

**FLÁVIA CRISTINA RODRIGUES LISONI**

**INVESTIGAÇÃO DA MODULAÇÃO DA EXPRESSÃO  
GÊNICA PELA ANEXINA A 1**



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**Tese apresentada para  
obtenção do Título de Doutor  
em Genética.**

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**São José do Rio Preto - SP  
2005**

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Doutor em Genética

Orientadora: Profa. Dra. Eloiza Helana Tajara da Silva

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GÊNICA PELA ANEXINA A1**

COMISSÃO JULGADORA

TESE PARA OBTENÇÃO DO TÍTULO DE DOUTOR

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São José do Rio Preto, \_\_\_\_/\_\_\_\_/\_\_\_\_

Dedico este trabalho aos meus grandes amigos.

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## LISTA DE ABREVIÇÕES

|                       |                                                                    |
|-----------------------|--------------------------------------------------------------------|
| ACTH                  | Hormônio adrenocorticotrófico                                      |
| ANXA1                 | Anexina A1                                                         |
| ANXA1 KO-/-           | <i>knockout</i> para o gene da ANXA1                               |
| ANXA1 <sub>2-26</sub> | Peptídeo da região N-terminal da proteína ANXA1                    |
| ATLa                  | Ligante <i>lipoxin A4</i>                                          |
| CCR10                 | Receptor 10 de quimiocina ( <i>motivo C-C</i> )                    |
| cPLA2                 | Fosfolipase citosólica A2                                          |
| CREB                  | Proteína ligadora do elemento respondedor de AMP cíclico           |
| EGF                   | Fator de crescimento epitelial                                     |
| EGFR                  | Receptor do fator de crescimento epitelial                         |
| ERK1 e 2              | Quinase regulada por sinais extracelulares                         |
| FARSLA                | Sintetase do RNAt-fenilalanina                                     |
| FGF                   | Fator de crescimento de fibroblasto                                |
| fMLP                  | Peptídeo formil-Met-Leu-Phe                                        |
| FPR                   | Receptor do peptídeo formil                                        |
| FPRL1                 | Receptor do peptídeo formil <i>like 1</i>                          |
| GC                    | Glicocorticóides                                                   |
| GHR                   | Receptor do hormônio de crescimento                                |
| Hep-2                 | Linhagem celular originalmente de carcinoma epidermóide de laringe |
| HGF                   | Fator de crescimento de hepatócito                                 |
| hrANXA1               | Anexina A1 recombinante humana                                     |



|                |                                                                          |
|----------------|--------------------------------------------------------------------------|
| IGF            | Fator de crescimento semelhante à insulina                               |
| I $\kappa$ B   | I Kappa B                                                                |
| IKK            | Quinase de I $\kappa$ B                                                  |
| ITGB1BP1       | Proteína 1 ligadora de integrina beta 1                                  |
| MAPK           | Proteína quinase ativada por mitógeno                                    |
| NF- $\kappa$ B | Fator nuclear de <i>kappa light polypeptide gene enhancer in B-cells</i> |
| PDFS           | Linhagem de células foliculoesteladas da pituitária humana               |
| PI3K           | Quinase de fosfoinosítídeos                                              |
| PKC            | Proteína kinase C                                                        |
| RaSH           | Hibridação subtrativa rápida                                             |
| SDF1 $\alpha$  | Fator derivado de células estromais $\alpha$                             |
| TGF $\beta$    | Fator de crescimento transformante $\beta$                               |
| TIFA           | Proteína ligadora de TRAF                                                |
| TNF            | Fator de necrose tumoral                                                 |
| TRADD          | Molécula associada com domínio de morte                                  |
| TRAF2          | Fator 2 associado ao receptor TNF                                        |
| VEGF           | Fator de crescimento endotelial vascular                                 |

## RESUMO

## ABSTRACT

A anexina A1 (ANXA1), uma proteína de 37kDa, é um mediador da ação de glicocorticóides nos sistemas neuroendócrino e imune e tem sido implicada na regulação do ciclo celular. Os mecanismos de ação dessa proteína são pouco entendidos e seu potencial na modulação da expressão gênica ainda não está esclarecido. No presente trabalho, utilizamos a técnica de Hibridação Subtrativa Rápida (RaSH) para avaliar os efeitos da anexina recombinante humana (hrANXA1) e seu peptídeo da região N-terminal (ANXA1<sub>Ac2-26</sub>) na expressão gênica. Para alcançar este objetivo, uma linhagem celular de carcinoma epidermóide de laringe (Hep-2) e outra de células foliculoesteladas da pituitária humana (PDFS) foram estimuladas por 72 horas com hrANXA1 e ANXA1<sub>2-26</sub>. A técnica de RaSH e sua validação por PCR semi quantitativa, *Western Blot* e imunocitoquímica mostraram 4 genes diferencialmente expressos: (a) *CCR10* (*GPR2*), o gene do receptor para citocina CCL27, que está envolvido na inflamação mediada por células T na pele e na neuroinflamação (b) *ITGBIBP1*, que codifica a proteína 1 ligadora da integrina beta 1 (ICAP-1A), (c) *FARSLA*, um gene que atua na regulação da diferenciação celular e (d) *TIFA*, um gene envolvido em vias de sinalização de sobrevivência celular. Além disso, camundongos *knockout* para a ANXA1 mostraram expressão reduzida de CCR10, tanto na pele como no trato respiratório. A função desses genes correlaciona-se com processos biológicos atribuídos à ANXA1 e reforçam o papel dessa proteína na resposta imune e na tumorigênese.

**ABSTRACT**

Annexin A1 (ANXA1), a 37kDa protein, is a mediator of glucocorticoid action in the neuroendocrine and host-defense systems, and has also been strongly implicated in the regulation of the cell cycle. The mechanism of ANXA1 action is poorly understood and its potential to influence gene expression has rarely been addressed. Accordingly, we have used rapid subtraction hybridization (RaSH) to examine the effects of human recombinant ANXA1 (hrANXA1) and an N-terminal ANXA1 peptide (ANXA1<sub>Ac2-26</sub>) on gene expression. To reach this aim, human larynx cell lines (Hep-2) and human pituitary folliculo stellate (PDFS) cell lines were stimulated for 72 hours with recombinant hrANXA1 and ANXA1<sub>Ac2-26</sub>. The RaSH analysis and the validation by semiquantitative PCR, western blot and immunochemistry showed four genes with strong differential expression: (a) *CCR10* (*GPR2*), a seven transmembrane receptor gene for chemokine CCL27, which is involved in T-cell mediated inflammation in the skin and in neuroinflammation, (b) *ITGB1BP1*, integrin beta 1 binding protein 1 gene (ICAP-1) (c) *FARSLA*, a tumor-selective regulator gene of cell differentiation and (d) *TIFA*, a cell survival signaling gene. Furthermore ANXA1 null mice showed a down regulation of *CCR10*, both in the skin and in the respiratory tract. The function of these genes correlate with biological properties ascribed to ANXA1 and further support a role for annexin A1 in host defense and tumorigenesis.



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## I. INTRODUÇÃO

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Nas últimas décadas, muitos trabalhos foram desenvolvidos para identificação de determinantes moleculares da tumorigênese e seus resultados permitiram a caracterização de um grande número de genes que atuam no controle do ciclo celular e em vários processos relacionados com a transformação de uma célula normal em maligna e com a invasão de outros tecidos pelo tumor (Hanahan, Weinberg, 2000). Além disso, esses resultados influenciaram o tratamento do câncer, com o desenvolvimento de terapias desenhadas para atingir um alvo molecular específico associado à doença (Sawyers, 2004).

Recentemente, surgiram muitas evidências de que o desenvolvimento e a progressão do câncer dependem não somente de suas características genéticas mas também de interações de suas células com as células do estroma, incluindo as endoteliais, as células do sistema imune, os fibroblastos, os adipócitos e as células inflamatórias (DeWever, Mareel, 2003). É possível que tais interações contribuam para o fenótipo neoplásico do mesmo modo que o desenvolvimento e a função de órgãos normais dependem da comunicação entre diferentes tipos celulares (Mueller, Fusenig, 2004).

O microambiente tumoral é muito diferente daquele observado em tecidos normais correspondentes, sendo caracterizado por composição alterada da matriz extracelular, densidade de microvasos elevada, abundância de células inflamatórias e fibroblastos com fenótipo ativado (Ronnov-Jessen, Petersen, 1996; Tlsty, Hein, 2001; DeWever, Mareel,





2003). Esse estroma “reativo” assemelha-se ao tecido de granulação formado durante a cicatrização (Dvorak, 1986), que também exhibe elementos capazes de migrar, proliferar e produzir fatores de crescimento importantes para o reparo tecidual (Powell et al., 1999).

Os detalhes dos mecanismos envolvidos na influência do estroma sobre o tumor não são bem conhecidos e alguns dados mostram resultados conflitantes como os experimentos de co-cultivo de estroma-tumor, que resultam em uma aparente perda de características malignas (Salgaller, 2003) ou em estímulo para proliferação (Coussens, Werb, 2002).

### **Elementos do estroma tumoral**

Os vasos sanguíneos são elementos críticos para o processo neoplásico, desde a progressão de uma condição benigna para maligna até o fenótipo invasivo. A indução de neovascularização por influxo de células endoteliais é realizada pelo tumor na tentativa de suprir suas necessidades metabólicas, com secreção de fatores de crescimento endoteliais, entre eles o fator de crescimento endotelial vascular/VEGF (Folkman, 2003), bem como pela indução da expressão de seus receptores nas células endoteliais do estroma, como o receptor 2 de VEGF (Mueller, Fusenig, 2002).

Os fibroblastos ativados do estroma são outros elementos com um papel importante em câncer, não apenas como fonte de fatores de crescimento parácrinos que atuam na proliferação das células neoplásicas, mas também como indutores de certos carcinomas. Por exemplo, os modelos *in vivo* revelam que a adição de fibroblastos ativados a epitélio iniciado de próstata aumenta dramaticamente a população de células epiteliais enxertadas em camundongos, ao contrário do que ocorre com a adição de fibroblastos normais (Izumi

et al., 2002). Um outro exemplo é o trabalho de Barcellos-Hoff e Ravani (2000). Esses autores mostraram que ocorre maior incidência de tumores de mama em camundongos transplantados com uma linhagem celular mamária não tumorigênica quando os fibroblastos mamários do local do transplante são irradiados previamente.

Vários fatores de crescimento derivados de fibroblastos estão envolvidos no processo neoplásico. Entre eles estão os fatores de crescimento de fibroblastos (FGF), semelhante a insulina (IGF), epitelial (EGF), de hepatócito (HGF) e transformante  $\beta$  (TGF $\beta$ ). A maioria é estimuladora de proliferação, exceto o TGF $\beta$  que é regulador negativo do crescimento celular mas também está envolvido positivamente no desenvolvimento de muitos carcinomas (Bhowmick et al., 2004).

Além da presença de fibroblastos ativados, a abundância de células inflamatórias é uma outra característica do microambiente tumoral, sendo seu papel na progressão neoplásica foco de muita discussão. A idéia de que a inflamação está envolvida na tumorigênese é apoiada pela observação de que o câncer surge freqüentemente em áreas de inflamação crônica. Os exemplos incluem a associação da pancreatite crônica e da colite ulcerativa com câncer de pâncreas e de cólon, respectivamente (Clevers, 2004; Farrow et al., 2004). Um outro exemplo é o desenvolvimento dependente de inflamação de tumores induzidos por injúria em galinhas infectadas pelo vírus do sarcoma de Rous (Martins-Green et al., 1994).

No início do processo neoplásico, as células inflamatórias e os fatores que produzem devem regular o crescimento, a migração e a diferenciação de todos os tipos celulares no microambiente tumoral incluindo as células neoplásicas, as endoteliais e os



fibroblastos. É possível que, posteriormente, as células neoplásicas utilizem alguns mecanismos inflamatórios para invasão de outros tecidos, como adesão de células à parede de vasos e migração ou diapedese por entre as células endoteliais. Perretti, em 1997, já havia destacado a semelhança da metastatização com esses estágios da inflamação.

As células inflamatórias secretam numerosas citocinas, fatores de crescimento e quimiocinas, que estimulam a proliferação, inibem a apoptose, induzem a morfogênese e geram espécies reativas de oxigênio capazes de lesar o DNA (Coussens, Werb, 2002). Em consequência, promovem um ambiente propício para o processo neoplásico, facilitando a instabilidade genômica (Ronnov-Jessen, Petersen, 1996; Tlsty, Hein, 2001). Além disso, como o VEGF, a angiopoetina e as metaloproteinases estão entre os fatores produzidos pelas células inflamatórias presentes no tumor, também ocorre estímulo para mitose de células endoteliais vasculares e remodelamento da matriz extracelular que contribuem para a angiogênese (Carmeliet, Jain, 2000).

Fica evidente desses dados que um grande número de padrões de sinalização devem participar das interações célula inflamatória-célula tumoral. Um exemplo é o padrão de sinalização do NF- $\kappa$ B, um fator de transcrição cuja ativação é dependente da quinase I $\kappa$ B (IKK) e ocorre em resposta a infecções virais e microbianas e a citocinas pró-inflamatórias. O NF- $\kappa$ B, que está relacionado com a transcrição de genes cujos produtos inibem a apoptose, já foi detectado ativado ou com expressão elevada em muitas neoplasias. Recentemente, foi reconhecido seu papel em tumores precedidos por inflamação crônica, que mostraram tamanho significativamente reduzido após inativação específica de IKK/NF- $\kappa$ B em células mielóides, provavelmente em consequência da expressão diminuída de



citocinas pró-inflamatórias (Greten et al., 2004).

Para entendimento do papel do estroma na tumorigênese, algumas abordagens envolvendo a análise da expressão gênica têm sido utilizadas e seus resultados têm revelado diferenças importantes entre os tipos celulares presentes no estroma tumoral e normal. O trabalho de Allinen e colaboradores (2004), por exemplo, identificou as quimiocinas CXCL14 e CXCL12 com expressão elevada em fibroblastos do estroma de carcinomas de mama. Esses autores observaram que tais quimiocinas ligam-se a receptores de células epiteliais e aumentam sua proliferação, migração e invasão. O trabalho de Oh e colaboradores (2004) também analisou o perfil protéico do estroma e comparou as proteínas expressas por membranas plasmáticas endoteliais de diferentes tumores e tecido normal adjacente. Os resultados chamaram a atenção para a expressão de uma proteína anti-inflamatória na superfície de células endoteliais, a anexina 1, que foi detectada apenas no material tumoral e não no tecido normal.

### **Anexinas: Aspectos gerais**

As anexinas (ANX) compreendem uma super-família bem conservada de proteínas, com pelo menos 13 membros descritos, relacionadas estruturalmente e caracterizadas por um domínio C-terminal homólogo com 4 a 8 repetições de 70-75 aminoácidos, responsável pelas suas propriedades ligadoras de cálcio e fosfolipídeos (Figura 1). Sua região variável N-terminal, que é única em comprimento e sequência para cada membro da família, inclui sítios potenciais de fosforilação, glicosilação e ação de peptidase (Gerke, Moss, 2002). São encontradas em muitos organismos, desde leveduras a plantas e mamíferos, preferencialmente no citosol, associadas à membrana plasmática ou ao citoesqueleto. Sua



função não está completamente esclarecida, mas existem evidências de sua participação em transdução de sinais (Alldridge et al., 1999; de Coupade et al., 2000), transporte de vesículas (Diakonova et al., 1997), inibição da inflamação (Getting et al., 1997), duplicação de DNA (Smith, Moss, 1994), transformação celular (Violette et al., 1990; Solito et al., 1998), formação de canais de ions, inibição de coagulação, interação célula-matriz (Croxtall et al., 1993) e apoptose (Raynal, Pollard, 1994; Moss, 1997).

A anexina 1 (ANXA1), originalmente conhecida como lipocortina 1, foi o primeiro membro dessa família que teve seu gene clonado e é considerada o seu protótipo (Wallner et al., 1986). O gene *ANXA1* está localizado no cromossomo 9, em 9q12-21.2, apresenta 13 exons e codifica uma proteína de 37kDa.

Além da função de inibidor da fosfolipase citosólica A2 (cPLA2), foi inicialmente verificado que os resíduos de tirosina da anexina 1 compreendiam um substrato para fosforilação pelo receptor do fator de crescimento epidérmico (Huang et al., 1986). Essas observações levaram à conclusão de que essa proteína representava um mediador da resposta anti-inflamatória, uma hipótese posteriormente apoiada por estudos mostrando sua expressão elevada após administração de esteróides durante a inflamação (Solito et al., 1991).

A atividade de inibição de cPLA2 está presente também em todos os outros membros da família das anexinas, cuja análise detalhada e de suas seqüências codificadoras resultaram em importantes informações sobre suas funções e sua regulação. Por exemplo, os dados de Antonicelli e colaboradores (2001) sobre seqüências do promotor de *anxa1* de camundongo forneceram evidências do envolvimento do fator de transcrição CREB





(proteína ligadora do elemento respondedor de AMP cíclico) na ativação desse gene por AMP cíclico ou glicocorticóides.

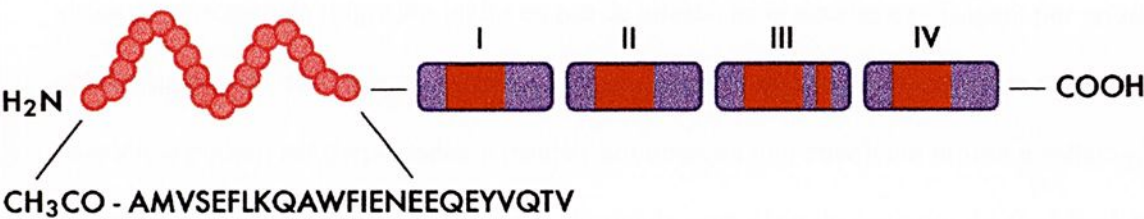
Alldrige e colaboradores (1999) observaram que a anexina regula os componentes proximais das cascatas de sinalização MAPK (proteína quinase ativada por mitógeno), o que pode explicar sua ação anti-proliferativa, talvez pela inibição da ciclina D1 ou pela indução da desorganização do citoesqueleto. Os elementos centrais da via de sinalização MAPK são proteínas quinases serina/treonina, que respondem a uma variedade de fatores de crescimento (como o fator de crescimento epidérmico) e seus receptores, citoquinas e hormônios. Uma das cascatas MAPK melhor caracterizadas compreende a das famílias ERK 1 e 2 (quinase regulada por sinais extracelulares), cuja ativação é importante para proliferação, desenvolvimento e diferenciação. Essa via é iniciada por receptores de fatores de crescimento ou receptores acoplados à proteína G, que desencadeiam uma série de fosforilações em mensageiros secundários culminando na translocação de um deles para o núcleo e conseqüente indução da fosforilação de fatores de transcrição e de outras proteínas associadas a genes de resposta imediata (Hazzalin, Mahadevan, 2002). As famílias ERK 1 e 2 participam de vias de sinalização que incluem famílias bem conhecidas em mamíferos, as quinases c-jun e as MAPKs p38, que são importantes para desenvolvimento, inflamação e apoptose (Pierce et al., 2002).

O mecanismo preciso pelo qual a ANXA1 inibe a proliferação celular não é bem conhecido, mas a maioria dos estudos indica seu papel na regulação de crescimento mediada pelos glicocorticóides (GC) (Buckingham, Flower, 1997; Alldrige, Bryant, 2003). Esses compostos induzem a expressão da ANX em vários tipos celulares, inclusive



em leucócitos (Perretti, Flower, 1996) e mastócitos (Oliani et al., 2000) e parecem levar à sua fosforilação e translocação para a superfície da célula (Solito et al., 2003b).

Vários trabalhos evidenciaram o papel das anexinas na exocitose. A ANXA1, por exemplo, foi detectada em grânulos contendo insulina e sua fosforilação mostrou-se concomitante à secreção desse hormônio (Ohnishi et al., 1995; Won et al., 2003). A ANXA2 é outro candidato para mediar a exocitose. Os dados de Liu (1999) e Konig e colaboradores (1998) revelaram sua importância na agregação de vesículas secretórias. A interação das anexinas com o citoesqueleto parece igualmente estar relacionada com esse processo, como sugerem estudos de Jones e colaboradores (1992) sobre a ligação da ANXA2 com a actina influenciando a secreção. A ANXA6, a única que contém 8 repetições no domínio C terminal, ao contrário, inibe o processo secretório. É possível que essa estrutura ímpar da ANXA6 permita a ocupação de sítios de interação, competindo com outras anexinas e bloqueando o processo (Donnelly, Moss, 1997).



**Figura 1.** Esquema da proteína anexina, mostrando os aminoácidos da região N terminal e as 4 repetições de 70-75 aminoácidos na região C terminal.

### **Anexinas e doenças humanas**

As anexinas, por causa de sua participação em padrões de sinalização, têm sido referidas na patogênese de algumas doenças, entre elas, processos crônicos decorrentes de inflamação, câncer e doenças auto-imunes. Realmente, auto-anticorpos contra várias anexinas foram detectados em pacientes com doenças auto-imunes como lúpus eritematoso sistêmico, artrite reumatóide e doença de Crohn. Entretanto, não está claro se sua presença reflete um mecanismo patogênético relevante ou representa uma expressão não específica de auto-imunidade aumentada nesses pacientes (revisto por Bastian, 1997).

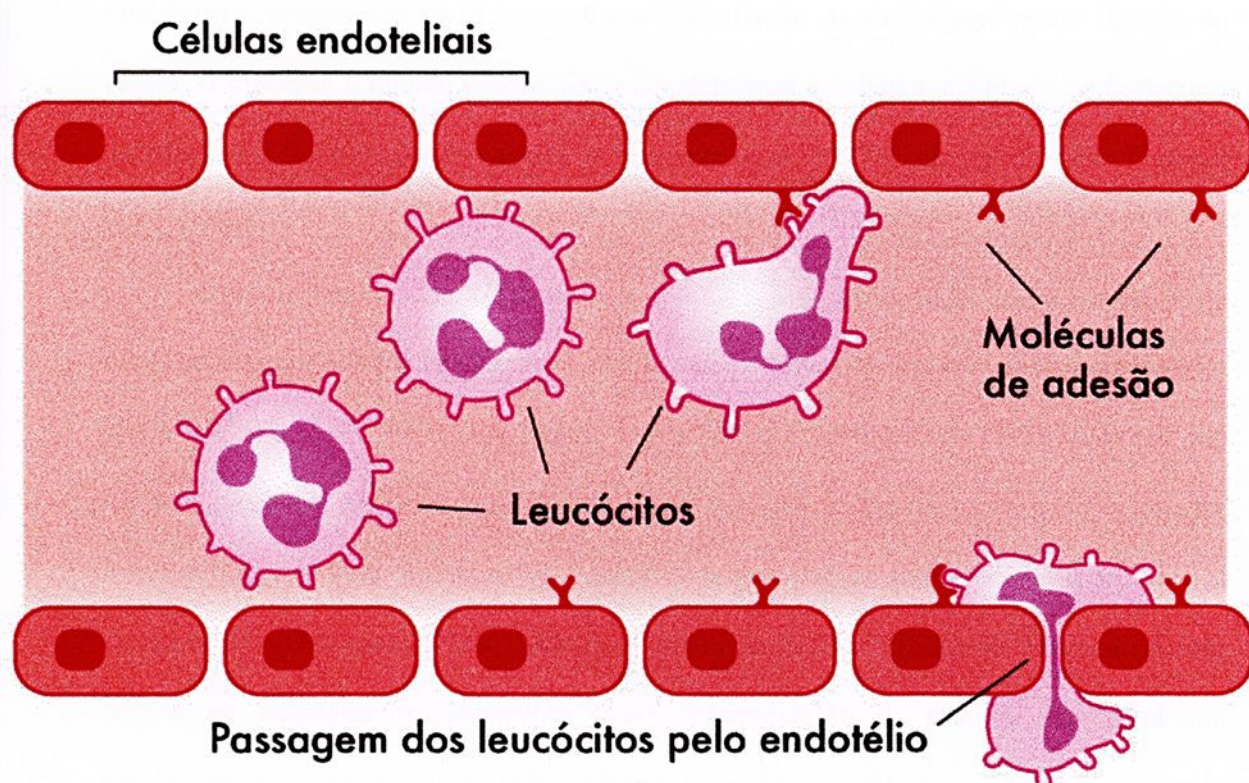
### **Anexinas e inflamação**

Sob condições fisiológicas, os leucócitos não extravasam. Após injúria, a inflamação é desencadeada e ocorre liberação de mediadores ou síntese de efetores como histaminas, ácido aracdônico, prostaglandinas e citocinas, cuja atuação supera o efeito inibidor de outros mediadores. Em consequência, os leucócitos movem-se para fora dos vasos. O processo de migração inclui etapas de adesão ao endotélio e passagem por entre as células da parede dos vasos sanguíneos (Figura 2). O acúmulo e a subsequente ativação de leucócitos podem ser perpetuados e resultar em doenças que envolvem injúria e inflamação crônicas. Para regulação desse processo, é ativada uma série de sistemas de *feed-back* que interrompem a migração leucocitária (Von Andrian et al., 1992; Mancuso et al., 1995; Panés et al., 1999). Entre esses estão os hormônios glicocorticóides, as citocinas IL10 e I4, a adenosina, o óxido nítrico, a prostaciclina, a catepsina G e a anexina 1 (Perretti, 1997).

Durante a inflamação, a liberação do ácido aracdônico a partir de fosfolipídeos celulares, e sua quebra em metabólitos, resulta em vários efeitos biológicos, entre eles a







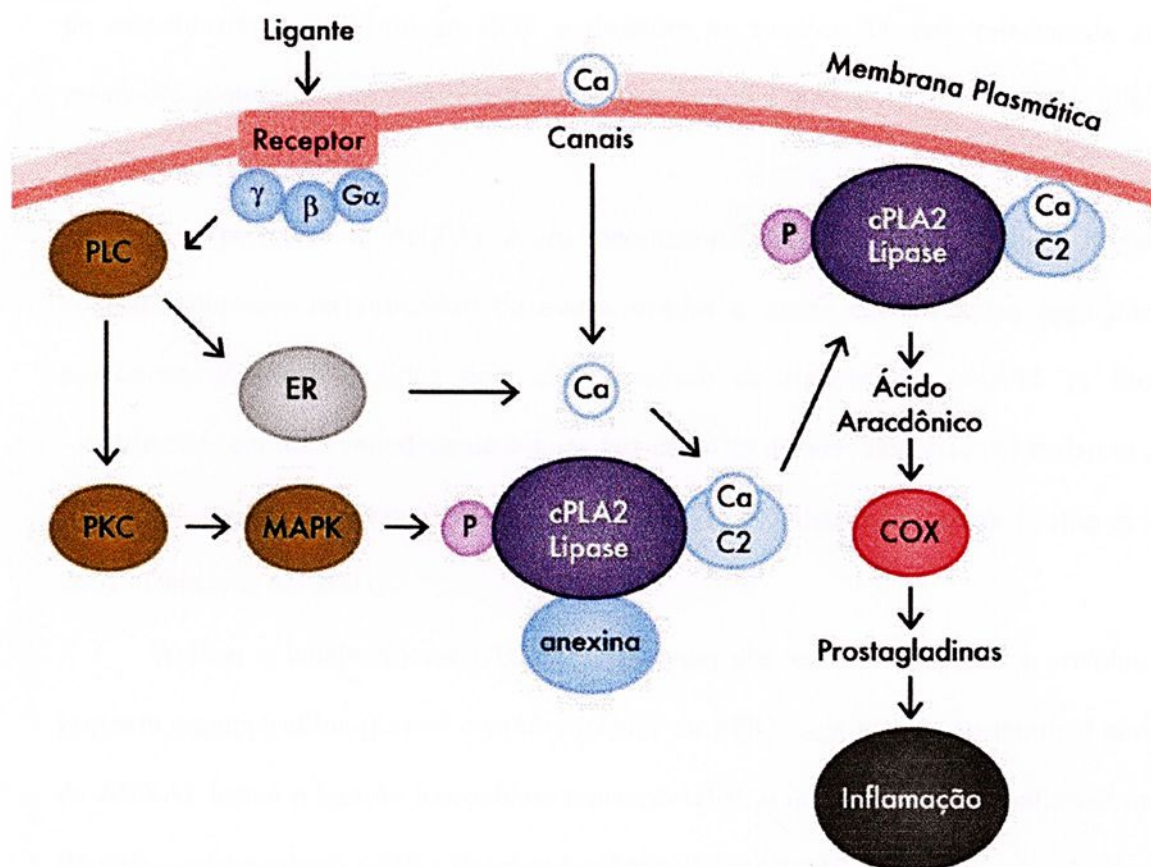
**Figura 2.** Esquema da diapedese: integrinas na superfície dos leucócitos ligam-se a moléculas de adesão na face interna das células endoteliais; os leucócitos aderidos passam por entre as células endoteliais e deixam os vasos sanguíneos.



vasodilatação (Figura 3). Essa liberação é decorrente da ação das fosfolipases A2 que, por sua vez, são ativadas por diferentes estímulos. Um deles envolve a cascata de sinalização desencadeada por recrutamento de Grb2 e p21<sup>ras</sup>, iniciada pelo receptor do fator de crescimento epidérmico, com conseqüente fosforilação da fosfolipase e sua ligação ao cálcio. O processo é inibido pelos GCs, que promovem a redução dos níveis de moléculas de adesão e a fosforilação da anexina 1 (Newman et al., 1997; Solito et al., 1998; Croxtall et al., 2000).

O efeito inicial dos GCs sobre a ANXA1 é a translocação dessa proteína do citoplasma para a superfície externa da célula, onde é retida por um mecanismo dependente de Ca<sup>2+</sup> (Philip et al., 1997; Taylor et al., 1993). Solito e colaboradores (1998) sugerem que a <sup>ANXA1</sup> forma citosólica bloqueia a cPLA2 e a forma extracelular também atua como regulador negativo do processo inflamatório. Oliani e colaboradores (2001) observaram que a isoforma citosólica (com 37kDa) está presente em neutrófilos circulantes. A adesão dessas células ao endotélio aparentemente causa externalização da proteína na membrana plasmática do leucócito. A segunda isoforma (com 33kDa) foi observada principalmente em neutrófilos extravasados e teve localização intracelular. A presença da ANXA1 na membrana plasmática (Diakanova et al., 1997) e sua habilidade para associação com a bicamada lipídica pode estar relacionada com o recrutamento de proteínas de sinalização para a membrana.

O processo de translocação da ANXA1 envolve fosforilação de um resíduo de serina e é dependente da proteína kinase C (PKC), do padrão MAPK (Canaider et al., 2000; John et al., 2002; Solito et al., 2003b) e, provavelmente, de um transportador ABC1 (John et al., 2002). A ANXA1 humana contém vários sítios de fosforilação canônicos (inclusive



**Figura 3.** Função da cPLA2 na ativação da inflamação. O cálcio se liga ao domínio C2 do cPLA2 fosforilado. Esse complexo se une à membrana intracelular e promove a liberação de ácido aracdônico, que é um precursor da prostaglandina resultando na vasodilatação, durante o início do processo inflamatório. Essa via é bloqueada pela proteína anexina, que representa um mediador da resposta anti-inflamatória.



Tyr<sup>21</sup>, Ser<sup>27</sup>, Ser<sup>44</sup>, Ser<sup>45</sup>, Thr<sup>24</sup>, Thr<sup>261</sup>, His<sup>246</sup> e His<sup>293</sup>) (Pepinsky, 1991; Haigler et al., 1992). A fosforilação dependente de PKC ou *src* nos resíduos de serina é crítica para sua exportação induzida por GC, em especial na Ser<sup>27</sup>. A fosforilação desencadeada pelo fator de crescimento semelhante ao EGF e presente na tirosina 21 está relacionada com promoção tumoral e com proliferação excessiva e a torna inativa como inibidor de cPLA2 (de Coupade et al., 2000).

A exportação de ANXA1 é um mecanismo importante que permite à proteína acessar receptores na superfície de outras células e assim exercer ações regulatórias parácrinas. Realmente, sítios com alta afinidade de ligação da ANXA1 já foram identificados em uma variedade de células tais como as de adenohipófise (Christian et al., 1997), em neutrófilos (Perretti et al., 2002) e em várias linhagens celulares (Solito et al., 2001; Tierney et al., 2003).

Walther e colaboradores (2000) descreveram um receptor acoplado à proteína G presente em neutrófilos (*formyl peptide receptor* ou FPR), cuja ligação ao terminal amino da ANXA1 leva à migração leucocitária transendotelial, o que foi também confirmado por Perretti e colaboradores (2001). Rescher e colaboradores (2002) verificaram que o FPR está igualmente presente em células de pulmão e sua ativação parece resultar na regulação de respostas da fase aguda da inflamação. Outros estudos revelaram que a ANXA1 também é ligante de outros receptores, como por exemplo, a lipoxina A4 (Gavins et al., 2005).

D'Amico e colaboradores (2000) observaram que a administração da ANXA1 inibe a injúria dependente do acúmulo de leucócitos em infarto do miocárdio em animais de laboratório. Sua ação nesse caso deve regular o recrutamento de leucócitos, sua adesão aos vasos e seu movimento em direção ao tecido lesado.





Recentemente, Solito e colaboradores (2003a) propuseram um outro mecanismo pelo qual a ANXA1 inibe a inflamação, que inclui a limitação do tempo de vida dos neutrófilos pela apoptose. Esses autores levantaram a hipótese de que a elevação dos níveis de ANXA1 e sua translocação para a membrana, durante a adesão dos leucócitos ao endotélio, provoca não apenas a redução dessa adesão, mas também o encaminhamento da célula para a apoptose. Da mesma maneira, a ANXA1 promoveria a apoptose de leucócitos no sítio da inflamação, quando sua síntese está aumentada. Realmente, a expressão elevada de ANXA1 já foi associada com o aumento da atividade da caspase 3, uma enzima chave no desencadeamento da apoptose (Solito et al., 2001). Como a cPLA2 é um substrato da caspase-3 (Atsumi et al., 1998), este mecanismo indireto anti-PLA da ANXA1 deve levar a uma diminuição da produção de prostaglandina E2 (PGE2), um metabólito pró-apoptose da cPLA2, o que reforça o potencial pró-apoptótico da ANXA1.

### **Anexina e câncer**

A idéia de que as anexinas participam da tumorigênese tem com base sua ligação com vias distintas de transdução de sinais (Bastian, 1997). Outro ponto importante é seu envolvimento na regulação de estágios do processo inflamatório, como adesão de células à parede de vasos e migração ou diapedese por entre as células endoteliais, que são muito semelhantes àqueles da metastatização de tumores (Perretti, 1997).

A sua relação com neoplasias benignas e malignas de diferentes origens tem recebido atenção nos últimos anos. Em alguns tumores, possuem ação supressora, enquanto em outros parecem estar envolvidas na progressão da doença. Por exemplo, a expressão elevada de ANXA1, 2, 4, 5 ou 6 foi observada em tumores de sistema nervoso periférico e

central, carcinoma espinocelular de pele, osteossarcoma, câncer de pâncreas, fígado e pulmão (Bastian, 1997). O aumento da expressão da ANXA1 é também referido em tumores de cabeça e pescoço (Wu et al., 2002). No caso da ANXA8, a elevação de expressão é referida em leucemia promielocítica aguda com translocação equilibrada t(15;17), embora o gene dessa anexina esteja localizado no cromossomo 10 e, portanto, não relacionado com o rearranjo cromossômico (Chang et al., 1992; Sarkar et al., 1994).

Outros autores fazem referência à expressão diminuída ou ausente de ANXA5 em carcinomas de colo de útero e endométrio (Karube et al., 1995), de ANXA1 em tumores de próstata (Cole et al., 1999; Kang et al., 2002), de esôfago (Emmert-Buck et al., 2000; Paweletz et al., 2000; Zhi et al., 2003) e de mama (Shen et al., 2005) e de ANXA2 e 7 em neoplasias de próstata (Banerjee et al., 2003; Liu et al., 2003; Srivastava et al., 2003).

A etiologia da expressão reduzida de anexina em câncer não é conhecida, mas alguns mecanismos são sugeridos, como deleções e mutações do gene, hipermetilação do promotor com subsequente perda de transcrição, alteração de outras proteínas que regulam a transcrição de ANX e alteração no processamento pós-traducional da proteína. Nesse último caso, a fosforilação de determinados resíduos da ANX pode ser implicada na liberação da cPLA2 e, conseqüentemente, na produção de prostaglandinas e na proliferação celular. Defeitos no transporte ou estocagem da proteína, que levam a níveis intracelulares reduzidos também são possíveis de ocorrer (Paweletz et al., 2000; Della-Gaspera et al., 2001). Outras explicações incluem a diminuição da frequência de apoptose causada pela perda de anexina ou o fato já observado em macrófagos da ativação prolongada de quinases relacionadas com sinais extracelulares na ausência dessa proteína (Alldridge et al., 1999; Kang et al., 2002).





Debret e colaboradores (2003) concluíram que a ANXA1 pode ter um papel importante na tumorigênese pela combinação de várias de suas propriedades: (1) na proliferação celular, graças ao seu efeito inibitório sobre quinases de vias de transdução de sinais, (2) na inflamação, pela sua atividade anti-PLA2 e (3) na apoptose, pela ativação da protease caspase-3.

É possível que algumas das ligações das anexinas com o processo neoplásico sejam também consequência de seu papel na diferenciação celular. Violette e colaboradores (1990), por exemplo, obtiveram evidências diretas da atividade da ANXA1 na indução da diferenciação de células malignas epidérmicas. Além disso, tem sido observado que essas proteínas são abundantes em tipos celulares diferenciados mas ausentes nos não diferenciados, por exemplo em células embrionárias e mesenquimais (Fava et al., 1989; Della-Gaspera et al., 2001). Entretanto, alguns dados são conflitantes, como os de Horlick e colaboradores (1991), mostrando inibição de diferenciação da glândula mamária pela ANXA1.

Em conclusão, a desregulação da anexina leva a mudanças biológicas importantes. Entretanto, embora seja um efector chave na regulação da inflamação, ainda não está claro se essa proteína pode afetar as células do sistema imune e impedir sua atuação na eliminação de patógenos ou de células neoplásicas. Os dados disponíveis sugerem que essa família de proteínas pode ter, além de seu importante papel no processo inflamatório, um envolvimento significativo em outras doenças, especialmente no câncer, através de cascatas de sinalização que incluem genes relacionados com o ciclo celular, com a diferenciação e com a apoptose.





## II. OBJETIVOS

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Considerando que os progressos na terapia do câncer dependem de um melhor entendimento dos mecanismos básicos relacionados com o desenvolvimento dessa doença, foi proposto o presente projeto que tem como objetivo geral investigar como as alterações nos níveis da anexina modulam a expressão gênica e como essas alterações podem participar do processo tumorigênico. Os objetivos específicos compreenderam:

1. Investigar alterações da expressão gênica em células neoplásicas e de glândulas pituitárias de camundongos tratadas com a ANXA1 recombinante humana (hrANXA1) e com o peptídeo da região N-terminal da ANXA1 (ANXA1<sub>Ac2-26</sub>), utilizando a técnica de hibridação subtrativa rápida (RaSH).
2. Validar os genes diferencialmente expressos encontrados pela técnica de RaSH por eletroforese 2D, PCR semiquantitativa, imunocitoquímica, *western blot* e citometria de fluxo.
3. Investigar vias de sinalização celular, das quais a ANXA1 participe, importantes para o processo inflamatório e para a tumorigênese.



Annexin A1 from gene organization to physiology

### III. RESULTADOS

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Artigo original submetido ao periódico *Memórias do Instituto de Física de São Carlos*







## Review

# Annexin A1

## From Gene Organization to Physiology

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## KEY WORDS

annexins, annexin A1, disease, neoplasms, inflammation

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## ABSTRACT

Annexin A1 is a member of the annexin calcium binding protein family that still lacks a clear physiological role. Gene evolution and organization are new factors which may give information about protein and their mechanism of action. We have attempted in this review to give an up-to date of the protein from gene organization and expression to its pathophysiological significance.

## INTRODUCTION

The Annexins (ANX) are a well-conserved superfamily of structurally related proteins characterized by a C-terminal homologous domain with 4 to 8 repeats of 70–75 aminoacids, responsible for calcium and phospholipid binding properties. Their variable N-terminal region is unique in length and sequence and includes potential sites of phosphorylation, glycosylation and peptidase action.<sup>1</sup> Annexins are present in many organisms, from molds (ANXE), to plants (ANXAD), fungi (ANXC) and mammals (ANXA). They are preferentially localized in the cytoplasm and associated with the plasma membrane or cytoskeleton, but some including ANXA1 have also been localized outside the plasma membrane.<sup>2,3</sup> The function of the annexins is far to be clear, they have been involved in signal transduction,<sup>4,5</sup> vesicle transport,<sup>6</sup> cell transformation,<sup>7,8</sup> inflammation,<sup>9</sup> cell matrix interaction and apoptosis.<sup>10-12</sup>

## ANNEXIN GENETIC BACKGROUND

The discovery of annexins in lower eukaryotes suggests that in evolutionary terms the origins of the family must indeed be ancient. This idea is consistent with the fact that the chromosomal loci of the human annexin genes are widely distributed within the genome. In respect of the structure of the annexin genes, it was expected that the repetitive structure of the annexins observed at the amino-acid level would be reflected in the organization of the exons. However, the human annexin 1 and 2 genes display a variety of intron-exon boundaries within the four repeats of the core domain. However, the positions of these boundaries are extremely well conserved between the corresponding repeats of the two proteins, suggesting that the evolution of the tetrad repeat preceded the divergence of the two annexins. Also, the differential expression of annexins indicates that, despite their similar structures, they have quite distinct modes of gene regulation.

The annexins gene superfamily comprises an excellent model for reconstructing eukaryotic genome history both because its evolutionary record has been preserved by strong selective constraint against mutations involving amino acid changes and because its paralogous genes have evolved independently at dispersed locations in eukaryotic genomes.<sup>13</sup> There has been an approximate quadrupling of the 3 to 5 annexin genes found in protist, plant and invertebrate animal species<sup>14</sup> to at least 12 paralogs in humans.

The vertebrate annexin family includes 12 annexins that have been confirmed to make up the complete family in mammals,<sup>15</sup> but the number of annexin family members varies in other vertebrates, as genes have been gained and lost. Ancient polyploidization events in bony fish, and more recent genome duplications in pseudotetraploid frogs (*Xenopus*), have duplicated many of the annexin genes. For example, annexin 1 has undergone two successive duplications to yield up to four copies in some fish, amphibians and birds.<sup>16</sup>

The 12 human annexin genes range in size from 15 Kb (*ANXA9*) to 96 Kb (*ANXA10*) and are dispersed throughout the genome on chromosomes 1, 2, 4, 5, 8, 9, 10 and 15.<sup>17</sup> Annexin genes from other vertebrates may vary slightly in size and chromosomal linkage, but orthologs are grossly similar in their sequence and splicing patterns.<sup>16</sup>



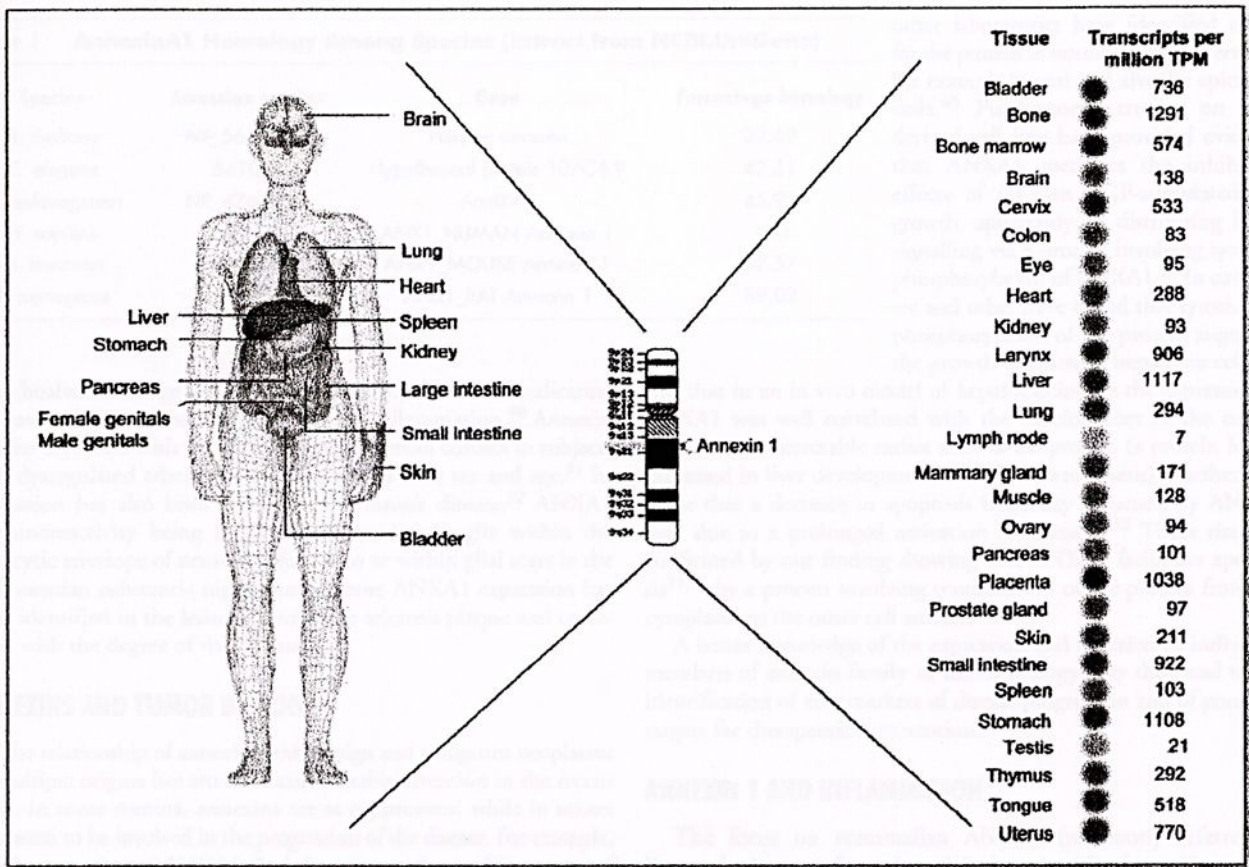


Figure 1. *ANXA1* chromosome localization and tissue distribution in human (extract from UNIGENE).

Annexin A1 (*ANXA1*), originally known as lipocortin 1, was the first member of this family to be cloned.<sup>18</sup> The *ANXA1* gene is located on human chromosome 9, at 9q12–21.2 and encodes a 38.71 kDa protein. The exon-intron organization of *ANXA1* genes in vertebrates have been described and are highly conserved,<sup>19</sup> each gene consisting of 13 exons of which the first and last are uncoding 5' and 3' sequences, with the translation initiator codon (AUG) found near the beginning of the second exon.<sup>20</sup> The conservation of the intron-exon organization of the *annexin* genes thus far analyzed is remarkable, for example the exons of the human and rat annexin 1 genes can be grouped into four size classes: exons 1, 8, 11 (61, 59, 57 bp); exons 2, 7 (77, 80 bp); exons 4, 6, 9, 10 (95, 91, 94, 96 bp); exons 3, 5, 12 (109, 114, 123 bp) whilst exon 13 is unique, with a size of 341 base pairs of which 56 base pairs are coding sequences. Moreover the two genes have an identical intron-exon arrangement and highly conserved promoter regions. This gene sequence homology is translated into protein with the rat, mouse and human sequence being 89% homologous (Table 1).

The exons containing highly conserved regions are repeated four times within the annexin A1 gene. This exon-intron structure suggests that the gene evolved through duplication of one functional domain, which consists of two exons.<sup>20</sup>

From a transcriptional regulation point of view the human *ANXA1* gene promoter contain consensus sequences for: glucocorticoids, AP-1 and NFIL-6. However the regulation from glucocorticoid

(GC) appear to be dependent upon an activation involving NFIL-6 or AP1.<sup>8,21</sup> The GC mediated regulations confirm the original studies which proposed *ANXA1* as mediator of the glucocorticoid action.<sup>22</sup>

In mouse, in contrast, GC regulation is mediated through two consensus sequences present in the upstream region of TATA box.<sup>20,23</sup> However the data of Antonicelli et al<sup>24</sup> regarding sequences of the mouse *Anxa1* promoter brought evidence of the involvement of the CREB transcription factor (the cyclic AMP-responding element-binding protein) in the activation of this gene by cyclic AMP and glucocorticoids. Studies from our lab have also shown opposite effects of dexamethasone and estradiol on the murine *Anxa1* promoter. These findings support our hypothesis that estrogens exert complex, tissue-specific effects on annexin A1 expression (Fig. 1), which may contribute to the aetiology of the sexual dimorphisms in neuroendocrine and host-defence function.<sup>25</sup>

## ANNEXIN A1 AND HUMAN DISEASES

Due to their participation in signalling patterns, annexins have been implicated in the pathogenesis of a number of diseases, including chronic processes caused by inflammation, cancer and auto-immune diseases.<sup>26</sup> Auto-antibodies against annexin A1 have been detected in patients with auto-immune diseases such as systemic lupus erythematosus, rheumatoid arthritis<sup>27,28</sup> and Crohn's disease.<sup>29</sup> Downregulation and degradation of Annexin 1 was also found in the



Table 1 **AnnexinA1 Homology Among Species (Extract from NCBI.UniGene)**

| Species                | Accession number | Gene                         | Percentage homology |
|------------------------|------------------|------------------------------|---------------------|
| <i>A. thaliana</i>     | NP_564920.1      | Putative annexin             | 32.69               |
| <i>C. elegans</i>      | S41022           | Hypothetical protein T07C4.9 | 42.31               |
| <i>D. melanogaster</i> | NP_476603.1      | AnnX-P2                      | 45.93               |
| <i>H. sapiens</i>      | P04083           | ANX1_HUMAN Annexin 1         | 100                 |
| <i>M. musculus</i>     | P10107           | ANX1_MOUSE Annexin 1         | 87.57               |
| <i>R. norvegicus</i>   | P07150           | ANX1_RAT Annexin 1           | 89.02               |

bronchoalveolar lavage fluid of patients with cystic fibrosis indicating the susceptibility of these patients to lung inflammation.<sup>30</sup> Annexin A1 also correlates with the altered level of serum cortisol in subjects with dysregulated adrenal function as well as with sex and age.<sup>31</sup> Its expression has also been linked to Parkinson's disease,<sup>32</sup> ANXA1 immunoreactivity being found in amoeboid microglia within the astrocytic envelope of neurons adjacent to or within glial scars in the parkinsonian substantia nigra. Furthermore ANXA1 expression has been identified in the lesions of multiple sclerosis plaque and correlated with the degree of the disease.<sup>33</sup>

## ANNEXINS AND TUMOR BIOLOGY

The relationship of annexins with benign and malignant neoplasms of multiple origins has attracted considerable attention in the recent years. In some tumors, annexins act as suppressors, while in others they seem to be involved in the progression of the disease. For example, a high expression of ANXA1, 2, 4, 5 or 6 was observed in tumors of the peripheral and central nervous system, spinocellular skin carcinoma, osteosarcoma, and cancers of the pancreas, liver and lung.<sup>26</sup> As for ANXA8, increased expression has been reported in acute promyelocytic leukemia with a translocation t(15;17), although the gene of this annexin is localized on chromosome 10 and, therefore, is not related to the chromosome rearrangement.<sup>34,35</sup> Similarly, ANXA2 is abnormally elevated in patients having the translocation t(15;17) form of acute promyelocytic leukemia and is associated with excessive fibrinolysis.<sup>36</sup>

Reduced expression of ANXA1 and 5 have been reported in malignant endometrial tissue;<sup>37</sup> the etiology of such reduced annexin expression is not known, but suggested mechanisms include gene deletions and mutations, hyper-methylation of the promoter with subsequent loss of transcription, and alterations in the post-translation processing (e.g., phosphorylation) of the protein involved in annexins regulation. In the latter case, the phosphorylation of certain annexins residues may be implicated in prostaglandin production and cell proliferation.<sup>5</sup> Indeed ANXA1 expression is significantly increased in malignant tumors of the breast,<sup>38,39</sup> liver,<sup>5,40</sup> stomach<sup>41,42</sup> and gliomas.<sup>43</sup> Such overexpression is may be related with the fact that ANXA1 is a substrate for epidermal growth factor (EGF-R)<sup>44,45</sup> and other factors which are involved in tumor development and progression.

Another possible link between ANXA1 over expression in neoplastic process is consequences of its role in cell differentiation and transformation.<sup>7,8</sup> It was further observed that this protein is abundant in differentiated cell,<sup>7</sup> but absent from nondifferentiated cell types,<sup>46,47</sup> although this data is apparently contradicted by finding that ANXA1 inhibits mammary gland differentiation.<sup>48</sup> However

other laboratories have identified a role for the protein in normal cells proliferation, for example buccal and alveolar epithelial cells.<sup>49</sup> Furthermore studies on lung derived cell line have provided evidence that ANXA1 mediates the inhibitory effects of GC on EGF-stimulated cell growth, apparently by disrupting EGF-signalling via a process involving tyrosine phosphorylation of ANXA1.<sup>50</sup> In contrast we and other have found that tyrosine 21 phosphorylation of the protein augments the growth of primary hepatoma cells.<sup>5,51</sup>

and that in an in vivo model of hepatocarcinoma the expression of ANXA1 was well correlated with the development of the tumor. ANXA1 was detectable earlier than  $\alpha$ -fetoprotein (a protein highly expressed in liver development and in cancerogenesis). Further data argue that a decrease in apoptosis frequency is caused by ANXA1 loss, due to a prolonged activation of kinases.<sup>4,52</sup> Those data are confirmed by our finding showing that ANXA1 facilitates apoptosis<sup>11,12</sup> by a process involving translocation of the protein from the cytoplasm to the outer cell surface.<sup>53-55</sup>

A better knowledge of the expression and function of individual members of annexin family in tumor biology may thus lead to the identification of new markers of disease progression and of potential targets for therapeutic intervention.

## ANNEXIN 1 AND INFLAMMATION

The focus on mammalian ANXA1 (previously referred as lipocortin 1) stems from the original work of Professor Flower and colleagues who demonstrated the role of this protein in mediating aspects of the anti-inflammatory actions of glucocorticoids.<sup>22</sup> Important work over the past decade has revealed a critical role for ANXA1 expressed in leukocytes in the adhesion of neutrophils to the endothelium.<sup>56</sup> Other data have shown that ANXA1 is also expressed in endothelial cells<sup>57</sup> and that exogenous recombinant ANXA1 blocks monocyte transmigration across human bone marrow microvascular endothelial cells via an interaction with the integrin  $\alpha 4 \beta 1$ .<sup>58</sup> In support of these findings, seminal in vivo studies in rodents on the anti-inflammatory actions of ANXA1 in the periphery have shown that administration of recombinant ANXA1, or peptides derived from its N-terminal region, inhibit the process of neutrophil extravasation and consequent tissue damage at sites of vascular injury following myocardial or splenic ischemia reperfusion.<sup>59-63</sup> In situation of peripheral inflammation ANXA1 has been shown to act as a functional antagonist of the chemo-attractant human formyl peptide receptor (FPR) or the closely related FPR-like 1 receptor (FRL1), both of which are expressed on mononuclear phagocytes<sup>64</sup> and serve to inhibit the migration of these cells into the extravascular tissue and to desensitize them to chemo-attractant challenge.<sup>65-67</sup>

There is no doubt that ANXA1 represent a good endogenous anti-inflammatory protein. Further studies will clarify if it may be used as a pharmacological anti-inflammatory agent.

## OVERALL CONCLUSIONS

Over a quarter of century, ANXA1 has been shown to play multiple role in different systems. We are still lacking of important information: how the alteration of such protein may determine a



pathological condition. Unfortunately our *in vivo* model of *ANXA1* null mice does not show a clear phenotype indicating that *ANXA1* may play a role as adaptor molecule. However, more human pathologies have been correlated with an alteration of *ANXA1* expression. The knowledge from future studies will clarify *ANXA1* importance in health and disease, opening possible avenues for therapeutic intervention.

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## Identification of genes regulated by annexin A1 using Rapid Subtraction

### Hybridisation (RaSH)

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## Identification of genes regulated by annexin A1 using Rapid Subtraction Hybridisation (RaSH)

**Running Title:** Annexin A1 modulates gene expression both positively (*CCR2*) and  
negatively (*ITGB1BP1*)

## Capítulo II

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**Key words:** gene expression, host defense, Annexin A1 and acute epithelial cells

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**Identification of genes regulated by annexin A1 using Rapid Subtraction****Hybridisation (RaSH)**

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**Running Title** Annexin A1 modulates gene expression both positively (*CCR10*) and negatively (*ITGB1BPI*).

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## ABSTRACT

Annexin A1 (ANXA1), a 37kDa protein, is a mediator of glucocorticoid action in the neuroendocrine and host-defense systems, and has also been strongly implicated in the regulation of the cell cycle. The mechanism of ANXA1 action is poorly understood and its potential to influence gene expression has rarely been addressed. Accordingly, we have used rapid subtraction hybridization (RaSH) to examine the effects of human recombinant ANXA1 (hrANXA1) and an N-terminal ANXA1 peptide (ANXA1<sub>Ac2-26</sub>) on gene expression.

Human larynx cell lines (Hep-2) and human pituitary folliculo stellate (PDFS) cell lines were stimulated for 72 hours with recombinant ANXA1 (100nM), and ANXA1<sub>Ac2-26</sub> (2μM). Total RNA was isolated and processed for RaSH analysis. Four genes showed strong differential expression after treatment with hrANXA1 and/or ANXA1<sub>Ac2-26</sub>. (a) *CCR10* (*GPR2*), a seven transmembrane receptor for chemokine CCL27, which is involved in T-cell mediated inflammation in the skin and in neuroinflammation, (b) *FARSLA*, a tumor-selective regulator of cell differentiation (c) *TIFA*, a cell survival signalling protein, and (d) *ITGB1BP1*, the accessory protein for integrin beta 1 (ICAP-1). Furthermore ANXA1 null mice showed a down regulation of *CCR10*, both in the skin and in the respiratory tract.

The identification of these genes correlate with certain biological properties ascribed to ANXA1 and further support a role for annexin A1 in host defense.

## INTRODUCTION

Annexin A1 is a member of a large family of  $\text{Ca}^{++}$  regulated lipid binding proteins (1), whose specific biological activity is believed to be conferred by the N-terminal domain (2). Calcium binding can alter the structure of the protein, triggering exposure of the N-terminal region and thereby influencing biological activity (3). Interestingly, the main function of ANXA1 has been related with its ability to interact with membranes and an ANXA1 receptor (formyl peptide receptor or FPR/FPRL1) has been identified (4, 5). Furthermore post-translational modification of ANXA1 has been related with the biological function both in vitro and in vivo (6, 7).

The role of ANXA1 in the regulation of cellular proliferation was first described by Sawyer and Cohen with the description of ANXA1 as a substrate for protein tyrosine kinase activity associated with epidermal growth factor receptor (EGFR) activation (8). In addition, ANXA1 has been shown to inhibit mitogen-induced cellular proliferation in T cells (9) and has also been reported to be involved in mitogenic signal transduction by hepatocyte growth factor (HGF) (10), glucocorticoids (11) and growth hormone receptor (GHR) (12). Differential expression of ANXA1 has also been observed in cells depending on their proliferative status or stage of differentiation (13, 14). More specifically Alldridge and Bryant have shown that ANXA1 regulates cell proliferation through the disruption of cell morphology and the inhibition of cyclin D1 (15).

Within the nucleus it has been proposed that ANXA1 tends to associate with ssDNA by binding replication protein A, itself a ssDNA binding protein (16), thus implying that ANXA1 can affect gene expression. Accordingly we have used rapid subtraction hybridisation (RaSH (17)) to investigate the effects of human recombinant ANXA1 (hrANXA1) and its N-terminal peptide (Ac2-26) on gene expression in cell



lines of human epithelial origin called Hep-2 and PDFS. The use of RaSH identified four genes whose expression was modulated (three down regulated and one up-regulated) in cells treated with ANXA1 peptide. Expression of genes downregulated by ANXA1 peptide include (a) *FARSLA*, a tumor-selective regulator of cell differentiation (b) *TIFA*, a cell survival signalling protein, and (c) the *ITGB1BP1* gene coding for accessory protein *ICAP-1A* for the integrin beta 1. Annexin A1 peptide upregulated the expression of CCR10, a seven transmembrane receptor for chemokine CCL27, which is involved in T-cell mediated inflammation in the skin and in neuroinflammation. The regulation of this latter gene product was also confirmed at protein level (western blot and FACS analysis) and furthermore CCR10 was abnormally expressed in the skin and respiratory tract of ANXA1 null animals. These data provide new insight into the role of ANXA1 in the modulation of host defence.

## MATERIALS AND METHODS

### *Cell culture and growth*

A human folliculo stellate cell line (PDFS), which developed spontaneously from a clinically nonfunctioning pituitary macroadenoma (18, 19) was cultured at 37°C in 5% CO<sub>2</sub> in DMEM-Glutamax (Invitrogen, Paisley, UK), pH 7.5, containing 10% fetal calf serum (PAA Laboratories, Teddington, Middlesex, UK), 1% nonessential amino acids, 0.1% transferrin, 0.1% selenium (Invitrogen), and 0.1% gentamycin (Sigma-Aldrich Corp.). Hep-2 cells (human larynx epidermoid carcinoma, ATCC Rockville, Maryland USA) were grown in the same medium but without transferrin and selenium. For experiments, the cells were plated at a density of 10<sup>6</sup> cells/10-cm plates (Corning, Ltd., High Wycombe, UK), with plates being poly-lysine coated for PDFS

cells only, and were incubated in the conditions described above until the cells attained 70% confluence. Ac2-26 peptide corresponding to the 2-26 N-terminal domain of human annexin A1 was synthesized at the Advance Biotechnology Center, Imperial College London, UK. Human recombinant annexin A1, (20) was purified by Scientific Proteins Witterswil Switzerland; fMLP was from Sigma and 15-epi-16-(para-fluoro)-phenoxy-ATLA-methyl ester (ATLa) was a generous gift from Professor C. Serhan (Harvard Medical School, Boston, U.S.A.)

#### *Rapid Substraction Hybridization (RaSH) and PCR analysis*

Hep-2 and PDFS were plated at a concentration of  $10^6$  cells/plate and stimulated the following day for 72 hours with recombinant ANXA1 (100nM), N-terminal peptide 2-26 (2 $\mu$ M) or the ANXA1 fragment 1-188 (430nM), and total RNA was isolated by RNeasy Qiagen. Twenty micrograms of total RNA from control cells and ANXA1 treated cells were used for double-stranded cDNA synthesis using a standard protocol (21). The DNA was then digested with Dpn II at 37°C for 3h followed by phenol/chloroform extraction and ethanol precipitation. The digested cDNAs were mixed with primers XPDN-14/XPDN-12 (final concentration 20 $\mu$ M) in ligation buffer, heated at 55°C for 1 min and cooled down to 14°C within 1h. Ligation was then set up overnight in presence of T4 ligase (5U/ml). After dilution in TE buffer, 40 $\mu$ l of the mixtures were used for PCR amplification. The PCR mixtures were set up in presence of 10M XPDN-18, 0.4 mM dNTPs, 10xPCR buffer, MgCl<sub>2</sub> and Taq DNA polymerase. 10 $\mu$ g of PCR purified product were digested with XhoI followed by extraction and precipitation. 100ng of *tester* cDNA (e.g untreated cells) was mixed with 5 $\mu$ g of *driver* cDNA (e.g ANXA1 treated cells, see also table 2) in hybridization solution and after



heating at 95°C was incubated at 42°C for 48h (17, 22). After extraction and precipitation the hybridization mixtures (1µg) were ligated to an XhoI digested pCDNA3 plasmid and transformed in competent bacteria. Bacterial colonies were randomly picked and PCR amplified. The sequences of these clones were determined using automated cycle sequencing at Advance Biotechnology Center, Imperial College London. Sequences were compared with the NCBI nucleotide bank (GenBank) allowing identification of any genes up or down regulated. Experiments were then set up (n=3) in the same conditions described above and RT-PCR with specific oligos (Table 1) for the identified gene was performed.

#### *Fixation, Processing and Embedding for Immunocytochemistry and Immunogold Labelling*

Hep-2 cells were maintained in MEM medium containing 5% fetal bovine serum in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. ANXA1 (5µg/ml) was added to the culture medium and maintained for 7 days. After 3 and 7 days, these cells were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde, 0.1M sodium cacodylate buffer, (pH 7.4) for 24 hours at 4°C. They were then washed in sodium cacodylate, dehydrated through a graded series of ethanol, and embedded in LRGold resin (London Resin Company, Reading, Berkshire, UK). For electron microscopy, sections (approximately 90-nm thick) were cut on an ultramicrotome (Reichert Ultracut; Leica, Austria) and placed on nickel grids for immunogold labelling. Ultrathin sections of LRGold resin Hep-2 embedded cells were prepared and incubated at room temperature with 0.1M phosphate buffer (PBS) containing 5% chicken egg albumin for 30 minutes (PBEA) followed by anti-ANXA1 antibody (1:200 in 1% chicken egg albumin in PBS) for 2 hours: raised in sheep against the N-terminal peptide of human ANXA1 (peptide Ac2-



26) (23). Sections incubated with non-immune sheep serum (diluted 1:200) were used as the control. After 20 minutes washing in PBEA, donkey anti-sheep IgG (Fc-fragment specific) antibody conjugated to 15nm colloidal gold (British BioCell International, Cardiff, UK) (1:50 dilution in PBEA) was added and sections were incubated for 1 hour at room temperature. Sections were extensively washed in PBEA and then in distilled water. Ultrathin sections were stained with uranyl acetate and lead citrate before examination on a ZEISS-LEO 906 electron microscope.

Analysis of ANXA1 protein expression in Hep-2 cells was performed in 30 electron micrographs of Hep-2 cells incubated with ANXA1 or control (unstimulated cells) for 3/7 days. The area of each cell compartment was determined with a software image analyzed Axiovision-ZEISS. The density of immunogold labelling (number of gold particles per  $\mu\text{m}^2$ ) was calculated and expressed for each compartment. Values are reported as mean  $\pm$  SEM of  $n$  number of electron micrographs. Statistical differences in the density of gold particles were determined by analysis of variance followed, if significant, by the Bonferroni test. A probability value less than 0.05 was taken as significant.

#### *Western blot analysis*

Expression of CCR10 (*GPR2*) was examined in duplicate by SDS PAGE. Briefly, total protein was extracted from the cells by freeze-thawing (14) and protein content was estimated according to Bradford (24). For analysis, samples (30 $\mu\text{g}$  protein) were loaded onto 10% acrylamide gels and separated by SDS-PAGE in denaturing conditions at 50 mA for 90 min. The separated proteins were then transferred electrophoretically (100mA per blot 70 min; 2117 MULTIFOR II, Pharmacia LKB,



Bromma, Sweden) to nitrocellulose paper (Hybond-P GE Healthcare) soaked in transfer buffer [25 mM Tris, 192 mM glycine (Sigma-Aldrich Corp.)] and 20% v/v methanol (VWR Int.). Nonspecific binding was blocked by incubation of the blots in 5% milk powder (Marvel, Knighton Edbaston, Stafford, UK) in TBS-Tween [50mM Tris; 150mM NaCl; and 0.1% v/v Tween (all from Sigma-Aldrich Corp.)] for 60 min. After washing, the blots were incubated overnight at 4° C in primary antibody (diluted 1:1000) (14) and then for 1 h at room temperature with the appropriate secondary antibody (diluted 1:25,000 goat anti rabbit peroxidase conjugated antiserum Sigma-Aldrich Corp.). Immunoreactive protein bands were detected by chemiluminescence using enhanced chemiluminescence reagents and exposed to Hyperfilm (both from GE Healthcare). The blots were then scanned and analyzed (HP Scanjet 5200 with Adobe Photodeluxe Business Edition, version 1.1; Cupertino, CA).

### *FACS analysis*

Aliquots of the cell suspension ( $10^5$  cells in 100  $\mu$ l) were added to a 96-flat well plate in triplicate and incubated at 4 °C for 1 h in the presence or absence (controls) of anti FPR antibody (mouse anti human 1:100 BD Pharmingen) or mouse IgG1,k clone NOPC-21 (control isotype 1:100, BD Pharmingen, UK), or a polyclonal antibody against formyl peptide receptor-like 1/FPRL1 (raised in rabbit against peptide TVWLKEMLFYGYKYIIDILVNP from 3rd extracellular domain of FPRL1 (Neosystem Strasburg, France) or a goat anti rabbit polyclonal antibody to CCR10 (GPR2, 1:100, Imgenex San Diego, Ca, USA) in buffer A; containing HEPES (25mM),  $\text{CaCl}_2$  (1 mM), and  $\text{MgCl}_2$  (1 mM); non-specific binding was minimized by the addition of human IgG (1.6mg/ml, Roche Diagnostics, Lewes, UK). The cells were then washed

in buffer A and incubated on ice for 30 min with FITC sheep conjugated anti rabbit or rabbit anti-mouse IgG (1:300, Serotec, Kidlington, Oxford UK). After further washing in buffer A, the cell surface fluorescence was analyzed immediately by flow cytometry. In some experiments, preimmune serum was employed as a further control. In all cases, a Becton Dickinson FACScan II analyser with air-cooled 100-mW argon ion laser tuned to 488 nm and Consort 32 computer running Lysis II software (Becton Dickinson and Co.) was used. At least 10,000 cells per sample were counted and characterized by dot plot according to their forward and side scatter characteristics. The data were analyzed as units of fluorescence measured in the FL1 channel (mean fluorescence intensity).

#### *Animal tissue collection and RNA extraction.*

Male C57bl/6 mice (purchased from Harlan-Olac Ltd, Nottingham, UK) and ANXA1 null mice were used (25). All animals were fed on a standard chow pellet diet with free access to water in a room with controlled lighting (lights on 08.00-20.00h) in which the temperature was maintained at 21-23°C. All procedures were carried out under licence in accord with the UK Animals (Scientific Procedures) Act, 1986. Tissue collected was frozen in liquid nitrogen and RNA extraction was carried out using the RNeasy Kit (Qiagen).

## RESULTS

### *Exogenous ANXA1 stimulates endogenous ANXA1 synthesis which modulates cell growth*

It is well known that ANXA1 inhibits cell growth in vitro (15, 26) but the mechanisms behind this effect are not well understood. Interestingly the ability of



ANXA1 to influence gene expression has scarcely been addressed. Figure 1 shows the inhibitory effect of ANXA1 and its N-terminal (Ac2-26) peptide on the growth of Hep-2 cells in normal (A) or semisolid medium (B). 3 days of treatment with ANXA1<sub>Ac2-26</sub> (2  $\mu$ M) induced a 60% reduction in cell growth and this effect was even greater (80%) after 7 days. Immunohistochemical analysis of Hep-2 cells treated with ANXA1<sub>Ac2-26</sub> showed immunoreactivity for ANXA1 within the nucleus, cytoplasm and membrane (Figures 2A – B). No gold labelling was detected in control sections incubated with non-immune sheep serum (Figure 2A panel c). ANXA1-treated cells induced a significant increase ( $P < 0.05$ ) in ANXA1 immunoreactivity when compared to the control cells (figure 2A, panel a, b, c). Furthermore semi quantitative RT-PCR (data not shown) and Western blot analysis (figure 2C) confirmed that stimulation of the cells with ANXA1 upregulates the endogenous expression of the protein.

#### *Screening of genes isolated by RaSH*

These data prompted us to investigate the effect of ANXA1 on gene expression. Accordingly we have used rapid subtractive hybridisation (RaSH) in a cell line of epithelial origin (Hep-2: human larynx carcinoma) to study these effects.

We found 7 down regulated sequences and 4 up regulated sequences. The sequences found were identified through the GenBank and among them 3 genes were down regulated by the treatment (a) TIFA, a Traf-2 binding protein, (b) FARSLA, coding for tRNA synthase and (c) ITGB1BP1, the accessory protein for integrin beta 1 also known as ICAP-1A (Table 2). The gene up-regulated was CCR10 a gene protein coupled receptor for chemokine CCL27 also known as GPR2 related to inflammation and cell signalling.

### *Confirmation of ANXA1 effects on gene expression using another cell line*

To confirm the RaSH data a human cell line derived from a pituitary adenoma, PDFS, was used in parallel with Hep-2 cells. Semi-quantitative PCR analysis of untreated or ANXA1 treated cells revealed that *CCR10 (GPR2)* and *ITGB1BP1* were equally up or down regulated by either Ac2-26 and ANXA1 (72 hours) treatment, independently of the cell line used (Figure 3). Furthermore western blot and FACS analysis of cells stimulated with ANXA1 and the peptide Ac2-26 clearly showed that *CCR10* expression was upregulated by either ANXA1 Ac 2-26 treatment for 72 hour confirming the data obtained by RaSH and RT-PCR (Figure 4).

### *FPR receptor ligands mimic the effects of ANXA1 on gene expression.*

Since ANXA1 and related peptides are potential ligands for members of the formyl peptide receptor (FPR) family we tested whether FPR ligands could modulate gene expression. First, we tested if both cell lines express the two FPR subtypes (FPR, FPRL1) by PCR and FACS analysis respectively. Figure 5 A-B demonstrates the presence of FPR and FPRL1 within PDFS cells (similar results were obtained with the Hep-2 cell line). Subsequently we used the FPR/FPRL1 ligands: fMLP and lipoxin A4 (ATLa) to determine whether they could modulate the expression of those genes influenced by ANXA1 treatment. Figure 6 showed that both fMLP and ATLa were able to stimulate the expression of *CCR10 (GPR2)*. In contrast neither ligand exerted any effect on the gene *ITGB1BP1*. These data show that the FPR/FPRL1 ligands partially mimic the effects of ANXA1 on gene expression suggesting a common receptor



mechanism pathway. Table 3 summarizes the effects of ANXA1 and its N-terminal peptide as well as fMLP and ATLa on gene expression within both cell lines.

These results provide evidence that ANXA1 modulates gene expression. Furthermore as reported in Table 3 (the peptide has no effect on the gene *TIFA*) they also show dissociation between the activities of ANXA1 and Ac2-26 in this regard, and indicate that the actions of Ac2-26, but not ANXA1, in the cell lines employed are akin to those of FPRs.

#### *GPR2 expression in ANXA1 KO animals*

In view of the fact that 1) *CCR10* was the only identified gene up regulated by ANXA1 treatment, 2) it plays a role in T-cell mediated skin inflammation (27) and 3) developmental studies indicate that the ANXA1 protein is abundantly expressed in the skin (28), we analysed by RT-PCR and western blot the expression of GPR2 in the respiratory tract (larynx and trachea) and skin of WT and ANXA1 null mice. Figure 7A and B shows that mice lacking the *anxa1* gene present a decreased expression of *CCR10* in both tissues.

## DISCUSSION

Since ANXA1 binding on target cells is mediated by the affinity of the protein for negatively charged phospholipids and by FPR receptor family members, it is theoretically possible that this is an important facet in mediating the biological activity of the protein. In fact previous evidence suggests that the biological mechanisms of ANXA1 action are fast (maximum effect in 10-30 minutes) and are related both with its membrane localization and its interactions with surface molecules such as  $\alpha 4\beta 1$  or L-



Selectin (29-32). However to the best of our knowledge we report here for the first time the effects of annexin A1 and its peptide N terminal (Ac 2-26) on gene expression, using both a genomic and an *in vivo* analysis on wild type vs annexin A1 null mice.

Despite the fact that ANXA1 modulates gene expression, this phenomenon been related with a late response in contrast to the previous ANXA1 effects (e.g. neutrophil detachment (2) or impairment of ACTH release (7)); it also regulates its own expression and interestingly, ANXA1 itself is localised in the nucleus. A few studies have previously demonstrated ANXA1 localisation within the eosinophils (33) and endothelial cells (34) suggesting a possible involvement of ANXA1 in gene activation (e.g. apoptosis). Moreover 2D gel analysis on ANXA1 treated cells vs untreated (data not shown) demonstrate a clear down regulation of a large number of proteins. These effects are comparable to the powerful actions of steroid treatment and reinforce the proposed role of ANXA1 as a second messenger of GC action (35).

With regard to its effects on gene transcription, ANXA1, which is largely expressed in epithelial cells and skin (13), positively regulates the expression of the *CCR10* gene, a skin homing receptor for chemokine CCL27 (27). Due to the fact that ANXA1 is commonly viewed as an anti-inflammatory protein one may expect a down regulation of this receptor, which modulates pro inflammatory effects, nonetheless *CCR10* has been proposed as a critical mediator of skin-specific lymphocyte homing in mice and humans, which seems to play a role in the movement of specialized effectors within epidermal microenvironments (36). Furthermore the expression of ANXA1 also changes during the proliferation processes of cell differentiation and embryonic development, and indeed, the skin, bone and respiratory tract were among several sites



that displayed strong *LacZ reporter* gene expression during embryonic development in *anxa1* null mice (28).

Glucocorticoids (GCs), as with all annexins, upregulate the expression of another pro-inflammatory chemokine receptor (CXCR4) in eosinophils but not in neutrophils, which may serve to sequester eosinophils from the circulation to non inflamed extravascular tissues (37). Thus, it may be that ANXA1 exerts a similar effect by positively modulating *CCR10* expression. Redundancy is one of the features of chemokine receptor and ligand super families, and thus different ligands may trigger different pathways through the same chemokine receptor. For instance, CCL11, a ligand for chemokine receptor CCR3, induces neither chemotaxis nor adhesion, but significant apoptosis in B cells (38). Also, stromal cell-derived factor (SDF1) $\alpha$ , a ligand for CXCR4 receptor, can signal for chemotaxis or cell death, maybe via the activation of different signalling pathways downstream of chemokine receptors (39).

Since ANXA1 1) is strongly expressed during development in the upper respiratory tract and in the skin (28) and 2) exhibits significant age-related changes in fibroblasts (40), being increased in human fibroblasts from adult donors comparing to old donors. This may reflect on the expression and functionality of *CCR10* as part of an age related general failure to respond to physiological and/or environmental stresses. Interestingly ANXA1, as a second messenger of glucocorticoids induces a dose and time dependent decrease in the expression of L-selectin in neutrophils (30) and monocytes (31) through the activation of a cell surface protease. This alteration may explain the modulation of factors involved in skin inflammation.

Subtractive hybridisation identified three different genes, down regulated by annexin A1 treatment. The first one was *ITGB1BP1* (previously named ICAP-1A, the



integrin cytoplasmic domain-associated protein 1A), a protein specifically associated with the  $\beta 1$  cytoplasmic domain of integrins (41) containing putative phosphorylation sites. The phosphorylated form probably impairs cell adhesion and spreading (42), but the overexpression of *ITGB1BP1* stimulates cell migration (43). Thus, the ability of ANXA1 to impair neutrophil migration and detachment from inflamed mesenterium (20) may be mediated through *ICAP-1A* downregulation. Further study is required to clarify such effect.

The second gene down-regulated by ANXA1 treatment was a phenylalanine-tRNA synthetase (*FARSLA*) expressed in a tumor selective and cell cycle stage and differentiation-dependent manner. Indeed, it has been shown that *FARSLA* is over expressed in the tumorigenic versus non- tumorigenic variant of the same cell line (44). Thus, ANXA1 may modulate cell growth through this gene. Although purely speculative at the moment such results suggest new possibilities for the mechanism of ANXA1 action on cell transformation and might explain why it is highly expressed in some cancers and not in others.

Finally, the third gene down modulated by ANXA1 was TRAF-interacting protein with a forkhead-associated domain (*TIFA* or *T2BP*). This is a small protein that interacts with TRAF2 and may be involved in tumor necrosis factor (TNF) mediated cell survival processes (45). TNF signalling is important for the regulation of diverse biological responses, ranging from inflammation to cell death, and TRAF2 is an adaptor protein involved in signal transduction and in activation of NF- $\kappa$ B and AP1 by the TNF receptor (45). The down-regulation of *TIFA* by ANXA1 suggests that annexin A1 may modulate inflammatory processes and cell death via TNF and *TIFA*, supporting our



previous findings on the role of ANXA1 in systemic endotoxemia in which ANXA1 treatment down regulated TNF $\alpha$  release (46).

In summary, we have used the RaSH approach as an efficient and rapid means of identifying ANXA1 modulated genes in cells of epithelial origin. For one of them (*CCR10*) we have clearly related its expression with the expression ANXA1 in mice. Despite influencing a number of genes, the lack of ANXA1 in the null mice does not compromise survival supporting an adaptive role for ANXA1 in a variety of pathways. Further experiments are necessary to determine if the other identified ANXA1-regulated genes are causally related to, or simply associated with, changes induced in these cell lines although this would seem unlikely as our findings are from two independent cell lines.

These studies, although challenging will provide a useful insight into the phenotype of the annexin A1 null mouse and hence into the physiological role of annexin A1 itself.

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**Table1.** Oligos for the amplification by PCR of the RaSH isolated genes

|                                 |                                                  |
|---------------------------------|--------------------------------------------------|
| <i>CCR10</i><br>( <i>GPR2</i> ) | F GCCCTTCGCGGCA<br>R GCGGCGGGGTGA                |
| <i>ITGB1BP1</i>                 | F GGAGCGGGAACGGG<br>R TCCCGCCCCCAGAC             |
| <i>TIFA</i>                     | F CCATACAGGTGCATGGTCA<br>R GAGCAGAGCGAATAAGTGCC  |
| <i>FARSLA</i>                   | F CCCTTCACTCCGGTCAAGTA<br>R CTGGGCTCTGTGTATGGGTT |



Table 2. Genes isolated by RaSH

|                                                                                                                           | Symbol                      | Locus ID | Function                                                                                              |
|---------------------------------------------------------------------------------------------------------------------------|-----------------------------|----------|-------------------------------------------------------------------------------------------------------|
| <b>Upregulated</b><br>100ng of <i>tester</i><br>(ANXA1<br>treated cells) vs<br>5 µg of <i>driver</i><br>(untreated cells) | <i>CCR10</i> (NM_016602)    | 2826     | a G protein coupled receptor for chemokine CCL27 mostly involved in T cell mediated skin inflammation |
|                                                                                                                           | <i>TIFA</i> (NM_052864)     | 92610    | a TRAF2 binding protein involved in the TNF-mediated signalling                                       |
|                                                                                                                           | <i>FARSLA</i> (NM_004461)   | 2193     | a tRNA synthetase family expressed in a cell cycle stage and differentiation-dependent manner         |
|                                                                                                                           | <i>ITGB1BP1</i> (NM_004763) | 9270     | accessory protein for the integrin beta 1                                                             |

**Table 3.** Comparison of the RaSH isolated gene expression in two different cell lines after 3 days stimulation with the above stimuli.

|              |                 | ANXA1 <sub>Ac2-26</sub> | ANXA1 | fMLP | ATLa |
|--------------|-----------------|-------------------------|-------|------|------|
| <b>Hep-2</b> | <i>CCR10</i>    | ↑                       | ↑     | ↑    | ↑    |
|              | <i>ITGB1BP1</i> | ↓                       | ↓     | ↔    | ↔    |
|              | <i>TIFA</i>     | ↓                       | ↔     | ↓    | ↔    |
|              | <i>FARSLA</i>   | ↓                       | ↓     | ↓    | ↓    |
| <b>PDFS</b>  | <i>CCR10</i>    | ↑                       | ↑     | ↑    | ↓    |
|              | <i>ITGB1BP1</i> | ↓                       | ↓     | ↔    | ↔    |
|              | <i>TIFA</i>     | ↓                       | ↔     | ↓    | ↔    |
|              | <i>FARSLA</i>   | ↓                       | ↓     | ↓    | ↓    |





## Figures Legend

### Figure 1

*Cell growth arrest in Hep-2 cells treated with ANXA1 or its N terminal peptide.*

(A) Growth Curve in normal and (B) semisolid medium, untreated cells or cells treated with ANXA1 or Ac2-26 were plated in semisolid medium using dilution factors of 1, 3, 9 and 27. Representative example of three independent experiments performed in triplicate \*; # $p < 0.0005$  ANXA1 and ANXA1<sub>Ac2-26</sub> vs untreated cells respectively.

### Figure 2

*Exogenous ANXA1 stimulates endogenous ANXA1 expression.*

Electron Micrograph of ANXA1 immunogold labelling in human larynx squamous carcinoma cell line Hep-2. **A:** Hep-2 cells (a) after treatment with ANXA1 showed intense ANXA1 immunoreactivity localized to the nucleus (N) and cytoplasm (arrow). Some gold particles were observed in the plasma membrane (arrowhead). (b) Untreated cells show immunoreactivity in the nucleus (N) and cytoplasm (arrows). (c) Absence of gold labelling in sections incubated with control non-immune sheep serum. Bars: (a-c) 1  $\mu$ m. **B:** *Density of ANXA1 immunogold particles in Hep-2 cells.* ANXA1 (5  $\mu$ g/ml) was incubated with the cells in culture for 7 days. Data are mean  $\pm$  SEM of 10 distinct cells. \*  $P < 0.05$  vs. control. **C:** Western blot analysis of ANXA1 content in Hep-2 cells stimulated with ANXA1 or the peptide Ac2-26.

**Figure 3**

*RT-PCR analysis of CCR10 and ITGB1BP1 in PDFS cells treated with ANXA1 (100 nM) and ANXA1<sub>Ac2-26</sub> (2  $\mu$ M).*

Cells were plated and treated as reported for RaSH analysis. After 72 hours of treatment total RNA was extracted and RT-PCR performed using specific oligos for the above genes. Semi-quantitative normalization was performed using GAPDH expression.

**Figure 4**

*(A) Western blot and (B) FACS analysis of CCR10 expression on PDFS cells treated with human recombinant ANXA1 and ANXA1<sub>Ac2-26</sub>.*

(A) Total protein cell extract from treated or untreated cells were analysed by western blot. (B) FACS analysis:  $10^6$  cells were incubated with a specific polyclonal antibody against CCR10 followed by incubation with a FITC anti rabbit secondary antibody. Histogram (intensity of fluorescence vs number of cells) showed the data of one of 3 independent experiments with similar results.

**Figure 5**

*PDFS cells express both FPR and FPRL1 receptors.*

(A) RT-PCR analysis for PDFS cells lane 1 and 4 represent the control amplified plasmids containing the entire coding sequences for FPR and FPRL1 respectively. Lanes 2 and 3 amplified product in PDFS cells for FPR and FPRL1 respectively. (B) FACS analysis of FPR and FPRL1 expression on the surface of PDFS cells.



**Figure 6**

*Effects of fMLP and ATLa on CCR10 and ITGB1BP1.*

RT-PCR analysis of CCR10 and ITGB1BP1 after stimulation with fMLP (100nM) and ATLa (100nM for 72 hours).

**Figure 7**

*CCR10 expression in ANXA1 null mice.*

(A) RT-PCR analysis and (B) western blot analysis of CCR10 in normal and annexin A1 null mouse skin and respiratory tract see material and methods for details.

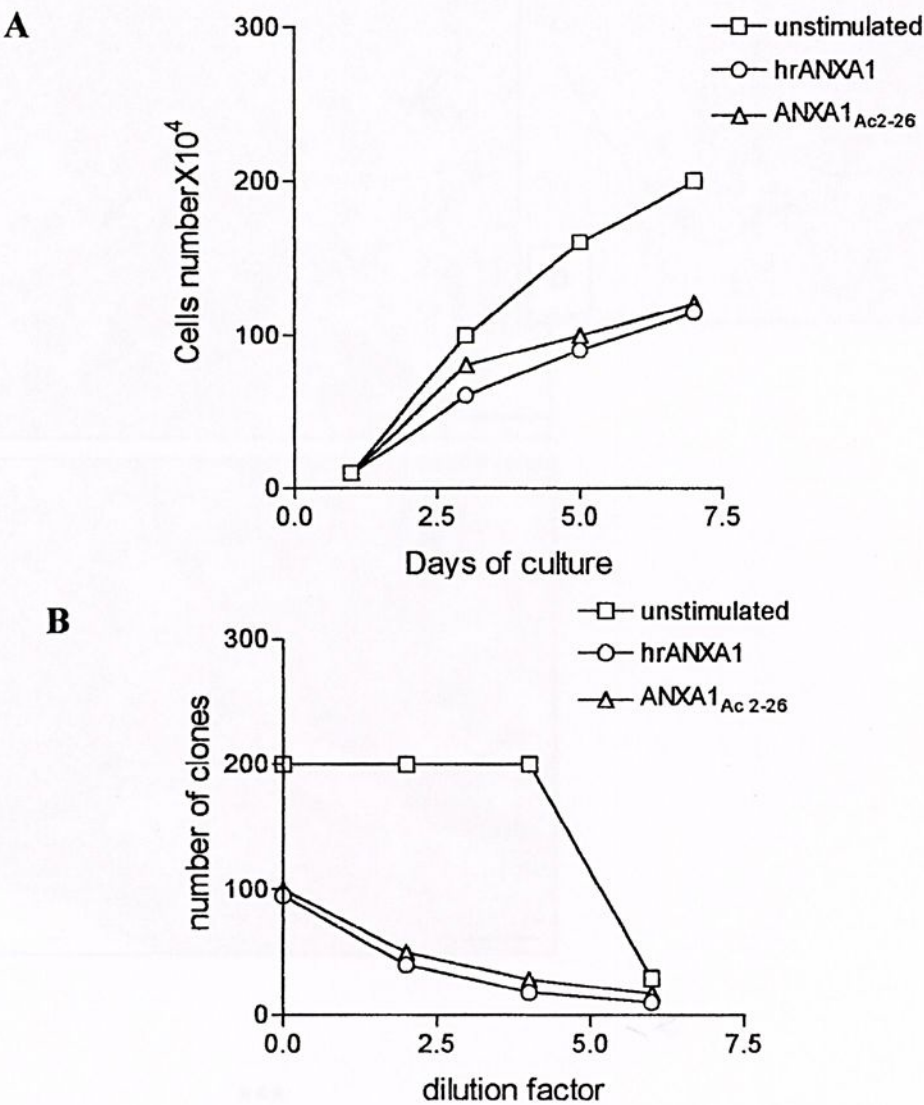
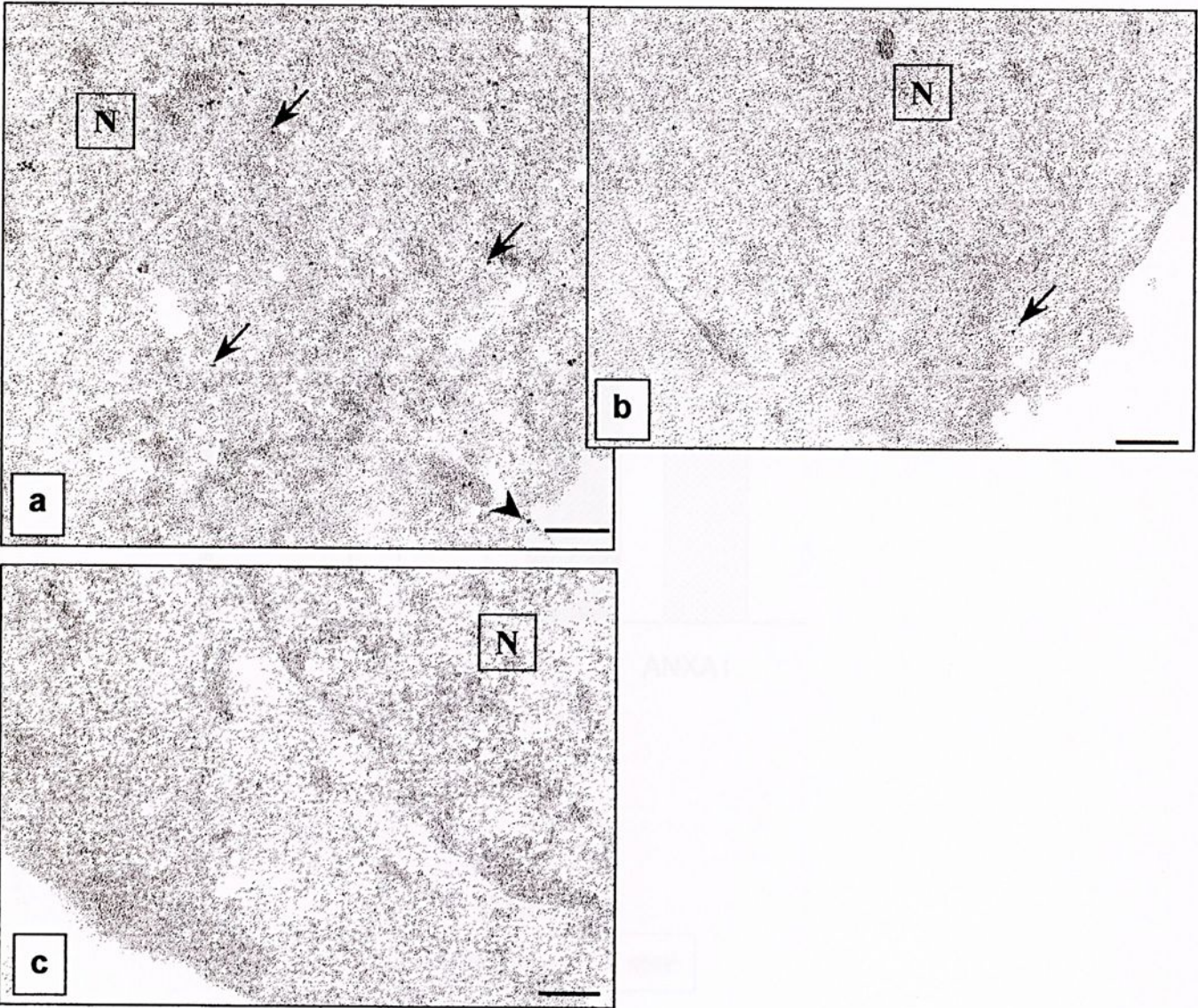


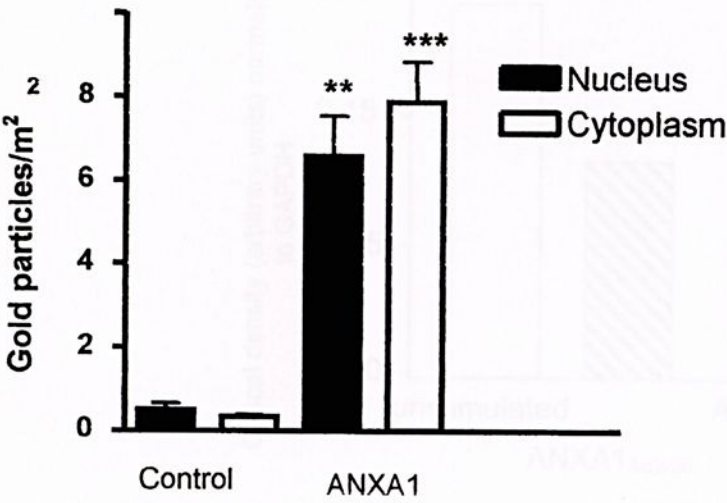
Figure 1



A.



B.



C.

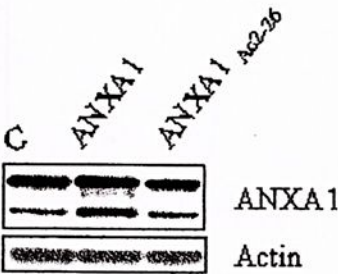


Figure 2

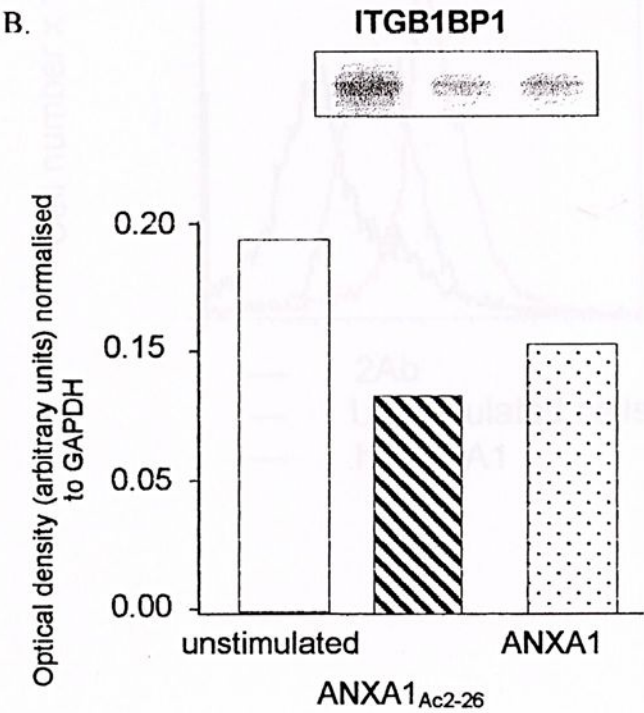
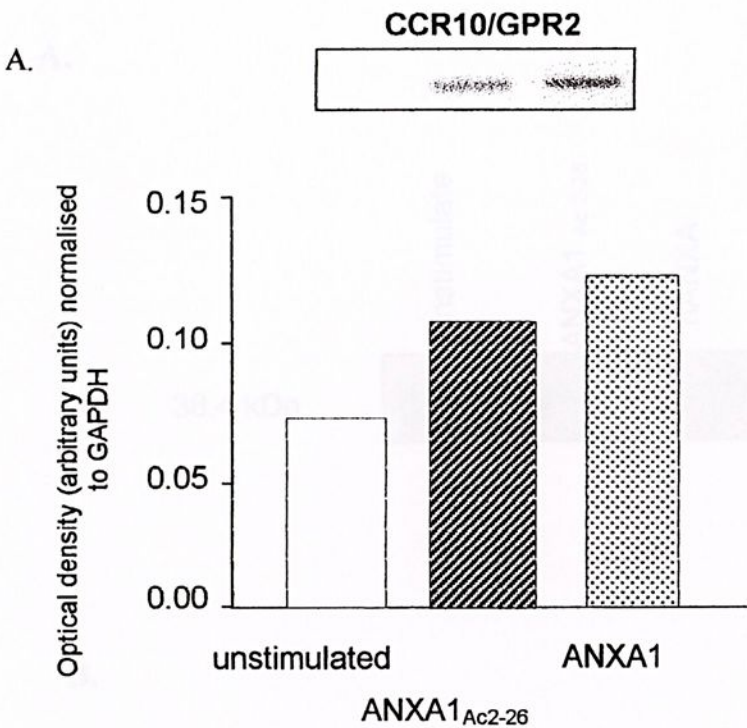
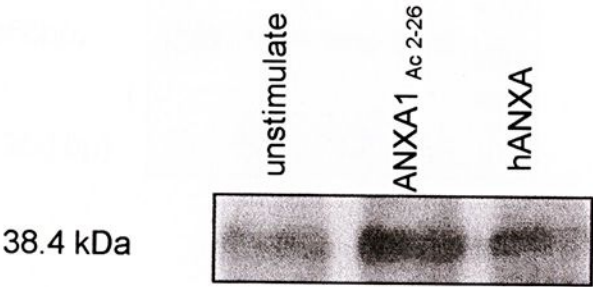


Figure 3



A.



B.

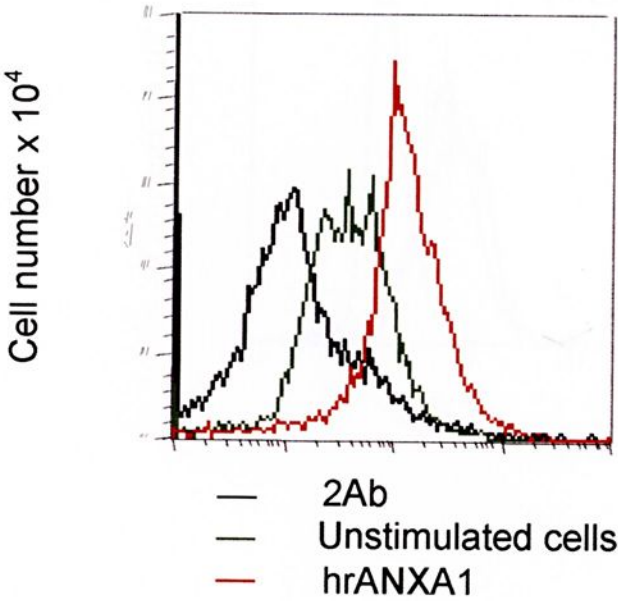
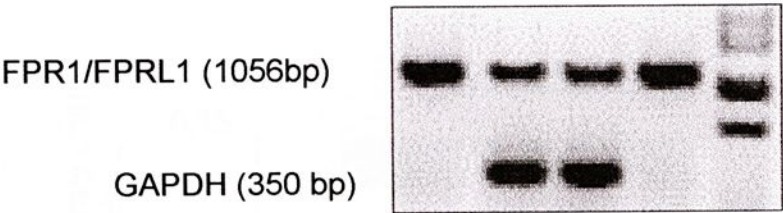


Figure 4

A



B

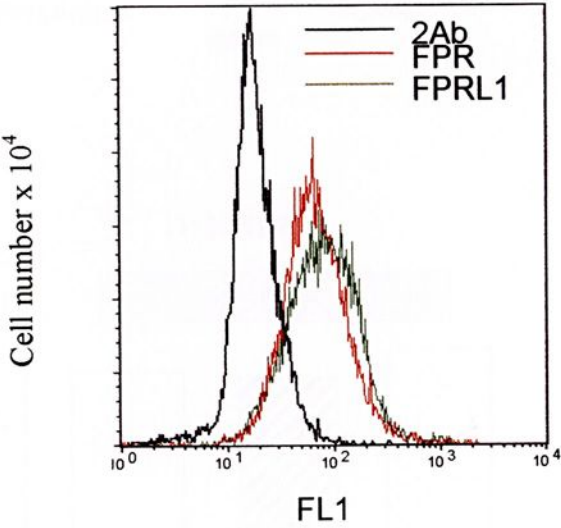


Figure 5



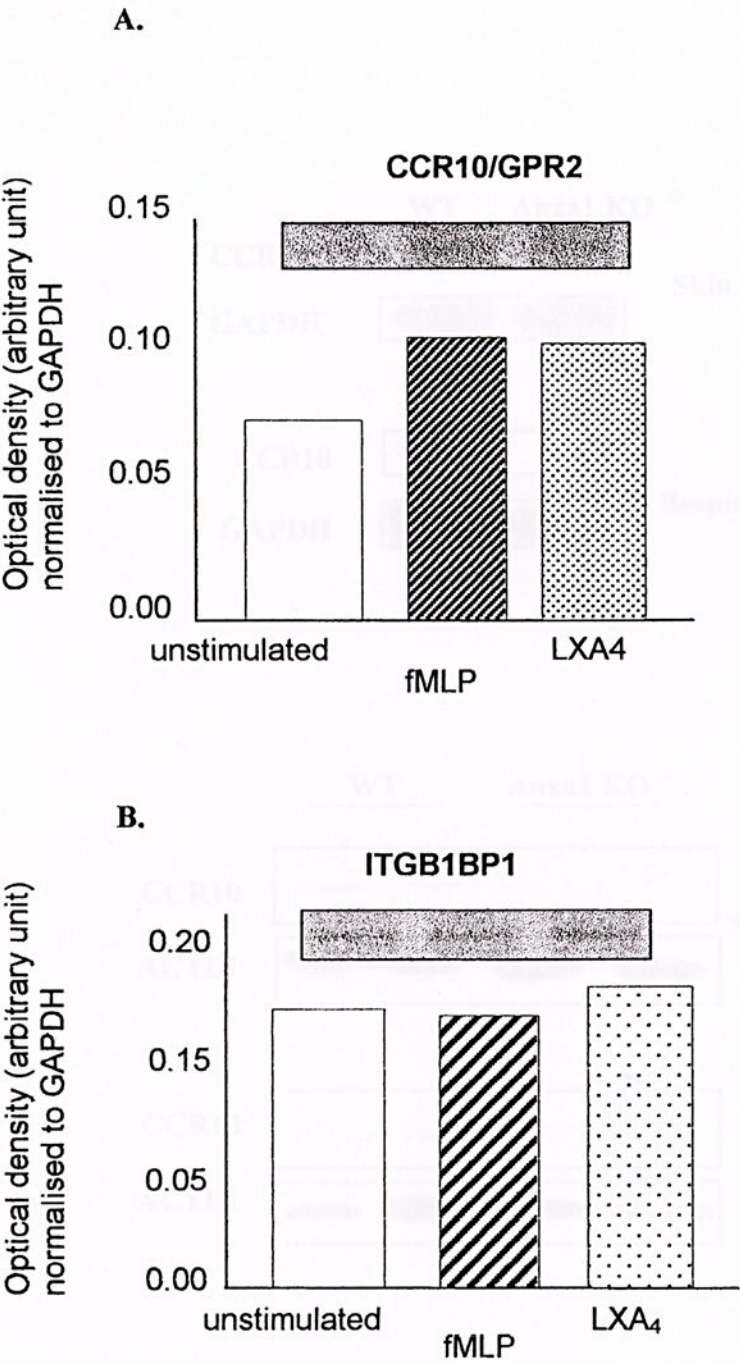


Figure 6

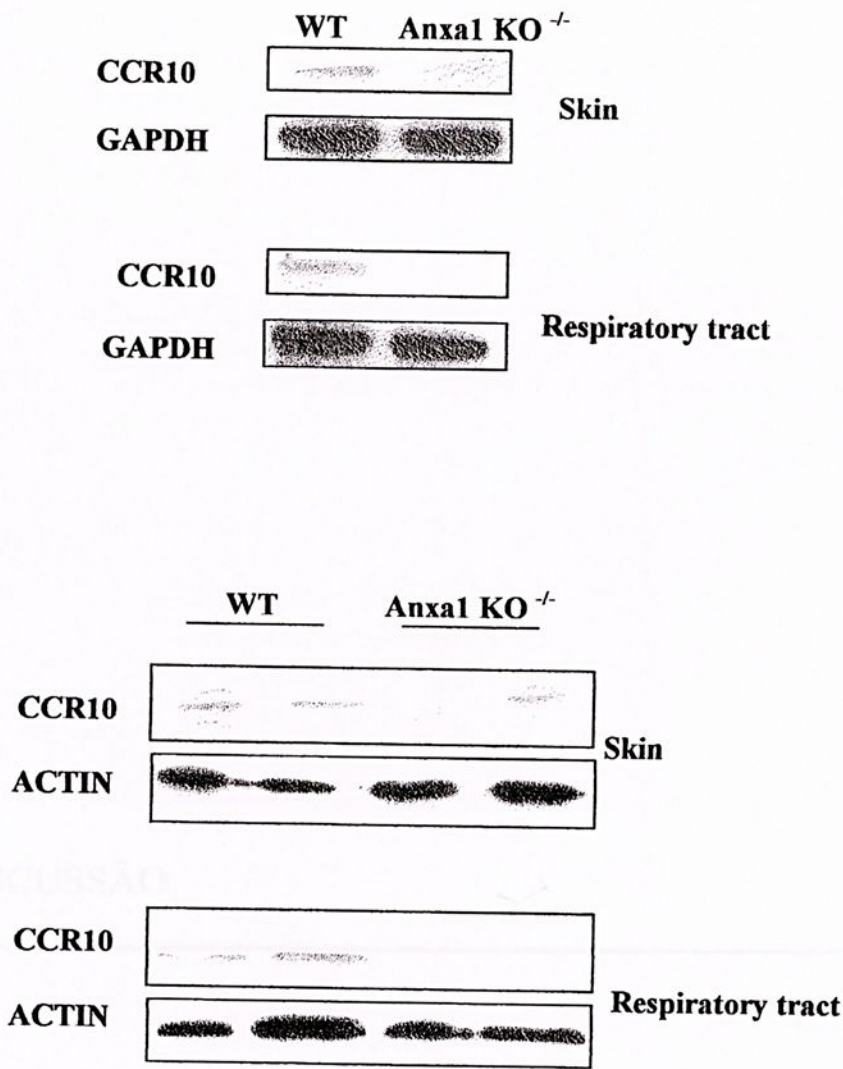


Figure 7





As anexinas compreendem uma super-família bem conservada de proteínas relacionadas com transdução de sinais (Alldridge et al., 1999; de Coupade et al., 2000), transporte de vesículas (Diakonova et al., 1997), inibição da inflamação (Getting et al., 1997), duplicação de DNA (Smith, Moss, 1994), transformação celular (Violette et al., 1990; Solito et al., 1998), formação de canais de íons, inibição de coagulação, interação célula-matriz (Croxtall et al., 1993) e apoptose (Raynal, Pollard, 1994; Moss, 1997).

Um dos membros dessa família é a anexina A1, que é abundante nas células do sistema imune, nos quais foi identificada como um mediador da ação anti-inflamatória dos glicocorticóides, e no sistema neuroendócrino, particularmente nas células foliculoesteladas da pituitária anterior (Taylor et al., 1993; Chapman et al., 2002). Os resultados de vários estudos sugerem que os mecanismos biológicos da ação da anexina estão relacionados com sua localização na membrana e sua interação com moléculas de superfície, como a L-selectina ou a integrina  $\alpha 4\beta 1$  (Solito et al., 2000; Strausbaugh, Rosen, 2001; de Coupade et al., 2003; Solito et al., 2003b), e com membros da família de receptores FPR (*formyl peptide receptor*).



A L-selectina é uma proteína altamente glicosilada, constitutivamente expressa em leucócitos, que media as interações de adesão dessas células e das plaquetas ao endotélio, na cascata precoce de eventos que levam aos processos de inflamação. Nessas etapas iniciais, o recrutamento de leucócitos é essencial para a geração de respostas de defesa do hospedeiro. Indivíduos que manifestam deficiência de recrutamento de neutrófilos, por exemplo, desenvolvem infecções severas e crônicas e cicatrização reduzida (Anderson et al., 1985). A migração dessas células para o sítio de injúria envolve uma sequência de eventos de sinalização e adesão da qual participam, além da L-selectina, citocinas e quimiocinas secretadas pelas células endoteliais, pelo tecido conjuntivo e por microorganismos invasores (Ebnet, Vestweber, 1999).

Os corticoesteróides e provavelmente seu efetor, a ANXA1, impedem o acúmulo de leucócitos nos tecidos inflamados. De Coupade e colaboradores (2003) sugerem que a translocação da ANXA1 endógena para a membrana celular facilita a inibição de L-selectina por uma interação dependente de cálcio e contribui para o controle da inflamação.

A integrina  $\alpha 4\beta 1$  e outras integrinas também atuam no recrutamento, migração e adesão de leucócitos por meio de sua interação com as moléculas de adesão ICAM-1 e VCAM-1, expressas na superfície de células endoteliais. Solito e colaboradores (2000) mostraram a colocalização de  $\alpha 4\beta 1$  e ANXA1 e a inibição desta integrina pela porção terminal da anexina.

Neste trabalho, relatamos, pela primeira vez, os efeitos da anexina A1 (ANXA1) e seu peptídeo N-terminal (ANXA1<sub>Ac2-26</sub>) na expressão gênica, utilizando análise genômica em linhagens de carcinoma e de células foliculoesteladas de adenoma pituitário. Também

foram utilizadas análises *in vivo* da pituitária de camundongos selvagens e *knockouts* para o gene da ANXA1.

A modulação da expressão gênica pela ANXA1 tem sido relatada como uma resposta tardia, em contraste com os seus efeitos precoces como, por exemplo, o destacamento de neutrófilos (Perreti et al., 1993) e a inibição da secreção na pituitária anterior (John et al., 2002; John et al., 2003).

Os dados do presente trabalho, obtidos por técnicas de eletroforese 2D, PCR semi-quantitativa, imuno-histoquímica, *western blot* e citometria de fluxo, mostraram que esta proteína regula sua própria expressão e inibe a expressão de um grande número de proteínas (dados não mostrados). Tais efeitos são comparáveis à ação poderosa do tratamento com esteróides e reforçam a função proposta da ANXA1 como um mensageiro secundário da ação de glicocorticóides (Flower, 1988).

Um dos resultados da técnica de RaSH em células tratadas e não tratadas com a proteína ANXA1 ou seu peptídeo foi a regulação positiva da expressão do gene *CCR10*, um receptor para citocina CCL27 (Homey et al., 2002). O mesmo resultado foi observado em camundongos *knockouts* para ANXA1. Tanto as análises por PCR semi quantitativa como por *Western Blot* mostraram que as células de pele e do trato respiratório desses animais exibem níveis reduzidos de CCR10 em relação aos camundongos selvagens.

A citocina CCL27 e seu receptor CCR10 mediam respostas quimiotáticas no recrutamento de células T *in vitro* (Leung, Soter, 2001; Soler et al., 2003). Alguns trabalhos sugerem que uma sub-população de células T na pele têm um papel principal na iniciação e na manutenção de doenças cutâneas auto-imunes e inflamatórias, como psoríase, dermatite atópica e dermatite de contato alérgica (Schon et al., 1997; Grabbe, Schwarz, 1998). A



CCL27, cuja expressão é constitutiva e elevada em pele normal e inflamada, é regulada positivamente pelo fator de necrose tumoral (TNF)  $\alpha$  e pelo *nuclear factor of kappa light polypeptide gene enhancer in B-cells* (NF- $\kappa$ B) *in vitro* e negativamente pelos glicocorticóides *in vivo* (Vestergaard et al., 2005; Homey et al., 2002).

Como a ANXA1 é vista freqüentemente como uma proteína anti-inflamatória, seria esperada uma baixa expressão do *CCR10*, que modula efeitos pró-inflamatórios. Entretanto, como a ANXA1 está presente na pele (Schlaepfer, Haigler, 1990), é possível que ela module positivamente a expressão desse receptor para facilitar o acúmulo local de linfócitos. Um efeito similar é observado em relação aos glicocorticóides, um outro grupo de fatores anti-inflamatórios que aumentam, em eosinófilos, a expressão de um dos membros desta família de receptores, o CXCR4, provavelmente resultando no seqüestro de eosinófilos circulantes para tecidos não inflamados (Nagase et al., 2000). Os eosinófilos, cuja migração é uma característica marcante da inflamação alérgica, possuem sítios ligadores de glicocorticóides, que parecem atuar diretamente sobre essas células e reduzir sua longevidade pela indução de apoptose (Wallen et al., 1991).

Uma outra explicação para ativação do *CCR10* pela anexina é a freqüente redundância nas famílias de ligantes e receptores de citocinas, com ligantes diferentes desencadeando vias de sinalização distintas utilizando o mesmo receptor. Por exemplo, o CCL11, um ligante para o receptor da quimiocina CCR3, induz a apoptose em células B (Jinquan et al., 2003) e o CCL28, um outro ligante para o mesmo receptor, mantém a migração de eosinófilos (Pan et al., 2000). Da mesma forma, o fator derivado de células estromais (SDF1)  $\alpha$ , um ligante para o receptor CXCR4, pode sinalizar para quimiotaxia

em células T CD4 ou morte celular em neurônios (Berndt et al., 1998; Vlahakis et al., 2001). Segundo Vlahakis e colaboradores (2002), os mecanismos moleculares de ativação do CXCR4 podem mediar sobrevivência ou morte celular pela ativação de diferentes vias de sinalização. Quando a via anti apoptótica da quinase fosfatidil-inositol 3 (PI3K) dependente de CXCR4-SDF $\alpha$  é inibida, é ativada a via MAPK/p38 independente de proteína G-1 $\alpha$ , que resulta em morte celular.

A ANXA1 está fortemente expressa durante o desenvolvimento do trato respiratório superior e da pele (Wells, et al. 2004) e exibe, em fibroblastos, significantes mudanças na expressão relacionadas com a idade (Boraldi et al., 2003), com níveis elevados em fibroblastos humanos de doadores adultos comparados a doadores idosos. Este fato pode se refletir na expressão e na funcionalidade do *CCR10* como parte de uma falha geral, relacionada com o envelhecimento, em responder ao estresse ambiental e/ou fisiológico. A mesma falha pode ocorrer em câncer, considerando a semelhança de muitos mecanismos envolvidos na senescência e na tumorigênese.

O fato do receptor *CCR10* ser um mediador do recrutamento de linfócitos específicos da pele e o fato da ANXA1 ser expressa na pele e inibir a L-selectina em neutrófilos (Strausbaugh, Rosen, 2001) e em monócitos (de Coupade et al., 2003) reforçam o papel dessa proteína na inflamação cutânea.

A técnica de hibridação subtrativa identificou três genes com expressão reduzida pelo tratamento com ANXA1. O primeiro deles foi o *ITGB1BP1* (anteriormente chamado *ICAP-1A*, *integrin cytoplasmic domain-associated protein 1A*), uma proteína especificamente associada com o domínio citoplasmático  $\beta 1$  de integrinas (Chang et al.,





1997). Sua fosforilação em sítios específicos parece ser um evento regulador crucial nos processos de adesão e migração celular mediados por integrina (Zhang, Hemler, 1999; Zawistowski et al. 2002; Bouvard et al., 2003). É possível, portanto, que a expressão reduzida de *ITGB1BP1* desencadeada pela ANXA1 leve à diminuição de adesão dos neutrófilos à parede de vasos em sítios de inflamação. Os dados de Lim et al. (1998), utilizando mesentério inflamado de camundongo tratado com ANXA1, estão de acordo com essa hipótese.

O segundo gene com expressão reduzida pelo tratamento com ANXA1 foi o *FARSLA* (*phenylalanine-tRNA synthetase*), que é transcrito consistentemente em alguns tumores e dependente da diferenciação e do estágio do ciclo celular. Esta enzima também tem mostrado expressão elevada em variantes tumorigênicas versus não tumorigênicas de linhagem celular de leucemia mielóide humana (Sen et al., 1997) e em outras linhagens de células neoplásicas. O gene *FARSLA* foi mapeado no cromossomo 19, que exibe freqüentes alterações em tumores sólidos e neoplasias hematológicas, conforme descrito no banco de dados "Mitelman Database of Chromosome Aberrations in Cancer", mantido pelo *The Cancer Genome Anatomy Project* (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>). A ANXA1 poderia, portanto, modular o crescimento celular através deste gene. Embora dependam de confirmação, esses resultados sugerem novas possibilidades do mecanismo da ação da ANXA1 na transformação celular. Nos processos neoplásicos envolvendo padrões dos quais participa o gene *FARSLA*, a ANXA1 poderia exibir elevação nos seus níveis como um mecanismo de controle desta enzima. Esta hipótese explica o porquê da ANXA1 estar altamente expressa em alguns tipos de câncer e não em outros.



Finalmente, o terceiro gene detectado por RaSH foi o *TIFA* (*TRAF-interacting protein with a forkhead-associated domain*), anteriormente chamado de *T2BP* (*TRAF 2 binding protein*). Esta pequena proteína interage com TRAF2 (*TNF receptor-associated factor 2*) e parece mediar processos de sobrevivência celular pela via do fator de necrose tumoral (TNF), que é importante para a regulação de diferentes respostas biológicas, desde inflamação até morte celular. Esta via é iniciada pelo receptor TNFR-1 e -2. O TNFR-1, que possui um domínio de morte, transduz tanto sinais de morte como de sobrevivência por dois complexos de sinalização. O primeiro complexo é formado pelo próprio receptor e por outras proteínas, como TRADD (*TNFRSF1A-associated via death domain*) e TRAF2, sendo responsável por ativar o NF- $\kappa$ B e, portanto, por sinais de sobrevivência (Wajant, Scheurich, 2001; Kanamori et al., 2002; Sheikh, Huang, 2003). No presente trabalho, a expressão reduzida de *TIFA* nas células tratadas, sugere que a ANXA1 pode modular processos inflamatórios e morte celular via TNF e TIFA. Essa hipótese está de acordo com os achados sobre a função da ANXA1 na endotoxemia sistêmica, na qual o tratamento com ANXA1 diminuiu a regulação da liberação de TNF $\alpha$ , um dos membros da superfamília TNF (de Coupade et al., 2001).

Em resumo, o presente trabalho mostrou a importância da técnica de RaSH como um método eficiente e rápido para identificar genes modulados pela ANXA1 em células de origem epitelial. Para o gene *CCR10*, sua expressão foi claramente relacionada com a presença da ANXA1 *in vivo*. Apesar da sua influência sobre um grande número de genes, a perda da ANXA1 em animais *knockout* não comprometeu sua sobrevivência, suportando uma função adaptativa para a ANXA1 em uma variedade de padrões metabólicos. Outros



experimentos são necessários para determinar se os demais genes regulados pela ANXA1 estão casualmente relacionados ou associados com mudanças induzidas pelo tratamento com esta proteína, embora isto pareça improvável, pois nossos achados foram observados em duas linhagens celulares independentes. Estes estudos, embora desafiantes, fornecerão informações importantes a respeito do fenótipo de organismos *knockout* para anexina A1 e, portanto, da função fisiológica desta proteína.

V CONCLUSÕES







O presente trabalho permitiu estabelecer as seguintes conclusões:

1. A técnica de Hibridação Subtrativa Rápida (RaSH) é um método eficiente e rápido para identificar genes modulados pela ANXA1 em células de origem epitelial.
2. O tratamento de linhagens celulares de carcinoma e de células foliculoesteladas de adenoma pituitário por ANXA1 resulta na elevação do nível de expressão do gene *CCR10* e na redução de expressão dos genes *ITGB1BP1*, *FARSLA* e *TIFA*.
3. A relação da ANXA1 com o gene *CCR10*, que está associado com as vias do fator de necrose tumoral  $TNF\alpha$  e do *nuclear factor of kappa light polypeptide gene enhancer in B-cells* NF- $\kappa$ B, sugere que ela modula o acúmulo local de linfócitos e reforça o seu papel na inflamação cutânea. Seus efeitos anti-inflamatórios de inibição de adesão de neutrófilos ao endotélio podem ser efetuados por *ITGB1BP1*, que atua na via das integrinas. Sobre a atuação da ANXA1 na tumorigênese, os dados obtidos levantam a possibilidade de que essa proteína module o crescimento celular pelo gene *FARSLA* e morte celular e inflamação pelo gene *TIFA*, este último também relacionado com a via do fator de necrose tumoral TNF.



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Apêndice B: Outra produção científica





## Apêndice I: Outra produção científica



# Large-scale Transcriptome Analyses Reveal New Genetic Marker Candidates of Head, Neck, and Thyroid Cancer

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## Abstract

A detailed genome mapping analysis of 213,636 expressed sequence tags (EST) derived from nontumor and tumor tissues of the oral cavity, larynx, pharynx, and thyroid was done. Transcripts matching known human genes were identified; potential new splice variants were flagged and subjected to manual curation, pointing to 788 putatively new alternative splicing isoforms, the majority (75%) being insertion events. A subset of 34 new splicing isoforms (5% of 788 events) was selected and 23 (68%) were confirmed by reverse transcription-PCR and DNA sequencing. Putative new genes were revealed, including six transcripts mapped to well-studied chromosomes such as 22, as well as transcripts that mapped to 253 intergenic regions. In addition, 2,251 noncoding intronic RNAs, eventually involved in transcriptional regulation, were found. A set of 250 candidate markers for loss of heterozygosity or gene amplification was selected by identifying transcripts that mapped to genomic regions previously known to be frequently amplified or deleted in head, neck, and thyroid tumors. Three of these markers were evaluated by quantitative reverse transcription-PCR in an independent set of individual samples. Along with detailed clinical data about tumor origin, the information reported here is now publicly available on a dedicated Web site as a resource for further biological investigation. This first *in silico* reconstruction of the head, neck, and thyroid transcriptomes points to a wealth of new candidate markers that can be used for future studies on the molecular basis of these tumors. Similar analysis is warranted for a number of other tumors for which large EST data sets are available. (Cancer Res 2005; 65(5): 1693-9)

## Introduction

Head and neck squamous cell carcinomas (HNSCC) are common and aggressive neoplasms, with an estimated annual incidence of 615,900 new cases worldwide (1) and an increasing incidence rate in many areas of the world (2-4). Thyroid cancer is the most common neoplasia from the endocrine system with an estimated annual incidence of 122,800 worldwide. Most of the incidence variations are probably due to ethnic or environmental factors such as radiation, dietary habits, or other external factors.

Cancer phenotype is defined by the accumulation of mutations and epigenetic changes that may alter protein function and/or cause alterations in transcriptional patterns. These events usually confer competitive advantages to a cell, ultimately leading to the malignant phenotype. Transcriptome wide analysis is an important tool to reveal some of these molecular mechanisms that underlie human malignancies (5, 6).

To generate a database of transcriptional profiles derived from tumor and nontumor tissues, a high-throughput cDNA-sequencing project, based on normalized mini-cDNA libraries generated with the open reading frame expressed sequence tags (ORESTES) methodology, was conducted by our group, yielding a set of ~1.2 million expressed sequence tags (EST) from diverse human tissues, from which a filtered set was deposited in public databases (6, 7). ORESTES sequences are predominantly derived from the central coding region of genes, thus favoring the identification of gene function based on protein similarity. In addition, this methodology partially normalizes the population of tagged genes, enabling the study of rare transcripts (8). The present study reports on a detailed informatics analysis of 134,495 ORESTES from head and neck as well as 79,141 ORESTES from thyroid tissues followed by experimental validation of new transcripts and new splicing isoforms and by an evaluation of putative markers for these malignancies.

## Materials and Methods

**Patients and Biological Samples.** Subjects were selected at random from surgical patients treated at the Head and Neck Surgery and Otorhinolaryngology Department of the Hospital do Câncer A.C. Camargo in São Paulo, Brazil. All patients were treated and followed up according to prevailing protocols. Eligible patients were asked to sign an informed

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consent previously approved by the hospital's institutional review board. The criteria for eligibility were determined to insure a good representation of the transcriptional diversity of the tissues studied. On this basis, selected patients had to be at least 18 years old and have a histologically confirmed diagnosis of a non-previously treated (a) oral, pharyngeal, hypopharyngeal, or laryngeal squamous cell carcinoma (31 samples) or (b) thyroid carcinoma, adenoma, or goiter (45 samples). Detailed composition of samples, in terms of tissue origin and pathologic state, is given in Supplementary Methods. Tumor and adjacent nontumor tissues were collected, snap-frozen, and stored; tumor margins that were considered disease free after histologic examination were used here as nontumor samples. Before RNA extraction, all pathologic diagnoses were reconfirmed and samples were macrodissected, reducing nontumor tissues to <20%.

**RNA Extraction and ORESTES.** PolyA<sup>+</sup> RNA was isolated, treated with DNase and purified as described before, (8) and used for constructing 1,576 ORESTES cDNA minilibraries (8) that were cloned and sequenced (7). The detailed composition of each of these cDNA minilibraries, in terms of tissue origin and pathologic state, is given in Supplementary Methods and in Supplementary Table 1. A total of 1,003 cDNA minilibraries were sequenced, generating 18,620 sequences from nontumor tissues, 190,253 from tumor tissues, and 4,763 from thyroid goiter samples; 134,495 sequences were derived from head and neck and 79,141 from thyroid tissues. mRNA derived from a second set of tumors and nontumor samples as well as from four human cell lines (FaDu, Hep2, HeLa, and SiHa) was extracted as described above and used for the experimental validation of new splicing isoforms, new genes, and potential tumor markers predicted by bioinformatics analyses.

**cDNA Sequence Analyses.** A total of 213,636 ESTs generated from head and neck and thyroid tissues were analyzed along with local copies of the human genome sequence draft (University of California at Santa Cruz, June 2002) and of the RefSeq and mRNA public data sets ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The complete set of 1,187,342 ESTs produced in the Human Cancer Genome Project (HCGP; ref. 7) from more than 20 tissues was filtered to reduce the presence of low-quality sequences or contaminants, and assembled in 956,456 reads, which included 173,640 sequences derived from head, neck, and thyroid tissues. The data set was clustered using BLAST (<http://www.ncbi.nlm.nih.gov/>) and assembled with Phrap (<http://www.phrap.org>). To further reduce the residual redundancy and to eliminate misassembled contigs or spurious sequences that passed the filtering step, all contigs and singlet sequences were aligned to the draft human genome sequence using BLAT (9). All mapped sequences that exhibited genomic overlap were merged into a unique genomic cluster. Only sequences that aligned with >90% identity to the draft sequence through at least 50% of their length were considered for further analyses. A subset of 119,552 ESTs that were used here and were not analyzed in previous publications (6–8) were now deposited in dbEST GenBank under accession numbers CV309219 to CV428770.

**Identification of Tissue-Specific Transcriptional Markers.** Genes that were preferentially expressed in certain head and neck sites (oral cavity, pharynx, and larynx) or thyroid were identified using a Bayesian model, as described in detail in Supplementary Methods. Using this approach, EST count thresholds were established to define 95% confidence intervals for the absence or presence of expression of a certain gene in each anatomic site.

**Analysis of New Splicing Isoforms and New Human Transcripts.** Four trained investigators, using the same criteria, visually inspected the genomic mapping of all ESTs with putative new splicing isoforms. For all new splicings approved by visual inspection, additional data were collected regarding splicing classification (10), size of the event in terms of nucleotides involved, donor/acceptor sites, and confirmation of the event by other cDNA sequences. Reverse transcription-PCR (RT-PCR) and cDNA sequencing were done to confirm a subset of these new gene isoforms. To reduce individual variations, isoform validations were done using human cell lines as well as pooled samples from different patients and topological locations.

Candidates for new human transcripts were identified by selecting clusters or single ESTs with no similarity to known human genes, as determined by BLASTN queries against a complete set of 162,104 human

mRNA full-length sequences deposited in GenBank (cutoff *E* value =  $10^{-15}$ ). In addition, clusters and singlets were identified that mapped to a 2.37-Mb region of chromosome 22 (22q11.2), flanking marker D22S421, previously shown to be deleted in larynx tumors (11). These were selected for manual annotation, which included sequence alignment with *in silico* gene predictions (GeneScan) and comparison with mouse full-length transcripts and ESTs, using the UCSC Genome Browser (<http://genome.ucsc.edu/>). A set of putative new transcripts was validated by RT-PCR, using pooled mRNAs from nontumor or tumor larynx and further evaluated in 45 different mRNA samples derived from larynx (14 nontumor and 13 tumor), tongue (9 nontumor and 3 tumor), and tonsil (4 nontumor and 2 tumor).

**Evaluation of Differential Gene Expression.** Experimental evaluation of differential expression was done for a subset of ORESTES contigs mapped to genomic regions that, according to the literature data, exhibit recurrent loss of heterozygosity or amplifications in the selected tumors. Three genes were selected for experimental validation of their differential expression by quantitative real-time PCR analysis in a GenAmp 5700 (Applied Biosystems, Foster City, CA). cDNA was produced from individual tumor and paired normal adjacent tissue samples from the larynx and oral cavity. To reduce individual sample variations possibly arising from contamination of normal tissue with adjacent tumor, the average expression level of each gene in all normal samples was used as a reference for each tissue examined. For each patient, fold change of expression was calculated as the ratio between the individual tumor sample and the average level of normal tissue samples. Each cDNA sample was analyzed in duplicate reactions using the SybrGreen PCR Core Reagent (Applied Biosystems), and normalization was done according to the manufacturer's instructions using *ACTB* or *GAPDH* as the reference gene.

**Web Site.** A dedicated Web site was prepared for data analysis, enabling visual inspection of the assemblies and of their genome map coordinates as well as searches using keywords, Gene Ontology classification, or chromosomal location of transcripts detected in head, neck, or thyroid tumor types under study. The site is publicly available at [http://verjo19.iq.usp.br/java/jsp/head\\_neck/](http://verjo19.iq.usp.br/java/jsp/head_neck/).

## Results and Discussion

**Assembly and Mapping the Head, Neck, and Thyroid EST Dataset.** A set of 213,636 EST sequences derived from head, neck and thyroid tumors was analyzed here, being part of the over 1.2 million ESTs that were generated by the HCGP (7). After filtering for contaminants, the remaining 173,640 ESTs (81%) were assembled together with all HCGP sequences as described under Materials and Methods. This assembly resulted in 32,576 contigs and 24,434 singlets that were aligned to the draft sequence of the human genome. All mapped sequences that exhibited genomic overlap were included in the same genomic cluster, thus reducing the data set to a nonredundant collection of 48,001 sequences (contigs plus singlets) grouped into 20,348 genomic clusters (Table 1). This corresponds to 38% of the total genomic groups formed by all ORESTES (20,348/53,105), and includes a number of clusters containing reads exclusively derived from head and neck (2,712) or thyroid (1,331) tissues.

**Patterns of Gene Expression and Tissue Markers.** A total of 10,228 distinct RefSeq genes were sampled by at least one EST derived from head and neck or thyroid, as determined by merging all nonoverlapping EST clusters that mapped within the same RefSeq gene sequence. Different amounts of ESTs were generated from each head and neck or thyroid tissue using different sets of primers, which preclude a direct assessment of genes differentially expressed at a distinct topology. Aiming to normalize these experimental factors, we applied a Bayesian statistical model (12) to select only EST counts that were





**Table 1.** Genomic mapping of all ORESTES sequences and of head and neck or thyroid assembled sequences

|                                                              | Assembled<br>contigs +<br>singlets | Percent<br>of total | Genomic<br>clusters | Percent<br>of total | Genomic<br>clusters<br>colinear<br>with RefSeqs* | Percent of<br>total in each<br>category |
|--------------------------------------------------------------|------------------------------------|---------------------|---------------------|---------------------|--------------------------------------------------|-----------------------------------------|
| Containing all ORESTES <sup>†</sup>                          | 173,329                            | 100                 | 53,105              | 100                 | 33,022                                           | 62                                      |
| Not containing head and neck<br>or thyroid sequences         | 125,328                            | 72                  | 32,742              | 62                  | 19,003                                           | 58                                      |
| Containing head and neck<br>or thyroid sequences             | 48,001                             | 28                  | 20,348              | 38                  | 14,019                                           | 69                                      |
| Containing only head and neck<br>or thyroid sequences        | 23,474                             | 14                  | 4,186               | 8                   | 2,162                                            | 52                                      |
| Containing only head and neck<br>sequences (normal or tumor) | 14,815                             | 9                   | 2,712               | 5                   | 1,396                                            | 4                                       |
| Containing only HNSCC sequences                              | 14,305                             | 8                   | 2,621               | 5                   | 1,338                                            | 4                                       |
| Containing only normal head<br>and neck sequences            | 473                                | 0.3                 | 66                  | 0.1                 | 38                                               | 0.1                                     |
| Containing only thyroid<br>sequences                         | 8,316                              | 5                   | 1,331               | 3                   | 651                                              | 2                                       |
| Containing only thyroid tumor<br>sequences                   | 6,591                              | 4                   | 1,099               | 2                   | 529                                              | 2                                       |
| Containing only normal thyroid<br>sequences                  | 1,651                              | 1                   | 208                 | 0.4                 | 106                                              | 0.3                                     |

\*A total of 20,602 RefSeqs mapped to the draft sequence of the human genome with 50% of their length with identity  $\geq 90\%$ .

<sup>†</sup>Only sequences aligned to the draft sequence of the human genome through at least 50% of their length with identity  $\geq 90\%$ .

detected within a confidence interval of 95% (see Materials and Methods). When this statistical model was applied, ESTs representing exons of 238 RefSeq genes were selected. The analysis suggested larynx/oral cavity as the most similar pair of tissues in terms of gene expression, followed by oral cavity/pharynx and larynx/pharynx (Fig. 1A). The same result was obtained when EST clusters that mapped to genomic regions with no RefSeq genes were included in the analysis (not shown). In addition, the trio larynx/oral cavity/pharynx contains more genes in common than trios that include thyroid (Fig. 1B). The 238 RefSeq genes that were identified as being differentially expressed in different topologies may be used as putative markers for identifying the tissue origin of circulating tumor cells, as well as for distinguishing primary tumor from metastatic lesions, a subject that deserves further evaluation.

This analysis revealed genes preferentially expressed in each head and neck site or in thyroid with statistical criteria, being a source of tissue-specific candidate markers for HNSCC or thyroid tumors that may be selected for further validation (list of genes is available as Supplementary Table 2).

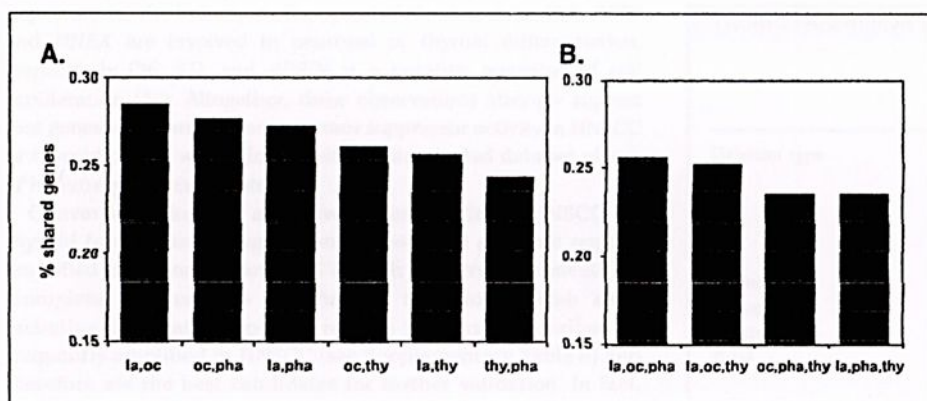
**New Genes and Noncoding RNAs.** Out of the total of 20,348 genomic clusters containing sequences from head and neck or thyroid, a significant fraction (25%, i.e., 5,038 clusters) represents possible new human transcripts because they have no significant similarities to previously known human genes (see Materials and Methods). Among these, a small fraction has evidence of splicing (16%, i.e., 835) and only 507 sequences (10%) have a normalized ESTscan score (13)  $>1$ , which is indicative of high protein coding potential. Nevertheless, none of them contain known protein motifs when compared with the PFAM data set. Among these new coding transcript fragments, 254 were found to map to intronic regions of RefSeq genes and may represent new coding exons (see

Supplementary Table 3). The remaining 253 map to intergenic regions and may represent fragments of new human genes (see Supplementary Table 4).

The remaining 4,531 clusters that represent new transcripts have a normalized ESTscan score lower than 1; that is, they have a low coding potential. Remarkably, a large fraction of these (50%, i.e., 2,251 clusters) map to intronic regions of RefSeq genes (Supplementary Table 5). Whereas some of these may reflect a certain degree of genomic DNA contamination that might have persisted even after DNase treatment and polyA<sup>+</sup> mRNA selection, it is likely that most of these ESTs represent bona fide mRNA transcripts. In fact, recent data showed that similar fractions of intronic and exonic transcripts are expressed in 47 samples of tumor and normal prostate, as detected by cDNA microarrays constructed with ORESTES clones (14).

Recent work describing the transcriptional output of the human genome points to the existence of a significant number of noncoding RNA transcripts (15, 16) and that 10% to 20% of human transcripts might form sense-antisense pairs (17, 18). Furthermore, comparable fractions of transcriptional activity were detected within exons or introns of annotated genes, and nearly half of these intronic transcripts were expressed antisense to their respective well-characterized introns (16, 19). Although the role of these antisense intronic noncoding messages in RNA regulation is not yet fully understood (20), it has been recently shown that intronic noncoding transcripts expressed antisense to their respective introns have their expression level significantly correlated to the degree of tumor differentiation in prostate cancer (14). Identification in the present work of a large set of intronic noncoding transcripts expressed in head-neck and thyroid suggests that noncoding intronic transcripts may also play an important role in HNSCC and thyroid tumors.





**Figure 1.** Transcriptome sharing between HNSCC and thyroid. 238 RefSeq genes detected with 95% confidence in the EST data set were scored (see Materials and Methods). Fractions of genes shared by each pair (A) or trio (B) of topologies are shown. *la*, larynx; *oc*, oral cavity; *pha*, pharynx; *thy*, thyroid.

Significantly, the majority of the new transcripts containing sequences from head and neck or thyroid (2,832 of 5,038, i.e., 56%) map to genomic regions previously associated to HNSCC or thyroid tumors (21). We found 12 putative new human transcripts mapping to a 2.37-Mb sequence from human chromosome 22q11-12, a region selected for its involvement with larynx tumors (11). These include three EST clusters containing head, neck, and thyroid sequences. All three had their expression confirmed in larynx by RT-PCR and were further verified by semiquantitative RT-PCR in 45 different mRNA samples obtained from pairs of nontumor/tumor tissues (see Materials and Methods). Positive amplification was obtained in 42 of 45 (LOC91353), 29 of 45 (LOC164615), and 21 of 45 (LOC91355) of the evaluated samples. Although no evidence for differential expression between nontumor and tumor samples was observed for this small set of genes, these results confirm the potential of this approach to identify new human genes.

**New Splicing Isoforms.** Genomic alignments of all EST clusters matching known human genes were visually inspected to evaluate the presence of putative new splicing isoforms. New events involving at least 10 nucleotides of deletions or insertions in previously known genes were considered as putative new splicing isoforms and were classified according to Wang et al. (10). Presence of conserved donor/acceptor sites as well as eventual confirmation of splicing events by other human ESTs or nonhuman transcripts in the public databases were recorded. A total of 788 new splicing events were identified in 748 different transcripts (see Supplementary Table 6).

Among the new splicing events observed here, 86% and 89% showed the canonical GT/AG donor and acceptor sites, respectively. The majority (75.3%) of new splicings was due to insertion events (Table 2). Overall, the most frequent events were exon extensions at 5' (type II) and insertions at 3' (type III). New splicing events could be confirmed by other human ESTs from GenBank in 28.6% of the cases and by sequences from other organisms in 6%.

New splicing isoforms were selected for experimental validation using a set of mRNAs different from that used for EST generation. From a total of 34 new splicing isoforms evaluated, the following 23 (68%) were confirmed by RT-PCR in *AF5Q31*, *CARD8*, *CCNL1*, *CTNNB1*, *CTPS*, *DNMT3A*, *EWSR1*, *MKNK1*, *NESHP*, *PDCD8*, *PFDN5*, *PORIMIN*, *PSMD10*, *PTK2*, *PTPN18*, *RABL2A*, *SMARCA4*, *SMARCB1*, *ST5*, *SULF2*, *TRIP11*, *TRRAP*, and *VAV2*. Examples of four novel splicing events are shown in Fig. 2. Additional examples are shown in Supplementary Fig. 1.

Many cases of splicing events affecting protein domains were observed, such as in *SMARCB1*, *PFDN5*, *AF5Q31*, and *EWSR1*.

*SMARCB1* is involved in chromatin remodeling and is mutated in a variety of human tumors of diverse tissue origins, including some very aggressive pediatric cancers (22, 23). The novel exon of *SMARCB1* identified here promotes an in-frame addition of 18 amino acids to the protein, with an unpredicted effect over its function. In *PFDN5*, the newly identified exon adds 101 amino acid residues (with putative phosphorylation sites and an amidation site) to the protein. Interestingly, isoforms of *PFDN5* transcripts were detected in Northern blots by Mori et al. (24) who also showed that this heterohexameric chaperone protein binds to c-Myc, repressing its transcriptional activity. The 101-amino-acid insertion may affect *PFDN5* binding capacity and interfere with its ability to repress the transcriptional activity of c-Myc.

Because the ORESTES database was shown to be enriched with rare messages (7), splicing variants described here may represent rare new splicing isoforms related to these tumors. Whereas some false-negative or positive splicing events may have occurred among the 788 new splicing events described, our validation rate (68%) suggests that true events are the majority. Indeed, the set of human ESTs available in public databases has recently been shown to provide potential markers for the classification of cancer (25). The new splicing isoforms reported here contribute to this notion and warrant further research aimed at the identification of tumor-specific transcriptional events.

**Finding Tumor Markers Using Transcript Analysis.** The complete HCGP data set is composed of a 9.8-fold excess of tumor-derived sequences. Despite this, a set of 8,988 genomic clusters exclusively from nontumor tissues sampled in the project was found. This set probably is enriched in genes down-regulated during tumor development and progression. Within this set, 270 genomic clusters contained sequences derived from normal head, neck, or thyroid tissues (the complete list can be searched at the project Web site). Twenty-three of these contain sequences from normal head and neck tissues and are of particular interest because they mapped to regions known to be frequently deleted in HNSCC (refs. 21, 26–28; Supplementary Table 7). Although we found several clusters composed exclusively from sequences derived from normal thyroid, only one of these (R2\_CLUSTER\_36019) mapped to a region (17p11) known to be deleted in thyroid tumors (21, 29, 30). Several of the genes included in this subset have antiproliferative, apoptotic, or differentiation-induction activities: *CASP10* is involved in the proximal pathway of Fas-mediated apoptosis and is down-regulated in lung cancer, neuroblastoma, and non-Hodgkin lymphomas (31–33). Two other genes are related to apoptosis: *CSEN*, a member of the neuronal calcium sensor family (34), and *MAPK8*, which is



important for the induction of apoptosis following stress (35). *RGS6* and *IIHEX* are involved in neuronal or thyroid differentiation, respectively (36, 37), and *APRIN* is a putative regulator of cell proliferation (38). Altogether, these observations strongly suggest that genes with a still unknown tumor suppressor activity in HNSCC or thyroid cancer will be found within this selected data set of loss of heterozygosity candidates.

Conversely, clusters exclusively derived from HNSCC or thyroid tumor tissues suggest oncogenes from genomic regions amplified in tumors. A set of 3,776 such clusters were identified (complete lists can be searched at the project Web site), including 144 that mapped to regions previously described as frequently amplified in HNSCC (see Supplementary Table 8) and therefore are the best candidates for further validation. In fact, 12 of these clusters are transcripts from known oncogenes, whereas another subset of about 30% of these clusters are from genes of unknown functions. Genes from this list may include potential antigens that could be used in immunodiagnosis or immunotherapeutic approaches, as well as tumor markers. However, due to the biased amount of sequences from tumor samples in our project, the list of tumor-only EST clusters identified here should be analyzed with caution.

Three transcripts were further investigated for differential expression in HNSCC by quantitative real-time PCR in paired patient samples (eight oral cavity tumors and nine larynx tumors). Selected genes mapped to genomic regions that were frequently amplified (*ZRF1* and *NDRG1*) or frequently lost (*RAP140*) in HNSCC. *ZRF1* mRNA levels were increased in most samples of

Table 2. Alternative splicing statistics

|                | Splicing events (%) | % of all 788 events |
|----------------|---------------------|---------------------|
| Deletion type  |                     |                     |
| I              | 68 (34.9)           | 8.6                 |
| II             | 15 (7.7)            | 1.9                 |
| III            | 17 (8.7)            | 2.2                 |
| IV             | 48 (24.6)           | 6.1                 |
| 2 events       | 24 (12.3)           | 3.1                 |
| 3 events       | 16 (8.2)            | 2.0                 |
| Multiple       | 7 (3.6)             | 0.9                 |
| Total          | 195 (100.0)         | 24.8                |
| Insertion type |                     |                     |
| I              | 103 (17.4)          | 13.0                |
| II             | 201 (33.9)          | 25.5                |
| III            | 158 (26.6)          | 20.1                |
| IV             | 59 (10.0)           | 7.5                 |
| 2 events       | 65 (11.0)           | 8.3                 |
| 3 events       | 4 (0.7)             | 0.5                 |
| Multiple       | 3 (0.5)             | 0.4                 |
| Total          | 593 (100.0)         | 75.3                |

NOTE: New splicing isoforms were classified according to Wang et al. (10). Insertions were divided into types I to IV: insertion of a new exon, 5' extension, 3' extension, and junction of two exons, respectively. Deletions were divided into types I to IV: exon skipping, 5' deletion, 3' deletion, and deletion of a central portion of the exon, respectively.

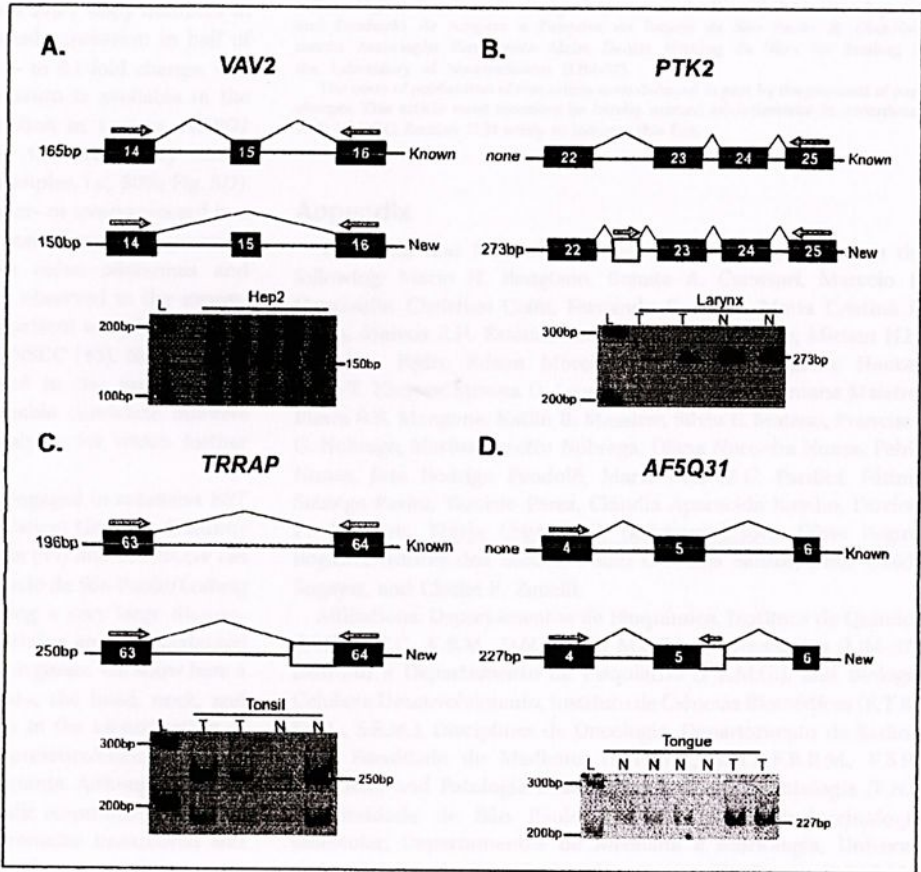
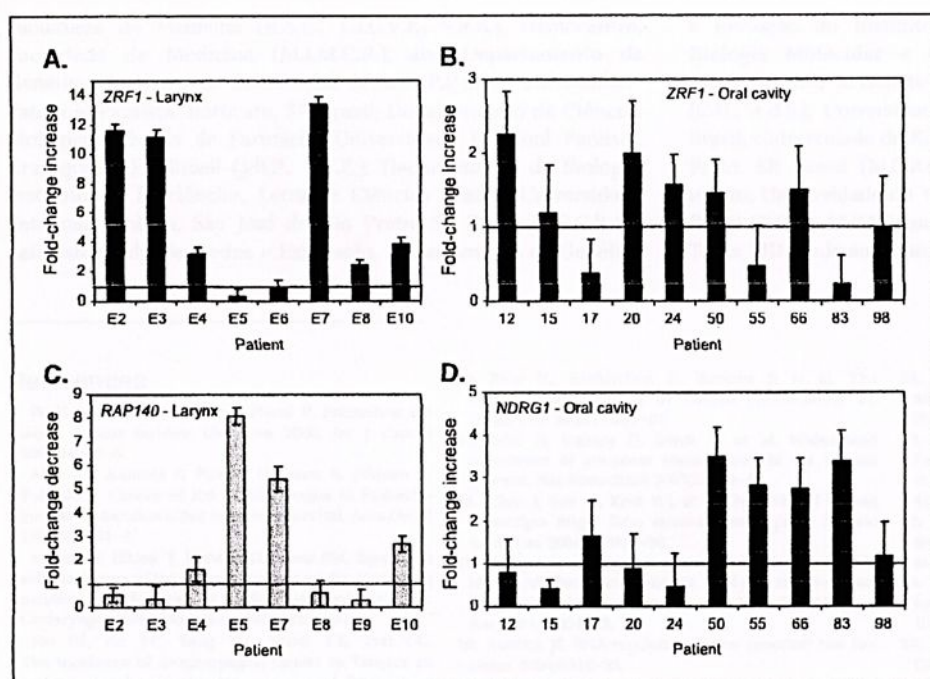


Figure 2. Experimental validation of new splicing isoforms using RT-PCR. A, type I deletion. B, type I insertion. C, type II insertion. D, type III insertion. Names of the genes are at the top of each panel. Arrows, primers; solid boxes, known exons with their corresponding exon numbers; open boxes, new exons. Horizontal lines, introns. Diagonal lines, alternative splicing events. For all gels, lane L corresponds to the molecular weight marker ladder (100 bp). The sources of template RNA samples used in the PCR reactions are indicated above the gel lanes. They are either replicates of cell line samples from Hep-2 (CCL-23, American Type Culture Collection, Manassas, VA), or normal (N) and tumor (T) tissue from Larynx, Tonsil, or Tongue, as indicated in each panel. All PCR products were confirmed by sequencing. Predicted band sizes (in base pairs) of PCR products amplified from Known and New mRNA splicing isoforms are indicated next to each diagram, and those predicted from genomic DNA are 2,618 bp for *VAV2*, 15,007 bp for *PTK2*, 1,560 bp for *TRRAP*, and 5,197 bp for *AF5Q31*.







**Figure 3.** Expression of putative tumor markers of HNSCC. Real-time quantitative PCR was used for the measurement of mRNA levels of candidate genes in paired samples of tumor and adjacent normal tissue from larynx (9 patients) or oral cavity (10 patients). Expression of *ZRF1* (A and B), *RAP140* (C), and *NDRG1* (D) in each individual tumor sample reflects the fold-change increase (black columns) or decrease (gray columns) relative to the average expression level of each gene in the normal samples. Bars, SE from two replicate experiments.

larynx (2.4- to 13.4-fold change, 6 of 8 samples, i.e., 75%) and of oral cavity tumors (1.5- to 2.3-fold change, 5 of 10 samples, i.e., 50%; Fig. 3A and B). This is in agreement with a previous study that showed an increase in chromosome 7 and *ZRF1* copy numbers in HNSCC (39). *RAP140* exhibited a decreased expression in half of the patient samples of larynx tumors (1.6- to 8.1-fold change, 4 of 8 samples, i.e., 50%; Fig. 3C). No information is available in the literature on *RAP140* expression or function in tumors. *NDRG1* mRNA levels were increased in half of the oral cavity tumor samples (1.7- to 3.5-fold change, 5 of 10 samples, i.e., 50%; Fig. 3D). *NDRG1* has been referred to be either under- or overexpressed in a variety of cancers, including lung, brain, melanoma, liver, prostate, breast, and renal cancers and also in colon adenomas and adenocarcinomas (40–42). The variability observed in the expression of cancer-related genes in individual patient samples probably reflects the biological heterogeneity of HNSCC (43). Nevertheless, the 50% to 75% validation rate achieved in the present work indicates that an informative list of possible candidate markers was generated in this transcriptome analysis, for which further experimental validation is warranted.

In conclusion, two large projects have engaged in extensive EST sequencing of human tumor tissues: the Cancer Genome Anatomy Project led by the National Cancer Institute (44) and the HCGP led by Fundação de Amparo à Pesquisa do Estado de São Paulo/Ludwig Institute for Cancer Research (7), providing a very large disease-oriented transcriptional database that contains an unprecedented amount of information on expressed human genes. We show here a detailed analysis of a subset of these data, the head, neck, and thyroid sequences, documenting its utility in the identification of new human genes, candidate tumor suppressors/oncogenes, and highlighting the structure of transcript variants. Although the EST data have been widely used by the scientific community, we show that a wealth of additional information remains unexplored and may be used to further the understanding of cancer biology.

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## Appendix

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