínas na célula (Tegnér *et al.*, 2003; Tabuchi *et al.*, 2006).

Por esse motivo é que há grande interesse na pesquisa do controle da síntese de RNAm em grande escala, definindo o perfil do transcriptoma. Os desenvolvimentos recentes de tecnologias tais como DNA *microarrays* fizeram possível obter um volume grande de perfis da expressão gênica, permitindo a mensuração simultânea de milhares de transcritos (Gatti *et al.*, 2007). Como a quantidade de informações geradas nesses estudos é enorme, programas computacionais específicos foram desenvolvidos, como o Cluster e Tree View, utilizado para traçar as chamadas assinaturas de expressão gênica, muito útil para o agrupamento hierárquico de amostras e de genes diferencialmente expressos (Eisen *et al.*, 1998) ou o programa SAM (*significance analysis of microarrays*) utilizado para identificar os genes diferencialmente expressos com base estatística (Tusher *et al.*, 2001).

Os programas citados são clássicos na bioinformática de *microarrays*, mas ainda não respondem como montar os dados de maneira a construir um modelo preditivo de interação entre os genes. Inferir a estrutura de uma rede de interações gênicas também passou a ser uma tarefa imperativa a fim avançar nossa compreensão sobre o mecanismo molecular do funcionamento das células ao nível genômico (Kim *et al.*, 2007).

A análise dos dados de *microarrays* incluiu agora um foco na reconstrução de redes regulatórias entre os genes, visando encontrar e entender as interações gene-a-gene a partir de dados de expressão (Wang *et al.*, 2006).

Atualmente concebe-se que a maioria dos relacionamentos bioquímicos entre genes, proteínas e outros substratos orgânicos devam ser *many-to-many*, significando que um componente pode ter muitas funções e uma função pode ser influenciada por muitos componentes. Para compreender estes relacionamentos complexos, a estrutura de um sistema biológico, tal como o relacionamento regulador entre os genes precisa ser identificado.

Os métodos de engenharia reversa fornecem uma boa maneira de se modelar interações baseadas em dados da expressão gênica durante o curso do tempo, cujos dados foram gerados por experimentos com *microarrays*. A rede gênica reconstruída terá por enquanto uma base puramente de probabilidade e estatística e é claro que deverá ser finalmente validada experimentalmente (Wu *et al.*, 2004).

Uma grande variedade de metodologias vem sendo proposta para inferir redes gênicas regulatórias a partir de dados temporais. Entre esses métodos estão modelos discretos, como as redes Booleanas e Bayesianas, modelos contínuos, como as redes neurais e equações diferenciais. Todos

esses modelos levam em consideração que o nível de expressão de um gene, num determinado intervalo de tempo é dado em função do nível de expressão dos outros genes nos tempos anteriores (Wang *et al.*, 2006).

A inferência da arquitetura de redes gênicas a partir de dados temporais obtidos de tecnologias experimentais, como os microarrays, ajuda a entender e esclarecer o comportamento do sistema em relação à expressão gênica e a regulação envolvida nessa expressão; fazendo uso da bioinformática.

Dentre os algoritmos propostos na literatura para a inferência de redes gênicas, existem aqueles baseados no formalismo matemático da teoria da informação, como o ARACNE (Basso *et al.*, 2005; Margolin *et al.*, 2006), os que consideram equações diferenciais ordinárias, como os programas NIR, NMI e TSNI (Gardner *et al.*, 2003; di Bernardo *et al.*, 2005; Bansal *et al.*, 2006), e também aqueles que solucionam o problema de inferência por meio de redes bayesianas, como o Banjo (Yu *et al.*, 2004) e o GeneNetwork (Wu *et al.*, 2003).

Comparando esses algoritmos, o Genenetwork foi escolhido para o presente trabalho devido suas características peculiares. O algoritmo realiza a interpolação de dados e tem habilidade de calcular a interação entre mais de mil genes. Além disso, a rede resultante é descrita por um gráfico orientado, no qual as setas positivas indicam indução e as negativas indicam repressão entre genes, respectivamente.

Num esforço para conseguirmos um melhor entendimento dos dados de *microarrays* sobre a PGE em células mTEC 3.10 evidenciando o efeito do gene Aire, construímos então as redes de interações baseadas na estatística Bayesiana.

Esse método de bioinformática nos ajudou testarmos a hipótese de que o gene Aire poderá atuar no controle da PGE de maneira indireta, ou seja, inicialmente controlando alguns genes-chave (nós gênicos) e estes por sua vez controlando um número maior de genes, ou seja, por meio de redes.

Hipóteses

2. HIPÓTESES

2.1. Células tímicas epiteliais medulares (mTECs) expressam, em cultura, grande quantidade de genes codificadores de antígenos relacionados a tecidos (expressão gênica promíscua).

2.2. O gene AIRE (*autoimmune regulator*) controla a expressão gênica promíscua nas células mTEC atuando por meio de interações em rede com outros genes.

Objetivos

3. OBJETIVOS

3.1. Avaliar a expressão gênica diferencial de TRAs (expressão gênica promíscua) em células tímicas epiteliais medulares (linhagem mTEC 3.10) usando a tecnologia dos cDNA microarrays.

3.2. Inibir o transcrito do gene *Aire* (*autoimmune regulator*) em células tímicas epiteliais medulares da linhagem mTEC 3.10 utilizando o método de RNA interferente (RNAi).

3.3. Avaliar o efeito da inibição do transcrito do gene *Aire* sobre a expressão gênica promíscua de células mTEC 3.10.

3.4. Construir redes de interações gênicas a partir de dados de *microarrays* de células tímicas epiteliais medulares (mTEC 3.10) após a inibição do transcrito do gene *Aire* com o intuito de observarmos as cascatas de regulação da expressão gênica promíscua.

Material e Métodos

4. MATERIAL E MÉTODOS

4.1. *Linhagem celular mTEC 3.10 (células tímicas epiteliais medulares)*

No presente trabalho utilizou-se a linhagem de células tímicas medulares primárias (mTEC 3.10), gentilmente cedida pelo Prof. Dr. Wilson Savino, coordenador do Laboratório de Pesquisas sobre o Timo da Fundação Instituto Oswaldo Cruz (FIOCRUZ) do Rio de Janeiro.

As células da linhagem 3.10 foram estabelecidas a partir do timo de camundongos da linhagem C57Bl/6 e o fenótipo de células medulares foi determinado por imunofenotipagem com anticorpo anti Th-4 (Mizuochi et al., 1992). Elas foram avaliadas usando um painel de anticorpos monoclonais anti-citoqueratina, confirmando o fenótipo medular distinto (Nihei et al., 2003). Além disso, confirmou-se pela técnica de FACS que estas células são CD80⁺ e, portanto, aptas a PGE (figura 6).

As células da linhagem 3.10 são aderentes e foram cultivadas em garrafas de cultura de 75 cm³ contendo 20 ml de meio RPMI 1640 e 10% de soro bovino fetal (SBF) a 37°C, em estufa com atmosfera de 5% CO₂.

Figura 6. Padrão de expressão de CD80 em mTEC 3.10

4.2. Inibição seletiva do transcrito do gene Aire com RNA interferente (RNAi).

4.2.1. Determinação da sequência de RNAi anti-Aire.

O ensaio de silenciamento do transcrito do gene Aire foi realizado utilizando o sistema de RNAi do Kit TriFECTa™ (IDT - Integrated DNA Technologies, Coralville, IA, USA). O kit inclui três seqüências diferentes de RNAi anti-Aire, as quais devem ser previamente testadas buscando a melhor eficiência de inibição do transcrito no modelo de estudo. Além das seqüências anti-Aire, outras três seqüências estavam incluídas no kit: 1) RNAi irrelevante marcado com Cy3 utilizado para medir a eficiência de transfecção (microscopia de fluorescência), 2) uma seqüência duplex de RNAi irrelevante, o qual não é capaz de alterar a expressão gênica nas células em estudo e 3) um controle positivo, constituída de uma seqüência RNAi anti-o transcrito do gene constitutivo HPRT (hypoxanthine guanine phosphoribosyl transferase), utilizada para avaliar se o processo de transfecção e o sistema modelo são adequados para inibição com RNAi.

O método escolhido para a transfecção das células mTEC em cultura foi o de lipofecção utilizando o Hiperfect Transfection Reagent® da Qiagen (Hilden, Alemanha), seguindo instruções do fabricante.

Para se determinar qual a melhor concentração e qual o melhor duplex para o silenciamento do transcrito de Aire, realizou-se um experimento piloto onde as três seqüências anti-Aire e as demais seqüências controle foram testadas nas concentrações de 2,5; 5,0 e 10,0 nM. Finalmente amostras de cDNA oriundas das células mTEC tratadas

com estes RNAs foram submetidas a reações de PCR (método de RT-PCR) para avaliarmos a melhor sequência inibitória.

As reações de RT-PCR do experimento piloto para a seleção da sequência de RNAi anti-Aire foram realizadas a partir de 2 µg de cada amostra de RNA, que foram convertidas a cDNA utilizando a enzima transcriptase reversa SuperScript II® (Invitrogen). Utilizamos então 5 µL do produto da reação e a amplificação por PCR foi feita em diluições sucessivas (1:1 a 1:128) em cada reação de RT-PCR semi-quantitativa. As sequências dos oligonucleotídeos *primers* utilizados encontram-se na Tabela I.

As condições de PCR foram: mix com volume final de 25 µl, contendo 200µM de dNTPs, 1,5 mM de MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8,4 a 25°C), 10 µM de cada *primer* e 2 U de Taq DNA polimerase. Ciclagem térmica: trinta e cinco ciclos foram realizados, cada um com três passos. Para o gene Aire: desnaturação a 94°C, 30 segundos; anelamento a 57°C, 30 segundos; extensão a 72°C, 30 segundos e para o gene HPRT: desnaturação a 94°C, 30 segundos; anelamento a 60°C, 30 segundos; extensão a 72°C, 30 segundos.

Os produtos da amplificação foram submetidos à eletroforese em gel de agarose 2% corado com brometo de etídeo.

Tabela I. Oligonucleotídeos *primers* utilizados nas reações de RT-PCR. (For = *forward*, Rev = *reverse*)

Gene	Seqüência dos <i>primers</i>	Produto PCR (pb)
HPRT(For)	5' GCTTGCTGGTGAAAAGGACCTCTCGAAG 3'	117
HPRT(Rev)	5'CCCTGAAGTACTCATTATAGTCAAGGGCAT3'	
Aire (For)	5' CATCCTGGATTCTCTGGAGGA 3'	253
Aire (Rev)	5' GCTCTTTGAGGCCAGAGTTG 3'	

4.2.2. Silenciamento do transcrito do gene *Aire* em cultura de células *mTEC* 3.10.

Ao atingirem semiconfluência as células da linhagem 3.10 foram destacadas das garrafas de cultura de 75 cm³ utilizando-se 9.5 ml de solução de tripsina 1x (2.5g de tripsina suína por litro em HBSS) + 0.5 ml de EDTA pH 8.0 por 5 minutos e as células foram contadas em câmara de *Newbauer*. As células foram colhidas por centrifugação a 1000xg durante 5 minutos. 5x10⁵ células foram semeadas em garrafas de cultura de 25 cm³ contendo 2,3 ml de meio RPMI e 10% de soro fetal bovino (SFB), uma hora antes do início do experimento com RNAi anti-Aire e colocadas em condições normais de cultura. Após colocar as culturas em estufa, procedeu-se com a preparação dos complexos RNAi-lipofectamina. Cinco nanomoles de RNAi foram gentilmente misturados à 100 µl de MEM filtrado sem SBF e então foram adicionados 6 µl de Hiperferct (Qiagen). Essa mistura foi vigorosamente agitada durante 10 segundos e em seguida incubadas 10 minutos à temperatura ambiente para a formação

dos complexos de transfecção RNAi-lipofectamina. Os complexos foram adicionados à cultura, permanecendo por 42 horas. Após 24 horas que as células foram semeadas, foram adicionados mais 2 ml de RPMI contendo 10% (SBF).

Foram utilizados 4 controles: 1) mTEC 3.10 sem RNAi; 2) mTEC 3.10 em presença apenas de lipofectamina; 3) mTEC 3.10 em presença de um RNAi irrelevante, sem a capacidade de silenciar qualquer gene de camundongo; 4) mTEC 3.10 na presença de um RNAi anti-HPRT, um controle positivo, que indica a funcionalidade do método.

Para a avaliação da eficiência da transfecção fez-se uso de microscopia fluorescente onde a seqüência duplex de RNAi irrelevante marcada com Cy3 foi testada em diferentes concentrações: 1; 2.5; 5 e 10 η M.

A avaliação da transfecção das células mTEC 3.10 utilizando microscopia de fluorescência foi realizada no Laboratório da Profa. Dra. Elza Tiemi Sakamoto-Hojo no Departamento de Genética da Faculdade de Medicina de Ribeirão Preto.

4.3. Extração de RNA total

A fim de prevenir da contaminação por ribonucleases durante a extração e manuseio dos RNAs, toda a vidraria, tubos plásticos, espátulas e pinças utilizadas foram previamente autoclavados. O material plástico (tubos e ponteiros) eram novos e previamente autoclavados. Todo o procedimento foi realizado usando luvas de látex sem talco e descartáveis.

Após 42 horas o RNA total de cada cultura foi extraído utilizando-

se o reagente MirVANA™ (Ambion), seguindo as instruções do fabricante. As células da linhagem 3.10 foram destacadas das garrafas de cultura de 25 cm³ utilizando-se 4.75 ml de solução de tripsina 1x (2.5g de tripsina suína por litro em HBSS) + 0.25 ml de EDTA pH 8.0 por 5 minutos, seguido de inativação da tripsina com meio de cultura + SBF e colhidas por centrifugação a 1000xg durante 5 minutos. As células foram lavadas duas vezes com PBS 1x, seguida de centrifugação a 1000 xg durante 5 minutos. Adicionou-se 1000 µl de solução de lise. Agita-se vigorosamente (pipetagem e 10 segundos no vórtex) para lisar completamente as células e obter um lisado homogêneo. Adiciona-se 100 µl de miRNA Homogenate Additive e mistura bem (vórtex 15 segundos). Deixa a mistura no gelo por 10 minutos. Adiciona-se 1000 µl de Ácido-Fenol:Clorofórmio e agita-se vigorosamente no vórtex por 60 segundos. O lisado é então centrifugado a 10000 xg por 5 minutos à temperatura ambiente. A fase aquosa é cuidadosamente transferida para um novo tubo e então é adicionado 1.25x volume recuperado após a centrifugação de ethanol 100% à temperatura ambiente. Para cada amostra é montada uma coluna em um tubo coletor. 700 µl da mistura foram pipetados na coluna e centrifugados 15 segundos à 10000xg. O líquido foi descartado e o procedimento foi repetido até que toda a mistura lisado/etanol tenha sido filtrado. Aplicou-se 700 µl de miRNA Wash Solution 1 à coluna e centrifugou-se por 10 segundos à 10000 xg. Descartou-se o líquido e 500 µl de miRNA Wash Solution 2/3 foram aplicados à coluna e centrifugados 10 segundos à 10000 xg. Repetiu-se o último passo e, após descartar o líquido, centrifugou-se 1 minuto para remover resíduos líquidos no filtro. A coluna foi transferida para um tubo novo coletor e a ela foram adicionados 100 µl de água DEPC pré-aquecida (95°C). Centrifugou-se 30 segundos para recuperar o RNA.

Ao RNA total recuperado na etapa anterior foram adicionados três volumes de etanol absoluto gelado a fim de precipitar e concentrar a amostra e a mistura deixada a -20°C por pelo menos 18 horas.

O RNA total foi coletado por centrifugação a frio e o precipitado foi lavado duas vezes com 1000 µL de etanol 70 % também a frio. O excesso de etanol foi removido do precipitado de RNA por evaporação em tubo aberto tomando cuidado para não ressecar as amostras. Finalmente as amostras de RNA foram dissolvidas em 13 µL de água Milli-Q estéril.

O grau de pureza das preparações de RNA total foi calculado por medidas espectrofotométricas (GENEQUANT - AMERSHAM PHARMACIA BIOTECH) calculando as razões entre as absorbâncias; $A_{260}/A_{280} \sim 2,0$ indicando que a preparação estava livre de proteínas e $A_{220}/A_{260} \sim 0,7$ livre de fenol. A integridade das preparações foi avaliada por meio de eletroforese em gel de agarose corado com brometo de etídeo seguindo protocolo convencional.

4.3.1. Quantificação de RNA total

O precipitado de RNA total era dissolvido em 13 µl de água Milli-Q previamente tratada com DEPC. A solução de RNA era então diluída 100X e submetida a dosagens em espectrofotômetro usando luz ultravioleta (GENEQUANT - AMERSHAM PHARMACIA BIOTECH). As dosagens tiveram como base a seguinte estimativa: 1U de $A_{260} = 40 \mu\text{g RNA/mL}$.

4.4. Eletroforese de RNA total em gel denaturante (Sambrook et al., 1989)

4.4.1. Preparação do gel

A agarose (70 ml) foi fundida em água Milli-Q autoclavada (1,5% de agarose), e quando à temperatura de 60°C, é adicionado 20 ml de formaldeído (37%) e 22 ml de tampão de migração 5X concentrado (20,6 g de MOPS [ácido propano sulfônico 3-(N-morfolino)], dissolvidos em 800 ml de acetato de sódio 50mM, pH 7,0 ajustado com NaOH 2N e adicionados 10 ml de EDTA 0,5 M pH 8,0, volume final ajustado para 1000ml). O gel foi solidificado em suporte de acrílico que foi, inicialmente, tratado com NaOH 0,5 M por 10 minutos, assim como a cuba e o pente para eliminação de RNAase sendo posteriormente lavado com água Milli-Q autoclavada.

4.4.2. Preparação das amostras de RNA

Em um tubo tipo *Eppendorf* novo e autoclavado colocado em banho de gelo foram adicionados 4,5 µl de uma solução de RNA (5 µg de RNA), 2 µl de tampão de migração (5X); 3,5 µl de formaldeído (37%) e 10 µl de formamida. A solução é, então, incubada a 65°C por 15 minutos, sendo posteriormente resfriado imediatamente em banho de gelo. Foi adicionado tampão de aplicação (1/10 do volume) e, as amostras aplicadas no gel. A eletroforese foi realizada à 80 V por cerca de 2,5 a 3 horas. Para o cálculo do peso molecular dos RNAs utilizamos RNAs ribossômicos 28 S (4,8 Kb) e 18 S (1,9 Kb) como marcadores.

4.5. *PCRs semi-quantitativas para os genes Aire e HPRT.*

Dois microgramas de cada amostra de RNA total foram convertidos a cDNA utilizando a enzima SuperScript II® (Invitrogen) em uma reação de rt-PCR. Cinco microlitros do produto da reação de rt-PCR foram amplificados em diluições sucessivas (1:2, 1:4, 1:8, até 1:1024) em cada reação de PCR semi-quantitativa. Foram usados pares de *primers* específicos (Tabela 1) para ambos os genes: Aire e HPRT. Um volume final de 25 µl de uma reação típica de PCR, contendo 200µM de dNTP, 1,5 mM de MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8,4 a 25°C), 10 µM de cada primer e 2 U de Taq DNA polimerase, foi submetida a ciclagem térmica. Trinta e cinco ciclos foram realizados, cada um com três passos. Para o gene Aire foi desnaturação: 94°C, 30 segundos; anelamento: 57°C, 30 segundos; extensão: 72°C, 30 segundos e para o gene HPRT foi desnaturação: 94°C, 30 segundos; anelamento: 60°C, 30 segundos; extensão: 72°C, 30 segundos. Os produtos da amplificação foram submetidos a uma eletroforese em gel de agarose 2% em paralelo ao lado de um marcador de peso molecular de 50 pb (Promega, EUA).

4.6. *Preparação de cDNA microarrays em lâminas de vidro*

4.6.1. *Biblioteca de cDNAs murinos*

Nosso laboratório mantém uma biblioteca de cDNA murino (Biblioteca MTB IMAGE), com cerca de 9.000 clones de timo provenientes do “IMAGE Consortium” (<http://image.llnv.gov>). São clones com seqüências “*expressed sequence tags*” (EST), cujos tamanhos moleculares

variam de 500 a 1.500 pb, e está totalmente caracterizada, cujos clones estão seqüenciados e catalogados. Os clones estão identificados com seus respectivos “*accession numbers*” (Acc) no *GenBank*, Clone ID, o nome do gene e a posição de cada clone nas placas de 384 poços em planilhas do programa Excel® Microsoft. Conservamos esta biblioteca em *E. coli* em placas de microtitulação (384 poços) a -80°C (material gentilmente cedido pela Dra. Catherine Nguyen, do INSERM-TAGC ERM 206, Marseille, França).

4.6.2. Amplificação de cDNA para a confecção de microarrays

Os clones de *E. coli* da biblioteca de cDNA IMAGE murina, repicados em placas de 384 poços contendo meio de cultura 2X LB + ampicilina foram incubados durante 16 horas, na proporção de 5 µl da cultura original diluídos em 45 µl de meio de cultura 2X LB (volume final em cada poço: 50 µl). A seguir uma alíquota de 8µl da cultura foi transferida para outra placa de 384 poços, contendo 72 µl de água deionizada estéril, e esta placa incubada a 95° C por 10 min. para lise das células. Após centrifugação a 4000xg por 5 min. foi retirado 10µl do sobrenadante e adicionado a 40µl de um *mix* para PCR contendo tampão da reação de PCR (10X), solução de dNTP (2mM), *primers* com seqüências consenso aos três vetores presentes na biblioteca (10µM), *primer* LBP 1S (5'TGTGGATTGTGAGCGGATAA3') e *primer* 1AS (5'GGGTTGAATTAGCGGAACG3'), Taq polimerase (5U/µl) e água deionizada estéril q.s.p 50 µl. O programa para amplificação dos insertos teve como base aquele descrito por Menossi *et al.*, 2000: 5 minutos a 94°C, seguidos de 30 ciclos (94°C, por 30 segundos; 55°C por 30 segundos, 72°C por 2 minutos), seguida por uma extensão final de 7 minutos a 72°C e 5

minutos a 4 °C. Foi realizada em um aparelho termociclador “*Mastercycler Gradient*” (Eppendorf), em placas de PCR seladas com adesivo laminado para evitar evaporação.

Cerca de 2 amplificações PCR de cada clone foram realizadas, sempre totalizando 50µl de volume.

A avaliação da qualidade dos produtos amplificados pela PCR foi feita em gel de agarose 1%, em tampão TAE 1X (Tris-acetato 0,04 M, EDTA 0,001 M) contendo brometo de etídio (10mg/ml) a 80V, durante 15 min. A visualização das bandas foi realizada utilizando transluminador U.V. e fotografados em Polaroid.

4.6.3. Purificação dos produtos de PCR

Para obtenção de uma eficiente fixação dos insertos de cDNA amplificados tanto nas membranas de náilon ou nas lâminas de vidro é necessária a remoção de nucleotídeos não incorporados e *primers* da reação de PCR (Hedge *et al.* 2000). Utilizamos a purificação dos produtos de PCR por precipitação com etanol.

Os produtos de PCR (50 µl) foram transferidos para placas de cultivo com fundo em “U” e foram adicionados 10 µl de acetato de sódio 3M pH 4,8 em cada poço de uma placa de 96 poços. Em seguida, os produtos das duas PCR foram agrupados, totalizando 100 µl, que foram transferidos para esta mesma placa. Após a adição de 100 µl de etanol absoluto, as placas foram incubadas por 16 horas (*overnight*) a -20°C. Após este tempo, as placas foram centrifugadas a 4000xg por 60 min. a 2-8°C. O sobrenadante foi desprezado e o *pellet* foi ressuspenso em 100 µl de etanol 70%. As placas foram novamente centrifugadas a 4000xg por 30

min a 2-8°C. O sobrenadante foi novamente desprezado e a placa secou à temperatura ambiente *overnight*. Após a placa seca, o *pellet* foi ressuspenso em 50 µl de água deionizada autoclavada.

Uma alíquota de 5 µl destes produtos foi aplicada em gel de agarose 1% contendo brometo de etídeo, visualizado em transluminador U.V. e fotografado em Polaroid para avaliar a qualidade dos produtos amplificados.

As placas foram secas em forno a 42°C e adicionou-se 27 µl de solução de spotagem (DMSO 50% - dimetil sulfoxido) por poço da placa. Em seguida, estas placas foram levadas ao freezer - 20°C e os produtos foram congelados e descongelados por 3 vezes para uma melhor dissolução do DNA concentrado. Posteriormente, os clones foram passados para as placas do robô, e estocadas a 4°C até a deposição dos produtos de PCR em lâminas de vidro.

4.6.4. *Confecção das lâminas de microarrays*

Amostras de produtos de PCR purificados foram preparadas para deposição em lâminas de vidro adicionando-se mesmo volume (1:1) de Reagente D (*GE Healthcare*) e transferidas para microplacas de 384 poços (*Genetix*). Por meio de um robô *Array Spotter III* (*Amersham Molecular Dynamics*) as amostras foram depositadas por um conjunto de 12 canetas que deposita um volume de 0,9 nl da amostra baseada na ação de capilaridade em superfície de lâminas de vidro (*Corning*® - *UltraGaps* 40015). Após a deposição de cada conjunto de amostras as canetas foram lavadas automaticamente em uma estação de lavagem que utiliza sucessivamente água purificada (18 megohm/cm), etanol absoluto

(Merck), solução 0,2 M de KOH e água novamente. As canetas foram secas com nitrogênio 5.0 analítico antes das próximas amostras serem carregadas. A câmara de deposição das amostras em lâminas do robô *Array Spotter III* possui temperatura e umidade controladas. A umidade relativa de deposição das amostras foi de $\approx 55\%$ e a temperatura foi de $\approx 25^{\circ}\text{C}$. Além disso, este robô está instalado numa sala especial com ar limpo (sala limpa classe 10.000).

Após a deposição e secagem de todas as amostras nas lâminas, o DNA foi fixado por “*cross-linking*” por meio de irradiação ultravioleta a 500 mJ de energia (*Hoefler UV Crosslinker*).

4.7. Delineamento das hibridações em microarrays

Uma escolha chave em um projeto que envolve a tecnologia de microarray é utilizar comparações que podem ser diretas ou indiretas isto é, estabelecer comparações dentro ou entre as lâminas. Existem três principais tipos de delineamento: 1) inversão de corantes (*dye-swap*), 2) experimentos em volta (*looping*) e 3) RNA de referência (figura 7). Mas, não existe um delineamento experimental ideal para todas as situações, ou seja, diferentes desenhos experimentais são necessários para contextos experimentais diferentes. Qualquer que seja o tipo de desenho utilizado será requerido o mesmo número de hibridações, e a decisão sobre o tipo de delineamento deve considerar a pergunta do trabalho.

Figura 7. Esquema dos principais tipos de delineamento experimental para *microarrays*. A, B e C - amostras controle; A', B' e C' - amostras teste; R - pool de RNA de referência.

O pool de RNA derivado de linhagens celulares é a amostra de referência mais utilizada atualmente. Para fornecer a cobertura ótima dos genes depositados na lâmina, as amostras de referência são freqüentemente constituídas de diferentes linhagens celulares oriundas de vários tecidos. Nos experimentos com *microarrays* do presente trabalho adotou-se a estratégia do RNA de referência ao invés do *dye-swap*, pois para realizar os experimentos com troca de corantes seria necessário marcar a mesma amostra de RNA com os dois corantes (Cy3 e Cy5). Porém, tal abordagem passa a ser inviável, pois a quantidade de RNA seria insuficiente para a repetição das hibridações.

O pool de RNA de referência neste caso foi uma mistura “equimolar” de RNA total de timos de camundongos C57BL/6 adultos cujas amostras foram extraídas exatamente nas mesmas condições das experimentais. Como neste trabalho escolheu-se o uso de RNA de referência, segue algumas considerações:

Ensaaios de co-hibridação diferencial usando *microarrays* medem a expressão gênica relativa de amostras emparelhadas e de uma amostra referência, sendo que o poder da análise de microarray vem da identificação de padrões informativos de expressão de um gene através das experiências múltiplas. O cumprimento destes objetivos é facilitado usando uma amostra de referência comum para todos os experimentos que forneça uma medida da expressão base para cada gene, permitindo a normalização e a comparação de experimentos independentes.

Um vasto número de linhagens celulares pode não melhorar necessariamente a representatividade total dos genes depositados no *array*, pois algumas linhagens celulares expressam significativamente mais genes do que outras e, nem todas as linhagens expressam todos os genes em níveis semelhantes. Misturar RNA de muitas linhagens celulares pode diluir os transcritos raros de modo que a sua representação no “*pool*” final do RNA corre o risco de ficar abaixo do limite detectável (Yang *et al.* 2002).

O uso de RNA de referência passou a ser uma abordagem bastante adequada eliminando assim a necessidade de repetir as marcações de uma mesma amostra com dois corantes (*dye-swap*) e, conseqüentemente evitando as diferenças de incorporação.

O delineamento das hibridações de *microarrays* consistiu em marcar todas as amostras de células mTECs em diferentes condições com fluorocromo Cy3 e combiná-los numa mesma lâmina com amostras de RNA (cDNA) de referência marcados com Cy5. Neste trabalho, o RNA de referencia utilizado foi um pool de RNAs de timos de seis camundongos da linhagem C57Bl/6.

As hibridações com cDNAs oriundos de RNA referência são importantes para os cálculos de normalização, pois reflete a estimativa da quantidade de material depositado em cada ponto do *microarray* (alvo).

4.8. Marcação das sondas complexas de cDNA com fluorocromos CY3 e CY5

Amostras de RNA foram convertidas a cDNA e marcadas utilizando o Kit *CyScribe Post-Labelling* (GE Healthcare - Buckinghamshire, UK) que envolve a preparação e adequação do cDNA em dois passos. No primeiro passo ocorre a síntese da primeira fita de cDNA com a incorporação de nucleotídeos amino- alil dUTP modificados, com posterior degradação da cadeia de RNAm e purificação do cDNA para remoção de nucleotídeos livres e oligômeros (figura 8). No segundo passo, o cDNA é marcado com formas reativas de ésteres NHS Cy3 e Cy5 que se ligam aos nucleotídeos modificados e após um processo de purificação para eliminação dos *CyDye* não incorporados a sonda está pronta para hibridação (figura 9).

Figura 8. Preparação da fita simples de cDNA incorporada com amino alil usando os reagentes do kit “CyScribe Post Labelling” (GE Healthcare).

Figura 9. Preparação do cDNA marcado com *CyDye* utilizando os reagentes do kit “*CyScribe Post Labelling*” (GE Healthcare).

a) Preparação da primeira fita de cDNA por incorporação de AAdUTP

Em um tubo *Eppendorf* de 1,5 µl imerso em gelo picado foram adicionados 10 µg de RNA total, 1 µl de *primers* randômicos, 3 µl de oligo (dT) e 0,5 µl de controle denominado “*spike mix*” para o Universal *ScoreCard* em um volume total de 11 µl (Proporção de 2 µl de *spike mix* para cada 1 µg de RNA marcado). A reação foi cuidadosamente montada e incubada a 70°C por 5 min, sendo posteriormente resfriada a 4°C durante 5 min. A extensão da cadeia de cDNA foi realizada utilizando 4 µl de tampão 5X *CyScript*, 2 µl de DDT 0,1 M, 1µl de nucleotídeo “*mix*”, 1µl de AA-dUTP (a quantidade de um tubo contendo AA-dUTP liofilizado foi ressuspenso em 30 µl de água livre de nucleases e mantido a -20°C por no máximo 30 dias), 1 µl de transcriptase reversa *CyScript*, em um volume final de reação de 20 µl. A reação foi incubada a 42°C por 1,5 hora. Procedemos à degradação do RNAm adicionando 2 µl de NaOH 2.5 M com incubação a 37°C durante 15 min, posteriormente foi adicionado 20 µl de HEPES 2M.

b) Purificação do cDNA com colunas de purificação CyScribe GFX

Para cada amostra de cDNA com volume entre 20 a 100 µl foram adicionados 500µl de solução “*capture buffer*” em uma coluna de purificação GFX colocada dentro de um tubo coletor. O produto de cDNA marcado com aminoalil não purificado foi adicionado à coluna e misturado 5 vezes. A centrifugação foi feita a 13800 xg por 30 seg. A coluna foi retirada e o líquido contido no tubo coletor foi descartado. A coluna foi novamente colocada no tubo coletor e foram adicionados 600µl de etanol 80%. A centrifugação foi feita a 13800 xg por 30 seg. A coluna foi retirada e o líquido contido no tubo coletor foi descartado. Esta

lavagem foi repetida mais duas vezes. Uma nova centrifugação foi feita a 13800 xg por 10 seg. para retirada do excesso de etanol 80% da amostra. O tubo coletor foi descartado e a coluna contendo o cDNA foi colocada em um tubo de 1,5 µl. Foi adicionada a coluna 60 µl de bicarbonato de sódio 0,1 M pH 9,0 e incubado durante 5 minutos. O material foi centrifugado a 13800 xg por 1 min.

c) Incorporação de Cy3 e Cy5

A amostra de cDNA marcada com aminoalil purificado foi adicionada a um tubo com a alíquota de CyDye NHS éster e ressuspensa várias vezes. O material foi centrifugado a 13800 xg por 1 min e incubado, no escuro, durante 1 hora. Posteriormente, foi adicionado 15 µl de hidroxilamina 4M seguida de incubação no escuro, durante 15 min a temperatura ambiente.

d) Purificação do cDNA marcado com CyDye com colunas de purificação CyScribe GFX

Para cada amostra de cDNA com volume entre 20 a 100 µl foram adicionados 500 µl de solução “capture buffer” em uma coluna de purificação GFX colocada dentro de um tubo coletor. O produto de cDNA marcado com CyDye não purificado foi adicionado à coluna e misturado 5 vezes. A centrifugação foi feita a 13800 xg por 30 segundos. A coluna foi retirada e o líquido contido no tubo coletor foi descartado. A coluna foi novamente colocada no tubo coletor e foram adicionados 600 µl da solução “wash buffer”. A centrifugação foi feita a 13800 xg por 30 segundos. A coluna foi retirada e o líquido contido no tubo coletor foi descartado. Esta lavagem foi repetida mais duas vezes. Uma nova

centrifugação foi feita a 13800 xg por 10 segundos para retirada do excesso da solução “wash buffer” da amostra. O tubo coletor foi descartado e a coluna contendo o cDNA foi colocada em um tubo de 1,5 µl. Foi adicionado a coluna 60 µl da solução “elution buffer” pré-aquecido à 65°C e incubado durante 5 minutos. O material foi centrifugado a 13800 xg por 1 minuto.

e) *Quantificação do CyDye incorporado no cDNA*

Após a purificação foi feita a monitoração de incorporação dos fluorocromos por meio de leitura em espectrômetro (*Ultrospec 2100, GE Healthcare*) em comprimento de onda a 550 nm para Cy3 e 650 nm para o Cy5 usando amostras diluídas 100X. A quantidade de Cy3 ou Cy5 incorporados no cDNA pode ser calculada através do seu coeficiente de extinção molar 150 000 l mol⁻¹ cm⁻¹ para Cy3 e 250 000 l mol⁻¹ cm⁻¹ para Cy5. As proporções de Cy3 e Cy5 incorporados no cDNA foram calculados pela fórmula: (A)/E x Z x fator de diluição x 10¹², onde:

A= absorbância de Cy3 a 550 nm ou Cy5 a 650 nm

E= coeficiente de extinção para Cy3 ou Cy5 x 10⁻⁶

Z= volume (µl) da sonda após purificação

Para hibridações com os dois fluorocromos foram adicionados os cDNA marcado com Cy3 e Cy5 em um tubo de microcentrífuga protegido da luz. A solução de cDNA foi concentrada no aparelho “*Speed Vacuum*”. A seguir o cDNA foi dissolvido em 6 µl de água livre de nuclease e desnaturado a 95°C por 2 min. A solução foi imediatamente resfriada no gelo por 30 seg. A essa reação adicionamos 1,5 µl de A₈₀ (1mg/ml) e incubamos a 75 °C por 45 min, estando assim pronta para o

processo de hibridação.

4.9. Hibridação das lâminas de microarrays

As lâminas de *microarrays* foram hibridadas utilizando um processador automático de lâminas, “*Lucidea Automated Slide Processor*” – ASP (Amersham Biosciences) que permite a hibridação e lavagens de lâminas em câmaras independentes (12 lâminas por vez). Este aparelho inclui um software que automatiza a injeção de amostras líquidas e soluções de lavagens ou ar dentro das câmaras e possui parâmetros de controle de temperatura e velocidade de injeção e circulação destas soluções no interior das mesmas.

As lâminas foram hibridadas por 15 horas a 42°C. As condições de lavagens foram: 1XSSC/0,2%SDS (2 x 20 seg. à temperatura ambiente); 0,1X SSC/0,2% SDS (2 x 20 seg. à temperatura ambiente); 0,1X SSC (2 x 20 seg. à temperatura ambiente). A última lavagem das lâminas foi feita com isopropanol, sendo em seguida aquecidas a 42°C, e novamente lavagem com isopropanol. Após as lavagens as lâminas foram aquecidas a 60°C para secagem. As lâminas estavam prontas para serem “lidas” em aparelho “*scanner*” a laser.

4.10. Aquisição de imagens de microarrays

As lâminas foram lidas num aparelho *Generation III “Array Scanner”* (Amersham Biosciences) com lasers de 532nm para o Cy3 (verde) e 633 nm para o Cy5 (vermelho). A leitura da lâmina gera dois arquivos com imagens separadas com os pontos em preto para os dois

canais (Cy3 e Cy5) e uma terceira imagem, agora colorida, sobrepondo Cy3 e Cy5 visualizadas usando o software *ImageQuant* (GE Healthcare).

4.11. Quantificação e normalização dos dados de microarrays

Após obtenção das imagens seguiu-se à análise realizada em duas etapas.

- Inicialmente, os dados contidos nas imagens foram transformados em dados numéricos, utilizando o programa *Spotfinder* (<http://www.tigr.org/software>). Esse programa, além de transformar as informações das imagens em valores numéricos, também analisa a qualidade dos pontos e calcula o "background". Dois parâmetros foram considerados para o controle de qualidade neste programa: os pontos de boa qualidade apresentam valores superiores e/ou igual a 1 valor "backgrounds" mais 1 valor desvio padrão.
- Em seguida, estes dados foram normalizados. A normalização retira os erros experimentais sistemáticos por balancear a intensidade dos dois fluorocromos. Esses erros podem ocorrer devido à diferença de incorporação dos corantes, efeitos espaciais na lâmina e diferenças durante a aquisição das imagens nos dois canais. Para esses ajustes, utilizou-se a plataforma R (www.r-project.org), com o pacote AROMA (<http://www.maths.lth.se/help/R/aroma/>), que retém as funções necessárias para a normalização dos dados de *microarrays*. Portanto, após a retirada do background pelo programa *Spotfinder*, os dados foram importados para o ambiente R e transformados para o formato de dados "M versus A", onde M é igual a $\log_2(R/G)$ e A é igual a

$1/2 \cdot \log_2(R/G)$. Em seguida, os métodos de normalização “*print-tip Lowess*” e “*absolute median deviation (MAD) re-scaling*” foram aplicados respectivamente. O primeiro método aplica uma regressão linear nos dados, para corrigir erros espaciais que possam ter sido gerados durante os experimentos. O segundo re-escala as razões de log para cada microarray, de maneira que cada slide adquira a mesma distribuição dos dados, de acordo com a MAD, capaz de estimar com robustez a variância de uma amostra. Na figura 10, observamos o fluxograma da “pipeline” desenvolvida para análise dos dados.

Utilizando o pacote LIMMA (Linear Models for Microarray Data) (Smith, 2004) foi aplicado o método Bayesiano empírico para análise estatística da expressão diferencial.

Figura 10. “Pipeline” utilizado na análise de dados de *microarrays* em lâminas de vidro.

A plataforma R irá gerar arquivos de extensão .TAV.MOD.MEV que serão utilizados no programa TIGR MEV (The Institute for Genomic Research MultiExperiment Viewer) (<http://www.tm4.org/mev.html>) que inclui o programa SAM (Significance Analysis of Microarrays) (Tusher et al, 2001) utilizado para a detecção dos genes diferencialmente expressos (Smyth, 2004). O programa TIGR MEV gera um *heat map* (mapa de cores relacionado com o nível de expressão dos genes) para os valores. O *heat map* é composto por um código de cores a fim de facilitar a associação visual dos níveis de expressão gênica.

O programa SAM (“Significance Analysis of Microarrays”) representa uma evolução dos softwares de análise estatística para a tecnologia de *microarrays* e encontra-se disponível no endereço (<http://www-stat.stanford.edu/~tibs/SAM/>). A análise baseia-se em uma série de testes-t específicos para cada gene, que são adaptados para a detecção da expressão gênica diferencial em larga escala. A partir da observação de que as flutuações casuais são específicas para cada gene, o teste SAM é baseado na razão entre a diferença das médias das situações, como por exemplo, mTECs controles (X_c) e mTEC em presença de RNAi anti-AIRE (X_p), e o desvio padrão de cada gene, calculados a partir de repetição experimental. A diferença relativa $d(i)$ na expressão gênica é então definida pela equação 1:

[EQUAÇÃO]

$$d(i) = \frac{X_p(i) - X_c(i)}{S(i) + S_0}$$

$$\left\{ \begin{array}{l} d(i) = \text{diferença relativa} \\ X_p(i) = \text{níveis médios da expressão dos genes} \\ \quad \text{no timo} \\ X_c(i) = \text{níveis médios da expressão dos genes} \\ \quad \text{no pool de órgãos} \\ S(i) = \text{desvio padrão de medidas repetidas de} \\ \quad \text{expressão} \\ S_0 = \text{constante positiva} \end{array} \right.$$

Onde $X_p(i)$ e $X_c(i)$ são definidos como níveis médios da expressão do gene nos estados p (timo) e c (pool de órgãos), respectivamente. A dispersão gene-específica $S(i)$ é o desvio padrão de medidas repetidas de expressão, e a constante positiva S_0 no denominador da equação acima, servem para certificação de que a variância de $d(i)$ é independente da expressão gênica. Para a determinação de genes com mudanças significativas na expressão, utilizou-se um gráfico de dispersão $d(i)$, em relação à diferença relativa esperada $d_E(i)$. Para uma grande maioria de genes $d(i) \cong d_E(i)$, mas alguns genes são representados por pontos distantes da linha $d(i) \cong d_E(i)$. As alterações das expressões dos genes que se encontram a uma distância maior do que o limiar (Δ) é então considerado significativo (figura 11).

Figura 11: Comparação entre a distância relativa observada $d(i)$ e esperada $d_E(i)$.

O limiar (Δ) determina dois cortes horizontais, ou seja, o menor valor de $d(i)$ indica que o gene seja considerado significativamente induzido (hiperexpresso), e o valor menos negativo de $d(i)$ indica que o gene está significativamente reprimido (hipoexpresso).

A porcentagem de genes identificados por mudanças aleatórias é chamada de Frequência de Descobertas Falsas (FDR), um método inicialmente idealizado por Benjamini & Hochberg (1995) e definido como a proporção esperada de rejeições falsas. O cálculo de FDR e o número de genes com mudanças significativas estão intimamente relacionados com o limiar Δ . À medida que o valor de Δ diminui, o número de genes significativamente alterados aumenta à custa de um aumento de um FDR. Essa determinação do nível de significância pelo limiar providencia cortes assimétricos para genes induzidos e genes reprimidos. Essa assimetria é desejável, posto que os genes induzidos e genes reprimidos podem se comportar de maneira diferente em alguns experimentos. Ao utilizar o SAM, o usuário pode escolher o limiar Δ mais conveniente com base no nível de significância estimado pelo FDR e no número de genes com os quais se pretende trabalhar.

O programa SAM estabelece automaticamente uma ligação entre o número de acesso das seqüências utilizadas com as páginas de informações sobre o clone em questão, situados no banco de dados S.O.U.R.C.E. (*"Stanford Online Universal Resource for Clones and ESTs"*) (<http://source.stanford.edu/cgi-bin/source/sourceSearch>). O S.O.U.R.C.E. compila informações de vários bancos de dados públicos (*UniGene, dbEST, Swiss-Prot, GeneMap99, RHdb, GeneCards e LocusLink*) e as disponibilizam de maneira a facilitar a identificação dos genes diferencialmente expressos.

O programa SAM permite ainda o agrupamento hierárquico, porém, o mais importante na elaboração deste agrupamento é a decisão sobre qual medida de similaridade será adotada, a necessidade de se transformar a escala dos valores de expressão (normalmente transformada em escala logarítmica) e a dependência dos genes entre si. O agrupamento hierárquico nos permite a definição de grupos de genes com o mesmo padrão de expressão, seja ele de indução ou de repressão.

Todas as informações citadas acima foram retiradas do trabalho de Tusher *et al.* 2001, do manual do software SAM e de relatórios técnicos publicados na página de Robert Tibshirani (<http://www-stat.stanford.edu/~tibs/SAM>).

4.11.1. Nomenclatura dos genes

Neste trabalho, optou-se por seguir a nomenclatura usual para genes de camundongos, ou seja, o símbolo do gene em inglês com a primeira letra maiúscula e as seguintes minúsculas (exemplo: Aire para **Autoimmune regulator**). Os nomes dos genes estão em inglês, pois toda a literatura e os bancos de dados internacionais como *Source*, *GenBank* etc. estão neste idioma. Além disso, todos os arquivos da biblioteca de cDNA estão em inglês.

4.12. Análise da expressão gênica promíscua

Os agrupamentos gênicos gerados durante o processamento dos dados no programa TIGR MEV foram analisados em termos de Expressão Gênica Promíscua (PGE) para avaliar a representatividade dos órgãos e

sistemas. A análise foi feita utilizando o banco de dados SymAtlas (<http://symatlas.gnf.org/SymAtlas/>) que permite a visualização da expressividade de cada gene em um gráfico de barras paralelas que apresenta 60 tecidos, que representam por sua vez 17 sistemas do organismo.

Visando a homogeneização dos dados optou-se por considerar como tecidos representados por um determinado gene, apenas aqueles tecidos que obtiveram expressividade superior à linha da mediana que se encontra presente em cada gráfico. Após a organização dos dados em sistemas, gerou-se uma planilha contendo o número de genes que caracterizavam cada sistema em particular e, posteriormente, gerou-se um gráfico de setores demonstrando a representatividade de cada sistema nos agrupamentos definidos.

4.13. GeneNetwork

O programa escolhido para a inferência de redes gênicas foi o GeneNetwork por ser aquele que melhor se aplica aos nossos conjuntos de dados. O GeneNetwork é um software livre e encontra-se disponível aos usuários no site: <http://genenetwork.sbl.bc.sinica.edu.tw/index.asp>.

O fluxo do trabalho para GeneNetwork é como segue: (1) input dos dados experimentais no formato de texto limitado por tabulações; (2) procede a interpolação dos dados através do controlador de interpolação se o número de pontos de dados experimentais forem insuficientes para iniciar os cálculos de inferência; (3) executar a aproximação da inferência por engenharia reversa através do “Modeling controller” para gerar a matriz gênica regulatória que descreve como os genes se regulam; (4)

extrair automaticamente a rede para a visualização, baseada na matriz regulatória; (5) comparar a rede intuitiva inferida com as bases de dados on-line tais como KEGG (<http://www.genome.jp/kegg/>), baseado na informação do GraphViewer Network e do Information Viewer; e (6) a revisão dos conjuntos de experiências propostos e geração da hipótese (Wu et al., 2004).

Para facilitar a interpretação dos grafos gerados pelo programa GeneNetwork um dos alunos de mestrado do nosso laboratório, Guilherme Silva Liberato desenvolveu o programa Mandala, ainda não publicado. Seu algoritmo, escrito em linguagem de programação C, decodifica a matriz regulatória proveniente do GeneNetwork, desvendando as interações gênicas existentes sem que seja necessário analisar o grafo correspondente à rede. O resultado é apresentado em formato textual.

DELINEAMENTO EXPERIMENTAL

5. DELINEAMENTO EXPERIMENTAL

Figura 12. Fluxograma do trabalho mostrando o sistema modelo experimental utilizando células tímicas epiteliais medulares (mTEC 3.10), a inibição do transcrito do gene Aire, as hibridações com *microarrays* e o uso dos programas de bioinformática.

Resultados

6. RESULTADOS

6.1. Inibição seletiva do transcrito do gene Aire com RNA interferente (RNAi).

O ensaio de silenciamento do transcrito do gene Aire foi realizado usando a sequência que apresentou melhor eficiência do Kit TriFECTa™ (IDT - Integrated DNA Technologies, USA).

Como já explicado em Material e Métodos, além das sequências anti-Aire, outras três sequências que estavam incluídas no kit foram utilizadas: 1) RNAi irrelevante marcado com Cy3 usado para medir a eficiência de transfecção (microscopia de fluorescência), 2) uma sequência duplex de RNAi irrelevante, o qual não é capaz de alterar a expressão gênica nas células em estudo e 3) um controle positivo, constituída de uma sequência RNAi anti-HPRT (hypoxanthine guanine phosphoribosyl transferase), um gene constitutivo, utilizada para avaliar se o processo de transfecção e o sistema modelo são adequados para inibição com RNAi.

6.1.1. Monitoramento da transfecção e determinação da sequência de RNAi anti-Aire.

Para o monitoramento do método de transfecção foi realizada uma cultura em presença de RNAi irrelevante marcado com Cy3. Como é possível observar na figura 13, o método de transfecção por lipofectamina (Hiperfect Transfection Reagent® da Qiagen) é adequado à transfecção de células mTEC 3.10 em cultura como observado por microscopia de

fluorescência. Os pontos em vermelho brilhante correspondem às moléculas de RNA no interior da célula.

Figura 13. Células mTEC 3.10 após 24 h em cultura em presença de RNAi irrelevante marcado com Cy3. Visualização por microscopia de fluorescência (aumento 20x).

O resultado do silenciamento foi mensurado por meio de rt-PCR semiquantitativa (figura 15) e a sequência 1 (figura 14) na concentração de 5 hM foi a que apresentou um melhor resultado.

Figura 14. Sequência do RNA interferente para o transcrito do gene Aire, selecionada para a realização do trabalho.

Figura 15. Eletroforese em gel de agarose de produtos de rt-PCR semiquantitativa para a detecção do transcrito do gene Aire. PCRs realizadas com as seguintes amostras: mTEC na ausência de RNA interferente e mTEC em presença das três diferentes seqüências de RNAi anti-Aire, cada uma em três concentrações (2.5, 5.0 e 10.0 nM). * RNAi e concentração escolhidos para a continuação dos experimentos.

6.2. Avaliação da integridade do RNA total

6.2.1. Amostras utilizadas como “pool” de referência.

A qualidade das amostras de RNA total proveniente de diferentes timos de camundongos C57Bl/6 adultos foi avaliada por eletroforese em gel de agarose 1% em condições denaturantes. As amostras que se apresentaram íntegras com relação às subunidades de RNAr 28S, 18S e 5S e RNAt, além das avaliações espectrofotométricas, foram selecionadas para compor o *pool* de referência (figura 16).

Figura 16. Eletroforese em gel de agarose de seis amostras de RNA total provenientes de timos de camundongos C57Bl/6 adultos.

6.2.2. RNA total de Amostras de células mTEC.

Assim como para as amostras de RNA total proveniente de timos as amostras de RNA total de células mTEC também foram submetidas à avaliação em gel de agarose 1% em condições denaturantes. As amostras que se apresentaram íntegras com relação às subunidades 28S, 18S e 5S e RNAt além das avaliações espectrofotométricas, foram selecionadas para marcação fluorescente (figura 17).

Figura 17. Eletroforese em gel de agarose de amostras de RNA total provenientes de células mTEC nas diferentes situações: 1) mTEC; 2) mTEC + lipofectamina; 3) mTEC + RNAi irrelevante; 4) mTEC + RNAi anti-HPRT; 5) mTEC + RNAi anti-Aire).

6.3. RT-PCRs semi-quantitativas para os transcritos dos genes Aire e HPRT.

Para avaliar o silenciamento dos transcritos dos genes Aire e HPRT foram realizadas duas rt-PCRs semi-quantitativas, uma para o transcrito HPRT (figura 18), um gene constitutivo contra o qual também foi usado um RNA interferente e outra para o próprio transcrito do gene Aire (figura 19). É possível observar que o silenciamento de ambos os transcritos foi parcial, pois ainda foi possível detectar seus respectivos transcritos, embora em menor concentração em relação aos respectivos controles (*; **).

Figura 18. Eletroforese de produtos de rt-PCR semiquantitativa para o gene HPRT realizada com as seguintes amostras: mTEC; mTEC + lipofectamina; mTEC + RNAi irrelevante (Cneg); mTEC + RNAi anti-HPRT (Cpos); mTEC + RNAi anti-Aire (RNAi). * É possível observar uma diminuição do gene HPRT na amostra submetida à presença de RNAi anti-HPRT.

Figura 19. Eletroforese de produtos de rt-PCR semiquantitativa para o gene Aire realizada com as seguintes amostras: mTEC; mTEC + lipofectamina; mTEC + RNAi irrelevante (Cneg); mTEC + RNAi anti-HPRT (Cpos); mTEC + RNAi anti-Aire (RNAi). ** É possível observar uma diminuição maior do gene Aire na amostra submetida à presença de RNAi anti-Aire.

6.4. Amplificação de cDNA para a confecção de microarrays

A primeira etapa para a construção dos microarrays consistiu na amplificação por PCR dos insertos de cDNA (biblioteca IMAGE) diretamente dos clones em cultura bacteriana.

Dos 624 clones selecionados da biblioteca murina e humana, 82,5% foram corretamente amplificados (uma única banda em gel), 6,5% não amplificaram e 11% apresentaram bandas múltiplas (Figura 20). O resultado típico destas amplificações pode ser observado na figura 21.

O microarray utilizado neste trabalho foi preparado com seqüências de cDNA de genes cujas funções são relacionadas ao sistema imune e também algumas ESTs (Expressed Sequence Tags) com função desconhecida.

Figura 20. Eficiência das amplificações por PCR dos clones de cDNA IMAGE utilizados no preparo do microarray.

Figura 21. Eletroforese em gel de agarose 1% dos produtos de PCR de alguns clones da biblioteca de cDNA IMAGE.

6.5. Aquisição de imagens de microarrays

A figura 22 ilustra uma imagem típica de hibridação de uma das lâminas microarray em vidro. Neste caso utilizamos sonda complexa marcada com Cy3 (células mTEC em diferentes situações) e o pool de referência (timo) foi marcado com Cy5. Os pontos vermelhos correspondem aos genes expressos no pool de referência, enquanto que os pontos verdes correspondem aos genes expressos somente nas células mTEC e os pontos amarelos correspondem aos genes expressos em ambos.

Figura 22. Imagem típica de hibridização de *microarray* com sondas fluorescentes (Cy3 e Cy5).

6.6. Quantificação e normalização dos dados de microarrays

Para análise dos dados dos microarrays em lâminas de vidro contendo 4.500 seqüências, foi utilizado inicialmente o tratamento estatístico SAM (*Significance Analysis of Microarrays*) com o qual se pode selecionar 1611 genes diferencialmente expressos ($FDR \leq 5\%$, $p\text{-value} \leq 0,005$), ou seja, com 95% de confiabilidade entre os cinco grupos de células analisadas. A figura 23 ilustra a matriz de expressão gênica com o dendrograma dos genes e amostras, com uma distinção entre os grupos amostrais de acordo com o tratamento. Os 1611 genes diferencialmente expressos estão listados na tabela II, do anexo I.

Figura 23. Agrupamento hierárquico mostrando o perfil de expressão gênica dos diferentes grupos de células mTEC. O agrupamento foi realizado utilizando correlação de Pearson e “average linkage” utilizando o programa TIGR MEV (<http://www.tm4.org/mev.html>). Verde representa genes reprimidos, vermelho genes induzidos, preto genes que não tiveram modulação com relação ao controle e cinza dados perdidos.

Para um melhor entendimento do que ocorre com a expressão gênica das células mTEC em presença de um RNA interferente para o transcrito do gene Aire, foi realizada uma nova análise, considerando apenas as amostras mTEC e mTEC transfectadas com RNAi anti-Aire.

A estatística SAM selecionou 212 genes diferencialmente expressos ($\text{FDR} \leq 15\%$, $\text{p-value} \leq 0,015$), ou seja, com 85% de confiabilidade. A figura 24 mostra um gráfico de dispersão gene-específico. A lista dos 212 genes diferencialmente expressos encontra-se na tabela III, do anexo II.

Dessa forma foi possível a elaboração de um *heat map* com os genes induzidos e reprimidos sob a influência de RNAi anti-Aire, gerando dendrogramas tanto dos genes quanto das amostras (figura 25).

Figura 24. Genes diferencialmente expressos em células mTEC 42 horas após a inibição do transcrito do gene Aire. Significância com base estatística no programa SAM. Comparação entre a distância relativa observada $d(i)$ e esperada $d_E(i)$. A linha contínua é a região onde $d(i) = d_E(i)$. As linhas tracejadas são cortes a uma distância Δ da linha contínua. Os pontos vermelhos são genes que se afastaram da linha $d(i) = d_E(i)$, a uma distância $>\Delta$, sendo considerados induzidos e os pontos verdes são genes que se afastaram da linha $d(i) = d_E(i)$, a uma distância $<\Delta$, sendo considerados reprimidos.

Figura 25. Agrupamento hierárquico mostrando o perfil de expressão gênica de células mTEC transfectadas com RNAi anti-Aire comparados ao grupo controle de células mTEC na ausência de qualquer tipo de RNA interferente. O agrupamento foi realizado utilizando correlação de Pearson e “average linkage” utilizando o programa TIGR MEV (<http://www.tm4.org/mev.html>). Verde representa genes reprimidos, vermelho genes induzidos, preto genes que não tiveram modulação com relação ao controle e cinza dados perdidos.

6.7. Análise da expressão gênica promíscua.

A PGE exibida pelas células mTEC controles e transfectadas com o RNAi anti-Aire foi analisada a partir dos genes diferencialmente expressos obtidos pelo programa SAM seguida de agrupamento hierárquico. Dos 212 genes diferencialmente expressos, 80 encontravam-se induzidos e 132 reprimidos em células mTEC transfectadas. Foi feita então uma consulta para cada um dos genes usando o banco de dados *Symatlas* (<http://symatlas.gnf.org/SymAtlas/>) que indica a representatividade do gene consultado em mais de 60 tecidos e/ou órgãos do camundongo (figura 26) os quais foram finalmente agrupados em 17 sistemas. As informações de cada gene encontrado no *Symatlas* possibilitaram a construção de gráficos de setores mostrando as porcentagens de representação dos 17 sistemas diferentes pelas células mTEC (figuras 27 e 28).

Como se pôde observar o silenciamento parcial de Aire desencadeou indução de vários genes e repressão de outros nas células mTEC, modulando assim os níveis de expressão dos genes TRAs (PGE) (figura 25). Esses dois conjuntos de genes serviram de base para se confeccionar dois gráficos de setores mostrando a modulação da PGE e, portanto, a modulação da representatividade de TRAs (figuras 27 e 28).

É importante salientar que a inibição parcial de Aire não alterou a diversidade deste conjunto gênico, mas sim seus respectivos níveis de expressão.

Figura 26. Gráfico ilustrativo gerado pelo banco de dados *SymAtlas* (<http://symatlas.gnf.org/SymAtlas/>) mostrando a representação tecidual do gene *Btg1*, com os nomes de cada tecido e/ou órgão.

Figura 27. Representação da expressão gênica específica de tecidos e órgãos em células mTEC. Os genes significativamente induzidos em células mTEC transfectadas com RNAi anti-Aire, dos 4500 analisados, foram anotados caracterizando a expressão promíscua, representando os antígenos relacionados a tecido.

Figura 28. Representação da expressão gênica específica de tecidos e órgãos em células mTEC. Os genes significativamente reprimidos em células mTEC transfectadas com RNAi anti-Aire, dos 4500 analisados, foram anotados caracterizando a expressão promíscua, representando os antígenos relacionados a tecido.

6.8. Influência do silenciamento do transcrito do gene Aire sobre as redes de interações gênicas das células mTEC

A figura 29 mostra a rede de interações gênicas das células mTEC controle e a figura 30 o mesmo tipo de rede sob a influência do silenciamento parcial do transcrito do gene Aire. É possível observar que as células mTEC controle mostraram o transcrito Gucy2d (*guanylate cyclase 2d*) que pode ser interpretado como um “nó gênico”, pois a grande maioria dos genes analisados exercem influência sobre este transcrito, tanto de indução como de repressão.

A rede de células mTEC tratadas com RNAi anti-Aire, ainda continuou exibir o transcrito Gucy2d como nó gênico. Entretanto, os tipos de interações se alteraram de maneira importante. Nota-se que a maioria das interações que partem do transcrito Gucy2d é de repressão sobre outros transcritos.

Os transcritos dos genes presentes nas redes bem como os tipos de interações entre eles, encontram-se listados nas tabelas IV e V do anexo III.

Figura 29. Rede de interações gênicas de células medulares epiteliais tímicas (mTEC linhagem 3.10) cultivadas sob condições controle.

Figura 30. Rede de interações gênicas de células medulares epiteliais tímicas (mTEC linhagem 3.10) transfetadas com RNAi anti-Aire.

Discussão

7. DISCUSSÃO

Este trabalho foi realizado tendo como base nosso interesse na investigação das bases genético-moleculares do controle imunológico do próprio-não-próprio ou da indução de tolerância, pois esta é uma das propriedades essenciais do sistema imune a qual contribui com a homeostase do corpo e impedindo manifestações auto-imunes.

Como discutem Kyewski e Derbinski, (2004) e Magalhães et al (2006), a idéia germinal da teoria da seleção clonal e da discriminação do próprio foi desenvolvida por Paul Ehrlich há mais de 100 anos (Ehrlich e Morgenroth, 1901) e representa até hoje a orientação conceitual básica de toda a investigação imunológica.

Neste contexto, a demonstração da deleção clonal de linfócitos auto-reativos é considerada como uma das mais importantes descobertas, a qual tem contribuído com nossa compreensão contemporânea do sistema imune adaptativo (Schwartz e Mueller, 2003) e inato (Medzhitov e Janeway, 1997).

As duas vias conhecidas da resposta imune são caracterizadas pelo uso limitado de receptores antimicrobianos *germline* no sistema imune inato ou por receptores de células T (TCR) ou imunoglobulinas (Ig) gerados somaticamente e altamente diversos no sistema imune adaptativo.

A molécula heterodimérica de TCR (TCR α/β) pode representar o conceito funcional do sistema imune devido a sua propriedade de reconhecer instantaneamente a molécula própria do complexo principal de histocompatibilidade (MHC) e o peptídeo antigênico não-próprio.

A tolerância do repertório das células T ao próprio é adquirida durante o desenvolvimento das células T imaturas, um fenômeno dependente da avidéz da interação entre um TCR específico, o MHC e o peptídeo próprio. O local no corpo onde ocorrem tais interações é o timo. O repertório de células T nascente no timo determina a diversidade de antígenos próprios e a especificidade da tolerância central.

Nós concordamos com Kyewski e Derbinski (2004) assumindo que tolerância central inclui os mecanismos intra-tímicos pelos quais as células T passam pelo reconhecimento dos antígenos próprios. De acordo com esse conceito, as células T reguladoras (T_{reg}) auto reativas são selecionadas no timo, mesmo se desempenharão seu papel na periferia.

De fato, todos os sub conjuntos de células apresentadoras de antígenos (APCs) tais como as células tímicas epiteliais corticais (cTECs), as epiteliais medulares (mTECs), células dendríticas tímicas e os macrófagos desempenhas seus papéis na apresentação de antígenos próprios no timo (Klein e Kyewski, 2000).

Entretanto, a expressão de antígenos restritos a tecidos (TRAs) no timo, a qual representa uma característica chave na efetivação da representação do próprio, foi reconhecida recentemente na história da imunologia.

A evidência da expressão intratímica de TRAs em camundongos e no homem, a qual foi referida de expressão gênica promíscua (PGE) (Jolicoeur et al., 1994; Derbinski et al., 2001; Gotter et al., 2004), reforçou o conceito de tolerância central a antígenos dos tecidos próprios.

Antes desta evidência, a tolerância periférica, isto é, mecanismos pelos quais a seleção das células T em direção aos TRAs ocorreriam fora

do timo, dominava o cenário na explicação da discriminação do próprio-não-próprio (Alferink et al., 1999; Walker & Abbas, 2002).

A PGE no timo está causando uma reversão no escopo do entendimento da tolerância central, levando a uma concepção não ortodoxa dos possíveis mecanismos da discriminação do próprio-não-próprio (Kyewski et al., 2002; Gallegos e Bevan, 2006).

A principal implicação desta expressão gênica heterogênea no timo está associada com a manutenção da homeostase imunológica no corpo, controlando as reações auto-imunes patogênicas.

As primeiras evidências desse fenômeno foram obtidas usando a técnica de *real-time PCR* (RT-PCR) focando antígenos de reações auto-imunes, como a insulina, o receptor de acetilcolina ou a proteína básica da mielina. Atualmente, a PGE é reconhecida como ao invés de seletiva, o conjunto de genes expressos é tão variado quanto possível, chegando a abranger de 5 a 10% do genoma funcional humano ou do camundongo (Derbinski et al., 2001; Gotter et al., 2004; Kyewski e Derbinski, 2004).

Atualmente, para explorar a expressão gênica em grande escala no timo, a estratégia de escolha é a tecnologia dos *microarrays*. O grupo de B. Kyewski e J. Derbinski do Centro Alemão de Pesquisas sobre o Câncer em Heidelberg, Alemanha utiliza os oligo *arrays* da Affymetrix, os quais permitiram uma extensa caracterização da PGE no timo murino (Kyewski & Derbinski, 2004).

De fato a técnica de *microarrays* seja em nylon ou lâminas de vidro, tem sido realizada por mais de 10 anos nas mensurações da expressão gênica em grande escala no timo (Nguyen et al, 1995; Espanhol et al., 2004; Puthier et al., 2004; Magalhães et al., 2005, Cardoso et al., 2006, Sousa Cardoso et al., 2006).

Com relação à técnica de cDNA *microarrays* desenvolvida no próprio laboratório para propósitos de análise de PGE no timo murino é de especial interesse a biblioteca de cDNA denominada “Soares thymus 2NbMT cDNA library” disponível no IMAGE Consortium (<http://image.llnl.gov>). Essa é uma biblioteca de ESTs (*expressed sequence tags*) normalizada, preparada do timo total de um camundongo macho adulto jovem de quatro semanas da linhagem C57Bl/6J (mesmo *background* genético da linhagem de células mTEC 3.10 utilizada neste trabalho).

Essa biblioteca é composta de mais de 25.000 clones de cDNA (re-sequenciados) representando a maioria, se não todos, os genes expressos do camundongo. Portanto, ela representa um recurso precioso para a preparação de cDNA *microarrays* “especializados em timo” muito úteis na pesquisa de PGE.

Por isso é que utilizamos essa biblioteca no presente estudo, além de dominarmos todas as etapas da tecnologia dos *microarrays* em nosso próprio laboratório.

Portanto, o sistema modelo estava organizado: de um lado as células mTEC em cultura e de outro o cDNA *microarray* tímico.

Nossa primeira questão foi avaliar se as células mTEC expressam em cultura os genes de TRAs, conferindo a expressão gênica promíscua.

Como a análise de dados de *microarrays* envolve o teste estatístico SAM (Tusher et al., 2001), o qual recupera somente os valores de expressão com alta significância, das 4.500 seqüências de cDNA presentes no *microarray* foram possíveis recuperar 1.611 por meio da função *multiclass* do programa SAM (figura 23) ou 211 seqüências usando a comparação pareada (figura 25).

Tais seqüências foram então comparadas com as do banco de dados GNF SymAtlas (<http://symatlas.gnf.org/SymAtlas>) (Su *et al.*, 2004) e, com a anotação minuciosa de cada um dos tecidos e/ou órgãos que são representados pelas seqüências, colocamo-las num contexto de PGE exibido pelas células mTEC normais.

O resultado mostrado na figura 28 mostra que foi possível associar a expressão gênica no timo com representação antigênica de tecidos e/ou órgãos.

Portanto, conseguimos validar a primeira hipótese do presente trabalho com a resposta de que as células mTEC em cultura reproduzem PGE.

Quanto à representação de tecidos e/ou órgãos observamos que há uma distribuição relativamente uniforme dos 17 sistemas, variando de quatro a nove por cento. Embora o silenciamento parcial de Aire tenha desencadeado a indução de vários genes e repressão de outros nas células mTEC (figura 25), modulando assim os níveis de expressão dos genes TRAs (PGE), ela não alterou a representatividade dos sistemas, a qual permaneceu praticamente inalterada (figuras 27 e 28).

Um fato que nos chamou a atenção foi o de que a PGE também é caracterizada pela expressão diferencial dos genes, ou seja, foi possível identificar um grupo de genes reprimidos e outro de induzidos, com as respectivas variações individuais nos níveis de expressão em cada grupo.

Nesse contexto, entra nossa segunda questão que foi referente ao controle da PGE nas células mTEC sendo o principal enfoque desta Tese.

Na realidade esse aspecto é foco de muito interesse na imunogenética atual. A descoberta mais relevante até o momento foi a do

gene *AIRE* (do inglês *autoimmune regulator*) no homem, o qual é considerado como principal controlador positivo da PGE em mTECs.

Esse gene foi identificado inicialmente como locus de susceptibilidade da doença autoimune APECED (*autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy*) também conhecida como APS1, a qual foi reconhecida como síndrome há mais de 60 anos atrás (Leonard, 1946).

Entretanto, o maior salto no entendimento da APECED começou em 1997 quando dois grupos de pesquisadores utilizaram estratégias de clonagem posicional para identificar o gene em questão (Finnish-German APECED Consortium, 1997; Nagamine et al., 1997).

Como discute Mathis e Benoist (2007), a proteína codificada por este locus foi denominada *AIRE*, a qual é uma grande proteína contendo 545 resíduos de aminoácidos com vários domínios remanescentes daqueles achados em reguladores transcricionais, notavelmente módulos do tipo *zinc fingers*, os quais servem como elementos de ligação com DNA em alguns fatores de transcrição. Mas notou-se que em *AIRE* faltam resíduos críticos de interação com DNA (Gibson et al., 1998).

Mais de 60 mutações foram localizadas no gene *AIRE* de diferentes pacientes com APECED, sendo que esse aspecto é também foco de intensas pesquisas, inclusive na tentativa de relacionar tais mutações com outras doenças autoimunes.

À época de sua descoberta, havia uma controvérsia significativa entre os autores acima citados a respeito do padrão da expressão de *AIRE*, reivindicando uma distribuição abrangente envolvendo diferentes órgãos (Finnish-German APECED Consortium, 1997), ou uma distribuição restrita aos órgãos linfóides, especialmente o timo

(Nagamine et al., 1997; Heino et al., 1999). Esta discrepância sem dúvida refletiu diferenças da especificidade das sondas moleculares e na confiabilidade da técnica sendo finalmente resolvida em favor da última teoria.

O conhecimento da estrutura e da expressão de AIRE levou a várias hipóteses de como ele pode operar para controlar a auto-imunidade. Estas hipóteses incluem: determinando a organização do estroma tímico; controlando a tolerância dos timócitos; regulando respostas das células B e das células T aos estímulos antigênicos; induzindo apoptose de células parenquimais e desse modo realçando a apresentação de seus antígenos; ou *impinging* na diferenciação de células T regulatórias CD4⁺CD25⁺(TReg) (Peterson et al., 1998).

Os estudos em camundongos permitiram uma dissecção extensiva de como Aire opera. Primeiramente, a disponibilidade de tecidos e de reagentes apropriados para a classificação celular permitiu um delineamento de onde Aire é expresso. Encontra-se em órgãos linfóides, especialmente o timo, dentro do qual é expresso na maior parte por células epiteliais medulares (MECs) mas também, embora em uns níveis muito mais baixos, por células dendríticas (DCs) (Mathis & Benoist, 2007).

Encontrar Aire em MECs (hoje sabemos que são as células mTEC que expressam Aire) era intrigante por causa das sugestões que este tipo células está envolvido na seleção negativa de timócitos auto-reativos (Kishimoto & Sprent, 1997) e por causa da emergência coincidente dos dados que estabelecem que os transcritos que codificam antígenos tecido específicos (TRAs) são expressos ectopicamente especificamente por mTECs (Kyewski & Klein, 2006). Assim, levantou-se a hipótese que Aire regula a transcrição tímica dos genes que codificam TRAs, e controla

desse modo a tolerância e conseqüentemente a auto-imunidade (Mathis & Benoist, 2007).

Esta hipótese pode ser avaliada diretamente em camundongos *Aire-knockout* (Anderson et al., 2002; Ramsey et al., 2002; Kuroda et al., 2005). Estes animais foram criados para ter um sistema imune relativamente normal, mas eram afetados com auto-imunidade de múltiplos órgãos, como indicado por infiltrados inflamatórios e por auto-anticorpos no soro.

A purificação de mTECs de camundongos tipo selvagem e *AIRE-knockout*, seguida do perfil da expressão gênica, revelou que certamente Aire promove a transcrição de um grande número genes que codificam antígenos relacionado a tecido (TRA) (Anderson *et al.*, 2002 e Anderson *et al.*, 2005) e associados com a seleção negativa de células T auto-reativas (Nagafuchi *et al.*, 2006). Aire também regula positiva ou negativamente a transcrição em mTECs de um conjunto de genes que não codificam TRAs (Mathis & Benoist, 2007).

Interessante é que a transcrição de um número de genes de TRAs, tais como aqueles codificadores da proteína C-reativa e da ácido glutâmico decarboxilase de 67kDa (GAD67), pareceu ser independente da expressão de Aire, e esta observação foi confirmada recentemente em um estudo mais amplo (Derbinski et al., 2005).

Aire também regula positivamente ou negativamente a transcrição, em mTECs, de genes que não codificam TRAs. O significado do controle por Aire da expressão de TRAs no timo em relação às manifestações auto-imunes observados em camundongos *Aire-knockout* foi substanciado associando a perda deste controle com o desenvolvimento de auto-anticorpos contra olho e estomago e, no primeiro caso, demonstrando

também que a falta da expressão de TRAs no timo era suficiente produzir a auto-imunidade (Mathis & Benoist, 2007).

Analisando camundongos duplo-transgênicos *Aire-knockout* e mutação num dos genes de receptor de célula T (TCR)-antígeno próprio, no qual células T antígeno-próprio-específicas podem ser seguidas, mostrou-se que, na ausência de Aire, os timócitos auto-reativos podem escapar da deleção clonal usual que os impede de migrar à periferia. (Anderson et al., 2005b; Liston et al., 2003; Liston et al., 2004).

Embora estes estudos ligassem claramente o controle da expressão de TRA por Aire com a deleção clonal, diversos trabalhos indicaram que a falta de Aire compromete a indução da tolerância sem afetar a transcrição dos genes de TRAs no timo (Anderson et al., 2005; Kuroda et al., 2005; Niki et al., 2006). A questão ainda permanece em aberto, havendo ainda interesse considerável na definição do papel de Aire na indução da tolerância central além de seu controle da expressão de TRAs.

Trabalhos recentes reportam que Aire liga-se diretamente à proteína ligante de CREB (CBP - *CREB-binding protein*) (Pitkanen et al., 2000). A proteína CBP funciona como um coativador transcricional para uma variedade de fatores de transcrição incluindo *Jun*, *Fos*, receptores nucleares, NFkB, e as proteínas STAT (Pitkanen et al., 2000). CBP apresenta três domínios ricos em histidina-cisteína (CH1, CH2 e CH3), envolvidos em interações específicas proteína-proteína (Janknecht & Hunter, 1996; Bhattacharya; 1996). Esses achados sugerem que CBP participa como integrador de vias de sinalização múltiplas (Pitkanen et al., 2003).

A proteína AIRE se liga a CBP nos domínios de interação CH1e CH3 (Pitkanen et al., 2000). No núcleo, Aire forma discretos corpos nucleares que parecem, mas são distintos dos corpos nucleares (NBs) da

PML (leucemia promielocítica). A significância funcional atual da interação entre AIRE e CBP e o papel dos NBs-AIRE na transcrição ainda não foi bem estudada. É possível que CBP forme a ligação entre AIRE, possíveis outras proteínas que interagem com AIRE e a maquinaria transcricional basal (Pitkanen et al., 2005).

AIRE e CBP sinergisticamente ativam a transcrição de dois repórteres diferentes: o elemento que contem o promotor de GAL4-ligante e o promotor mínimo de IFN β . Além disso, AIRE e CBP induzem colaborativamente a expressão endógena do mRNA de IFN β .

As análises da localização subnuclear sugerem que os corpos de AIRE não são locais da transcrição. Em adição, ao ocorrer no mesmo NBs, AIRE, e CBP têm a posição espacial diferente dentro dos corpos (Pitkanen et al., 2005).

Pitkanen et al. (2005) em seu trabalho suportam fortemente a idéia que AIRE pode provocar diretamente a expressão dos antígenos-próprios como um regulador transcricional em colaboração com CBP.

Estudos adicionais são necessários para identificar como AIRE seleciona seus genes do alvo e para caracterizar quais são os mecanismos moleculares da ação colaborativa de AIRE e de CBP na transcrição.

As proteínas PIAS também foram identificadas como novos parceiros na interação com AIRE (Ilmarinen et al., 2007). A expressão de AIRE realça a formação de PIAS1 NBs. PIAS1 pode atrair AIRE em complexos contendo SUMO1 e o processo é dependente de SIM e de PIAS1. PIAS1 e AIRE ativam simultaneamente o promotor humano da insulina, um gene conhecido como alvo de AIRE, e SP-RING é requerida para esta ativação. Além disso, AIRE reprime e PIAS1 ativa o promotor de CSTB, usado como um modelo para um promotor *housekeeping*, e

ambos SP-RING e SIM são necessários para sua ativação por PIAS1. Desta forma, acredita-se que AIRE e PIAS1 interagem funcionalmente para regular as atividades dos genes do alvo de AIRE (Ilmarinen et al., 2007).

Aire interage com e é fosforilado pelo complexo DNA-PK de proteínas DNA-PKcs, Ku70 e Ku80. Ensaio de fosforilação *in vitro* demonstraram que DNA-PK fosforila a proteína AIRE nos resíduos Thr68 e Ser156, e mutações nesses sítios de fosforilação diminuem significativamente a atividade transcricional de AIRE (Liiv et al., 2007).

Modificações pós-transcricionais, como fosforilação, acetilação e ubiquitinação, são comumente usados para regular a atividade de fatores e reguladores de transcrição. Livv et al. (2007) descrevem a fosforilação de sítios de AIRE e propõem que este estado de fosforilação é importante para sua atividade transativadora. Entretanto, várias questões permanecem, inclusive quais são os sinais que levam a fosforilação de AIRE e como essa fosforilação está conectada à sua função no timo.

Outros fatores adicionais devem estar envolvidos na regulação da PGE. Giraud et al. (2007) descrevem um mecanismo que controla a transcrição tímica de um gene de auto-antígeno humano restrito a tecido, o CHRNA1. Este gene codifica a subunidade α do receptor de acetilcolina muscular, o qual é o principal alvo dos auto-anticorpos patogênicos na *myasthenia gravis* auto-imune. Foi observado que uma variante deste gene previne a ligação do IRF8 (*interferon regulatory factor 8*) e revogando a atividade do promotor CHRNA1 em células epiteliais tímicas *in vitro*, revelando uma função crítica de AIRE e a via de sinalização do interferon na regulação da expressão quantitativa deste auto-antígeno no timo, sugerindo que eles estejam juntos no ponto de partida para a tolerância *versus* a auto-imunidade.

Boehm et al. (2003) afirmaram que o sinal via linfotoxina (LT) regula a composição e organização do timo, particularmente do compartimento medular epitelial. Enquanto isso uma série de trabalhos tem indicado sinais de que a LT controla a expressão de AIRE, mediando a transcrição ectópica dos genes que codificam uma variedade de antígenos relacionados a tecidos e promovendo a deleção clonal de timócitos auto-reativos e não a composição/estrutura do epitélio tímico (Chin et al., 2003; Chin et al., 2006; Zhu M et al., 2006). Para tentar elucidar essa questão, Venanzi et al (2007) examinou o papel da via da LT no timo e sua relação com AIRE e a transcrição gênica de TRAs, usando uma combinação de ferramentas de histologia, citometria de fluxo e perfis de expressão gênica. Concluíram que a via LT permanece sendo importante no domínio do desenvolvimento/organização do timo e parece não participar como um protagonista crítico na tolerância mediada por AIRE.

As observações acima relatadas mostram claramente que *AIRE* (Aire) é um controlador central, mas não atua sozinho, necessitando outros genes (ou suas respectivas proteínas) no mecanismo da modulação da expressão de genes de TRAs.

Neste ponto é que procuramos inserir o presente trabalho, formulando a hipótese de que Aire é um controlador *upstream* de uma rede gênica de TRAs.

Para começar a testar tal hipótese, utilizamos o método de silenciamento gênico (RNA interferente ou RNAi) dirigido contra o transcrito do gene Aire.

A utilização de RNAi na pesquisa tem sido amplamente difundida, pois a possibilidade de “silenciar” a expressão de genes pode levar a aplicações na pesquisa básica como no seu uso clínico (Chen et al, 2003;

Strillacci et al, 2006).

Nossos resultados mostram que o silenciamento do transcrito de Aire foi parcial (figura 19), mas mesmo assim, foi possível identificar perfis de expressão gênica distintos comparando células mTEC controle e as transfectadas com RNAi anti-Aire (figura 23). O silenciamento parcial passou inclusive a ser de interesse, pois nos permitiu evidenciar os genes de TRAs com forte dependência de Aire.

Para a análise da PGE escolhemos trabalhar somente com um dos grupos controle (mTEC) *versus* células mTEC transfectadas com RNAi anti-Aire e, conseqüentemente, analisando aquele grupo de genes altamente dependentes de Aire.

Como é possível observar na figura 25, houve uma separação das amostras de acordo com o grupo de células e uma modulação dos níveis de expressão dos genes TRAs após a transfecção com RNA interferente. Dos 212 genes diferencialmente expressos, 80 encontravam-se induzidos (dos quais 37 ainda representam ESTs ou *expressed sequence tags*) e 132 reprimidos (17 ESTs) em células mTEC transfectadas, indicando uma diminuição do número de genes de TRAs expressos após o silenciamento de Aire (figuras 27 e 28).

Os sistemas circulatório, digestivo, olhos, tecido adiposo e sistema linfóide tiveram sua representatividade diminuída após a transfecção com RNAi anti-Aire. Já os sistemas glandular, nervoso periférico, reprodutor, órgãos sensoriais e sistema urinário apresentaram uma representatividade aumentada enquanto que nos sistemas nervoso central, locomotor, muscular, respiratório, epiderme/pele, intestino e placenta ela permaneceu inalterada.

Para analisar as interações em rede entre os genes diferencialmente expressos após a transfecção com o RNAi anti-Aire, escolhemos o

programa Genenetwork (Wu et al., 2004) devido as suas características peculiares que melhor se enquadram aos nossos tipos de dados.

Dentre os quatro modelos de inferência diferentes que o programa GeneNetwork oferece para extrair a “matriz de regulação gênica” dos dados de expressão gênica, o modelo linear foi escolhido para análise dos dados. Este é um método contínuo que usa equações diferenciais ordinárias lineares para descrever o sistema. Embora seja a metodologia mais simplista que o programa apresenta, permite observar as principais e mais importantes informações que envolvem os genes em questão. Para a obtenção da rede gênica representando as interações entre os genes, é assumido que o nível de expressão de um componente num determinado tempo é dado pela somatória dos pesos de todos os outros genes. Assim, o nível de expressão neste tempo é influenciado pelo nível de expressão dos tempos anteriores.

Nessas redes é possível estabelecer cinco tipos de interação entre os genes: interação de ativação, inibição, auto-regulação negativa e positiva e dupla interação (dois sentidos) permitindo esclarecer como é a regulação das interações na situação em estudo.

A rede de interações gênicas das células mTEC controle apresentada na figura 29 mostra o transcrito Gucy2d (*guanylate cyclase 2d*) como único nó gênico, ou seja, um gene que participa de um número maior de interações. O mesmo tipo de rede, porém sob a influência do silenciamento parcial do transcrito do gene Aire está apresentada na figura 30. Nesta última, o transcrito do gene Gucy2d também se apresenta como único nó gênico.

Comparando-se as duas redes, observamos que, embora apresentem o mesmo único nó gênico, as relações entre Gucy2d e os demais genes se alteraram de maneira significativa.

Na rede de células controle observamos que há uma regulação de diversos genes sobre Gucy2d, sendo que este, por sua vez, exerce influência sobre um número relativamente pequeno de genes (tabela IV, anexo III). Já na rede das células sob influência do silenciamento parcial do transcrito do gene Aire notou-se que a maioria das interações partem de Gucy2d e são, em sua maioria, de inibição sobre outros transcritos (tabela V, anexo III).

Estes resultados mostram que sob diminuição do transcrito de Aire, o gene Gucy2d passa a ser um controlador negativo sobre genes de TRAs.

Embora o gene Aire não esteja representado no *microarray* usado nesse estudo e, conseqüentemente não apareça nas redes, ficou clara sua influência sobre as interações com os demais transcritos na célula mTEC.

As observações das redes são probabilísticas e precisamos partir para métodos de validação, tais como a utilização do algoritmo de Markov (Ellrott et al., 2002), com o qual podemos avaliar seqüências de DNA quanto ao seu potencial de interagir com fatores de transcrição como Aire.

Conclusões

8. CONCLUSÕES

- ✦ Células tímicas epiteliais medulares (mTECs) expressam, em cultura, genes codificadores de antígenos relacionados a tecidos (expressão gênica promíscua);
- ✦ Genes que caracterizam a PGE estão conectados em rede nas células mTEC;
- ✦ Um nó gênico pode agir como intermediário no seu controle e
- ✦ O gene AIRE (*autoimmune regulator*) em redes PGE controla a expressão gênica promíscua nas células mTEC, numa posição *upstream*.

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Anexo I

Tabela II. Genes diferencialmente expressos comparando-se os cinco grupos de células analisadas(FDR $\leq$ 5%, p-value $\leq$ 0,005).

<i>Gene Symbol</i>	<i>Clone ID</i>	<i>Gene Name</i>	<i>Expected score (dExp)</i>	<i>Observed score(d)</i>	<i>q-value (%)</i>
EST	IMAGE:640793	Expressed sequence tag	0.19811828	0.33850563	5.28
Herc4	IMAGE:583062	Hect domain and RLD 4	0.20369264	0.33869174	5.28
A230061C15Rik	IMAGE:583262	Human immunodeficiency virus type I enhancer binding protein 2	0.14123869	0.33878335	5.28
EST	IMAGE:640001	Expressed sequence tag	0.19408871	0.33931252	4.84
Hnrpl	IMAGE:641039	Heterogeneous nuclear ribonucleoprotein L	0.48570776	0.3393502	4.84
Dock7	IMAGE:583775	Dedicator of cytokinesis 7	0.35370365	0.3394484	4.84
Ywhag	IMAGE:583389	3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	0.26358023	0.33949354	4.84
EST	IMAGE:583009	Expressed sequence tag	0.11662422	0.33949926	4.84
Btbd1	IMAGE:582942	BTB (POZ) domain containing 1	0.20342788	0.3395322	4.84
Txk	IMAGE:583533	TXK tyrosine kinase	0.05148377	0.3397727	4.84
Eml4	IMAGE:577678	Echinoderm microtubule associated protein like 4	0.25414103	0.3398389	4.84
Il12b	IMAGE:750641	Interleukin 12b	0.1708792	0.34007028	4.84
EST	IMAGE:583272	Expressed sequence tag	0.08268311	0.34009635	4.84
Trim24	IMAGE:576305	Tripartite motif protein 24	0.13736697	0.34015146	4.84
EST	IMAGE:640911	Expressed sequence tag	0.16771524	0.3402881	4.84
Sypl	IMAGE:640468	Synaptophysin-like protein	0.18131702	0.34053463	4.84
D030051D21	IMAGE:1226420	Centrosomal protein 164	0.13844152	0.34055072	4.84
Setd8	IMAGE:640985	SET domain containing (lysine methyltransferase) 8	0.1984913	0.34055364	4.84
EST	IMAGE:582875	Expressed sequence tag	0.33123899	0.3405946	4.84
EST	IMAGE:640180	Expressed sequence tag	0.17984709	0.34068993	4.84
Hspa14	IMAGE:574381	Heat shock protein 14	0.13240105	0.3408499	4.84
Iars2	IMAGE:583626	Isoleucine-tRNA synthetase 2, mitochondrial	0.20717484	0.34086606	4.84
Rab10	IMAGE:573868	RAB10, member RAS oncogene family	0.18625002	0.34088877	4.84

EST	IMAGE:582370	Expressed sequence tag	0.3197391	0.34095538	4.84
Casp2	IMAGE:573760	Caspase 2	0.17146178	0.34144688	4.84
Dnajc7	IMAGE:573602	DnaJ (Hsp40) homolog, subfamily C, member 7	0.114779234	0.34149453	4.84
Ttc27	IMAGE:582965	Tetratricopeptide repeat domain 27	0.18838125	0.34173462	4.84
Zfp251	IMAGE:639625	Zinc finger protein 251	0.1229852	0.34176758	4.84
Ncoa2	IMAGE:640607	Nuclear receptor coactivator 2	0.40649518	0.34202594	4.84
Gzma	IMAGE:572830	Granzyme A	0.02663688	0.34218764	4.84
EST	IMAGE:582322	Expressed sequence tag	0.3184161	0.34235486	4.84
Cecr5	IMAGE:640582	Cat eye syndrome chromosome region, candidate 5 homolog (human)	0.307422	0.34307024	4.84
Dbp	IMAGE:583416	D site albumin promoter binding protein	0.08370037	0.343139	4.84
1810074P20Rik	IMAGE:583188	RIKEN cDNA 1810074P20 gene	0.08466934	0.34323442	4.84
EST	IMAGE:639811	Expressed sequence tag	0.2251166	0.34348485	4.84
EST	IMAGE:583900	Expressed sequence tag	0.1769163	0.34348944	4.84
EST	IMAGE:583025	Expressed sequence tag	0.18899988	0.34358582	4.84
Bud31	IMAGE:583158	BUD31 homolog (yeast)	0.2039092	0.34375015	4.84
EST	IMAGE:640841	Expressed sequence tag	0.19822282	0.3437841	4.84
Rpl22	IMAGE:640141	Ribosomal protein L22	0.12672086	0.3438051	4.84
EST	IMAGE:583212	Expressed sequence tag	0.084830716	0.3439196	4.84
Ift52	IMAGE:639742	Intraflagellar transport 52 homolog (Chlamydomonas)	0.2990289	0.34397003	4.84
Cuedc2	IMAGE:640434	CUE domain containing 2	0.21135248	0.34416264	4.84
EST	IMAGE:582442	Expressed sequence tag	0.32029676	0.34437323	4.37
1110002B05Rik	IMAGE:639950	RIKEN cDNA 1110002B05 gene	0.14564832	0.34442365	4.37
Septin 2	IMAGE:640553	Septin 2	0.19696581	0.34444684	4.37
EST	IMAGE:575853	Expressed sequence tag	0.15325369	0.3446287	4.37
EST	IMAGE:583338	Expressed sequence tag	0.20570286	0.34473073	4.37
Tmem183a	IMAGE:583868	Transmembrane protein 183A	0.2423611	0.3448882	4.37
Centb2	IMAGE:582978	Centaurin, beta 2	0.20423083	0.344948	4.37
Rab2	IMAGE:573835	RAB2, member RAS oncogene family	0.17177582	0.34508854	4.37
Shmt1	IMAGE:573743	Serine hydroxymethyl transferase 1 (soluble)	0.057885677	0.3451006	4.37

EST	IMAGE:583133	Expressed sequence tag	0.18863866	0.3455151	4.37
EST	IMAGE:640614	Expressed sequence tag	0.21096689	0.34611577	4.37
Irf3	IMAGE:574768	Interferon regulatory factor 3	0.25552166	0.34618762	4.37
EST	IMAGE:573496	Expressed sequence tag	0.045981497	0.3463354	4.37
EST	IMAGE:640778	Expressed sequence tag	0.14950752	0.34707937	4.37
Ly9	IMAGE:577717	Lymphocyte antigen 9	0.23151654	0.34723872	4.37
Pias1	IMAGE:577047	Protein inhibitor of activated STAT 1	0.18718278	0.34730554	4.37
Rps20	IMAGE:575123	Ribosomal protein S20	0.28337494	0.3473207	4.37
Pank4	IMAGE:639921	Pantothenate kinase 4	0.26785523	0.34741336	4.37
EST	IMAGE:582940	Expressed sequence tag	0.17372158	0.3475094	4.37
BC031353	IMAGE:583717	CDNA sequence BC031353	0.1222328	0.34773922	4.37
EST	IMAGE:583293	Expressed sequence tag	0.26324844	0.3482185	4.37
Smg5	IMAGE:582898	Smg-5 homolog, nonsense mediated mRNA decay factor (C. elegans)	0.28726238	0.3487496	4.08
Adcy7	IMAGE:583381	Adenylate cyclase 7	0.11992148	0.34893504	4.08
Btrc	IMAGE:639945	Beta-transducin repeat containing protein	0.2679437	0.34907925	4.08
EST	IMAGE:583776	Expressed sequence tag	0.08847373	0.34909162	4.08
Dstn	IMAGE:640056	Destrin	0.09598648	0.34943917	4.08
Pbx2	IMAGE:574114	Pre B-cell leukemia transcription factor 2	0.20126277	0.34957504	4.08
Tcf25	IMAGE:583391	Transcription factor 25 (basic helix-loop-helix)	0.3436306	0.34966034	4.08
Rnf181	IMAGE:639924	Ring finger protein 181	0.095251635	0.34988308	4.08
EST	IMAGE:575632	Expressed sequence tag	0.13451812	0.35004562	4.08
Cdk4	IMAGE:640626	Cyclin-dependent kinase 4	0.21173468	0.3500662	4.08
EST	IMAGE:640399	Expressed sequence tag	0.22745329	0.35056454	4.08
Col6a1	IMAGE:1281632	Procollagen, type VI, alpha 1	0.17330185	0.35064837	4.08
EST	IMAGE:641034	Expressed sequence tag	0.21294843	0.3506621	4.08
EST	IMAGE:640491	Expressed sequence tag	0.16514237	0.35066614	4.08
Atpaf1	IMAGE:639636	ATP synthase mitochondrial F1 complex assembly factor 1	0.093742035	0.3509819	4.08
EST	IMAGE:640924	Expressed sequence tag	0.18304045	0.35103709	4.08
EST	IMAGE:583855	Expressed sequence tag	0.2227512	0.35148782	4.08
Lat	IMAGE:582840	Linker for activation of T cells	0.27982166	0.35173225	4.08

Dhps	IMAGE:583332	Deoxyhypusine synthase	0.08561945	0.35173878	4.08
EST	IMAGE:640489	Expressed sequence tag	0.12778933	0.3517643	4.08
EST	IMAGE:640041	Expressed sequence tag	0.26974627	0.35210213	4.08
Pank2	IMAGE:583251	Pantothenate kinase 2 (Hallervorden-Spatz syndrome)	0.15816931	0.3523138	4.08
Sepp1	IMAGE:639638	Selenoprotein P, plasma, 1	0.14488117	0.35247594	4.08
Hspa8	IMAGE:575712	Heat shock protein 8	0.18424597	0.35282058	3.73
Smndc1	IMAGE:639687	Survival motor neuron domain containing 1	0.1624431	0.35287923	3.73
Transcribed locus	IMAGE:639984	CDNA sequence BC052328	0.09360567	0.35355002	3.73
Ankib1	IMAGE:640020	Ankyrin repeat and IBR domain containing 1	0.09766332	0.35360998	3.73
Klf13	IMAGE:573768	Kruppel-like factor 13	0.1715122	0.35394427	3.73
Dleu2	IMAGE:582992	Deleted in lymphocytic leukemia, 2	0.23698056	0.35420838	3.73
EST	IMAGE:640289	Expressed sequence tag	0.19555312	0.35443944	3.73
EST	IMAGE:639903	Expressed sequence tag	0.1629262	0.35444814	3.73
Transcribed locus	IMAGE:639922	Baculoviral IAP repeat-containing 6	0.29835656	0.35482526	3.73
Mre11a	IMAGE:641065	Meiotic recombination 11 homolog A (S. cerevisiae)	0.13049798	0.35485992	3.73
Klhl20	IMAGE:582908	Kelch-like 20 (Drosophila)	0.23769656	0.3549872	3.73
EST	IMAGE:640770	Expressed sequence tag	0.21206549	0.35519534	3.73
Zranb1	IMAGE:640533	Zinc finger, RAN-binding domain containing 1	0.27456436	0.3553344	3.73
Eif3s5	IMAGE:575801	Eukaryotic translation initiation factor 3, subunit 5	0.11314324	0.3553901	3.73
EST	IMAGE:641139	Expressed sequence tag	0.16739805	0.35551375	3.73
Transcribed locus	IMAGE:640197	Transcribed locus, weakly similar to XP_322061.1 ENSANGP00000024462 [Anopheles gambiae]	0.2717655	0.35554564	3.73
Scmh1	IMAGE:583929	Sex comb on midleg homolog 1	0.26551953	0.35558715	3.73
EST	IMAGE:640624	Expressed sequence tag	0.18235385	0.3556204	3.73
Letm2	IMAGE:1224844	Leucine zipper-EF-hand containing transmembrane protein 2	0.11511838	0.3556223	3.73
EST	IMAGE:640144	Expressed sequence tag	0.18058197	0.3558154	3.73

EST	IMAGE:583777	Expressed sequence tag	0.121242285	0.3562752	3.73
Herc1		Hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1			
	IMAGE:583423		0.22075455	0.35633478	3.73
Maea	IMAGE:583828	Macrophage erythroblast attacher	0.055177603	0.35687596	3.44
Ap3d1		Adaptor-related protein complex 3, delta 1 subunit			
	IMAGE:573056		0.07284064	0.35687634	3.44
In multiple clusters	IMAGE:639506	Expressed sequence tag	0.28487006	0.3569772	3.44
Slc7a11		Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11			
	IMAGE:575418		0.13413575	0.356981	3.44
Edar	IMAGE:3661299	Ectodysplasin-A receptor	0.28670955	0.3571688	3.44
EST	IMAGE:639948	Expressed sequence tag	0.09538322	0.35730532	3.44
EST	IMAGE:582360	Expressed sequence tag	0.31916782	0.35732123	3.44
EST	IMAGE:583048	Expressed sequence tag	0.17435218	0.35736024	3.44
Transcribed locus		Transcribed locus, strongly similar to NP_848844.1 and leucine-rich repeat protein 5 [Mus musculus]			
	IMAGE:639657		0.26698583	0.3584844	3.44
Arl1	IMAGE:640580	ADP-ribosylation factor-like 1	0.24855761	0.35894233	3.44
EST	IMAGE:640586	Expressed sequence tag	0.1481716	0.35902396	3.44
Aatf	IMAGE:639661	Apoptosis antagonizing transcription factor	0.12425462	0.35919055	3.44
Il1b	IMAGE:3989461	Interleukin 1 beta	0.21389844	0.35946178	3.44
In multiple clusters	IMAGE:573897	Expressed sequence tag	0.106632546	0.35959485	3.44
Man2b1	IMAGE:640168	Mannosidase 2, alpha B1	0.1806364	0.35970306	3.44
EST	IMAGE:640943	Expressed sequence tag	0.46805397	0.3600849	3.44
EST	IMAGE:640939	Expressed sequence tag	0.23051128	0.36065164	3.44
Pgam5	IMAGE:640720	Phosphoglycerate mutase family member 5	0.18256535	0.360898	3.15
Mapk9	IMAGE:583487	Mitogen activated protein kinase 9	0.3448025	0.36091268	3.15
2210404O07Rik	IMAGE:573122	RIKEN cDNA 2210404O07 gene	0.11244351	0.36114112	3.15
BC002199	IMAGE:583821	CDNA sequence BC002199	0.26647037	0.36150783	3.15
EST	IMAGE:639675	Expressed sequence tag	0.16157694	0.36193871	3.15
Socs1	IMAGE:582855	Suppressor of cytokine signaling 1	0.15766856	0.36274025	3.15
EST	IMAGE:640613	Expressed sequence tag	0.19628459	0.3628432	3.15

Mapkapk2	IMAGE:572979	MAP kinase-activated protein kinase 2	0.036123693	0.36288625	3.15
Myg1	IMAGE:583514	Melanocyte proliferating gene 1	0.1409242	0.36304536	3.15
Traf7	IMAGE:640688	Tnf receptor-associated factor 7	0.24777286	0.36311918	3.15
EST	IMAGE:639725	Expressed sequence tag	0.19273359	0.36314243	3.15
Nxf1	IMAGE:574499	Nuclear RNA export factor 1 homolog (S. cerevisiae)	0.13267353	0.36322436	3.15
Rab2	IMAGE:573835	RAB2, member RAS oncogene family	0.10643569	0.3633285	3.15
E530011L22Rik	IMAGE:583156	RIKEN cDNA E530011L22 gene	0.17414281	0.36366665	3.15
EST	IMAGE:640846	Expressed sequence tag	0.311438	0.36384338	3.15
1700011J10Rik	IMAGE:640963	RIKEN cDNA 1700011J10 gene	0.23057146	0.36455745	3.15
Il16	IMAGE:573050	Interleukin 16	0.039014325	0.3649915	2.91
Dnajb7	IMAGE:1263299	DnaJ (Hsp40) homolog, subfamily B, member 7	0.15621331	0.3650117	2.91
Hint1	IMAGE:583258	Histidine triad nucleotide binding protein 1	0.28990576	0.3653645	2.91
EST	IMAGE:640033	Expressed sequence tag	0.12527369	0.3654705	2.91
EST	IMAGE:640344	Expressed sequence tag	0.0974249	0.36594763	2.91
EST	IMAGE:640118	Expressed sequence tag	0.14699501	0.36603355	2.91
Rab19	IMAGE:640004	RAB19, member RAS oncogene family	0.24463102	0.3660546	2.91
EST	IMAGE:583346	Expressed sequence tag	0.14049973	0.36615703	2.91
LOC100043018	IMAGE:583525	Hypothetical protein LOC100043018	0.120380186	0.36637902	2.91
Chic2	IMAGE:640156	Cysteine-rich hydrophobic domain 2	0.1797974	0.36661118	2.91
LOC100039015	IMAGE:640715	Similar to Unknown (protein for MGC:117841)	0.39596888	0.36676776	2.91
EST	IMAGE:640766	Expressed sequence tag	0.14945124	0.3670429	2.91
Bcl11b	IMAGE:576116	Transcribed locus	0.18465409	0.36709076	2.91
In multiple clusters	IMAGE:581967	Expressed sequence tag	0.21751688	0.3671049	2.91
Rcor1	IMAGE:575972	REST corepressor 1	0.15359582	0.3674069	2.91
Rab28	IMAGE:1328024	RAB28, member RAS oncogene family	0.18774924	0.36742395	2.91
1500034J01Rik	IMAGE:640967	RIKEN cDNA 1500034J01 gene	0.47321466	0.36755872	2.91
EST	IMAGE:583506	Expressed sequence tag	0.20608248	0.3678798	2.91
EST	IMAGE:640274	Expressed sequence tag	0.14646642	0.36792225	2.91
Gfm2	IMAGE:640159	G elongation factor, mitochondrial 2	0.22601599	0.36802432	2.91

P2rx1	IMAGE:576538	Purinergic receptor P2X, ligand-gated ion channel, 1	0.15559824	0.36858922	2.91
Plxnc1	IMAGE:583517	Plexin C1	0.18999496	0.36862567	2.91
EST	IMAGE:640024	Expressed sequence tag	0.18031982	0.3688207	2.91
Rasa2	IMAGE:574375	RAS p21 protein activator 2	0.13233405	0.36894882	2.69
Cd24a	IMAGE:576831	CD24a antigen	0.21317054	0.3695614	2.69
EST	IMAGE:583006	Expressed sequence tag	0.28896558	0.36963874	2.69
9430029L20Rik	IMAGE:640104	RIKEN cDNA 9430029L20 gene	0.09622878	0.37007475	2.69
EST	IMAGE:640881	Expressed sequence tag	0.2758818	0.37014169	2.69
EST	IMAGE:641033	Expressed sequence tag	0.19859414	0.37106466	2.69
Mll	IMAGE:640194	Myeloid/lymphoid or mixed-lineage leukemia 1	0.2103087	0.3717319	2.69
Map3k7ip1	IMAGE:639878	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	0.14546908	0.37182847	2.69
Strbp	IMAGE:640364	Spermatid perinuclear RNA binding protein	0.24678503	0.3722977	2.69
In multiple clusters	IMAGE:583518		0.20527217	0.3723412	2.69
2810046L04Rik	IMAGE:1282289	CDNA, clone:Y1G0106K14, strand:plus, reference:ENSEMBL:Mouse-Transcript-ENST:ENSMUST00000058577, based on BLAT search	0.18743837	0.37244222	2.5
EST	IMAGE:583928	Expressed sequence tag	0.24147595	0.37254235	2.5
Mpp1	IMAGE:583178	Membrane protein, palmitoylated	0.14007208	0.3727963	2.5
EST	IMAGE:583741	Expressed sequence tag	0.12230929	0.37300166	2.5
Bag2	IMAGE:577004	Bcl2-associated athanogene 2	0.2134527	0.3730878	2.5
EST	IMAGE:639767	Expressed sequence tag	0.36326018	0.3735364	2.5
Mical1	IMAGE:583371	Microtubule associated monooxygenase, calponin and LIM domain containing 1	0.15844448	0.37381506	2.5
Ext1	IMAGE:640618	Exostoses (multiple) 1	0.3055139	0.37390387	2.5
Ube2v1	IMAGE:583840	Ubiquitin-conjugating enzyme E2 variant 1	0.17761478	0.37402195	2.5
EST	IMAGE:3586130	Expressed sequence tag	0.23207933	0.37424892	2.5
Rps27l	IMAGE:640461	Ribosomal protein S27-like	0.2742857	0.37425935	2.5
EST	IMAGE:640906	Expressed sequence tag	0.30953735	0.37426174	2.5

Gm608	IMAGE:583350	Gene model 608	0.20488659	0.37445837	2.5
EST	IMAGE:640814	Expressed sequence tag	0.15042305	0.3744847	2.5
0610038F07Rik	IMAGE:583455	RIKEN cDNA 0610038F07 gene	0.15949105	0.37466523	2.5
Csnk1a1	IMAGE:641129	Casein kinase 1, alpha 1	0.19881043	0.37483838	2.5
Usp25	IMAGE:583604	Ubiquitin specific peptidase 25	0.2416167	0.37563157	2.5
EST	IMAGE:583244	Expressed sequence tag	0.23954351	0.37575468	2.5
MSR1	IMAGE:4453348	Macrophage scavenger receptor 1	0.15398371	0.3759334	2.5
EST	IMAGE:640977	Expressed sequence tag	0.27625927	0.3765886	2.26
Eif4ebp2	IMAGE:583329	Eukaryotic translation initiation factor 4E binding protein 2	0.26217997	0.37679127	2.26
D4Wsu53e	IMAGE:583562	DNA segment, Chr 4, Wayne State University 53, expressed	0.14204179	0.37683904	2.26
Rps6kb1	IMAGE:583236	Ribosomal protein S6 kinase, polypeptide 1	0.08499483	0.37691882	2.26
EST	IMAGE:640589	Expressed sequence tag	0.19622897	0.37704912	2.26
Cbx3	IMAGE:583742	Chromobox homolog 3 (Drosophila HP1 gamma)	0.14337903	0.37713507	2.26
EST	IMAGE:639639	Expressed sequence tag	0.16233143	0.377684	2.26
Atp2a2	IMAGE:583700	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	0.24189112	0.37771693	2.26
EST	IMAGE:640479	Expressed sequence tag	0.16595668	0.3777229	2.26
EST	IMAGE:641144	Expressed sequence tag	0.2502757	0.37778413	2.26
EST	IMAGE:583340	Expressed sequence tag	0.23981206	0.3778922	2.26
EST	IMAGE:640812	Expressed sequence tag	0.10454907	0.3780873	2.26
Fau	IMAGE:1328685	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (fox derived)	0.18785195	0.37815145	2.26
Tcrg-C	IMAGE:640129	T-cell receptor gamma, constant region	0.12556937	0.37820753	2.26
Gtpbp6	IMAGE:640177	GTP binding protein 6 (putative)	0.1257116	0.3785117	2.26
EST	IMAGE:576406	Expressed sequence tag	0.20004235	0.3785163	2.26
Sec31l1	IMAGE:583334	SEC31 homolog A (S. cerevisiae)	0.1414248	0.3789015	2.26
Nol14	IMAGE:1447151	Nucleolar protein 14	0.25879025	0.379031	2.26
EST	IMAGE:640840	Expressed sequence tag	0.18356669	0.37930754	2.26

Lat	IMAGE:582840	Linker for activation of T cells	0.07671909	0.37948382	2.26
EST	IMAGE:640789	Expressed sequence tag	0.111658454	0.3798207	2.26
Rela	IMAGE:574694	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	0.25498712	0.38000986	2.26
Slbp	IMAGE:575728	Stem-loop binding protein	0.13490704	0.38011533	2.26
Mapk1	IMAGE:574250	Mitogen activated protein kinase 1	0.13206095	0.38047567	2.26
Cd3e	IMAGE:576441	CD3 antigen, epsilon polypeptide	0.19902663	0.38092244	2.07
In multiple clusters	IMAGE:641019		0.1671865	0.38106275	2.07
Arhgap19	IMAGE:583932	Rho GTPase activating protein 19	0.09150129	0.3812569	2.07
EST	IMAGE:641120	Expressed sequence tag	0.25020286	0.3814518	2.07
Rnaseh2b	IMAGE:583708	Ribonuclease H2, subunit B	0.17660268	0.38206822	2.07
Transcribed locus	IMAGE:1225946	Transcribed locus, weakly similar to XP_001074405.1 similar to zinc finger protein 709 [Rattus norvegicus]	0.13831413	0.3822267	2.07
Cotl1	IMAGE:641101	Coactosin-like 1 (Dictyostelium)	0.13165681	0.3828425	2.07
Ints10	IMAGE:583281	Integrator complex subunit 10	0.26201698	0.3829644	2.07
EST	IMAGE:639759	Expressed sequence tag	0.16260336	0.38311413	2.07
Prkd2	IMAGE:582778	Protein kinase D2	0.11029921	0.38332644	2.07
EST	IMAGE:577582	Expressed sequence tag	0.25369206	0.38344187	2.07
Lta	IMAGE:751270	Lymphotoxin A	0.199669	0.3834567	2.07
EST	IMAGE:640503	Expressed sequence tag	0.1660084	0.3840424	2.07
1700021K02Rik	IMAGE:577779	RIKEN cDNA 1700021K02 gene	0.2863832	0.3843776	2.07
EST	IMAGE:582338	Expressed sequence tag	0.3187861	0.38449496	2.07
Sirt3	IMAGE:640394	Sirtuin 3 (silent mating type information regulation 2, homolog) 3 (S. cerevisiae)	0.14770485	0.3845391	2.07
EST	IMAGE:640689	Expressed sequence tag	0.27372587	0.38456708	2.07
EST	IMAGE:641021	Expressed sequence tag	0.19785608	0.38470018	2.07
EST	IMAGE:640835	Expressed sequence tag	0.41914478	0.38489947	1.98
Irf3	IMAGE:576736	Interferon regulatory factor 3	0.19924013	0.3850657	1.98
Ankrd17	IMAGE:582866	Ankyrin repeat domain 17	0.13907702	0.38530305	1.98
Rapgef1	IMAGE:640093	Rap guanine nucleotide exchange factor (GEF) 1	0.12658244	0.38559288	1.98

Ccdc72	IMAGE:640628	Coiled-coil domain containing 72	0.24869469	0.38605982	1.98
Wfikkn2	IMAGE:583320	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2	0.08301636	0.3860893	1.98
Il12rb1	IMAGE:582118	Interleukin 12 receptor, beta 1	0.2813599	0.3863084	1.98
EST	IMAGE:640972	Expressed sequence tag	0.1831426	0.38672358	1.98
In multiple clusters	IMAGE:640200	Expressed sequence tag	0.09672052	0.38694626	1.98
EST	IMAGE:639899	Expressed sequence tag	0.3596155	0.3869807	1.98
Prrg2	IMAGE:640686	Proline-rich Gla (G-carboxyglutamic acid) polypeptide 2	0.2111313	0.38747868	1.98
Foxj3	IMAGE:640143	Forkhead box J3	0.1643275	0.38806814	1.98
EST	IMAGE:640644	Expressed sequence tag	0.10230052	0.3881982	1.98
Spna2	IMAGE:583512	Spectrin alpha 2	0.084340475	0.3881995	1.98
Gcc2	IMAGE:575478	GRIP and coiled-coil domain containing 2	0.06938428	0.38822982	1.98
A630034I12Rik	IMAGE:640302	RIKEN cDNA A630034I12 gene	0.20980926	0.38826653	1.98
Cxcl14	IMAGE:583442	Chemokine (C-X-C motif) ligand 14	0.14074096	0.3884111	1.98
EST	IMAGE:640564	Expressed sequence tag	0.18151987	0.38856086	1.98
Cdk2ap1	IMAGE:640009	CDK2 (cyclin-dependent kinase 2)-associated protein 1	0.12520646	0.38856375	1.98
EST	IMAGE:583782	Expressed sequence tag	0.20673692	0.38879347	1.98
Tnfrsf4	IMAGE:573690	Tumor necrosis factor receptor superfamily, member 4	0.17125042	0.38893205	1.98
EST	IMAGE:640598	Expressed sequence tag	0.14904618	0.3891659	1.77
5830405N20Rik	IMAGE:583659	RIKEN cDNA 5830405N20 gene	0.15998822	0.38950008	1.77
Transcribed locus	IMAGE:639941	Transcribed locus	0.19315057	0.39022473	1.77
Calm1	IMAGE:577674	Calmodulin 1	0.21678129	0.39035785	1.77
Ly6d	IMAGE:581909	Lymphocyte antigen 6 complex, locus D	0.25690898	0.39068517	1.77
EST	IMAGE:583299	Expressed sequence tag	0.1582784	0.39118844	1.77
EST	IMAGE:640796	Expressed sequence tag	0.13543391	0.39150044	1.77
EST	IMAGE:641012	Expressed sequence tag	0.25102594	0.3915085	1.77
EST	IMAGE:1281355	Expressed sequence tag	0.17314598	0.39151922	1.77
DCLRE1C	IMAGE:1743667	DNA cross-link repair 1C (PSO2 homolog, S. cerevisiae)	0.21400993	0.3916138	1.77

Tpr	IMAGE:576622	Translocated promoter region	0.21503003	0.39219284	1.77
Transcribed locus	IMAGE:574942	Transcribed locus	0.2826275	0.39224002	1.77
EST	IMAGE:640349	Expressed sequence tag	0.19487499	0.39230144	1.77
Rhoa	IMAGE:583485	Ras homolog gene family, member A	0.2639187	0.39250165	1.77
EST	IMAGE:640440	Expressed sequence tag	0.099690616	0.3926225	1.77
EST	IMAGE:640376	RIKEN cDNA 4930432K21 gene	0.24573953	0.39331234	1.64
Zfp131	IMAGE:572856	Zinc finger protein 131	0.06827795	0.39385924	1.64
Nkx6-2	IMAGE:576409	NK6 transcription factor related, locus 2 (Drosophila)	0.15520824	0.39392173	1.64
EST	IMAGE:640828	Expressed sequence tag	0.18283015	0.39415282	1.64
Cox17	IMAGE:583452	Cytochrome c oxidase, subunit XVII assembly protein homolog (yeast)	0.08636887	0.3945485	1.64
Ly6e	IMAGE:640890	Lymphocyte antigen 6 complex, locus E	0.133406	0.39520568	1.64
EST	IMAGE:640654	Expressed sequence tag	0.30787432	0.39615345	1.64
EST	IMAGE:581675	Expressed sequence tag	0.32438198	0.3968295	1.51
Fasl	IMAGE:3417263	Fas ligand (TNF superfamily, member 6)	0.17109413	0.39707422	1.51
Cxcl10	IMAGE:581882	Chemokine (C-X-C motif) ligand 10	0.2567548	0.39722618	1.51
EST	IMAGE:640864	Expressed sequence tag	0.18361919	0.39728165	1.51
Tagap1	IMAGE:639758	T-cell activation GTPase activating protein 1	0.14517656	0.39740938	1.51
Gtf2i	IMAGE:639942	General transcription factor II I	0.20845036	0.3974217	1.51
EST	IMAGE:640036	Expressed sequence tag	0.17954084	0.39757675	1.51
Pkp1	IMAGE:583760	Plakophilin 1	0.24099706	0.39757895	1.51
EST	IMAGE:640904	Expressed sequence tag	0.24955747	0.3979872	1.51
EST	IMAGE:582858	Expressed sequence tag	0.20406908	0.39799318	1.51
Exdl2	IMAGE:640270	Exonuclease 3'-5' domain-like 2	0.30362678	0.39818573	1.51
EST	IMAGE:639734	Expressed sequence tag	0.1451156	0.39847633	1.51
Klhdc5	IMAGE:640756	Kelch domain containing 5	0.18193711	0.39860782	1.51
EST	IMAGE:583699	Expressed sequence tag	0.22328195	0.39863968	1.51
Mxd4	IMAGE:640190	Max dimerization protein 4	0.14717604	0.3988511	1.51
Clec1b	IMAGE:574821	C-type lectin domain family 1, member b	0.15156524	0.39935553	1.51
Transcribed locus	IMAGE:640165	Transcribed locus	0.12679222	0.39939374	1.51
Cys1	IMAGE:582804	Cystin 1	0.07945655	0.39942518	1.51

Transcribed locus		Transcribed locus, moderately similar to XP_001103164.1 similar to 60S ribosomal protein L12 [Macaca mulatta]			
	IMAGE:582892		0.17361486	0.40003896	1.51
EST	IMAGE:583695	Expressed sequence tag	0.1608681	0.4003079	1.51
Eif2a	IMAGE:582939	Eukaryotic translation initiation factor 2a	0.15705606	0.40112728	1.37
Ccdc58	IMAGE:582929	Coiled-coil domain containing 58	0.18879382	0.4011698	1.37
Btg2	IMAGE:639859	B-cell translocation gene 2, anti-proliferative	0.22523461	0.4013058	1.37
Taf15		TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor			
	IMAGE:639860		0.24421383	0.4013146	1.37
EST	IMAGE:583906	Expressed sequence tag	0.2946352	0.40145835	1.37
In multiple clusters	IMAGE:641096	Expressed sequence tag	0.2501315	0.40156463	1.37
Uqcrrf1		Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1			
	IMAGE:640752		0.101116255	0.40176198	1.37
Dhrs13		Dehydrogenase/reductase (SDR family) member 13			
	IMAGE:576046		0.11375762	0.4019725	1.37
Fyb	IMAGE:1264938	Prohibitin	0.17272258	0.40204743	1.37
Rpain	IMAGE:573267	RPA interacting protein	0.13627423	0.40244517	1.37
2500002K03Rik		CDNA, clone:Y0G0124L21, strand:unspecified			
	IMAGE:583524		0.086820185	0.40269917	1.37
Trim17	IMAGE:1263520	Transcribed locus	0.15638469	0.40282822	1.37
Bid	IMAGE:640130	BH3 interacting domain death agonist	0.1461156	0.40290505	1.37
Clec14a	IMAGE:576574	C-type lectin domain family 14, member a	0.21469185	0.40293807	1.37
Arpc3	IMAGE:640677	Actin related protein 2/3 complex, subunit 3	0.2750277	0.40315855	1.37
EST	IMAGE:640868	Expressed sequence tag	0.2505796	0.40386733	1.37
EST	IMAGE:640319	Expressed sequence tag	0.38339138	0.404027	1.37
EST	IMAGE:583103	Expressed sequence tag	0.33644107	0.4041983	1.37
EST	IMAGE:640173	Expressed sequence tag	0.1080738	0.4047628	1.26
C77370	IMAGE:583793	Expressed sequence C77370	0.19215246	0.40533575	1.26
5330438D12Rik	IMAGE:640084	RIKEN cDNA 5330438D12 gene	0.17964597	0.40572393	1.26
EST	IMAGE:583673	Expressed sequence tag	0.1918779	0.40591183	1.26
Arntl		Aryl hydrocarbon receptor nuclear translocator-like			
	IMAGE:582931		0.21954234	0.4060044	1.26

Transcribed locus	IMAGE:640397	Transcribed locus	0.19581184	0.4068279	1.26
1700106N22Rik	IMAGE:640218	RIKEN cDNA 1700106N22 gene	0.21035998	0.4069919	1.26
EST	IMAGE:583595	Expressed sequence tag	0.34630018	0.4074916	1.26
1810059G22Rik	IMAGE:583234	RIKEN cDNA 1810059G22 gene	0.2897901	0.40794325	1.18
EST	IMAGE:583474	Expressed sequence tag	0.29084098	0.40822262	1.18
Grcc10	IMAGE:641036	Atrophin 1	0.25110045	0.40835306	1.18
Chchd1	IMAGE:583655	Coiled-coil-helix-coiled-coil-helix domain containing 1	0.3520113	0.40859562	1.18
Kctd10	IMAGE:640583	Potassium channel tetramerisation domain containing 10	0.40539417	0.4089895	1.18
AI194318	IMAGE:583276	3 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A630066K17 product:unclassifiable, full insert sequence	0.17482738	0.4091023	1.18
Stat1	IMAGE:583302	Signal transducer and activator of transcription 1	0.048201974	0.40922952	1.18
EST	IMAGE:640849	Expressed sequence tag	0.12987265	0.4096255	1.18
Transcribed locus	IMAGE:583764	Transcribed locus	0.090552844	0.4096824	1.18
CXCL10	IMAGE:2069299	Chemokine (C-X-C motif) ligand 10	0.17077018	0.41001084	1.18
EST	IMAGE:582106	Expressed sequence tag	0.28126255	0.4101807	1.18
Mbnl1	IMAGE:583553	Muscleblind-like 1 (Drosophila)	0.19088008	0.41020763	1.18
Transcribed locus	IMAGE:640052	Similar to ariadne homolog 2	0.24588272	0.410971	1.18
Atp5c1	IMAGE:640931	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1	0.42529702	0.41185647	1.07
EG666113	IMAGE:641052	Predicted gene, EG666113	0.10545162	0.4122206	1.07
Amica1	IMAGE:640276	Adhesion molecule, interacts with CXADR antigen 1	0.18005581	0.41252714	1.07
Ltb	IMAGE:577871	Lymphotoxin B	0.23434414	0.41267005	1.07
Tcrg-C	IMAGE:640129	T-cell receptor gamma, constant region	0.10797722	0.41389433	1.07
EST	IMAGE:639831	Expressed sequence tag	0.16276084	0.4139148	1.07
Anks1b	IMAGE:573163	Ankyrin repeat and sterile alpha motif domain containing 1B	0.112788066	0.41394684	1.07

EST	IMAGE:583221	Expressed sequence tag	0.2630039	0.41415575	1.07
1700097N02Rik	IMAGE:1329935	RIKEN cDNA 1700097N02 gene	0.20256568	0.41428274	1.07
Cfb	IMAGE:583545	Complement factor B	0.26284125	0.41430536	1.07
Plekha1	IMAGE:583794	Pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	0.20750478	0.41514707	1.07
Transcribed locus	IMAGE:2410765	Transcribed locus	0.17082557	0.41538087	0.99
Angel2	IMAGE:640692	Angel homolog 2 (Drosophila)	0.10251409	0.41552565	0.99
Rock2	IMAGE:583652	Rho-associated coiled-coil containing protein kinase 2	0.24175228	0.41553754	0.99
Il2	IMAGE:750652	Interleukin 2	0.31681585	0.4160047	0.99
Aak1	IMAGE:640223	AP2 associated kinase 1	0.3808932	0.41619292	0.99
EST	IMAGE:640966	Expressed sequence tag	0.3120666	0.4163041	0.99
Mrps28	IMAGE:582901	Mitochondrial ribosomal protein S28	0.117291525	0.41632688	0.99
Aim1	IMAGE:640153	Absent in melanoma 1	0.12563856	0.41637465	0.99
ITGA5	IMAGE:135671	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	0.16914217	0.41653648	0.99
EST	IMAGE:641158	Expressed sequence tag	0.31270653	0.4166488	0.99
EST	IMAGE:640527	Expressed sequence tag	0.16606297	0.41682085	0.99
Ube2i	IMAGE:576391	Ubiquitin-conjugating enzyme E2I	0.1999881	0.41685176	0.99
Tnfrsf13b	IMAGE:574040	Tumor necrosis factor receptor superfamily, member 13b	0.10711665	0.41689742	0.99
Mecp2	IMAGE:574625	Methyl CpG binding protein 2	0.061005175	0.4169386	0.99
Pdlim1	IMAGE:583846	PDZ and LIM domain 1 (elfin)	0.29626772	0.41696715	0.99
Nkx6-2	IMAGE:576409	NK6 transcription factor related, locus 2 (Drosophila)	0.19897287	0.41724557	0.99
EST	IMAGE:640333	Expressed sequence tag	0.12728567	0.41790578	0.99
Malat1	IMAGE:640016	Metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	0.24469773	0.41803008	0.99
EST	IMAGE:583352	Expressed sequence tag	0.23881117	0.41838577	0.99
Il4	IMAGE:578022	Interleukin 4	0.23466878	0.4184573	0.99
Jtv1	IMAGE:583892	JTV1 gene	0.2424275	0.41855776	0.99

Tbc1d10c	IMAGE:583160	TBC1 domain family, member 10c	0.2373676	0.41888836	0.99
Flna	IMAGE:640772	Filamin, alpha	0.2491305	0.41957656	0.99
Rab22a	IMAGE:640602	RAB22A, member RAS oncogene family	0.2116796	0.41959673	0.99
Myc	IMAGE:574902	Myelocytomatosis oncogene	0.15184784	0.41983315	0.92
EST	IMAGE:639951	Expressed sequence tag	0.1630376	0.42079803	0.92
Grk6	IMAGE:640178	G protein-coupled receptor kinase 6	0.14622976	0.4208583	0.92
EST	IMAGE:583803	Expressed sequence tag	0.16026035	0.42093688	0.92
Ptpa	IMAGE:583653	Protein tyrosine phosphatase, receptor type, A	0.26585543	0.4210699	0.92
5730453I16Rik	IMAGE:583134	RIKEN cDNA 5730453I16 gene	0.20385604	0.42134878	0.92
Tnrc6	IMAGE:577267	Trinucleotide repeat containing 6a	0.2019609	0.42153668	0.92
In multiple clusters	IMAGE:574025		0.10702222	0.42173398	0.92
Fos	IMAGE:5376627	FBJ osteosarcoma oncogene	0.2534694	0.42192382	0.92
Transcribed locus	IMAGE:583612	Mus musculus, clone IMAGE:1329925, mRNA	0.25752932	0.4222022	0.92
H13	IMAGE:640912	Histocompatibility 13	0.18372406	0.42224127	0.92
Nrip1	IMAGE:582877	Nuclear receptor interacting protein 1	0.11721584	0.4225895	0.92
Tardbp	IMAGE:583625	TAR DNA binding protein	0.1917746	0.42298526	0.92
Transcribed locus	IMAGE:1381297	Transcribed locus	0.17098534	0.42305145	0.92
Becn1	IMAGE:576646	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	0.21531317	0.42308939	0.92
EST	IMAGE:640738	Expressed sequence tag	0.3062471	0.42310855	0.92
MacroD2	IMAGE:640244	MACRO domain containing 2	0.24643749	0.42325637	0.92
EST	IMAGE:639810	Expressed sequence tag	0.20899235	0.4232903	0.92
FAS	IMAGE:5222802	Fas (TNF receptor superfamily, member 6)	0.15381682	0.42384133	0.85
EST	IMAGE:583366	Expressed sequence tag	0.29202715	0.424191	0.85
CCR5	IMAGE:2119876	Chemokine (C-C motif) receptor 5	0.15370564	0.4249536	0.85
EST	IMAGE:583515	Expressed sequence tag	0.15877527	0.4252803	0.85
Ifit2	IMAGE:574088	Interferon-induced protein with tetratricopeptide repeats 2	0.201051	0.42539278	0.85
Cenpf	IMAGE:583540	Centromere protein F	0.17540695	0.4257368	0.85
Rab1	IMAGE:582445	RAB1, member RAS oncogene family	0.2785224	0.4257654	0.85
Fbxo3	IMAGE:639967	F-box protein 3	0.22459362	0.42609283	0.85

Transcribed locus	IMAGE:640755	Transcribed locus, strongly similar to XP_900360.2 similar to fetal Alzheimer antigen isoform 2 isoform 2 [Mus musculus]	0.16574147	0.4263404	0.85
AW061096	IMAGE:639792	Expressed sequence AW061096	0.09256457	0.42658284	0.85
Rpl27a	IMAGE:640012	Ribosomal protein L27a	0.17948692	0.42683122	0.85
Polr3d	IMAGE:573740	Polymerase (RNA) III (DNA directed) polypeptide D	0.17140786	0.42746672	0.85
EST	IMAGE:640811	Expressed sequence tag	0.4177818	0.4275684	0.77
Prkcq	IMAGE:583229	Protein kinase C, theta	0.3152362	0.42793712	0.77
Transcribed locus	IMAGE:583571	RIKEN cDNA 2410017I17 gene	0.34600005	0.42815882	0.77
Msi2	IMAGE:639887	Musashi homolog 2 (Drosophila)	0.36542237	0.42951286	0.77
EST	IMAGE:640574	Expressed sequence tag	0.1489878	0.43028948	0.77
Rabac1	IMAGE:583923	Rab acceptor 1 (prenylated)	0.16053703	0.43038416	0.77
EST	IMAGE:640568	Expressed sequence tag	0.24741884	0.43061203	0.77
EST	IMAGE:582987	Expressed sequence tag	0.15716521	0.43074083	0.77
Cd27	IMAGE:575719	CD antigen 27	0.13484305	0.43084687	0.77
Foxo3	IMAGE:577721	Forkhead box O3a	0.28379744	0.43173894	0.73
Ankrd37	IMAGE:576620	Ankyrin repeat domain 37	0.2149755	0.43190283	0.73
Transcribed locus	IMAGE:640544	Transcribed locus, strongly similar to XP_001481228.1 hypothetical protein [Mus musculus]	0.2473489	0.4328049	0.73
Transcribed locus	IMAGE:575368	Transcribed locus	0.109926365	0.43281043	0.73
Rc3h2	IMAGE:583335	Ring finger and CCH-type zinc finger domains 2	0.15922098	0.43286303	0.73
EST	IMAGE:583071	Expressed sequence tag	0.15789115	0.43303695	0.73
D2Ert750e	IMAGE:583587	DNA segment, Chr 2, ERATO Doi 750, expressed	0.15982248	0.43304643	0.73
Bid	IMAGE:583029	BH3 interacting domain death agonist	0.2611936	0.43314427	0.73
Lef1	IMAGE:575374	Lymphoid enhancer binding factor 1	0.3224128	0.43357465	0.73
Gucy2d	IMAGE:582819	Guanylate cyclase 2d	0.15677238	0.4341715	0.73
Prkdc	IMAGE:1345935	Protein kinase, DNA activated, catalytic polypeptide	0.19977558	0.4341767	0.73

Ccl6	IMAGE:576828	Chemokine (C-C motif) ligand 6	0.17229344	0.43438098	0.73
Transcribed locus	IMAGE:640298		0.14652362	0.43467996	0.73
EST	IMAGE:583784	Expressed sequence tag	0.24106531	0.4351152	0.64
Copg2	IMAGE:583460	Coatomer protein complex, subunit gamma 2	0.24014038	0.43526316	0.64
Nebi	IMAGE:639873	Nebulette	0.26767907	0.43534335	0.64
Cacnb3	IMAGE:583697	Calcium channel, voltage-dependent, beta 3 subunit	0.19193478	0.43561983	0.64
Cdc2a	IMAGE:581926	Cell division cycle 2 homolog A (S. pombe)	0.32476765	0.43566224	0.64
Ywhah	IMAGE:640494	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	0.21079752	0.4360397	0.64
Cetn3	IMAGE:640413	Centrin 3	0.2741018	0.4362668	0.64
Transcribed locus	IMAGE:640003	Phosphorylase kinase alpha 2	0.22559364	0.43629718	0.64
EST	IMAGE:640029	Expressed sequence tag	0.2711319	0.43635	0.64
EST	IMAGE:583363	Expressed sequence tag	0.22151221	0.43642572	0.64
Pmpcb	IMAGE:583586	Peptidase (mitochondrial processing) beta	0.14209922	0.43652424	0.64
Ly6a	IMAGE:574422	Lymphocyte antigen 6 complex, locus A	0.13253751	0.43691057	0.64
EST	IMAGE:583174	Expressed sequence tag	0.2894368	0.43719336	0.64
Cdk9	IMAGE:641150	Cyclin-dependent kinase 9 (CDC2-related kinase)	0.15116554	0.4372174	0.64
Clip4	IMAGE:583743	CAP-GLY domain containing linker protein family, member 4	0.16098022	0.43724516	0.64
5830404H04Rik	IMAGE:577432	RIKEN cDNA 5830404H04 gene	0.23251173	0.43745306	0.64
Wdr40a	IMAGE:640023	WD repeat domain 40A	0.16405813	0.43802822	0.64
B3gnt1	IMAGE:640940	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2	0.25080135	0.43824345	0.64
D4Wsu53e	IMAGE:639889	DNA segment, Chr 4, Wayne State University 53, expressed	0.12380852	0.43843865	0.64
Dock11	IMAGE:583239	Dedicator of cytokinesis 11	0.15899843	0.438704	0.64
Gnai2	IMAGE:640167	Guanine nucleotide binding protein, alpha inhibiting 2	0.16438067	0.43874565	0.64
ERCC3	IMAGE:1655018	Excision repair cross-complementing rodent	0.19956277	0.4390542	0.64

		repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)			
Fdx1	IMAGE:583500	Ferredoxin 1	0.086673975	0.4390946	0.64
Lrmp	IMAGE:583128	Lymphoid-restricted membrane protein	0.07908369	0.4391222	0.64
EST	IMAGE:640419	Expressed sequence tag	0.16497816	0.4394148	0.61
Itsn2	IMAGE:583881	Intersectin 2	0.26534885	0.43961382	0.61
TLR4	IMAGE:6047339	Toll-like receptor 4	0.15409493	0.43962702	0.61
Grlf1	IMAGE:640573	Glucocorticoid receptor DNA binding factor 1	0.12905097	0.4396407	0.61
2310065K24Rik	IMAGE:583706	RIKEN cDNA 2310065K24 gene	0.14239968	0.43988585	0.61
Cant1	IMAGE:1379942	Calcium activated nucleotidase 1	0.23620737	0.43988624	0.61
Ccdc41	IMAGE:639929	RIKEN cDNA 4921537D05 gene	0.19393376	0.44014138	0.61
Suz12	IMAGE:640237	Suppressor of zeste 12 homolog (Drosophila)	0.12700497	0.44020954	0.61
EST	IMAGE:641111	Expressed sequence tag	0.51701295	0.4404906	0.61
EST	IMAGE:640047	Expressed sequence tag	0.16411279	0.44056523	0.61
In multiple clusters	IMAGE:574076		0.107304975	0.44075873	0.61
In multiple clusters	IMAGE:577750		0.25468263	0.44120133	0.61
2810026P18Rik	IMAGE:1224948	RIKEN cDNA 2810026P18 gene	0.11520675	0.44122702	0.61
Mmp14	IMAGE:573066	Matrix metalloproteinase 14 (membrane-inserted)	0.040244598	0.4415797	0.61
EST	IMAGE:640634	Expressed sequence tag	0.14828616	0.4420567	0.61
EST	IMAGE:639660	Expressed sequence tag	0.10750361	0.44263285	0.61
Mrpl3	IMAGE:583898	Mitochondrial ribosomal protein L3	0.14282548	0.44307244	0.61
EST	IMAGE:583688	Expressed sequence tag	0.2407978	0.4431002	0.61
1110006G06Rik	IMAGE:582920	RIKEN cDNA 1110006G06 gene	0.23678574	0.44330415	0.61
EST	IMAGE:640866	Expressed sequence tag	0.2126287	0.44408375	0.52
EST	IMAGE:640431	Expressed sequence tag	0.16584426	0.44449207	0.52
Il18rap	IMAGE:640615	Interleukin 18 receptor accessory protein	0.1331393	0.44458842	0.52
Cxcl4	IMAGE:573339	Chemokine (C-X-C motif) ligand 4	0.043369222	0.4449553	0.52
EST	IMAGE:583780	Expressed sequence tag	0.17675592	0.44537395	0.52
Atg2b	IMAGE:640202	ATG2 autophagy related 2 homolog B (S. cerevisiae)	0.14629018	0.44544846	0.52

Sdc1	IMAGE:577122	Syndecan 1	0.21361962	0.44550738	0.52
Ostf1	IMAGE:641061	Osteoclast stimulating factor 1	0.2779244	0.44555533	0.52
EST	IMAGE:640075	Expressed sequence tag	0.22673777	0.44578466	0.52
D10Wsu102e	IMAGE:583685	DNA segment, Chr 10, Wayne State University 102, expressed	0.19114035	0.4459335	0.52
EST	IMAGE:582250	Expressed sequence tag	0.28209364	0.44596457	0.52
EST	IMAGE:640449	Expressed sequence tag	0.27278548	0.44724146	0.53
AI481772	IMAGE:583909	Expressed sequence AI481772	0.12283737	0.4475117	0.53
Transcribed locus	IMAGE:583612	Mus musculus, clone IMAGE:1329925, mRNA	0.1763957	0.4483368	0.53
Hook3	IMAGE:640124	Hook homolog 3 (Drosophila)	0.24609293	0.44875866	0.53
Rragc	IMAGE:640581	Ras-related GTP binding C	0.27475202	0.44900617	0.53
EST	IMAGE:640360	Expressed sequence tag	0.18105556	0.44908473	0.53
VEGFA	IMAGE:34778	Vascular endothelial growth factor A	0.15252505	0.44951892	0.53
EST	IMAGE:583096	Expressed sequence tag	0.17440337	0.44983044	0.53
Pcnt	IMAGE:640026	Pericentrin (kendrin)	0.20997316	0.4500112	0.53
Transcribed locus	IMAGE:575780	Chemokine (C-C motif) ligand 25	0.15291804	0.4503173	0.53
Stat6	IMAGE:5349719	Signal transducer and activator of transcription 6	0.2804397	0.4504837	0.53
EST	IMAGE:639855	Expressed sequence tag	0.16281806	0.45090765	0.53
EST	IMAGE:639747	Expressed sequence tag	0.1617378	0.4509311	0.53
2610209M04Rik	IMAGE:640363	RIKEN cDNA 2610209M04 gene	0.22733416	0.45204553	0.49
Cd83	IMAGE:574651	CD83 antigen	0.15128258	0.45249233	0.49
TGFB1	IMAGE:136821	Transforming growth factor, beta 1 (Camurati-Engelmann disease)	0.16908714	0.45257518	0.49
Ep400	IMAGE:639900	E1A binding protein p400	0.09512772	0.45283258	0.49
Evl	IMAGE:582896	Ena-vasodilator stimulated phosphoprotein	0.23671931	0.4530029	0.49
Gm237	IMAGE:639696	Gene model 237, (NCBI)	0.09202822	0.45314112	0.49
Il4	IMAGE:574369	Interleukin 4	0.21605256	0.453198	0.49
EST	IMAGE:576452		0.20019776	0.45330137	0.49
2310035C23Rik	IMAGE:573037	RIKEN cDNA 2310035C23 gene	0.11218727	0.4534347	0.49
Nr4a1	IMAGE:582922	Nuclear receptor subfamily 4, group A,	0.2873756	0.4536512	0.49

		member 1			
EST	IMAGE:583824	Expressed sequence tag	0.088761464	0.4540717	0.49
EST	IMAGE:639691	Protein phosphatase 4, catalytic subunit	0.22482716	0.4540784	0.49
Cd4	IMAGE:640273	CD4 antigen	0.108447455	0.4543197	0.49
H2afz	IMAGE:575684	H2A histone family, member Z	0.1346452	0.45442256	0.49
Ppp2ca	IMAGE:581653	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0.21711949	0.455006	0.44
Btbd1	IMAGE:640060	BTB (POZ) domain containing 1	0.17958812	0.4552251	0.44
EST	IMAGE:640073	Expressed sequence tag	0.19508456	0.45522803	0.44
Csnk1a1	IMAGE:640022	Casein kinase 1, alpha 1	0.14676546	0.45591566	0.44
IRAK2	IMAGE:4450379	Interleukin-1 receptor-associated kinase 2	0.15387209	0.45648474	0.44
EST	IMAGE:640783	Expressed sequence tag	0.111119255	0.45692128	0.44
1200014M14Rik	IMAGE:583885	RIKEN cDNA 1200014M14 gene	0.122763	0.45700908	0.44
EST	IMAGE:582514	Expressed sequence tag	0.06277903	0.45704105	0.44
Pkp3	IMAGE:583696	Plakophilin 3	0.17729883	0.45723903	0.44
Fbxo34	IMAGE:1362061	F-box protein 34	0.21797502	0.4580247	0.44
Dph4	IMAGE:640955	DPH4 homolog (JJJ3, <i>S. cerevisiae</i>)	0.42702895	0.45818985	0.44
Psap	IMAGE:1264859	Prosaposin	0.15665899	0.45820612	0.44
EST	IMAGE:582899	Expressed sequence tag	0.3314561	0.45821428	0.44
Lsm1	IMAGE:640072	LSM1 homolog, U6 small nuclear RNA associated (<i>S. cerevisiae</i>)	0.18042335	0.4583143	0.44
Cd24a	IMAGE:576831	CD24a antigen	0.17234898	0.45846042	0.44
Nup50	IMAGE:639698	Nucleoporin 50	0.14409864	0.45861965	0.44
Bptf	IMAGE:583596	Bromodomain PHD finger transcription factor	0.089597665	0.45881146	0.44
2410018C17Rik	IMAGE:640132	RIKEN cDNA 2410018C17 gene	0.1797458	0.4588168	0.44
Transcribed locus	IMAGE:581923	Transcribed locus, moderately similar to XP_001096337.1 similar to 60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (CPN60) (Heat shock protein 60) (HSP-60) (Mitochondrial matrix protein P1) (P60 lymphocyte protein)	0.25213364	0.45904043	0.44

		(HuCHA60) [Macaca mulatta]			
AI316807	IMAGE:575276	Expressed sequence AI316807	0.10928467	0.45915985	0.44
In multiple clusters	IMAGE:640368		0.097541355	0.45921302	0.44
EST	IMAGE:639723	Expressed sequence tag	0.16168493	0.45930547	0.44
Syngn2	IMAGE:640773	Synaptogyrin 2	0.27540338	0.45931292	0.44
Hivep2	IMAGE:1282235	Human immunodeficiency virus type I enhancer binding protein 2	0.17335606	0.45987818	0.4
In multiple clusters	IMAGE:640263		0.3268622	0.45998484	0.4
Capn7	IMAGE:583039	Calpain 7	0.21901226	0.45999777	0.4
Adfp	IMAGE:583345	Adipose differentiation related protein	0.118576825	0.4603393	0.4
Il5	IMAGE:972705	Interleukin 5	0.1993991	0.46035945	0.4
EST	IMAGE:582312	Expressed sequence tag	0.31806487	0.46052033	0.4
EST	IMAGE:640393	Expressed sequence tag	0.1275003	0.46071306	0.4
Tfam	IMAGE:1329916	Transcription factor A, mitochondrial	0.20251001	0.46080697	0.4
Ptp4a3	IMAGE:583209	Protein tyrosine phosphatase 4a3	0.26177517	0.46132147	0.4
EST	IMAGE:641020	Expressed sequence tag	0.18325152	0.4620093	0.4
Stom	IMAGE:640728	Stomatin	0.10100498	0.46256894	0.4
Elk4	IMAGE:583566	ELK4, member of ETS oncogene family	0.20624699	0.46271864	0.4
EST	IMAGE:640272	Expressed sequence tag	0.09707037	0.46344548	0.36
1700012G19Rik	IMAGE:582884	Jagged 2	0.23763284	0.46395567	0.36
Ly6a	IMAGE:581749	Lymphocyte antigen 6 complex, locus A	0.2518387	0.46415645	0.36
Ppp2r5e	IMAGE:640259	Protein phosphatase 2, regulatory subunit B (B56), epsilon isoform	0.37304685	0.4642693	0.36
EST	IMAGE:640373	Expressed sequence tag	0.19492763	0.46454036	0.36
Lypla1	IMAGE:641132	Lysophospholipase 1	0.25139084	0.4656194	0.36
EST	IMAGE:640785	Expressed sequence tag	0.27550352	0.46572915	0.36
D14Ert449e	IMAGE:640010	Placenta specific 9	0.14582492	0.46619272	0.36
Ide	IMAGE:574949	Insulin degrading enzyme	0.15190135	0.46653748	0.36
EST	IMAGE:583462	Expressed sequence tag	0.29250666	0.46668932	0.36
Sec24c	IMAGE:640591	SEC24 related gene family, member C (S. cerevisiae)	0.22793202	0.46680376	0.36
Capn2	IMAGE:575158	Calpain 2	0.108809896	0.4668477	0.36

Transcribed locus	IMAGE:583004	Transcribed locus	0.23788959	0.46791652	0.31
TLR9	IMAGE:5495717	Toll-like receptor 9	0.15432386	0.4679352	0.31
Sf3b1	IMAGE:640508	Splicing factor 3b, subunit 1	0.24834006	0.46861404	0.31
TLR2	IMAGE:4753177	Toll-like receptor 2	0.15421213	0.4686194	0.31
Rnf4	IMAGE:640415	Ring finger protein 4	0.3985474	0.46888396	0.31
Hdlbp	IMAGE:640671	High density lipoprotein (HDL) binding protein	0.16637671	0.47082257	0.31
Insl5	IMAGE:575182	Insulin-like 5	0.15201358	0.47085735	0.31
Rela	IMAGE:574694	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	0.15139265	0.47125432	0.31
Ube1x	IMAGE:575553	Ubiquitin-activating enzyme E1, Chr X	0.13445424	0.47157744	0.31
Tpp1	IMAGE:641000	Tripeptidyl peptidase I	0.24984907	0.471742	0.31
Nrbf2	IMAGE:583296	Nuclear receptor binding factor 2	0.0474979	0.47185194	0.31
Zfp213	IMAGE:583538	Zinc finger protein 213	0.14098835	0.47202215	0.31
Rbm18	IMAGE:640326	RNA binding motif protein 18	0.20986354	0.47325337	0.31
Taf3	IMAGE:640587	TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor	0.16535634	0.4735352	0.31
Serinc5	IMAGE:583286	Serine incorporator 5	0.14130089	0.47354332	0.31
Rmi1	IMAGE:640893	RMI1, RecQ mediated genome instability 1, homolog (S. cerevisiae)	0.27723488	0.47381207	0.31
EST	IMAGE:583264	Expressed sequence tag	0.17561679	0.47466543	0.26
HSPD1	IMAGE:133099	Transcribed locus, weakly similar to XP_360622.1 protein MG03165.4 [Magnaporthe grisea 70-15]	0.16903718	0.47474864	0.26
Gtf2b	IMAGE:640038	General transcription factor IIB	0.20932186	0.47519597	0.26
EST	IMAGE:640895	Expressed sequence tag	0.45987147	0.47538242	0.26
EST	IMAGE:583447	Expressed sequence tag	0.22081181	0.4754048	0.26
EST	IMAGE:640717	Expressed sequence tag	0.12945664	0.47556496	0.26
BC036313	IMAGE:639985	CDNA sequence BC036313	0.12410744	0.47563264	0.26
Pdha1	IMAGE:583224	Pyruvate dehydrogenase E1 alpha 1	0.082349576	0.47570455	0.26
Tdg	IMAGE:642198	Thymine DNA glycosylase	0.11503782	0.4759388	0.26
Cd300d	IMAGE:582766	Cd300D antigen	0.2796216	0.47678146	0.26

Cdc42se2	IMAGE:583723	CDC42 small effector 2	0.22334374	0.4768892	0.26
EST	IMAGE:641087	Expressed sequence tag	0.5034835	0.47725055	0.26
Inpp5f	IMAGE:640224	Inositol polyphosphate-5-phosphatase F	0.09684145	0.47747043	0.26
Vamp1	IMAGE:573892	Vesicle-associated membrane protein 1	0.18635565	0.4775774	0.26
2810403A07Rik	IMAGE:583082	RIKEN cDNA 2810403A07 gene	0.13951267	0.47772956	0.26
EST	IMAGE:583002	Expressed sequence tag	0.20428687	0.47783142	0.26
EST	IMAGE:1362232	Expressed sequence tag	0.21803206	0.4778885	0.26
Pias1	IMAGE:577047	Protein inhibitor of activated STAT 1	0.21350627	0.47827592	0.27
Pscd3	IMAGE:583138	Pleckstrin homology, Sec7 and coiled-coil domains 3	0.28793588	0.47852045	0.27
Gprc5b	IMAGE:583550	RIKEN cDNA 2310008H09 gene	0.14197764	0.47888368	0.27
EST	IMAGE:640787	Expressed sequence tag	0.4164065	0.4790333	0.27
EST	IMAGE:640781	Expressed sequence tag	0.19749016	0.47980005	0.27
Gnb2-rs1	IMAGE:574006	Guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1	0.18687387	0.48002723	0.27
Ap2a2	IMAGE:639674	Adaptor protein complex AP-2, alpha 2 subunit	0.14403972	0.48081723	0.27
Ccnb1	IMAGE:576166	Cyclin B1	0.17034668	0.4815708	0.27
Dlgh4	IMAGE:640255	Discs, large homolog 4 (Drosophila)	0.22625417	0.48200703	0.21
EST	IMAGE:639787	Expressed sequence tag	0.22505805	0.48223203	0.21
Cdc37	IMAGE:640301	Cell division cycle 37 homolog (S. cerevisiae)	0.19477247	0.48236358	0.21
E230029C05Rik	IMAGE:639804	RIKEN cDNA E230029C05 gene	0.094641104	0.4823713	0.21
Slc30a7	IMAGE:640436	Solute carrier family 30 (zinc transporter), member 7	0.24812809	0.4824453	0.21
Cd28	IMAGE:576501	CD28 antigen	0.15554333	0.4829441	0.21
EST	IMAGE:582907	Expressed sequence tag	0.21948357	0.4829645	0.21
Glrx2	IMAGE:640163	Glutaredoxin 2 (thioltransferase)	0.3709704	0.48300242	0.21
EST	IMAGE:640455	Expressed sequence tag	0.16590142	0.48344278	0.21
Pomgnt1	IMAGE:640648	Protein O-linked mannose beta1,2-N-acetylglucosaminyltransferase	0.18240888	0.48422137	0.21
Pts	IMAGE:639817	6-pyruvoyl-tetrahydropterin synthase	0.12358407	0.48432413	0.21
EST	IMAGE:583145	Expressed sequence tag	0.18916145	0.4853606	0.21

Tfdp1	IMAGE:640119	Transcribed locus	0.164275	0.48550537	0.21
XRCC4	IMAGE:2383277	X-ray repair complementing defective repair in Chinese hamster cells 4	0.19961604	0.48570868	0.22
EST	IMAGE:640909	Expressed sequence tag	0.13112594	0.48674083	0.22
Pofut2	IMAGE:575950	Protein O-fucosyltransferase 2	0.15353853	0.48702434	0.22
Mtdh	IMAGE:640549	Metadherin	0.12898394	0.4873163	0.22
EST	IMAGE:640416	Expressed sequence tag	0.09957807	0.48751202	0.22
EST	IMAGE:640370	Expressed sequence tag	0.14670296	0.48756543	0.22
Acbd3	IMAGE:583413	Acyl-Coenzyme A binding domain containing 3	0.26366648	0.48767176	0.22
Hrh3	IMAGE:573677	Histamine receptor H 3	0.17119707	0.48873988	0.22
Cyb561d2	IMAGE:640083	Cytochrome b-561 domain containing 2	0.16336505	0.4891482	0.22
EST	IMAGE:641115	Expressed sequence tag	0.16734755	0.48946708	0.22
Zcchc11	IMAGE:582979	Zinc finger, CCHC domain containing 11	0.21965896	0.48990157	0.22
EST	IMAGE:583097	Expressed sequence tag	0.18905367	0.49028417	0.22
Prp19	IMAGE:583380	PRP19/PSO4 pre-mRNA processing factor 19 homolog (S. cerevisiae)	0.08591524	0.49032435	0.22
Mex3b	IMAGE:1264089	Mex3 homolog B (C. elegans)	0.15660278	0.49146432	0.22
EST	IMAGE:640228	Expressed sequence tag	0.17994799	0.49154043	0.22
EST	IMAGE:640612	Expressed sequence tag	0.18162496	0.49155793	0.22
Rgs1	IMAGE:573637	Regulator of G-protein signaling 1	0.1548263	0.49187157	0.22
C1qb	IMAGE:583277	Complement component 1, q subcomponent, beta polypeptide	0.18947515	0.49202642	0.22
EST	IMAGE:583884	Expressed sequence tag	0.091222055	0.4920771	0.22
Aars	IMAGE:640995	Alanyl-tRNA synthetase	0.16713159	0.4921125	0.22
Tagap	IMAGE:639756	T-cell activation GTPase activating protein 1	0.094393566	0.49229187	0.22
4930427A07Rik	IMAGE:1225262	RIKEN cDNA 4930427A07 gene	0.11545827	0.49275184	0.22
Btg2	IMAGE:574837	B-cell translocation gene 2, anti-proliferative	0.15162195	0.49278197	0.22
EST	IMAGE:583630	Expressed sequence tag	0.29512927	0.493871	0.17
Transcribed locus	IMAGE:639995		0.36118522	0.4940328	0.17
Sox4	IMAGE:573051	SRY-box containing gene 4	0.112269714	0.4944265	0.17
Dmtf1	IMAGE:640217	RIKEN cDNA E030031F02 gene	0.19540049	0.4944575	0.17

Nos2	IMAGE:4978648	Nitric oxide synthase 2, inducible, macrophage	0.3178935	0.49450976	0.17
Psmb9	IMAGE:641076	Proteosome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2)	0.10554914	0.49461052	0.17
E2f2	IMAGE:582911	E2F transcription factor 2	0.33447585	0.49500376	0.17
Zwint	IMAGE:1327907	ZW10 interactor	0.18769568	0.49506673	0.17
Rbm39	IMAGE:582834	RNA binding motif protein 39	0.20401609	0.49532282	0.17
EST	IMAGE:640658	Expressed sequence tag	0.14834438	0.49566662	0.17
EST	IMAGE:640388	Expressed sequence tag	0.24685533	0.49604195	0.17
Mpg	IMAGE:640382	N-methylpurine-DNA glycosylase	0.14764671	0.49756774	0.17
Tcf12	IMAGE:583795	Transcription factor 12	0.22352651	0.49792054	0.17
Jak1	IMAGE:639840	CDNA, clone:Y1G0138P08, strand:plus, reference:ENSEMBL:Mouse-Transcript-ENST:ENSMUST00000030255, based on BLAT search	0.1075994	0.49799746	0.17
Rhoa	IMAGE:572818	CDNA, clone:Y0G0104B14, strand:minus, reference:ENSEMBL:Mouse-Transcript-ENST:ENSMUST00000007959, based on BLAT search	0.021658225	0.49844038	0.17
1700021C14Rik	IMAGE:1363064	RIKEN cDNA 1700021C14 gene	0.2180898	0.498499	0.17
Cd97	IMAGE:573301	CD97 antigen	0.05658531	0.49895424	0.17
Ptgs2	IMAGE:5355607	Prostaglandin-endoperoxide synthase 2	0.18593664	0.49955586	0.17
EST	IMAGE:583344	Expressed sequence tag	0.08318424	0.4996085	0.17
EST	IMAGE:583844	Expressed sequence tag	0.24229437	0.49980402	0.17
EST	IMAGE:576500	Expressed sequence tag	0.20051917	0.49998504	0.17
EST	IMAGE:640451	Expressed sequence tag	0.38748118	0.5006749	0.17
Chd9	IMAGE:639708	Chromodomain helicase DNA binding protein 9	0.094131924	0.500939	0.17
Birc3	IMAGE:640942	Baculoviral IAP repeat-containing 3	0.31191644	0.50129527	0.17
Ly86	IMAGE:583305	Lymphocyte antigen 86	0.04890407	0.5015906	0.17
Rasgef1a	IMAGE:640917	RasGEF domain family, member 1A	0.27733576	0.5016265	0.17
Cldnd1	IMAGE:583307	Claudin domain containing 1	0.33875048	0.50193053	0.17
EST	IMAGE:583100	Expressed sequence tag	0.23808527	0.5019472	0.17

EST	IMAGE:583902	Expressed sequence tag	0.20700927	0.50209224	0.17
Lss	IMAGE:641986	Lanosterol synthase	0.076114014	0.50237995	0.17
Brd9	IMAGE:577648	Bromodomain containing 9	0.21666881	0.5027735	0.17
Rsrc2	IMAGE:640246	Arginine/serine-rich coiled-coil 2	0.3034885	0.5034631	0.17
Arl1	IMAGE:640580	ADP-ribosylation factor-like 1	0.32748058	0.5034973	0.17
2310066E14Rik	IMAGE:574836	RIKEN cDNA 2310066E14 gene	0.25583035	0.5037302	0.17
Rpl27a	IMAGE:640214	Ribosomal protein L27a	0.14723425	0.50412285	0.17
Hmox2	IMAGE:583257	Heme oxygenase (decycling) 2	0.26193693	0.50438005	0.17
EST	IMAGE:639769	PRP38 pre-mRNA processing factor 38 (yeast) domain containing B	0.12343413	0.50537276	0.12
Misc12	IMAGE:583930	MIS12 homolog (yeast)	0.29476365	0.50541264	0.12
Taf8	IMAGE:582835	TAF8 RNA polymerase II, TATA box binding protein (TBP)-associated factorq	0.21930796	0.5062212	0.12
EST	IMAGE:641099	Expressed sequence tag	0.43921557	0.5064951	0.12
EST	IMAGE:576189	Expressed sequence tag	0.17039932	0.5067498	0.12
EST	IMAGE:639732	Expressed sequence tag	0.09426703	0.5073724	0.12
EST	IMAGE:640934	Expressed sequence tag	0.15071239	0.50820905	0.12
Transcribed locus	IMAGE:639795	CDNA clone IMAGE:3493537	0.16184732	0.50837034	0.12
Pnpla8	IMAGE:639891	Patatin-like phospholipase domain containing 8	0.16206753	0.508754	0.12
EST	IMAGE:583439	Expressed sequence tag	0.34422663	0.5092995	0.12
EST	IMAGE:640213	Expressed sequence tag	0.12693521	0.51080287	0.12
Ccdc94	IMAGE:583256	Coiled-coil domain containing 94	0.23853765	0.5108386	0.12
EST	IMAGE:640525	Expressed sequence tag	0.12891454	0.51094645	0.12
IL8	IMAGE:5760258	Interleukin 8	0.1537609	0.5110281	0.12
In multiple clusters	IMAGE:640006		0.30036062	0.5112326	0.12
Transcribed locus	IMAGE:640973		0.19775125	0.511581	0.12
Med23	IMAGE:640123	Mediator complex subunit 23	0.22685882	0.51175964	0.12
Gpr108	IMAGE:583925	G protein-coupled receptor 108	0.19161469	0.51239294	0.12
EST	IMAGE:640383	Expressed sequence tag	0.16486622	0.5125301	0.12
EST	IMAGE:640797	Expressed sequence tag	0.13549757	0.51266927	0.12
Jak1	IMAGE:639840	CDNA, clone:Y1G0138P08, strand:plus,	0.09283215	0.5131256	0.12

		reference:ENSEMBL:Mouse-Transcript-ENST:ENSMUST00000030255, based on BLAT search			
Bcl6	IMAGE:573624	B-cell leukemia/lymphoma 6	0.1060376	0.51329064	0.12
IL18	IMAGE:5766881	Interleukin 18 (interferon-gamma-inducing factor)	0.15403911	0.51355904	0.12
Wdr43	IMAGE:640730	WD repeat domain 43	0.14852192	0.5140816	0.12
EST	IMAGE:577724	Expressed sequence tag	0.2544516	0.5143954	0.12
Lpin2	IMAGE:640879	Lipin 2	0.2296519	0.51505584	0.12
Dgkd	IMAGE:640601	Diacylglycerol kinase, delta	0.19706884	0.5157428	0.12
3200002M19Rik	IMAGE:640622	RIKEN cDNA 3200002M19 gene	0.14910631	0.515929	0.12
Errfi1	IMAGE:640765	ERBB receptor feedback inhibitor 1	0.129597	0.5159792	0.12
Txnrd1	IMAGE:583921	Thioredoxin reductase 1	0.12170483	0.5161739	0.12
EST	IMAGE:583519	Expressed sequence tag	0.22098556	0.5165068	0.12
Ddr1	IMAGE:575615	Discoidin domain receptor family, member 1	0.16893026	0.5171872	0.12
Hsp110	IMAGE:582878	Heat shock protein 110	0.31304258	0.5174871	0.12
EST	IMAGE:583504	Coiled-coil domain containing 98	0.17613299	0.5177578	0.12
Stat1	IMAGE:583302	Signal transducer and activator of transcription 1	0.2048296	0.51852375	0.12
EST	IMAGE:640932	Expressed sequence tag	0.10495185	0.5188277	0.12
Becn1	IMAGE:582959	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	0.3349716	0.51915944	0.12
Dnajb11	IMAGE:641016	DnaJ (Hsp40) homolog, subfamily B, member 11	0.10393543	0.51942676	0.12
Fgfr2	IMAGE:641147	Fibroblast growth factor receptor 2	0.44419205	0.5195918	0.12
EST	IMAGE:640570	Expressed sequence tag	0.3052207	0.5196621	0.12
Skap1	IMAGE:573833	Src family associated phosphoprotein 1	0.17172296	0.52073675	0.12
Tcf3	IMAGE:639972	Transcription factor 3	0.09549857	0.5209563	0.12
Insig2	IMAGE:640096	Insulin induced gene 2	0.1804777	0.52283347	0.12
EST	IMAGE:640055	Expressed sequence tag	0.37683028	0.52298284	0.12
Pdcd7	IMAGE:640487	Programmed cell death protein 7	0.40137583	0.5231999	0.12
Lpin1	IMAGE:581906	Lipin 1	0.21746156	0.52368134	0.12

2410022M11Rik	IMAGE:640727	RIKEN cDNA 2410022M11 gene	0.41250664	0.52394694	0.12
Slc39a7	IMAGE:575017	Solute carrier family 39 (zinc transporter), member 7	0.2829419	0.523961	0.12
Prkcq	IMAGE:582973	Protein kinase C, theta	0.11744608	0.52403384	0.12
EST	IMAGE:583127	Expressed sequence tag	0.33669275	0.52410597	0.12
EST	IMAGE:583426	Expressed sequence tag	0.29061174	0.524814	0.12
EST	IMAGE:583010	Expressed sequence tag	0.13933095	0.52546763	0.12
EST	IMAGE:639700	Expressed sequence tag	0.17803162	0.5256474	0.12
Hadh	IMAGE:639882	Hydroxyacyl-Coenzyme A dehydrogenase	0.20910299	0.5259876	0.12
9630055N22Rik	IMAGE:583600	RIKEN cDNA 9630055N22 gene	0.17707624	0.52682585	0.06
Rnf166	IMAGE:640507	Ring finger protein 166	0.2286626	0.5273612	0.06
Transcribed locus	IMAGE:639833	Transcribed locus	0.19372703	0.52794564	0.06
EST	IMAGE:640186	Expressed sequence tag	0.30130044	0.52802306	0.06
1110018J18Rik	IMAGE:582921	RIKEN cDNA 1110018J18 gene	0.25965518	0.52867585	0.06
EST	IMAGE:640948	Expressed sequence tag	0.18309069	0.5294974	0.06
EST	IMAGE:583549	Expressed sequence tag	0.120459504	0.52961504	0.06
EST	IMAGE:583215	Expressed sequence tag	0.15894194	0.52973616	0.06
Gfi1	IMAGE:640751	Growth factor independent 1	0.41377366	0.53016007	0.06
EST	IMAGE:640860	Expressed sequence tag	0.10475012	0.53028804	0.06
Icam2	IMAGE:574542	Intercellular adhesion molecule 2	0.23364852	0.5309473	0.06
EST	IMAGE:582865	Expressed sequence tag	0.11613484	0.53098875	0.06
EST	IMAGE:582941	Expressed sequence tag	0.18832797	0.5313243	0.06
Hgs	IMAGE:1362037	HGF-regulated tyrosine kinase substrate	0.21791725	0.5324976	0.06
Plekha1	IMAGE:640919	Pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	0.46373412	0.53276396	0.06
EST	IMAGE:640822	Expressed sequence tag	0.31127465	0.53357553	0.06
EST	IMAGE:640952	Expressed sequence tag	0.24970433	0.53416634	0.06
EST	IMAGE:640992	Expressed sequence tag	0.10382806	0.5344075	0.06
A130026P03Rik	IMAGE:639979	RIKEN cDNA A130026P03 gene	0.2255342	0.5344217	0.06
EST	IMAGE:640486	Expressed sequence tag	0.30699024	0.53462267	0.06
EST	IMAGE:576382	Expressed sequence tag	0.19993486	0.53532344	0.06

Zmat5	IMAGE:574272	Zinc finger, matrin type 5	0.21565072	0.53540295	0.06
Cacnb3	IMAGE:583865	Calcium channel, voltage-dependent, beta 3 subunit	0.19231188	0.5355396	0.06
Erh	IMAGE:583664	Enhancer of rudimentary homolog (Drosophila)	0.2407312	0.53561157	0.06
Pcgf5	IMAGE:582943	Polycomb group ring finger 5	0.21878202	0.5356839	0.06
EST	IMAGE:640855		0.22958882	0.5357983	0.06
Strap	IMAGE:583713	Serine/threonine kinase receptor associated protein	0.26475862	0.5367087	0.06
EST	IMAGE:583691	Expressed sequence tag	0.3475069	0.5367163	0.06
Ppp1r9b	IMAGE:640962	Protein phosphatase 1, regulatory subunit 9B	0.2127891	0.5371898	0.06
Vps36	IMAGE:640102	Vacuolar protein sorting 36 (yeast)	0.30279672	0.53748107	0.06
EST	IMAGE:639733	Expressed sequence tag	0.1244709	0.538895	0.06
Bag2	IMAGE:573075	Bcl2-associated athanogene 2	0.112358525	0.5394721	0.06
EST	IMAGE:583800	Expressed sequence tag	0.088619255	0.5395805	0.06
Tbcel	IMAGE:583526	Tubulin folding cofactor E-like	0.14191842	0.5398346	0.06
EST	IMAGE:640609	Expressed sequence tag	0.12813959	0.54037684	0.06
EST	IMAGE:640788	Expressed sequence tag	0.10444761	0.5406272	0.06
EST	IMAGE:583290	Expressed sequence tag	0.2055962	0.5413343	0.06
Mtdh	IMAGE:583311	Metadherin	0.15916356	0.54190296	0.06
EST	IMAGE:576502	Expressed sequence tag	0.20057273	0.542172	0.06
Rnf5	IMAGE:640281	Ring finger protein 5	0.27066663	0.5422499	0.06
EST	IMAGE:582864	Expressed sequence AU020206	0.076923795	0.5423601	0.06
D10Bwg1070e	IMAGE:583610	DNA segment, Chr 10, Brigham & Women's Genetics 1070 expressed	0.14215766	0.5430077	0.06
Cope	IMAGE:574058	Coatomer protein complex, subunit epsilon	0.13787648	0.5434906	0.06
EST	IMAGE:640794	Expressed sequence tag	0.1353031	0.54441935	0.06
Clone DB.1	IMAGE:573388	Clone DB.1 T cell receptor delta chain	0.1366657	0.54525334	0.06
Pctk1	IMAGE:640693	PCTAIRE-motif protein kinase 1	0.12938836	0.54581887	0.06
EST	IMAGE:639736	Expressed sequence tag	0.17886813	0.54591256	0.06
EST	IMAGE:583528	Expressed sequence tag	0.17618664	0.546018	0.06
Wdsub1	IMAGE:640769	WD repeat, SAM and U-box domain	0.19743752	0.5460643	0.06

		containing 1			
Kif14	IMAGE:583647	Kinesin family member 14	0.16075969	0.5461313	0.06
Btg1	IMAGE:582825	B-cell translocation gene 1, anti-proliferative	0.25934076	0.5464542	0.06
EST	IMAGE:640861	Expressed sequence tag	0.13098764	0.5467287	0.06
Coro1a	IMAGE:583431	Coronin, actin binding protein 1A	0.15943696	0.546795	0.06
EST	IMAGE:583050	Expressed sequence tag	0.20433937	0.5469681	0.06
EST	IMAGE:639775	Expressed sequence tag	0.22411872	0.5470471	0.06
Gga1	IMAGE:640935	Golgi associated, gamma adaptin ear containing, ARF binding protein 1	0.16776794	0.54727286	0.06
Grb2	IMAGE:575387	Growth factor receptor bound protein 2	0.16850777	0.54767483	0.06
EST	IMAGE:583218	Expressed sequence tag	0.20543016	0.54796785	0.06
EST	IMAGE:575820	Expressed sequence tag	0.15308909	0.54812384	0.06
EST	IMAGE:640231	Expressed sequence tag	0.22619554	0.5483948	0.06
EST	IMAGE:640794	Expressed sequence tag	0.21251145	0.5491544	0.07
EST	IMAGE:640309	Expressed sequence tag	0.12721592	0.5491956	0.07
EST	IMAGE:583007	Expressed sequence tag	0.33545718	0.5496869	0.07
Dgkd	IMAGE:583692	Diacylglycerol kinase, delta	0.090142116	0.54977095	0.07
Twf2	IMAGE:640339	Twinfilin, actin-binding protein, homolog 2 (Drosophila)	0.22727431	0.5499401	0.07
Hmgn2	IMAGE:640742	High mobility group nucleosomal binding domain 2	0.14939407	0.5500161	0.07
Acat2	IMAGE:583609	Acetyl-Coenzyme A acetyltransferase 2	0.12070159	0.5504583	0.07
Zcchc7	IMAGE:575396	Zinc finger, CCHC domain containing 7	0.13400303	0.5505079	0.07
4930523C07Rik	IMAGE:583877	RIKEN cDNA 4930523C07 gene	0.1915109	0.55073535	0.07
EST	IMAGE:640816		0.18351197	0.55085975	0.07
Map2k1ip1	IMAGE:575958	Mitogen-activated protein kinase kinase 1 interacting protein 1	0.11349201	0.5510415	0.07
Cebpg	IMAGE:640947	CCAAT/enhancer binding protein (C/EBP), gamma	0.1670212	0.5510602	0.07
Bnip2	IMAGE:640945	BCL2/adenovirus E1B interacting protein 1, NIP2	0.13014787	0.5517748	0.07
EST	IMAGE:640922	Expressed sequence tag	0.14985436	0.5521537	0.07

EST	IMAGE:582894	Expressed sequence tag	0.20331979	0.55236584	0.07
Ttc3	IMAGE:583646	Tetratricopeptide repeat domain 3	0.1431372	0.5533183	0.07
Cops2	IMAGE:583870	COP9 (constitutive photomorphogenic) homolog, subunit 2 (Arabidopsis thaliana)	0.2963936	0.5548976	0.07
Mapk9	IMAGE:582919	Mitogen activated protein kinase 9	0.21872519	0.55608267	0.07
EST	IMAGE:639909	Expressed sequence tag	0.26919284	0.5569017	0.07
EST	IMAGE:641107	Expressed sequence tag	0.23082368	0.55736643	0.07
EST	IMAGE:583034	Expressed sequence tag	0.13939174	0.5574827	0.07
C230085N15Rik	IMAGE:639894	RIKEN cDNA C230085N15 gene	0.20834012	0.55755836	0.07
Rabl4	IMAGE:639631	RAB, member of RAS oncogene family-like 4	0.22394477	0.5576526	0.07
Nol7	IMAGE:640296	Nucleolar protein 7	0.09718718	0.558025	0.07
Nfe2l1	IMAGE:640629	Nuclear factor, erythroid derived 2,-like 1	0.27493533	0.5585609	0.07
EST	IMAGE:640359	Expressed sequence tag	0.16481401	0.5587113	0.07
Hipk1	IMAGE:583858	Homeodomain interacting protein kinase 1	0.29437843	0.55886424	0.07
EST	IMAGE:639849	Expressed sequence tag	0.267597	0.5589586	0.07
Stambpl1	IMAGE:640611	Stam binding protein like 1	0.16541195	0.55895954	0.07
Tcea3	IMAGE:573147	Transcription elongation factor A (SII), 3	0.112612486	0.5604485	0.07
Nol5a	IMAGE:640557	Nucleolar protein 5A	0.27465722	0.5611505	0.07
EST	IMAGE:583399	Expressed sequence tag	0.22069606	0.56347096	0.07
Il4	IMAGE:640300	Interleukin 4	0.18010898	0.563516	0.07
Dusp1	IMAGE:583905	Dual specificity phosphatase 1	0.26543644	0.56353015	0.07
EST	IMAGE:583052	Expressed sequence tag	0.23801969	0.5637954	0.07
Mxd4	IMAGE:583632	Max dimerization protein 4	0.087568805	0.56434244	0.07
Gpr114	IMAGE:583744	G protein-coupled receptor 114	0.17740531	0.56446487	0.07
Nap1l1	IMAGE:583645	Nucleosome assembly protein 1-like 1	0.12200394	0.5646603	0.07
EST	IMAGE:576020	Expressed sequence tag	0.16971181	0.5651494	0.07
Prkrir	IMAGE:640886	Protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of (P58 repressor)	0.15059586	0.5652854	0.07
5830436I19Rik	IMAGE:583418	RIKEN cDNA 5830436I19 gene	0.14067939	0.5653354	0.07
Ebi3	IMAGE:1265208	Epstein-Barr virus induced gene 3	0.1728854	0.5657158	0.07
Parp3	IMAGE:640154	Poly (ADP-ribose) polymerase family,	0.14617608	0.56579113	0.07

		member 3			
In multiple clusters	IMAGE:639744		0.09229713	0.5661505	0.07
Gnb4	IMAGE:639662	Guanine nucleotide binding protein, beta 4	0.1449375	0.56653166	0.07
E2f2	IMAGE:640754	E2F transcription factor 2	0.14857896	0.5666933	0.07
D10Wsu52e	IMAGE:1265174	DNA segment, Chr 10, Wayne State University 52, expressed	0.1728301	0.56701624	0.07
Ccl3	IMAGE:1330016	Chemokine (C-C motif) ligand 3	0.20261806	0.56718755	0.07
Adck4	IMAGE:583043	AarF domain containing kinase 4	0.33281875	0.5671911	0.07
Sar1b	IMAGE:640897	SAR1 gene homolog B (S. cerevisiae)	0.13000837	0.5674497	0.07
Mapk8	IMAGE:575689	Mitogen activated protein kinase 8	0.07236491	0.567604	0.07
EST	IMAGE:641013	Expressed sequence tag	0.27772212	0.5678571	0
Neu1	IMAGE:583136	Neuraminidase 1	0.2373021	0.5678653	0
Ppp3cb	IMAGE:583231	Protein phosphatase 3, catalytic subunit, beta isoform	0.22028783	0.56876785	0
EST	IMAGE:640823		0.44978333	0.56938916	0
Ppfia1	IMAGE:640478	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein, alpha 1	0.14881063	0.56975424	0
EST	IMAGE:640635	Expressed sequence tag	0.16547036	0.5710804	0
EST	IMAGE:583470	Expressed sequence tag	0.20516384	0.57154936	0
EST	IMAGE:640422	Expressed sequence tag	0.2107416	0.57185537	0
Apitd1	IMAGE:1225768	Apoptosis-inducing, TAF9-like domain 1	0.13806783	0.57199794	0
EST	IMAGE:640779	Expressed sequence tag	0.16664648	0.57216036	0
Megf9	IMAGE:577718	Multiple EGF-like-domains 9	0.2836924	0.57236755	0
Fmn13	IMAGE:573632	Formin-like 3	0.15477121	0.5725871	0
EST	IMAGE:640050		0.21002796	0.57260156	0
FASLG	IMAGE:5178596	Fas ligand (TNF superfamily, member 6)	0.15426768	0.57383657	0
R3hdm	IMAGE:640077	R3H domain 1 (binds single-stranded nucleic acids)	0.27131394	0.57387805	0
Zdhhc20	IMAGE:640059	Zinc finger, DHHC domain containing 20	0.16330983	0.57393575	0
Ythdf1	IMAGE:573974	YTH domain family 1	0.1866697	0.5748813	0
Mbp	IMAGE:639805	Myelin basic protein	0.1246879	0.57503706	0

Akap11	IMAGE:640000	A kinase (PRKA) anchor protein 11	0.1794353	0.5753789	0
Rad9	IMAGE:583606	RAD9 homolog (S. pombe)	0.2950098	0.57556844	0
Tmed2	IMAGE:639673	Transmembrane emp24 domain trafficking protein 2	0.12313386	0.57559407	0
Ppp1r14b	IMAGE:639973	Protein phosphatase 1, regulatory (inhibitor) subunit 14B	0.1250631	0.5758613	0
EST	IMAGE:640543		0.2278111	0.57607156	0
Ncf4	IMAGE:576061	Neutrophil cytosolic factor 4	0.18445186	0.57679147	0
Cops5	IMAGE:573699	COP9 (constitutive photomorphogenic) homolog, subunit 5 (Arabidopsis thaliana)	0.17130366	0.5770635	0
Myb	IMAGE:575702	Myeloblastosis oncogene	0.18419547	0.57722646	0
Elavl1	IMAGE:583934	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	0.14385645	0.57739806	0
AI451557	IMAGE:583669	Expressed sequence AI451557	0.12208138	0.57757556	0
2700049A03Rik	IMAGE:640874	RIKEN cDNA 2700049A03 gene	0.14973748	0.57807255	0
Dlst	IMAGE:583304	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	0.23867688	0.57818896	0
1700106N22Rik	IMAGE:640240	RIKEN cDNA 1700106N22 gene	0.18079656	0.5786328	0
Lims1	IMAGE:583635	LIM and senescent cell antigen-like domains 1	0.15993533	0.5801778	0
Transcribed locus	IMAGE:583689		0.26467103	0.58060867	0
EST	IMAGE:641125	Expressed sequence tag	0.13172472	0.5810095	0
Atp6ap1	IMAGE:583065	ATPase, H ⁺ transporting, lysosomal accessory protein 1	0.2601336	0.5810896	0
Maea	IMAGE:583828	Macrophage erythroblast attacher	0.17680933	0.5817139	0
Bbs5	IMAGE:640968	Bardet-Biedl syndrome 5 (human)	0.10373003	0.58217555	0
EST	IMAGE:640843	Expressed sequence tag	0.23026723	0.58259135	0
0610007P14Rik	IMAGE:583161	RIKEN cDNA 0610007P14 gene	0.26045766	0.5831315	0
EST	IMAGE:576194	Expressed sequence tag	0.18470694	0.58368045	0
Rabgap1	IMAGE:641029	RAB GTPase activating protein 1	0.1314601	0.5841514	0
EST	IMAGE:640275	Expressed sequence tag	0.16379203	0.5845397	0
Lrpap1	IMAGE:639699	Low density lipoprotein receptor-related protein associated protein 1	0.16163208	0.58459306	0

EST	IMAGE:639632	Expressed sequence tag	0.24262908	0.58497113	0
Foxo1	IMAGE:574862	Forkhead box O1	0.1517315	0.5855997	0
Cntrob	IMAGE:583719	Centrobins, centrosomal BRCA2 interacting protein	0.16092543	0.58563435	0
Eif4a1	IMAGE:640577	Eukaryotic translation initiation factor 4A1	0.19701757	0.5862689	0
Xcl1	IMAGE:576815	Chemokine (C motif) ligand 1	0.17224295	0.5865847	0
Prkar2b	IMAGE:640704	Protein kinase, cAMP dependent regulatory, type II beta	0.10089621	0.5868613	0
Nsd1	IMAGE:641011	Nuclear receptor-binding SET-domain protein 1	0.23069587	0.5871623	0
Stip1	IMAGE:583886	Stress-induced phosphoprotein 1	0.05564601	0.58742905	0
Transcribed locus	IMAGE:3574183		0.17114542	0.5886168	0
Park7	IMAGE:640406	Parkinson disease (autosomal recessive, early onset) 7	0.14863753	0.5886708	0
Transcribed locus	IMAGE:583162		0.28804773	0.5889592	0
4933415E08Rik	IMAGE:640993	RIKEN cDNA 4933415E08 gene	0.13028908	0.58942217	0
EST	IMAGE:582857	Expressed sequence tag	0.18874207	0.5898468	0
EST	IMAGE:639876	Expressed sequence tag	0.09500626	0.5905874	0
EST	IMAGE:640837	Expressed sequence tag	0.13092123	0.59077686	0
A130075G07	IMAGE:583493	16 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A130075G07 product:unclassifiable, full insert sequence	0.1899414	0.59106964	0
Gabarapl2	IMAGE:583190	Gamma-aminobutyric acid (GABA-A) receptor-associated protein-like 2	0.14105347	0.5911539	0
EST	IMAGE:640377	Expressed sequence tag	0.27104014	0.5916175	0
H2-Q2	IMAGE:573667	Histocompatibility 2, K1, K region	0.1550985	0.5917366	0
EST	IMAGE:583557	Expressed sequence tag	0.26416802	0.59210694	0
Lck	IMAGE:577700	Lymphocyte protein tyrosine kinase	0.2542196	0.5926691	0
Wdr61	IMAGE:640880	WD repeat domain 61	0.24948633	0.5928021	0
Map3k7ip1	IMAGE:639878	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	0.10769057	0.5934566	0
Serpnb2	IMAGE:573970	Serine (or cysteine) peptidase inhibitor, clade	0.18661867	0.5945947	0

		B, member 2			
EST	IMAGE:583321	Expressed sequence tag	0.11849164	0.59506106	0
EST	IMAGE:583490	Expressed sequence tag	0.1408627	0.59517026	0
Cerk	IMAGE:583388	Ceramide kinase	0.23994374	0.596567	0
Xrcc4	IMAGE:574242	X-ray repair complementing defective repair in Chinese hamster cells 4	0.13199781	0.59714526	0
Trim8	IMAGE:573726	Tripartite motif protein 8	0.17135528	0.5982586	0
EST	IMAGE:640825	Expressed sequence tag	0.12980765	0.59830797	0
E430004N04Rik	IMAGE:576548	RIKEN cDNA E430004N04 gene	0.21446314	0.5987843	0
Rad21	IMAGE:583531	RAD21 homolog (S. pombe)	0.22192219	0.5991422	0
EST	IMAGE:640193	Expressed sequence tag	0.19534622	0.59980005	0
EST	IMAGE:639875	Expressed sequence tag	0.3592195	0.60040456	0
Ssbp2	IMAGE:641018	Single-stranded DNA binding protein 2	0.15007697	0.6031685	0
Klf6	IMAGE:639648	Kruppel-like factor 6	0.09176337	0.60394967	0
Sh3yl1	IMAGE:641043	Sh3 domain YSC-like 1	0.16724242	0.60536087	0
Tmpo	IMAGE:582754	Thymopoietin	0.0674344	0.60545945	0
A630042L21Rik	IMAGE:583599	RIKEN cDNA A630042L21 gene	0.16064806	0.60598564	0
Dtnb	IMAGE:583405	Dystrobrevin, beta	0.1200001	0.6062134	0
EST	IMAGE:582824	Expressed sequence tag	0.2365286	0.6068175	0
Transcribed locus	IMAGE:576524	CDNA clone IMAGE:30293206	0.20062548	0.60701203	0
Slk	IMAGE:583214	STE20-like kinase (yeast)	0.14111699	0.6076623	0
EST	IMAGE:640621	Expressed sequence tag	0.129189	0.6079374	0
Trp53inp2	IMAGE:640719	Transformation related protein 53 inducible nuclear protein 2	0.16648273	0.6086512	0
Psm8	IMAGE:582981	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	0.26102397	0.6098178	0
Pank3	IMAGE:641037	3 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A630012F14 product:unclassifiable, full insert sequence	0.27782476	0.61046827	0
4921505C17Rik	IMAGE:576048	RIKEN cDNA 4921505C17 gene	0.11384423	0.6109068	0
EST	IMAGE:641113	Expressed sequence tag	0.130638	0.61104083	0
EST	IMAGE:576644	Expressed sequence tag	0.21525626	0.6110576	0

Ankrd10	IMAGE:583740	Ankyrin repeat domain 10	0.09041374	0.6112935	0
EST	IMAGE:640351	Expressed sequence tag	0.22649825	0.6113829	0
EST	IMAGE:639762	Expressed sequence tag	0.20888554	0.6116807	0
EST	IMAGE:640105		0.12549669	0.6118786	0
Ogt	IMAGE:583678	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	0.29538405	0.61315095	0
EST	IMAGE:583240	Expressed sequence tag	0.17556734	0.61382604	0
EST	IMAGE:640027	Expressed sequence tag	0.22661947	0.6140328	0
Lef1	IMAGE:575374	Lymphoid enhancer binding factor 1	0.1101145	0.6141012	0
2810442I21Rik	IMAGE:1226803	RIKEN cDNA 2810442I21 gene	0.13863099	0.61464864	0
EST	IMAGE:583529	Expressed sequence tag	0.19082807	0.615881	0
5730508B09Rik	IMAGE:639881	RIKEN cDNA 5730508B09 gene	0.19383171	0.61591375	0
EST	IMAGE:640341	Expressed sequence tag	0.27233028	0.61604214	0
Tmie	IMAGE:640505	Transmembrane inner ear	0.19686215	0.6163534	0
Prkag1	IMAGE:640537	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	0.12792921	0.616834	0
Got1	IMAGE:640640	Glutamate oxaloacetate transaminase 1, soluble	0.24763133	0.6174141	0
Suv39h1	IMAGE:640238	Suppressor of variegation 3-9 homolog 1 (Drosophila)	0.1472913	0.61868167	0
Srpk2	IMAGE:640659	Serine/arginine-rich protein specific kinase 2	0.16552153	0.61934835	0
5930434B04Rik	IMAGE:583644	RIKEN cDNA 5930434B04 gene	0.08988068	0.6212908	0
Selenoprotein 15	IMAGE:582910	Selenoprotein	0.2886248	0.62209004	0
TNF	IMAGE:5213134	Tumor necrosis factor (TNF superfamily, member 2)	0.17071606	0.622866	0
Fancc	IMAGE:582993	Fanconi anemia, complementation group C	0.25989532	0.6244712	0
Tusc4	IMAGE:640378	Tumor suppressor candidate 4	0.3023817	0.62480545	0
Xpot	IMAGE:639640	Exportin, tRNA (nuclear export receptor for tRNAs)	0.17870922	0.62504584	0
Rbm22	IMAGE:583263	RNA binding motif protein 22	0.15905473	0.62546045	0
Sox4	IMAGE:640239	SRY-box containing gene 4	0.16454339	0.6260612	0

Abce1	IMAGE:583670	ATP-binding cassette, sub-family E (OABP), member 1	0.1431964	0.62674534	0
Jakmip1	IMAGE:583827	Janus kinase and microtubule interacting protein 1	0.16031556	0.62733376	0
Jak3	IMAGE:576670	Janus kinase 3	0.1991874	0.6275021	0
EST	IMAGE:583292	Expressed sequence tag	0.23967974	0.62786347	0
EST	IMAGE:582961	Expressed sequence tag	0.11645908	0.62795234	0
Transcribed locus	IMAGE:577462	Transcribed locus, weakly similar to XP_001061311.1 hypothetical protein [Rattus norvegicus]	0.23263651	0.6281541	0
Rbm4	IMAGE:640067	RNA binding motif protein 4	0.36906374	0.62835467	0
EST	IMAGE:583267	Expressed sequence tag	0.22127491	0.6285686	0
NKRF	IMAGE:21961	NF-kappaB repressing factor	0.15229517	0.6287052	0
EST	IMAGE:641085	Expressed sequence tag	0.2780205	0.6290783	0
Spnb2	IMAGE:582801	Spectrin beta 2	0.25926423	0.62924385	0
EST	IMAGE:583063	Expressed sequence tag	0.2190707	0.6299588	0
Anapc5	IMAGE:582154	Anaphase-promoting complex subunit 5	0.28156555	0.63081324	0
G3bp1	IMAGE:640974	Ras-GTPase-activating protein SH3-domain binding protein 1	0.13380834	0.6308355	0
Ncaph2	IMAGE:583463	Non-SMC condensin II complex, subunit H2	0.34451017	0.63102627	0
C330043M08Rik	IMAGE:583132	RIKEN cDNA C330043M08 gene	0.17409138	0.6310858	0
Nrip1	IMAGE:582841	Nuclear receptor interacting protein 1	0.11604868	0.6318484	0
EST	IMAGE:640791	Expressed sequence tag	0.13510104	0.6320719	0
EST	IMAGE:640305	Expressed sequence tag	0.27075502	0.6327355	0
EST	IMAGE:583864	Expressed sequence tag	0.17766881	0.63287437	0
Rpl26	IMAGE:577223	Ribosomal protein L26	0.20185018	0.6333289	0
Eif1b	IMAGE:639988	Eukaryotic translation initiation factor 1B	0.17865722	0.6335299	0
AK129128	IMAGE:583761	CDNA sequence AK129128	0.2649242	0.6337239	0
Nab2	IMAGE:640749	Ngfi-A binding protein 2	0.2753119	0.6338504	0
B3gnt1	IMAGE:574207	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2	0.21542658	0.63397986	0
Cxcr7	IMAGE:1263525	Chemokine (C-X-C motif) receptor 7	0.1564405	0.6341011	0

EST	IMAGE:641035	Expressed sequence tag	0.23075894	0.63505423	0
EST	IMAGE:640173	Expressed sequence tag	0.2716774	0.63578355	0
Akt1	IMAGE:575486	Thymoma viral proto-oncogene 1	0.16861643	0.6369869	0
EST	IMAGE:640562	Expressed sequence tag	0.14811382	0.6371415	0
Slc3a2	IMAGE:582928	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	0.17424825	0.6373064	0
EST	IMAGE:639713	Expressed sequence tag	0.19346057	0.63736546	0
Tgm4	IMAGE:640389	Transglutaminase 4 (prostate)	0.2725148	0.6382008	0
Tlk1	IMAGE:640295	Golgi reassembly stacking protein 2	0.3827594	0.63850456	0
Atp6v0e	IMAGE:583287	ATPase, H ⁺ transporting, lysosomal V0 subunit E	0.1591082	0.6394574	0
Rmnd5a	IMAGE:582813	Required for meiotic nuclear division 5 homolog A (<i>S. cerevisiae</i>)	0.2605371	0.6400216	0
Slc22a7	IMAGE:574190	Solute carrier family 22 (organic anion transporter), member 7	0.21537092	0.64038783	0
Tbce	IMAGE:582730	Tubulin-specific chaperone e	0.066864535	0.6423231	0
Hrb1	IMAGE:573414	HIV-1 Rev binding protein-like	0.13673037	0.6430164	0
Ggcx	IMAGE:582706	Gamma-glutamyl carboxylase	0.06628617	0.64316064	0
Dock8	IMAGE:639971	Dedicator of cytokinesis 8	0.36079308	0.6432137	0
Sit	IMAGE:576098	Suppression inducing transmembrane adaptor 1	0.18460535	0.64334905	0
EST	IMAGE:582956	Expressed sequence tag	0.23776491	0.644441	0
EST	IMAGE:641121	Expressed sequence tag	0.2768426	0.6453938	0
Fbxo46	IMAGE:1330038	F-box protein 46	0.2026733	0.6454447	0
EST	IMAGE:640630	Expressed sequence tag	0.30772632	0.6462859	0
Seh1l	IMAGE:583520	SEH1-like (<i>S. cerevisiae</i>)	0.23928645	0.6464102	0
EST	IMAGE:583668	Expressed sequence tag	0.09001157	0.64697737	0
Alg9	IMAGE:639947	Asparagine-linked glycosylation 9 homolog (yeast, alpha 1,2 mannosyltransferase)	0.36041862	0.6470013	0
FASLG	IMAGE:5178596	Fas ligand (TNF superfamily, member 6)	0.17103939	0.6473672	0
EST	IMAGE:639794	Expressed sequence tag	0.1443428	0.6474242	0
EST	IMAGE:583220	Expressed sequence tag	0.23947747	0.6474561	0

Ctla4	IMAGE:575713	Cytotoxic T-lymphocyte-associated protein 4	0.072606966	0.64802665	0
Transcribed locus	IMAGE:640371		0.16400498	0.64805055	0
Slamf7	IMAGE:1263387	SLAM family member 7	0.15627268	0.64808863	0
Frag1	IMAGE:639841	FGF receptor activating protein 1	0.12365905	0.64864206	0
ENSMUSG00000073044	IMAGE:640716	Predicted gene, ENSMUSG00000073044	0.1026207	0.649474	0
EST	IMAGE:582948	Expressed sequence tag	0.080558665	0.6498827	0
EST	IMAGE:640847	Expressed sequence tag	0.4528077	0.65050757	0
Rnpep	IMAGE:582632	Arginyl aminopeptidase (aminopeptidase B)	0.06509324	0.65082055	0
2310004I24Rik	IMAGE:583374	RIKEN cDNA 2310004I24 gene	0.20494172	0.65160793	0
Bcl7a	IMAGE:582794	B-cell CLL/lymphoma 7A	0.138885	0.6517982	0
Jak2	IMAGE:5068946	Janus kinase 2	0.31558356	0.6518644	0
Stau1	IMAGE:583155	Staufen (RNA binding protein) homolog 1 (Drosophila)	0.1575544	0.6519792	0
Rab27a	IMAGE:640750	RAB27A, member RAS oncogene family	0.3084801	0.6524004	0
EST	IMAGE:639737	Expressed sequence tag	0.19351713	0.6525292	0
Transcribed locus	IMAGE:583603		0.22304592	0.6539815	0
EST	IMAGE:639711	Expressed sequence tag	0.16249497	0.65516764	0
Trmt5	IMAGE:583471	TRM5 tRNA methyltransferase 5 homolog (S. cerevisiae)	0.22087015	0.65747476	0
EST	IMAGE:583432	Expressed sequence tag	0.17597587	0.65784013	0
EST	IMAGE:640225	Expressed sequence tag	0.12585647	0.6582008	0
In multiple clusters	IMAGE:641343		0.07439952	0.6583419	0
Smardc2	IMAGE:582859	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2	0.21936552	0.65860844	0
EST	IMAGE:640518	Expressed sequence tag	0.21085075	0.6588071	0
Cldn10	IMAGE:583554	Claudin 10	0.2061936	0.6598668	0
EST	IMAGE:576428	Diacylglycerol kinase, gamma	0.20009434	0.6601858	0
EG544864	IMAGE:577654	Predicted gene, EG544864	0.2540683	0.66027	0
Dync2li1	IMAGE:639709	Dynein cytoplasmic 2 light intermediate chain 1	0.12439899	0.660859	0

Vcam1	IMAGE:576563	Vascular cell adhesion molecule 1	0.21457873	0.6613954	0
EST	IMAGE:640656	Expressed sequence tag	0.10067886	0.6622571	0
Adss	IMAGE:640646	Adenylosuccinate synthetase, non muscle	0.14916323	0.66248	0
Ptgs1	IMAGE:5041952	Prostaglandin-endoperoxide synthase 1	0.28086063	0.6636433	0
Iqcb1	IMAGE:640287	IQ calmodulin-binding motif containing 1	0.16465275	0.66415644	0
Rap2a	IMAGE:583613	RAS related protein 2a	0.1909839	0.6652152	0
Irf8	IMAGE:640774	Interferon regulatory factor 8	0.30863506	0.6663607	0
Nfkb1	IMAGE:575033	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	0.28305134	0.66741085	0
Rab11a	IMAGE:574089	RAB11a, member RAS oncogene family	0.059888612	0.6679615	0
EST	IMAGE:640015	Expressed sequence tag	0.22565359	0.66812897	0
2900076A07Rik	IMAGE:583769	RIKEN cDNA 2900076A07 gene	0.19209681	0.6698119	0
Sh3glb1	IMAGE:577165	SH3-domain GRB2-like B1 (endophilin)	0.21373369	0.67040926	0
EST	IMAGE:583516	Expressed sequence tag	0.17535445	0.6709999	0
Pdha1	IMAGE:583233	Pyruvate dehydrogenase E1 alpha 1	0.26185915	0.6717668	0
Flt3	IMAGE:576267	FMS-like tyrosine kinase 3	0.13730833	0.6721882	0
Hsd17b4	IMAGE:574568	Cd63 antigen	0.2337761	0.67238903	0
Gbp4	IMAGE:639885	Guanylate nucleotide binding protein 4	0.2690983	0.67246294	0
Cd97	IMAGE:573301	CD97 antigen	0.13640434	0.6727853	0
Rpp30	IMAGE:638902	Ribonuclease P/MRP 30 subunit (human)	0.25808272	0.6730167	0
Transcribed locus	IMAGE:583508		0.24026999	0.67327994	0
Asxl2	IMAGE:1281412	Additional sex combs like 2 (Drosophila)	0.1732522	0.67330325	0
EST	IMAGE:640417	Expressed sequence tag	0.12757416	0.673491	0
Tollip	IMAGE:4951445	Toll interacting protein	0.317535	0.6737925	0
Psm5	IMAGE:639989	Proteasome (prosome, macropain) subunit, alpha type 5	0.19325256	0.6740738	0
EST	IMAGE:639763	Expressed sequence tag	0.22499973	0.6743433	0
C130036K02	IMAGE:583575	Transcribed locus	0.16059211	0.6743841	0
Slc3a2	IMAGE:583139	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	0.33351967	0.67508745	0
AI481772	IMAGE:583909	Expressed sequence AI481772	0.25776374	0.67527413	0
Mthfr	IMAGE:640110	5,10-methylenetetrahydrofolate reductase	0.20943232	0.67529535	0

EST	IMAGE:640956	Expressed sequence tag	0.1050524	0.6754842	0
EST	IMAGE:640839	Expressed sequence tag	0.16755901	0.6756553	0
C230096C10Rik	IMAGE:583779	RIKEN cDNA C230096C10 gene	0.1602062	0.6758635	0
Obfc2a	IMAGE:582845	Oligonucleotide/oligosaccharide-binding fold containing 2A	0.18811317	0.67758733	0
Snapc3	IMAGE:583409	Small nuclear RNA activating complex, polypeptide 3	0.19056232	0.6776366	0
EST	IMAGE:640391	Expressed sequence tag	0.38533962	0.67856294	0
Dusp2	IMAGE:640913	Dual specificity phosphatase 2	0.1983864	0.6785886	0
EST	IMAGE:582346	Expressed sequence tag	0.31897455	0.6791632	0
Transcribed locus	IMAGE:641142	Transcribed locus	0.21245591	0.6792396	0
3110037I16Rik	IMAGE:640889	RIKEN cDNA 3110037I16 gene	0.19833039	0.6795188	0
EST	IMAGE:583288	Expressed sequence tag	0.17566766	0.68066347	0
EST	IMAGE:640666	Expressed sequence tag	0.30580163	0.6809548	0
D4Wsu53e	IMAGE:583358	DNA segment, Chr 4, Wayne State University 53, expressed	0.14148726	0.6811138	0
Cct6a	IMAGE:582061	Chaperonin subunit 6a (zeta)	0.2175738	0.68172115	0
In multiple clusters	IMAGE:640134		0.20948781	0.6818743	0
EST	IMAGE:577604	Expressed sequence tag	0.25376618	0.68210185	0
Gtl3	IMAGE:640245	Gene trap locus 3	0.27194953	0.6831312	0
EST	IMAGE:640385	Expressed sequence tag	0.19575894	0.68394053	0
EST	IMAGE:583310	Expressed sequence tag	0.14136209	0.6840975	0
1600020E01Rik	IMAGE:640044	RIKEN cDNA 1600020E01 gene	0.097787276	0.6842015	0
Gpatc2	IMAGE:639834	G patch domain containing 2	0.20904918	0.68440264	0
EST	IMAGE:641090	Expressed sequence tag	0.1501903	0.68510705	0
EST	IMAGE:639969	Expressed sequence tag	0.2680322	0.68578446	0
Uqcrcq	IMAGE:640529	Ubiquinol-cytochrome c reductase, complex III subunit VII	0.1969121	0.68606955	0
Fbxl11	IMAGE:639916	F-box and leucine-rich repeat protein 11	0.17850156	0.68700635	0
Yeats4	IMAGE:582836	YEATS domain containing 4	0.23749758	0.6873498	0
Cd97	IMAGE:641135	CD97 antigen	0.536733	0.68741673	0
Tulp3	IMAGE:583226	Tubby-like protein 3	0.14019334	0.687433	0

Tegt	IMAGE:639877	Testis enhanced gene transcript	0.124761075	0.6875081	0
Fancc	IMAGE:583016	Fanconi anemia, complementation group C	0.23704857	0.6888247	0
Zwilch	IMAGE:639781	Zwilch, kinetochore associated, homolog (Drosophila)	0.12461598	0.6890666	0
Nck2	IMAGE:640608	Non-catalytic region of tyrosine kinase adaptor protein 2	0.10045737	0.6894222	0
Cops3	IMAGE:583648	COP9 (constitutive photomorphogenic) homolog, subunit 3 (Arabidopsis thaliana)	0.17718685	0.68978983	0
EST	IMAGE:641100	Expressed sequence tag	0.10564747	0.6903736	0
Jmjd2b	IMAGE:583085	Jumonji domain containing 2B	0.1885369	0.69182485	0
Psip1	IMAGE:583537	PC4 and SFRS1 interacting protein 1	0.119220965	0.6920932	0
Il18rap	IMAGE:640615	Interleukin 18 receptor accessory protein	0.227993	0.69217515	0
Ccdc41	IMAGE:639871	Coiled-coil domain containing 41	0.22435208	0.69284207	0
Hs3st3b1	IMAGE:640008	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	0.09574361	0.6930942	0
EST	IMAGE:639724	Expressed sequence tag	0.17808397	0.6942962	0
EST	IMAGE:583598	Expressed sequence tag	0.143015	0.6944292	0
Xpot	IMAGE:639806	Exportin, tRNA (nuclear export receptor for tRNAs)	0.14529587	0.69512993	0
EST	IMAGE:583051	Expressed sequence tag	0.21982904	0.6955135	0
EST	IMAGE:583788	Expressed sequence tag	0.09068557	0.69597137	0
Cybb	IMAGE:583187	Cytochrome b-245, beta polypeptide	0.31505698	0.69895315	0
Hltf	IMAGE:640694	Helicase-like transcription factor	0.14927734	0.69905347	0
Cd164	IMAGE:640054	CD164 antigen	0.30266228	0.6998011	0
Ndufa11	IMAGE:640357	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11	0.12735748	0.7014962	0
Zzz3	IMAGE:583815	Zinc finger, ZZ domain containing 3	0.16114314	0.702413	0
Anxa1	IMAGE:640741	Annexin A1	0.12952639	0.70246565	0
EST	IMAGE:640588	Expressed sequence tag	0.18157241	0.70268375	0
EST	IMAGE:640782	Expressed sequence tag	0.11102761	0.7033643	0
EST	IMAGE:582849	Expressed sequence tag	0.25942096	0.705071	0
Cd4	IMAGE:640273	CD4 antigen	0.12600295	0.70600426	0

Pcbp1	IMAGE:640145	Poly(rC) binding protein 1	0.1952406	0.70607436	0
B2m	IMAGE:576472	Beta-2 microglobulin	0.20036045	0.7065583	0
Abca5	IMAGE:582867	ATP-binding cassette, sub-family A (ABC1), member 5	0.1568846	0.7071335	0
Def6	IMAGE:573651	Differentially expressed in FDCP 6	0.15498956	0.70748454	0
EST	IMAGE:583193	Expressed sequence tag	0.1901005	0.7103242	0
Wdr12	IMAGE:640744	WD repeat domain 12	0.18261509	0.71045685	0
Col27a1	IMAGE:1378843	Procollagen, type XXVII, alpha 1	0.23594911	0.7116088	0
Glr5	IMAGE:640672	Glutaredoxin 5 homolog (<i>S. cerevisiae</i>)	0.18245952	0.7133583	0
Tap1	IMAGE:576775	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	0.1992924	0.7138117	0
C130022K22Rik	IMAGE:583889	RIKEN cDNA C130022K22 gene	0.19236284	0.71382004	0
Nol7	IMAGE:641133	Nucleolar protein 7	0.27821597	0.71395254	0
Elp4	IMAGE:583768	Elongation protein 4 homolog (<i>S. cerevisiae</i>)	0.17745937	0.7140481	0
Msn	IMAGE:583860	Moesin	0.09109095	0.7153999	0
Socs1	IMAGE:583890	Suppressor of cytokine signaling 1	0.20773016	0.71598846	0
Rbms1	IMAGE:583875	RNA binding motif, single stranded interacting protein 1	0.16042644	0.7162843	0
EST	IMAGE:639785	Expressed sequence tag	0.19362487	0.71663874	0
CCR7	IMAGE:5180758	Chemokine (C-C motif) receptor 7	0.15438387	0.7166572	0
Anxa3	IMAGE:573265	Annexin A3	0.13620813	0.71678513	0
Rpl24	IMAGE:641145	Ribosomal protein L24	0.2769403	0.7171816	0
2610207I05Rik	IMAGE:640606	RIKEN cDNA 2610207I05 gene	0.3075754	0.7184207	0
Atf4	IMAGE:583297	Activating transcription factor 4	0.118415415	0.7193015	0
Cep78	IMAGE:641590	Centrosomal protein 78	0.07483943	0.719304	0
Rpl27a	IMAGE:640775	Ribosomal protein L27a	0.41503644	0.7194412	0
EST	IMAGE:583874	Expressed sequence tag	0.14276372	0.72275186	0
Adfp	IMAGE:640464	Adipose differentiation related protein	0.09979873	0.7235365	0
Zfp35	IMAGE:641159	Zinc finger protein 35	0.580739	0.7240021	0
Brpf1	IMAGE:640234	Bromodomain and PHD finger containing, 1	0.30156377	0.72466093	0
EST	IMAGE:583910	Expressed sequence tag	0.14379641	0.7260223	0
LOC625360	IMAGE:640316	Similar to 2-cell-stage, variable group, member	0.24664533	0.72602725	0

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Ehmt2	IMAGE:582419	Euchromatic histone lysine N-methyltransferase 2	0.25264516	0.7278524	0
EST	IMAGE:640367	Expressed sequence tag	0.38464978	0.7281571	0
EST	IMAGE:640445	Expressed sequence tag	0.19591807	0.7283839	0
Tcrg-V4	IMAGE:640763	T-cell receptor gamma, variable 4	0.39765054	0.7284448	0
EST	IMAGE:582626	Expressed sequence tag	0.06477621	0.729255	0
EST	IMAGE:583291	Expressed sequence tag	0.2213351	0.73048735	0
EST	IMAGE:575704	Expressed sequence tag	0.13471045	0.73086864	0
2310028N02Rik	IMAGE:583941	RIKEN cDNA 2310028N02 gene	0.26681283	0.73115087	0
EST	IMAGE:640645	Expressed sequence tag	0.12925385	0.731651	0
Ly6a	IMAGE:574422	Lymphocyte antigen 6 complex, locus A	0.21610725	0.7323237	0
Ier3ip1	IMAGE:583721	Immediate early response 3 interacting protein 1	0.19199292	0.7327444	0
A230070E04Rik	IMAGE:641084	RIKEN cDNA A230070E04 gene	0.25124466	0.73326975	0
Ebag9	IMAGE:641072	Estrogen receptor-binding fragment-associated gene 9	0.25006145	0.73356116	0
Znrf2	IMAGE:640555	Zinc and ring finger 2	0.22878706	0.73370904	0
Rap1a	IMAGE:583523	RAS-related protein-1a	0.34087414	0.7340795	0
EST	IMAGE:640885	Expressed sequence tag	0.13105536	0.737274	0
In multiple clusters	IMAGE:640735		0.22829701	0.73733836	0
EST	IMAGE:583243	Expressed sequence tag	0.22121713	0.7376358	0
EST	IMAGE:576044	Expressed sequence tag	0.1698138	0.738246	0
H2-Eb1	IMAGE:574155	Histocompatibility 2, class II antigen E beta	0.13192831	0.7383201	0
D430001N20	IMAGE:583191	Hypothetical protein D430001N20	0.15888494	0.73980457	0
Erf	IMAGE:583589	Ets2 repressor factor	0.19093207	0.74099076	0
Itm2b	IMAGE:640094	Integral membrane protein 2B	0.14694008	0.7415464	0
Ogt	IMAGE:583774	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	0.29589108	0.74195945	0
EST	IMAGE:640921	Expressed sequence tag	0.1300807	0.74228555	0

EG629595	IMAGE:640390	Predicted gene, EG629595	0.30434254	0.7452167	0
Uros	IMAGE:583476	Uroporphyrinogen III synthase	0.0865208	0.7453261	0
Irf8	IMAGE:640774	Interferon regulatory factor 8	0.13327445	0.74581957	0
Anxa1	IMAGE:640284	Annexin A1	0.09892646	0.74629813	0
EST	IMAGE:640176	Expressed sequence tag	0.09659772	0.7465617	0
Jmjd3	IMAGE:640268	Jumonji domain containing 3	0.24650778	0.74755526	0
In multiple clusters	IMAGE:640212		0.09858139	0.7478466	0
Transcribed locus	IMAGE:583356		0.08576527	0.7495674	0
Calcoco1	IMAGE:640758	Calcium binding and coiled coil domain 1	0.2112402	0.7508538	0
Transcribed locus	IMAGE:204515	Similar to C-C chemokine receptor type 2 (C-C CKR-2) (CC-CKR-2) (CCR-2) (CCR2) (Monocyte chemoattractant protein 1 receptor) (MCP-1-R) (CD192 antigen)	0.17055681	0.7520735	0
Spg20	IMAGE:573449	Spastic paraplegia 20, spartin (Troyer syndrome) homolog (human)	0.11443134	0.75315756	0
Psmb1	IMAGE:640335	Proteasome (prosome, macropain) subunit, beta type 1	0.16476169	0.7539268	0
EST	IMAGE:575350	Expressed sequence tag	0.1098296	0.754293	0
Transcribed locus	IMAGE:640171	Transcribed locus, weakly similar to NP_039499.1 c oxidase 1 [Schizosaccharomyces pombe]	0.22697778	0.75461566	0
Rab22a	IMAGE:640602	RAB22A, member RAS oncogene family	0.13300219	0.75471085	0
Ccr9	IMAGE:573572	Heterogeneous nuclear ribonucleoprotein A1	0.10594203	0.7552143	0
2010106G01Rik	IMAGE:575420	RIKEN cDNA 2010106G01 gene	0.13419901	0.7565941	0
Tada2l	IMAGE:583605	Transcriptional adaptor 2 (ADA2 homolog, yeast)-like	0.26568365	0.7566177	0
Transcribed locus	IMAGE:5181343	Transcribed locus, strongly similar to NP_001036107.1 protein precursor [Macaca mulatta]	0.17050588	0.7569159	0
Rrp1b	IMAGE:575876	Ribosomal RNA processing 1 homolog B (S. cerevisiae)	0.15342484	0.7581555	0
Rnf185	IMAGE:583933	Ring finger protein 185	0.12291178	0.75857556	0

EST	IMAGE:583217	Expressed sequence tag	0.19015172	0.7589768	0
EST	IMAGE:640392	Expressed sequence tag	0.09947279	0.75992495	0
Pnrc1	IMAGE:639793	Proline-rich nuclear receptor coactivator 1	0.123510204	0.7605269	0
9130004C02Rik	IMAGE:640356	RIKEN cDNA 9130004C02 gene	0.099256575	0.76088154	0
Ect2	IMAGE:583377	Ect2 oncogene	0.26233613	0.7609333	0
Cdkn2c	IMAGE:640222	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.303349	0.76103824	0
A430107D22Rik	IMAGE:574964	RIKEN cDNA A430107D22 gene	0.28273493	0.76106584	0
EST	IMAGE:583268	Expressed sequence tag	0.23961218	0.76146907	0
EST	IMAGE:583501	Expressed sequence tag	0.120302334	0.7620668	0
EST	IMAGE:641131	Expressed sequence tag	0.23088832	0.76224345	0
Nf2	IMAGE:640467	Neurofibromatosis 2	0.1650886	0.76268274	0
Rpl15	IMAGE:583166	Ribosomal protein L15	0.14001003	0.7632721	0
Trappc5	IMAGE:640891	Trafficking protein particle complex 5	0.23039232	0.7652603	0
EST	IMAGE:583216	Expressed sequence tag	0.17551346	0.7667527	0
Bccip	IMAGE:640916	BRCA2 and CDKN1A interacting protein	0.2507315	0.7671389	0
Dnajb4	IMAGE:583480	DnaJ (Hsp40) homolog, subfamily B, member 4	0.17608087	0.7673634	0
EST	IMAGE:641075	Expressed sequence tag	0.43694702	0.7679423	0
Khdrbs1	IMAGE:641002	KH domain containing, RNA binding, signal transduction associated 1	0.3100019	0.76888686	0
Tube1	IMAGE:1447102	Epsilon-tubulin 1	0.2587101	0.7691424	0
EST	IMAGE:575548	Expressed sequence tag	0.06989316	0.76957285	0
EST	IMAGE:583337	Expressed sequence tag	0.190408	0.76996815	0
EST	IMAGE:575870	Expressed sequence tag	0.15336819	0.7701433	0
EST	IMAGE:640815	Expressed sequence tag	0.16750668	0.77286106	0
Art4	IMAGE:583437	ADP-ribosyltransferase 4	0.26374847	0.7731826	0
1300012G16Rik	IMAGE:640297	RIKEN cDNA 1300012G16 gene	0.12607153	0.77359635	0
EST	IMAGE:582988	Expressed sequence tag	0.17382735	0.7741013	0
In multiple clusters	IMAGE:640331		0.3746578	0.77417654	0
EST	IMAGE:640900	Expressed sequence tag	0.18298908	0.774818	0
1700029I01Rik	IMAGE:640379	RIKEN cDNA 1700029I01 gene	0.3757439	0.7750295	0

AI450540	IMAGE:583622	Expressed sequence AI450540	0.14307564	0.7752005	0
EST	IMAGE:640865	Expressed sequence tag	0.1982755	0.7752754	0
5730494M16Rik	IMAGE:583861	RIKEN cDNA 5730494M16 gene	0.12268458	0.77829164	0
EST	IMAGE:640121	Expressed sequence tag	0.19518688	0.77886623	0
Mre11a	IMAGE:639936	Meiotic recombination 11 homolog A (S. cerevisiae)	0.0933463	0.7790241	0
D3Ucla1	IMAGE:640021	DNA segment, Chr 3, University of California at Los Angeles 1	0.12636012	0.7799295	0
Csad	IMAGE:640308	Cysteine sulfinic acid decarboxylase	0.0990381	0.7802706	0
Top2a	IMAGE:582997	Topoisomerase (DNA) II alpha	0.117531955	0.78138226	0
Cd24a	IMAGE:574099	CD24a antigen	0.20120902	0.7819296	0
Transcribed locus	IMAGE:583650	Transcribed locus	0.20722556	0.783015	0
EST	IMAGE:583269	Expressed sequence tag	0.26316804	0.78336143	0
EST	IMAGE:640424	Expressed sequence tag	0.24700123	0.78427637	0
Ier5	IMAGE:574793	Immediate early response 5	0.15150793	0.7849595	0
IL12A	IMAGE:1912458	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	0.17066334	0.7860652	0
EST	IMAGE:640619	Expressed sequence tag	0.39266124	0.78737324	0
Gmpr	IMAGE:640348	Guanosine monophosphate reductase	0.18021631	0.7894533	0
EST	IMAGE:583245	Expressed sequence tag	0.2630846	0.7897177	0
Ifit1	IMAGE:575371	Interferon-induced protein with tetratricopeptide repeats 1	0.16845484	0.79002476	0
C330006K01Rik	IMAGE:640249	RIKEN cDNA C330006K01 gene	0.125929	0.7903798	0
Slc35a4	IMAGE:583816	Solute carrier family 35, member A4	0.17756383	0.7914161	0
Arih2	IMAGE:583839	Ariadne homolog 2 (Drosophila)	0.16119698	0.7943986	0
D6Bwg1452e	IMAGE:583527	12 days embryo spinal ganglion cDNA, RIKEN full-length enriched library, clone:D130035P07 product:unclassifiable, full insert sequence	0.15965864	0.79527813	0
EST	IMAGE:640372	Expressed sequence tag	0.18026958	0.7958939	0
EST	IMAGE:639757	Expressed sequence tag	0.12454497	0.797681	0

EST	IMAGE:640851	Expressed sequence tag	0.16680586	0.7979852	0
Ssr3	IMAGE:583461	Signal sequence receptor, gamma	0.26383346	0.79902655	0
EG665189	IMAGE:640668	Predicted gene, EG665189	0.1024133	0.79938745	0
Rab3d	IMAGE:575981	RAB3D, member RAS oncogene family	0.18439856	0.8029781	0
Eef1a1	IMAGE:1265434	Eukaryotic translation elongation factor 1 alpha 1	0.17304425	0.8034982	0
Csnk1d	IMAGE:573689	Casein kinase 1, delta	0.05747467	0.80771637	0
Ccnd3	IMAGE:575230	Cyclin D3	0.10900051	0.80834854	0
EST	IMAGE:583385	Expressed sequence tag	0.19051276	0.8088961	0
Usp19	IMAGE:640652	Ubiquitin specific peptidase 19	0.24876826	0.81150454	0
Wdr1	IMAGE:1226712	WD repeat domain 1	0.13856903	0.8118112	0
Paqr8	IMAGE:575398	Progestin and adipoQ receptor family member VIII	0.1340689	0.8122714	0
H2-Q8	IMAGE:583608	Histocompatibility 2, Q region locus 8	0.087416485	0.81369483	0
Skp2	IMAGE:583572	S-phase kinase-associated protein 2 (p45)	0.08946324	0.81454104	0
Stx18	IMAGE:640560	Syntaxin 18	0.10023864	0.81456774	0
D030066L21	IMAGE:583940	9 days embryo whole body cDNA, RIKEN full-length enriched library, clone:D030066L21 product:unclassifiable, full insert sequence	0.24256238	0.81533676	0
4933439C20Rik	IMAGE:573956	RIKEN cDNA 4933439C20 gene	0.058695585	0.81758904	0
Rbm4b	IMAGE:575510	RNA binding motif protein 4B	0.1343891	0.8180169	0
Septin 1	IMAGE:583672	Septin 1	0.17724204	0.8196671	0
Thap2	IMAGE:641049	THAP domain containing, apoptosis associated protein 2	0.276549	0.8208455	0
Mrpl34	IMAGE:575652	Mitochondrial ribosomal protein L34	0.13457982	0.82162803	0
Sfrs1	IMAGE:639830	Splicing factor, arginine/serine-rich 1 (ASF/SF2)	0.14535399	0.8220042	0
EST	IMAGE:583315	Expressed sequence tag	0.22139253	0.8229378	0
EST	IMAGE:583101	Expressed sequence tag	0.2614432	0.8255884	0
EST	IMAGE:639938	Expressed sequence tag	0.14470191	0.826303	0
Bcap31	IMAGE:640282	B-cell receptor-associated protein 31	0.30183372	0.8275663	0
E030031F02Rik	IMAGE:639701	RIKEN cDNA E030031F02 gene	0.19268036	0.8284439	0

EST	IMAGE:640791	Expressed sequence tag	0.16745125	0.82896	0
EST	IMAGE:640795	Expressed sequence tag	0.23020369	0.8294383	0
EST	IMAGE:582983	Expressed sequence tag	0.33520988	0.8352107	0
Ly6e	IMAGE:640890	Lymphocyte antigen 6 complex, locus E	0.2126839	0.83536303	0
7030402D04Rik	IMAGE:583375	RIKEN cDNA 7030402D04 gene	0.22063796	0.83641315	0
Ankrd16	IMAGE:583852	Ankyrin repeat domain 16	0.17686474	0.8378268	0
Chic2	IMAGE:582852	Cysteine-rich hydrophobic domain 2	0.07983024	0.83809674	0
EST	IMAGE:640429	Expressed sequence tag	0.12863469	0.838762	0
EST	IMAGE:583503	Expressed sequence tag	0.15960543	0.8442098	0
Wdr53	IMAGE:1330385	WD repeat domain 53	0.20277601	0.8468846	0
2810021B07Rik	IMAGE:640500	RIKEN cDNA 2810021B07 gene	0.10165408	0.84793746	0
Transcribed locus	IMAGE:583154	Transcribed locus	0.13970225	0.84813267	0
EST	IMAGE:640789	Expressed sequence tag	0.13078244	0.8493136	0
2610307P16Rik	IMAGE:639668	RIKEN cDNA 2610307P16 gene	0.24365145	0.84994435	0
2010001A14Rik	IMAGE:640493	RIKEN cDNA 2010001A14 gene	0.19601828	0.8510261	0
Transcribed locus	IMAGE:640343	Transcribed locus, strongly similar to XP_001083304.1 similar to UPF3 regulator of nonsense transcripts homolog B isoform 1 isoform 4 [Macaca mulatta]	0.3840242	0.8511316	0
AW146242	IMAGE:583111	Expressed sequence AW146242	0.21919075	0.8546919	0
Btf3	IMAGE:583772	Basic transcription factor 3	0.2420932	0.8551778	0
Tmem115	IMAGE:582843	Transmembrane protein 115	0.15682927	0.8560752	0
Mettl3	IMAGE:575022	Methyltransferase-like 3	0.061360545	0.85629964	0
Transcribed locus	IMAGE:640294		0.3037683	0.857805	0
Fkrp	IMAGE:640283	CDNA, clone:Y2G0144I15, strand:unspecified	0.3735732	0.85855323	0
Sh3gl1	IMAGE:582991	SH3-domain GRB2-like 1	0.21889967	0.86197454	0
Ddx39	IMAGE:583491	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	0.15872298	0.86287564	0
Tubgcp4	IMAGE:583551	Tubulin, gamma complex associated protein 4	0.15971231	0.8637202	0
Tpm3	IMAGE:640122	Tropomyosin 3, gamma	0.21019569	0.8644068	0
EST	IMAGE:639766	Expressed sequence tag	0.29916385	0.8671045	0
9130404D08Rik	IMAGE:583561	RIKEN cDNA 9130404D08 gene	0.12054103	0.86745816	0
Tomm20	IMAGE:640575	Translocase of outer mitochondrial membrane	0.16616605	0.8684453	0

		20 homolog (yeast)			
Ube1c	IMAGE:640462	Ubiquitin-activating enzyme E1C	0.30683884	0.8689302	0
Fbxl20	IMAGE:582950	F-box and leucine-rich repeat protein 20	0.13988739	0.87042886	0
Transcribed locus	IMAGE:640175		0.3797492	0.87240785	0
Ptpdc1	IMAGE:1263784	Protein tyrosine phosphatase domain containing 1	0.15654548	0.87248284	0
EST	IMAGE:639710	Expressed sequence tag	0.14505656	0.8735055	0
Hnrpul2	IMAGE:576590	Heterogeneous nuclear ribonucleoprotein U-like 2	0.21474604	0.8776049	0
Cd8a	IMAGE:1247019	CD8 antigen, alpha chain	0.25294292	0.87801075	0
Nol7	IMAGE:583408	Nucleolar protein 7	0.1759257	0.8787466	0
Hisppd1	IMAGE:583887	Histidine acid phosphatase domain containing 1	0.16130574	0.8788877	0
Rpl18a	IMAGE:583588	Ribosomal protein L18A	0.17634466	0.8791649	0
EST	IMAGE:640540	Expressed sequence tag	0.18147059	0.8798733	0
Phtf1	IMAGE:583390	Putative homeodomain transcription factor 1	0.29214954	0.8806779	0
Transcribed locus	IMAGE:583382	Transcribed locus, moderately similar to NP_001039318.1 protein LOC564838 [Danio rerio]	0.14154886	0.8816358	0
EST	IMAGE:639823	Expressed sequence tag	0.22423749	0.88319343	0
Sp1	IMAGE:583862	Trans-acting transcription factor 1	0.14367822	0.88493025	0
EST	IMAGE:583270	Expressed sequence tag	0.29155937	0.8851008	0
Sfrs1	IMAGE:639976	Splicing factor, arginine/serine-rich 1 (ASF/SF2)	0.17938285	0.88733906	0
Rps25	IMAGE:583911	Ribosomal protein S25	0.16135903	0.8895052	0
EST	IMAGE:583671	Expressed sequence tag	0.16081488	0.89549106	0
Fgfr1	IMAGE:575608	Fibroblast growth factor receptor 1	0.07117962	0.89566976	0
EST	IMAGE:640957	Expressed sequence tag	0.13125779	0.89618623	0
Cd34	IMAGE:573426	CD34 antigen	0.045163922	0.89677894	0
Ercc5	IMAGE:642033	Excision repair cross-complementing rodent repair deficiency, complementation group 5	0.114865534	0.8987005	0
Bcap31	IMAGE:574060	B-cell receptor-associated protein 31	0.10720919	0.89920884	0

EST	IMAGE:640428	Expressed sequence tag	0.10133358	0.89969724	0
Sema7a	IMAGE:583574	Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	0.14295398	0.9024824	0
Commd9	IMAGE:640506	COMM domain containing 9	0.21151488	0.90260774	0
EST	IMAGE:640420	Expressed sequence tag	0.18121293	0.9036803	0
Rab14	IMAGE:640958	RAB14, member RAS oncogene family	0.1507684	0.9052191	0
EST	IMAGE:583897		0.12162696	0.9080433	0
Transcribed locus	IMAGE:639874	Transcribed locus, strongly similar to NP_796188.2 snRNP-specific protein, 200 kDa [Mus musculus]	0.29808673	0.90835416	0
Ctsl	IMAGE:640032	Cathepsin L	0.095869504	0.9086081	0
EST	IMAGE:582879	Expressed sequence tag	0.15772521	0.91267884	0
2610318N02Rik	IMAGE:1263539	RIKEN cDNA 2610318N02 gene	0.15649268	0.9130029	0
EST	IMAGE:639739	Expressed sequence tag	0.22494186	0.91341853	0
EST	IMAGE:639735	Expressed sequence tag	0.16254744	0.9137181	0
Aldh9a1	IMAGE:641104	Aldehyde dehydrogenase 9, subfamily A1	0.18404077	0.9152881	0
D2Ertd750e	IMAGE:575429	DNA segment, Chr 2, ERATO Doi 750, expressed	0.16855976	0.9155457	0
Hdgf	IMAGE:639890	Hepatoma-derived growth factor	0.14458501	0.9161266	0
EST	IMAGE:640681	Expressed sequence tag	0.12835144	0.91866237	0
1700047I17Rik	IMAGE:583576	Signal recognition particle 54	0.17702304	0.91978014	0
EST	IMAGE:583274	Expressed sequence tag	0.1403133	0.92276156	0
Transcribed locus	IMAGE:641128	Transcribed locus, weakly similar to NP_006283.1 tumor susceptibility gene 101 [Homo sapiens]	0.18409316	0.9232936	0
Rps13	IMAGE:640463	Ribosomal protein S13	0.40038946	0.92348844	0
In multiple clusters	IMAGE:573361		0.13653407	0.9253338	0
Pthr1	IMAGE:583513	Peptidyl-tRNA hydrolase 1 homolog (S. cerevisiae)	0.11914009	0.92612064	0
Psmc3	IMAGE:583912	Proteasome (prosome, macropain) 26S subunit, ATPase 3	0.17777064	0.92723286	0
Triobp	IMAGE:640097	TRIO and F-actin binding protein	0.19513556	0.92870533	0

Rasa3	IMAGE:582174	RAS p21 protein activator 3	0.25257158	0.92892563	0
Gbbp1	IMAGE:583064	GC-rich promoter binding protein 1	0.23717466	0.92908806	0
Sema4d	IMAGE:583259	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	0.33824086	0.92932725	0
Cd27	IMAGE:583084	CD antigen 27	0.1739877	0.9302863	0
EST	IMAGE:640040	Expressed sequence tag	0.2447632	0.93166554	0
LOC433894	IMAGE:639996	Hypothetical LOC433894	0.09562077	0.9323258	0
AI662270	IMAGE:583873	Expressed sequence AI662270	0.12155324	0.9327311	0
EST	IMAGE:640767	Expressed sequence tag	0.16659042	0.9332772	0
Fen1	IMAGE:639819	Flap structure specific endonuclease 1	0.16190428	0.9359021	0
EST	IMAGE:640884	Expressed sequence tag	0.10485231	0.93814737	0
EST	IMAGE:583936	Expressed sequence tag	0.17782243	0.93868136	0
EST	IMAGE:575844	Expressed sequence tag	0.1531439	0.9395625	0
Transcribed locus	IMAGE:2410765		0.17093271	0.93994594	0
Taf6	IMAGE:640288	TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor	0.18090148	0.9403317	0
Ppp2ca	IMAGE:583384	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0.17587511	0.94072795	0
EST	IMAGE:576526	Expressed sequence tag	0.20067982	0.94323826	0
Transcribed locus	IMAGE:640725		0.27521336	0.9439663	0
D4Wsu53e	IMAGE:583453	DNA segment, Chr 4, Wayne State University 53, expressed	0.12015055	0.9442281	0
Ndufab1	IMAGE:639895	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	0.22441117	0.946922	0
Ppp3cb	IMAGE:583115	Protein phosphatase 3, catalytic subunit, beta isoform	0.33328447	0.9492225	0
EST	IMAGE:583031	Expressed sequence tag	0.33570895	0.9518504	0
EST	IMAGE:640384	Expressed sequence tag	0.18110855	0.9521329	0
EST	IMAGE:640664	Expressed sequence tag	0.24770363	0.95290923	0
Apobec3	IMAGE:582807	Apolipoprotein B editing complex 3	0.15761167	0.95400316	0
EST	IMAGE:640876	Expressed sequence tag	0.18293494	0.9558044	0

Numb	IMAGE:640667	Numb gene homolog (Drosophila)	0.39430717	0.95620507	0
LOC100043591	IMAGE:583285	Hypothetical protein LOC100043591	0.11961005	0.9582426	0
EST	IMAGE:583261	Expressed sequence tag	0.11953098	0.9592889	0
Pltp	IMAGE:640088	Phospholipid transfer protein	0.2449041	0.96018493	0
clone:F830035G06	IMAGE:640191	Activated spleen cDNA, RIKEN full-length enriched library, clone:F830035G06 product:unclassifiable, full insert sequence	0.16443421	0.961305	0
EST	IMAGE:583313	Expressed sequence tag	0.1903567	0.9657844	0
Smardc1	IMAGE:639851	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1	0.35883695	0.9669286	0
FCRH3	IMAGE:5229383	Fc receptor-like 3	0.1541532	0.9674967	0
Pgm1	IMAGE:583548	Phosphoglucomutase 1	0.086973496	0.968313	0
Dnpep	IMAGE:641031	Aspartyl aminopeptidase	0.16792856	0.9717757	0
Cx3cl1	IMAGE:1329058	Chemokine (C-X3-C motif) ligand 1	0.18800981	0.9718362	0
Mapk9	IMAGE:583552	Mitogen activated protein kinase 9	0.17623904	0.9732266	0
2610207I05Rik	IMAGE:640678	RIKEN cDNA 2610207I05 gene	0.30802563	0.97422063	0
Transcribed locus	IMAGE:583451	Ras responsive element binding protein 1	0.34033206	0.97497404	0
EST	IMAGE:582951	Expressed sequence tag	0.15783565	0.97708565	0
Afmid	IMAGE:582985	Arylformamidase	0.11653907	0.97849864	0
LOC625360	IMAGE:575653	Similar to 2-cell-stage, variable group, member 3	0.07166224	0.9805407	0
D930015E06Rik	IMAGE:639854	RIKEN cDNA D930015E06 gene	0.14541331	0.98077303	0
In multiple clusters	IMAGE:583656		0.087721646	0.98327965	0
Transcribed locus	IMAGE:640317	Transcribed locus	0.27223167	0.98339844	0
EST	IMAGE:583505	Expressed sequence tag	0.1907765	0.9907677	0
EST	IMAGE:582680	Expressed sequence tag	0.06599352	0.99207526	0
EST	IMAGE:583265	Expressed sequence tag	0.1902574	0.9922045	0
LOC100043339	IMAGE:582955	Hypothetical protein LOC100043339	0.21960132	0.9929837	0
Mat2b	IMAGE:583812	Methionine adenosyltransferase II, beta	0.09081751	0.99832153	0
Ddx3x	IMAGE:639697	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked	0.12320918	0.9985023	0

EST	IMAGE:640642	Expressed sequence tag	0.30565807	0.9985243	0
Anxa5	IMAGE:576596	Annexin A5	0.21480387	1.0008115	0
Mel13	IMAGE:575321	Melanoma nuclear protein 13	0.32203823	1.0029927	0
EST	IMAGE:640396	Expressed sequence tag	0.18116067	1.0056394	0
Ccnl2	IMAGE:583850	Cyclin L2	0.1427058	1.0061259	0
Rpl7a	IMAGE:582916	Ribosomal protein L7a	0.17367032	1.0064209	0
EST	IMAGE:583481	Expressed sequence tag	0.19072291	1.0064325	0
Tapt1	IMAGE:576636	Transmembrane anterior posterior transformation 1	0.2150888	1.0071121	0
Ankrd10	IMAGE:583837	Ankyrin repeat domain 10	0.12260576	1.0080744	0
EST	IMAGE:583053		0.26127622	1.009449	0
Transcribed locus	IMAGE:582947	Transcribed locus	0.33190817	1.0095013	0
LOC626877	IMAGE:640460	Similar to zinc finger protein 709	0.24819928	1.0097693	0
EST	IMAGE:583497	Expressed sequence tag	0.2626778	1.0108321	0
Transcribed locus	IMAGE:582793	Transcribed locus	0.11587919	1.0117749	0
Kpna2	IMAGE:640444	Karyopherin (importin) alpha 2	0.181264	1.0162456	0
EST	IMAGE:583241	Expressed sequence tag	0.19020322	1.0179682	0
Rbm39	IMAGE:583614	RNA binding motif protein 39	0.20635769	1.0184754	0
Cdca8	IMAGE:576454	Cell division cycle associated 8	0.20025322	1.0235033	0
Rbbp4	IMAGE:583477	Retinoblastoma binding protein 4	0.12022709	1.0249457	0
Prmt3	IMAGE:583383	Protein arginine N-methyltransferase 3	0.15932877	1.0288887	0
Cd72	IMAGE:573570	CD72 antigen	0.15460835	1.0293872	0
EST	IMAGE:640472	Expressed sequence tag	0.24713854	1.0327979	0
E130112N10Rik	IMAGE:640155	RIKEN cDNA E130112N10 gene	0.16352412	1.0332108	0
Tuba2	IMAGE:582927	Tubulin, alpha 2	0.15778112	1.0343908	0
Cebpg	IMAGE:641074	Transcribed locus	0.15212524	1.0360912	0
EST	IMAGE:583044	Expressed sequence tag	0.081285626	1.0366639	0
Mecp2	IMAGE:576274	Methyl CpG binding protein 2	0.18573175	1.0384046	0
EST	IMAGE:640838	Expressed sequence tag	0.15048277	1.0384777	0
EG665189	IMAGE:582828	Predicted gene, EG665189	0.079648115	1.0400876	0
Nde1	IMAGE:640079	Nuclear distribution gene E homolog 1 (A nidulans)	0.37741485	1.0413917	0

EST	IMAGE:640800	Expressed sequence tag	0.103029124	1.0455887	0
EST	IMAGE:582869	Expressed sequence tag	0.1881667	1.0476729	0
EST	IMAGE:583360	Expressed sequence tag	0.17582336	1.0504134	0
Zmynd10	IMAGE:1225083	Zinc finger, MYND domain containing 10	0.11537553	1.0510406	0
Mrpl3	IMAGE:583309	Mitochondrial ribosomal protein L3	0.11968966	1.0513797	0
Ctsl	IMAGE:583530	Cathepsin L	0.20613752	1.0514634	0
Fstl1	IMAGE:583623	Follistatin-like 1	0.16070542	1.0539619	0
Golt1b	IMAGE:639999	Golgi transport 1 homolog B (<i>S. cerevisiae</i>)	0.16314551	1.0546305	0
Samhd1	IMAGE:640697	SAM domain and HD domain, 1	0.1972764	1.0547925	0
Ormdl1	IMAGE:640700	ORM1-like 1 (<i>S. cerevisiae</i>)	0.24891211	1.0555212	0
Stip1	IMAGE:583886	Stress-induced phosphoprotein 1	0.14373931	1.0614493	0
Pot1a	IMAGE:640128	Protection of telomeres 1A	0.0963532	1.0616719	0
Tbl1xr1	IMAGE:583935	Transducin (beta)-like 1X-linked receptor 1	0.16141373	1.0632274	0
EST	IMAGE:640248	Glycosylphosphatidylinositol specific phospholipase D1	0.09695831	1.0643129	0
EST	IMAGE:583813	Expressed sequence tag	0.12253151	1.0655004	0
EST	IMAGE:583336	Expressed sequence tag	0.17577156	1.0658431	0
EST	IMAGE:582796	Expressed sequence tag	0.17340605	1.0707246	0
Arhgap4	IMAGE:582914	Rho GTPase activating protein 4	0.1391398	1.0729194	0
Epc1	IMAGE:583089	Enhancer of polycomb homolog 1 (<i>Drosophila</i>)	0.26021516	1.0760452	0
Sfrs1	IMAGE:583110	Splicing factor, arginine/serine-rich 1 (ASF/SF2)	0.20380194	1.076277	0
Fxyd2	IMAGE:640559	FXDY domain-containing ion transport regulator 2	0.40434322	1.0858704	0
EST	IMAGE:583428	Expressed sequence tag	0.086212896	1.0912954	0
In multiple clusters	IMAGE:583796		0.24216327	1.0917478	0
EST	IMAGE:640477	Expressed sequence tag	0.12877384	1.0925419	0
1700029I01Rik	IMAGE:583028	RIKEN cDNA 1700029I01 gene	0.23795384	1.0932949	0
Anxa4	IMAGE:583430	Annexin A4	0.14167187	1.0968199	0
0610009K11Rik	IMAGE:582885	RIKEN cDNA 0610009K11 gene	0.26077446	1.0974934	0
Pkig	IMAGE:583036	Adenosine deaminase	0.1738811	1.0975935	0

4930566A11Rik	IMAGE:583433	RIKEN cDNA 4930566A11 gene	0.19061698	1.0984402	0
D4Wsu53e	IMAGE:583112	DNA segment, Chr 4, Wayne State University 53, expressed	0.23724103	1.0998384	0
EST	IMAGE:641097	Expressed sequence tag	0.27674356	1.1003822	0
Transcribed locus	IMAGE:1226515		0.1385048	1.1007899	0
EST	IMAGE:640539	Expressed sequence tag	0.1652494	1.105236	0
Hexb	IMAGE:575852	Hexosaminidase B	0.15319869	1.1071315	0
Cebpg	IMAGE:641074	Transcribed locus	0.3104874	1.1078917	0
EST	IMAGE:583087	Expressed sequence tag	0.21913026	1.108813	0
C230075L07	IMAGE:583521	0 day neonate cerebellum cDNA, RIKEN full-length enriched library, clone:C230075L07 product: unclassifiable, full insert sequence	0.26276082	1.1094133	0
EST	IMAGE:575254	Expressed sequence tag	0.10909994	1.1111922	0
EST	IMAGE:640241	Expressed sequence tag	0.19545148	1.1112188	0
EST	IMAGE:583502	Expressed sequence tag	0.14185527	1.1160715	0
EST	IMAGE:583581	Expressed sequence tag	0.26560113	1.1213808	0
Ap2b1	IMAGE:583107	Adaptor-related protein complex 2, beta 1 subunit	0.15744251	1.1231766	0
Zzz3	IMAGE:576612	Zinc finger, ZZ domain containing 3	0.21491918	1.1241798	0
EST	IMAGE:583908	Expressed sequence tag	0.09136899	1.1254189	0
EST	IMAGE:583130	Expressed sequence tag	0.13963741	1.1279197	0
Transcribed locus	IMAGE:640334		0.1475255	1.1293075	0
Ankrd10	IMAGE:583182	Ankyrin repeat domain 10	0.2045607	1.1305966	0
Cmpk	IMAGE:640631	Cytidylate kinase	0.4075747	1.1318333	0
Spg20	IMAGE:582846	Spastic paraplegia 20, spartin (Troyer syndrome) homolog (human)	0.2032074	1.1364282	0
Capn2	IMAGE:583248	Calpain 2	0.08251614	1.1409152	0
Ntn1	IMAGE:583376	Netrin 1	0.23888218	1.1454443	0
A430079K02	IMAGE:640409	0 day neonate thymus cDNA, RIKEN full-length enriched library, clone:A430079K02 product:unclassifiable, full insert sequence	0.19665025	1.1476575	0
EST	IMAGE:583192	Expressed sequence tag	0.17546168	1.1511065	0

Ndst1	IMAGE:639716	N-deacetylase/N-sulfotransferase (heparan glucosaminy) 1	0.24378918	1.1523278	0
Chd1	IMAGE:640262	Chromodomain helicase DNA binding protein 1	0.14735462	1.1564187	0
Tmem48	IMAGE:640512	Transmembrane protein 48	0.10002733	1.1573735	0
Pdcd2	IMAGE:583406	Programmed cell death 2	0.141611	1.1582869	0
Taf12	IMAGE:583488	TAF12 RNA polymerase II, TATA box binding protein (TBP)-associated factor	0.08417694	1.1589596	0
Transcribed locus	IMAGE:639923	Transcribed locus	0.36000997	1.1657805	0
Rps3a	IMAGE:639738	Ribosomal protein S3a	0.20882998	1.1669494	0
EST	IMAGE:583597	Expressed sequence tag	0.12185238	1.167814	0
EST	IMAGE:640633	Expressed sequence tag	0.12821132	1.1689384	0
Mtmr11	IMAGE:1265247	Myotubularin related protein 11	0.17293817	1.1706191	0
Lrba	IMAGE:573828	LPS-responsive beige-like anchor	0.17166992	1.1735682	0
Prkra	IMAGE:583424	Protein kinase, interferon inducible double stranded RNA dependent activator	0.23901932	1.1739807	0
4931406I20Rik	IMAGE:583790	RIKEN cDNA 4931406I20 gene	0.14349651	1.1748334	0
Slc12a7	IMAGE:639998	Solute carrier family 12, member 7	0.14576584	1.1837113	0
EST	IMAGE:640280	Expressed sequence tag	0.24546485	1.1873623	0
EST	IMAGE:583289	Expressed sequence tag	0.19030617	1.1915681	0
Ccl20	IMAGE:1226828	Chemokine (C-C motif) ligand 20	0.13869393	1.1922461	0
D4Wsu53e	IMAGE:583478	DNA segment, Chr 4, Wayne State University 53, expressed	0.14179797	1.1961561	0
EST	IMAGE:640261	Ssu72 RNA polymerase II CTD phosphatase homolog (yeast)	0.12707685	1.1981158	0
Mis12	IMAGE:583716	MIS12 homolog (yeast)	0.090277486	1.2016376	0
Itpkb	IMAGE:639740	Inositol 1,4,5-trisphosphate 3-kinase B	0.24385644	1.2046329	0
Abce1	IMAGE:583718	ATP-binding cassette, sub-family E (OABP), member 1	0.14331894	1.207865	0
EST	IMAGE:583814	Expressed sequence tag	0.14355832	1.2079262	0
Stard9	IMAGE:640768	START domain containing 9	0.1826697	1.210068	0
2810455D13Rik	IMAGE:582820	RIKEN cDNA 2810455D13 gene	0.17345755	1.2141036	0

EST	IMAGE:640824	Expressed sequence tag	0.10313236	1.2171714	0
EST	IMAGE:640252	Expressed sequence tag	0.18000229	1.2227986	0
EST	IMAGE:640795	Expressed sequence tag	0.13536878	1.2259285	0
4932433N03Rik	IMAGE:640355	PR domain containing 10	0.37522268	1.2281287	0
EST	IMAGE:640990	Expressed sequence tag	0.31221637	1.2286857	0
EST	IMAGE:640313	Expressed sequence tag	0.19560425	1.2315154	0
Tuba1b	IMAGE:639633	Tubulin, alpha 2	0.26689938	1.232416	0
Eif4g2	IMAGE:640625	Eukaryotic translation initiation factor 4, gamma 2	0.19712143	1.2330875	0
Rps6kb1	IMAGE:640345	Ribosomal protein S6 kinase, polypeptide 1	0.12621732	1.2400026	0
EST	IMAGE:583422	Expressed sequence tag	0.20505205	1.2448963	0
Lck	IMAGE:640179	Lymphocyte protein tyrosine kinase	0.16357891	1.2512286	0
EST	IMAGE:583106	Expressed sequence tag	0.13957363	1.2535152	0
Glr5	IMAGE:583838	Glutaredoxin 5 homolog (S. cerevisiae)	0.14361861	1.2561904	0
EST	IMAGE:640848	Expressed sequence tag	0.10323192	1.2563207	0
Cyb5b	IMAGE:640762	Cytochrome b5 type B	0.30639276	1.2566386	0
EST	IMAGE:583149	Expressed sequence tag	0.2615292	1.2611681	0
Tcf2a	IMAGE:583333	Transcription factor E2a	0.11976735	1.2694123	0
EST	IMAGE:582966	Expressed sequence tag	0.20347823	1.2760503	0
EST	IMAGE:583429	Expressed sequence tag	0.120075494	1.2829258	0
Sfrs5	IMAGE:583131	Splicing factor, arginine/serine-rich 5 (SRp40, HRS)	0.15749812	1.2834913	0
3110001D03Rik	IMAGE:641030	RIKEN cDNA 3110001D03 gene	0.15093622	1.2862916	0
Egr1	IMAGE:576356	Early growth response 1	0.18588513	1.2877073	0
Ndufab1	IMAGE:583543	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	0.22104767	1.3321646	0
H2-K1	IMAGE:640137	Histocompatibility 2, K1, K region	0.27011466	1.3347989	0
2410131K14Rik	IMAGE:583765	RIKEN cDNA 2410131K14 gene	0.12238359	1.3359904	0
Tmco6	IMAGE:583157	Transmembrane and coiled-coil domains 6	0.18868993	1.3363899	0
EST	IMAGE:575672	Expressed sequence tag	0.16898367	1.3396982	0
Nap1l1	IMAGE:583621	Nucleosome assembly protein 1-like 1	0.12192973	1.353248	0
2410127E18Rik	IMAGE:583407	RIKEN cDNA 2410127E18 gene	0.1593836	1.3600667	0

EST	IMAGE:640872	Expressed sequence tag	0.10333227	1.3640214	0
EST	IMAGE:583189	Expressed sequence tag	0.1192999	1.367478	0
Kbtbd8	IMAGE:582871	Kelch repeat and BTB (POZ) domain containing 8	0.21860829	1.3820908	0
Mll5	IMAGE:583357	Myeloid/lymphoid or mixed-lineage leukemia 5	0.11984163	1.3827217	0
Pex12	IMAGE:640739	Peroxisomal biogenesis factor 12	0.3967752	1.3871258	0
EST	IMAGE:583361	Expressed sequence tag	0.1904587	1.3879939	0
In multiple clusters	IMAGE:577177	Expressed sequence tag	0.21379174	1.3921089	0
Nol6	IMAGE:583756	Nucleolar protein family 6 (RNA-associated)	0.17670348	1.3955104	0
EST	IMAGE:576310	RIKEN cDNA 2810021B07 gene	0.18578498	1.3972731	0
Sema7a	IMAGE:583573	Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	0.121779755	1.4004148	0
Myh11	IMAGE:583021	Myosin, heavy polypeptide 11, smooth muscle	0.117609486	1.4048539	0
Lsp1	IMAGE:640873	Lymphocyte specific 1	0.12994066	1.4113812	0
Pbrm1	IMAGE:640336	Polybromo 1	0.18100296	1.4261597	0
Lsp1	IMAGE:640873	Lymphocyte specific 1	0.1333418	1.4281049	0
EST	IMAGE:641098	Expressed sequence tag	0.3106367	1.4292423	0
Transcribed locus	IMAGE:583181		0.18926719	1.4303479	0
D10Ert610e	IMAGE:576334	DNA segment, Chr 10, ERATO Doi 610, expressed	0.18583731	1.4371021	0
OTTMUSG00000015615	IMAGE:573984	Predicted gene, OTTMUSG00000015615	0.18677296	1.4386475	0
Rpl27a	IMAGE:583237	Ribosomal protein L27a	0.11945638	1.4410703	0
EST	IMAGE:640636	Expressed sequence tag	0.18167754	1.4471576	0
Ndufa11	IMAGE:640025	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11	0.1949766	1.453027	0
Sfrs1	IMAGE:640898	Splicing factor, arginine/serine-rich 1 (ASF/SF2)	0.14979707	1.4552816	0
Rpl6	IMAGE:583113	Ribosomal protein L6	0.2602955	1.4553547	0
4933411K20Rik	IMAGE:641854	RIKEN cDNA 4933411K20 gene	0.07570058	1.4596387	0
EST	IMAGE:583924	Expressed sequence tag	0.17696965	1.4621696	0

EST	IMAGE:582998	Expressed sequence tag	0.13994968	1.4656422	0
EST	IMAGE:640563	Expressed sequence tag	0.16530347	1.477091	0
EST	IMAGE:582954	Expressed sequence tag	0.20417933	1.4864513	0
Transcribed locus	IMAGE:640718	Predicted gene, EG329160	0.14933823	1.5124165	0
EST	IMAGE:583114	Expressed sequence tag	0.28782284	1.5153432	0
Pcbp3	IMAGE:583766	Poly(rC) binding protein 3	0.14343545	1.517046	0
Pdk3	IMAGE:578105	Pyruvate dehydrogenase kinase, isoenzyme 3	0.23485824	1.5186045	0
EST	IMAGE:583400	5'-3' exoribonuclease 1	0.23895131	1.5261348	0
Sfrs1	IMAGE:583015	Splicing factor, arginine/serine-rich 1 (ASF/SF2)	0.21895544	1.5297421	0
Rbm39	IMAGE:640923	RNA binding motif protein 39	0.16696563	1.5497329	0
Tapt1	IMAGE:640665	Transmembrane anterior posterior transformation 1	0.27362913	1.5509655	0
EST	IMAGE:583200	Expressed sequence tag	0.08217306	1.5649662	0
EST	IMAGE:640981	Expressed sequence tag	0.13132267	1.5652509	0
Hist1h2bc	IMAGE:640662	Histone cluster 1, H2bc	0.2110765	1.5770954	0
Fip1l1	IMAGE:640279	FIP1 like 1 (<i>S. cerevisiae</i>)	0.2263168	1.5827358	0
EST	IMAGE:583001	Expressed sequence tag	0.18894938	1.5916317	0
1500032D16Rik	IMAGE:583789	RIKEN cDNA 1500032D16 gene	0.122455634	1.6036406	0
EST	IMAGE:572934	Expressed sequence tag	0.11174699	1.6059886	0
Eif5	IMAGE:582912	Eukaryotic translation initiation factor 5	0.07731564	1.6264099	0
EST	IMAGE:576256	Expressed sequence tag	0.18558083	1.658207	0
EST	IMAGE:640521	Expressed sequence tag	0.2730733	1.6630465	0
Adhfe1	IMAGE:640899	Alcohol dehydrogenase, iron containing, 1	0.16691019	1.6720959	0
EST	IMAGE:583675	Expressed sequence tag	0.22322412	1.6949353	0
9430015G10Rik	IMAGE:583737	RIKEN cDNA 9430015G10 gene	0.26484284	1.7045926	0
Myg1	IMAGE:640034	Melanocyte proliferating gene 1	0.14588425	1.731795	0
D14Ert449e	IMAGE:577726	Transcribed locus	0.25452778	1.7631146	0
Zhx1	IMAGE:640278	Zinc fingers and homeoboxes protein 1	0.20975778	1.7648783	0
EST	IMAGE:641143	Expressed sequence tag	0.23014303	1.7739022	0
Transcribed locus	IMAGE:640875	Transcribed locus	0.16685823	1.7804123	0
EST	IMAGE:640984	Expressed sequence tag	0.18383066	1.7832661	0

Es2el	IMAGE:583108	Expressed sequence 2 embryonic lethal	0.17403734	1.7871766	0
EST	IMAGE:640337	Expressed sequence tag	0.19565637	1.8290895	0
EG621205	IMAGE:640982	Predicted gene, EG621205	0.15082297	1.8387327	0
Ttll4	IMAGE:582821	Tubulin tyrosine ligase-like family, member 4	0.18806146	1.8657908	0
Hist1h2bc	IMAGE:640662	Histone cluster 1, H2bc	0.07307641	1.8738339	0
EST	IMAGE:583213	Expressed sequence tag	0.11937629	1.9202455	0
Rnf187	IMAGE:640076	Ring finger protein 187	0.24595205	1.9577457	0
Ncf1	IMAGE:5371913	Neutrophil cytosolic factor 1	0.25331965	1.972282	0
EST	IMAGE:583636	Expressed sequence tag	0.17644823	1.978074	0
Mycbp	IMAGE:583915	Ras-related GTP binding C	0.22382583	1.9908214	0
Sfrs1	IMAGE:583038	Splicing factor, arginine/serine-rich 1 (ASF/SF2)	0.20363934	1.9977032	0
EST	IMAGE:640817	Expressed sequence tag	0.19817033	1.9988683	0
Cib1	IMAGE:583939	Calcium and integrin binding 1 (calmyrin)	0.22388275	2.0442023	0
Sfrs1	IMAGE:582848	Splicing factor, arginine/serine-rich 1 (ASF/SF2)	0.23659152	2.2645428	0
Dtymk	IMAGE:583035	Deoxythymidylate kinase	0.15727472	2.8086443	0
EST	IMAGE:639730	Expressed sequence tag	0.2974373	3.5508795	0

Anexo II

Tabela III. Genes diferencialmente expressos comparando-se células mTEC controles *versus* células mTEC transfectadas com RNAi anti-Aire (FDR $\square$ 15%, p-value $\square$ 0,015).

<i>Gene Symbol</i>	<i>CloneID</i>	<i>Gene Name</i>	<i>Fold change</i>	<i>q-value (%)</i>
EST	IMAGE:640598	Expressed sequence tag	1.0673177	14.5946665
EST	IMAGE:640817	Expressed sequence tag	1.091488	14.5946665
Gclm	IMAGE:639968	Glutamate-cysteine ligase , modifier subunit	1.1114856	12.903029
EST	IMAGE:640144	Expressed sequence tag	1.0997492	12.903029
E2f2	IMAGE:640754	E2F transcription factor 2	1.1284692	12.903029
2610040C18Rik	IMAGE:122576 8	Apoptosis-inducing, TAF9-like domain 1	1.06998	12.903029
EST	IMAGE:640272	Expressed sequence tag	1.1294833	12.903029
EST	IMAGE:640451	Expressed sequence tag	1.0881842	12.903029
EST	IMAGE:583312	Expressed sequence tag	1.0601645	12.903029
EST	IMAGE:639827	Expressed sequence tag	1.1019533	12.085992
Myg1	IMAGE:583514	Melanocyte proliferating gene 1	1.1121532	12.085992
Klhl7	IMAGE:583190	Gamma-aminobutyric acid (GABA-A) receptor-associated protein-like 2	1.0723306	12.085992
Sh3d1B	IMAGE:583881	Intersectin 2	1.0822399	12.085992
EST	IMAGE:640792	Expressed sequence tag	1.0899262	12.085992
Epc1	IMAGE:583089	Enhancer of polycomb homolog 1 (Drosophila)	1.1037792	12.085992
AI452102	IMAGE:639804	RIKEN cDNA E230029C05 gene	1.0712574	12.085992
Txnrd1	IMAGE:583921	Thioredoxin reductase 1	1.0930426	12.085992
EST	IMAGE:583308	Expressed sequence tag	1.0649359	12.085992
EST	IMAGE:641140	Expressed sequence tag	1.0681186	12.085992
EST	IMAGE:640351	Expressed sequence tag	1.1232543	12.085992
1110008H02Rik	IMAGE:640371	Transcribed locus	1.0701028	12.085992
H13	IMAGE:640912	Histocompatibility 13	1.0737796	12.085992
EST	IMAGE:640865	Expressed sequence tag	1.1486661	12.085992

1500002B03Rik	IMAGE:133038 5	WD repeat domain 53	1.1449281	12.085992
A430107D22Rik	IMAGE:574964	RIKEN cDNA A430107D22 gene	1.1061863	12.085992
EST	IMAGE:582951	Expressed sequence tag	1.1534444	10.909013
Idh3b	IMAGE:640557	Nucleolar protein 5A	1.1218609	10.909013
EST	IMAGE:640943	Transcribed locus	1.1063399	10.909013
Nde1	IMAGE:640079	Nuclear distribution gene E homolog 1 (A nidulans)	1.1183379	10.943718
EST	IMAGE:640860	Expressed sequence tag	1.091564	10.134216
HSPD1	IMAGE:133099	Transcribed locus, weakly similar to XP_360622.1 protein MG03165.4 [Magnaporthe grisea 70-15]	1.1233237	10.134216
EST	IMAGE:583031	Expressed sequence tag	1.1456674	10.134216
Bcap31	IMAGE:640282	B-cell receptor-associated protein 31	1.0808171	10.134216
Mmp14	IMAGE:573066	Matrix metalloproteinase 14 (membrane-inserted)	1.1140016	10.134216
Hist1h2bc	IMAGE:640662	Histone cluster 1, H2bc	1.1471213	10.134216
1110037N09Rik	IMAGE:583451	Ras responsive element binding protein 1	1.1069003	10.134216
EST	IMAGE:582879	Expressed sequence tag	1.2095115	10.134216
Rpl27a	IMAGE:640775	Ribosomal protein L27a	1.1284332	10.134216
Cd97	IMAGE:573301	CD97 antigen	1.064621	10.134216
Cd28	IMAGE:576501	CD28 antigen	1.0784754	9.724821
4921537D05Rik	IMAGE:639871	RIKEN cDNA 4921537D05 gene	1.1277622	9.724821
EST	IMAGE:640381	Expressed sequence tag	1.1173592	9.724821
EST	IMAGE:640299	Expressed sequence tag	1.0907763	9.724821
3110037I16Rik	IMAGE:640889	RIKEN cDNA 3110037I16 gene	1.1730123	9.1921625
EST	IMAGE:583900	Expressed sequence tag	1.1453668	9.1921625
EST	IMAGE:138013	Expressed sequence tag	1.08235	9.1921625
EST	IMAGE:641123	Expressed sequence tag	1.1028172	8.697915
Eaf1	IMAGE:640226	ELL associated factor 1	1.070029	7.769566
EST	IMAGE:640838	Expressed sequence tag	1.15003	6.6869216

Dusp2	IMAGE:640913	Dual specificity phosphatase 2	1.1603421	6.6869216
EST	IMAGE:639637	Expressed sequence tag	1.0655534	4.846363
2410024A21Rik	IMAGE:640202	RIKEN cDNA 2410024A21 gene	1.0942664	4.846363
EST	IMAGE:641137	Expressed sequence tag	1.0804759	4.846363
EST	IMAGE:640341	Expressed sequence tag	1.128608	4.846363
EST	IMAGE:640121	Expressed sequence tag	1.0771161	4.846363
EST	IMAGE:640280	Expressed sequence tag	1.1345134	4.846363
EST	IMAGE:640664	Expressed sequence tag	1.1900343	4.846363
EST	IMAGE:583290	Expressed sequence tag	1.0764095	4.846363
Prkdc	IMAGE:134593 5	Protein kinase, DNA activated, catalytic polypeptide	1.1635538	4.3860455
6330415G19Rik	IMAGE:583538	Zinc finger protein 213	1.0928538	4.7988496
Myc	IMAGE:574902	Myelocytomatosis oncogene	1.1011529	3.979534
Mrpl34	IMAGE:575652	Mitochondrial ribosomal protein L34	1.0884858	4.0286636
EST	IMAGE:583053	Expressed sequence tag	1.1728827	4.0286636
EST	IMAGE:641113	Expressed sequence tag	1.0897497	4.0286636
Maea	IMAGE:575996	Macrophage erythroblast attacher	1.1015344	4.0286636
EST	IMAGE:640837	Expressed sequence tag	1.1438826	4.0286636
Jmjd3	IMAGE:640268	Jumonji domain containing 3	1.1368691	4.0286636
Syng2	IMAGE:640773	Synaptogyrin 2	1.1226083	3.352621
Rps13	IMAGE:640463	Ribosomal protein S13	1.1350834	3.352621
EST	IMAGE:640521	Expressed sequence tag	1.1595614	3.352621
EST	IMAGE:640602	Expressed sequence tag	1.1412392	3.352621
2900076A07Rik	IMAGE:583769	RIKEN cDNA 2900076A07 gene	1.1678199	3.708202
EST	IMAGE:583456	Expressed sequence tag	1.1011477	3.199233
H2-K1	IMAGE:640137	Histocompatibility 2, K1, K region	1.1671582	3.199233
EST	IMAGE:582983	Expressed sequence tag	1.1746738	3.199233
EST	IMAGE:576044	Expressed sequence tag	1.1233515	3.3991852

EST	IMAGE:640173	Expressed sequence tag	1.1573058	3.5469759
Numb	IMAGE:640667	Numb gene homolog (Drosophila)	1.1834155	0
AA960558	IMAGE:583794	Pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	1.1686431	0
Zhx1	IMAGE:640278	Zinc fingers and homeoboxes protein 1	1.1978401	0
FASLG	IMAGE:517859 6	Fas ligand (TNF superfamily, member 6)	0.7508043	0
Rab3d	IMAGE:575981	RAB3D, member RAS oncogene family	0.78198445	0
Akt3	IMAGE:583636	Serologically defined colon cancer antigen 8	0.8486121	0
Ptgs2	IMAGE:535560 7	Prostaglandin-endoperoxide synthase 2	0.8196461	0
Bag2	IMAGE:573075	Bcl2-associated athanogene 2	0.8758778	0
FCRH3	IMAGE:522938 3	Fc receptor-like 3	0.7801735	0
EST	IMAGE:640539	Expressed sequence tag	0.862176	0
In multiple clusters	IMAGE:573361		0.8615393	0
Fos	IMAGE:537662 7	FBJ osteosarcoma oncogene	0.8363829	0
IL12A	IMAGE:191245 8	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	0.8045339	0
Transcribed locus	IMAGE:204515	Similar to C-C chemokine receptor type 2 (C-C CKR-2) (CC-CKR-2) (CCR-2) (CCR2) (Monocyte chemoattractant protein 1 receptor) (MCP-1-R) (CD192 antigen)	0.85200554	0
TNF	IMAGE:521313 4	Tumor necrosis factor (TNF superfamily, member 2)	0.81887746	0
Grb2	IMAGE:575387	Growth factor receptor bound protein 2	0.8562132	0
CCR7	IMAGE:518075	Chemokine (C-C motif) receptor 7	0.7866933	0

	8			
Spg20	IMAGE:573449	Spastic paraplegia 20, spartin (Troyer syndrome) homolog (human)	0.8325986	0
In multiple clusters	IMAGE:573897		0.8534656	0
NOX1	IMAGE:357418 3	Transcribed locus	0.85462606	0
Myb	IMAGE:575702	Myeloblastosis oncogene	0.90313786	0
Ifit1	IMAGE:575371	Interferon-induced protein with tetratricopeptide repeats 1	0.8970446	0
Lims1	IMAGE:583635	LIM and senescent cell antigen-like domains 1	0.8956814	0
EST	IMAGE:639730	Expressed sequence tag	0.8338604	0
EST	IMAGE:576310	Expressed sequence tag	0.84747314	0
D10Ertd610e	IMAGE:576334	DNA segment, Chr 10, ERATO Doi 610, expressed	0.8760762	0
Rela	IMAGE:574694	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	0.89305645	2.4721346
Ifit2	IMAGE:574088	Interferon-induced protein with tetratricopeptide repeats 2	0.890293	2.4721346
Transcribed locus	IMAGE:575254		0.81951696	2.3994248
Ccr9	IMAGE:573572	Heterogeneous nuclear ribonucleoprotein A1	0.8542561	2.2661235
Zwilch	IMAGE:639781	Zwilch, kinetochore associated, homolog (Drosophila)	0.9069887	2.2661235
AI662270	IMAGE:583873	Expressed sequence AI662270	0.91842496	2.204877
Clec2	IMAGE:574821	C-type lectin domain family 1, member b	0.8481572	2.0918062
EST	IMAGE:583181	Expressed sequence tag	0.90183985	2.0918062
EST	IMAGE:640424	Expressed sequence tag	0.89434534	2.0395112
Tbx21	IMAGE:138129 7	Transcribed locus	0.86731595	1.989767
Chd9	IMAGE:639708	Chromodomain helicase DNA binding protein 9	0.91148907	1.9423915
Mecp2	IMAGE:576274	Methyl CpG binding protein 2	0.915491	3.6257975
Cdca8	IMAGE:576454	Cell division cycle associated 8	0.89017075	3.6257975
Polr3d	IMAGE:573740	Polymerase (RNA) III (DNA directed) polypeptide D	0.9152492	3.6257975
2810475A17Rik	IMAGE:640512	Transmembrane protein 48	0.90270126	3.4715083

Cnot10	IMAGE:576406	CCR4-NOT transcription complex, subunit 10	0.91797125	2.9665616
5930434B04Rik	IMAGE:583644	RIKEN cDNA 5930434B04 gene	0.9124771	2.9665616
EST	IMAGE:582312	Expressed sequence tag	0.8512796	2.9665616
D14Ert449e	IMAGE:577726	DNA segment, Chr 14, ERATO Doi 449, expressed	0.9229027	2.9665616
EST	IMAGE:575208	Expressed sequence tag	0.8866187	4.012153
Stx18	IMAGE:640560	Syntaxin 18	0.8706409	4.012153
Ap2a2	IMAGE:639674	Adaptor protein complex AP-2, alpha 2 subunit	0.90311587	4.012153
3110031B13Rik	IMAGE:583227	RIKEN cDNA 3110031B13 gene	0.9055848	4.012153
Sfrs1	IMAGE:583015	Splicing factor, arginine/serine-rich 1 (ASF/SF2)	0.7740291	4.012153
CD80	IMAGE:518134 3	CD80 molecule	0.88077635	3.7652512
Tusc2	IMAGE:583440	Hyaluronoglucosaminidase 2	0.9165395	3.7652512
2410016F01Rik	IMAGE:640076	Ring finger protein 187	0.92492443	3.7652512
Vamp1	IMAGE:573892	Vesicle-associated membrane protein 1	0.892336	3.6528556
EST	IMAGE:575844	Expressed sequence tag	0.91412157	3.5469759
Arl12	IMAGE:581822	ADP-ribosylation factor-like 5C	0.87418383	3.5469759
FASLG	IMAGE:517859 6	Fas ligand (TNF superfamily, member 6)	0.8506223	4.409754
2010106G01Rik	IMAGE:575420	RIKEN cDNA 2010106G01 gene	0.9245134	4.350957
EST	IMAGE:640562	Expressed sequence tag	0.90648896	3.9315877
2610510L01Rik	IMAGE:574272	Zinc finger, matrin type 5	0.9106585	4.7988496
Bcas3	IMAGE:583447	Breast carcinoma amplified sequence 3	0.9313725	4.583171
EST	IMAGE:575796	Expressed sequence tag	0.93010813	4.583171
Nol6	IMAGE:583756	Nucleolar protein family 6 (RNA-associated)	0.94241685	4.583171
Il16	IMAGE:573050	Interleukin 16	0.8774276	4.583171
Anxa4	IMAGE:583430	Annexin A4	0.92426145	4.532247
Btg1	IMAGE:574654	B-cell translocation gene 1, anti-proliferative	0.7811802	4.482442
Misc12	IMAGE:583716	MIS12 homolog (yeast)	0.89525664	4.4337196

Gzma	IMAGE:572830	Granzyme A	0.8852119	5.4909916
1700095N21Rik	IMAGE:640611	Stam binding protein like 1	0.9253962	5.4909916
Rab10	IMAGE:573868	RAB10, member RAS oncogene family	0.8931526	5.4909916
EST	IMAGE:640027	Expressed sequence tag	0.93267274	6.1570144
Hspa8	IMAGE:575712	Heat shock protein 8	0.92555046	6.1570144
2810455D13Rik	IMAGE:582820	RIKEN cDNA 2810455D13 gene	0.91810757	6.0994725
Rhebl1	IMAGE:576372	Ras homolog enriched in brain like 1	0.8832183	6.042996
EST	IMAGE:583299	Expressed sequence tag	0.903509	6.6146307
Tmem 60	IMAGE:639684	Transmembrane protein 60	0.9299942	6.6146307
H2-Eb1	IMAGE:574155	Histocompatibility 2, class II antigen E beta	0.8886254	6.6146307
Dibd1	IMAGE:639947	Asparagine-linked glycosylation 9 homolog (yeast, alpha 1,2 mannosyltransferase)	0.9231943	6.5555716
EST	IMAGE:582894	Expressed sequence tag	0.9101722	6.4405613
Abca5	IMAGE:582867	ATP-binding cassette, sub-family A (ABC1), member 5	0.91500676	6.4405613
EST	IMAGE:575632	Expressed sequence tag	0.93499315	6.3845563
Btg1	IMAGE:582825	B-cell translocation gene 1, anti-proliferative	0.9065552	6.9726872
EST	IMAGE:640794	Expressed sequence tag	0.88052225	6.9726872
2210404O07Rik	IMAGE:573122	RIKEN cDNA 2210404O07 gene	0.9250655	6.6869216
EST	IMAGE:640040	Expressed sequence tag	0.911263	6.6869216
B930006L02Rik	IMAGE:640875	Transcribed locus	0.86167	6.6869216
Lef1	IMAGE:575374	Lymphoid enhancer binding factor 1	0.90452534	6.6325564
Pank2	IMAGE:583251	Pantothenate kinase 2 (Hallervorden-Spatz syndrome)	0.9153259	7.8317227
EST	IMAGE:640717	Expressed sequence tag	0.888545	7.8317227
EST	IMAGE:640843	Expressed sequence tag	0.92703193	7.5888786
Ankrd10	IMAGE:583182	Ankyrin repeat domain 10	0.916508	7.5888786
Cd8a	IMAGE:124701 9	CD8 antigen, alpha chain	0.93137217	7.5888786
Sit	IMAGE:576098	Suppression inducing transmembrane adaptor 1	0.9147729	8.095769

EST	IMAGE:576142	Expressed sequence tag	0.9321476	8.095769
Mre11a	IMAGE:639936	Meiotic recombination 11 homolog A (<i>S. cerevisiae</i>)	0.8711923	8.034437
6820402O20Rik	IMAGE:640923	RNA binding motif protein 39	0.86235416	8.587415
Cybb	IMAGE:583187	Cytochrome b-245, beta polypeptide	0.89804614	8.762344
Rbl2	IMAGE:576225	Retinoblastoma-like 2	0.918051	8.762344
Cd83	IMAGE:574651	CD83 antigen	0.89952916	9.1921625
EST	IMAGE:641087	Expressed sequence tag	0.9061627	9.1921625
Hel308	IMAGE:581959	Helicase, mus308-like (<i>Drosophila</i>)	0.90254235	9.1921625
Klhl20	IMAGE:582908	Kelch-like 20 (<i>Drosophila</i>)	0.9313501	9.724821
EST	IMAGE:582322	Expressed sequence tag	0.9115154	9.724821
Atpaf1	IMAGE:639636	ATP synthase mitochondrial F1 complex assembly factor 1	0.9414004	9.724821
Park7	IMAGE:583034	Parkinson disease (autosomal recessive, early onset) 7	0.9238755	9.724821
Rhoa	IMAGE:572818	CDNA, clone:Y0G0104B14, strand:minus, reference:ENSEMBL: Mouse-Transcript-NST:ENSMUST00000007959, based on BLAT search	0.896466	9.724821
Transcribed locus	IMAGE:575780	Chemokine (C-C motif) ligand 25	0.93027353	10.575243
EST	IMAGE:136223 2	Expressed sequence tag	0.9037807	10.943718
C730042F17Rik	IMAGE:640700	ORM1-like 1 (<i>S. cerevisiae</i>)	0.8992265	10.909013
In multiple clusters	IMAGE:573267	RPA interacting protein	0.9084455	10.909013
Dbp	IMAGE:583416	D site albumin promoter binding protein	0.9236056	10.909013
Actr1a	IMAGE:583567	ARP1 actin-related protein 1 homolog A (yeast)	0.90226614	10.909013
Dnajc7	IMAGE:573602	DnaJ (Hsp40) homolog, subfamily C, member 7	0.8710109	10.909013
Nlk	IMAGE:582998	Nemo like kinase	0.9272456	11.317518
Ddx3x	IMAGE:639697	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked	0.9183363	12.837532
Elk4	IMAGE:583566	ELK4, member of ETS oncogene family	0.8972935	12.987931
EST	IMAGE:583908	Expressed sequence tag	0.8932793	12.987931

EST	IMAGE:576476	Expressed sequence tag	0.9310019	12.987931
Gucy2d	IMAGE:582819	Guanylate cyclase 2d	0.9221744	12.987931
Ncf4	IMAGE:576061	Neutrophil cytosolic factor 4	0.9115133	14.5946665
Pot1	IMAGE:640128	Protection of telomeres 1A	0.92453456	14.5946665
Btbd1	IMAGE:582942	BTB (POZ) domain containing 1	0.9308438	14.5946665
E130112N10Rik	IMAGE:640155	CD antigen 27	0.8934426	14.5946665
EST	IMAGE:641158	Expressed sequence tag	0.8800882	14.5946665
EST	IMAGE:576500	Expressed sequence tag	0.94008034	14.5946665
Slc22a7	IMAGE:574190	Solute carrier family 22 (organic anion transporter), member 7	0.9212938	14.5946665
EST	IMAGE:583342	Expressed sequence tag	0.9084793	14.5946665
Sh3gl1	IMAGE:582991	SH3-domain GRB2-like 1	0.90447086	14.5946665
Ier5	IMAGE:574793	Immediate early response 5	0.9171551	14.5946665
EST	IMAGE:640956	Expressed sequence tag	0.8923168	14.5946665
EST	IMAGE:640851	Expressed sequence tag	0.9186528	14.5946665
EST	IMAGE:583661	Expressed sequence tag	0.9314311	14.5946665
Brpf1	IMAGE:640234	Bromodomain and PHD finger containing, 1	0.93281096	14.5946665
Hexb	IMAGE:575852	Hexosaminidase B	0.9403777	14.5946665

Anexo III

Tabela IV. Genes da rede controle que fazem interação com o nó gênico Gucy2d e o tipo de interação.

Regulados positivamente por Gucy2d	Regulados negativamente por Gucy2d	Regulam positivamente Gucy2d	Regulam negativamente Gucy2d
Gclm Wdr53 IMAGE:640838 Prkdc Ccde41 IMAGE:582951 IMAGE:582879 3110037I16Rik IMAGE:640865 Dusp2 IMAGE:583053 IMAGE:640664 IMAGE:640521 H2-K1 Sfrs1 IL12A Ormdl1 Dnajc7 Cd27	Hist1h2bc Arl5c IMAGE:639730 IMAGE:575254 IMAGE:640875 Adhfe1 Rbm39 IMAGE:582312 Gucy2d	IMAGE:640272 IMAGE:583031 IMAGE:640280 IMAGE:640860 Hist1h2bc IMAGE:640351 1110037N09Rik Txnrd1 Gclm A430107D22Rik E230029C05Rik Nol5a IMAGE:640173 Rab22a Maea Cd97 Ifngr2 Cnot10 Anxa4 Hel308 Fos Mis12 Zmat5 Cd8a IMAGE:583908 Sfrs1 IMAGE:639730 Il16 IMAGE:575844 Sh3gl1 Cdca8 Gzma IMAGE:575254 IMAGE:640851 Adhfe1 Rbm39 Stx18 Ifit2 3110031B13Rik IL12A IMAGE:576142 IMAGE:573897	E2f2 Rpl27a IMAGE:640451 Mmp14 Wdr53 IMAGE:640838 Prkdc IMAGE:639827 Ccde41 IMAGE:582951 3110037I16Rik IMAGE:640865 Dusp2 Jmjd3 IMAGE:640837 2900076A07Rik IMAGE:583456 IMAGE:640667 Atg2b IMAGE:640341 IMAGE:582983 Plekha1 IMAGE:640598 H13 IMAGE:640121 Syngr2 Bcap31 IMAGE:583312 Cd28 Zhx1 FASLG FASLG CCR7 FCRH3 AI662270 LOC729230 IMAGE:639684 IMAGE:641087 IMAGE:1362232 IMAGE:640027 Nlk IMAGE:640717

		Ifit1	IMAGE:640843
		Rela	Arl5c
		IMAGE:575632	Lef1
		Grb2	2210404O07Rik
		Ccl25	Sit
		IMAGE:583299	IMAGE:640875
		Btbd1	IMAGE:639781
		Clec1b	IMAGE:640539
		IMAGE:583181	Stambpl1
		Slc22a7	Nol6
		Hnrpa1	Ptgs2
		IMAGE:575796	IMAGE:577726
		Bcas3	IMAGE:573361
		Myb	Ap2a2
		Polr3d	Abca5
		Rab10	Cd83
		Vamp1	Lims1
		Actr1a	2810455D13Rik
		IMAGE:582312	Spg20
		IMAGE:582322	CD80
		Rbl2	H2-Eb1
		IMAGE:583661	Ier5
		IMAGE:572818	Gucy2d
		Hspa8	Ormdl1
		Hexb	IMAGE:641158
			Dnajc7
			Cd27
			IMAGE:640956
			Mre11a
			Pot1a
			Btg1

Tabela V. Genes da rede experimental que fazem interação com o nó gênico Gucy2d e o tipo de interação.

Regulados positivamente por Gucy2d	Regulados negativamente por Gucy2d	Regulam positivamente Gucy2d	Regulam negativamente Gucy2d
IMAGE:640144	FASLG	Gclm	E2f2
E2f2	FASLG	Wdr53	IMAGE:640272
IMAGE:640272	CCR7	Prkdc	IMAGE:583031
HSPD1	FCRH3	IMAGE:582951	IMAGE:640280
IMAGE:583031	Cnot10	2900076A07Rik	IMAGE:640860
Rpl27a	Rnf187	IMAGE:583456	Itsn2
IMAGE:640280	Anxa4	Atg2b	Hist1h2bc
IMAGE:640860	AI662270	IMAGE:640598	Epc1
Itsn2	Chd9	H13	IMAGE:640351
Hist1h2bc	LOC729230	IMAGE:640121	1110037N09Rik
Epc1	IMAGE:639684	Eaf1	Txnrd1
IMAGE:640351	Hel308	FASLG	IMAGE:640943
1110037N09Rik	IMAGE:641087	FASLG	IMAGE:640817
IMAGE:640451	Klhl20	CCR7	A430107D22Rik
Mmp14	Fos	FCRH3	Ccdc41
Txnrd1	IMAGE:583342	Rnf187	IMAGE:582879
IMAGE:640943	IMAGE:1362232	AI662270	IMAGE:583053
IMAGE:640817	Mis12	Chd9	E230029C05Rik
Gclm	Zmat5	LOC729230	IMAGE:640664
Wdr53	IMAGE:640027	IMAGE:639684	IMAGE:640521
IMAGE:640838	Cd8a	IMAGE:641087	H2-K1
Prkdc	Nlk	Klhl20	Nde1
A430107D22Rik	IMAGE:640717	IMAGE:1362232	Nol5a
IMAGE:639827	IMAGE:640843	IMAGE:640027	IMAGE:582983
Ccdc41	IMAGE:583908	Nlk	IMAGE:640173
IMAGE:582951	Arl5c	IMAGE:640717	Myc
IMAGE:582879	IMAGE:639730	IMAGE:640843	IMAGE:641140
3110037I16Rik	Lef1	Arl5c	Gabarapl2
IMAGE:640865	IMAGE:575844	Lef1	IMAGE:138013
Dusp2	D10Ertd610e	D10Ertd610e	Apitd1
Jmjd3	2210404O07Rik	2210404O07Rik	Rab22a
IMAGE:583053	Alg9	Alg9	Mrpl34
IMAGE:640837	IMAGE:576500	IMAGE:576500	IMAGE:576044
2900076A07Rik	2810021B07Rik	2810021B07Rik	Maea
E230029C05Rik	IMAGE:575208	IMAGE:575208	Cd97
IMAGE:640664	Sit	Sit	IMAGE:641137
IMAGE:641123	Sh3gl1	IMAGE:575254	Ifngr2
IMAGE:640521	Cdca8	IMAGE:576476	Cnot10
H2-K1	Gzma	IMAGE:640794	Anxa4
IMAGE:583456	IMAGE:575254	IMAGE:639781	Hel308
IMAGE:640667	IMAGE:576476	IMAGE:640539	Fos
Atg2b	IMAGE:640794	Stambpl1	Mis12

Nde1	IMAGE:640875	IL12A	Zmat5
Nol5a	Adhfe1	IMAGE:3574183	5930434B04Rik
IMAGE:640341	Rbm39	Nol6	Cd8a
Rps13	IMAGE:639781	Ptgs2	IMAGE:583908
IMAGE:582983	Stx18	IMAGE:577726	Sfrs1
IMAGE:640173	Ifit2	Sdccag8	IMAGE:639730
Myg1	3110031B13Rik	Bag2	Il16
Myc	IMAGE:640539	IMAGE:573361	IMAGE:575844
IMAGE:583290	IL12A	IMAGE:640040	Sh3gl1
Plekha1	IMAGE:576142	Park7	Gzma
IMAGE:640598	IMAGE:3574183	IMAGE:640562	IMAGE:640851
IMAGE:641140	IMAGE:1381297	Abca5	IMAGE:640875
IMAGE:640299	IMAGE:573897	IMAGE:583182	Adhfe1
H13	Ifit1	Cd83	Rbm39
IMAGE:640381	Ptgs2	2810455D13Rik	Stx18
IMAGE:583308	IMAGE:577726	Btg1	Ifit2
IMAGE:640121	Sdccag8	Spg20	IMAGE:576142
Syng2	Bag2	CD80	IMAGE:573897
Gabarapl2	IMAGE:573361	Hyal2	IMAGE:575632
IMAGE:583900	Rela	Dbp	Ccl25
Bcap31	IMAGE:575632	Tmem48	Btbd1
IMAGE:138013	IMAGE:640424	H2-Eb1	Pank2
Apitd1	IMAGE:640040	Rhebl1	IMAGE:583181
1110008H02Rik	Grb2	Elk4	Lims1
Zfp213	Ap2a2	Ier5	Slc22a7
Rab22a	Park7	Ddx3x	IMAGE:575796
IMAGE:583312	Ccl25	Gucy2d	Myb
Mrpl34	IMAGE:583299	Ncf4	Polr3d
Eaf1	Btbd1	Ormdl1	Rab10
IMAGE:576044	Pank2	IMAGE:641158	Vamp1
Cd28	Clec1b	Dnajc7	Actr1a
IMAGE:641113	IMAGE:583181	Cd27	IMAGE:582312
Maea	IMAGE:640562	IMAGE:640956	Rpain
IMAGE:639637	Abca5	Brpf1	IMAGE:583661
IMAGE:641137	IMAGE:583182	Mre11a	IMAGE:572818
Zhx1	Cd83	Pot1a	Hspa8
Gucy2d	Lims1	Btg1	
	2810455D13Rik		
	Btg1		
	Spg20		
	Slc22a7		
	CD80		
	Hnrpa1		
	Mecp2		
	2010106G01Rik		
	Hyal2		
	IMAGE:575796		

	Dbp Bcas3 Myb Polr3d Tmem48 Rab10 H2-Eb1 Vamp1 Actr1a IMAGE:582312 Rhebl1 Rpain IMAGE:582322 Elk4 Cybb Ier5 Ddx3x Rbl2 Ncf4 IMAGE:583661 Ormdl1 IMAGE:641158 Dnajc7 Cd27 IMAGE:640956 Mre11a IMAGE:582894 Pot1a IMAGE:572818 Hspa8 Hexb Btg1		
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Manuscrito da Tese

Promiscuous gene expression is connected in network

Promiscuous gene expression in medullary thymic epithelial cells is connected in network where the Aire gene is an upstream modulator

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Abstract

The expression of peripheral tissue antigens (PTAs) in the thymus by medullary thymic epithelial cells (mTECs) is essential for the central self-tolerance of T cells. Due to heterogeneity of autoantigen representation this phenomenon has been termed promiscuous gene expression (PGE), in which the autoimmune regulator (Aire) gene plays a role as main transcriptional activator on a large set of Aire-dependent PTAs. Aire protein is able in binding to specific DNA sequence motifs and plays a role as a direct regulator. Here we used the cDNA microarray method to access PGE in murine CD80⁺ 3.10 mTEC cell strain cultured *in vitro*. Hierarchical clustering of the data allowed observation that PTA genes were differentially expressed being possible to found those induced or repressed. To further investigate the control of PGE, we hypothesize that PTA genes establish networks, reflecting the existence of a feedback-like control of the transcriptional levels. Aire in this case plays a role as upstream modulator. To test this hypothesis, initially we silenced Aire by gene knockdown (RNA interference) in 3.10 mTEC cells. Hierarchical clustering of cDNA microarray data showed a set of Aire-dependent PTA genes, which were downregulated after Aire silencing. Gene networks reconstructed from these data allowed the identification of a gene node (Gucy2d) establishing positive regulation upon downstream genes in normal mTECs. Nevertheless, under silencing of Aire, Gucy2d has become a repressor on PTA genes. These findings evidentiate that genes featured in PGE are connected in network, a gene node may act as intermediate in their control and that Aire in PGE network plays a role as an upstream regulator.

Introduction

Self-tolerance of the T cell repertoire is acquired during the development of immature T cells, a phenomenon dependent on the avidity of the interaction between specific T cell receptor (TCR) and self-peptide-MHC ligands. The nascent T cell repertoire in the thymus determines the diversity of self-antigens and the specificity of central-tolerance. We agree with Kyewski and Derbinski (2004) in subsuming under central tolerance the intrathymic mechanisms, which T cells undergo in recognition of self-antigens. Accordingly, self-reactive regulatory T (T_{reg}) cells are selected in the thymus, even though these cells play their role in the periphery.

In fact, all subsets of antigen-presenting cells (APCs), such as cortical thymic epithelial cells (cTECs), medullary thymic epithelial cells (mTECs), thymic dendritic cells and macrophages play their roles in presenting unique sets of self-peptides, contributing to the diversity of self-antigens displayed in the thymus (Klein and Kyewski 2000; Anderson et al., 2007).

However, the expression of PTAs in the thymus, which represents a key feature for effecting self-representation, was only recently recognized in the immunologic research. The evidence of thymic expression of PTAs in mice and humans, which has been referred to as promiscuous gene expression (PGE) (Jolicoeur et al., 1994; Derbinski et al., 2001; Gotter et al., 2004), reinforced the conception of central tolerance of tissue-restricted self-antigens.

Prior to this evidence, the peripheral tolerance, that is, the mechanisms by which T cell selection towards PTAs occurs outside the thymus,

dominated the scenario in explaining self-non-self discrimination (Alferink et al., 1999; Walker and Abbas 2002).

Promiscuous gene expression in the thymus is causing a reversal in the scope of central tolerance understanding, allowing for an unorthodox conception of the possible mechanisms of self-non-self discrimination (Kyewsky et al., 2002, Gallegos and Bevan, 2006; Magalhães et al., 2006; Sousa Cardoso et al., 2006).

The main implication of this heterogeneous gene expression in the thymus is associated with the maintenance of the immunological homeostasis in the body, controlling the pathogenic autoimmune reactions.

Evidence for this phenomenon was obtained using the reverse transcription-PCR (RT-PCR) method, which was biased towards antigens in autoimmune reactions, such as insulin, acetylcholine receptor or myelin basic protein. Today it is recognized that rather than selective, the set of expressed genes is as broad as possible, and involves up to 5-10% of the all known mouse genes (Derbinski et al., 2001; Gotter et al., 2004; Kyewski and Derbinski, 2004).

The extent of self-representation of most parenchymal tissues and organs is guaranteed by PGE in the thymus, a phenomenon exhibited by mTECs. Accordingly, the complexity of PGE increases in ascending order, from cortical thymic epithelial cells (cTECs) to immature mTECs to mature CD80+ mTECs. These different gene pools are not complementary but additive, that is, there is no apparent association between the respective molecular/biological function of the genes in parenchymal organs. The significance of PGE in the thymus is associated with central tolerance of T

cells (Sospedra et al., 1998; Bruno et al., 2002; Kyewski and Derbinski, 2004). While PGE was well characterized in mTECs by the authors above cited and in intact thymus by our group (Sousa Cardoso et al., 2006; Magalhães et al., 2006), their detailed molecular control requires further exploration.

A gene identified and cloned in 1997 was termed *AIRE* (autoimmune regulator) due to their association with autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy (APECED) syndrome, a human autoimmune disease, (Finnish-German APECED Consortium, 1997; Nagamine et al., 1997). Studies in mice have permitted an extensive mechanistic dissection of how Aire operates. It was found that Aire is expressed primarily in lymphoid organs, especially the thymus. Within the thymus it is expressed mostly by medullary epithelial cells (MECs) strongly by mTECs but also although at much lower levels, by dendritic cells (Anderson et al., 2002; Anderson et al., 2005).

Identifying Aire expression in MECs was intriguing because of suggestions that this cell-type is involved in the negative selection of self-reactive thymocytes (Kishimoto and Sprent, 1997) and because of the coincident emergence of data establishing that transcripts encoding PTAs are ectopically expressed specifically by MECs (Kyewski and Klein, 2006).

These evidences allowed the formulation the hypothesis that Aire regulates the thymic transcription of PTA genes and thus thymocyte tolerization and therefore autoimmune reactions.

The Aire-knockout mice harbored a normal immune system but featured multi-organ autoimmunity, as indicated by both inflammatory infiltrates and

serum autoantibodies, allowing confirming this hypothesis (Anderson et al., 2002; Ramsey et al., 2002; Kuroda et al., 2005).

Expression profiling of MECs isolated from Aire-knockout mice allowed the identification of autoantigen-encoding genes, such as insulin, salivary protein-1 and fatty-acid-binding protein, which are under its control (Anderson et al., 2002, 2005). Meanwhile, other PTA genes, such as C-reactive protein and glutamic acid decarboxylase of 67 kDa (GAD67), appeared to be independent of Aire expression (Derbinski et al., 2005). Aire also regulates the transcription of genes that do not encode PTAs.

As discussed by Mathis and Benoist (2007), a broad question still remain to be clarified on the molecular mechanisms underlying the function of Aire, that is, precisely how the protein coded by this gene promotes ectopic transcription of genes encoding PTAs and certain other proteins while repressing other loci.

Bioinformatic re-evaluations have shown that Aire transcription might influence a large number of genes, in the order of thousands rather than hundreds, representing a significant fraction of the genome (Derbinski et al., 2005).

Aire has many features of a transcription factor, sharing several domains with known transcriptional control elements (Bottomley et al., 2001; Isaac et al., 2006) and can also associate with other transcription factors, such as CERB-binding protein (CBP), at least *in vitro* (Pitkanen et al., 2000, 2005).

Other several characteristics of AIRE distinguish it from a “conventional” transcription factor that binds to a well defined DNA motif in an enhancer or promoter regions of a co-ordinately regulated genes. Certainly the effect of

AIRE on ectopic PTA gene expression is not that of a simple on/off operon; its effect on most genes is only partial, and a number of PTAs are not affected by Aire (Mathis and Benoist, 2007).

In addition the wideness of its transcriptional impact weakens the notion of a classical site-specific control element, as it is not easy to envision that an Aire-binding site occurs in thousands of promoters of such disparate structure (Mathis and Benoist, 2007).

These considerations may suggest an indirect mode of action for Aire. Here, we hypothesize that PTA genes in mTECs establish networks, reflecting the existence of a feedback-like control of the transcriptional levels. Aire in this case plays a role as upstream modulator.

Material and Methods

Medullary thymic epithelial cell line

Medullary 3.10 TEC line was kindly provided by Willem van Ewijk (Rotherdam University, The Netherlands) to one of us (W.S.). This cell line was established from C57BL/6 mice and medullary phenotype was determined by immunostaining with Th-4 antibody. They were further evaluated in one of our laboratory (W. Savino) using a panel of anti-cytokeratin monoclonal antibodies, which confirmed the original distinct medullary phenotype. Cells were cultured in 10% fetal bovine serum-supplemented RPMI 1640 medium at 37°C in a 5% CO₂ atmosphere.

Silencing Aire gene transcript

We used the TriFectaTM (IDT, Integrated DNA Technologies, USA) anti-Aire siRNA sequence (GGAUUCUCUUUAAGGACUACAAUCTAGAUUGUAGUCCUUAAGAGAAUCCUC) to silence Aire mRNA. Confluent cultures of 3.10 mTEC cell line

were transfected with 10 nM of anti-Aire siRNA using Hiperfect reagent (Qiagen) following manufacturer's instructions.

After transfection, cells were cultured during 24 h in RPMI medium as above mentioned and total RNA was extracted using the mirVana kit (Ambion), which served as template for cDNA synthesis. Gene knockdown was confirmed by semi-quantitative reverse transcription PCR (RT-PCR) using the primers 5' CATCCTGGATTCTGGAGGA 3' forward and 5' GCTCTTTGAGGCCAGAGTTG 3' reverse, which allowed amplification of a 253 bp PCR product corresponding to a segment of the Aire mRNA (cDNA). The PCR mix contained 200 μ M dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM TRIS-HCl (pH 8.4), 10 μ M each primer and 2 U Taq DNA polymerase. The cycling temperature was: 35 x 30 sec each (94°C, 57°C and 72°C).

An anti-HPRT siRNA included in this kit but whose sequence was not furnished was used in parallel to evaluate the efficiency of the gene knockdown assay on 3.10 mTEC cell line (data not shown).

cDNA microarray

The gene expression of 3.10 mTEC cell line was assessed using glass slide cDNA microarrays prepared on silane-coated UltraGAPS slides (# 40015, Corning®, New York, NY, USA) containing a total of 4,500 target tissue restricted antigen cDNA sequences representing most of murine tissue and organs. These were from the Soares thymus 2NbMT normalized library, representing expressed sequence tag (ESTs) cDNA clones prepared from the thymus of a C57BL/6J 4-week-old male mouse, and available at the IMAGE Consortium (<http://image.llnl.gov/image/html/iresources.shtml>). The cDNA inserts were homogeneous in size (near 1 kb), cloned in three vectors (pT7T3D,

pBluescript and Lafmid) and were amplified in 384- or 96-well plates using vector-PCR amplification with the following primers, which recognize the three vectors: LBP 1S GTGGAATTGTGAGCGGATACC forward and LBP 1AS GCAAGGCGATTAAGTTGG reverse.

The microarrays were prepared based on published protocols using PCR products from the cDNA clones [Hedge et al., 2000] using a Generation III Array Spotter (Amersham Molecular Dynamics, Sunnyvale, CA, USA). A complete file providing all genes and ESTs present in the microarrays used in this study is available on line at (www.rge.fmrp.usp.br/passos/mmu_array)

Complex cDNA probe preparation and hybridization

The cDNA complex probes derived from total RNA obtained of control or Aire silenced 3.10 mTECs were prepared by reverse transcription using 10 µg of total RNA. For this study, we opted for monocolour labeling and then the cDNA samples were labeled with Cy3 fluorochrome using the CyScribe post labeling kit (GE Healthcare Biosciences). The 15 hour period required for hybridization was allowed to elapse, followed by washing, using an automatic slide processor system (ASP, Amersham Biosciences) and microarrays were scanned using a Generation III laser scanner (Amersham Biosciences).

As a reference for the hybridization procedure, we used equimolar quantities of cDNAs obtained from unrelated total RNA (mouse thymus total RNA). This approach allowed estimation of the amount of cDNA target in each microarray spot.

cDNA microarray data analysis

The microarray image quantification was performed using the Spotfinder software (<http://www.tm4.org/spotfinder.html>). The normalization process was carried out using the R platform (<http://www.r-project.org>) and statistical data

were analyzed using the Multiexperiment Viewer (MeV) software (version 3.1; available online at (<http://www.tm4.org/mev.html>) [Smyth, 2004].

The differentially expressed genes (induced or repressed) between control and Aire silenced mTECs were identified by using the significance analysis of microarrays program (SAM) (Tusher et al., 2001) considering only those genes presenting both FDR (false discovery rate) and p value ≤ 0.01 .

To analyze the gene expression profiling we used an unsupervised hierarchical clustering method that grouped genes on the vertical axis and samples on the horizontal axis, on the basis of similarity in their expression patterns. The similarities and dissimilarities in gene expression are presented as dendrograms, in which the pattern and length of the branches reflect the relatedness of the samples or genes, and as heat maps (Eisen et al., 1989) (Cluster version 3.0 and Java Tree View (<http://rana.lbl.gov/EisenSoftware.htm>).

Determination of PGE in mTECs

PGE was identified on the basis of data from microarray analysis of the different mouse organs using combined information from the public database GNF Gene expression Atlas (<http://symatlas.gnf.org/SymAtlas>). This data bank shows gene expression in more than 60 mouse tissue/organs, as assessed by gene array analysis using Affymetrix microarrays. Data information includes GenBank accession, chromosomal location, tissue/organ representation and molecular/biological function of each gene analyzed.

In this study, only the promiscuous genes of which the expression was detected in different organs or tissues and of which the expression levels were greater than median in relation to all other organs which appear in the GNF Atlas were considered. The modulation of transcription levels (repression or

induction) of these genes was evaluated comparing control and Aire silenced 3.10 mTECs.

Reconstruction of gene networks

The GeneNetwork 1.2 algorithm [Wu et al., 2004] was used to compare means of different gene expression values whose standard deviation did not overlap, whose objective was to compute a network that established relationships between all genes. The linear interpolation for the network organization was used. The software for this algorithm can be obtained from the authors (<http://idv.sinica.edu.tw/hchuang/GeneNetwork1.2Setup.exe>).

In order to organize networks only with genes whose expression values were significant, values for the induced and repressed genes selected by the SAM statistics were used. In this case, of the 4,500 sequences present on the microarray, a total of 212 from control and 212 from Aire silenced 3.10 mTECs differentially expressed genes were included in the calculations of the GeneNetwork software due to their statistical significance.

Results and Discussion

PGE exhibited by mTECs in the thymus is a complex phenomenon until now observed in humans and mice and involves 5-10% of all known genes of these species. This process is a guarantee of self-representation of most parenchymal tissues and organs during the central tolerance of T cells.

Using RT-PCR, some of the most important autoantigens mRNAs, such as thyroglobulin, CRP, GAD67, insulin and albumin were detectable only in mTECs

purified from *ex vivo* mouse thymic tissue at embryonic day 15 (E15) (Derbinski et al., 2001).

In view of that, the complexity of PGE increases in, from cTECs to immature mTECs to mature CD80^{hi} mTECs. As the PGE is a phenomenon associated to autoantigen representation, the different gene pools are not complementary, but additive, that is, there is no apparent association between the molecular/biological functions of the genes representing parenchymal organs and tissues. The significance of PGE in the thymus is related with the central tolerance to self antigens (Kyewski and Derbinski, 2004; Sospedra et al., 1998; Bruno et al., 2002; Bruno et al., 2004; Derbinski et al., 2005; Gallegos et al., 2006).

In a recent study made by our group and using RT-PCR, we observed that the Aire gene start its expression in embryonic mouse thymus at E16 stage and by cDNA microarray method, it was observed that PGE emerges as high throughput gene expression at E20 stage (Sousa Cardoso et al., 2006).

PGE is today a well documented phenomenon, but several of their aspects are still unexplored, as for example, its evaluation in the thymus of autoimmune and/or knockout mouse strains, its modulation by means of cytokines or other molecules interfering in the gene expression, such as RNA interference.

In the present study, we used a purified CD80⁺ mature mTEC cell line growing *in vitro* to further explore the role of the Aire gene in the control of PGE.

Initially we demonstrated by RT-PCR that the 3.10 mTEC cell line expresses Aire gene corroborating previous data from the literature (Figure 1).

We were able in the silencing this gene by RNA interference (RNAi) (Figure 2), which although partial, allowed to evidentiate the PTA genes, which were

down regulated in mTEC cells. Figure 3 shows the gene expression hierarchical clustering of statistically significant (SAM program) microarray data of control and Aire-silenced mTEC cells. It was possible to identify 212 differentially expressed PTA genes, which 135 were induced in control mTECs and repressed after Aire silencing.

This gene data set was compared with GNF database allowing picturing the PGE featured by mTECs following the effect of Aire silencing. The PTA genes were grouped in 17 body systems, which are represented in this mTEC cell line (Figure 4). It was possible to find that these 135 PTA genes are under control of the Aire gene, since its silencing caused their repression.

Nevertheless, still remained the question about if the genes, which feature PGE are connected in networks and what is the role of the Aire gene in their control.

Two widely used methods for microarray data analysis are the Cluster-and-Tree View (Eisen et al., 1989), which is based on a mathematical description of similarity and the organization of gene expression data is realized by grouping together genes with similar patterns of expression (hierarchical clustering), and the Significance Analysis of Microarray (SAM) (Tusher et al., 2001), which is based on *t* test statistics specifically developed for high-throughput microarray data.

Although these methods are useful tools for the identification of differentially expressed genes, they not permit the determination of interactions between genes.

There are several computational solutions proposed in the literature to infer gene networks; the approaches based on the mathematical formalism of the

information theory as ARACNE program (Basso et al., 2005; Margolin et al., 2006), the algorithms that consider ordinary differential equations as NIR, NMI and TSNI programs (Gardner et al., 2003; Di Bernardo et al., 2005; Bansal et al., 2006) and those which solve the inference problem by means of Bayesian networks as Banjo (Yu et al., 2004) and GeneNetwork (Wu et al., 2004) programs.

Comparing the algorithms, GeneNetwork was chosen for the analysis purposes due to its differential features. The algorithm realizes the interpolation of data and is able to handle with more than one thousand of genes. Moreover, the resulting network is described by an oriented graph in which positive arrows indicate induction and negative ones denote repression between genes.

In an effort to provide a clearer understanding of the high-throughput data resulting from microarray experiments and to establish regulatory interactions in PGE, normalized data were analyzed by the GeneNetwork program (Wu et al., 2004), which is based on dynamic Bayesian statistics inferring gene networks.

Figure 5a shows that PTA genes featuring PGE in mTECs are in fact connected in network. It was found that the *Gucy2d* (guanylate cyclase 2d) gene, which was induced in control mTECs, plays a role as a gene node due to its numerous interactions, mostly as a positive controller, established among other genes.

Silencing Aire in mTECs, the *Gucy2d* gene was subsequently repressed but still played a role as a gene node, which instead positive turned a negative controller, repressing mostly of PTA genes (Figure 5b).

These findings are important firstly to evidenciate that PTA genes in mTECs are connected in network, which might reflect the existence of a feedback-like

control of their transcriptional levels, secondly that a gene, such as *Gucy2d*, may act as additional positive controller upon downstream genes besides Aire in the control of PGE.

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Figures

Figure 1. rt-PCR to Aire gene in mTEC 3.10 cells and the negative control.

Figure 2. Semi-quantitative rt-PCR to Aire gene.

Figure 3. The gene expression hierarchical clustering of statistically significant (SAM program) microarray data of control and Aire-silenced mTEC cells. The clustering was performed using Pearson correlation and average linkage, in TIGR MEV program (<http://www.tm4.org/mev.html>).

Figure 4. The 17 systems and its representation in mTEC cells.

Súmula Curricular

CURRICULUM VITAE (Janeiro 2008)

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FUNÇÃO ATUAL

Doutorado: Departamento de Genética, Faculdade de Medicina de Ribeirão Preto-USP, Campus de Ribeirão Preto, com bolsa de pesquisa CNPq. Área de concentração: **Genética**.

ESCOLARIDADE

- ✓ **Doutor em Ciências (em andamento)** pela Faculdade de Medicina de Ribeirão Preto. Área de concentração Genética: sub-área: Imunogenética Molecular – Projeto de Tese: *O papel modulador do gene Aire (autoimmune regulator) sobre redes de expressão gênica em células tímicas epiteliais medulares.*
- ✓ **Bacharel em Ciências Biológicas** pela Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (USP), no período de 1996 a 2000 – Diploma Registrado sob Nº 1274474, processo 2001.1.325.59-0.
- ✓ **Licenciada em Ciências Biológicas** pela Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (USP), no período de 1996 a 2000 – Diploma Registrado sob Nº 1274475, processo 2001.1.325.59-0.

CURSOS DE EXTENSÃO UNIVERSITÁRIA

- ✓ *"Transcriptome Analysis: Theoretical Basis and Applications"*, realizado no período de 29 de novembro a 2 de dezembro de 2005, INSERM Research Director Catherine Nguyen – Marseille, França, Departamento de Genética da Faculdade de Medicina de Ribeirão Preto-USP cumprindo carga horária total de 16 horas.
- ✓ *"Utilizando a técnica de RNA interference (RNAi) para silenciamento gênico"*, realizado durante o 49º Congresso Nacional de Genética em Águas de Lindóia, SP, de 16 a 19 de setembro de 2003, com carga horária de 3 horas.
- ✓ *"Update on Genome Projects and Genomics"*, realizado no período de 25 a 28 de Setembro de 2000, CNRS emeritus Research Director Bertrand Jordan – Marseille, França, Departamento de Genética, FMRP-USP, Ribeirão Preto, SP.
- ✓ Curso e Simpósio *"Tecnologia de microarrays – abordagem teórico-prática"*, realizado nos dias 6 e 7 de julho de 2000, IBiLCE – UNESP, São José do Rio Preto, SP.
- ✓ *"Large Scale Gene Expression Measurement Using DNA-arrays"*, realizado no período de 2 a 6 de agosto de 1999, INSERM Research Catherine Nguyen – Marseille, França, Departamento de Genética da Faculdade de Medicina de Ribeirão Preto-USP cumprindo carga horária total de 48 horas.
- ✓ *"Diversidade Genética e Evolução do Sistema Imune"*, realizado durante o 44º Congresso Nacional de Genética em Águas de Lindóia, SP, de 9 a 12 de setembro de 1998, com carga horária de 3 horas.
- ✓ *"Genética Molecular do Sistema Imune e Engenharia de Anticorpos"*, realizado durante o 44º Congresso Nacional de Genética em Águas de Lindóia, SP, de 9 a 12 de setembro de 1998, com carga horária de 3 horas.
- ✓ *"Aspectos Moleculares da Regulação Gênica"*, realizado durante a Semana de Bio-estudos 1997, na FFCLRP-USP, de 3 a 7 de novembro, com carga horária de 20 horas.
- ✓ *"Interface Imunologia Genética"*, realizado durante a Semana de Bio-estudos 1996, na FFCLRP-USP, de 4 a 8 de novembro, com carga horária de 9 horas.
- ✓ *"Imunologia Básica"*, realizado na FFCLRP-USP, em novembro de 1996, com carga horária de 12 horas.

ESTÁGIOS

- **Laboratório de Imunogenética Molecular** do Departamento de Genética da Faculdade de Medicina de Ribeirão Preto-USP – sob orientação do Prof. Dr. Geraldo A.S. Passos, no período de julho de 1997 a dezembro de 2000.

ESTÁGIO NO EXTERIOR

- **Laboratório INSERM ERM 206**, (TAGC – Technologies Avancées pour le Génome et la Clinique) localizado na cidade de Marseille, França e dirigido por Catherine Nguyen Com linha de pesquisa voltada para estudos de Transcriptoma, utilizando a tecnologia dos Microarrays Humanos e Murinos. Fevereiro a outubro de 2006.

MONITORIAS E AULAS PRÁTICAS

- 1) Instituição: FMRP-USP
XIII Curso de Verão de Genética: Departamento de Genética (graduação)
Data: 14 janeiro a 25 de Janeiro de 2008.
Título: Noções Práticas sobre a aplicação de cDNA-microarrays.
- 2) Instituição: FMRP-USP
X Curso de Verão de Genética: Departamento de Genética (graduação)
Data: 31 janeiro a 06 de fevereiro de 2005.
Título: Noções Práticas sobre a aplicação de cDNA-microarrays.
- 3) Instituição: FMRP-USP
VIII Curso de Verão em Genética: Departamento de Genética (graduação)
Data: 3 a 7 de fevereiro de 2003.
Título: Análise da expressão Gênica em larga escala utilizando DNA-arrays.
- 4) Instituição: CRBio-1ª Região
13º Encontro de Biólogos do CRBio-1 (SP,MT,MS)
Data: 25 a 28 de março de 2002.
Monitora.
- 5) Instituição: CRBio-1ª Região
11º Encontro de Biólogos do CRBio-1 (SP,MT,MS)
Data: 17 a 20 de abril de 2000.
Monitora.
- 6) Instituição: FFCLRP-USP
Disciplina “Genética Molecular e Citogenética Básica e Aplicada”.
Data: 2º semestre de 1999.
Monitora Voluntária de Estágio teórico-prático.
- 7) Instituição: FFCLRP-USP
Disciplina “Genética”.
Data: 1º semestre de 1999.
Monitora Voluntária de Estágio teórico-prático.
- 8) Instituição: FFCLRP-USP
Disciplina “Elementos de Anatomia e Fisiologia Humana”.
Data: 1º semestre de 1997.
Monitora Voluntária de Estágio teórico-prático.

PALESTRAS PROFERIDAS

- Palestra intitulada “Uso de cDNA-microarrays na identificação de genes envolvidos no processo de carcinogênese”. Apresentada durante o XIII Curso de Verão de Genética no Departamento de Genética da Faculdade de Medicina de Ribeirão Preto – USP, no período de 14 a 25 de janeiro de 2008.
- Palestra intitulada “Uso de cDNA-microarrays na identificação de genes envolvidos no processo de carcinogênese”. Apresentada durante o X Curso de Verão de Genética no Departamento de Genética da Faculdade de Medicina de Ribeirão Preto – USP, no período de 17 a 28 de janeiro de 2005.
- Palestra intitulada “Análise da expressão gênica em larga escala utilizando cDNA-arrays. Apresentada durante o VIII Curso de Verão de Genética no Departamento de Genética da Faculdade de Medicina de Ribeirão Preto – USP no período de 20 a 31 de janeiro de 2003.

EXPERIÊNCIA DIDÁTICA

Estágio de Licenciatura com carga horária de 360 horas distribuídos em colégios de Ensino Fundamental e Médio, da rede pública de ensino de Ribeirão Preto, São Paulo, com aplicações práticas das teorias dos Cursos de Graduação: “Didática I e II”, “Programação de Curso”, “Prática de Ensino em Ciências” e “Prática de Ensino em Biologia”.

PRODUÇÃO CIENTÍFICA

A) TRABALHOS PUBLICADOS EM PERIÓDICOS (COMPLETO)

- 1) Sousa-Cardoso R, Magalhães DAR, Baião AMT, Junta CM, **Macedo C**, Marques MMC, Sakamoto-Hojo ET, Donadi EA, Passos GAS Onset of Promicuous Gene Expression in Murine Fetal Thymus Organ Culture (FTOC). Immunology. Londres.Vol aceito. 2006.
- 2) Cardoso RS, Junta CM, **Macedo C**, Silveira ELV, Paula MO, Marques MMC, Magalhães DAR, Mello SS, Záráte-Bladés CR, Nguyen C, Houlgate R, Donadi EA, Sakamoto-Hojo ET, Passos, GAS Hybridization Signatures of Gamma-Irradiated Murine Fetal Thymus Organ Culture (FTOC) Reveal Modulation of Genes Associated with V (D) J Recombination and DNA Repair. Mollecular Immunology. Oxford: v. 43, p. 464-472 , 2006.
- 3) Magalhães DAR, **Macedo C**, Junta CM, Mello SS, Marques MMC, Cardoso RS, Sakamoto-Hojo ET, Donadi EA, Passos, GAS Hybridization Signatures During Thymus Ontogeny Reveals Modulation of Genes Coding for T-cell Signaling Proteins. Molecular Immunology. Oxford: v.42, n.9, p.1043 - 1048, 2005.
- 4) **Macedo C**, INITIATIVE, The Ludwig Fapesp Transcript Finishing. A

transcript finishing initiative for closing gaps in the human transcriptome.. Genome Research. , v.14, p.1413 - 1423, 2004.

5) Espanhol AR, **Macedo C**, Junta CM, Cardoso RS, Victorero G, Lloriod B, Nguyen C, Jordan B, Passos GAS. Gene expression profiling during thymus ontogeny and its association with TCRVB8.1-DB2.1 rearrangements of inbred mouse strains. Molecular and Cellular Biochemistry. , v.252, p.223 - 228, 2003.

6) Sandrin-Garcia P, **Macedo C**, Martelli LR, Ramos ES, Guion-Almeida ML, Richieri-Costa A, Passos GAS. Recurrent 22q11.2 deletion in a sibship suggestive of parental germline mosaicism in velocardiofacial syndrome.. Clinical Genetics. , v.61, p.380 - 383, 2002.

7) **Macedo C**, Junta CM, Passos GAS. Onset of T-cell receptor V 8.1 and D 2.1 V(D)J recombination during thymus development of inbred mouse strains.. Immunology Letters. , v.69, p.371 - 373, 1999.

B) TRABALHOS SUBMETIDOS

1) **Macedo C**; Magalhães DAR; Tonani M; Marques MMC; Junta CM; Passos GAS. Genes coding for T cell signaling proteins establish transcriptional regulatory networks during thymus ontogeny. Artigo Submetido.

C) TRABALHOS PUBLICADOS EM ANAIS (RESUMOS) EM CONGRESSOS NO BRASIL

1) **Macedo C**; Magalhães DAR; Tonani M; Marques MMC; Junta CM; Passos GAS. Transcriptional regulation during thymus ontogeny reveals networks between genes coding for T cell signaling proteins. 53º Congresso Brasileiro de Genética, 2007, Águas de Lindóia.

2) Magalhães DAR; **Macedo C**; Joly F; Lloriod B; Boulanger N; Victorero G; Puthier D ; Nguyen C; Passos GAS. Molecular Dissection of the Mouse Thymus Based on Cell-Type Specific Gene Expression. 53º Congresso Brasileiro de Genética, 2007, Águas de Lindóia.

3) Magalhães DAR; **Macedo C**; Joly F; Puthier D; Lloriod B; Boulanger N; Victorero G; Nguyen C; Passos GAS. Identification of Specific T-Cell Gene Expression Profiles During the Fetal Thymus Maturation. 52º Congresso Brasileiro de Genética, 2006, Foz do Iguaçu.

4) **Macedo C**; Junta CM; Sandrin-Garcia P; Fachin AL; Sakamoto-Hojo ET; Donadi EA; Passos GAS. Assinaturas de expressão gênica de células mononucleares sanguíneas e sua relação com a indução de câncer pelo metilcolantreno. 51º Congresso Brasileiro de Genética, 2005, Águas de Lindóia - SP.

- 5) Magalhães DAR; Junta CM; **Macedo C**; Sandrin-Garcia P; Mello SS; Fachin AL; Sakamoto-Hojo ET; Donadi EA; Passos GAS . Identification of genes preferentially expressed by the thymus during ontogeny. 51º Congresso Brasileiro de Genética, 2005, Águas de Lindóia - SP.
- 6) Lobo, CH; Magalhães DAR; Franco, TM; **Macedo C**; Brassesco, MS. Presença de NORs em uma linhagem celular de *Apis mellifera* testada por FISH e AgNO₃. 51º Congresso Brasileiro de Genética, 2005, Águas de Lindóia - SP.
- 7) Paula MO; Zárate-Bladés CR; Silveira EL; Fachin AL; Junta CM; Mello SS; Magalhães DAR; **Macedo C**; Sandrin-Garcia P; Silva CL; Donadi EA; Sakamoto-Hojo ET; Passos GAS . Interleukin 7 modulates the gene expression in murine adult thymus organ culture. XXIX Meeting of Brazilian Society of Immunology, 2004, Ouro Preto - MG.
- 8) Magalhães DAR; Junta CM; **Macedo C**; Sandrin-Garcia P; Mello SS; Fachin AL; Silveira EL; Paula MO; Zárate-Bladés CR; Sakamoto-Hojo ET; Donadi EA; Passos GAS . cDNA MICROARRAYS MAY POTENTIALLY IDENTIFY NEW CANDIDATES GENES THAT MODULATE THE IN VIVO T CELL MATURATION. XXIX Meeting of Brazilian Society of Immunology, 2004, Ouro Preto - MG.
- 9) Zárate-Blades CR; Silveira EL; Paula MO; Fachin AL; Junta CM; Magalhães DAR; **Macedo C**; Donadi EA; Sakamoto-Hojo ET; Passos GAS. High-Troughput gene expression profile of *Mycobacterium tuberculosis* infected mice treated with two distinct hsp65-DNA vaccines. XXIX Meeting of the Brazilian Society of Immunology, 2004, 2004, Ouro Preto.
- 10) INITIATIVE, The Ludwig Fapesp Transcript Finishing ; **Macedo C**. Transcript Finishing Initiative (Tfi): New Genes Identification And Annotation.. XXXII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2003, Caxambu.
- 11) Magalhães DAR; Junta CM; **Macedo C**; Mello SS; Marques MMC ; Cardoso RS; Sakamoto-Hojo ET; Donadi EA; Passos GAS. Uso de cDNA microarrays na identificação de novos genes candidatos à modulação da recombinação V(D)J dos receptores de células T (TCR). 49º Congresso Nacional de Genética, 2003, Águas de Lindóia - SP.
- 12) Magalhães DAR; **Macedo C**; Junta CM; Passos GAS . Emergência da recombinação V(D)J de TCRV 8.1 e análise da expressão gênica no timo de heteozigotos entre linhagem isogênica de camundongos. 13º Encontro de Biólogos - CRBio1, 2002, São Pedro.
- 13) Magalhães DAR; **Macedo C**; Junta CM; Passos GAS . Detecção do início da recombinação V(D)J durante a ontogenia do timo de heterozigotos entre

linhagens isogênicas de camundongos. 48º Congresso Nacional de Genética, 2002, Águas de Lindóia. Genetics end Molecular Biology, 2002.

14) INITIATIVE, The Ludwig Fapesp Transcript Finishing; **Macedo C** The Transcript Finishing Initiative (Tfi) Project Transcript Finishing Consortium. XXXI Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular (SBBq), 2002, Caxambu-MG.

15) Passos GAS; Junta CM; Espanhol AR; **Macedo C**; Bertrand J; Nguyen C; Victorero G; Loriod B; Granjeaud, S. Using DNA-arrays to search new genes expressed in the thymus during V(D)J recombination of T-cell receptor (TCR V 8.1). 45º Congresso Nacional de Genética., 1999, Gramado. Genetics and Molecular Biology.

16) **Macedo C**; Junta CM; Passos GAS. Onset of T-cell receptor Vb8.1 and Db2.1 V(D)J recombination during thymus development of inbred mouse strains. In: International Meeting on Vaccines, 1998, Salvador-BA.

D) TRABALHOS PUBLICADOS EM CONGRESSOS E/OU REUNIÕES CIENTÍFICAS NO EXTERIOR

1) Magalhães DAR, **Macedo C**, Junta CM, Mello SS, Marques MMC, Cardoso RS, Sakamoto-Hojo ET, Donadi EA, Passos GAS. Association of T-cell receptor beta (TRBV8.1-DB2.1) rearrangement with hybridization Signatures During Thymus Ontogeny. In: 6th International Meeting of Microarrays Gene Expression Data Society (MGED), 2003, Aix en Provence-France, 2003.

2) Espanhol AR; **Macedo C**; Junta CM; Cardoso RS; Victorero G; Loriod B; Nguyen C; Bertrand J; Passos GAS . Genes Differentially Expressed during TCR V 8.1-D 2.1 rearrangements in vivo in the thymus of inbred mouse strains.. In: 11th International Congress of Immunology. 2001, Stockholm. Scandinavian Journal of Immunology. Stockholm: Blackwell Science, 2001. v. 54. p. 9-9.

PRÊMIOS E TÍTULOS

1º Prêmio de Apresentação de Trabalhos concedido pela Comissão de Avaliação de Painéis do Conselho Regional de Biologia - 1ª região, com o trabalho intitulado “Emergência da recombinação V(D)J de TCRVB8.1 e análise da expressão gênica no timo de heterozigotos entre linhagens isogênicas de camundongos” apresentado durante o 13º Encontro de Biólogos do CRBio-1 realizado em São Pedro - SP de 25 a 28 de março de 2002.

RELATOR DE TRABALHOS

Atuação como avaliadora na área de Genética, no 12º SICUSP- Simpósio de Iniciação Científica da Universidade de São Paulo- Áreas Ciências Biológicas,

realizado pela Comissão de Pesquisa da Faculdade de Filosofia, Ciências e Letras da USP no Centro de Convenções de Ribeirão Preto em 23 de dezembro de 2004, com carga horária de 3 horas.

PARTICIPAÇÃO EM CONGRESSOS E REUNIÕES CIENTÍFICAS NO BRASIL

- 53º Congresso Brasileiro de Genética, realizado em Águas de Lindóia-SP no período de 2 a 5 de setembro de 2007.
- 51º Congresso Brasileiro de Genética, realizado em Águas de Lindóia-SP no período de 7 a 10 de setembro de 2005.
- VI Simpósio Nacional de Biologia Molecular Aplicada à Medicina, realizado em Santos-SP de 8 a 9 de abril de 2005
- 49º Congresso Nacional de Genética, realizado em Águas de Lindóia-SP de 16 a 19 de setembro de 2003.
- 48º Congresso Nacional de Genética, realizado em Águas de Lindóia-SP de 17 a 20 de setembro de 2002.
- 13º Encontro de Biólogos - CRBio1, realizado em São Pedro-SP de 25 a 28 de março de 2002.
- Simpósio dos 30 anos da Pós-Graduação em genética da FMRP-USP, dias 14 e 15 de dezembro de 2001.
- Brazilian International Genome Conference, realizado em Angra dos Reis-RJ de 26 a 29 de março de 2001.
- 11º Encontro de Biólogos - CRBio1, realizado em São Pedro-SP de 17 a 20 de abril de 2000.
- 45º Congresso Nacional de Genética, realizado em Gramado-RS de 3 a 6 de outubro de 1999.
- International Meeting on Vaccines, realizado em Salvador-BA de 29 de novembro a 3 de dezembro de 1998.
- 44º Congresso Nacional de Genética, realizado em Águas de Lindóia-SP de 9 a 12 de setembro de 1998.

BOLSAS DE ESTUDO

- **Bolsa de Iniciação científica** concedida pela FAPESP Fundação de Amparo à Pesquisa do Estado de São Paulo), para o desenvolvimento do projeto de iniciação científica: *Emergência da recombinação V(D)J no locus TCRB durante a ontogenia do timo*, sob a orientação do Prof. Dr Geraldo A.S. Passos.
- **Bolsa de Doutorado** concedida pelo CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) na área de Genética, para o desenvolvimento do projeto da tese de Doutorado, sob a orientação do Prof. Dr Geraldo A.S. Passos .
- **Bolsa de Estágio de Doutorado (Doutorado-Sanduiche)** concedida pelo CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) na área de Genética, pelo período de fevereiro a outubro de 2006, sob a orientação do Prof. Dr Geraldo A.S. Passos, no Brasil, e supervisão da Dra. Catherine Nguyen, diretora da unidade INSERM ERM 206, Marseille – França.

Proficiência em Línguas

- **Certificado de Proviência em Francês** “Test de Français” aplicado pela “Délégation Générale de L’Alliance Française au Brésil” com nível bom no francês oral e escrito, realizado no dia 19 de agosto de 2005.
- **TEAP – Test of English for Academic Purposes** na área de Saúde/ Biológicas e obtido pontuação (0-10) 7,5 - Tese Prime - TB03/005 em 05 de novembro de 2002, Ribeirão Preto-SP.

Claudia Macedo

Publicações

**Genes coding for T cell signaling proteins establish
transcriptional regulatory networks during thymus ontogeny**

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Abstract

The definition of gene expression profiling by cDNA microarrays during murine thymus ontogeny has contributed to dissecting the large-scale molecular genetics of T cell maturation. Gene profiling, although useful for characterizing the thymus developmental phases and identifying the differentially expressed genes, does not permit the determination of possible interactions between genes. In order to reconstruct genetic interactions during a murine thymus period of development, which is important for thymocyte differentiation (14-17 days of gestation), a pair of microarrays was used containing a total of 1,576 cDNA sequences derived from the IMAGE MTB library, whose data were analyzed using the GeneNetwork program. Characteristic interactions involving genes were observed during thymus ontogeny, which were previously identified as differentially expressed and once included in a network, showed their relationships with several other genes. The present method provided the detection of gene nodes implicated in the calcium signaling pathway, such as *Prr2* and *Stxbp3*, and in protein transport towards the cell membrane, such as *Gosr2*. The results demonstrate the feasibility of reconstructing networks based on cDNA microarray gene expression determinations, contributing to a clearer understanding of the complex interactions between genes involved in thymus/thymocyte development.

Keywords Thymus development, gene expression, microarray, gene networks

Introduction

The thymus is an important organ implicated in the control of the main functions of the immune system, providing a specialized microenvironment for the maturation of thymocytes into positive TCR α/β T cells [1,2]. Organ functioning begins early during fetal development, when immature thymocytes from the bone marrow reach the thymus and thousand of genes are simultaneously modulated in both, with thymocytes and thymic stroma contributing to tolerance induction to self-antigens [3-8].

Deciphering these genes is a complex task and DNA microarray technology, including mathematical and statistical algorithms for data analysis, such as hierarchical clustering [9] and significance analysis of microarrays (SAM) [10], have permitted the description of the modulation of several of such genes, featuring gene expression signatures [6-8,11] .

Recent subsuming realized that most biochemical relationships among genes act in a synchronized fashion, involving many genes at once, in which one gene can exert many functions and one function can be exerted by many genes [12].

Accordingly, a biological phenomenon needs to be interpreted as a whole biochemical system and in order to grasp it, a comprehensive data set needs to be produced.

One example of a highly complex biochemical system is thymocyte differentiation into mature T cells. It occurs within the fetal thymus and all developmental stages are distinguishable by their expression of a combination of clusters of differentiation (CD) cell-surface markers.

This is a highly modulated phenomenon, whose central molecular machinery is formed by the recombinase complex (RAG-1 and RAG-2) directly implicated

in the V(D)J recombination of T cell receptor gene segments (TRA, TRB, TRG and TRD) [13].

The early fetal thymus has homogeneous cell populations composed of lymphoid progenitors derived from the bone marrow, which is formed by double-negative (DN1), CD44^{high} CD25⁺ (DN2), CD44^{low} CD25⁺ (DN3), and CD44^{low} CD25⁻ (DN4) thymocytes. During DN to CD4⁺ CD8⁺ double-positive (DP) transition, thymocytes relocate to the thymic cortical region, where most do not recognize the available MHC molecules expressed by thymic stromal cells and die as a result.

The remaining DP thymocytes undergo negative selection occurring both in the cortex and in medulla, leading to the deletion of autoreactive clones and favoring MHC-restricted CD4 and CD8 single-positive T cells (CD4⁺ SP and CD8⁺ SP), which finally gain the periphery [14,15].

In contrast, the newborn and adult thymus is the arrival place of new colonizing precursors, which contribute to the formation of heterogeneous cell populations, including macrophages and dendritic cells [16].

Studies on temporal V(D)J recombination of TRA and TRB genes [17,18] and differential expression of immune system-related genes, such as interleukins and the major histocompatibility complex (MHC) [19] during thymus ontogeny, showed that the genetic background of different inbred mouse strains plays an important role in the modulation of the emergence of T cell maturation.

The cDNA microarray, as a high-throughput functional genomic technology, provides comparative analysis between the different phases of thymus ontogeny, yielding quantitative expression data of hundreds or thousands of genes in a single experiment. Research on gene expression during thymus

ontogeny using such technology has been the subject of extensive studies [6-8,11,20-22].

Nevertheless, current understanding regarding the molecular pathways of thymocyte maturation is still considered as fragmented and the results often remain difficult to situate in an integrated and dynamic view of T cell differentiation [6].

Recently a high-throughput data set containing thousands of gene expression values during murine thymus ontogeny, including nonmanipulated and knockout strains, was generated by cDNA microarray technology and analyzed by SAM or hierarchical clustering programs [6-8,11], which permitted the monitoring of numerous differentially expressed genes simultaneously.

The differentially expressed genes identified by SAM statistics and grouped by hierarchical clustering provided a valuable data set for new insights into how these genes could interact with each other.

In this study, the interactive relationships of constructing networks among these differentially expressed genes of thymus development observed during the gestational period of (Balb-c x C57Bl/6) F₁ hybrid mice were evaluated.

Hybridization data from nylon cDNA microarrays containing a total of 1,576 thymus IMAGE sequences were analyzed using the GeneNetwork 1.2 software [12], whose results showed that each phase of thymus development exhibited a characteristic interaction between genes, permitting the identification of important genetic nodes, i.e., genes that exert an inductive/repressive effect on other genes or contrary to this, are the targets of several other genes.

Among the genetic nodes observed, those implicated in intracellular protein transport were the most interactive, which may represent important genetic cascades during the development of the thymus/thymocytes.

Material and methods

Fetal thymus and RNA preparation

The (♀Balb-c x ♂C57Bl/6) F₁ hybrid mice were obtained in our own laboratory. The animals were maintained in a 0.45μ air filtered isolator, on a 12 hour light/dark cycle, a temperature of 23°C ± 2 °C and feed and water *ad libitum*. Pregnant Balb-c females were sacrificed by cervical dislocation and the fetuses were surgically collected from the uterus. The age of the fetuses (in days post coitum, p.c.) was confirmed on the basis of the morphological characteristics of each development phase [23] (Rugh, 1968). Thymic samples collected from 14 to 17 days p.c. were used, a stage when the maturation of the thymocytes and the emergence of the T cell receptor V beta genes occur [7,18].

Fetal thymus tissue (1 mg containing approximately 1 x 10⁷ cells) was obtained by surgery under a stereomicroscope and immediately processed for total RNA extraction using Trizol® reagent (Invitrogen). Only protein- and phenol-free RNA preparations were used, as determined by UV spectrophotometry, and those featuring native undegraded RNA species in standard denaturing agarose gel electrophoresis.

cDNA microarray method

A pair of cDNA microarrays was used, containing a total of 1,576 clones in the form of PCR products, spotted in duplicate on 2.5 x 7.5 cm Hybond N+ nylon membranes (GE Healthcare). The arrays were prepared in our own

laboratory using a Generation III Array Spotter (Amersham-Molecular Dynamics, Sunnyvale, CA, USA). The complete file providing all genes present in the microarrays used in this study is available online at (http://rge.fmrp.usp.br/passos/nylon_array/mtb1).

Mouse expressed sequence tag (EST) cDNA clones were obtained from the Soares thymus 2NbMT normalized library, prepared from a C57Bl/6J 4-week-old male thymus, and available at the I.M.A.G.E. Consortium (<http://image.llnl.gov/image/html/iresources.shtml>).

cDNA inserts were homogeneous in size (approximately 1 Kb) and cloned in three vectors (pT7T3D, pBluescript and Lafmid) and were amplified in 384- or 96-well plates using vector-PCR amplification with the following primers, which recognize the three vectors, LBP 1S 5'-GTGGAATTGTGAGCGGATACC-3' forward and LBP 1AS 5'-GCAAGGCGATTAAGTTTGG-3' reverse.

The membranes were first hybridized with the LBP 1AS[γ -³³P]dCTP (GE Healthcare, USA) labeled oligonucleotide (vector hybridization). Quantification of the signals obtained permitted the estimation of the amount of DNA deposited in each spot. After stripping, the membranes were subsequently used for hybridization with thymus cDNA complex probes (sample hybridization).

The annotation of each cDNA sequence was updated using S.O.U.R.C.E. (<http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch>) and DAVID (<http://david.abcc.ncifcrf.gov>) databases, allowing for a set of information: gene name, sequence, biological and molecular functions.

Complex cDNA probe preparation and hybridization

In this study, we refer to the radioactive cDNA originated from the thymus RNA samples as the complex probe and the PCR product originated from the clones and deposited on the nylon microarrays as the target.

The ^{33}P -labeled cDNA complex probes were prepared by reverse transcription of 10 μg of thymus total RNA using oligo dT₁₂₋₁₈ as a primer. One hundred microliters of ^{33}P -cDNA complex probe containing 30- 50-million cpm was hybridized with nylon microarrays, as previously described [24-27].

Imaging acquisition

Imaging plates and a phosphor imager storage system (Cyclone model, Packard Instruments, USA) were used to capture the hybridization signals and the Array Vision ® (Imaging Research Inc. USA) was used to quantify the signals with local background subtraction, whose spots were matched with a template grid.

The ratio between vector probe hybridization values and complex probe hybridization values for each spot was used as a reference normalization value. Total intensity normalization, using the median expression value, was also used [28].

cDNA microarray data analysis

Statistics

The gene expression data analyzed here were obtained from three independent determinations for each day of fetal development.

The SAM method (Significance Analysis of Microarrays), available online at <http://www-stat.stanford.edu/~tibs/SAM/index.html> [10], was used to analyze the significant variations in the gene expression. This method is based on *t* test statistics, specially modified for high-throughput analysis. Significant variations in gene expression of the developing thymus were compared with that found at 14 days p.c. (14 vs 15, 14 vs 16 and 14 vs 17 days of gestation). The program calculates the chance of global error and the false discovery rate (FDR) and, in this study, data from genes with $FDR \leq 0.05$ was used.

Gene networks

The GeneNetwork 1.2 algorithm [12] was used to compare means of different gene expression values whose standard deviation did not overlap, whose objective was to compute a network that established relationships between all genes. The linear interpolation for the network organization was used. The software for this algorithm can be obtained from the authors (<http://idv.sinica.edu.tw/hchuang/GeneNetwork1.2Setup.exe>).

In order to organize networks only with genes whose expression values were significant, values for the induced and repressed genes selected by the SAM statistics were used. In this case, of the 1,576 sequences present on the microarrays, a total of 104 (from 14 vs 15 days p.c.), 63 (from 14 vs 16 days p.c.) and 39 (from 14 vs 17 days p.c.) differentially expressed genes were included in the calculations of the GeneNetwork software due to their statistical significance.

Finally, to evaluate the consistency of the results obtained, the networks developed by our group were compared to those reported in the literature and compiled by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>).

Results

Differentially expressed genes

To identify significant changes in gene expression during fetal development of the thymus, the stage when the thymocytes mature and the onset of T cell receptor V beta 8.1 (TRBV8.1) recombination occurs in the (♀Balb-c x ♂C57Bl/6) F₁ hybrid mice (at 14 days of gestation p.c.) was compared with those observed on subsequent days (14 vs 15, 14 vs 16 and 14 vs 17 days p.c.). The demarcation of the emergence of TRBV8.1 recombination in these hybrid mice was realized in a previous study by our group [7] and confirmed in the samples used in this study (data not shown).

A scatter plot of the observed relative difference $d(i)$ versus the expected relative difference $d_E(i)$ was used, as shown by the SAM program.

Most of the 1,576 sequences tested presented $d(i) \approx d_E(i)$, i.e., their expression pattern did not change; however, some genes were significantly modulated during thymus development, which were then analyzed in the GeneNetwork program (Fig. 1).

Gene networks

As previously mentioned, the data of the significantly expressed genes revealed by the SAM statistics were input into the GeneNetwork 1.2 program to visualize their possible relationships during thymus development.

The early thymus (14 vs 15 days p.c.) was the most dynamic in terms of gene interactions. Of the 104 differentially expressed genes in this phase, a set of 21 were found to participate in this network; these represent gene nodes (Fig. 1a).

The subsequent days of development featured 12 gene nodes at 14 vs 16 days p.c. (Fig. 1b) and eight gene nodes at 14 vs 17 days p.c. (Fig. 1c), participating in their respective networks. The remaining 83 (14 vs 15 days p.c.), 51 (14 vs 16 days p.c.) and 31 genes (14 vs 17 days p.c.), although differentially expressed, do not appear in these networks.

Four possible types of interactions were observed between gene nodes participating in the networks. The first type was positive interaction, i.e. when a gene g positively regulated a gene h ($g \rightarrow h$), the second was a negative interaction, i.e. when a gene g negatively regulated a gene h ($g \rightarrow h$), the third featured simultaneous positive interactions, i.e. when a gene g positively regulated a gene h and vice-versa ($g \leftrightarrow h$) and the fourth was a positive self-interaction, i.e. when a gene g positively regulated itself ($g \leftrightarrow g$).

Discussion

Two widely used methods for microarray data analysis are the Cluster-and-Tree View [9], which is based on a mathematical description of similarity and the organization of gene expression data is realized by grouping together genes with similar patterns of expression (hierarchical clustering), and the Significance

Analysis of Microarray (SAM) [10], which is based on t test statistics specifically developed for high-throughput microarray data.

Although these methods are useful tools for the identification of differentially expressed genes, they not permit the determination of interactions between genes.

There are several computational solutions proposed in the literature to infer gene networks; the approaches based on the mathematical formalism of the information theory as ARACNE proram [29,30], the algorithms that consider ordinary differential equations as NIR, NMI and TSNi programs [31-33] and those which solve the inference problem by means of Bayesian networks as Banjo [34] and GeneNetwork [12] programs.

Comparing the algorithms, GeneNetwork was chosen for the analysis purposes due to its differential features. The algorithm realizes the interpolation of data and is able to handle with more than one thousand of genes. Moreover, the resulting network is described by an oriented graph in which positive arrows indicate induction and negative ones denote repression between genes.

In an effort to provide a clearer understanding of the high-throughput data resulting from microarray experiments and to establish regulatory gene interactions during murine thymus ontogeny, normalized data were analyzed by the GeneNetwork program [12], which is based on dynamic Bayesian statistics inferring gene networks.

Recently, gene networks were reconstructed based on microarray data studying model organisms, such as *S. cerevisiae* [35], *E. coli* [36] and *Drosophila* [37]. Additionally, gene networks were reconstructed for mammalian

cells, including human herpesvirus-infected T cells [38] and astrocyte differentiation in rat cells [39].

To the best of our knowledge, this is the first study showing gene network reconstruction from microarray data during normal thymus development. Three networks were reconstructed, corresponding to a period of thymus ontogeny important for the differentiation of thymocytes to mature T cells, in which it was possible to indicate gene nodes, i.e., genes establishing positive and/or negative interactions with other genes, including self-regulation. It is subsuming that nodes represents the main points in the control of genetic cascades.

We agree with the discussion of de La Fuente that networks obtained with only part of a genome, as is the case of the set of genes examined in this study, are still useful representation of the underlying gene regulatory structure. Accordingly, if a gene A influences the expression of gene B through the action of gene C, but gene C is not known (or its oligo/cDNA sequence was not included in the microarray used), the gene network algorithm will find a regulatory effect of A on B [40].

For this reason, and trying to minimize this oversight, in this study only gene nodes establishing self-regulation and/or at least three interactions with other genes and whose pathways were confirmed in the KEGG pathway data base were selected for discussion.

The significantly modulated genes during thymus ontogeny featured nodes in the three networks establishing positive or negative interaction among them.

As mentioned, 14-15 days of gestation p.c. was the most dynamic phase in terms of gene interactions (Fig. 1A). Genes implicated in the calcium signaling pathway, such as the Proline-rich Gla (G-carboxyglutamic acid) polypeptide 2

gene, *Prr2*, (mRNA accession number NM_022999, ID 640686) and the Syntaxin binding protein 3 gene, *Stxbp3*, (mRNA accession number NM_011504, ID 640484), whose respective coded proteins play a role in the inhibition of the calcium influx pathway via calcineurin, correspond to important nodes in this and in subsequent phases.

These genes participate in the control of calcium influx pathway mediated by calcineurin, which is important in the activation of the nuclear factor of activated T cells (*Nfat*), a T cell transcription factor. As calcium signaling is implicated in the induction of T cell anergy mediated through calcineurin and NFAT [41-43], the nodes identified could be useful to provide a clearer understanding of the gene interactions implicated in the immunological tolerance induction.

Twenty-one gene nodes were identified at 14 vs 15 days p.c. and eight were selected for discussion:

1) The *Prr2* is a gene node featuring self positive regulation, was positively regulated by 19 other genes (of the 21 found in the network) and was indirect-positively regulated by two others, i.e. two genes interacted first and then positively regulated the *Prr2* gene; the *Cope* gene positively regulated the *Prmt3* gene, which positively regulated the *Prr2* gene: *Cope* → *Prmt3* → *Prr2*. This strongly suggests the importance of maintaining the *Prr2* gene transcription levels. A negative feedback-like regulation, between the *Prr2* gene and Golgi SNAP receptor complex 2 gene, *Gosr2*, (mRNA accession number NM_019650, ID 640152) was found. While the *Gosr2* gene positively regulates *Prr2*, the latter presented an inhibitory effect on *Gosr2*.

2) The *Stxbp3a* gene node also featured self positive regulation and was positively regulated by three genes participating in the network. However, this

gene was mostly indirectly positively regulated. Even so, this strongly suggests the importance of maintaining the *Stxbp3a* transcription levels. Moreover, this gene presented an inhibitory effect on *Gosr2*.

3) The Protein arginine N-methyltransferase 3 enzyme gene, *Prmt3*, (mRNA accession number NM_133740, ID 639644) was another main node identified, participating in tryptophan and androgen/estrogen metabolism. This gene was positively self-regulated and positively regulated by 11 genes, including the *Prr2* and *Stxbp3a* genes discussed above, and the Anaphase promoting complex subunit 2 gene, *Anapc2*, (mRNA accession number NM_175300, ID 640882), whose protein product is an essential component of the SCF (skp1-cul1-f-box protein) e3 ubiquitin ligase complex, which mediates the ubiquitylation of proteins involved in cell cycle progression, signal transduction and transcription.

4) The *Zfp420* gene also participates as an important node, featuring positive self-regulation and positively regulated by other genes, such as *Prmt3*, *Prrg2* and *Stxbp3a*. Interestingly, the positive interactions with these genes were bidirectional, i.e. $Zfp420 \leftrightarrow Prmt3$, $Zfp420 \leftrightarrow Prrg2$ and $Zfp420 \leftrightarrow Stxbp3a$, featuring a feedback-like interaction. *Zfp420* gene presented an inhibitory effect on *Gosr2*.

5) The *Gosr2* gene, whose coded protein is a SNARE member involved in intracellular protein transport, was also identified as a node. *Gosr2*, in its turn, was negatively regulated by the four genes discussed above (*Stxbp3a*, *Prmt3*, *Prrg2* and the Zinger finger protein 420 gene, *Zfp420*, mRNA accession number NM_172740, ID 640711).

6-8) The participation of three transcribed loci was identified, none with an assigned function to date, characterizing three nodes. One of them (ID 639992)

was positively self-regulated and was positively regulated by several other genes, including *Prmt3*, *Prrg2* and *Stxbp3a* and these interactions were bidirectional, as mentioned above. The second transcribed locus (ID 574022) positively regulated *Prmt3* and *Prrg2* genes and was positively regulated by seven genes and indirectly regulated by several others. The third transcribed locus (ID 640082) defines a node positively regulated by three genes, *Prrg2*, *Prmt3* and *Zfp420*. This strongly suggests that these sequences, although still anonymous, could play an important regulatory role during thymus development.

It was possible to define 12 nodes at 14 vs 16 days p.c., of which seven were chosen for discussion (Fig. 1B).

1) The Lunatic fringe gene homolog (*Drosophila*), *Fringe* (*Lnfg*), (mRNA accession number NM_008494, ID 640133), which participates in the Notch signaling pathway via delta receptor and is important for T cell differentiation, was identified as a gene node that positively regulated two genes (*Dock8* and *Cast*) and two transcribed loci (ID 640292 and ID 640674).

2) The next gene node identified was the *Gosr2* and, similar to that observed at 14 vs 15 days p.c., it was negatively regulated, in this case by two genes (*Cast* and *Fringe*) and by one transcribed locus (ID 640674).

3) The Cyclin-dependent kinase 4 gene, *Cdk4*, (mRNA accession number NM_009870, ID 640338) also was identified as a negatively regulated gene node, interacting with three genes (*Dock8*, *Cast* and *Fringe*) and two transcribed loci (ID 640292 and ID 640674). CDK4 is a late element participating in the T cell receptor signaling pathway, which starts with the attachment of CAM ligands of antigen presenting cells on TCR α/β on

lymphocytes and finally associates with proliferation-differentiation molecules, such as *Cdk4*.

4) The Calpastatin gene, *Cast*, (mRNA accession number NM_009817, ID 640722) represents a dynamic gene node that was positively self-regulated, negatively regulated the *Cdk4* gene and positively regulated by four genes (*Fringe*, *Dock8*, *Emb* and *Kif3c*) and two transcribed loci (ID 640292 and ID 640710). Their coded protein, calpastatin is a natural inhibitor of the enzyme calpain, which catalyzes protein cleavage.

5-6) The transcribed locus (ID 640674), although anonymous, represents another dynamic gene node, which was positively self-regulated, regulated by five genes (*Cast*, *Lnfg*, *Dock8*, *Emb* and *Kif3c*) and by two anonymous transcribed loci (ID 640292 and ID 640643). The anonymous transcribed locus (ID 640292) also featured a gene node, which was positively self-regulated, regulated by two genes (*Cast* and *Lnfg*) and by the anonymous transcribed locus (ID 640674). It positively regulated the *Dock8* gene and negatively regulated the *Cdk4* gene.

7) The Dedicator of cytokinesis 8 gene, *Dock8*, (mRNA accession number NM_028785, ID 639971), whose coded protein is a guanyl-nucleotide releasing factor, featured another gene node. Of the five gene interactions, four were positive, including a feedback-like interaction with the *Cast* gene (*Dock8* ↔ *Cast*) and three others; one with the *Fringe* gene and two with the transcribed loci, ID 640292 and ID 640674, respectively. Moreover, *Dock8* negatively regulated the *Cdk4* gene.

The network of 14 vs 16 days p.c. is particularly interesting, due to the presence of sequences uncharacterized in terms of their biological function,

opening a new perspective for the discovery of genes participating in T cell differentiation.

Finally, 14 vs 17 days p.c. exhibited eight gene nodes, of which four were chosen for discussion (Fig. 1C).

1) The first gene node is represented by the *Stxbp3a* gene, which participates in intracellular protein traffic and transport. It was negatively regulated by itself, by the *Tacc1* gene and by the transcribed locus (ID 640711) and positively regulated the Capicua homolog (*Drosophila*) gene, *Cic* (mRNA accession number NM_027882).

2) The *Cic* gene was also identified as a node, which was positively regulated by the *Stxbp3a* and *Tacc1* genes and by the transcribed locus (ID 640711). This indicates that *Cic* gene node could play a role in the late phase of thymus development.

3) Another gene node identified was the Transforming acidic coiled-coil-containing protein 1 gene, *Tacc1* (mRNA accession number NM_177089, ID 640235), which is expressed at high levels during early embryogenesis and whose function is likely involved in the processes that promote cell division prior to the formation of differentiated tissues. This gene node was positively regulated by four others; the transcribed locus ID 640711 and the *Prrg2*, *Gosr2* and *Triobp* genes, in addition to positive self-regulation. It established negative regulation with the *Stxbp3a* gene in both directions (*Tacc1* ↔ *Stxbp3a*) and positively regulated the *Cic* gene. Due to its positively regulated profile and biological function, this gene node represents an important candidate for a role in thymus/thymocyte development.

4) Finally, *Prrg2* was also identified as a gene node, which was negatively regulated by five (*Itm2b*, *transcribed locus ID 640711*, *Stxbp3a*, *Triobp* and *Gosr2*) of the eight gene nodes in this phase and also exhibited self-negative regulation. However, it positively regulated the *Tacc1* node.

Taken together, these findings indicate that thymus ontogeny (14-17 days of gestation) features dynamic gene networks, involving pathways implicated in cell signaling and protein transport, including T cell receptor chains towards the cell membrane.

However, is important to consider that the networks reconstructed in this study are the result of specialized statistical microarray data analysis. Further investigations are necessary to determine their precise molecular mechanisms, i.e. involvement of RNA-RNA or DNA-RNA associations controlling the networks.

The probabilistic gene interactions observed in this work open new perspective for further molecular/biochemical approaches to confirm the role of genetic cascades in thymus development.

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Figure legend

Figure 1. Gene networks during thymus development reconstructed from cDNA microarray gene expression data. A) 14 vs 15 days p.c. B) 14 vs 16 days p.c. and C) 14 vs 17 days p.c. The italicized symbols represent those discussed gene nodes.

Onset of promiscuous gene expression in murine fetal thymus organ culture

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Summary

T-cell differentiation and induction of tolerance to self-antigens occurs mainly in the thymus. Thymic stromal cells, specifically medullary thymic epithelial cells, express a diverse set of genes encoding parenchymal organ-specific proteins. This phenomenon has been termed promiscuous gene expression (PGE) and has been implicated in preventing organ-specific autoimmunity by inducing T-cell tolerance to self antigens. Early thymopoiesis and the critical factors involved in T-cell differentiation can be reproduced *in vitro* by murine fetal thymus organ culture (FTOC), which mimics the natural thymic microenvironment. To evaluate the occurrence of PGE in FTOC, gene expression profiling during *in vitro* thymic development in BALB/c mice was performed using a set of nylon cDNA microarrays containing 9216 sequences. The statistical analysis of the microarray data (SAM program) revealed the temporal repression and induction of 57 parenchymal and seven lymphoid organ-specific genes. Most of the genes analysed are repressed during early thymic development (15–17 days post-coitum). The expression of the autoimmune regulator (*AIRE*) gene at 16 days post-coitum marks the onset of PGE. This precedes the induction of parenchymal organ genes during the late developmental phase at 20 days post-coitum. The mechanism of T-cell tolerance induction begins during fetal development and continues into adulthood. Our findings are significant because they show a fine demarcation of PGE onset, which plays a central role in induction of T-cell tolerance.

Keywords: cDNA microarray; fetal thymus organ culture; promiscuous gene expression; thymus; T-cell tolerance

Introduction

The thymus is the key primary lymphoid organ that is mainly involved in the progressive differentiation of thymocytes to mature T cells in which all developmental stages are distinguishable by their expression of a combination of clusters of differentiation (CD) cell-surface markers. Studies with freshly obtained fetal thymus allowed for the demarcation of T-cell receptor β V(D)J recombination during *in vivo* thymus ontogeny among different inbred mouse strains; this contributed to the revealing of the effect of the genetic background on T-cell development.^{1–3} The control of gene expression during the development of this organ has gained priority among

several research groups, including our own, leading to the identification of candidate genes involved in thymopoiesis. Using the cDNA microarray method, a dozen expressed sequence tags have been found that are modulated during the *in vivo* development of the thymus.⁴ Using the same kind of analysis, the *in vivo* modulation of several cell-signalling genes was identified, including those of the calcium cascade pathway, which is important for individual stages of T-cell maturation and the control of energy during murine thymus ontogeny.⁵

Meanwhile, a better understanding of the central tolerance mechanism, that is, all the mechanisms by which T cells contribute to self-tolerance by intrathymic recognition of self-antigens, emerged from evidence that

tissue-specific antigens are expressed in the thymus and contribute to the selection of the T-cell repertoire. These tissue-specific antigens are expressed as a normal physiological property of thymic epithelial cells (TECs).⁶ This phenomenon was termed promiscuous gene expression (PGE), which includes self antigens that represent most of the parenchymal organs.^{7–14} Evidence of PGE in the thymus is causing a reversal in our understanding of central tolerance, allowing for an unorthodox conception of the possible mechanism of self–non-self discrimination.^{15,16}

The initial evidence for promiscuously expressed genes was biased towards antigens involved in autoimmune reactions, such as insulin, acetylcholine receptor or myelin basic protein. Today it is recognized that rather than being selective, the set of expressed genes is as broad as possible, estimated to include up to 5–10% of all currently known mouse genes.⁹ Thus, the main implication of this heterogeneous gene expression in the thymus is associated with maintenance of the immunological homeostasis in the body, controlling pathogenic autoimmune reactions.

Studies on thymus gene expression in the broadest aspect, including PGE, are normally designed to be performed immediately using samples collected by surgery, which must reflect the short-term *in vivo* situation.

Nevertheless, fetal thymus organ culture (FTOC), introduced by DeLuca's group,¹⁷ which is based on the use of early fetal thymus tissue, preserves the original architecture of the thymus, with its cellularity formed of stromal TECs and homogeneous thymocyte population double-negative cells. This is a singular, powerful culture model system reproducing intrathymic T-cell development *in vivo*, making it easier to study the candidate genes implicated in normal thymus development. Using the cDNA microarray method, our group recently showed the modulation of gene expression in murine FTOCs at the transcriptome level and revealed an overlap between genes associated with T-cell receptor V(D)J recombination and DNA repair. We showed that the association of FTOC and cDNA microarray technology allows sufficient accuracy to uncover the participation of essential genes implicated in thymus development.¹⁸

In the present study, cDNA microarrays were employed to observe the onset of PGE during the *in vitro* development of murine thymus in FTOCs. Comparing the time of thymus gestation as a result of fetal age and culture duration, it was possible to discover the onset of gene induction, representing 57 different parenchymal and seven lymphoid organs. Some coded proteins are considered to be tissue-specific antigens, thus indicating that FTOCs reproduce promiscuous gene expression.

The FTOC transcriptome profiling was assessed using a set of six nylon cDNA microarrays containing a total of 9216 IMAGE thymus sequences. PGE was identified on the basis of gene expression data for different parenchymal

organs. To our knowledge, these findings represent the first demonstration of the temporal beginning of PGE in murine FTOC. Moreover, the importance of this event is associated with the fact that this model system could be a useful tool for testing cytokines, RNA interference or other compounds that directly control gene expression in the thymus, as possible modulators of PGE.

Material and methods

FTOCs and total RNA preparation

BALB/c mice were bred in an isolator with 0.45- μ m pore-size air filtered in our own breeding facility. To obtain timed pregnancies, the mice were mated and the day when the vaginal plug was observed at 7:00 hr was considered to be day zero of gestation post-coitum (p.c.). The pregnant mice were killed by ether inhalation and the fetuses were collected via surgery of the uterus. The p.c. age of the fetuses was confirmed by the morphological characteristics of each developmental phase.^{18,19} The thymi were removed from the fetuses under a stereomicroscope and cultured according to the organ culture technique previously described.¹⁷

Briefly, the organs were dissected and cleaned from the adjacent tissue and placed on the surface of Millipore filters (0.45 μ m pore size) pre-embedded with culture medium. These filters were supported on plastic grids in 2 ml Dulbecco's modified Eagle's medium/HAM F-10 culture medium (Gibco, Gaithersburg, MD, USA) supplemented with 20% heat inactivated fetal bovine serum (Biobrás, Montes Claros, MG, Brazil) in 24-well tissue culture plates. The medium was also supplemented with 100 μ g/ml streptomycin, 250 U/ml penicillin, 10 μ g/ml gentamicin, 1 mM sodium pyruvate, 20 μ M 2-mercaptoethanol and 3.4 g/l sodium bicarbonate. The incubation time of the organ culture varied from 2 to 5 days at 37° in a 5% CO₂ incubator.

Thymi were dissected from fetuses obtained at 13, 14 and 15 days of gestation and cultured for 2 days, so mimicking 15, 16 and 17 days p.c. of *in vivo* development, respectively. Thymi from 15-day p.c. fetuses were also cultured for 5 days, mimicking the 20th day of *in vivo* development, and in this case, the culture medium was changed on the third day of incubation.

To evaluate the viability of FTOCs, three randomly selected thymi from each group were used for the single cell suspension preparations, which were separately stained with acridine orange (100 μ g/ml) or with ethidium bromide (100 μ g/ml) and analysed on an Axiophot II fluorescence microscope (Carl Zeiss, Oberkochen, Germany) to evaluate the frequency of apoptosis and necrosis, respectively. Total RNA samples were prepared from FTOCs using Trizol[®] reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

cDNA microarray method

A set of six microarrays were used containing a total of 9216 cDNA sequences (1536 sequences each) in the form of polymerase chain reaction (PCR) products, spotted in duplicate on 2.5×7.5 cm Hybond N + nylon membranes (GE Healthcare, Amersham, UK). The arrays were prepared in our laboratory using a Generation III Array Spotter (Amersham Biosciences-Molecular Dynamics, Sunnyvale, CA, USA).

Mouse thymus expressed sequence tag cDNA clones were obtained from the Soares thymus 2NbMT normalized library, prepared from the thymus of a C57BL/6J 4-week-old male mouse, and available from the IMAGE Consortium (<http://image.llnl.gov/image/html/iresources.shtml>) and from the RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (<http://www.rzpd.de>).

The cDNA inserts were homogeneous in size (near 1 kb), cloned in three vectors (pT7T3D, pBluescript and Lafmid) and were amplified in 384- or 96-well plates using vector-PCR amplification with the following primers, which recognize the three vectors: LBP 1S GTGGAATTGTGAGCGGATACC forward and LBP 1AS GCAAGGCGATTAAGTTGG reverse. This set of cDNAs included sequences of some known autoantigens, such as small nuclear ribonucleoprotein SMD1, lupus Ku protein p70, p80 and p86 and PM-SCL; the full list of cDNA sequences used to prepare the microarrays can be retrieved online at http://rge.fmrp.usp.br/passos/mtb_library.

The membranes were first hybridized with LBP 1AS [γ - ^{33}P]dCTP-labelled oligonucleotide (vector hybridization). Quantification of the signals obtained allowed for the estimation of the amount of cDNA insert fixed in each spot. After stripping, the membranes were used for hybridization with cDNA complex probes (sample hybridization). The characterization of each cDNA sequence was updated using the ELOGE[®] (Ipsogen, Marseille, France, <http://www.ipsogen.fr>) software, which runs on our local server and links each clone ID with the genome data banks (<http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch>), providing information such as DNA and protein sequences, biological and molecular functions and chromosomal location.

Complex cDNA probe preparation and hybridization

In this study, we refer to the radioactive cDNA originated from the thymus RNA samples (FTOCs) as complex probe and to the PCR product originated from the clones and deposited on nylon microarrays as target.

The ^{33}P -labelled cDNA complex probes were prepared by reverse transcription of 10 μg thymus total RNA using oligo-dT(12-18) as a primer; 100 μl ^{33}P -cDNA complex probe containing 30–50 million counts per minute was hybridized with nylon microarrays as previously described.^{20–22}

cDNA microarray image acquisition

Imaging plates and a phosphor imager apparatus (Packard Phosphor Imager, model Cyclone, Packard Instruments, Downers Grove, IL, USA) were used to capture the hybridization signals and the BZScan software²³ was used to quantify the signals with local background subtraction, whose spots were matched with a template grid. The ratio between vector probe hybridization values and complex probe hybridization values for each spot was used as a reference normalization value. Total intensity normalization was also employed, using the median expression value.²⁴

Significance analysis of microarray data

The gene expression data analysed here were obtained from six independent determinations for each day of fetal development in FTOC. The SAM method²⁵ (Significance Analysis of Microarrays, available at <http://www.stat.stanford.edu/~tibs/SAM/index.html>) was used to analyse significant variations in gene expression. This method is based on *t*-test statistics, specially modified for high throughput analysis. The significant variations in the gene expression of developing thymus in FTOCs were compared using the 15-day p.c. as reference (15 versus 16, 15 versus 17 and 15 versus 20 days of development). In this study, only those genes presenting a two-fold change of expression (either repressed or induced) and a gene error chance (*q*-value) equal to 0.01 (see Supplementary Table S1) were considered.

Determination of PGE in the thymus

PGE was identified on the basis of data from microarray analysis of the different mouse organs using combined information from the public database GNF Gene Expression Atlas²⁶ (<http://symatlas.gnf.org/SymAtlas>). This data bank shows gene expression in more than 60 mouse tissues/organs, as assessed by gene array analysis using Affymetrics microarrays. Data information includes GenBank accession, chromosomal location and the molecular/biological function of each gene analysed (Table S1).

In this study, only the promiscuous genes of which the expression was detected in different organs or tissues, besides the thymus, and of which the expression levels were greater than median in relation to all other organs which appear in the GNF Atlas were considered. The modulation of transcription levels (repression or induction) of these genes was evaluated during FTOC development.

Semi-quantitative reverse transcription-PCR (SQ RT-PCR)

The zinc finger protein 369 gene (ZNF 369; acc NM_178364, clone ID 573600) that was found to be

induced in the microarray experiments and the *AIRE* gene (acc NM_009646) were tested by SQ RT-PCR; 1 µg total RNA from each developmental phase in FTOC was copied to cDNA by reverse transcription using oligo-dT(12-18) as a primer. One microlitre of successive dilutions of the cDNA (1 : 2 to 1 : 128) were used as a PCR template. PCRs were performed in a final volume of 25 µl containing 100 µM each dNTP, 1 µM each primer and 1 U *Taq* DNA polymerase (Amersham Biosciences).

The amplification protocol for the *ZNF 369* and *AIRE* genes assayed was as follows; one cycle at 94° for 5 min, 30 cycles of 94° for 1 min, 55° for 1 min, then 72° for 1 min, and then one cycle at 72° for 10 min then 4° for 10 min. The amplification was terminated with a final incubation step at 72° for 10 min.

Aliquots of the PCR products were mixed with 1 µl of a diluted solution of ethidium bromide, resolved on 2% agarose gel electrophoresis using 1 × TAE buffer. The gels were visualized on a UV transilluminator and the images were captured using a digital camera.

The PCR primers were identified using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) on the cDNA sequences retrieved from GenBank (<http://www.ncbi.nlm.nih.gov>) for each gene. The forward and reverse sequences are given in the 5' to 3' orientation; *ZNF 369* (clone ID 573600, GenBank acc NM_178364) (GGGAGGACCTTATGTGTGT and GGGGCTCTCCTTATCCAAAAG) and the autoimmune regulator gene, *AIRE* (GenBank acc NM_009646), although not included in the microarrays, was also assayed with the following primers (CATCCTGGATTCTCTGGAGGA and GCTCTTTGAGGC CAGAGTTG).

Results

Genes differentially expressed during FTOC development

To identify significant changes in gene expression during fetal development in FTOCs, the early thymus at 15 days was compared with those observed on the subsequent days (15 versus 16, 15 versus 17 and 15 versus 20 days p.c. FTOC). A scatter plot of the observed relative difference $d(i)$ against the expected relative difference $d_E(i)$ was used, as shown by the SAM program.

Most of the 9216 sequences tested presented $d(i) \cong d_E(i)$, indicating that their expression pattern remained unchanged; however, some genes were significantly repressed (154 genes) or induced (38 genes) during FTOC development (Fig. 1).

Interestingly the induction of the 38 genes that include representation of parenchymal organs in the thymus, thus characterizing PGE, was observed at late FTOC (20 days p.c.) (Table S1).

Figure 1. Differential gene expression during the development of FTOC showing that induction of tissue-specific genes emerges at 20 days. Staging corresponds to development of fetal thymus in culture; 15 days FTOC = 13 days gestation thymus plus 2 days in FTOC.

Gene expression assessed by SQ RT-PCR

The *ZNF 369* gene (acc NM_178364) was selected to confirm the respective result obtained with the cDNA microarray. SQ RT-PCR evaluation confirmed that this gene was induced at 20 days p.c. FTOC (Fig. 2a). Moreover, the *AIRE* gene (acc NM_009646) began its expression at 16 days p.c. FTOC (Fig. 2b).

Parenchymal organ representation in the thymus

The 38 genes identified as significantly induced at 20 days p.c. in FTOC were assigned to 57 parenchymal and seven lymphoid organs according to their predominant expression, which were subgrouped in 17 systems. Interestingly, central nervous and reproductive systems and glands are the predominant parenchymal organs represented in the thymus at this phase of development, followed by the lymphoid system (Fig. 3). Four genes; small nuclear ribonucleoprotein D1, acc NM_009226; CD27 antigen, acc L24495; Riken cDNA 1110065L07, acc AA119642; and D-amino-acid oxidase, acc A1447515; code for tissue-specific antigen proteins, because they are expressed in only five or six tissues (Table S1).

Figure 2. Semi-quantitative reverse transcription-PCR. Confirmation of *ZNF* gene as induced at 20 days FTOC (a) and emergence of *AIRE* gene expression at 16 days FTOC (b).

Figure 3. Representation of tissue/organ system-specific gene expression in 20 days FTOC.

Figure 4. Chromosomal distribution of the repressed and induced genes considering all FTOC development (15–20 days). NF, not found.

Chromosomal location of differentially expressed genes

The genomic distribution of the 192 genes modulated (repressed or induced) during the 5 days of FTOC development allowed for the co-ordinated expression of organizing chromosomal clusters.

Figure 4 shows the frequency distribution of the repressed and induced genes among chromosomes. Taking all the developmental phases together, the number of repressed genes (154) was greater than that of induced genes (38). All chromosomes, except Y, harboured differentially expressed genes, with a slightly biased distribution on chromosomes 2, 5, 11 and 19 for repressed genes and on chromosomes 3, 4, 11, 15 and 19 for induced genes.

Discussion

PGE in the thymus is a complex phenomenon observed in humans and mice, which is exhibited by TECs (mTECs)

and involves 5–10% of all known genes of these species. This process is a guarantee of self-representation of most parenchymal tissues and organs during the central tolerance of T cells.

Using PCR, Derbinski's group previously demonstrated that the expression of five tissue-specific genes, such as thyroglobulin, CRP, GAD67, insulin and albumin, was detectable only in mTECs purified from *ex vivo* thymic tissue at embryonic day 15 (E15).⁷

Accordingly, the complexity of PGE increases in, from cTECs to immature mTECs to mature CD80^{hi} mTECs. These different gene pools are not complementary, but additive, that is, there is no apparent association between the respective molecular/biological functions of the genes in parenchymal organs. The significance of PGE in the thymus is associated with the central tolerance of T cells.^{9–14}

While PGE by mTECs was well documented by the authors cited above, many aspects of this phenomenon remain unexplored, for example, its evaluation in the thymus of autoimmune strains and/or knockout mice, its modulation by means of cytokines or other molecules interfering in gene expression, such as RNA interference, and its time-course during the ontogenetic development of the thymus.

Molecular characterization of PGE in the FTOC model system is a relevant approach in immunobiology, because the functional analysis of T-cell development has become possible after the introduction of techniques in which the thymic microenvironment is mimicked, such as FTOC. This method is based on the use of early fetal thymus tissue, which is composed of a homogeneous thymocyte population (double-negative cells).

In this study, the occurrence of PGE *in vitro* in FTOC is described for the first time. To evaluate whether PGE in this model system is a development-dependent phenomenon, we regarded the differential gene expression during ontogeny by comparing days of gestation (p.c.), as the most informative in the delineation of the gene pool. Use of the FTOC model system was chosen, rather than compare the changes in expression profiles in thymus tissue obtained at different gestational days, to approach three important aspects simultaneously. Since FTOC reproduces the *in vivo* thymus development, this model system represented a useful tool first, for the fine demarcation of PGE onset and, second, to demonstrate that PGE is a phenomenon that can be reproduced *in vitro*. The third aspect was a beneficial possibility of the FTOC model system, comparing the same pool of thymus tissue at day 15 with later time-points.

We demonstrated that PGE in FTOC, which is characterized by the overexpression of parenchymal organ and tissue-specific antigen genes, emerges at 20 days p.c. (Fig. 1). Since this *in vitro* organ culture mimicked the thymus gestation *in vivo*, including the maturation of T

cells,¹⁸ these results strongly suggest that PGE is dependent on thymus maturation. The significance of these findings is associated with the timing of T-cell tolerance induction during ontogeny. T-cell receptor β rearrangements emerge at 16 days p.c. *in vivo* and in FTOC in BALB/c mice.^{1–3,18}

The early fetal thymus, by 13–15 days p.c., is composed of homogeneous double-negative CD4[–]CD8[–] T-cell precursors; however, by day 18 p.c. this population gradually acquires the CD4 marker resembling the adult CD4^{low} precursor.^{26,27} These features allow for T-cell receptor–major histocompatibility complex peptide recognition and enable the positive/negative selection of T cells in fetal thymus. Our evidence for the occurrence of PGE in late fetal thymus is associated with the timing of the molecular events of T-cell tolerance induction during ontogeny.

The data collected here were obtained using the cDNA microarray method, with the expression of the 9216 sequences analysed by the SAM algorithm.²⁵ Considering 15 days p.c. FTOC as a reference, it was possible to make comparisons with the subsequent days of development. A statistically significant set of 154 repressed genes were found between 15 and 17 days p.c. and 38 genes were considered as overexpressed at 20 days p.c. FTOC, indicating the emergence of PGE (Table S1).

Moreover, these findings are important to the PGE differentiation model in the thymus and the concomitant increased *AIRE* gene expression in the most mature mTECs. In the FTOC model system studied, *AIRE* gene expression begins at 16 days p.c., before a significant induction of parenchymal and tissue-specific antigen genes (Fig. 2b), suggesting that this gene could be associated with the control of the parenchymal organ gene expression observed.²⁸

Although the experiments were not conducted with purified mTECs, this caused no problems regarding PGE detection in the thymus. To bypass this potential difficulty, a cDNA microarray method was used, including a dedicated statistical algorithm for data analysis (SAM algorithm), which presented sufficient accuracy to distinguish and quantify parenchymal organ gene expression originating from thymic epithelial cells, especially mTECs.^{8,9,13}

The cDNA microarray data mining has permitted the virtual dissection of the mouse thymus into its principal cellular components by means of the identification of the specific cellular transcripts (mRNAs).²⁹ These observations demonstrate the feasibility for the use of whole thymus as starting material in PGE studies.

Moreover, we selected a gene found in the microarray experiments (*ZNF 369*) that is representative of parenchymal organs, whose modulation in the expression was confirmed by SQ-RT-PCR (Fig. 2a).

In agreement with previous observations,¹³ the molecular/biological function of promiscuously expressed genes

found in the present model system showed no interrelationship (Table S1).

Regarding the chromosomal localization of the repressed and induced genes, no important preferential distribution was identified. All chromosomes harbour promiscuously expressed genes with a slightly biased distribution on chromosomes 2, 5, 11 and 19 among the repressed genes and on chromosomes 3, 4, 11, 15 and 19 among the induced genes. The exceptions were chromosome Y, on which no repressed or induced genes were identified, and chromosome 2, on which no induced genes were positioned (Fig. 4).

This feature of random PGE distribution in the genome strongly suggests an uncommon model of gene regulation found in the thymus, which requires further investigation, including the elucidation of the molecular mechanism of the *AIRE* gene action.

The model system presented here was important to demonstrate that PGE is a differentially controlled phenomenon,⁹ beginning in BALB/c mice at 20 days p.c. (FTOC), and because this phenomenon can be reproduced *in vitro*, these findings raise the possibility of testing the induction of gene expression alteration caused by FTOC cytokine treatment or selected gene transfections that could modulate PGE. Recent evidence for a second thymus in mice³⁰ increase the possibility of further research into their contribution to self-tolerance mechanisms, including determination of occurrence of the PGE in this organ.

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Supplementary material

The following supplementary material is available online for this article:

Table S1. Promiscuously induced genes in 20 days p.c. Balb-c thymus (FTOC).

This material is available as part of the online article from <http://www.blackwell-synergy.com>

Hybridization signatures of gamma-irradiated murine fetal thymus organ culture (FTOC) reveal modulation of genes associated with T-cell receptor V(D)J recombination and DNA repair

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Abstract

In this study, we observed the occurrence of TRBV8.1-DB2.1 V(D)J recombination in murine fetal thymus organ culture (FTOC), in which the thymic microenvironment is mimicked. Since ionizing radiation affects T-cell development, we irradiated FTOCs with gamma rays to evaluate the modulation of genes implicated in TRBV8.1-DB2.1 rearrangements. The nylon cDNA microarray method was employed to monitor the expression of 9216 genes, which were organized in coexpression clusters. Clustering analysis showed similar expression profiling of genes implicated in the V(D)J recombination and DNA double strand break (DSB) repair processes such as XRCC4, RAG-2, Artemis and DNA-PK-cs, thus suggesting overlap between the two processes. The RUNX3 gene, whose coded protein binds to the enhancers of TR genes, was also modulated and the DNA cross-linking LR1 gene, which plays a role in the opening of hairpin DNA structures and whose expression pattern is similar to Artemis, may play a role in the control of V(D)J recombination. Furthermore, our data demonstrate that the FTOC model system and cDNA microarray method are useful tools to evidenciate genes that may play a role in both processes V(D)J recombination and DNA repair. © 2005 Elsevier Ltd. All rights reserved.

Keywords: FTOC; T-cell receptor beta; V(D)J recombination; DNA repair; cDNA microarray; Transcriptome profiling; Ionizing radiation

1. Introduction

The differentiation of thymocytes to mature T-cells occurs within the fetal thymus and all developmental stages are distinguishable by their expression of a combination of CD cell-surface markers. This is a highly modulated phenomenon whose central molecular machinery is formed by the recombinase complex (recombination activating genes,

RAG-1 and RAG-2) directly implicated in the V(D)J recombination of T-cell receptor (TRA, TRB, TRG and TRD) and immunoglobulin (Ig) gene segments (Lefranc and Lefranc, 2001).

The occurrence of the V(D)J reaction is important for triggering the maturation of T-cells and is also regulated by several other gene products such as interleukins (Muegge et al., 1993; Sollof et al., 1997; Fink and McMahan, 2000; Fugmann, 2002; Espanhol et al., 2003). Using the cDNA array method, we have previously found that during a fetal phase of the in vivo development of the murine thymus, when

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the TR gene rearrangements begin, several genes related to DNA synthesis, protein metabolism, oncogenes, transcription factors and signal transduction are modulated in concert (Espanhol et al., 2004; Magalhães et al., 2005).

Functional analysis of T-cell development has become possible after the introduction of techniques in which the thymic microenvironment is mimicked, such as fetal thymus organ culture (FTOC). This method is based on the use of early fetal thymus tissue, which is composed of homogeneous thymocyte population (double-negative cells), and on the possibility of testing the induction of alterations in gene expression during T-cell development caused by chemical and physical agents (DeLuca et al., 1995).

In the present study, we demonstrated the occurrence of the TRBV8.1-BD2.1 rearrangement in FTOCs, indicating the occurrence of T-cell maturation *in vitro*. Moreover, the FTOC transcriptome profiling was accessed using a set of six nylon cDNA microarrays containing a total of 9216 thymus IMAGE sequences.

Comparing time of thymus gestation as a result of age of fetuses and length of cultures, versus gamma-irradiated and unirradiated cultures, it was possible to found genes differentially expressed, several among them were considered to be novel candidates participating simultaneously in the control of T-cell receptor V(D)J recombination and DNA repair.

Each phase of thymus development in FTOCs exhibited a characteristic gene expression pattern involving genes of known functions, permitting the identification of those implicated in T-cell maturation, including the V(D)J recombination process.

Since in the both processes, DNA repair of damage caused by ionizing radiation and T-cell receptor V(D)J recombination occurs repair of DNA double-strand breaks (DSBs) (Prise et al., 2001; Toki et al., 2003), we irradiated FTOCs with gamma rays to evaluate the effects on TRB V(D)J recombination and on modulation of genes implicated in these mechanisms.

Among the genes which were found to be differentially expressed, several were considered to be novel candidates participating simultaneously in the control of T-cell receptor V(D)J recombination and DNA repair.

2. Material and methods

2.1. Fetal thymus organ culture, total RNA and genomic DNA preparations

BALB-c mice were bred in an 0.45 μ m pore size air filtered isolator in our own breeding facility. To obtain timed pregnancies, mice were mated and the day when the vaginal plug was obtained at 7:00 am was considered to be day zero of gestation post coitum (pc). Pregnant mice were sacrificed by ether inhalation and the fetuses collected by surgery of the uterus. The pc age of fetuses was confirmed by the morpho-

logical characteristics of each developmental phase (Rugh, 1968).

Thymi were removed from the fetuses under a stereomicroscope and cultured according to the organ culture technique described by DeLuca et al., (1995). The organs were dissected and cleaned from adjacent tissue and placed on the surface of Millipore filters (0.45 μ m pore size) pre-embedded with culture medium. These filters were supported on plastic grids in 2 ml Dulbecco's modified Eagle's Medium/HAM F-10 culture medium (GIBCO, USA) supplemented with 20% heat inactivated fetal bovine serum (Biobrás, Brazil). The medium was also supplemented with 100 μ g/ml streptomycin, 250 U/ml penicillin, 10 μ g/ml gentamicin, 1 mM sodium pyruvate, 20 μ M 2-mercaptoethanol and 3.4 g/L sodium bicarbonate. The incubation time of the organ culture varied from 2 to 5 days at 37 °C in a 5% CO₂ incubator.

Thymi were dissected from fetuses obtained at 13, 14 and 15 days of gestation and cultured for two days, respectively, mimicking 15, 16 and 17 days of *in vivo* development. Thymi from 15 days pc fetuses were also cultured for 5 days, mimicking the 20th day of *in vivo* development, and in this case, the culture medium was changed on the 3rd day of incubation.

Immediately after culture preparation, some FTOCs were irradiated with 4 Gy of gamma rays from ⁶⁰Co source using a Gammatron S-80 apparatus (Siemens, Germany) at the dose of 0.53 Gy/min. Control cultures were sham irradiated.

In order to evaluate the viability of FTOCs before and after irradiation, three randomly selected thymus of each group was used for the single cell suspensions preparation which were separately stained with acridine orange (100 μ g/ml) or with ethidium bromide (100 μ g/ml) and analyzed on a Axioophot II fluorescence microscope (Carl Zeiss, Germany) in order to evaluate the frequency of apoptosis and necrosis, respectively.

Total RNA and genomic DNA were prepared from FTOCs using Trizol[®] reagent according to manufacturer instructions (Invitrogen, USA).

2.2. Semiquantitative TRBV8.1-BD2.1 recombination assay

T-cell maturation was monitored by detection of rearrangements between segments TRBV8.1 and BD2.1 during FTOC development using PCR as previously described (Muegge et al., 1993). The oligonucleotide primers were the BV8.1 forward primer (primer 1, CAGGCTGCAGT-CACCCAAAGTCCAA), the BV8.1 reverse primer (primer 2, ACAGAAATATACAGCTGTCTGAGAA) and the BJ2.1 reverse primer (primer 3, TGAGTCGTGTTCTGGTCC-GAAGAA). The PCR mixture contained 35 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 100 μ g/ml bovine serum albumin, nucleotide triphosphates (NTPs) (2 μ M each), the primers (0.5 μ M each) and 1 U Taq polymerase (Amersham Biosciences, Buckinghamshire, UK), with a constant amount of genomic DNA (1 μ g) from each developmental stage in FTOC. The PCR program consisted of 30 cycles (1 min at

94 °C, 2.5 min at 54 °C and 1.5 min at 70 °C, with a 7-min extension period).

In order to make this PCR a semiquantitative method, we included in the mix an internal amplification control of the β -actin gene (β -actin forward ATGGATGACGATATCGCT and β -actin reverse ATGAGGTAGTCTGTCA). The adequate number of PCR cycles to reach the log-phase amplification was determined in experiments with 25, 30 and 35 PCR cycles. The signal generated by the 30-cycle PCR was significantly lower than that generated by the 35-cycle PCR. This result demonstrates that at 30-cycle PCR, the amplification of the TRBV8.1-BD2.1 rearranged segment (and the beta-actin segment) was at log-phase and was adopted in this study (data not shown).

After cycling, 15 μ l of PCR products were resolved by 1.8% agarose gel electrophoresis and transferred (blotting) to a nylon Hybond N+ membrane (Amersham Biosciences). The PCR products were identified by hybridization with 7–15 million cpm of each T-cell receptor beta (TRBV8.1) and β -actin 32 P radiolabeled probe.

Imaging plates and a phosphor imager apparatus (Cyclone model, Packard Instruments, USA) were used to capture the hybridization signals and the OptiQuant[®] software (Packard) was used to quantify the signals with local background subtraction. The adequate level of image saturation was ensured by serial dilution of the positive control (data not shown).

For the data from V(D)J recombination experiments we applied *t*-test as statistical method of analysis and a *P*-value < 0.05 was considered to be significant from three independent determinations.

2.3. cDNA microarray method

We used a set of six microarrays containing a total of 9216 cDNA sequences in the form of PCR products, spotted in duplicate on 2.5 cm $\times$ 7.5 cm Hybond N+ nylon membranes (Amersham Biosciences). The arrays were prepared in our laboratory using a Generation III Array Spotter (Amersham Biosciences—Molecular Dynamics, USA).

Mouse thymus expressed sequence tag (EST) cDNA clones were obtained from the Soares thymus 2NbMT normalized library, prepared from a C57Bl/6J 4-week-old male thymus, and available from the I.M.A.G.E. Consortium (<http://image.llnl.gov/image/html/iresources.shtml>) and from the RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (www.rzpd.de).

The cDNA inserts were homogeneous in size (near 1 kb) and cloned in three vectors (pT7T3D, pBluescript and Lafmid) and were amplified on 384- or 96-well plates using vector-PCR amplification with the following primers, which recognize the three vectors: LBP 1S GTGGAATTGTGAGCGGATACC forward and LBP 1AS GCAAGGCGATTAAGTTGG reverse.

The membranes were first hybridized with the LBP 1AS [γ - 33 P]dCTP-labeled oligonucleotide (vector hybridization).

Quantification of the obtained signals allowed the estimation of the amount of cDNA insert deposited in each spot. After stripping, the membranes were used for hybridization with cDNA complex probes (sample hybridization).

The characterization of each cDNA sequence was updated using the Elogé[®] (Ipsogen, France, www.ipsogen.fr) software, which runs on our local server and links each clone ID with the genome data banks GenBank (www.ncbi.nlm.nih.gov) and S.O.U.R.C.E. (<http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch>), providing information such as DNA and protein sequences, biological and molecular functions and chromosomal location.

2.4. Complex cDNA probe preparation and hybridization

In this study, we refer to the radioactive cDNA originating from the thymus RNA samples (FTOCs) as complex probe and to the PCR product originating from the clones and deposited on nylon microarrays as target.

The 33 P-labeled cDNA complex probes were prepared by reverse transcription of 10 μ g of thymus total RNA using oligo dT_{12–18} as primer. One-hundred μ l 33 P-cDNA complex probe containing 30–50 million cpm was hybridized with nylon microarrays as previously described (Nguyen et al., 1995; Bertucci et al., 2002; Verdeil et al., 2002).

2.5. cDNA microarray image acquisition

We used imaging plates and a phosphor imager apparatus (Packard model Cyclone) to capture the hybridization signals and the BZScan software (available from the authors at <http://www.tagc.univ-mrs.fr>) to quantify the signals with local background subtraction, whose spots were matched with a template grid.

The ratio between vector probe hybridization values and complex probe hybridization values for each spot was used as reference normalization value. We also employed total intensity normalization using the median expression value (Quackenbush, 2002).

2.6. Hierarchical clustering

We used a hierarchical clustering algorithm, comparing means of different genes whose standard deviation did not overlap, and whose objective was to compute a dendrogram that assembles all elements into a single tree. The software for this algorithm can be obtained from the authors (Eisen et al., 1998) at (<http://rana.stanford.edu/clustering>). Before clustering, the gene expression values were median-centered and log transformed using the Pearson correlation distance metrics and average linkage for clustering organization from six independent determinations of each situation studied.

3. Results

3.1. Monitoring thymocyte maturation in FTOCs

The detection of the TRBV8.1-BD2.1 rearranged DNA segment was evidence for thymocyte maturation in FTOCs. Using a semiquantitative PCR method, it was possible to detect significant amounts ($P < 0.05$) of amplification products for both the β -actin internal control and the TRBV8.1-BD2.1 segment (Fig. 1). The onset of V(D)J recombination occurred at the 14 day pc fetal thymi after 2 days in FTOC (these 2 days of incubation time correspond to the 16th day of gestation in vivo).

Gamma irradiation caused a 1.5-fold significant increase ($P < 0.05$) of the TRBV8.1-BD2.1 segment in 15 day pc fetal thymus incubated for five days in FTOC (corresponding to the 20th day pc in vivo); however, organ irradiation did not change the onset of recombination, which also occurred in

irradiated 14 day pc fetal thymus (corresponding to the 16th day of gestation in vivo). This dose of gamma irradiation did not significantly changed cell viability (t -test, $P < 0.05$) of FTOCs as evaluated by determination of frequency of apoptotic and necrotic cells (Table 1).

3.2. Hierarchical clustering

The hierarchical cluster analysis of the results obtained for a total of 9216 sequences by comparison between irradiated and non-irradiated FTOCs showed variability in the hybridization signatures of gene expression presented by different cultures. The dendrogram shows that this variability allowed the distinction of samples according to incubation time, as well as the distinction between control and irradiated FTOCs (Fig. 2).

It was possible to identify several clusters of repressed and induced genes. However, six of them which included

Fig. 1. Semiquantitative detection of V(D)J recombination between TRBV8.1 and BD2.1 during thymus development in control and gamma-irradiated FTOCs. Polymerase chain reactions were performed using TRBV8.1 and BD2.1 specific primers together with beta-actin primers as internal control in order to monitor reactions and normalize data. The 30-cycle PCR allowed amplification of the TRBV8.1-BD2.1 rearranged segment (and beta-actin segment) at log-phase. (A) Southern-blot of PCR products. (B) Quantification of hybridizations using a phosphor-imager. Arbitrary normalized values (on the basis of beta-actin values) expressed as digital light units (DLU) as displayed by the OptiQuant® software.

Table 1
Frequency (%) of apoptotic and necrotic cells in 4 Gy irradiated and control FTOCs

	Apoptosis		Necrosis	
	Staging		Staging	
Control	15	1.2 ± 0.5	15	4.1 ± 1.5
	16	1.0 ± 0.5	16	3.0 ± 1.0
	17	3.2 ± 1.0	17	5.2 ± 1.5
	20	2.0 ± 1.0	20	2.7 ± 1.0
Irradiated	15	1.8 ± 1.0	15	4.1 ± 0.5
	16	1.9 ± 1.0	16	4.2 ± 1.0
	17	3.0 ± 0.5	17	2.3 ± 0.5
	20	2.1 ± 1.0	20	5.8 ± 1.5

Mean and standard deviation of three independent determinations. Data analyzed by *t*-test ($P < 0.05$).

Staging corresponds to development of fetal thymus in culture; 15 FTOC = 13 day gestation thymus plus two days in FTOC.

genes implicated in cell cycle control, DNA repair, T-cell differentiation and V(D)J recombination were selected for discussion. The complete color heat-map indicating the six gene clusters is available online at (www.rge.fmrp.usp.br/passos/FTOCcluster/9216).

Among the genes differentially expressed, we selected 42 to discuss from six clusters whose functions are associated with cell cycle control, transcription factors, immune regulation, T-cell V(D)J recombination and DNA repair (Table 2).

4. Discussion

4.1. T-cell receptor and TRBV8.1 rearrangement occurs in vitro in FTOC

The IL-7 is a cofactor associated with modulation of V(D)J recombination of TCR genes (Muegge et al., 1993; Sollof et al., 1997).

In this study, we described for the first time the occurrence of TRBV8.1-BD2.1 V(D)J recombination in vitro in FTOC.

The onset of TRBV8.1-BD2.1 V(D)J recombination in vitro mimicked the thymus gestation in vivo, demonstrating that this model system is similar to the in vivo development of

the thymus in inbred mouse strains, as previously described by our group (Espanhol et al., 2003; Macedo et al., 1999).

On the basis of a variety of criteria, FTOC reproduces the normal thymic development; however, the results can be influenced by the length of culture, and mouse strain and age (DeLuca et al., 1995).

In addition, it was shown that there are differences in temporal rearrangements at the TRBV locus, including the TRBV8.1 segment, among BALB-c, C57Bl/6 and CBA/J inbred mouse strains, probably due to the different genetic backgrounds of the different strains (Espanhol et al., 2003; Macedo et al., 1999; Junta and Passos, 1998).

For this reason, the fetuses used in this study were obtained within the age range of 13–15 days of gestation pc, which permitted comparisons with the data obtained in vivo with the BALB-c strain.

4.2. Hierarchical clustering

The expression patterns disclosed several clusters of co-expressed genes, six of which (clusters 1–6) were selected for discussion on the basis of the biological functions of the most relevant genes investigated in this study.

Cluster 1 (correlation = 0.81) harbors genes which were induced in irradiated cultures. This cluster displayed genes involved in cell cycle/cell division and DNA repair. Among them, we pinpointed MYB (myeloblastosis oncogene, accession no. NM010848, chromosome 10), which has a role in transcriptional activation and in the control of proliferation and differentiation of hematopoietic progenitor cells, and Kirsten rat sarcoma oncogene 2 (KRAS2, accession no. NM021284, chromosome 6). This oncogene belongs to the small GTPase superfamily (ras family), whose proteins bind to GDP/GTP and possess intrinsic GTPase activity. Its biological function is associated with control of the cell cycle. The induction of these oncogenes suggests triggering cell division by irradiation in FTOCs.

Interestingly, the cell division cycle 42 homolog *S. cerevisiae* gene (CDC42, accession no. NM009861, chromosome 4) which plays a role in GTPase-mediated signal transduction and participates in cytokinesis, presented an expression profile similar to that of the KRAS2 oncogene, suggesting that GTPase activity seems to be required after exposure to ionizing radiation.

The X-ray repair complementing defective repair in Chinese hamster cells 5 gene (Ku80) (XRCC5, accession no. NM009533, chromosome 1) participates in the mechanism of DNA double-strand break repair (DSB repair) via NHEJ. The induction of the XRCC5 gene suggests that NHEJ may constitute an important process of DNA repair in irradiated FTOCs.

Cluster 2 (correlation = 0.71) encompasses genes involved in the regulation of transcription in T-cells such as signal transducer and activator of transcription 4 gene (STAT4, accession no. NM011487, chromosome 1), whose activities are associated with a transcription factor and

Fig. 2. Clustering RNA (cDNA) samples and 9216 genes of each developmental phase of control and gamma-irradiated FTOCs. The hierarchical clustering algorithm compared means of the different genes and RNA (cDNA) samples whose standard deviations did not overlapped computing the dendrogram that assembled all elements into a tree. Complete color heat-map indicating the repressed, induced and unmodulated genes among all situation studied is available online at (www.rge.fmrp.usp.br/passos/FTOCcluster9216).

Table 2

Genes differentially expressed (induced) in gamma-irradiated compared to control FTOCs as displayed by the Cluster and TreeView program

Cluster	Gene name	Accession no.	Chromosome	Function
1	Myeloblastosis oncogene	NM10848	10	Oncogene
	Kirsten rat sarcoma oncogene 2	NM021284	6	Oncogene
	Cell division cycle 42	NM009861	4	GTPase-signal transduction
	Ku 80, X-ray repair, XRCC5	NM009533	1	DSB repair
2	Signal transducer activation of transcription	NM011487	1	Transcription factor
	Neuroprotective protein gene	AK129214	2H3	Transcription factor
	Nuclear factor of activated T-cells	AK048610	18	Induction of cytokines
3	Transcription elongation factor 1	AK048564	1H2	RNA pol II transcription
	Transcription elongation factor 3	AJ223472	4D3	RNA pol II transcription
	Runt-related transcription factor 3	AK053910	4	Enhancer binding protein
	Thyroid autoantigen Ku70	BC051085	15	DSB repair
	Janus kinase 3	L40172	8	IL2/IL4 signaling
	Interleukin receptor IL-11TRA2	X98519	4	Immune response/V(D)J recombination
	Interleukin receptor IL-7R	NM008372	15	TCR V(D)J recombination
	Tumor necrosis factor, member7	L24495	6	Survival of activated T-cells
	Granzyme A	M13226	13	Targeting cell lysis
4	Fibroblast growth factor receptor 1	BC010200	8	Embriogenesis
	Vascular cell adhesion molecule 1	AK089320	3	Cell adhesion
	Retinoblastoma binding protein 7	BC003785	XF4	Negative regulation of transcription
	SRY-box-SOX-4	AK028989	13	Binding to T-cell enhancer
	Artemis protein	AK052369	2A1	TCR V(D)J recombination
5	Mitogen activated protein kinase 7	BC070467	8A1.1	Activation of Jun kinases
	Insulin-like growth factor binding protein 7	AB012886	5D	Regulation of cell growth
	A-disintegrin and metalloprotease domain 8	BC025584	7F3-F5	Extravasation of leukocytes
	Nuclear transcription factor Y-gamma	BC053723	4	Transcription factor
	Tripartite motif protein 28	NM011588	7A1	Transcription factor
	Protease, serine, 16 (thymus)	AK088019	13	Protease, T-cell development
	Calpain 8	NM130890	1H5	Endopeptidase
6	X-ray repair, XRCC4	AK038105	13C3	DSB repair
	Heat shock protein 1-like	D85732	17	Cooperation with chaperones
	XPC, xeroderma pigmentosum C	AK028595	6D	DNA excision repair (NER)
	Interleukin IL-1B	NM008361	2	Immune response
	Interleukin IL-2	X01772	3	Immune response
	Interleukin IL-6	AK089780	5	Immune response
	Interleukin IL-7	AK041403	3	Immune response/ V(D)J recombination
	Lymphotoxin A	NM10735	17	Cytotoxic for tumor cells
	Cyclin D3	AK046638	17	Protein kinase
	EGR1, early growth response 1	M22326	18	Transcriptional regulator
	RAG-2, recombination activator gene 2	AK040375	2	V(D)J recombination
	DNA-activated protein kinase	AK088981	16	DSB repair and V(D)J recombination
	MMTV endogenous retrovirus	B4515481	19	Superantigen
	DNA cross-link LR1	BC011094	3F2.2	Nucleotide excision repair

a cytokine- and chemokine-mediated signaling pathway. Activity-dependent neuroprotective protein gene (ADNP, accession no. AK129214, chromosome 2H3) encodes a DNA-binding protein with transcription factor activity and nuclear factor of activated T-cells, cytoplasmic 1 gene (NFATC1, accession no. AK048610, chromosome 18) plays a role in the inducible expression of cytokine genes in T-cells, especially, in the induction of the IL-2 or IL-4 gene transcription.

The expression patterns of these genes in non-irradiated FTOCs suggest a tendency of induction at the early stages and repression at the late stages.

Cluster 3 (correlation = 0.71) also encompasses genes involved in the regulation of transcription such as TCEA1 and TCEA3 (transcription elongation factor gene 1, accession

no. AK048564, chromosome 1A2 and gene 3, accession no. AJ223472, chromosome 4D3), necessary for efficient RNA polymerase II transcription elongation past template-encoded arresting sites. Cleavage of the nascent transcript by S-II allows the resumption of elongation from the new 3' terminus. The Runt-related transcription factor 3 gene (RUNX3 gene, accession no. AK053910, chromosome 4) codes for a protein that binds to the enhancers of T-cell receptor genes. As enhancers of TRs play a role in the control of gene rearrangements (Hempel et al., 1998), RUNX3 could participate in the control of TR V(D)J recombination.

G22P1 (Ku70) (thyroid autoantigen gene, accession no. BC051085, chromosome 15) plays a role in DSB repair via nonhomologous end-joining. Ku70/80 heterodimer is the

DNA-binding subunit of DNA-PK, an important component of the repair of DSB via NHEJ (Lees-Miller and Meek, 2003).

Absence of Ku70 leads to hypersensitivity to ionizing radiation. Janus kinase 3 gene (JAK3, accession no. L40172, chromosome 8) codes for a tyrosine kinase of the non-receptor type involved in the IL-2 and IL-4 signaling pathway. This gene belongs to the Tyr family of protein kinases and is implicated in lymph gland development. The interleukin receptor genes IL-11RA2 (accession no. X98519, chromosome 4) and IL-7R (accession no. NM008372, chromosome 15) are also present in cluster 3 and may later be associated with IL-7 signaling, which plays a role in the V(D)J recombination of TRs (Muegge et al., 1993; Sollof et al., 1997).

In addition, we observed genes associated with apoptosis such as tumor necrosis factor receptor superfamily, member 7 gene (TNFRSF7, accession no. L24495, chromosome 6) and GZMA (granzyme A gene, accession no. M13226, chromosome 13). TNFRSF7 encodes a receptor for tnfsf7/cd271, possibly playing a role in survival of activated T-cells, while GZMA codes for a granzyme A precursor, an enzyme necessary for targeting cell lysis in cell-mediated immune responses.

Therefore, the induction of several genes in cluster 3 suggests the occurrence of T-cell differentiation following the irradiation stimulus in irradiated FTOCs.

Cluster 4 (correlation = 0.81) contains genes with induced expression in irradiated FTOCs similar to cluster 3. Among the modulated genes, two may play a role in thymus morphogenesis in FTOC, the fibroblast growth factor receptor 1 gene (FGFR1, accession no. BC010200, chromosome 8) which may be essential for generation of mesodermal and endodermal layers and invaginations of various types of cells, and vascular cell adhesion molecule 1 gene (VCAM1, accession no. AK085320, chromosome 3) which is important in cell–cell recognition and appears to function in leukocyte–endothelial cell adhesion.

Two genes implicated in the control of transcription such as retinoblastoma binding protein 7 gene (RBBP7, accession no. BC003785 chromosome XF4) which plays a role in the negative regulation of transcription from the polymerase II promoter and SRY-box containing gene 4 (SOX4, accession no. AK028989, chromosome 13) which codes for a transcriptional activator that binds with high affinity to the T-cell enhancer 5'-AACAAAG-3' motif, may be associated with the control of V(D)J recombinations in FTOCs, in concert with the Artemis gene accession no. AK052369, chromosome 2A1, which is directly associated with T-cell receptor gene rearrangements since these genes were included in the same cluster.

Cluster 5 (correlation = 0.78) harbors genes, which were induced in irradiated FTOCs. Among these genes, we observed those associated with cell proliferation such as mitogen activated protein kinase 7 gene (MAP2K7, accession no. BC070467, chromosome 8A1.1) which has dual kinase activities involving the activation of jun kinases mapk8 (jnk1) and mapk9 (jnk2); as well as insulin-like growth factor

binding protein 7 gene (IGFBP7, accession no. AB012886, chromosome 5D) that plays a role in the regulation of cell growth.

A gene associated with cell organization and biogenesis, A-disintegrin and metalloprotease domain 8 gene (ADAM8, accession no. BC025584, chromosome 7F3-F5), which is possibly involved in extravasation of leukocytes, was induced. As mature T-cells migrate from the thymus to the periphery, this gene could be implicated in such process.

Transcription factors such as nuclear transcription factor-Y gamma gene (NFYC, accession no. BC053723, chromosome 4), 3FP62 (tripartite motif protein 28 gene) (TRIM 28, accession no. NM011588 chromosome 7A1) form a complex with a krab-domain transcription factor and increase the efficiency of krab-mediated repression by recruiting setdb1 to histone H3 (by similarity).

We also observed induction of those genes implicated in protein metabolism such as PRSS16 [protease, serine, 16 (thymus) gene, accession no. AK088019, chromosome 13] which codes for a protease that may play a role in T-cell development and is specifically expressed in the developing thymus, and calpain 8 gene (CAP8, accession no. NM130890, chromosome 1H5) that codes for a cysteine-type endopeptidase and whose enzyme is involved in proteolysis and peptidolysis.

Cluster 6 (correlation = 0.77) is formed by genes whose functions are directly related to the response to DNA damage stimulus such as X-ray repair complementing defective repair in Chinese hamster cells 4 gene (XRCC4, accession no. AK038105, chromosome 13C3) involved in DSB repair and heat shock protein 1-like gene (HSC70T, accession no. D85732, chromosome 17) whose coded protein in cooperation with other chaperones, for example HSP70, stabilizes pre-existing proteins against aggregation and mediates the folding of newly translated polypeptides in the cytosol, as well as within organelles. These chaperones participate in the recognition of non-native conformations of proteins. Another gene present in the cluster 6 was xeroderma pigmentosum, complementation group C gene (XPC, accession no. AK028595, chromosome 6D), which encodes a DNA repair protein, which is involved in DNA excision repair (NER) and may play a role in DNA damage recognition. XPC seems to recognize DNA helix distortions (Gontijo et al., 2003) and is responsible by opening the DNA helix in response to DNA damage (Tapias et al., 2004).

Moreover, as the DNA repair XPC protein may alter chromatin structure, it is plausible that its action could facilitate the accessibility of the V(D)J machinery to the TR locus.

We also found genes directly associated with immune response/activation and with the V(D)J recombination process. There are four interleukin genes, IL-1B, accession no. NM008361, chromosome 2; IL-2, accession no. X01772, chromosome 3; IL-6, accession no. AK089780, chromosome 5 and IL-7, accession no. AK041403, chromosome 3. The IL-7 is a cofactor associated with modulation of V(D)J

recombination of TRB8.1 [2,3]. The lymphotoxin A gene, accession no. NM010735, chromosome 17, codes for the tumor necrosis factor produced by lymphocytes. This protein is cytotoxic for a wide range of tumor cells *in vitro* and *in vivo*.

Two genes in cluster 6 are implicated in immune cell activation, cyclin D3 gene (CCND3, accession no. AK046638, chromosome 17) codes for a cdk6 protein kinase essential for the control of the cell cycle at the G1/S transition and early growth response 1 gene (EGR1, accession no. M22326, chromosome 18) codes for a transcriptional regulator (early growth response protein 1) that activates the transcription of target genes whose products are required for mitogenesis and differentiation.

Finally, we found genes in this cluster closely implicated in the V(D)J recombination mechanism of T-cell receptor genes and immunoglobulins (Ig). The recombination activating gene 2 (RAG-2, accession no. AK040375, chromosome 2) codes for recombinase 2, a component of the rag enzymatic complex (rag complex formed by rag-1 and rag-2 recombinases). This complex recognize and binds to the recombination signal sequences (RSSs) at the unrearranged TR or Ig loci, consisting of conserved heptamer and nonamer motifs separated by a spacer region of 12 or 23 bp.

V(D)J recombination results in a precise head-to-head ligation of two signal sequences in a joint signal, which may contain additions or deletions of a few bp. The reaction is initiated by the formation of DSB, which is resolved by the DNA repair machinery, followed by an exonuclease and/or by terminal deoxynucleotidyl transferase (TdT) before formation of a coding joint.

Mice that do not express RAG-1 or RAG-2 genes are unable to form DSB and there is evidence that DNA DSB repair and V(D)J recombination share a partially common mechanism (Schlissel et al., 1993; Chaudhuri and Alt, 2004; Jeggo and Concannon, 2001).

DNA-activated protein kinase gene (DNA-PK_{cs}, accession no. AK088981, chromosome 16), also present in cluster 6, codes for a large polypeptide DNA-dependent protein kinase catalytic subunit and its kinase activity is involved in DNA NHEJ required for DSB repair and V(D)J recombination. DNA-PK_{cs} forms a complex with two subunits of the heterodimeric Ku protein (Ku 70 and Ku 80) and must be bound to DNA to express its catalytic properties; cells that lack DNA-PK_{cs} are radiosensitive and defective in DNA repair, while DNA-PK_{cs} null mice are viable but immunodeficient, due to inability to complete V(D)J recombination (Lees-Miller and Meek, 2003; Pastwa and Blasiak, 2003).

The assembly of the DNA-PK_{cs} complex to DNA ends is required for NHEJ binding step. Interestingly, the XRCC4 repair gene was also observed in cluster 6, and the product of this gene was found to interact with DNA ligase IV stimulating the ligase activity, in order to complete the NHEJ mechanism and probably the V(D)J recombination process (Lees-Miller and Meek, 2003; Grawunder et al., 1997).

Interestingly, the MMTV endogenous retrovirus (accession no. BU515481, chromosome 19) is also present in this cluster and displayed overexpression in irradiated FTOCs. We have previously shown an association between the expression of MMTV and the parallel reduction of the TRBV8.1 rearranged segment *in vivo* during the thymus ontogeny of the CBA/J strain (Espanhol et al., 2003).

Finally, the DNA cross-link LR1 gene (DNA cross-link repair 1B, accession no. BC011094, chromosome 3F2.2), which codes for a protein involved in nucleotide excision repair, was induced in irradiated cultures. The deduced amino acid sequence of its coded protein was about 30% similar to that of the Artemis protein (Moshous et al., 2001) and its expression pattern was similar to that of genes implicated in V(D)J recombination, suggesting a possible role for this gene in this process.

In conclusion, our results showed for the first time that the FTOC model system reproduces the *in vivo* development of the thymus regarding TRBV8.1-BD2.1 V(D)J recombination.

Ionizing radiation, a potent physical agent capable of inducing DNA DSB, did not change the onset of recombination but increased the amount of the TRBV8.1-BD2.1 rearranged segment, suggesting a modulation of genes involved in such process.

The hybridization signatures obtained with cDNA microarrays permitted the identification of genes modulated during gamma irradiation of FTOCs. Among them, we found some genes related to the V(D)J recombination mechanism and we were able to pinpoint the DNA cross-link LR1 gene (DNA cross-link repair 1B) whose deduced protein sequence is similar to that of the Artemis protein, and the RUNX3 and SOX4 genes whose coded proteins bind to the enhancers of T-cell receptor genes.

The present data obtained by application of the cDNA microarray method indicated several genes participating in the mechanism of V(D)J recombination and while some genes are novel candidates, some others are important components for the repair of DSB via NHEJ, thus strongly suggesting an overlap between the two processes. These results open new perspectives for further studies on the specific roles of these genes, using strategies of gene silencing, such as RNA interference.

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Hybridization signatures during thymus ontogeny reveals modulation of genes coding for T-cell signaling proteins

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Abstract

Non-manipulated inbred mouse strains constitutes an interesting model-system for *in vivo* studies on thymus ontogeny due to the possibility to observe the molecular events of the thymocyte maturation. In previous studies, using RT-PCR method, we have found that several immune system genes such as interleukins and MHC are differentially expressed during ontogeny of the thymus whose genes act as modulators of T-cell differentiation. To determine which other genes are modulated on a large-scale basis, we measured the levels of mRNA expression in mouse fetal thymus (14–17 days of gestation) by hybridization with cDNA microarrays containing 1,576 cDNA sequences derived from the IMAGE MTB library. T-cell maturation was monitored by detection of the T-cell receptor beta TRBV8.1-BD2.1 rearranged DNA segment. Each developmental phase of thymus, displayed a characteristic expression profile, as evaluated by the Cluster and Tree-View softwares. Genes differentially and significantly expressed were selected on the basis of significance analysis of the microarray data (SAM program). With the reclustering of only significantly expressed genes, it was possible to characterize the phases of thymus ontogeny, based on the differential profile of expression. Our method provided the detection of genes implicated in the cell signaling, such as the hematopoietic cell signal transducer gene, genes implicated in T-cell calcium influx (tyrosine phosphatase) and calcium signaling proteins (vesicle transport binding protein 3, proline rich Gla, casein kinase alpha 1 and Down syndrome homolog protein 1) and a gene important for the protein transport, including T-cell receptors chains, towards the cell membrane (Golgi SNAP receptor complex member 2). The results demonstrate that the cDNA microarray used to explore the gene expression was useful for understanding the modulation of several cell-signaling genes, including the calcium cascade pathway, which is important for individual stages of T-cell maturation and control of anergy during thymus ontogeny.

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1. Introduction

The differentiation of thymocytes to mature T-cells occurs within the fetal thymus and all developmental stages are distinguishable by their expression of combination of CD cell-surface markers. This is a highly modulated phenomenon,

whose central molecular machinery is formed by the recombinase complex (RAG-1 and RAG-2) directly implicated in the V(D)J recombination of T-cell receptor gene segments (TRA, TRB, TRG and TRD) (Lefranc and Lefranc, 2001). The occurrence of the V(D)J reaction is important for triggering the maturation of T-cells and is also regulated by several other gene products such as interleukins (Fink and McMahon, 2000; Fugmann, 2002; Muegge et al., 1993; Sollof et al., 1997).

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The timing of T-cell maturation during thymus ontogeny is different among inbred mouse strains, strongly suggesting a role for the genetic background in the modulation of this phenomenon (Junta and Passos, 1998; Macedo et al., 1999).

Using the reverse-transcription-PCR (RT-PCR) method we have previously found that several immune system-related genes such as interleukins and the major histocompatibility complex (MHC) are differentially expressed during thymus ontogeny of inbred mouse strains (Espanhol et al., 2003).

Although the knockout mice have been used as a valuable tool for defining the role of a set of immune system-related genes (Mak et al., 2001), non-manipulated mouse strains still constitute a classical model-system useful for studies on the *in vivo* modulation of several other genes acting in cascade during thymocyte development.

The new functional genomic technology using the cDNA microarray method provides the opportunity to perform a comparative analysis between the different phases of the thymus ontogeny, yielding quantitative expression data of hundreds or thousands of genes in a single experiment.

The early fetal thymus has homogeneous cell populations composed of double negative (DN) thymocytes, whose proportion changes to double positive (DP) in the late stages of fetal development. In contrast, the newborn and adult thymus is the place of arrival of new colonizing precursors, which contribute to the formation of heterogeneous cell populations including macrophages and dendritic cells (Shortman and Wu, 1996).

In this study, we evaluated the dynamic transcriptional profile of thymus development observed during the gestational period of (Balb-c $\times$ C57Bl/6) F₁ hybrid mice using a set of two nylon cDNA microarrays containing a total of 1,576 thymus IMAGE sequences. Each phase of thymus development exhibited a characteristic gene expression pattern involving genes of known functions, permitting the identification of those implicated in the T-cell signaling pathway and expressed sequence tags (ESTs) whose functions are still unknown. The differentially and significantly expressed genes and ESTs observed here may be considered as novel markers characterizing individual stages of thymus ontogeny.

2. Material and methods

2.1. Fetal thymus, RNA and DNA preparation

The (Balb-c $\times$ C57Bl/6) F₁ hybrid mice were obtained in our own animal facilities. Pregnant Balb-c females were sacrificed by ether inhalation and the fetuses were surgically collected from the uterus. The age of the fetus (in days post coitum, p.c.) was confirmed on the basis of the morphological characteristics of each development phase (Rugh, 1968).

Fetal thymus tissue (1 mg containing approximately 1×10^7 cells) was obtained by surgery under a stereomicroscope and immediately processed for total RNA and genomic

DNA extractions using Trizol[®] reagent, following the manufacturer's instructions (Invitrogen).

2.2. TRBV8.1-BD2.1 recombination assay

T-cell maturation was monitored by detection of rearrangements between segments TRBV8.1-BD2.1 during thymus development using PCR (Muegge et al., 1993). The oligonucleotide primers were the VB8.1 forward primer (primer 1, GAGGCTGCAGTCACCCAAAGTCCAA) the VB8.1 reverse primer (primer 2, ACAGAAATATACAGCTGTCTGAGAA), and the JB2.1 reverse primer (primer 3, TGAGTCGTGTTCTGTCGCAAGAA). The PCR reaction mixture contained 35 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, bovine serum albumin (100 μ g/ml), nucleotide triphosphates (NTPs) (2 μ M each), the primers (0.5 μ M each), 1 U Taq polymerase (Amersham Biosciences, Buckinghamshire, England), with a constant amount of genomic DNA (1 μ g) from each developmental stage. The PCR program consisted of 30 cycles (1 min at 94 °C, 2.5 min at 54 °C, and 1.5 min at 70 °C, with a 7-min extension period).

The PCR product obtained with primers 1 and 3 was resolved on 1.2% agarose gel and blotted on Hybond Nylon+ (Amersham Biosciences). The rearranged VB8.1-BD2.1 330 bp segment was detected by Southern hybridization with the ³²P-labeled PCR product of 100 bp obtained with primers 1 and 2, and visualized using a phosphor imager system (Cyclone, Packard Co. USA).

2.3. cDNA-microarray method

We used a pair of cDNA microarrays containing a total of 1,576 clones in the form of PCR products, spotted in duplicate on 2.5 $\times$ 7.5 cm Hybond N+ nylon membranes (Amersham Biosciences). The arrays were prepared in our laboratory using a Generation III Array Spotter (Amersham-Molecular Dynamics). A complete file providing all genes present in the microarrays used in this study is available on line at http://rge.fmrp.usp.br/passos/nylon_array/mtb1.

Mouse EST cDNA clones were obtained from the Soares thymus 2NbMT normalized library, prepared from a C57Bl/6J 4-week-old male thymus, and available in the I.M.A.G.E. Consortium (<http://image.llnl.gov/image/html/iresources.shtml>).

cDNA inserts were homogeneous in size (near 1 Kb) and cloned in three vectors (pT7T3D, pBluescript and Lafmid) and were amplified in 384- or 96-well plates using vector-PCR amplification with the following primers, which recognize the three vectors, LBP 1S 5'-GTGGAA-TTGTGAGCGGATAACC-3' forward and LBP 1AS 5'-GCAAGGCGATTAAAGTTGG-3' reverse.

The membranes were first hybridized with the LBP 1AS [γ -³³P]dCTP (Amersham Biosciences) labeled oligonucleotide (vector hybridization). Quantification of the obtained signals allowed the estimation of the amount of DNA

deposited in each spot. After stripping, the membranes were subsequently used for hybridization with cDNA complex probes (sample hybridization).

The characterization of each cDNA sequence was updated using the Elogé® (Ipsogen, France, www.ipsogen.fr) software, which runs in our local server and links each clone ID with the genome data banks (GenBank, www.ncbi.nlm.nih.gov and S.O.U.R.C.E., <http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch>), allowing a set of information such as sequence, biological and molecular functions, chromosomal location, percent identity with humans and expression in different tissues.

2.4. Complex cDNA probe preparation and hybridization

In this study, we refer to the radioactive cDNA originated from the thymus RNA samples as complex probe and the PCR product originated from the clones and deposited on the nylon microarrays as target.

The ^{33}P -labeled cDNA complex probes were prepared by reverse transcription of 10 μg of thymus total RNA using oligo dT_{12–18} as primer. One hundred microliters of ^{33}P -cDNA complex probe containing 30–50 million cpm was hybridized with nylon microarrays as previously described (Bertucci et al., 2002; Nguyen et al., 1995; Verdeil et al., 2002).

2.5. Imaging acquisition

We used imaging plates and a phosphor imager (Cyclone, Packard Instruments, USA) to capture the hybridization signals and the Array Vision® (Imaging Research Inc. USA) to quantify the signals with local background subtraction, whose spots were matched with a template grid.

The ratio between vector probe hybridization values and complex probe hybridization values for each spot was used as reference normalization value. We employed also total intensity normalization, using the median expression value (Quackenbush, 2002).

2.6. cDNA-microarray data analysis

The gene expression data analyzed here were obtained from three independent determinations for each day of fetal development.

We used the SAM method (Significance Analysis of Microarrays available at <http://www-stat.stanford.edu/~tibs/SAM/index.html>) Tusher et al. (2001) to analyze the significant variations in the gene expression. This method is based on *t*-test statistics, specially modified to high throughput analysis. The significant variations in the gene expression of the developing thymus were compared with the 14 days p.c. (14 versus 15, 14 versus 16 and 14 versus 17 days of

gestation). The program calculates a global error chance, the false discovery rate (FDR) and a gene error chance (*q*-value).

2.7. Hierarchical clustering

We used a hierarchical clustering algorithm, comparing means of different genes whose standard deviation did not overlap, whose objective was to compute a dendrogram that assembles all elements into a single tree. The software for this algorithm can be obtained from the author (<http://rana.stanford.edu/clustering>) (Eisen et al., 1998). Before clustering, the gene expression values were median-centered and converted to log. We used the Pearson correlation distance metrics and average linkage for clustering organization.

From the 1,576 sequences present on the microarrays, 315 (20%) were excluded from the calculations by the software due to their low statistical significance (in this case were analyzed 1,261 sequences, including 200 named genes and 1,061 ESTs).

In order to cluster only genes whose expression values were significant, we used the values for the induced and repressed genes detected by the SAM method.

3. Results

3.1. Monitoring thymocyte maturation

The detection of the T-cell receptor TRBV8.1-BD2.1 rearranged DNA segment served as an indication of thymocyte maturation during the fetal development of the thymus. We detected the onset of TRBV8.1 V(D)J recombination at 14 days gestation in the heterozygous (Balb-c $\times$ C57Bl/6) F₁ fetuses whose amount of the rearranged DNA fragment increased during thymus ontogeny (Fig. 1).

3.2. Hierarchical clustering of significant genes

The hierarchical cluster analysis, computing all the 1,261 sequences and probes from thymi of fetuses of different

Fig. 1. Detection of the recombined TRBV8.1-BD2.1 segment in genomic DNA during thymus ontogeny of (Balb-c $\times$ C57Bl/6) F₁ hybrid mice. (–) = PCR without DNA, A31 = murine Balb/3T3 derived fibroblast cell line DNA.

Fig. 2. Clustering samples of each day of thymus gestation of the (Balb-c $\times$ C57Bl/6) F₁ hybrid mice considering the raw normalized expression data of the 1,261 genes. (www.rge.fmrp.usp.br/passos/TRBV81/cluster1261).

ages p.c., showed that there is variability in the patterns of gene expression of the thymus within mice for each day of gestation. This variability caused shuffling in the clustering of the RNA samples (cDNA probes) of fetuses (Fig. 2) (www.rge.fmrp.usp.br/passos/TRBV81/cluster1261). We used the approach of clustering the significant genes proposed by the SAM program, i.e. only those induced or repressed genes whose variability was statistically significant. This approach successfully distinguished the samples of thymi according to days of gestation (Fig. 3) (www.rge.fmrp.usp.br/passos/TRBV81/reclustering/SAM).

3.3. Genes differentially expressed in the developing thymus

To identify significant changes in gene expression during fetal development of the thymus, we compared the stage when the onset of TRBV8.1 recombination occurs, at 14 days of gestation, with those observed on the subsequent days (14 versus 15, 14 versus 16 and 14 versus 17 days p.c.). We used a scatter plot of the observed relative difference $d(i)$ versus the expected relative difference $d_E(i)$ as shown by the SAM program.

Most of the 1,261 genes tested presented $d(i) \cong d_E(i)$, i.e. their expression pattern did not change; however, some genes were significantly induced or repressed during thymus development (Table 1) (www.rge.fmrp.usp.br/passos/TRBV81/SAM/table1).

Fig. 3. Reclustering samples of each day of thymus gestation of (Balb-c $\times$ C57Bl/6) F₁ hybrid mice considering only the significant expressed genes reported by the SAM program. (a) 14 vs. 15, (b) 14 vs. 16 and (c) 14 vs. 17 days of gestation. (www.rge.fmrp.usp.br/passos/TRBV81/reclustering/SAM).

Table 1

Number of genes differentially expressed during thymus development of (Balb-c $\times$ C57Bl/6) F₁ hybrid mice as reported by the SAM program^a

Thymus development (in days p.c.)	Significant genes		FDR
	Induced	Repressed	
14–15	31	73	48
14–16	16	47	22
14–17	18	21	64

^a Complete file of gene names available online (www.rge.fmrp.usp.br/passos/TRBV81/SAM/table1), FDR = false discovery rate (median).

4. Discussion

The aim of the present study was to perform a comparative large-scale gene expression analysis of thymocyte maturation during ontogeny of the thymus of (Balb-c $\times$ C57Bl/6) F₁ hybrid mice to gain new insights into the modulation of gene transcriptions which could be correlated with T-cell maturation.

In previous observations, we described the different timing of T-cell maturation first by means of detection of the onset of the T-cell receptor TRA and TRB V(D)J recombinations in Balb-c, C57Bl/6 and CBA/J strains (Junta and Passos, 1998; Macedo et al., 1999) and second by showing that several immune system-related genes such as interleukins and MHC displayed a differential expression pattern among these strains during thymus ontogeny (Espanhol et al., 2003).

T-cell differentiation and all developmental stages within the thymus are distinguishable by the expression of CD cell-surface marker combination. The V(D)J recombination of the TRA/TRB and TRG/TRD segments is a central phenomenon defining the T-cell fate, which is mediated by recombinase complex from *RAG-1* and *RAG-2* genes and modulated by several other gene products (Muegge et al., 1993; Sollof et al., 1997).

In the present study we showed that the emergence of the TRBV8.1 rearrangement during fetal development of the heterozygous (Balb-c $\times$ C57Bl/6) F₁ mouse thymus occurs on day 14 p.c., similar to the parental strain C57Bl/6 (Espanhol et al., 2003). This suggest a participation of the genetic background of the C57Bl/6 strain in the control of T-cell maturation.

Despite the relevance of transgenic and knock-out mice to immunological research, the classical inbred non-manipulated mouse strains constitute a model-system for studies on the effects of different genetic backgrounds on the modulation of T-cell maturation. In recent years, our group has pursued this approach with the classical inbred strains (Espanhol et al., 2003, 2004; Junta and Passos, 1998; Macedo et al., 1999) and in the present study we are reporting the first large-scale hybridization signatures of the thymus of heterozygous mice resulted from the fusion of two inbred different genetic backgrounds.

Two approaches were used to analyze the large-scale gene expression profile of the developing thymus of F₁ hybrid

mice, encompassing a previous analysis of the genes which were only differentially expressed (Cluster and Tree-View method), and a second analysis clustering only those genes which were differentially and significantly expressed (SAM and Cluster and Tree-View methods).

By exploring a large set of genes, we intended to identify novel differentially expressed genes that could be used as markers for the individual stages of thymus ontogeny.

However, the clustering of raw expression data using the set of 1,261 genes caused a shuffling among RNA samples (cDNA probes) from each day of gestation (Fig. 2).

Considering the possibility that the similarity between samples from two distant days of gestation, such as 14 and 17 days p.c., as seen in the dendrogram of Fig. 2, might not be statistically significant, we applied a second round of clustering, now using the differentially and significantly expressed genes. Using this approach, it was possible to distinguish the days of gestation (Fig. 3), demonstrating the importance, in this study, of a previous statistical treatment instead of using only raw normalized microarray data to run the Cluster and Tree-View method.

Reclustering showed that the constitution of the gene cluster diverged for each stage of thymus development, i.e. each cluster harbored different genes with different expression patterns.

The 14th day p.c. RNA sample was considered to be the test sample for comparisons with the subsequent days of gestation (14 versus 15, 14 versus 16 and 14 versus 17 days p.c.); however, in a given cluster, the repressed genes in a given day of gestation presented as induced on the following day. This is evidence that the genes used for data reclustering may play a role in thymus ontogeny and may represent novel candidate genes participating in the control of T-cell maturation.

It was possible to point out seven genes implicated in cell signaling. The hematopoietic cell signal transducer gene, *Hcst*, (accession number NM011827, ID 640698) was induced in the early stages of thymus development (14–16 days p.c.) and repressed at 17 days p.c. The *Hcst* protein is located outside the plasma membrane and is implicated in the coupling of receptor stimulation to downstream activation of GTPases. As the maturation of the thymocytes within the thymus depends on the participation of other cell types, such as stroma (Gill et al., 2003), this could represent evidence for a role of the *Hcst* gene in cellular communication via molecular signaling in the early thymus.

Genes implicated in the calcium signaling pathway were also modulated, such as the proline-rich Gla (G-carboxyglutamic acid) polypeptide 2 gene, *Prrg2*, (accession number NM022999, ID 640686), the Down syndrome critical region homolog 1 gene, *Dscr1*, (accession number NM019466, ID 640638), and the syntaxin binding protein 3 gene, *Stxbp3*, (accession number NM011504, ID 640484), whose proteins have a role in the inhibition of the calcium influx pathway via calcineurin. These genes were repressed in the early (15 days p.c.) and late (17 days p.c.) thymus. The casein kinase 1, alpha 1 gene, *Csnk1a1* (accession number

Table 2

Number of ESTs differentially expressed during thymus development of (Balb-c × C57Bl/6) F₁ hybrid mice as reported by the SAM program^a

Thymus development (in days p.c.)	Significant ESTs		FDR
	Induced	Repressed	
14–15	20	49	48
14–16	10	30	22
14–17	10	14	64

^a Complete file of ESTs available online (<http://www.rge.fmrp.usp.br/passos/TRBV81/SAM/table2>), FDR = false discovery rate (median).

NM146087, ID 640022) whose protein is an inhibitor of calcineurin, was induced at 16 days p.c. These data, suggest that the calcium influx pathway could be activated in the thymus with 15 and 17 days p.c. and down-regulated with 16 days p.c.

All these genes above mentioned participates in the control of calcium influx pathway mediated by calcineurin, which is important in the activation of the nuclear factor of activated T-cells (NFAT), an transcription factor of T-cells.

As calcium signaling is implicated in the induction of T-cell anergy mediated through calcineurin and NFAT, our results could be useful to know the genes that induce tolerance (Feske et al., 2001, 2003; Heissmeyer et al., 2004).

The Golgi SNAP receptor complex member 2 gene, *Gosr2*, (accession number NM019650, ID 640152) was induced in the thymus from 15 to 17 days p.c. This gene has a role in the intracellular transport of newly synthesized proteins from the endoplasmic reticulum to their destination in the cell. Since we showed in this study that T-cells within the thymus begin to mature from 14 days p.c., the activation of the *Gosr2* gene in this phase is of particular importance due to the necessity of delivering the T-cell receptors chains on the cell surface.

Finally, we have shown that the protein tyrosine phosphatase 4a3 gene, *Ptp4a3*, (accession number NM008975, ID 640437), was induced in the thymus with 17 days p.c. There is evidence for a role of this gene in humans in colorectal cancer metastasis (Saha et al., 2001), i.e. cell migration. As the mature thymocytes should migrate from the thymus to blood stream, this may be evidence for the participation of the *Ptp4a3* gene in the late stages of T-cell maturation including migration from the thymus.

The 133 EST sequences reclustered in the dendrograms of Fig. 3, whose functions were not yet assigned, presented a differential pattern of expression and were reclustered together with named genes (Table 2) (www.rge.fmrp.usp.br/passos/TRBV81/SAM/table2). This can be evidence for the participation of these ESTs in biological processes similar of those of the known genes. These ESTs are being studied by our group with the aim of associating their expression profile with gene function.

As the microarrays used in this study were prepared with thymus sequences, we explored their expression levels during the development of this organ. Although, the thymus harbors different cell types including macrophages and dendritic

cells, we have found statistically significant expression of genes whose functions are associated with thymocytes.

The genes selected for discussion in this study may have a broader functions(s) during thymus development in cell types other than thymocytes. However, these aspects are still an open matter.

Taken together, our findings demonstrate that the cDNA microarrays and the bioinformatics programs employed here were useful tools to demonstrate the changes in the gene expression pattern of developing thymus which may reflect specific organ transcriptome programs.

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Gene expression profiling during thymus ontogeny and its association with TCRV β 8.1-D β 2.1 rearrangements of inbred mouse strains

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Abstract

The V(D)J recombination of TCR α and β in early developing T-cells is a highly modulated phenomenon initiated and completed by recombinase complex (RAG-1 and RAG-2), and regulated by other gene products such as interleukins. To further evaluate the association of several other gene products with the evolution of TCRV β 8.1 V(D)J rearrangements *in vivo*, the mRNA expression levels of seven interleukins, three cytokines, receptors TCRV β 8.1 and IL-2R β , MHC-I/MHC-II, RAG-1/RAG-2 and retroviral superantigen MMTV(SW) were measured by RT-PCR during the fetal development of the thymus of three inbred mouse strains (Balb-c, C57Bl/6 and CBA/J). Clustering using the Tree View software, was used to organize these genes based on similarity of expression patterns. Each strain displayed a different expression profile during thymus ontogeny.

During the late developmental stage the most evident association was the kinetics of MMTV(SW) retrovirus, IL-2R β and IL-7 overexpression with reduction of TCRV β 8.1-D β 2.1 rearrangement in the thymus of CBA/J mice. These data suggest a susceptibility of this strain to expression of MMTV(SW) upon reduction of the rearranged TCRV β 8.1-D β 2.1 segment in developing thymocytes, with parallel IL-7 overexpression. (Mol Cell Biochem **252**: 223–228, 2003)

Key words: TCRV β 8.1, V(D)J recombination, gene expression profiling, cytokine, cluster analysis.

Introduction

Non-manipulated inbred mouse strains constitute a model system for studies on the effects of different genetic backgrounds on the *in vivo* maturation of thymocytes. In previous work, we have observed that onset of T-cell receptor (TCR α/β) V(D)J recombination differs among the Balb-c, C57Bl/6 and CBA/J strains [1, 2].

The V(D)J recombination in early developing T-cells is a highly modulated phenomenon. The rearrangements of T-cell

receptor genes (TCR α and β) is initiated and completed by the recombinase enzymatic complex (RAG-1 and RAG-2), but is regulated by other gene products such as interleukins [3, 4].

Stromal cells from the thymus or bone marrow are very important for development of T-cells producing cytokines [5, 6]. In this context, interleukin (IL)-7 in particular appears to serve as a proliferative agent before and after gene rearrangements and possibly as a survival factor during rearrangements [7].

Deletion of the IL-7 gene delays V(D)J recombination of the TCR β , γ and δ loci during fetal development in mice [6]. Recent studies have shown that IL-7 plays a decisive role in thymus maturation and T-cell differentiation modulating TCR α/β and γ/δ locus accessibility to the V(D)J recombinase [8, 9].

The function of T-cell receptors (TCR α/β) is related to the recognition of a processed non-self antigen together with a self molecule of class I or class II MHC. It is plausible to consider that these molecules expressed in the thymus environment may play a role in T-cell maturation [10].

The TCR β receptor is of particular interest because there is evidence for its essential role in TCR α/β differentiation. Thymuses of mice with a mutation in TCR genes contain 10-fold fewer cells than thymuses of wild-type mice and 50% of these cells are double positive, indicating that the recombination and/or expression of TCR β is required for maturation [11]. Moreover, the TCR β enhancer controls the recombinations of its own locus [12].

To further evaluate the association of several other gene products with the evolution of TCRV β 8.1 V(D)J rearrangements *in vivo*, the mRNA expression levels of seven interleukins (IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-7), three cytokines (TNF- β , IF- γ and GM-CSF), receptors IL-2R β and TCRV β 8.1, MHC-I and MHC-II, RAG-1 and RAG-2 and retroviral superantigen MMTV(SW) were measured by RT-PCR during the *in vivo* fetal development of the thymus of three inbred mouse strains (Balb-c, C57Bl/6 and CBA/J).

The expression profiles were examined using the Gene Cluster method and visualized with the TreeView software [13, 14]. The inbred strains displayed distinct gene expression signatures.

These data, when compared with the evolution of TCRV β 8.1 rearrangements, which were also distinct among strains, permitted a direct visualization of the effect of genetic background on the modulation of V(D)J recombination.

Materials and methods

TCRV β 8.1-D β 2.1 V(D)J recombination assay

Rearrangements between segments TCRV β 8.1-D β 2.1 during thymus development were investigated by PCR as previously described [3]. The oligonucleotide primers used were the V β 8.1 forward primer (primer 1, GAGGCTGCAGTCACC-CAAAGTCCAA) the V β 8.1 reverse primer (primer 2, ACA-GAAATATACAGCTGTCTGAGAA) and the J β 2.1 reverse primer (primer 3, TGAGTCGTGTTCTGTCGTCGAAGAA). The PCR reaction mixture contained 35 mM TRIS (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, bovine serum albumin (100 μ g/ml), nucleotide triphosphates (NTPs) (2 μ M each), the primers (0.5 μ M each), 1U Taq polymerase (Amersham Bio-

sciences), with a constant amount of genomic DNA (1 μ g) from each developmental stage. The PCR program consisted of 30 cycles (1 min at 94°C, 2.5 min at 54°C, and 1.5 min at 70°C, with a 7-min extension period). The PCR product obtained with primers 1 and 3 was resolved on 1.2% agarose gel and blotted on Hybond Nylon + (Amersham Biosciences). The rearranged V β 8.1-D β 2.1 330 bp segment was detected by Southern hybridization with the ³²P-labeled PCR product of 100 bp obtained with primers 1 and 2 and quantified using a phosphor imager system (Cyclone, Packard Co., USA) and the OptiQuant® software.

Reverse transcription-PCR (RT-PCR)

The gene transcriptions were quantified with cDNA prepared from total thymus RNA.

Balb-c, C57Bl/6 and CBA/J mice were mated by isogenic crosses, pregnant females were sacrificed by ether inhalation and fetuses surgically collected from the uterus. The age of the fetuses (as days p.c.) was confirmed on the basis of the morphological characteristics of each phase of development [15].

Thymus tissue (one milligram containing approximately 1×10^7 cells) from fetuses at different developmental stages (14–17 days p.c.) and from new born pups and adults was obtained by surgery under a stereomicroscope and immediately processed for total RNA using Trizol® reagent (Invitrogen).

The cDNA samples were obtained by reverse transcription from thymus total RNA using the Super Script II enzyme and oligo dT_{12–18} to prime the reaction according to the instructions of Invitrogen. The cDNA was used as template for PCR (RT-PCR) using the primers for genes listed in Table 1. The PCR mixture was as described for the V(D)J recombination assay with a constant amount of cDNA from each developmental stage. The PCR program consisted of one cycle at 94°C for 1 min, 30 cycles (1 min at 94°C, 1 min at 60°C (except for IL-1 α at 55°C), 1 min at 72°C), and a 1-min extension period.

These conditions permitted proportional amplifications according to the expression levels, enough to be detected by the oligonucleotide hybridizations and quantified using the phosphorimager system.

The RT-PCR products were blotted on nylon Hybond N+ (Amersham Pharmacia Biotech) using a 96 well Hybri-Dot Manifold (Life Technologies, Inc.). The mRNA expression levels were detected by hybridization with ³²P-labeled specific oligonucleotides internal to each cDNA (Table 1), except for TCRV β 8.1, RAG-1 and RAG-2, MHC-I and MHC-II, whose probes were the ³²P labeled PCR product obtained with forward and reverse primers and quantified using the phosphor storage system as mentioned above. The values were normalized on the basis of β -actin expression in each total RNA sample.

Table 1. Target cDNA sequences amplified by RT-PCR, oligonucleotide primers and probes^a

Target	Forward primer	Reverse primer	Internal probe
IL-1 α	TTACAGTGAACGAAGA	TGTTTGTCACATCCTG	GAGAACCTCTGAAACGTC
IL-1 β	GCAACTGTTCTGAACTC	CTCGGAGCCTGTAGTGCA	ATTGTGGCTGTGGAGAA
IL-2	AACAGCGCACCCACTTCAA	TTGAGATGATGCTTTGACA	GTTTCATCTTCTAGGCACTG
IL-3	GCCAGCTCTACCACCAGCA	AACATTCCACGGTTCCACG	AGGTTCTGGGAGCTTCCC
IL-4	TAGTTGTCATCCTGCTCTT	CTACGAGTAATCCATTTCG	GATGATCTCTCTCAAGTG
IL-6	TTCCTCTCTGCAAGAGACT	TGTATCTCTCTGAAGGACT	CATTTCCACGATTTCCCA
IL-7	ACATCATCTGAGTGCCACA	CTCTCAGTAGTCTCTTTAG	TGCCTTGTGATACTGTTAG
TNF- β	TCAGAAGCACTTGACCCAT	AAGTCCCAGGATACACAGACT	CAAAGTAGAGGCCACTGG
IF- γ	AACGCTACACACTGCATCT	TGCTCATGTGAATGCTTGG	TCGCCTTGCTGTTGCTGT
GM-CSF	TTCCTGGGCATTGTGGTCT	TGGATTCCAGAGCTGGCCTGG	ACCTCTTCATTCAACGTG
IL2-R β	CCAGCTGCTTCACCAACCA	AGAAGAGCAGCAGGTCATC	CCTGCCAGGTGTACTT
RAG-1	ATGGCTGCCTCCTTGCCGTCT	GTATCTCCGGCTGTGCCCCGC	-----
RAG-2	ATGTCCTGCAGATGGTAACA	TAAATCTTATCGGAAAGCTCA	-----
MHC-I	GGGGATGGAACCTTCCAGAAGTG	CACAAAAGCCACCACAGCTCCA	-----
MHC-II	CGACTGTAGAACCTTAGCCTG	GTGGACACAATTCCTGTTTT	-----
MMTV	GTAGACCTAATTGTATTATAAATGTT	GGACTTATAGGGGACCTTACAT	TGCTATAGCTCCCTCCCTCTTCG
β -ACTIN	ATGGATGACGATATCGCT	ATGAGGTAGTCTGTCAGGT	AGCAAGAGAGGTATCCT

^aProbes for cytokines, β -actin and MHC-I and MHC -II see ref. [26]; for RAG-1 and RAG-2 see [27] and for MMTV see [28].

Hierarchical clustering

The gene expression data analyzed here were collected, from three independent determinations, on blotted RT-PCR of several gene products from different developmental stages of the thymus of Balb-c, C57Bl/6 and CBA/J strains. We used a hierarchical clustering algorithm, comparing means of different genes whose standard deviation do not overlaps and, whose objective, is to compute a dendrogram that assembles all elements into a single tree. The software for this algorithm can be obtained from the author <http://rana.stanford.edu/clustering> [13, 14].

Results

Quantification of TCRV β 8.1 rearrangement in developing thymus

We detected temporal and quantitative differences in the onset of V(D)J recombination of TCRV β 8.1-D β 2.1 DNA segments among the strains studied.

The Balb-c strain started recombination at 16–17 days p.c., CBA/J at 16 days p.c and C57Bl/6 at 14–15 days p.c. (Fig. 1).

The CBA/J strain drastically reduced the rearranged DNA segment at 17 days p.c., reappearing in newborn and adult thymus.

Waves of gene expression

The developing thymus of the three strains displayed three gene clusters according to their waves of expression levels

A)

B)

Fig. 1. V(D)J recombination between TCRV β 8.1 and D β 2.1 during thymus development investigated by PCR. (A) Southern-blot of PCR products. (B) Quantification of hybridizations using a phosphor-imager. Arbitrary values expressed in DLU (digital light units) as displayed by the OptiQuant® software.

as organized by the Tree View software. However, the constitution of these clusters diverged among strains, i.e. each cluster harbor different genes with different expression patterns.

*Balb-c Strain (Fig. 2a)**

Cluster 1 comprised the IL-4 gene which was not modulated remaining expressed at low levels during thymus development.

Cluster 2 comprised MHC-I, RAG-2, IF- γ , IL-6, MHC-II, MMTV(SW), IL-1 β , RAG-1, IL-2, IL-1 α and TCRV β genes. We observed waves of transcriptional activity at 15–16 days p.c. involving MHC-I, RAG-2, IF- γ , IL-6 and MHC-II. The adult thymus overexpressed IF- γ , IL-6, MHC-II, MMTV(SW), IL-1 β , RAG-1 and IL-2.

Cluster 3 comprised IL-3, IL-2R β , TNF- β , IL-7 and GM-CSF genes which displayed a peak of expression at 14 days p.c. remaining unmodulated and at low levels during development.

Taken together, the genes of the cluster 2 were usually more transcribed at 16 days p.c. and in newborn pups and adults.

CBA/J Strain (Fig. 2b)

Cluster 1 comprised MHC-II, IL-6, MMTV(SW), IL-2R β and IL-7 genes displaying a peak of expression in the late thymus (17 days p.c. and newborn pups).

Cluster 2 comprised IF- γ , IL-1 α , IL-3, IL-2, MHC-I, TNF- β , IL-1 β , TCRV β , RAG-2 and RAG-1 genes and showed a more equilibrated transcriptional activity, i.e. waves of up- and down-regulation distributed during development.

Cluster 3 comprised IL-4 and GM-CSF genes which, in relation to the other strains, were not modulated remaining expressed at low levels during thymus development.

C57Bl/6 Strain (Fig. 2c)

Cluster 1 also comprised only the IL-4 gene which was not modulated, remaining expressed at low levels during thymus development.

Cluster 2 comprised IL-1 β , IL-3, TCRV β , MMTV(SW), IL-1 α , RAG-2 and TNF- β genes. These genes were usually down-regulated except MMTV(SW) and TNF- β which displayed two peaks of expression at 16 days p.c. and newborn pups.

Cluster 3 comprised RAG-1, IL-6, IL-7, MHC-I, IL-2, IL-2R β , MHC-II, IF- γ and GM-CSF genes. Taken together, the genes of the cluster 3 were more transcribed displaying a peak of expression at 17 days p.c. except GM-CSF which was overexpressed in the early thymus (14–15 days p.c.) and down-regulated later.

Discussion

Our aim was to compare the evolution of TCR V β 8.1-D β 2.1 V(D)J rearrangements and transcription of several genes important in T-cell differentiation during the thymus ontogeny of normal inbred mouse strains. We observed that emergence of V(D)J recombination and transcription of TCR β locus differ among these strains (Fig. 1), strongly suggesting an effect of genetic background as previously discussed [1, 2].

T-cell differentiation occurs within the thymus and all developmental stages are distinguishable by their expression of combination of CD cell-surface markers. The V(D)J recombination of the TCR α/β and γ/δ segments is a central phenomenon defining the T-cell fate, which is mediated by the recombinase complex from RAG-1 and RAG-2 genes and modulated by several other gene products [3, 4].

Transcriptional regulation studies have monitored expression of RAG-1, RAG-2, transcription factors and cytokine genes during T-cell development *in vitro* and *in vivo*. T-cell specific transcription factors such as TCF-1, LEF-1, Sox-4, CREB and GATA-3 modulate the expression of several other important genes such as TCRs, RAG-1, IL-4 and CD4/CD8 α markers [16]. Data obtained by reverse transcription-polymerase chain reaction (RT-PCR) with total thymocytes showed that expression of RAG-1 begins at a high level and is then developmentally down-regulated, and IL-2 and IL-4 are both expressed at a low levels [17].

The mouse strains we studied displayed different expression patterns involving the 17 genes assayed (Fig. 2). These findings demonstrate that the RT-PCR method followed by hierarchical clustering can be used to visualize the effect of genetic background on the expression profiling of the thymus. Moreover, associations between the expression data can be established.

Several studies using *in vitro* cultures of fetal liver cells or fetal thymocytes have suggested that IL-7 signaling could specifically induce RAG-1 and RAG-2 and thus have an effect on TCR β rearrangements. Moreover, as thymocytes differentiate further, IL-7 becomes the dominant cytokine that appears to serve as a proliferative agent before and after rearrangement, and possibly as a survival factor during rearrangement [5, 7].

Clustering (Fig. 2) showed that the peak of IL-7 gene expression occurred before (Balb-c and C57Bl/6) or usually

*Figure 2 is available at: www.rge.fmrp.usp.br/passos/RT_PCR

coincided (CBA/J) with expression of the RAG-1 and RAG-2 genes.

In agreement with previous observations [17, 18] we observed that the overall level of RAG-2 expression was lower than that of RAG-1 in Balb-c and C57Bl/6 mice, whereas the inverse situation was observed in CBA/J mice.

Germline transcripts of TCRV β were detected before the TCR β locus rearrangements [1, 19–22] and our results with the CBA/J strain agree these observations. However, TCRV β was one among the lesser transcribed genes.

MHC-I and MHC-II displayed different expression patterns among strains and there was a suggestion that their contribution to the termination of TCR rearrangements is unlikely [23].

The clustering of IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IF- γ and IL-2R β genes diverged among strains; however, we observed a common tendency of their expression after (Balb-c) and before RAG-1 and RAG-2 genes (C57Bl/6 and CBA/J strains).

We were able to quantify the expression levels of all these genes implicated in T-cell proliferation, V(D)J recombination or T-cell clonal deletion, such as MMTV(SW) retroviral superantigen, during thymus ontogeny (14–17 days p.c.) in newborn pups and adults. The onset of the TCRV β 8.1-D β 2.1 rearrangement in fetal thymus was correlated with a previous expression of germline TCRV β 8.1 in the CBA/J strain (15–16 days p.c.). The most evident feature demonstrated was the kinetics of MMTV(SW) mRNA overexpression and parallel reduction of the TCRV β 8.1-D β 2.1 rearrangement in the 17 days p.c. thymus DNA of the CBA/J strain (Fig. 1), suggesting a susceptibility of this strain to the expression of MMTV(SW) superantigen in combination with its genetic background, including MHC H-2^k, upon reduction of T-cell clones bearing the rearranged V β 8.1 DNA segment, while coinciding with overexpression of IL-7 gene that plays a role in T-cell proliferation.

Expression of superantigen proteins from endogenous MMTV proviruses carried by mouse germline usually results in the deletion of self-reactive T-cells during negative selection in the thymus and the elimination of T-cells required for infection by specific milk-borne MMTVs [24, 25].

Our data contribute to establishing a direct correlation between the different expression profiling, including cytokines, and backgrounds of the strains with evolution of TCRV β 8.1-D β 2.1 rearrangements, of particular interest for characterization of CBA/J mice, since the expression of these genes are not typically studied in this strain.

However, due to the complexity of this phenomenon involving the participation of a range of known and unknown genes, our model-system with inbred mouse strains open perspectives for further studies on large-scale gene expression profiling using DNA-arrays.

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